

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

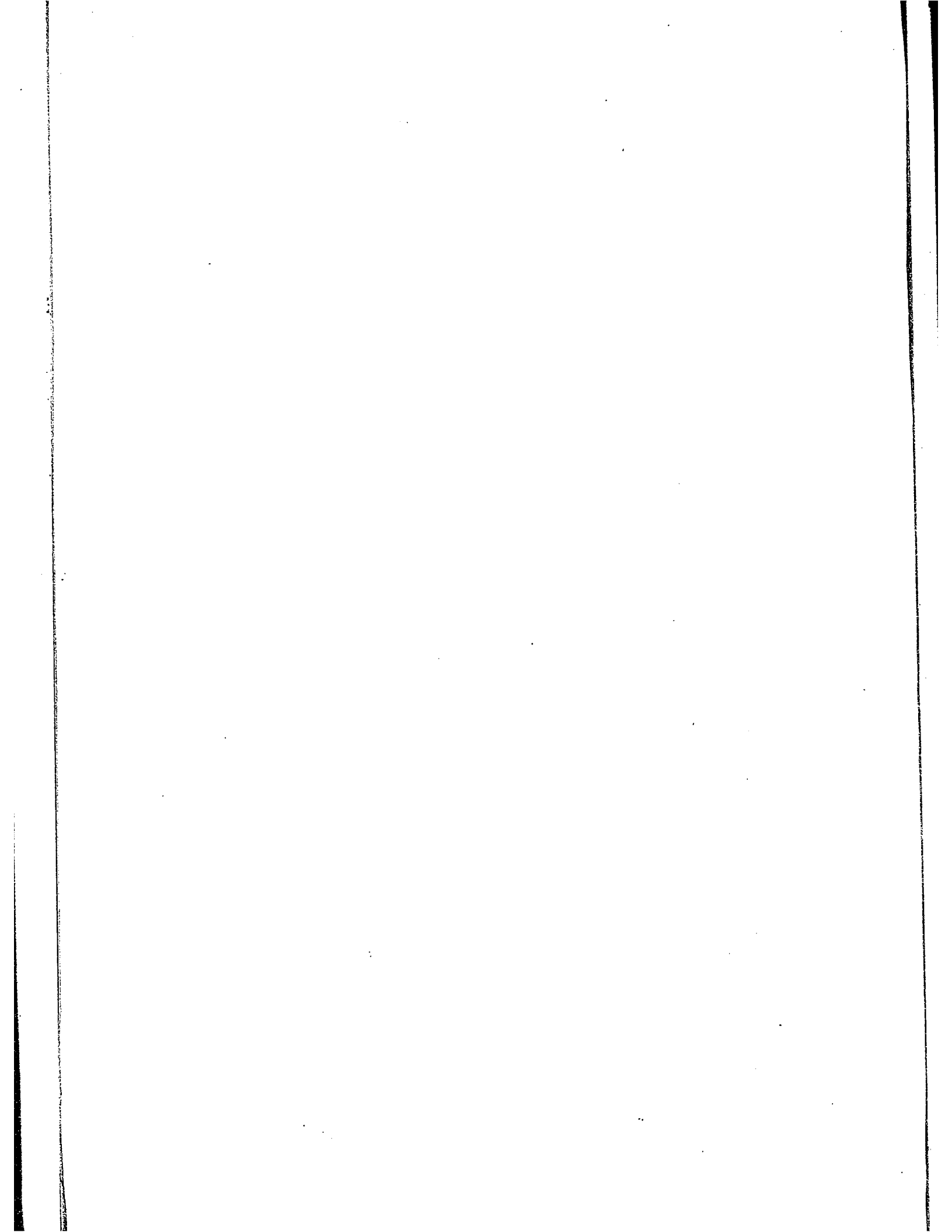
The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI[®]



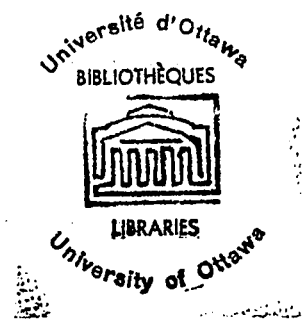
HS

STUDIES ON THE BRAIN SALIVARY GLANDS
AND SALIVA OF MICE EXPERIMENTALLY INFECTED
WITH STREET MABIES VIRUS

by

Michel Beaugregard

A Thesis



Presented to the
Ottawa University in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

March 1964

UMI Number: EC52211

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI[®]

UMI Microform EC52211
Copyright 2007 by ProQuest LLC
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

TABLE OF CONTENTS

	<u>Page</u>
<u>ABSTRACT</u>	1
<u>INTRODUCTION</u>	3
<u>LITERATURE REVIEW</u>	5
<u>HISTORY</u>	5
<u>GEOGRAPHICAL DISTRIBUTION</u>	6
<u>SEASONAL PREVALENCE</u>	11
<u>ETIOLOGY</u>	12
<u>SUSCEPTIBILITY AND TRANSMISSION</u>	13
<u>INCUBATION PERIOD</u>	14
<u>CLINICAL COURSE</u>	14
<u>PUBLIC HEALTH SIGNIFICANCE</u>	17
<u>VETERINARY SIGNIFICANCE</u>	25
<u>SUSCEPTIBILITY OF VARIOUS ANIMAL SPECIES</u>	26
<u>SYMPTOMLESS CARRIERS OF RABIES</u>	27
<u>LABORATORY METHODS FOR THE STUDY OF RABIES</u>	29
<u>EXPERIMENTAL STUDIES</u>	52
<u>MATERIALS AND METHODS</u>	53
<u>Strain of Street Rabies Virus</u>	53
<u>Inoculation of Experimental Animals</u>	55
<u>Collection and Preservation of Tissues</u>	56
<u>Microscopic Examination of Tissues by Standard</u> <u>Impression Smear Technique</u>	58
<u>Examination of Tissues for Evidence of</u> <u>Infectivity for Mice</u>	58
<u>Microscopic Examination of Tissues by</u> <u>Fluorescent Antibody Technique</u>	60
<u>Field Specimens</u>	66
<u>RESULTS</u>	67
<u>Infection by the Intra-Cranial Route</u>	67
<u>Infection by the Intra-Muscular Route</u>	73
<u>Field Specimens</u>	82
Domestic Animals	82
Wild Animals	89
<u>DISCUSSION</u>	92
<u>SUMMARY</u>	100
<u>REFERENCES</u>	101
<u>ACKNOWLEDGMENTS</u>	115

LIST OF TABLES

<u>TABLE</u>		<u>Page</u>
I	Results of examinations of brains from mice inoculated by the intra-cranial route	69
II	Results of examinations of salivary glands from mice inoculated by the intra-cranial route	71
III	Results of examination of saliva from mice inoculated by the intra-cranial route	72
IV	Results of examinations of brains from mice inoculated by the intra-muscular route	74
V	Results of examinations of salivary glands from mice inoculated by the intra-muscular route	73
VI	Results of examination of saliva from mice inoculated by the intra-muscular route	81
VII	Results of examinations of field brain specimens from domestic animals	83
VIII	Results of examinations of field brain specimens from wild animals	90

LIST OF ILLUSTRATIONS

<u>PLATE</u>		<u>Page</u>
1	Intra-cranial inoculation of an experimental mouse	116
2	Experimental mice one day after intra-cranial inoculation	117
3	Experimental mice 11 days after intra-cranial inoculation	118
4	Brain of an experimental mouse from which the right hippocampus has been dissected and is shown in the upper right quarter	119
5	Experimental mouse whose sub-maxillary salivary glands have been exposed for dissection.	120
6	Preparation of impression smears from the salivary gland of an experimental mouse	121
7	Williams' stained impression smear of a Negri-negative salivary gland from an experimental mouse. 700X.	122
8	Williams' stained impression smear of a Negri-negative hippocampus from an experimental mouse. 700X.	123
9	Williams' stained impression smear of the hippocampus from a rabid experimental mouse. Negri bodies are indicated by arrows. 700X.	124
10	Williams' stained impression smear of the hippocampus from a rabid bovine field specimen. Negri bodies are indicated by arrows. 700X.	125
11	Williams' stained impression smear of the hippocampus from a dog field specimen showing distemper inclusions, as indicated by arrows. 700X.	126
12	Negri bodies and dust particles as shown by the fluorescent antibody technique in the hippocampal tissue of a rabid experimental mouse. 400X.	127
13	Negri bodies and dust particles as shown by the fluorescent antibody technique in the brain tissue of a rabid skunk field specimen. 400X. ...	128

ABSTRACT

The objective of this investigation was to compare the sensitivity of the intra-cranial and intra-muscular routes of inoculation of street rabies virus. This was done by determining the time of appearance of Negri bodies and/or infectivity in the brain and salivary glands of experimentally infected Swiss white mice. Individual groups of five mice inoculated by either of these routes were sacrificed at daily intervals until they died of the infection. Brain and salivary glands from each mouse were examined by three methods: a) standard impression smear technique, b) mouse infectivity test, and c) fluorescent antibody technique. When obtainable, saliva samples were also examined by the mouse infectivity test.

