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Studies on brain specificity

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BY

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Introduction

The work in this thesis was stimulated by the 1961 paper of Mihailovic and Jankovic (118) which stated that when the anatomically distinct structures of caudate nucleus and hippocampus were injected into rabbits, immunologically distinct antibodies resulted. The specificities of these antibodies were only demonstrated by their ability to <u>specifically</u> inhibit EEG activity of caudate or hippocampus when injected intracisternally into cats. The purpose of this thesis was to investigate <u>in vitro</u> with the Ouchterlony technique, the claimed <u>in vivo</u> EEG specificity of antibodies to caudate nucleus and hippocampus.

This paper will be divided into an historical and experimental section. In the first three historical chapters, the association of immunology and neurology is developed from the approach of allergic encephalomyelitis and allergic neuritis, (Chapter I), through various kinds of brain antigens, (Chapter II), and through the application of antibrain antibody to nervous systems, (Chapter III). In the experimental section, (Chapters IV and V), the production of antisera to brain antigens is described and the results of Ouchterlony testing recorded. In the final chapter test results will be discussed.

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HISTORICAL SECTION

Chapter I. The Injection of Heterologous and Homologous Nervous Tissue into Animals

The Problem of Paralytic Accidents

The impetus to investigate the immunological functions of nervous tissue can be said to have begun in 1885 with Pasteur's publication of a method for the prevention of rabies by the injection of a vaccine prepared from dried rabbit spinal cord in which "fixed" rabies virus had been grown (1). Variations of Pasteur's original method amounted to using combinations of killed vaccines, live vaccines and heated vaccines in varying dosage schedules. Common to all the therapies the patient received many injections of heterologous nervous tissue and usually appreciated effective rabies prophylaxis.

Some patients however apparently failed to receive rabies protection and went on to develop a paralysis which was often fatal. There are three documented cases of persons who were bitten by non-rabid dogs and then developed paralysis during the course of the injections (2, 3, 4). In each case it was possible to isolate rabies virus from the brain after death. Death in these cases was most probably the result of inadequate attenuation of the fixed virus.

However, in the majority of these so-called paralytic accidents occurring during the course of rabies prophylaxis, the brains were free of rabies virus and the symptoms of ascending paralysis with urinary and fecal incontinence differed considerably from hydrophobia, respiratory distress, dysphagia, and salivation so typical of classical rabies. Furthermore, histopathology of the central nervous system

in these cases uniformly revealed inflammation, degeneration, demyelination, the absence of Negri bodies, and an equal distribution of lesions in white and grey matter, with a predominance of cured lesions in the dorsal columns. This was not the pathology of rabies.

In 1940 the Bulletin of the Health Organization of the League of Nations reported on the administration of rabies vaccine to 1,060,832 people in the various Pasteur Institutes of the world from 1890 to 1937 (5). The number of paralytic accidents in that series was 181, a ratio of 1:5861 with one third of the cases fatal and the others recovering. Although the rate approximates the current anesthesia mortality, administration of rabies vaccine was usually an elective procedure, especially when it was not possible to examine the brain of the biting animal. Under these conditions, even a risk of 1:6000 became a significant contraindication and the phenomenon of an occasional person developing paralysis after an injection of foreign nervous tissue an important practical and academic question.

Many theories were generated to explain paralytic accidents. Pasteur and others (6, 7, 8, 9, 10, 11, and 12) were convinced that the attenuated fixed virus of the vaccine "cured" the street virus infection and modified its symptoms to a new form, dumb rabies. Papamarku (13) thought the fixed virus itself was responsible for the paralysis, (the "rage de labatorie"). The concept of a paralytic toxin produced by the rabies virus had much appeal (16) because of the pathological similarity exhibited in the brains of fatal paralytic accidents and in the lesions of encephalitis which occasionally

complicated smallpox (16), measles (17) and smallpox vaccination (18).
A humoral antibody theory of "defensive ferments" directed against
nervous tissue was considered by Stuart (19) but not pursued. The
theory which many proposed was that paralytic accidents were unrelated
to the presence of fixed virus. Rather the injection of normal nervous
tissue could in some inknown manner induce a paralysis.

In 1898 Centanni (20) observed that rabbits tolerated injections
of brain substance poorly, developing weakness, emaciation and abscesses.
Aujeszky (21) attempted to immunize dogs against rabies with injections
of ox brain emulsions using 10 cc of 1:10 ox-cord daily for 18 days.
The dogs became thin and weak; one of eight animals developed an abscess,
and another developed convulsions. He also injected rabbits with
homologous brain **emulsion** and observed these animals lost weight
rapidly during the first week. His conclusion was that rabbits were
more sensitive than dogs to the injection of foreign nervous tissue.

Delezenne (22) and Armand-Delille (23) while attempting to
prepare a neurotoxic serum noted that rabbits often died when injected
with emulsions of dog brain.

In 1904 Heller and Bertarelli (24) injected various nervous
preparations (filtrates, alcohol and ether extracts) in many routes
(intravenous, intraperitoneally, intracerebrally) into mice, rabbits,
guinea pigs and dogs. They described the development of fever, weakness,
emaciation, paralysis and occasionally death in their animals.

According to Hurst (25), Müller's paper in 1908 (26) was the
first paper to consider the forgoing evidence and suggest that
paralysis was due to the "toxic action" of foreign nervous tissue.

In 1920 Remlinger (27) injected a rabbit with a total of 14 grams of nervous tissue varying from a 2-20% suspension in six graded doses. On the 55th experimental day the animal developed a lower paralysis and died three days later. At autopsy Remlinger reported "congestion" of the lumbar cord. With Bailly (28) he attempted unsuccessfully to produce paralytic accidents in dogs by prolonged anti-rabic treatment, (101 days). They concluded that it was individual susceptibility rather than length of treatment which caused paralysis.

In an extensive study in 1925 Koritschuner and Schweinburg (30) reported upon 8 fatal cases of paralytic accidents in their Vienna clinic. They carried out an extensive experiment to duplicate in rabbits, the various methods then current for treating human rabies. Rabbits were treated with human brain for 14 days prepared according to the methods of Pasteur, Babes-Puscariu and Hoegyes.* All animals injected with brain tissue lost weight and 16 of 78 animals treated with emulsions prepared by the first two methods became paralyzed. Animals injected with material prepared according to Hoegyes did not become paralyzed. Histological examination of the paralyzed animals showed hyperemia, neuronal degeneration, perivascular infiltration, and hemorrhage in the spinal cord. Animals dying of other causes

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*Pasteur's original method consisted of air-drying spinal cords for varying lengths of time to attenuate the virus. In the first 10 days of treatment, cords which had been dried 14 days were used. At the end of treatment 5 day cords were used. The Babes-Puscariu modification was to use heat attenuated virus in large immunizing doses. Hoegyes achieved viral attenuation by dilution of the live virus and injected very small amounts of virus and nervous tissue.

and controls did not present such lesions. They concluded that
injections of foreign nervous tissue caused paralytic accidents in
man. Their paper presented the highest experimental mortality then
published. Many criticized their interpretation and invoked a
neurotropic virus (31) or additional factors, (32). However, that
same year (1926) Miyagawa and Ishii (33) reported the development
of paralysis, paresis, circular movements and ataxia in 37 of 47
white rats which were injected with 10 per cent suspensions of rabbit
and ox brains. Their experiments were controlled with liver injections
which produced no paralysis. Histologically they observed hemorrhage,
and neuronal and nuclear degeneration.

Cornwall and Beer (32) conducted experiments to determine the
minimally effective dose of nervous tissue required to produce paralysis.
They reported human cases of paralysis with a total dose of 18 mg/kg
dried cord. In their experiments they were able to paralyze rabbits
with 94 mg/kg and 500 mg/kg but a rabbit which received 800 mg/kg of
nervous tissue remained well. Two years later in 1928 Stuart and
Krikorian (19) produced paralysis in three rabbits with doses of
128, 528, and 900 mg/kg respectively. On histological examination
the spinal cords of these animals showed an acute myelitis. They
also produced anaphylaxis with jackal-brain-sensitized rabbits and
were able to show that the clinical and pathological picture of
anaphylaxis induced by brain injections was very different from
paralytic accidents. They concluded that anaphylaxis was not the
mechanism of paralysis and that an individual predisposition rather
than the amount of nervous tissue injected was at the root of these accidents.

Beginning in 1933 workers at the Hospital of the Rockefeller Institute published an important series of papers which finally explained the problem of paralysis during rabies treatment. Rivers, Sprunt, and Berry (34) first designed experiments to discover the relation of vaccine virus to postvaccinal encephalomyelitis. No evidence was found to support the idea that vaccine virus placed in the cisterna magna of monkeys was capable of producing an acute disseminated encephalomyelitis with perivascular demyelination. However repeated intramuscular injections of brain extracts and brain emulsions into monkeys resulted in 2 of 8 monkeys developing an inflammatory reaction with central nervous demyelination. The authors, however, wer not willing to accept the 25 per cent disease rate as proof of the provocative role of the brain tissue in the origin of paralysis.

Two years later Rivers and Schwentker (35) injected 8 monkeys intramuscularly, alternating between aqueous emulsions and alcohol-ether extracts of normal rabbit brain. The animals were injected three times a week and received from 46 (minimum) to 85 (maximum) injections. Six of eight monkeys developed signs of central nervous involvement. Ptosis of the eyelids, a mask-like expression of the face, facial paralysis, abnormal position of the head, blindness and ataxia were common clinical manifestations. Seven of the eight monkeys had histological evidence of inflammation, generalized and perivascular, and demyelination. Cultures from all brains were sterile and all intracerebral innoculations of emulsions from the involved brains were negative. This was the final bit of evidence to prove

paralytic accidents were in some manner, directly or indirectly, the result of injecting foreign nervous tissue into an animal. The role of virus was not settled.

Such experiments were soon repeated and verified by Ferraro and Jervis (36) who injected seven monkeys with a minimum of 29 and maximum of 103 injections using the same material as Rivers and Schwentker. Six of seven animals showed clinical disease and all seven presented the appropriate pathological findings.