Out of 60 mice injected by the intra-cranial route, 38 showed Negri bodies when brains were examined by the standard impression smear technique; in one mouse they were detected as early as the fifth post-inoculation day. The salivary glands failed to show Negriogenesis when examined by this method. The presence of infectivity was demonstrated in the brain of 43 mice and preceded by one day the appearance of Negri bodies. Only three mice were found to excrete virus in salivary glands: the first on the eighth, the second on the ninth and the third on the tenth post-inoculation day. Saliva from the second of these three mice proved infectious. Rabies viral antigen was revealed by the fluorescent antibody technique in the brain of all five mice sacrificed on the second post-inoculation day, in that of two of the five killed on the third post-inoculation day and in that of every mouse thereafter. This method, like the direct smear examination, did not detect evidence of infection in any of the salivary glands.

The lower sensitivity of the intra-muscular route of injection was evidenced by the late appearance and the marked irregularity of both lesions and infectivity in the 125 mice thus inoculated. Only 19 developed Negriogenesis in the brain; the earliest by the 10th post-inoculation day. Thirty-three mouse brains proved to harbour virus by the mouse infectivity test. The appearance of infectivity did not precede that of Negri bodies in the brain tissue. Only four mice excreted virus in salivary glands. Two saliva samples exhibited infectivity. The fluorescent antibody technique detected rabies viral antigen in 16 Negri-positive and 20 Negri-negative brains, seven of the latter being also non-infective for mice. Four brains found to contain virus were missed by this technique, although three of the four disclosed Negriogenesis. Positive results were obtained by the fluorescent antibody technique with all mouse-infective salivary glands.

Among field brain specimens from 100 domestic and wild animals, 11 revealed evidence of infection by the standard impression smear technique, 54 by the mouse infectivity test and 50 by the fluorescent antibody technique.

The results of these studies indicate that the intra-cranial route of inoculation is more sensitive than the intra-muscular one for the rapid detection of street rabies virus infection in Swiss white mice. Brain also proved to be the most satisfactory tissue for this purpose. Of the methods employed, the standard impression smear technique was found the least sensitive. In contrast to what was observed in experimental mice, the mouse infectivity test appeared more sensitive than the fluorescent antibody technique for the examination of field brain specimens.

STUDIES ON THE BRAIN SALIVARY GLANDS
AND SALIVA OF WICE EXPERIMENTALLY INFECTED
WITH STREET RABIES VIRUS

INTRODUCTION

"Rabies has been known in animals and man for centuries, and its present character appears unchanged from that described in early Greek and Roman Times" (1). However, the etiology and prophylaxis of the disease remained a mystery until the end of the 19th century. In 1881, Pasteur et al (2) proved that the causative agent of the disease is present in the central nervous system in a pure and concentrated form. A few years later, Pasteur (3) reported the finding of a method for the attenuation of the etiological agent of rabies. With this discovery, the problem of practical immunization against the disease appeared solved at last.

In 1903, it was shown by Reulinger and Riffat-Bey (4) that the causative agent of rabies can pass through a Berkefeld V filter. In the same year, Negri (5) demonstrated the presence of specific cellular inclusions in the central nervous system of rabid animals. These inclusions, called "Negri bodies" in commemoration of their discoverer, have considerably facilitated the post-mortem identification of the disease.

In Canada, the diagnosis of rabies in animals has become a responsibility assumed by veterinary laboratories. The susceptibility of man to rabies calls for a close collaboration between veterinarians and medical health officers. It also necessitates the application of adequate and rapid methods of diagnosis to avoid delay in the

treatment of persons exposed to suspected animals. When Negri bodies are found in the brain of such animals, a positive diagnosis of rabies can be issued immediately from the laboratory, and appropriate prophylactic measures undertaken (6). If these specific lesions cannot be demonstrated, laboratory animals are inoculated as a further means of finding out if the suspected animal was harbouring the virus. It has been observed by Negri-Luzziani (6), Leach (7), Damon and Sellers (8), Johnson (9) and other workers that approximately 5 to 15 per cent of the cases submitted for laboratory diagnosis may prove to be "positive" on animal inoculation, although microscopic examination of the brain had failed to reveal the presence of Negri bodies. Our personal observations are in general agreement with those mentioned above. During the last five years, 9.1 per cent of the specimens submitted to the mouse inoculation test following a negative microscopic examination were found to be infected with rabies virus.

Many factors have to be considered when determining which laboratory animal is best suited for experimental inoculation purposes. First of all, this species must be very susceptible to rabies. It must also be readily available in large quantities, economical to keep, easy and safe to handle, and require minimal housing space so that several animals may be used for one specimen. Since all these requirements are best fulfilled by Swiss white mice, they were proposed as experimental animals by Webster and Dawson (10), following their success in obtaining positive results as early as seven days after intra-cranial inoculation of street rabies virus in this line of mice.

At the Animal Diseases Research Institute, Hull, P.C., Swiss white mice are also used as experimental animals when virus isolation has to be attempted from the brain tissue of suspected animals in which Negri bodies cannot be demonstrated. Therefore, it appeared to be of interest to carry out studies on brain and salivary gland tissues of mice experimentally infected with street rabies virus by the intra-cranial and intra-muscular routes. The two main objectives of these studies were to determine the time of infectivity of these tissues and the time of appearance of Negri bodies in the same tissues. The infectivity of saliva was also investigated in order to obtain information on the possible hazard involved in the handling of those animals by laboratory personnel. The following methods of study were used: (a) microscopic examination of impression smears by the standard method, (b) mouse inoculation test, and (c) microscopic examination of impression smears by the fluorescent antibody technique.

LITERATURE REVIEW

HISTORY

Webb (11) states in his resume of the history of rabies:

"It is mentioned by Plutarch who asserts that, according to Athenodorus, it was first observed in mankind in the days of the Asclepiadae, the descendants of Aesculapius the god of medicine. The disease was known to Homer, and was alluded to by Hippocrates and Democritus. Allusions to it are also found in the works of Virgil, Horace and Ovid.

The disease was described by Aristotle in the Fourth Century B.C., who writes: 'Dogs suffer from madness, which puts them in a state of fury, and all the animals that they bite when in this condition become also attacked with rabies'. In the first part of the Christian era, Cornelius Celsus referred to human rabies and employed the term hydrophobia."

According to Kelsner (12), in the first century A.D., Celsus stated that the disease in humans is caused by a bite of a rabid animal. Therefore the wound must be thoroughly washed with water and burned with a hot iron to prevent the development of the disease, for after the symptoms develop there is no cure and death always follows. Galen (A.D. 200), favoured surgical resection of the wound area, as mentioned by Johnson (13).

However, up to the end of the last century, the exact cause of rabies was unknown and many people believed in the spontaneous development of the disease. In fact, it was supposed that hot food, lack of drinking water, unsatisfied sexual desire and violent nervous excitement might cause rabies (14).

GEOGRAPHICAL DISTRIBUTION

According to Kelsner (12), rabies knows no geographic boundaries and its presence has been reported from the Arctic regions to the Tropics. However, Australia, Hawaii and Panama have been kept free of the disease. England is also free of infection at present and has been so for considerable periods of time since 1902 owing to a strict system of quarantine.