Experimental Allergic Encephalomyelitis

The next significant development in this history of the paralysis resulting from the injection of foreign nervous tissue hereforth to be called experimental allergic encephalomyelitis followed upon a discovery by Jules Freund that an animal's antibody response to an antigen could be greatly hastened and augmented if the eliciting antigen was combined in an emulsion with paraffin oil, Aquaphor and heat killed tubercle bacilli (37, 38, 39). Moreover, a single injection of antigen in this emulsion (or adjuvant) would elicit high titer antibody for up to one year, whereas in the past multiple injections had been required for the production of high titer antibody and continuing injections required to sustain the titer.

It had already been established that animals dying of allergic encephalomyelitis had antibrain antibodies in their sera when Kabat (40, 41) advanced the hypothesis in 1946 that the characteristic paralysis and microscopic pathology of this disease were caused by antibody which formed in response to the injected brain emulsion, and

then reacted with the host animal's own central nervous tissue. With this hypothesis, Kabat turned to Freund's new adjuvant as the material very likely to produce the hypothesized antibrain antibody rapidly, and thus reduce the lengthy innoculation time.

He injected one group of four monkeys with rabbit brain tissue in adjuvant, and another group with rabbit lung in adjuvant. Three of four monkeys in the first group became ill with the classic signs and histology of an acute encephalomyelitis with generalized and perivascular demyelination 25 to 33 days after the first injection. The lung injected animals remained healthy. In another experiment (42) he was able to produce paralysis in monkeys with the spinal cord of a three day old rabbit, however, brain from the third day of life would not do the same until the rabbit was 12 days old. Fish and frog brain, as well as monkey peripheral nerve was found to be inactive. For those materials which were found to be encephalitogenic, neither formalin fixation, boiling nor treatment with ultrasound altered its activity. Kabat attempted, unsuccessfully, to transfer the disease to healthy monkeys injecting intravenously up to 200 ml of serum withdrawn from paralyzed animals. He also injected serum intracisternally and injected serum of rabbits immunized with monkey brain tissue. Although all attempts failed Kabat did not give up the antibody hypothesis. He concluded his paper with the possibility that the central nervous system of the donor animal had absorbed the antibody, and suggested that 200 ml was probably not enough serum for transfer.

At the same time Kabat was injecting his animals, Morgan (43) was attempting to use the new adjuvant technique of Freund to enhance

the antigenicity of poliomyelitis virus which she was growing in
monkey spinal cord. She innoculated monkeys with polio infected
homologous spinal cord and in 3-5 weeks the majority of animals
developed the classical paralysis. Animals injected with brain or
spinal cord showed an equally high rat of disease. Monkeys injected
with homologous grey matter (estimated to be 10 per cent contaminated
with white matter) showed only an occasional paralysis. Monkey
peripheral nerve or kidney was found to be non-paralytic.

Production of EAE in Animals other than Monkeys

Once experimental allergic encephalomyelitis (EAE) had been
established in monkeys, investigators turned toward other animals
to extend this experimental phenomena.

In 1947 Freund, Stein and Pisani (44) were able to report the
production of EAE in guinea pigs with one injection of homologous
brain in adjuvant. This was immediately confirmed by Kopeloff and
Kopeloff (45), Jervis and Koprowsky (46), Alvord (47), and Cazzulo
and Ferraro (48). Morrison (49) reported EAE in rabbits in 1947 with
homologous tissue.

Production of EAE in mice was more difficult but was finally
achieved after several injections of nervous tissue in adjuvant by
Olitsky and Yager (50). Moreover, Olitsky, Casal and Tal (51) found
that susceptibility to EAE varied in different stocks of mice.

Lumsden unsuccessfully attempted to produce EAE in Albino rats
with homologous brain in Freund's adjuvant injected by the subcutaneous
route (52). This was finally achieved by Lipton and Freund (53) using

nervous tissue from rat and guinea pig injected with adjuvant by the intracutaneous route. The authors observed that an emulsion containing rat spinal cord and adjuvants induced symptoms and lesions in 90-100 per cent of the rats when injected into the skin, whereas the same material failed to produce symptoms or lesions when injected into the subcutaneous tissue. In guinea pigs it did not matter which route was employed; both produced 100 per cent paralysis.

In the realm of larger animals Innes (54) injected eight goats, eight sheep and nine lambs with an emulsion of normal sheep brain in Freund's adjuvant. None of the goats became ill but four of eight sheep and six of nine lambs developed paralysis leading to death after an unusually long delay for animals treated with Freund's adjuvant. For the sheep 53, 74, 132, and 151 days elapsed between the injections and death; the period for the lambs was similarly prolonged, 40-118 days.

Transfer of EAE

A continuous problem in the studies of EAE has always been one of definition. Is EAE an immunological disease? has never been completely answered. Lipton (55) summing up the arguments in 1953 stated, "The experimental disease has many aspects in common with accepted types of allergy. It is characterized by specificity; the encephalitogenic material is organ specific, albeit not species-specific, and the manifestations of the disease are limited to the CNS. The provoking material used is antigenic, inducing the formation of complement-fixing antibodies whose specificity is similar to that of the encephalitogenic property. Moreover, CNS material of very young rabbits or mice (containing

scant amounts of myelin) is ineffective in evoking the disease or

formation of complement-fixing antibodies . . . the significance of

the antibody associated with the disease is not clear. Furthermore,

there is a discrepancy between the presence of circulating antibodies

and encephalitis. Only a portion of animals responding with formation

of these antibodies develop the disease." Having considered the

previous attempts to transfer EAE with serum antibody, Lipton and

Freund (55) decided a more vigorous approach was needed. In their

first experiment 23 pregnant guinea pigs were given an encephalitogenic

injection of homologous nervous tissue in adjuvant with the hope that

the disease if produced by circulating antibody, would pass across

the placenta and involve the offspring. No lesions were seen in the

CNS of the infant guinea pigs.

Considering the evidence that sensitization of the skin to tuber-

culin or simple chemicals could be transferred by living white cells

but not by serum (56), Lipton and Freund immunized a group of rats

with intracutaneous CNS tissue in adjuvant. At varying intervals

after immunization, these "donor" rats were joined together parabiotically

with matched "recipient" littermates. Of 18 donor animals with moderate

to sever lesions in the CNS, five recipient parabionts had moderate

to severe lesions in the spinal cord. From this experiment the authors

had demonstrated that a union which allows free transfer of cells

and serum, (i.e. parabiosis) would allow for transfer of EAE. Whether

cells, serum, or both were required to transfer EAE was not answered

(and has not yet been completely answered).

The transfer problem was finally solved by Paterson (57) in 1959 working with rats. Lymph node cells were obtained from donor rats sensitized to spinal cord and injected into recipient rats which had been treated with normal rat spleen cells in the neonatal period, thereby rendered immunologically tolerant. He found that the CNS lesions in positive recipients were indistinguishable from acute EAE lesions found in rats injected with spinal cord adjuvant.

This work was soon confirmed by Stone (58) using lymph node cells in histocompatible guinea pigs. He found that for optimum induction of passive disease cells must be transferred before the day on which the disease becomes manifest. Lymphoid cells from a donor guinea pig of strain 13 would not produce disease in Hartley guinea pigs, who have been shown to have the highest susceptibility to actively induced allergic encephalomyelitis.

Experimental Allergic Neuritis

An experimental allergic disease of this peripheral nervous system was produced by Waksman and Adams (59) following the injection of rabbit sciatic nerve or spinal ganglia into rabbits with adjuvant. This new disease, experimental allergic neuritis, (EAN) differed clinically and histologically from EAE. It presented itself as a symmetrical, ataxic paresis of the extremities without fecal or urinary incontinence and was rarely fatal. Histologically the spinal cord, brain and meninges were always normal. The spinal ganglia, dorsal roots and peripheral nerves shared the pathology which included inflammation with lymphocytes and histiocytes, neurophagia and demyelination. As in EAE, which many believe to be the experimental model for multiple sclerosis and post vaccinal encephalitis; EAN was proposed as the model

for "acute infectious polyneuritis" or the Guillain-Barre-Landry
syndrome.

Waksman and Adams were able to demonstrate this disease in
guinea pigs, rabbits and mice (60) and Astrum and Waksman (61) were
able to transfer it with lymph node cells. To the present time
neither EAE or EAN has been transferred by serum.

Recent Progress in EAE

The lesions of EAE have been shown to preferentially appear in
areas of cyanide encephalopathy (62) or where an electrolytic lesion
has been produced (63). In both examples local alterations in the
blood-brain barrier have been evoked as the probable explanations.

Scheinberg and Lee (64) reported on the production of EAE in
mice induced with a mouse glioma adjuvant mixture. The tumor which
was a methylcholanthrene induced ependymoblastoma did not demonstrate
myelin by the usual stains. H. pertussis vaccine was injected
intraperitoneally prior to immunization. This work has not yet been
replicated.

Studies have been made on the differential susceptibility of
various rat strains to develop EAE without the aid of adjuvant. With
a pretreatment injection of H. pertussis vaccine four days before the
injection of brain tissue, Lewis CD F, Fischer 334, Buffalo, M 520/N
and Wistar/Furth rats showed a clinical incidence of EAE of 86, 68, 25,
75, 17, and 0 per cent respectively. The histological severity
paralleled the clinical observations (65). It was also possible to
produce EAE in CD F rats without adjuvant or pertussis pretreatment
using brain alone, but only with a 31 per cent incidence and mild

histopathology, (66). The same authors were also able to produce EAN in CD F rats using Freunds adjuvant without tubercle bacilli (67).

Most recently, Levine and Wenk have described a hyperacute form of allergic encephalomyelitis in Lewis rats (68). This experimental disease has a reproducible onset of 6 days in adrenalectomized rats and 7-8 days in unoperated animals. The hyperacute form is induced only when aqueous spinal cord and aqueous pertussis vaccine are injected together. When Freund's adjuvant was used, the onset of disease was 9-10 days. Moreover, the histopathology of the hyperacute form of EAE is characterized by large numbers of neutrophils, (not usually seen in ordinary EAE) as well as mononuclear leukocytes, fibrin and edema fluid.

Chapter II. The Specificity of the Nervous System

Having now dealt with the essentially clinical and pathological effects of injections of gross nervous tissue, let us examine some of the work which has been done in the area of separating and analyzing central nervous tissue with respect to its antigenicity, the search for the EAE antigen being a very small part of this area of investigation.

A convenient manner of subdividing the antigens of the CNS which also has an historical basis, is to consider the alcohol soluble, alcohol insoluble, water soluble and EAE antigens separately.

Alcohol Soluble Antigens of the CNS

The use of alcoholic extracts of brain in the early German investigations of nervous tissue specificity has its roots in the history of German immunology. Soon after Landsteiner had produced a hemolytic serum it was shown that an alcoholic extract of erythrocytes would block his hemolytic reaction (69). It was also demonstrated that hemolysins could be produced by the injection of alcoholic extracts of red cells alone (70).

The work of Wasserman (71) on the serodiagnosis of syphillis also contributed to the use of alcoholic extracts. After observing that the serum from a syphilitic patient would fix complement with an alcoholic extract of syphilitic tissue, Wasserman discovered that the "lipoid" or alcohol soluble antigen of even normal tissue would react with syphilitic sera.

In 1911 Forssman (72) discovered an <u>alcohol soluble</u> hapten initially in guinea pig tissue, (and later in alcoholic extracts throughout the plant and animal kingdom) which would lyse sheep erythrocytes. These alcohol soluble F (Forssman) antigens were also found to be resistent to boiling.

The application of "lipoid" antigens to brain immunology was first described by Brandt, Guth, and Müller in 1926 (73). They immunized rabbits with 18 daily injections of beef brain lipoid mixed with pig serum. The resulting antiserum fixed complement with alcoholic extracts of beef, human, pig and guinea pig brain and was negative or weakly positive with similar extracts of other organs.

Further experiments by Witebsky and Steinfield (74) Hermann and Steinfield (75) and Plaut and Kassowitz (76) firmly established the <u>organ specificity</u> of anti brain-lipoid antibodies prepared with the injection of alcohol extracts. An antibody to brain lipoid was found to react well with <u>all</u> brain lipoids.

When Witebsky immunized rabbits with <u>saline suspensions</u> of brain he obtained two classes of antibodies. Some sera reacted with all species of boiled or native mammalian brain. Other sera only reacted with unheated homologous brain suspensions. Those sera which were organ specific contained antibodies against lipoid antigens. The sera whose reaction was limited to homologous antigen did not contain antibodies to lipoid antigen (74).

Using antibodies to lipoid antigens, Witebsky and Behrens (77) demonstrated an immunologic difference between the anterior and

posterior pituitary and with Klinke, Witebsky was able to differentiate between the cortex and medulla of the adrenal gland (78).

In 1934 Reichner and Witebsky (79) immunized rabbits with boiled suspensions of selected brain regions. White matter was obtained from the centrum semiovale and sciatic nerve while grey matter was removed from the hippocampus and the caudate nucleus. Antisera produced to these boiled suspensions were found to react organ-specifically with an alcoholic extract of whole brain. In the complement fixation reaction antibodies to the grey areas could be distinguished from those prepared against white matter by reactions with "grey" and "white" antigens respectively. Anti caudate nucleus and anti hippocampus antisera fixed complement avidly with both grey matter alcoholic extracts; each antisera reacted weakly with both white matter extracts. In similar manner, the anti white matter antibodies reacted strongly with both of the white matter alcoholic extracts and weakly with the grey matter extracts. Antibodies prepared against boiled suspensions of medualla oblongata, cerebellum and sacral spinal cord were found to behave as white matter antibodies. When an embryonic mammalian brain and a fish brain were tested against the two species of antibodies (anti white and anti grey) it was found that the anti white matter antibodies reacted with fish brain but not with the embryonic mammalian brain and conversely, the anti grey matter antibodies reacted with the mammalian embryonic brain and not the fish brain. Today, 32 years later, this experiment remains one of the most significant demonstrations of regional brain specificity.

Lewis (80) immunized rabbits with twice a week intravenous injections of brain suspensions (saline) and brain alcoholic extracts. Beef, rat, rabbit, and guinea pig brains were used. The saline suspensions were mixed with normal rabbit serum before injection whereas the lipoid extracts were combined with or "activated" with pig serum. All preparations were found to be antigenic in the rabbit except for rabbit brain suspension in normal rabbit serum. The resulting antibodies were indistinguishable when tested against homologous and heterologous antigens from both methods of preparation, using the complement fixation technique. All anti brain antisera were brain specific (organ specific) when tested with homologous and heterologous organs (heart, liver, spleen, kidney, liver) except that each sera displayed a weaker cross-reaction with testicle antigens of all species. Lewis extended Witebsky's observation of organ specificity by the demonstration of an equally strong reaction of antibrain sera against the saline suspensions and the alcoholic extracts of brains from 8 mammals, 4 birds, 2 fish and a frog. When antibrain sera was tested against brain parts (cortex, thalamus, basal ganglion, corpora quadrigemina, cerebral peduncles, pons, cerebellum, medulla and cord) no significant difference was found in the ability of any region to fix complement with antibrain sera. It was also observed that antibrain sera fixed complement markedly in the presence of cholesterol but not with lecithin.

Neither Witebsky and Steinfield (74) nor Lewis (80) were able to produce antibodies in the rabbit to suspensions of homologous brain.

More generally, most alcoholic extracts of antigenic tissues were found to be non-antigenic until mixed with a foreign protein, often pig serum. This enhancement phenomena was first described by Landsteiner (81) (82) while working with the Forssman hapten. The hapten extracted from horse kidney with alcohol was observed to have a strong affinity for Forssman antibodies but was unable to elicit antibody formation when injected alone. Landsteiner was able to endow immunizing capacity to the hapten by mixing it with a foreign protein.

The production of antigenicity with the addition of a foreign serum was called the conveyer method, "Kombinationsimmunisierung" or the "Schlepper Function." Landsteiner (81) thought that the hapten might enter into a loose combination with the conveying protein. Sachs (83) and Weil (84) suggested that the foreign protein served as an envelope to allow the non-protein matter in enter the cells. However, none of the theories were able to explain other observations that in some cases the conveyor could be replaced by adsorbants like kaolin or $Al(OH)_3$, (85, 86, 87, 88).

However, without a complete explanation, addition of a foreign protein allowed the reproducible production of antisera to alcoholic extracts of brain, testicle, blood cells, etc. Moreover, Sachs (83) demonstrated that when a rabbit was injected with an ethanolic extract of organs (homologous or heterologous) by the above method, the resulting serum exactly mimiced the reactivity of a positive syphilitic serum in the Wassermann reaction.

A special investigation of the antigenicity of homologous brain preparations in the rabbit was designed by Schwentker and Rivers (89). Five rabbit brain innoculants were prepared:

1. Emulsions of fresh brain.

2. Emulsions of fresh brain with pig serum.

3. Emulsions of autolyzed brain prepared by allowing sterile normal rabbit brain to stand at room temperature for 5 to 30 days.

4. Emulsions of brain infected with vaccine virus.

5. Alcoholic extracts of brain with pig serum.

Rabbits received 10 intraperitoneal injections of 5 cc of one of the above preparations. Their sera was then collected and tested by complement fixation against both emulsions and alcoholic extracts of rabbit brain. High titer antibodies (1:80) were produced in the rabbits injected with emulsions #3 and #4. Low titer antibodies (1:20) were produced in animals receiving #2 and #5. No antibodies were produced in rabbits injected with #1.

When tested for specificity, the sera resulting from the injection of autolyzed brain fixed complement organ specifically only with alcoholic brain extracts. When aqueous emulsions were substituted, the sera reacted with all organs. These non-specific antibodies were absorbable with an aqueous emulsion of rabbit kidney. The absorbed antisera then tested brain specifically with both aqueous and ethanolic antigens. In converse fashion it was possible to absorb the specific anti brain antibodies and leave behind only the non-specific reactivity. Such production of non-specific antibodies is in agreement with other experiments in which heterologous antigens in aqueous form were used for immunization. It must be expected

that water soluble antigens of connective tissue, ground substance, blood vessels, etc., would be included in an aqueous emulsion of brain tissue and that antibodies to these "contaminants" would fix complement when such an aqueous emulsion is the antigen.

Further experiments were conducted by Schwentker and Rivers to better locate the brain antigen. When aqueous emulsions of known amounts of white and grey matter were tested against the antibrain sera it was found that white matter was six times more antigenic than grey. Moreover, when a series of rabbit brains from fetus to maturity were tested, the "brain antigen" was found to parallel development and was not present in fetal brains. It was the authors' conclusion that the antigen concentration paralleled the development of myelin in brain.

In the tradition of Reichner and Witebsky, Jankovic, Isakovic and Mihailovic (93) removed frontal cortex, occipital cortex, temporal cortex, cerebellar cortex, caudate nucleus, thalamus, cerebral white matter, medulla and spinal cord from cat, dog, ox, monkey and human. Each of these brain regions was extracted with ethanol and the lipid residues were combined with Freund's adjuvant and injected into rabbits. Antisera to all the lipid extracts were found to be highly organ specific, but no regional specificity was found.

The most recent contributions to the nature of the "lipid" organ specific antigen of brain comes from the work of Niedieck and Pette (94), Rapport, Graf, Autilio and Norton (95), and Joffe and Rapport (96). These authors have shown that the organ specific

alcohol soluble thermostabile antigen of brain is galactocerebroside.
When an animal was immunized with galactocerebroside isolated from
bovine spinal cord all of the activity in the antisera could be
absorbed with either myelin or galactocerebroside.

It was also found that the antibody which reacted with galacto-
cerebroside was identical with the antibody which reacted with myelin.
A significant difference however, was that for the complete absorption
of galactocerebroside the presence of the auxillary lipid lecithin
was required, whereas myelin, a complete lipid, reacted alone. With
a sensitivity of three complement units, 7 ug of myelin was detectable.
Antisera prepared against particulate fractions of bovine brain con-
tained the anti galactocerebroside antibodies but in addition, contained
other antibodies against non-galactocerebroside determinants.