As early as 1271, rabies was known to occur in western Europe being prevalent among wolves in France. Indeed, as Johnson (13) states, prior to the 18th century rabies was considered to be a disease of wild canines with domestic dogs playing no significant part in its maintenance and spread. The first recorded epizootic among domestic dogs in urban centers occurred in Italy during 1708. By 1728, the disease had appeared in epizootic proportions in most of the major cities of Hungary, Germany and France. Although rabies was known in England as early as 1613, it did not occur in epizootic proportions among dogs until 1734.

Soon it spread to America since the archives of the State of Virginia contain references to rabies in dogs as early as 1753, and those of North Carolina as early as 1762. Rabies appeared in skunks of lower California in 1826. Since that time, the United States has experienced other outbreaks in wild animals such as foxes, coyotes, striped and spotted skunks. Rabies was not recognized in South America until 1803 when it appeared among dogs in Peru and in 1806 among hunting dogs imported into La Plata, Argentina.

Rabies exists throughout southwest Asia. It is especially common in India where the more important vectors are the jackal, fox and wild dog, although mongooses and civets probably also constitute an important wildlife reservoir of the disease in that country and elsewhere in Asia. McKendrick (15) mentions that in Japan a very severe epizootic of canine rabies broke out in 1923 and lasted a few years. In 1908, Dudley (16) reported that canine rabies was of common occurrence in the Philippine Islands.

Hudson, quoted by Johnson (13), mentions that rabies has been present for a long time in Kenya and occurred in epizootic proportions among jackals in the period of 1912 to 1916. In South Africa, rabies appeared for the first time in 1892 among dogs from Port Elizabeth area. In 1933, Nicolau et al (17) identified as rabies a peculiar disease of dogs from West Africa known as "oulou-fato".

Plummer (18) states that in Canada rabies was first shown to occur in dogs in 1906. Two outbreaks were then noted: one in Alberta and Saskatchewan, the other in the Niagara peninsula of Ontario. During the period of 1926 to 1931 another epizootic of canine rabies occurred in the Eastern Townships of Quebec, the infection spreading to Montreal and Ottawa. From 1944 to 1947, canine rabies was present around Windsor, Ontario. In 1947, the infection was diagnosed for the first time in foxes, wolves and sled dogs in the Northwest Territories. By 1953, rabies had spread over the province of Alberta and then took an easterly direction, being disseminated by foxes and other wild animals. At the present time infection is still present in the southern part of Manitoba, Ontario, Quebec, and in the Northwest Territories.

Lassen (19) states that, for at least a hundred years, epizootics of a disease resembling rabies have raged among the sled dogs of Northern Greenland. It has been noted that very often the disease broke out a few days or weeks after fights between dogs and "mad" polar foxes. Ferenbaugh, quoted by Johnson (13), states that rabies was known to be epizootic among foxes in Alaska as early as 1915. Chadwick (20) reports that rabies was identified in dogs and foxes of Alaska in 1956.

The transmission of the disease by bats was first recognized approximately 50 years ago when Carini (21), in 1911, identified as rabies a paralytic disease of cattle and other livestock, called "mal de caderas", which was then prevalent in the southern Brazilian state of Santa Catharina. He suspected bats as the possible vector, since some of these nocturnal animals had been seen attacking cattle in broad daylight. As mentioned by Pawan (22), this was verified in 1916 by Haupt and Nehaag who succeeded in producing rabies in a rabbit and a guinea-pig with brain material from a frugivorous bat of the genus *Phyllostoma* which had been caught biting at a bovine in South Brazil. In 1925, a disease of livestock, characterized by paralysis, made its appearance in Trinidad and was identified as rabies by Metivier (23). A few years later, Hurst and Pawan (24) established that an acute ascending myelitis of the Landry's type which had first appeared in humans in Trinidad in 1929 was rabies. There was strong evidence that the vector of the disease in both humans and livestock was the vampire bat, since canine rabies had disappeared from the Island after 1912. In 1936, Torres and Queiroz Lima, as mentioned by Williams (25), proved conclusively that bovine rabies in Brazil was carried by blood-lapping Chiroptera, especially by *Desmodus rotundus*, the most prevalent species of the family *Desmodontidae*. These results were confirmed by Pawan (22) in Trinidad, who incriminated the same vector in paralytic rabies affecting humans. Other investigations soon demonstrated similar situations occurring in Argentina, Paraguay, British Guiana, Venezuela, Central America and Mexico (26).

Tierkel (27) mentions that vampire bats are found from northern Argentina and southern Brazil northward to about 100 miles south of the Rio Grande river in Mexico. These bats are not known to occur in the United States or in the eastern hemisphere. Williams (25) states that the family Desmodontidae comprises three genera, each with a single species: (a) Desmodus rotundus, the common vampire bat; (b) Dicoemus youngi, the white-spotted vampire; and (c) Diphylla ecaudata, the hairy-legged vampire. Only the first two occur in Trinidad. These three bats are sanguivorous or hematophagous in their feeding habits and may inadvertently transmit paralytic rabies. Desmodus bats usually live in groups or colonies in roosts which may be caves, hollow trees, culverts and the like. He also states that vampire bats have been known in Trinidad for over 100 years, although it is uncertain for how long they have been infected with rabies. However, he quotes the following statement from de Verteull's history of Trinidad, which was first published in 1856: "The loss of blood from numerous or repeated biting is, at times, so great, that large animals, such as oxen, become immediately enfeebled and may die within two to three weeks; this, however, happens only at intervals of several years, when great loss in livestock is occasioned to proprietors of estates".

The natural occurrence of rabies in insectivorous bats was reported for the first time from the island of Trinidad by Pawan (22) in 1936. Seventeen years later, Venters et al (28) isolated the virus of rabies from an insectivorous yellow bat (Dasypterus floridanus) which had bitten a child near Tampa, Florida. Since then over 600 cases of bat rabies have been reported from 39 states, involving 25 of the 36 insectivorous species

distributed throughout the United States (29, 30, 31). The greatest number of virus isolations have been made from the Mexican free-tailed bat (Tadarida brasiliensis mexicana) in the southwestern United States (32). In 1954, Veeraraghavan, quoted by Burke-Gaffney (33), reported from India the first case of hydrophobia following a bat bite. In Yugoslavia, Nikolic and Jelesic (34) isolated the rabies virus from three insectivorous bats of the Nyctalus noctula species in 1956. A few years ago, rabies was also diagnosed in insectivorous bats of Turkey (27). In Canada, rabies was reported for the first time in insectivorous bats in 1957 when Avery and Tallyour (35) isolated the rabies virus from a big brown bat (Eptesicus fuscus) and a small brown bat (Myotis lucifugus) in British Columbia. In 1958, they diagnosed rabies in another big brown bat and in a silver-haired bat (Lasiurus noctivagans) from the same province. In 1960, they found two big brown bats infected with rabies, again in British Columbia. Beauregard and Stewart (36) have isolated the virus of rabies from five big brown bats of the province of Ontario, since 1961.

SEASONAL PREVALENCE

According to Kelser (12), the old idea that rabies is a summer disease was based on the fact that during the months of July and August the dog-star Sirius rises with the sun. In ancient times this was believed to be the cause of dogs going mad; hence these two months were called "Dog Days". One can now understand how this erroneous association was based on the fact that dogs have much greater freedom in summer than in winter, and thus more facility for contact with each other, other

animals and man. In Canada, for example, where rabies is spread by wild animals such as foxes and skunks, the peak of infection takes place annually from early fall through late winter.