Alcohol Insoluble Antigens of CNS

Bailey and Gardner (90, 91) used the anaphylactic response of
guinea pigs to demonstrate the organ specificity of the brain antigen.
They immunized rabbits with a sedimented, heat killed vaccine of
Pasteurella boviseptica grown in an infusion broth prepared from
rabbit or rat brain. The antiserum resulting was used to produce
passive sensitivity in guinea pigs 24 hours before testing with
various autoclaved organ preparations.

When guinea pigs were sensitized with anti rabbit brain broth
18 rabbit tissues tested negative except for brain which produced
a fatal anaphylaxis. A slight reaction was observed with testicle.
The autoclaved broths of 14 other mammalian brains produced a fatal

anaphylactic reaction; chicken brain produced only a slight response and fish, duck and frog brain were inactive.

When the guinea pigs were sensitized with anti rat brain broth only rat brain, sciatic nerve and a carcinoma derived from rat mammary gland produced death. All other rat tissues were inactive. (Note, sciatic nerve was not tested in the first experiment.)

In another experiment the anaphylactogen was demonstrated in rat brain beginning at 17 days of age in bovine white matter but not in grey matter. Fetal brains from rat, guinea pig or rabbit were inactive. It was also possible to show an increasing severity of the anaphylactic response as a guinea pig brain matured from 2 days to 6 months. The anaphylactogen was found to be alcohol insoluble, thermostabile, and water soluble. It was not dialyzable. Bailey and Gardner did not consider their antigen to be a lipoid because an autoclaved alcoholic extract of brain was negative in their hands. Rather, they felt their antigen was a polysaccharide or an altered protein produced by the effects of steam and pressure.

Milgram, Tuggac, Cambell and Witebsky (92) have recently defined organ specific antigens of brain and adrenal which are saline soluble, alcohol insoluble and stable at 100°C. They referred to them as BE preparations for their resistance to boiling and ethanol insolubility. This group prepared antisera in rabbits to BE of bovine, porcine and human brain. Antisera to human and bovine brain BE reacted with its homologous brain BE but not with the BE of other homologous organs.

Anti porcine brain BE had to first be absorbed with porcine kidney BE for its similar specificity to be established.

The BE of brain was not found as universally antigenic as brain lipid. The three brain BE preparations were cross tested against their corresponding antisera. The anti human brain serum combined with the homologous brain preparation only, anti bovine brain serum combined with bovine and porcine brain preparations and anti porcine brain serum combined with all three BE preparations. These reactivities were confirmed with inhibition studies.

Whatever the BE antigen may be, it is apparently present in native brain suspensions. Anti bovine brain BE sera reacted with suspensions of bovine brain exclusively and anti bovine brain suspension reacted in similar manner with bovine brain BE. When the BE of parts of bovine brain were tested with the anti bovine brain BE serum, the BE of grey matter had a reaction titer 30 times higher than the BE of white matter. Thus, the BE factor must be different from Bailey and Gardners' anaphylactogen which was mostly in white matter (91) even though both antigens are alcohol insoluble and thermostable.

Milgram's group also showed that the antibody to brain BE is very different from the antibody to brain lipid. Anti porcine and bovine brain BE did not react with their homologous alcoholic brain extracts, whereas antisera to procine and bovine brain suspensions reacted and cross reacted with the alcoholic extracts.

Water-Soluble Protein Antigens of the CNS

Investigations of the water-soluble protein antigens of the nervous system were delayed until electrophoresis and immunoelectrophoresis became firmly established as practical laboratory techniques. In the early 1960's Schalekamp and Kuyken (97) obtained 13 electrophoretic fractions of whole brain extract and Van Sande (98) obtained 17 fractions using high voltage electrophoresis.

Employing a starch gel medium, Bailey and Heald (99) performed electrophoresis on protein extracts from different parts of human and guinea pig brain. With this technique, human cerebral cortex yielded 10 components while cerebellum yielded only eight. Moreover, at pH 8.3, 0.04 M borate buffer, human cortex displayed three negatively charged components which were not present in the cerebellum. Proteins of the guinea pig brain were markedly different from human brain by electrophoresis. The authors searched for changes in protein patterns following anesthesia, electroshock and metrazol shock, but none were found (100).

The most complete separation of cerebral proteins to date has been achieved by Bogoch, Rajan and Belval (101) with the combined methods of column chromatography and disk gel-electrophoresis. These workers exhaustively extracted the proteins from human grey matter and then collected 50 fractions, classified unique by protein, hexose, hexosamine and neuraminic acid analysis, from a diethyl amino ethyl (DEAE) cellulose column. Each of these 50 chromatographic fractions were then examined by disk acrylamide gel-electrophoresis, resolving between two and eleven clearly separated protein zones for each of

the 50 fractions. Allowing for overlap the authors claimed to have

identified over 100 cerebral proteins. Curiously, one of these

sub-subfractions was found to be greatly elevated in a brain from

a patient with Tay Sachs disease (102).

Moore and McGregor (103) also chromatographed the soluble

proteins of human brain on DEAE cellulose and followed with starch

gel electrophoresis of each column fraction. They too obtained from

70-100 fractions, however, these workers also studied the soluble

proteins of liver as a control. From 100-200 discretely staining

bands on starch electrophoresis, these investigators found one constant

fast moving acidic protein present only in brain and absent in a liver

of rat, rabbit, beef and monkey. This protein was called S 100 by

Moore because it was soluble in saturated ammonium sulfate solution.

Further investigations (104) identified this protein uniquely

in the brains of: hog, hamster, guinea pig, mouse, dog, human, turkey,

eagle, alligator, black snake, turtle, pompano and red snapper. It

was found in several parts of the nervous system of the rabbit: whole

brain, grey and white matter, cerebrum, cerebellum, brain stem, spinal

cord, sciatic nerve and vagus nerve. It was also found in beef retina

and in sciatic nerve and spinal cord of rat. Its concentration has

been estimated to be 0.6 per cent of the total soluble protein of

beef brain. S 100 has not yet been located in any non-nervous tissue!

Using antisera prepared to the S 100 protein, it was estimated

that brain soluble extracts contain from 1000 to 10,000 times as much

S 100 as other organ extracts. Moore has estimated the molecular

weight of the protein to be about 30,000. On the basis of its
distribution, he has suggested that S 100 is probably neuronal
protein and not part of the myelin sheath.

What is the role of S 100 in the nervous system? Rubin and
Stenzel (105) prepared a brain cell-free actively synthesizing system
which was allowed to incorporate C^{14} leucine. After one hour of
active synthesis Moore's S 100 protein and its antibody were added
to the reaction system to allow co-precipitation with any radioactive
acidic proteins which were being newly synthesized. After four days
of incubation in the cold 21 per cent of the soluble protein synthesized
in the cell-free system had bound in immune co-precipitation to the
S 100-anti S 100 complex. In control co-precipitations 9.2 per cent
of the protein bound non specifically to RNase-anti RNase and 7.0
per cent to egg albumin-anti egg albumin.

Hyden and McEwen (106) have localized S 100 principally in
glial cells. They extracted equal amounts of soluble proteins from
50 rabbit Dieters' nerve cells, and the corresponding amount of glia
and brain stem homogenates. With a micro capillary gel-diffusion
method, immune precipitation occurred only when the proteins of
brain homogenate and glia were tested with the anti-S 100 sera. No
precipitation occurred with neuronal proteins. With the sandwich
technique, fluorescent antibody analysis was used to locate S 100
antigen in the rabbit brain stem. Specific fluorescence was seen
in the glia, but also in the big Dieter's nerve cells. The neuronal
nucleolus and cytoplasm did not display specific fluorescence.

Attempts at quantitation revealed that the concentration of S 100 in neurons was <u>at least</u> less than one fifth that of the proteins of the whole Dieter's nucleus.

The most recent studies of S 100 (107) have shown that this assumedly homogenous protein can be separated into five components with electrophoresis in 15 per cent acrylamide gel. Only two of the five bands react with antibody prepared against the unseparated S 100.

Encephalitogenic Antigens of Brain

There are so many apparently unrelated subfractions of brain with encephalitogenic activity which have been proposed as "the antigen" that a discussion of this area is not only confusing but contradictory. The history of the problem is best summed up by Lumsden (122) in his recent paper which appears to have explained the ubiquitous nature of the EAE antigen.

> "Firstly there was the evidence of Kabat, Wolf and Bezer (41) relating the appearance of encephalito-genicity to myelinogenesis. Laatsch, Kies, Gordon and Alvord (122), using an ultracentrifugation technique controlled by electron microscopy, have clearly confirmed that the active substance is related to lamellar myelin. Chemically, the early evidence (123) that at least some encephalitogenic substance resisted autoclaving and treatment with formalin, alcohol, chloroform and ether, and that some was extractable with the sphingomyelin and cerebroside fraction, was overshadowed by the later enthusiasm for soluble protein, which might have been expected to be more labile. At the Bethesda symposium (124) it was widely agreed that there were three groups of EAE antigens: (i) water-soluble proteins, (ii) fat-soluble proteolipids, and (iii) a water-insoluble glycoprotein or 'collagen-like' protein. The prevailing conclusion of Waksman (125) was that 'several substances' were responsible."

Lumsden and his group set to testing the biologic activity
(in the sense of EA-encephalitogenicity with Freund's adjuvant)
of <u>all</u> extracts and residues throughout all major extraction
schedules of basic proteins, proteolipids, and collagen-like proteins,
(the three major candidates for the EAE antigen). Altogether, 173
fractions and residues were tested, each in groups of ten guinea
pigs at different dose levels. According to his paper, the common
denominator of EAE-activity in <u>every</u> fraction which was active was
a dialysable basic small polypeptide (BSP) with the following
properties:

1. "It comprises a specific polypeptide or polypeptide
   species responsible is some as yet undetermined way for
   the encephalogenicity of all EAE-active compounds in
   CNS tissue.

2. It is released by spontaneous breakdown of larger
   basic polypeptides and basic protein.

3. It is itself highly basic, migrating rapidly to the
   cathode on electrophoresis on acrylamide gel in formic-
   acetic acid buffer at pH 2.

4. In addition to its association with basic protein it may
   also be capable of binding with at least one type of
   anodic protein and with the proteins constituting the
   neurokeratin moiety of proteolipid.

5. It may exist in bound or complexed forms with lipids,
   phosphatides and gangliosides.

6. In all its forms so far examined it has given a faint
   anthrone reaction carbohydrate.

7. Preliminary molecular weight studies give a value of about
   4000. Current studies of more highly purified material
   indicates an even lower molecular weight."

At present this appears to be the best statement about the EAE antigen.

Chapter III. The Application of Antibody to Functioning Nervous
              Systems

The application of antibrain antibody to functional studies of the

nervous system is in this author's view, the most exciting activity in

this recently named field of immuno-neurology. The hope of producing

a specific lesion in the nervous system with antibody, and the further

hope that very specific antibodies will produce similarly specific

lesions has concerned some investigators enough to consider antibody

for a new tool. At present in the field of neurophysiology the non-

behavioral methods available for studying brain function include

stimulation and recording via implanted electrodes, chemical

alterations with local or systemic application of metabolic antagonists

or hormones, or mechanical interference either by heat coagulation, cryo-

techniques, alcohol necrosis, ultrasound, x ray, or surgery. Except

for metabolic antagonists, none of these methods contain the quality

of specificity which is implicit in the concept of antibody function.

In the next group of experiments some of the published applications

of antibody to functioning nervous systems will be presented.

Early Animal Injections

The earliest whole animal experiments with "neurotoxic" sera

were conducted by Delezenne in 1900 (22). In this study antibody

was produced in ducks who had received intraperitoneal injections

of dog forebrain, cerebellum, brain stem, or spinal cord over a period

of two months. Those ducks which survived this procedure were bled

between 8-10 days after the last injection. In preparation for

injecting the sera into dogs it was first determined that dogs could tolerate the instillation of 0.5-0.6 ml/kg of normal duck serum into their frontal lobes without any signs of behavioral abnormalities. However, when similar amounts of test serum were injected most of the animals becam quickly paralyzed, rarely showed convulsions and died in respiratory arrest. Animals given a smaller amount, 0.3-0.4 ml/kg, "collapsed, showed convulsive movements that were rarely generalized, became paralyzed slowly, breathed stertorously and died in respiratory arrest several hours later. Animals given 0.1-0.2 ml/kg rarely demonstrated any effect in the first few minutes after the injection except that most of them tried to run away and if they were not held ran into a corner. Very soon they began scratching and walking became difficult. About a half hour later paralysis began and they had great difficulty standing. Some dogs now showed clear epileptic signs: loud crying, salivation, clonic and tonic convulsions which were repeated several times before being followed by clonic convulsions that intensified with time. Throughout this period the dogs continually moved their heads and limbs in an uncoordinated manner. This excitatory period lasted 7 to 8 hours and was followed by a period of spastic paralysis similar to that shown by the animals receiving high doses and ended in death."

This work was repeated by Centanni who in the same year immunized a lamb with 7 months of intraperitoneal injections of rabbit brain. The lamb serum killed a rabbit in 48 hours when 0.5 ml was injected intracerebrally. Intravenous injections produced no effect (108).

In Delezenne's laboratory, Armand-Dellile prepared "neurotoxic"
serum against dog brain in the guinea pig. His macroscopic obser-
vations of the killed dogs' brains revealed congestion and occasional
ecchymosis of the pia but curiously never any bleeding at the needle
sight. Microscopically the most significant lesion was the extreme
degree of congesting and dilatation of the blood vessels. Chromatolysis
was present in all of the motor nuclei. None of these effects occurred
in the control (23).

These kinds of experiments died out for forty years to reappear
in the late 1940's when investigators attempted unsuccessfully to
transfer the clinical and pathological manifestations of EAE with
serum, (42, 43, 44). The conclusions of the EAE investigators was
that the sera of EAE animals did not cause any disturbance when
injected intraperitoneally, intravenously, or intracisternally.
Hurst argued however, that the sera of EAE animals did not constantly
show complement fixing antibrain antibodies, whereas when animals
were injected with suspensions or alcoholic extracts of brain they
always produced brain-specific antisera. He suggested that this might
explain the negative transfer results.

Hurst himself injected monkeys intra-arterially and intra-
cisternally with goat anti-monkey brain sera and anti-monkey spleen
sera. His two papers carefully describe the necro-pathology resulting
from the injections but he failed to demonstrate that intracisternal
or intra-arterial injections of "neurotoxic" sera differed in
activity from anti-spleen sera. Moreover, the anti-spleen sera was
more noxious by the arterial route than the anti-brain sera (116, 117).

Antibody In Tissue Cultures

There were also investigators who put antibody into their
tissue cultures. Lambert and Hanes, reporting on the effect of
immune serum on the growth of a mouse sarcoma in 1911, were probably
the first of this group (109). The first application of antibrain
serum to cultures of neuronal cells was in Kimura's laboratory in
1928. In a brief report a specific cytotoxic effect of rabbit anti-
brain sera upon cells of the CNS was described (111).

Twenty years later Grunwalt decided that tissue culture would
be a valuable tool for evaluating anti-brain sera (110). He prepared
antisera in rabbits to three different preparations of newly hatched
chicken brains: whole brain in saline, the alocohol-soluble fraction,
and the alcohol insoluble residue. These three antisera were then
added in 25 per cent concentration to hanging drop cultures of 9
and 13 day chick embryo spinal cords and their effects upon cell
growth observed.

The effects of the anti-whole brain and the anti-alcohol insoluble
residue were indistinguishable: both inhibited the outgrowth of
fibers and cells in the 9 and 13 day cultures. However, the anti-brain
sera obtained from injection of the alcohol soluble fraction of
brain inhibited growth in the 13 day culture but had no effect upon
growth in the 9 day culture. The suggestion was made that there must
be two different antigens in the brain of the chick, one is alcohol
insoluble and appears early; the other is alcohol soluble and appears
four days later.

More recently, Bornstein and Appel, in their attempt to set
up a model system to study demyelination, have described a specific
and characteristic reaction in myelinated cultures of newborn
rat cerebellum which occurs whenever the serum from animals with
experimental allergic encephalomyelitis or from humans with active
multiple sclerosis is introduced (112, 113, 114).

"The reaction may begin within an hour after
exposure to high concentrations of EAE serum. The
first observable alteration appears as a swelling of
neuroglia, visualized directly with time-lapse
cinematography, or as a displacement or deviation of
myelinated axons in the more usual photomicrography.
At about the same time, the myelin sheaths appear
brighter and more distinct. Neuroglia continue to
swell throughout the acute course of the reaction.

"The myelin sheaths soon become disfigured by
fusiform swellings at irregular intervals along the length
of the axons. Many swellings enlarge further to protrude
grotesquely into surrounding areas. They may break
away to become myelin fragments. The fragmentation,
however, does not seem to be the only manifestation of
myelin damage, for whether or not this myelinolysis has
occurred, the myelin seems simply to melt away. Thus,
apparently intact sheaths as well as isolated myelin
fragments more or less rapidly lose their characteristic
refringence, become less and less distinct, and finally
fade into a background of neuroglia, neurons, and small
fat granules.

"Meanwhile, some neuroglia have become enormously
swollen. Their pycnotic nuclei frequently lie against
the cell membrane with retracted elements of the cytoplasm,
while the main body of the cell is filled with fluid and
particles in Brownian movement.

"At this time, neurons seem little affected. Some
nuclei tend to assume eccentric positions; such neurons
stain less intensely with cresyl violet than do the normal
controls. The axis cylinders appear intact when viewed in
the living state with bright field or phase contrast
illumination or when fixed and stained with Bodian's
silver impregnation technique.

> "Phagocytic activity is remarkably slight at this
> time and ependymal cells seem totally unaffected.
>
> "The tissue is far from overwhelmed by the reaction.
> Removal of the foreign serum and its replacement by
> normal nutrient medium halts the process. Further
> studies, moreover, demonstrated that remyelination may
> occur after a short recovery period.
>
> "Myelinated cultures of fetal rat spinal ganglia
> failed to react in any way during a 48 hour exposure to a
> 20 per cent concentration of EAE acute or its paired
> normal serum. Al aliquot of the former, simultaneously
> applied to cultures of rat cerebellum, produced a total
> demyelination of the central nervous tissue in less than
> 12 hours."

The demyelinating activity has been found to be complement dependent

and resides within the gamma-2 globulin fraction, one microgram of

which is sufficient to cause demyelination in a culture in 24 hours.

Exposure to homologous or heterologous brain removes the activity

whereas non-nervous tissue does not. Fluorescent antibody studies

have located the EAE globulin on the myelin sheath and glial

membrane during the process of demyelination.

Bornstein and Crain (115) have also applied antibody to myelinated

cultures of cerebral neocortex and spinal cord in which the response

to electrical stimulation was measured. Exposure to antibody not

only produced the characteristic demyelination, but produced

extensive alteration in the bioelectric properties of the tissue

long before any morphological changes had been detected. The first

responses to disappear were those electrical patterns characteristic

of snyaptic transmission, often within a few minutes of application,

leaving the axon spikes. With time, the threshold for direct

stimulation of spikes rose; when the serum was replaced normal

function returned. Human serum from patients with acute multiple sclerosis also blocked intraneuronal transmission but a 50 per cent concentration was required to equal the effect of 10 per cent rabbit EAE serum.

The last group of experiments to be described represent what may be a major breakthrough in the application of antibodies to the nervous system. These experiments propose, that in addition to the specificities of white matter, grey matter, peripheral nerves and central nerves, and general brain, there may be an intraregional specificity among grey matter structures.