ETIOLOGY

Rabies is an infectious disease caused by a filterable virus which has a predilection for nervous tissue (27). Nicolau et al (37) state that the virus of rabies is strictly neurotropic, spreading along the nervous axis in a centripetal direction when introduced peripherally. On the other hand, the introduction of the rabies virus in the organism through the intra-cranial route results in the centrifugal diffusion of the virus again along the nervous axis. Fagan (38) states that the virus of rabies is about 125-150 mu. in diameter, which lies about halfway between the smallest viruses such as that of foot-and-mouth disease and the largest viruses of the psittacosis group. According to Lepine (39), the virus of rabies is very resistant to cold, dessication and glycerol, and somewhat resistant to alcohol, ether and acetone. It is destroyed by heating for 15 minutes at 50°C., for five minutes at 60°C., or for two minutes at 100°C. It is inactivated by ultra-violet rays, proteolytic enzymes and antiseptics such as phenol and formol.

Pasteur et al (40, 41) designated as "street rabies virus" the various strains of the agent isolated from field cases of the disease in dogs. On the other hand, they used the term "fixed rabies virus" to indicate the particular strains of rabies virus obtained following repeated intra-cerebral passages of the "street rabies virus" in the same species of animals, especially the rabbit. The characteristics of the fixed

virus are summarized by Havens and Hayfield (42) who state that rabies virus adapted to the rabbit by a long series of passages differs in several respects from the strains found in dogs and other animals in nature. Not only is it characterized by a uniform period of incubation, as contrasted with the wide variability of street viruses in this respect, but it is said to be more neurotropic, infection occurring less regularly following injection elsewhere than directly into the central nervous system. Another difference is the rarity or complete absence of Negri bodies. Even the occasional ones seen are not typical, being smaller and containing fewer basophilic granules than the characteristic ones found in the brains of rabid dogs. Furthermore, these workers have observed that street viruses are more antigenic than fixed viruses.

SUSCEPTIBILITY AND TRANSMISSION

According to Mohler (43), all warm-blooded animals including man are susceptible to rabies infection, the disease usually being transmitted from animal to animal or to man through bites of rabid animals excreting the virus in their saliva. Remlinger (44, 45) points out that rabies may also develop from the contamination of open wounds, abraded mucous membranes and even apparently intact mucous membranes by virulent saliva. Evidence of transplacental transmission of rabies in dogs, guinea-pigs and rabbits was also demonstrated by Konradi (46), and identical findings were obtained in insectivorous bats by Sims et al (47). Constantine (48) considered

aerosols as the probable mechanism of rabies transmission to experimental carnivores in Frio Cave, Texas. On the other hand, ticks were found by Bell et al (49) to be of no significance in the transmission of rabies.

INCUBATION PERIOD

Stimson (50) states that the incubation period of rabies is remarkable for its length and variability. It is seldom shorter than 10 days, but it may extend over many months, even a year or more. The length of the incubation period may be influenced by factors such as: the species of animal affected, the site of inoculation, the severity of the wound, certain conditions which tend to weaken the resistance of the nervous system, the treatment and any variations in the virulence of different strains of street virus. Stimson quotes Bauer's observations on 537 cases of human rabies in which the incubation period varied from less than 20 days to a little more than a year, with an average of 72 days. He also lists the average incubation period in domestic animals, as given by Friedberger and Frohner: cattle and horse 4-8 weeks, cat 2-4 weeks, pig 2-3 weeks, sheep 3-4 weeks, poultry 6 weeks to 11 months.

CLINICAL COURSE

As mentioned by Gamaleia (51), the two clinical forms of rabies, that is the furious and the paralytic forms, were known to occur in animals and man at the time of Pasteur's experimental work on the disease. Gibbons (52) divides the symptomatology of rabies into three phases: 1- a premonitory stage, 2- a stage of excitement, and 3- a paralytic stage. Sagan and Bruner (53) note that domestic and wild

carnivorous animals affected with rabies first exhibit vague temperament changes, which are followed in furious rabies by the uttering of strange cries and howls and by the development of a very dangerous ferocity. This corresponds to the excitation stage in which affected animals may travel considerable distances, biting and snapping at anything that attracts their attention. If restrained, they will often chew metal chains or cage bars, which results in damages to their teeth and buccal mucosa with the appearance of blood in the drooling saliva. Sometimes these animals do not feel pain or display a perverted appetite. Finally, the paralytic stage appears and may last up to 48 hours before the animal dies. In paralytic or dumb rabies, paralysis usually appears first in the muscles of the head and neck. As a result, the animal cannot chew its food, swallow water, or does so with difficulty. The animal being unable to close its mouth, the lower jaw hangs down. Within a few hours evidence of paralysis develops in other parts of the body. This is soon followed by general paralysis and death.

According to Raymond (54), furious rabies is manifested in herbivorous animals by sexual excitement, greatly increased pugnacity and sometimes a tendency to bite. In the paralytic form, these animals are not aggressive at all. They only appear more dull and stupid than usual until paralysis sets in and is followed by recumbency and death.

Hemlinger and Bailly (55) consider as exceptional the occurrence of avian rabies under natural circumstances. However, they describe paralytic, furious and abortive experimental cases of the disease in chicken. Paralytic rabies in this species is characterized by feather

bristling, inappetence, impaired gait and progressive paralysis resulting in decubitus and death. In furious rabies birds exhibit agitation, convulsive seizures, tendency to attack other birds, animals and even man. They also display a change in the voice and inability to swallow liquids. The disease ends in general paralysis and death. Abortive forms of the disease mimic paralytic rabies, but they are characterized by uneventful recovery.

Johnson (13) states that in man the onset of rabies is marked by two to four days of prodromal symptoms such as intermittent fever, headache, malaise, shallow respirations, nausea and sore throat. The early symptom of most diagnostic significance is a painful sensation at the site of infection. The onset of the excitement phase is gradual and marked by increasing nervousness, insomia and apprehension. The outstanding clinical symptom of rabies consists of a marked difficulty in swallowing. The sight, smell or sound of liquids often precipitates spasms of the muscles of the throat, hence the term "hydrophobia". As the disease progresses, periods of intense excitement alternate with periods of quietness. Most often patients die in the acute excitement phase of the disease, during a convulsion. In other instances, death follows the terminal paralytic stage.

In paralytic rabies there may be constipation, urine retention or incontinence or abnormal stimulation of the sex organs. Difficulty in swallowing usually does not appear until the terminal phase of the disease. Consciousness is also retained until late in the course of the disease. Finally progressive paralysis appears and precedes death.

In addition to the various symptoms listed above, Remlinger (56) points out that glycosuria is of frequent occurrence in rabid carnivores and of constant occurrence in rabid herbivores. In experimental rabbits however it appears late in the disease. This glycosuria would result from subnormal utilization of sugar by the rabid animals. Sugar is abundant in the blood, and hypoglycogenia rather than hyperglycogenia goes on in the liver. Johnson (13) mentions that a reaction for glucose and acetone is given by the urine of most rabid persons.

Despite the irregular length of the incubation period of rabies, the clinical course of the disease is always relatively short, usually not more than a week or so (51, 53). A few reports such as those of Pasteur et al (57) and Remlinger (58) indicate possible recoveries in natural and experimental rabies in animals. Roder, quoted by Camaleira (51), stated that he knew of five unequivocal recoveries in human rabies. However, such cases are so rare that rabies can be considered as an invariably fatal disease. There is no specific treatment for the disease once the symptoms have developed (13).

PUBLIC HEALTH SIGNIFICANCE

Human rabies occurs in areas where the disease is prevalent in animals and is usually transmitted through bites of rabid animals. Butyra et al (14) mention that the virulence of the saliva of rabid dogs was first demonstrated by Zinke in 1804, and that of herbivorous animals by Berndt in 1822. It has been shown by Roux and Nocard (59) that infected dogs may excrete virus in the saliva at least three days before the appearance of any symptoms. Konradi (46) states that the bite of

a dog might be dangerous as early as 14 days before the appearance of the symptoms of rabies. Remlinger (60) demonstrated the virulence of the saliva of an experimental dog five days after its recovery. It was found by Bequignon et al (61, 62) that the diffusion of the rabies virus excreted in the saliva of rabid dogs is enhanced by an enzymatic factor similar to hyaluronidase, which is normally present in the saliva of dogs. The infectivity of saliva in rabies is attributed by Manouelian (63) to the escape of virus from the minute nerve cells of the salivary glands, tongue and mucous membrane of the mouth.