Functional Regional Specificity

The first experiment of this kind was performed by Mihailovic and Jankovic in 1961 (118). For this study whole cat caudate nucleus was carefully dissected, homogenized, mixed with Freund's adjuvant and injected subcutaneously into rabbits (1.0 g. wet tissue/kg body weight). The rabbit serum was ammonium sulfate fractionated and the immune globulin tested by complement fixation and gel-diffusion against the saline extract of cat caudate. The reaction between the rabbit gamma globulin and the caudate yielded 2-3 lines in gel and a titer of 1:128-1:256 in complement fixation. The antisera was apparently not tested with any other brain antigens.

The antibody was then tested in cats who were prepared with a cannula inserted into the lateral ventricle on one side, and electrodes implanted in frontal cortex, frontal white matter, caudate nucleus, hippocampus, hypothalamus, thalamus, and brain

stem reticular formation in the opposite side. Cats were injected
intraventricularly with 0.3 ml of either immune gamma globulin,
normal gamma globulin, or normal saline two or three times a day
for a period of four days. Both control groups demonstrated no
significant changes in their electroencephalogram during a month
of observation. For those animals injected with the immune
globulin the following changes were noted:

> "The first stage was characterized by the
> appearance of spikes and high-voltage sharp waves
> localized in the caudate. These transient irritative
> abnormalities became apparent on the third day of
> antibody administration, and disappeared two or
> three days afterwards. The second, also transient
> stage was characterized by slight general accentuation
> of background activity and by sporadic appearance of
> diffuse abnormalities consisting of bursts of 4-6/sec
> high voltage waves, in all leads. Such activity
> outlasted the disappearance of spikes and sharp waves
> in the caudate for several days. The third stage was
> characterized by gradual slowing down, progressive
> decrease in the amplitude and almost complete dis-
> appearance of spontaneous electrical activity of the
> caudate nucleus within a month after antibody
> administration."

No change in the activity of the caudate nucleus could be observed in
the animals treated similarly with anti-hippocampus antibody. Incidental
observations showed that evoked potentials to auditory stimuli were
modified as was the activity propogated to the caudate during
seizures induced by electrical stimulation of the hippocampus.

In another in vivo experiment by the same group (119) cats were
injected intraventricularly with anti-caudate, anti-hippocampus,
or normal rabbit gamma globulin. One hour after the last injection
the animals were sacrificed and samples of the caudate nucleus,

hippocampus and frontal cortex were taken for analysis of histamine-like substances, under the assumption that elevated histamine-like activity would be indicative of an antigen-antibody reaction. The results of this study revealed a statistically significant increase ($P = 0.01$) of histamine-like substances in the caudate only when anti-caudate antibody was injected and a corresponding specific increase in those substances in the hippocampus when its antibody was injected.

If these experiments can be confirmed a tool is available to induce specific inhibition of electrical activity in specified brain regions and perhaps to selectively inhibit a myriad of additional "specific" brain functions not yet completely identified.

In an investigation of neuronal electrogenesis, Mihailovic et al. (120) applied anti-lobster nerve antibody to the giant axon of lobsters and observed the action potential and resting potential at hourly intervals. The most significant changes attributed to the action of the antibody were the progressive decrease in the resting potential and the deterioration of the action potential. It was also demonstrated that nerves bathed in an immune gamma globulin lost twice as much potassium as nerves bathed in normal gamma globulin. Hence, a suggestion was made that antibody may increase the nerve membrane's permeability to potassium. Similar electrical changes were found by Huneeus-Cox (121) who perfused giant squid axons with antiserum to squid axoplasm.

EXPERIMENTAL SECTION

In this section antibody was prepared to whole unperfused cat caudate, cat hippocampus, and pig caudate according to the technique of Mihailovic and Jankovic (118); antibody was also prepared to saline-perfused cat caudate and hippocampus. All antisera were tested by Ouchterlony gel immunodiffusion. Precipitin activity, specific to the caudate or hippocampus was searched for in all Ouchterlony plates.

Chapter IV. Preparation of Antisera

Antigens

All cats used in this study were obtained from Yale University Department of Animal Care. Early in the work cat brains were collected from animals which had been used in other experiments, terminally or soon after death. Many brains came from cats used by Yale medical students in their Physiology course.

When perfused brains were specifically desired, animals were anesthetized with pentobarbital 25 mg/kg and a thoracotomy performed. Normal saline at a pressure of 2-4 feet was administered through a cannula inserted into the left ventricle; the descending aorta was clamped and the right heart was opened. The absence of visible blood in the fine capillaries of the choroid plexus and the lack of a precipitin reaction when the antigen suspension was tested with Hyland rabbit anti-cat serum, were used as indices of an adequate perfusion.

The cat brains were stored in ice immediately after removal from the skull. The caudate and hippocampus were freed by blunt dissection. White matter was obtained from the corpus callosum and internal capsule by careful sharp dissection and samples of cortical grey were removed in thin slices with a razor.

Unperfused pig brains were obtained from the Sperry Barnes Packing Company, formerly of New Haven, and from Connecticut Packers, Bloomfield, Connecticut. Pigs were stunned by electroshock or by a bullet shot into the head, and then bled from the neck. The skull was divided by a cleaver and the brain always removed in two pieces. All brains were put into ice within one hour of he animal's death. Caudate and hippocampus were bluntly dissected; the white matter was removed by sharp dissection.

All antigens were stored in small vials at -20°C. for periods of up to one year.

Immunization Methods

The antigen for immunization was homogenized with known amounts of saline, usually at 20 per cent w/v, in a glass Potter-Elvehjem tissue grinder with a teflon pestle. An equal amount of Freund Complete Adjuvant, (Difco, Detroit, Michigan), was added to the homogenate which was mixed to emulsion. New, white, New Zealand rabbits received a total of 1.5 ml of immunizing emulsion, 0.15-0.20 ml in each of four toe pads, the remainder of the emulsion was injected subcutaneously into multiple sites on the rabbit's back. Booster injections contained the antigen in saline or in a

few cases in Freund adjuvant. In recent experiments rabbits were first anesthetized with a combination of pentobarbital and ethyl ether before receiving the painful toepad injections.

The sheep were initially immunized with antigen in Freund complete adjuvant injected into four sites on the back subcutaneously and boosted with an intravenous injection of the antigen in saline. Control bleedings were obtained from all animals prior to immunization. A detailed immunization for the experimental animals is presented.

Bleedings of Animals

Rabbits were bled at two to three week intervals from the ear. Following vasodilatation induced by topical xylene a razor cut through a major vein provided 30 to 60 ml of whole blood. The sheep were bled 50-150 ml every two weeks with a #13 x 3" needle in the jugular vein. All animals received Imferon (Lakeside Laboratories), a parenteral iron-dextran preparation after each 3-4 month bleeding period.

Immunization Schedule

Rabbit 33

Date	Events
4/6/64	Injected with 250 mg. unperfused cat caudate homogenized in Freund complete adjuvant. Total volume 1.5 ml; 0.15 ml in one toe pad of each foot, remainder intradermally and subcutaneously into multiple sites on back.
4/20/64	Test bleed, neg.
5/6/64	Boosted with 200 mg cat caudate in saline (s.c.).
5/28/64	Boosted with 250 mg cat caudate in saline (s.c.).
6/3/64	Test bleed, active sera.
6/17/64	Boosted with 250 mg cat caudate in saline (s.c.).
7/3/64	Bleed (from 7/3-11/11 boosting data not available).
7/9/64	Bleed
7/28/64	Bleed
* 8/4/64	Bleed
*11/11/64	Bleed

*These sera were used for experiments reported in this paper.

Immunization Schedule

Sheep 209-210

Date	Event
2/17/65	2.5 g unperfused cat caudate in FCA (ID).
3/1/65	Bleed and boost, 0.75 g cat caudate in saline (s.c.).
3/18/65	Bleed, Boost, 0.75 g cat caudate in saline (s.c.).
3/25/65	Bleed and boost, 1.0 g cat caudate in saline (IV).
4/2/65	Animal ataxic, impairment of righting reflex.
4/8/65	Bleed and boost, 1.5 g cat caudate in FCA, (weak but alert)
4/16/65	Sacrificed by exsanguination.

Serum from final bleeding was used in experiments.

Immunization Schedule

<div align="center">Sheep 5-6</div>

Date	Event
11/14/64	Inject 0.7 g pig caudate FCA (s.c.), 0.7 g pig caudate in saline (IV).
12/3/64	Bleed and boost, 0.5 g pig caudate in saline (IV) (s.c.).
12/16/64	Bleed and boost, 0.5 g pig caudate in saline (IV) (s.c.).
1/13/65	Bleed and boost, 1.0 g pig caudate in saline (IV) (s.c.).
2/9/65	Bleed and boost, 3.0 g pig caudate in FCA (IM).
2/17/65	Bleed and boost, 2.5 g pig caudate, 2.0 ml mouse serum* in FCA (s.c.).
3/1/65	Bleed and boost, 1.0 g pig caudate, 1.0 ml mouse serum* in FCA (s.c.).
3/2/65	Animal weak, often falling to ground has tonic, clonic movements of all four extremities.
3/5/65	Animal sacrificed by exsanguination.

Serum from final bleeding was used in experiments.

*It was attempted to use the "Schlepper Function" of Landsteiner.

Immunization Schedule

Rabbit 23

Date	Event
4/27/65	Injected with 350 mg of perfused cat hippocampus homogenate in Freund complete adjuvant (FCA). Total volume 1.5 ml. 0.15 ml injected into one toe pad on each food, remainder injected into multiple sites on back intradermally.
5/14/65	Boosted with 350 mg hippocampus in saline (s.c.).
7/1/65	Boosted with 350 mg hippocampus in FCA (s.c.).
7/12/65	Test bleed, negative.
7/14/65	Boosted with 350 mg hippocampus in FCA (s.c.).
* 7/27/65	Bleed, positive.
8/13/65	Bleed
10/19/65	Bleed
*10/28/65	Bleed
*11/2/65	Bleed

*These sera were used for experiments reported.