However, rabies virus is not always excreted in the saliva of rabid animals. Indeed, Ouchakoff, quoted by McKendrick (64), claims that the possibility of human infection following exposure to saliva of rabid herbivorous animals is less than one-sixtieth per cent.

Veeraraghavan et al (65) showed the presence of virus in the submaxillary glands of only 36 in a group of 61 rabid dogs. In the same study, these workers found that saliva may be virulent in both furious and dumb rabies, and in approximately the same percentage of cases. Through experimental investigation Sikes (66) demonstrated that only 10 of 24 rabid foxes, and 15 of 18 rabid skunks excreted virus in their saliva. Furthermore, not all animals with virus-positive salivary glands excreted detectable amounts of virus in their saliva.

According to Lepine (39), dogs are responsible for 86 per cent of the human cases of rabies, cats for five per cent, wild carnivores for 3.5 per cent and ruminants for two per cent. The same author points out that the likelihood of developing infection following bites by domestic and wild carnivores depends on the site and depth of the wounds as well as on the diffusion property of hyaluronidase which is

normally present in the saliva of these animals. Although wolves are responsible for only 0.1 per cent of all bites inflicted to humans, yet their severity is so great that it results in 6.8 per cent of the human rabies fatalities.

Fortunately human cases of rabies have been relatively rare in Canada. The first recorded fatality occurred in 1819 when the fourth Duke of Richmond died of hydrophobia after being bitten by his pet fox (67). Wells (68) lists 14 human fatalities due to rabies infection between 1925 and 1944 in Quebec, Ontario, Saskatchewan and Alberta. McLean et al (69) report that a seven-year old boy of Port Perry, Ontario, died of rabies in 1959, approximately six weeks after being bitten by a skunk. In the same year a man of Peterborough, Ontario, also died of the disease (70).

In some instances human rabies has been found to be an occupational disease. Ostertag, as mentioned by Mohler (43), records the case of a veterinary student at Copenhagen who infected a wound on his finger while making an autopsy on a rabid dog and died of the disease. Humphrey et al (71) mention that an entomologist and a consultant mining engineer died of rabies after working in Frio Cave, Texas, where bats are very numerous and known to be infected with the virus.

Hemlinger (56) says that rabies contamination from human to human is open to question. However, as mentioned by Meyer (72), Fermi states that the mother of Malpighi died of rabies in the 17th century after a bite inflicted by her daughter. Mohler (43) recalls the case of

a Cuban woman in the early stages of rabies who transmitted the disease to her nursing baby apparently through her milk.

The susceptibility of humans to rabies infection raises the problem of the disposition of meat and milk from infected animals. Bohler (43) considers that rabies cannot be transmitted to normal animals through virulent food unless lesions are present in the alimentary canal. He also mentions Wyrzykowski's findings that gastric juice has a deleterious effect upon the virus of rabies. However, Peral, quoted by Marie (73), succeeded experimentally in infecting rats and mice by the oral route at the rate of 78 and 42 per cent respectively. The infectivity of milk and mammary gland tissue of rabid dogs, guinea-pigs and rabbits was demonstrated by Henlinger and Bailly (74), and by Johnson (75). However, Nicolas (76) failed to isolate virus from either the milk or mammary gland tissue of herbivorous animals affected with clinical rabies or inoculated by the intra-venous route with virulent emulsions. In spite of this, Schoening (77) maintains that the milk from a rabid or suspected rabid cow should be condemned as unfit for consumption. With regard to the disposition of suspect meat, he quotes the recommendation of the International Rabies Conference, held in Paris in 1927: "Animals bitten by rabid animals, whether treated or not after the bite, should not be butchered between the eighth day and, at the very least, the end of the third month following the bite".

Leblanc, quoted by Courmont and Panisset (78), states that before the discovery of the Pasteurian treatment approximately 16 per cent of the persons bitten by rabid animals were dying of the disease.

Lepine and Cruveilhier (79) report that during the first 50 years following the introduction of anti-rabies vaccination, 51,057 persons were treated at the Pasteur Institute in Paris, of which only 151 or 0.29 per cent died of the disease.

According to Pasteur's original method (3), patients were given approximately 14 sub-cutaneous injections of vaccine in as many days. The vaccine consisted of an emulsion of dessicated spinal cord tissue from rabbits infected with fixed rabies virus. The segment of spinal cord used for the first injection had been previously dessicated for 14 days in dry air in large bottles containing caustic potash. The segment used for the second injection had been dessicated for 13 days, and so on until the last injection consisted of almost fresh material. This method was submitted to various modifications as described by Lepine and Cruveilhier (79), and Hagan and Bruner (53). In spite of this, Habel (80) still could write in 1957: "The currently used rabies vaccine has always been a crude biological product. This vaccine consists of a heavy suspension of rabies-infected rabbit brain in which the virus has been inactivated by various chemical or physical agents".

During or shortly after vaccination, some patients have developed neuro-paralytic symptoms. Remlinger (81) mentioned the occurrence of 40 cases, two of which were fatal, among 107,712 vaccinated people. He incriminated both the toxin contained in the vaccine and the idiosyncrasy of the people being immunized. The same author (82) also described the various clinical manifestations of these accidents which may appear as an acute ascending myelitis or Landry's type, a sub-acute

dorso-lumbar myelitis, attenuated myelites, characterized by paresis of the inferior extremities as well as transient urine retention, or nevretic affections of the facial musculature. Whereas approximately 30 per cent mortality occurs in the first form and five per cent in the second, the two others are followed by complete recoveries. Stuart and Krikorian (83) state that the incidence of these accidents has been very low over the years. In studying the histopathology of a number of cases, they observed lesions similar to those seen in post-vaccinal encephalomyelitis. They also found that the cause of paralytic accidents lies in the protective inoculations themselves, presumably in some constituent of normal nerve substance unavoidably introduced during the process of immunization. Similar accidents can be produced experimentally by repeated injections of nerve substance, normal or rabid, homologous or heterologous. Olitsky and Tal (84) found that proteolipides A and B of Folch and Lees are capable of bringing about in mice an acute disseminated encephalomyelitis indistinguishable from the affection induced by the injection of whole brain tissue. These two substances contain practically all of the encephalitogenic agent present in brain material. According to Folch and Lees (85), these proteolipides would be components of myelin, since they are absent from unmyelinated brain.

In addition, cases of so-called "rage de laboratoire" have been found to be associated with anti-rabies vaccination in various European countries, Lebanon and Brazil. Remlinger (82, 86) states that such fatalities are caused by the fixed virus in defective vaccines rather than by the street virus excreted in the saliva of the biting

animals. Approximately 163 cases in toto have been reported in the literature, and it would appear that the use of a vaccine prepared according to the Hogeny-Philipps dilution procedure has been incriminated in many instances. The diagnosis of the condition is usually based on the identification of the fixed virus through inoculation of rabbits and histopathological study of the central nervous system of these experimental animals. However, an accurate diagnosis might be impossible since the condition can take the form of a "neuro-infection mortelle auto-stérilisable", as indicated by Levaditi et al (87), and Kemlinger and Bailly (88).

Attempts to eliminate severe neurological reactions to rabies vaccine have stimulated research with two types of vaccine produced from avian embryos. Koprowski et al (89, 90) have developed a vaccine by adapting the Flury strain of rabies virus to fertile hens' eggs. Fox et al (91) reported the possibility of immunizing humans with a live attenuated rabies virus vaccine. Kuessegger et al (92) presented data indicating that not only does such a vaccine produce a good antibody titer but it also eliminates systemic reactions and causes only a few local reactions. Komarov and Hornstein (93) reported good results following the immunization of animals with the Kelev strain of rabies virus adapted to the developing chick embryo. Furthermore, Powell and Culbertson (94, 95) who succeeded in cultivating fixed rabies virus in embryonated duck eggs found such a source to be adequate for the production of potent antirabies vaccine. MacFarlane and Culbertson (96) showed duck embryo suspensions and vaccines to be almost devoid of the

encephalomyelitis producing factor when tested with Freund's adjuvant in guinea-pigs. In view of the antigenic similarity of duck and chicken egg albumin demonstrated by Wetter et al (97), the admonition of Hatner and Untracht (98) that persons sensitive to chicken egg albumin should not receive duck embryo vaccine must not be overlooked. Whereas Schnurrenberger et al (99) estimated chick and duck-embryo vaccines to be of comparable value for the immunization of man, Greenberg and Childress (100) found the duck-embryo vaccine to be definitely superior when used in a comparative study with brain tissue rabies vaccine (Semple vaccine).