Immunization Schedule

Rabbit 25

Date	Event
4/29/65	Injected 500 mg perfused cat caudate homogenate in equal amounts of Freund complete adjuvant. Total volume 1.5 ml. 0.15 ml injected into one toe pad on each foot. Remainder into multiple sites on back intradermally.
5/14/65	Boosted with 500 mg cat caudate in saline, subcutaneously.
7/1/65	Test bleed, negative.
7/2/65	Boosted with 500 mg cat caudate in FCA (s.c.).
7/12/65	Test bleed, negative.
7/14/65	Boost with 250 mg cat caudate in FCA (s.c.).
* 7/26/65	Bleed, positive.
8/13/65	Bleed
10/19/65	Bleed and boosted (no data).
*10/28/65	Bleed
*11/4/65	Bleed
12/14/65	Bleed
1/5/66	Boosted with 100 mg cat caudate IV, and 100 mg (s.c.) with FCA.
1/10/66	Bleed

*These sera were used for experiments reported.

Ouchterlony Double Immunodiffusion

"Immunoplates," patterns A and C were obtained from Hyland

Laboratories (Los Angeles, California). These micro agar-diffusion

plates contained one central well and six equidistant peripheral wells

with a center-to-center distance between central and peripheral wells

of 4 mm, 5 mm and 7 mm. The 4 and 6 mm distances were usually optimal

for maximum resolution; many lines did not appear at 7 mm separation.

Antigens and antibody were put into the wells with micropipettes

drawn from melting point capillary tubes. Plates were stored overnight

in a moist chamber and examined the next morning for lines of antigen-

antibody precipitation.

When lines were faint, concentration of the antigen by dialysis

against Carbowax flakes or 30 per cent solution (Union Carbide) often

served to intensity the reaction. A concentration of two to five times

was regularly achieved but because of the small volume and high viscosity

of the concentrate, accurate measurements were not possible.

In early experiments with Rabbit 33, Ouchterlony plates were

visualized in the oblique light of a fluorescent desk lamp and

photography was not possible. Following the construction of a darkfield

illumination all results were photographed.

Visualization of Ouchterlony Lines--Darkfield Illumination

A major problem in the use of the Ouchterlony double diffusion

technique in agar has been the lack of an appropriate optical system

which would allow accurate recording of even the faintest precipitin

bands in their nature unstained state. Workers have attempted to solve

this problem by various methods of direct illumination of the agar plate, none of which have been very successful. It is only with oblique or "darkfield" illumination that the almost invisible bands of the precipitin reaction are visualized against the colorless agar. The illuminator employed in these experiments was a refinement of a prototype conceived by Dr. Nobuya Ohtoma working in the laboratory of Dr. Henry Treffers.

The illuminator is housed within a square box 14 x 14 x 8 inches. A round aperture 4 1/2 inches in diameter is present in the center of its hinged lid. Aperture inserts were designed to accommodate Immunoplates and microscope slides. The light source is a commercially available 8 inch circular fluorescent tube which supports an overlapping 45 degree truncated aluminum cone. In the central axis of the box, an adjustable baffle has been placed whose function is to block all direct illumination from appearing at the aperture. With these conditions, the only light reaching the aperture is oblique.

Photography

Photographic recording of results was obtained with a Bessler 4 x 5 enlarger equipped with a 75 mm Polaroid lens in combination with a close up lens, (Kodak plus 3, 5, 10). This enlarger was adapted to accommodate the Polaroid 4 x 5 film holder; type P/N 55 film was used with its advantage of a negative which could be stored for later reproduction of results.

Chapter V. Testing of Sera

I. Experiments with Unperfused Brain Antigens

When unperfused brain regions were used as antigen according to the techniques of Mihailovic and Jankovic, the resulting antisera reacted strongly with the immunizing antigen in many different lines in the Ouchterlony plate. The strongest reaction was always seen with serum from the antigen donor, and the continuity of these reaction lines identified serum as the major contaminant in all preparations of regional brain antigens. In addition to serum lines, one to three other continuous lines were found only with brain tissue antigens. These lines were found to be continuous between species, (i.e. pig and cat, rabbit and cat) identifying the antibrain antibody as organ specific. Occasionally, in well-resolved Ouchterlony plates an additional line precipitated with caudate, but not with hippocampus, callosum, or any other antigen tested, however, this observation was not routinely reproducible. Variations in antigen concentration were also observed to alter the number of lines of visible precipitation.

A. Rabbit 33--Immunized with unperfused cat caudate.

Rabbit 33 had antibodies to cat caudate, hippocampus, superior and inferior colliculus, spinal cord, kidney and serum. It also reacted with white and grey antigens from the brains of rat, rabbit, pig and monkey.
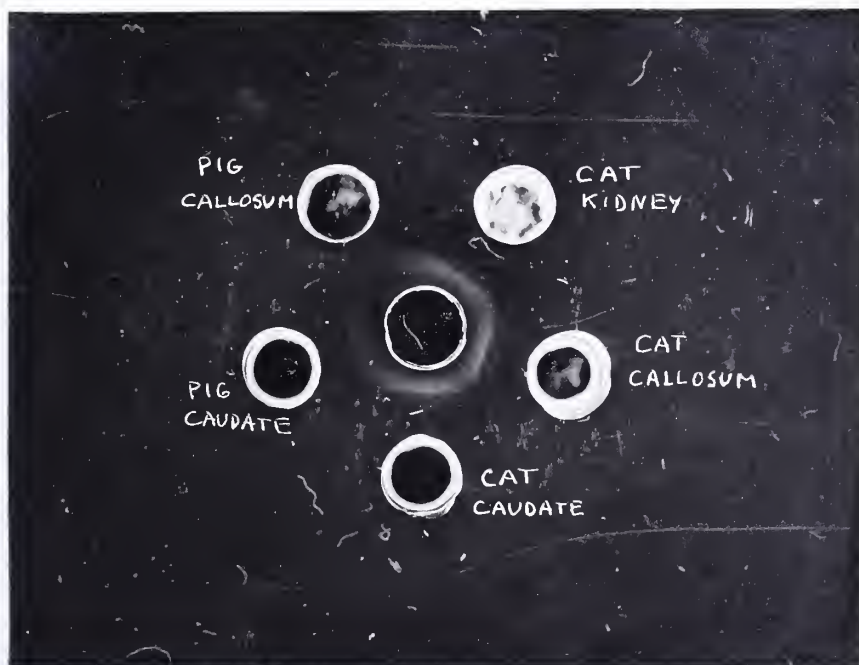
Figure I

Figure I demonstrates some reactions of R 33. There are definitely two lines shared by the 3 cat antigens and questionably, by pig caudate. An additional line with cat caudate and cat callosum are faintly visible. There is no visible reaction with pig callosum. Most of the reactions of R 33 have not been photographed.

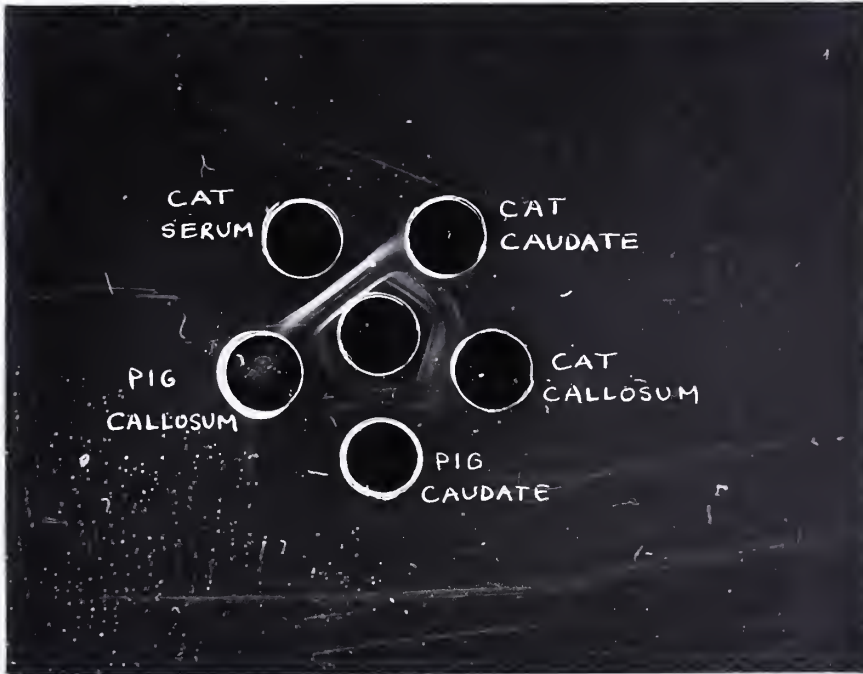B. Sheep 209-210--Immunized with unperfused cat caudate.

Figure II

Figure II demonstrates the significant antibody response of the sheep

to the small amounts of cat serum contained in the immunizing antigens.

It also shows the presence of cat serum proteins in the cat caudate

and cat callosum antigens. A system of two antibrain antibodies

also appears to be present in both cat and pig antigens. There is an

additional sharp line (innermost) opposite the cat brain antigens

which may be a brain antigen or cat tissue antigen.

The problem of interpreting the Ouchterlony plates when antigens are serum contaminated is illustrated in Figures III and IV. In Figure III the four lines with antiserum and hippocampus A each cross the cat serum line. Therefore, each of these lines must be interpreted as representing an antigen-antibody reaction different from any of the many cat serum protein-anti cat serum protein reactions.

Figure III

However, when the reagents were rearranged as in Figure IV, six lines with serum appeared, each looking suspiciously similar to the five and four lines obtained with caudate and hippocampus

respectively. Reliable information could not be obtained from such inconsistent Ouchterlony plates.

Figure IV

The observation that the number of lines obtainable with a regional brain antigen decrease with storage is demonstrated in Figure III. The A and B suspensions of cat caudate and hippocampus were both made at 20 per cent w/v. Suspension B was one month old, A was freshly prepared for the test.