Habel (101) reports that many scientists attempted with inconsistent results to develop seroprophylaxis in animals following the first report on the preparation of immune rabies serum by Babes and Lepp in 1889. The same author also mentions that more recently Proca, Babes and Jonesco demonstrated some protection to be afforded by topical or subcutaneous serotherapy in experimental rabbits and guinea-pigs. These workers also used seroprophylaxis alone or in combination with vaccination in man and found it beneficial. The possibility that seroprophylaxis might be useful in the prevention of rabies in man aroused great interest in the light of the findings of Baltazard and Ghodssi (102) that Iranian people severely bitten by rabid wolves developed rabies at the same high rate (40 per cent) whether given a full course of potent vaccine or no treatment. In 1954, Baltazard and Bahmanyar (103) had an opportunity to study the value of serotherapy in persons severely exposed to rabies infection.

Their results were sufficiently encouraging to prompt the World Health Organization Committee (104) to recommend the routine use of a single dose of antiserum of equine origin followed by a full course of vaccine for all severe exposures. When feasible, the infiltration of wounded tissues with hyperimmune antirabies serum is also recommended. Recently, Soloviev and Kobrinski (105) have found that the local application of antirabies gamma-globulin in the dried form on experimental animals is more effective and less harmful than parenteral serotherapy. Burns et al (106) report that cortisone and corticotropin have been widely and successfully used in controlling neuromuscular and anaphylactoid reactions induced by the vaccine containing rabbit brain tissue. However, they point out that cortisone is contraindicated, as it appears to prevent the production of active immunity to the virus as well. Antihistamine drugs have been recommended for the treatment of the so-called "serum sickness" which often follows the use of equine hyperimmune antirabies serum (107).

VEETERINARY SIGNIFICANCE

Rabies is not of major economic importance in large domestic animals as is the case with brucellosis, vesicular diseases, hog-cholera and other dreaded diseases of livestock. Animals other than domestic and wild carnivores do not usually transmit the disease to one another (50). This may be explained by their relative lack of ability, opportunity, or tendency to bite when rabid. However, individual flocks and herds may suffer significant losses in areas where the reservoir of infection resides in the wildlife population. During the last 10 years, for example, 7,311 fatalities have been reported among

farm animals in the United-States (29), and 2,909 in Canada (108). An epidemic of the disease, vehiculated by vampire bats, killed 20 per cent of livestock in Trinidad, in 1925 (25).

SUSCEPTIBILITY OF VARIOUS ANIMAL SPECIES

Although rabies may affect all warm-blooded animals, especially mammals, it is usually more common in wild and domestic carnivores than in other animals. Recent studies have demonstrated relatively marked differences in the susceptibility of various wild species. Sikes (66, 109) used a strain of street rabies virus isolated from the salivary glands of a fox to infect by the intramuscular route foxes, striped skunks, racoons and opossums. The fox was found to be the most susceptible animal, the opossum the most resistant. Less than 10 mouse LD₅₀ were required to kill 50 per cent of the former, whereas the latter resisted 80,000 mouse LD₅₀. The striped skunks were next most susceptible, followed closely by the racoons. Approximately 500 mouse LD₅₀ were required to kill 50 per cent of the skunks, and 1,000 for the racoons.

Among domestic animals, cattle are observed to be one of the most susceptible species (27). These are quite curious about strange animals and investigate by smelling. If the strange animal happens to be a rabid carnivore, bites on the muzzle, face or legs are the usual consequence (110). During the last 10 years, 92 per cent of the fatalities reported among farm animals in the United States, and 78 per cent of those reported in Canada, occurred in the bovine species.

Thiery (111) demonstrated that the susceptibility of animals to rabies is inversely proportional to the content of their serum in non-specific gamma-globulins. The fact that the amount of these globulins increases with the aging of animals accounts for the relative resistance of the old ones to rabies. The folliculin hormone was also found by this worker to enhance susceptibility to rabies in females, in contrast to progesterone.

Reulinger and Bailly (55) state that although most species of birds can be experimentally infected with rabies, the results are quite inconsistent. The natural occurrence of the disease in these animals is however exceptional and of no significant importance. According to the same authors (112), cold-blooded animals such as turtles, snakes and frogs are refractory to the disease.

SYMPTOMLESS CARRIERS OF RABIES

In 1936, Torres and Queiroz Lima, quoted by Williams (25), reported that the vampire bat, especially Desmodus rotundus, naturally infected with rabies, might resist the infection and become a carrier. In this state it could transmit the virus to livestock or other vampires for a period of up to 106 days. In the same year, Fawan (113) confirmed the above findings and showed that rabies in the experimentally infected vampire may manifest itself in one or another of the following forms: a) furious rabies followed by paralysis and death; b) paralytic rabies followed by death; c) furious rabies followed by recovery; d) abrupt death without evidence of illness; e) subclinical or latent infection without apparent departure from normal.

It is now generally recognized that nonsanguivorous bats are susceptible to rabies and that some may harbour the virus as symptomless carriers, since their saliva has been found to be infectious (114). In fact, fatal cases of rabies in animals and man have been found to result from bites of such animals (27, 71, 114). Sulkin (115) presented evidence to indicate that the brown adipose tissue of the bat, sometimes referred to as the "hibernating gland", provides a "reservoiring mechanism" for storage of rabies virus during prolonged periods of latency.

It has been shown by Broz and Phan-Trinh (116), Andral and Serie (117), and Yurkovsky (118) that dogs may, in certain instances, be apparently healthy carriers of rabies virus. In such cases they may contaminate other animals or persons and constitute especially dangerous vectors because they are not suspected of excreting the virus in their saliva.

Kantorovich (119) claimed that he succeeded in isolating many strains of rabies virus from the brains of "healthy" polar foxes killed without any signs of disease. He concluded that it is possible to assume preservation of madness virus in polar foxes carriers under conditions of mild or asymptomatic infection.

Hemlinger (120) attempted to isolate the rabies virus from the brain of 13 rats captured in Tanager sewers. He succeeded in three instances and was inclined to believe that these animals could be carriers as are bats from Trinidad. It has been shown by Svet-Moldavskaya (121) that in rats inoculated with street rabies virus,

either subcutaneously or by different undamaged mucous epithelial routes, the virus may penetrate into the salivary glands, the animals remaining without clinical signs of rabies for long periods of time.

LABORATORY METHODS FOR THE STUDY OF RABIES

Before the advent of the various laboratory techniques now available for the study of rabies, the diagnosis of the disease in animals and humans was based on the following criteria: bites by suspect or rabid animals, clinical symptoms exhibited by affected individuals and certain macroscopic autopsy findings such as the presence of inflammation or foreign bodies in otherwise empty stomachs of dogs. Hyperemia of the brain and meninges was also considered significant (50, 122, 123). These empirical methods resulted in many false diagnoses because other animal or human diseases of the central nervous system were mistaken for rabies (45).

Experimental Inoculation

As early as 1879, Galtier (124) reported that the rabbit should be used to demonstrate the virus in the saliva and other fluids from suspected animals. From his experience, rabbits showed a constant susceptibility and an average incubation period of 18 days which was shorter than in any other species of animals. Pasteur et al (40, 41) also made an extensive use of rabbits as experimental animals. According to Webster and Dawson (10), rabbits and guinea-pigs had been the experimental animals most commonly used for the isolation of the rabies virus until they discovered that Swiss white mice were even more susceptible. In these animals the incubation period was found to be

as short as seven days, and a constant Negri-genesis in the brain followed intra-cranial inoculation of street rabies virus. The white mouse is at present the experimental animal of choice, as recommended by the World Health Organization (125).