C. Sheep 5-6--Immunized with unperfused pig caudate.

The use of pig caudate for antigen was undertaken because of
the abundance of fresh pig brains in New Haven. Since all pig
brains used were grossly bloody it was not unexpected that the
major precipitating activity of the sera was against pig serum
(Fig. V). However, strong antibrain antibody was evident after
the antisera was absorbed with pig serum (Fig. VI). Although it
does not show well, pig caudate and pig callosum were found to
share three antigens in common, each different from pig serum. The
sheep serum was also observed to react with other brain antigens, cat
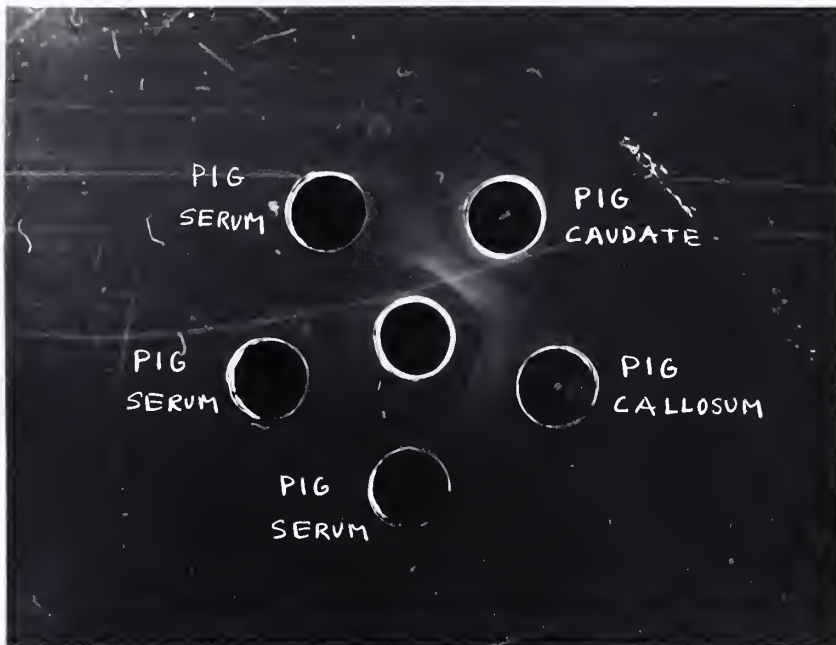caudate and callosum.
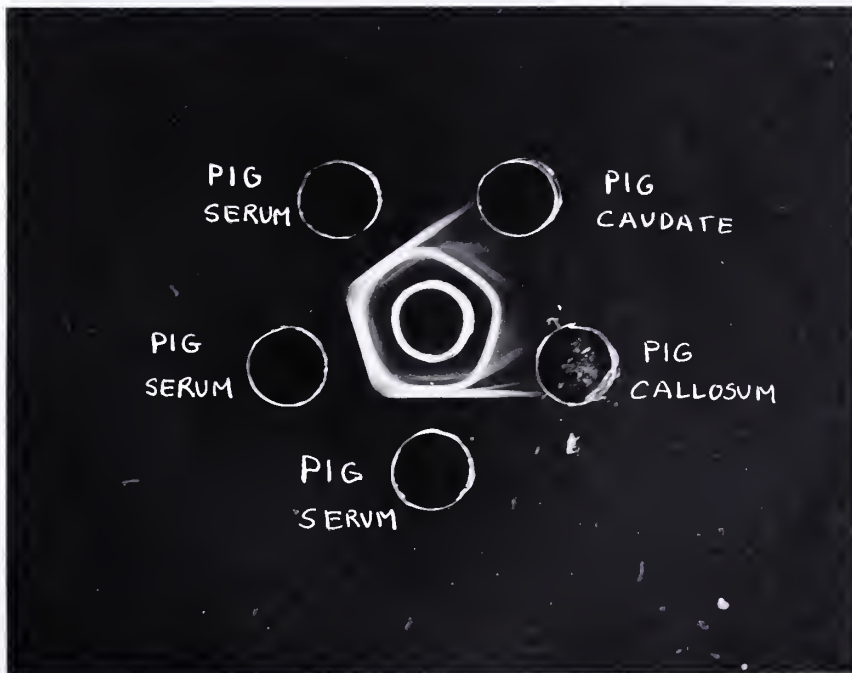
Figure V

Figure VI

Figure VI

II. The Problem of Serum Contamination

The massive antibody response of rabbit and sheep to small amounts of serum protein in the brain antigens quickly became the limiting factor in the interpretation of Ouchterlony data. Since all brain antigens used for immunization contained highly antigenic serum proteins, there was no doubt that along with anti-brain antibodies, anti-serum antibodies were being produced. Moreover, since these contaminated brain antigens were also being used for testing, except for absorptions (see Figure VI), it became impossible to positively identify a line as belonging to the brain or serum system. The solution to this problem was to eliminate as much serum protein as possible from the brain antigens. Accordingly in the following experiments cat brains were saline perfused, and tested for the presence of serum, before their use as immunizing or test antigens.

III. Experiments with Saline-Perfused Brain Antigens

In these experiments rabbits were immunized with essentially serum-free, saline-perfused, cat caudate and cat hippocampus. The change to perfused antigens resulted in the absence of precipitating anti-cat serum activity in the early rabbit bleedings, and only one or two faint lines after many booster injections. At the same time, one, and sometimes two, concentric lines of identity precipitated with all the cat brain regions. No difference was seen between the sera resulting from caudate or hippocamus injections.

A. <u>Rabbit 23</u>--Immunized with perfused cat hippocampus.

B. <u>Rabbit 25</u>--Immunized with perfused cat caudate.
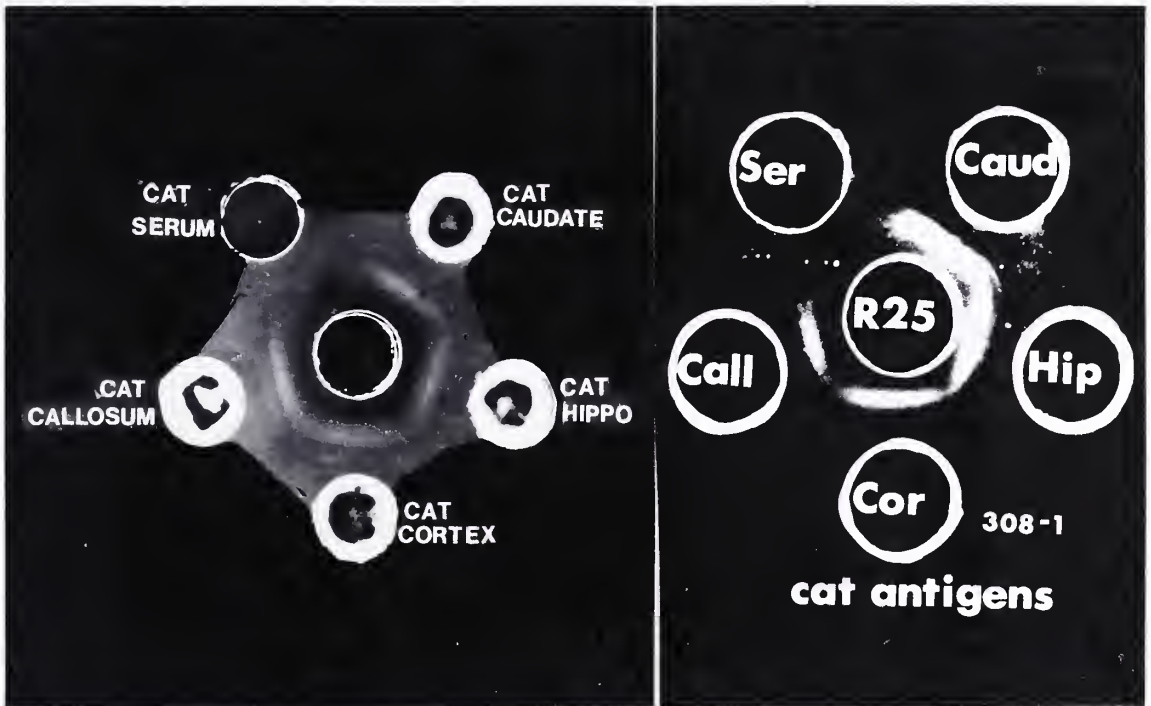

<u>Figure VII--Rabbit 23</u>   <u>Figure VIII--Rabbit 25</u>

There was no difference between the sera of the two rabbits. Both
reacted in two concentric lines with all cat brain antigens.
Significantly, the usual lines with cat serum were absent. Cat
caudate and cat hippocampus were not distinguishable.

Chapter VI. <u>Conclusion</u>

Mihailovic and Jankovic reported on the production of antibody
to the cat caudate and cat hippocampus <u>specific</u> in its ability
to extinguish the EEG activity and to increase the histamine-like
content in the homologous brain region. Although "two to three
lines" in immunodiffusion were reported when cat caudate was
tested with its antiserum, no other brain regions were tested, so
it was not known if <u>precipitating specificity</u> was actually obtained.

In this thesis antibody was first prepared without perfusion
of brain tissue. Sera manufactured in this manner contained high
titer antibodies to the traces of serum proteins which were unavoidably
present in the injections of brain homogenates. Moreover, when these
brain homogenates were used for Ouchterlony testing, at least
two separate antigen-antibody systems were found to be operating:
serum protein- anti serum protein, and brain-anti brain. It was only
with exhaustive absorption of the anti serum-protein activity,
(which was done with Sheep 5-6), that other non-serum-protein reactions
could be definitely uncovered.

Because of this complication, antisera was prepared and tested
using saline-perfused brain antigens. This new sera no longer reacted
with serum proteins, but neither did it demonstrate any caudate or
hippocampus specificity. The antibody that was produced reacted
equally well with all parts of the cat brain: caudate nucleus, hippocampus,
corpus callosum, and cortex, in at least two continuous precipitation
lines.

It has not been possible to demonstrate specific precipitating antibody to unique water-soluble, gel-diffusible substances of the caudate nucleus. Ouchterlony tests were not able to distinguish the caudate nucleus from any other brain region with antibody. Whether other species of antibody will harbor the claimed EEG and histamine-increase specificity remains to be tested, although to date, workers have failed to demonstrate caudate uniqueness with complement fixation or the fluorescent antibody technique (126).

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