Histopathology

According to Van Gehuchten and Nelis (123), the first publications on the histopathology of the central nervous system of animals and persons having died of rabies began to appear around 1875, and described degenerative and proliferative inflammatory lesions. Until then, hyperemia of the brain and meninges had been the only abnormality observed. These authors also mentioned that Benedikt and Kolesnikoff had come to the conclusion that rabies is an acute and diffuse encephalomyelitis. Distension of blood vessels, extravasation of erythrocytes and invasion of perineuronal and perivascular spaces by round cells are quite pronounced in the rhombencephalon. The other portions of the brain and the spinal cord are less severely affected. In 1892, Babes (122) described encephalitic foci now known as "Babes nodules" or "rabid tubercles". These consisted of areas of gliosis and neuronophagia localized in any part of the brain parenchyma, but particularly in the neighbourhood of the central nuclei and in the subcortical regions. This author also referred to the presence of perivascular sleeves, i.e. round cell capillary cuffing, which become more marked the longer the disease persists. He finally described a meningitis, sometimes intense, which predominates at the base of and along the principal septa, and a myelitis affecting the anterior horns of the spinal cord.

After studying hundreds of cranial, spinal and sympathetic ganglia, Van Gehuchten and Nelis (123) reported as a constant finding the complete disappearance of many ganglionic cells following the proliferation of their endothelial capsule. The severity of these lesions was found to vary according to the species of animal involved, the stage of the clinical course of the disease and the ganglia studied. The ganglion nodosum of the vagus nerve was found to be the most susceptible to the action of the rabies virus. According to these workers, other histopathologists who previously had studied various ganglia had found similar lesions, but they felt that these changes were not directly connected with rabies. For example, spinal ganglia had been examined by Kolesnikoff and Golgi, the Gasserian ganglion by Nepveu, and sympathetic ganglia by Kolesnikoff. Later it was established that the lesions occurring in the brain, spinal cord and nervous ganglia as a result of rabies infection constitute only a presumptive indication of the disease without being pathognomonic, since other viral infections of the central nervous system and even old age may produce essentially similar changes (27, 39, 56, 126). As pointed out by Raymond (54), Frothingham (127), Herzog (128), and Lapi et al (129), the histological examination of the nodosum and Gasserian ganglia may be of value when animals are killed in the early stages of the disease, and when traumatic destruction or putrefactive changes of the brain substance make an examination for Negri bodies unsatisfactory or impossible.

Although non-suppurative encephalitis and Negri bodies are usually found in the brain tissue of rabid humans and animals, atypical cases have been reported which were characterized by the absence of one or even both types of lesions. Boiron and Camain (130) did not succeed finding either Negri bodies or non-suppurative encephalitic lesions in the brain of an African child who had died of rabies. Tuncman and Aksel (131) found Negri bodies only in the brain of a 25 year-old rabid man. Ninomiya (132) and Thiery (133) observed similar findings in the brain tissue of rabid animals. A few years ago, we found Negri bodies but no encephalitic lesions in the brain of a two-year-old rabid heifer. On the other hand, we found non-suppurative encephalitis but no Negri bodies in the brain of a rabid horse.

As rabies develops following bites by rabid animals only when virus is excreted in their saliva, various workers have studied the histopathology of the salivary glands of rabid animals. Ninomiya (132) mentioned that Orth, Gruber and Aschoff considered the histological changes in the salivary glands of rabid dogs as "alterative-degenerative". For these scientists, the stromal cellular response was secondary to the destruction of the glandular epithelium. Ninomiya (132), however, attributed both types of changes to the action of the virus. In addition to the mandibular salivary glands, this worker also studied the parotid in which he found atrophy only. It would appear that the above described lesions, just as those of the central nervous system, constitute no more than a presumptive indication of rabies.

In 1903, Negri (5) reported the finding of specific intracytoplasmic corpuscles in the central nervous system of rabid animals and humans.

More precisely, he found these lesions in the pyramidal cells of the hippocampi and cerebral cortex, and in the Purkinje cells of the cerebellum. According to this worker, identical lesions could not be seen in the brains of animals and men, either healthy or that had died of a disease other than rabies. Negri described these corpuscles as round or oval intracytoplasmic structures of approximately 4 to 25 μ . in diameter, which stained deep red with Mann's technique and displayed individual or multiple small chromatin dots "Innenkorpers" in their matrix. Negri's finding has been confirmed by many other scientists (44), and the inclusion bodies which now bear his name have been used the world over in the diagnosis of rabies (126).

Negri and his disciples believed that the intracytoplasmic inclusion bodies seen in rabies represented one stage in the development of a Protozoan parasite "Glugea Lyssae", whereas other scientists favoured the opinion that rabies was caused by an ultramicroscopic organism (44). Subsequent studies of the inclusions found in rabies or in other viral diseases indicate that, while they may contain infective units of the virus, they are composed largely of a matrix derived from the cytoplasm of degenerating cells. The absence of thymonucleic acid from the Negri body, as shown by the Fuchsen stain, conforms with the characteristics of other inclusion bodies due to viral infections (13).

Negri-Luzziani, quoted by Sargent (134), mentions Negri's statement that the distribution of Negri bodies in the central nervous system of rabid animals or persons is the same, but differs according to the clinical form of the disease. In furious rabies these inclusions

are particularly numerous in the pyramidal cells of hippocampi and cerebral cortex, and in the Purkinje cells of the cerebellum. In paralytic or dumb rabies they are more numerous in the spinal cord and spinal ganglia.

Negri, quoted by Negri-Luzziani (6), demonstrated the presence of Negri bodies in the Gasserian ganglia of experimentally infected rabbits. Negri-Luzziani, quoted by Sergent (134), encountered similar lesions in the Gasserian and plexiform ganglia of a rabid boy. Hurst and Pawan (135) discovered Negri bodies in the Gasserian ganglia of experimental monkeys. Goodpasture (136) claims to have found Negri bodies invariably in the Gasserian ganglia of experimental rabbits. Lapi et al (129), however, did not observe the inclusions invariably when studying the Gasserian ganglia of 95 dogs affected with clinical rabies.

Poor (137) did not succeed finding Negri bodies in either the parotid or sub-maxillary glands of rabid dogs, guinea-pigs or rabbits infected with street or fixed rabies virus. Stefanescu (138) claims to have found Negri bodies in the glandular cells of the parotid of a dog suffering from furious rabies. Manouelian (139), on the other hand, states that Negri bodies can be seen only in the cytoplasm of the nervous cells of the ganglia present in the interstitial tissue of the parotid and sub-maxillary glands of rabid dogs.

Attempts to demonstrate the possible occurrence of Negri bodies in non nervous tissues were undertaken by Levaditi and Schoen (140). Rabbits and Swiss white mice were inoculated with street rabies virus in various organs of ectodermic, mesodermic and entodermic origin but the epithelium of the cornea proved to be the

only tissue in which Negri bodies could subsequently be found. A similar experiment repeated with cats, dogs, guinea-pigs and monkeys failed to reveal the presence of Negri bodies even in the epithelium of the cornea.

Although Negri bodies have been recognized as the pathognomonic lesion of the street rabies virus infection (5), these inclusions cannot be found invariably. As mentioned earlier, approximately 5 to 15 per cent of rabid animals do not develop a Negriogenesis (6, 7, 8, 9). Volpino, quoted by Sergent (141), stated that Negri bodies are to be found in the hippocampi of rabid dogs a day or two before the appearance of symptoms. However, he claimed that they are smaller, less deeply stained and less numerous than when the symptoms have developed. Negri-Luzziani (6), on the other hand, was unable to find Negri bodies in the brain tissue of rabid animals before the appearance of symptoms. Leach (7) claimed that Negri bodies are more difficult to demonstrate in brain material of rabid animals that had been vaccinated against rabies, paralytic rabies being the usual form of the disease in such animals. Similar findings have been observed at our laboratory in experimental cattle. Gerlach and Schweinberg, Lubinski and Prausnitz, quoted by Hagan and Evans (126), also found Negri bodies much less frequently in rabid humans which have been immunized than in those who have not received such a treatment.

Bongiovanni, quoted by Salimbeni (142), stated that Negri was the first worker to have noticed the absence of Negri bodies in the brain tissue of rabbits infected with fixed rabies virus and obtained

the same results himself on repeating Negri's experiment. Nevertheless, after modifying Mann's staining method, Manouelian (143) was able to demonstrate a few small Negri bodies in the brain tissue of rabbits infected with fixed rabies virus. Negri-Luzziani (6) observed that Negri bodies are small, scarce and therefore more difficult to find in animals dying of fixed rabies virus infection. Nicolau and Kopcziowska (144) also demonstrated a few small Negri bodies in Ammon's horns and cerebral cortex of rabbits infected with fixed rabies virus when using the staining method of Giemsa. However, they claimed that Negri bodies occur constantly in the optic basal nucleus of such animals. According to Lepine and Sautter (145), Negri bodies are always smaller, less numerous and devoid of internal structure in animals infected with fixed rabies virus. These inclusions can be demonstrated with greater facility when using Lepine's stain (146). Moreover these authors observed oxyphilic nuclear inclusions in pyknotic neurones, which consisted of round or oval corpuscles of irregular size seen in an acidophilic mass representing the remains of the nucleus. They were hyperchromatic and mainly basophilic, although some were strongly acidophilic. Intra-cytoplasmic granular formations were also found irregularly by these workers in fixed rabies virus infection.

Other types of inclusion bodies are sometimes seen in the brain tissue of rabies suspected animals. Because of certain similarities, these inclusions may be mistaken for Negri bodies. As early as 1907, Lentz, quoted by Marie (147), reported the presence of such inclusions in the hippocampi of dogs affected with distemper, a finding confirmed by Negri-Luzziani (6) a few years later. According to Tierkel (27),

the acidophilic inclusion bodies of canine distemper or of Rubarth's disease are occasionally encountered in the brains of dogs, foxes, skunks and other wild carnivores. Schipper et al (148) on examination of Saller's stained brain smears prepared from cattle affected with mucosal disease observed inclusion bodies similar to Negri bodies. These workers recalled the fact that similar inclusions had been described by Stenius in bovine malignant catarrhal fever.

Inclusions resembling Negri bodies have also been reported as occurring in the brain tissue of normal animals. In 1904, Negri-Luzziani (6) described for the first time the presence of such inclusions in the nervous cells of normal cats. In a later paper (6), she mentioned that her finding had been confirmed by Jastremsky. In 1933, Nicolau et al (149) reported the presence of cytoplasmic inclusions similar to Negri bodies in the brain tissue of normal wild and Swiss mice.

As stated by Tierkel (27), these non-specific inclusions all have the same staining characteristics with Saller's stain and cannot be differentiated from one another. However, they can be differentiated from Negri bodies by using the following criteria: Negri bodies have a very definite internal structure, characterized by basophilic inner granules, and a heterogeneous or mottled matrix; they are not refractile and have a definite purplish tinge. The non-specific inclusions lack an internal structure and have a homogenous or smooth matrix; they are highly refractile and somewhat more acidophilic giving a pinker appearance. Furthermore, Nicolau (150) agreed

with Lentz that distemper inclusions can occur in the cytoplasm as well as in the nucleus of both neurones and glial cells.

Serology

Complement-fixation test

According to Ando et al (151), the first attempts to use the complement-fixation test as a rapid method for the diagnosis of rabies were carried out in 1907 by Heller and Tomarkin, and by Friedberger. Unfortunately, the results proved to be non-specific. Similar non-specific findings were obtained by Nedrigalloff and Sawtschenko in 1910, Kraus and Takaki, and Kraus and Michalka in 1926, Marie and Urbain in 1929, and Kondo and Obana in 1930. Schultz, Bullock and Brewer (152) also demonstrated that an antiserum showing complement-fixation with the virus of rabies was just as likely to react with the virus of vaccinia and with normal brain as well. Havens and Mayfield (153), on the other hand, found that the serum of rabies-immune guinea-pigs possesses specific complement-fixing antibodies, but that the rabbit immune serum is unsatisfactory because of its anti-complementary properties. Casals and Palacios (154) also obtained a specific complement-fixation test and claimed that complement-fixing antibodies were present in high titre in the sera of dogs, guinea-pigs, mice and rabbits immunized with rabies virus. Ando et al (151, 155) developed a new complement-fixation test for the diagnosis of rabies in suspect animals, using an antigen derived from deep frozen brain tissue of rabid dogs. A 40 per cent emulsion was prepared in saline at

pH 7.2, by grinding with sand or in a Waring blender. The emulsion was held at 40°C. for one hour, centrifuged at 10,000 r.p.m. for 15 minutes, and the supernatant fluid used as antigen. Salivary gland antigen was prepared in the same way except that a 33 per cent suspension was prepared for the blender by cutting the gland into pieces of about 1 mm.³ and adding twice the amount of saline. The inhibitory action of mucin had to be neutralized before the test could be performed. The antigens were tested against the sera of guinea-pigs immunized with either fixed or street rabies viruses. Only guinea-pigs free from antibodies to Japanese B encephalitis were used for this purpose. The complement-fixation method employed two full units of complement. On the basis of their results these authors claimed that their method was of greater value than either the histological examination of brain tissue for the detection of Negri bodies or the mouse inoculation test followed by the serum-virus neutralization test, because a final diagnosis could be reached within five hours. In addition, the test could be carried out successfully on putrid material which was unsuitable for the detection of Negri bodies or the mouse inoculation test. It also detected the presence of extremely small amounts of antigenic substance in specimens which did not always contain an amount of active virus sufficient to infect mice. Approximately 200 dogs were examined by this method as well as by the usual histological techniques and the mouse inoculation test. In no case was a positive virus isolation associated with a negative complement-fixation test. Indeed, in three instances in which the virus isolation was negative,

the complement-fixation test was weakly positive. Depoux and Merveille (156) carried out the complement-fixation test as described by Ando et al (151, 155), except that they used rabbit instead of guinea-pig hyperimmune serum. They obtained less a satisfactory result: as compared with the histological techniques and the mouse inoculation test, the complement-fixation method showed only 66 per cent accuracy. Furthermore, putrid brains exhibited increased anti-complementary activity rather than higher antigenic titre. Sethna and Soman (157) carried out the complement-fixation test on 101 brains and 49 salivary glands, following the methods of preparing the antigenic suspension and guinea-pig antiserum recommended by Ando et al (151, 155). In order to verify the titre of the immune serum, a positive sheep brain antigen (fixed rabies virus) was always put up with each routine test. The following controls were also included in the main test: antigen control, positive and negative serum controls, complement controls for two, one and $\frac{1}{2}$ unit, and the sheep cell suspension control. These workers were of the opinion that the complement-fixation test has a definite place in the rapid diagnosis of rabies in animals, especially in the Negri-negative ones, because the results of the complement-fixation test can be obtained within 24 hours. However, two brains and five salivary glands found to be positive by the mouse inoculation test were missed by the complement-fixation test in this study. Therefore they concluded that, when Negri bodies cannot be demonstrated on preliminary examination, the last word can only be obtained by the mouse inoculation test. As to the specificity of the test, it is noteworthy that not a

single case of non-specific positive reaction was observed throughout their work.

Plate gel diffusion precipitin test

Following Oudin-Ouchterlony's method, as modified by Mansi, the plate gel diffusion precipitin test was applied by Villemot and Provost (158) to the study of 64 rabies suspect brains. Of these, 24 dog brains and one human brain were found positive by the agar precipitation test as well as by the classical tests. Two lines of precipitation were obtained by the agar precipitation test, indicating the presence of two soluble antigens which had formed complexes with precipitating antibodies. These two lines of precipitation were assumed to be specific for the virus of rabies as they never appeared when rabies anti-serum was added to the virus of canine distemper. Sera of donkeys, immunized with formalized suspensions of infected rabbit brain tissue (fixed virus), were used as the source of rabies anti-serum. Precipitating antibodies were found to be particularly abundant in the sera of these hyperimmunized donkeys.

Polson and Wessels (159) demonstrated in infected suckling mouse brains the presence of a soluble rabies antigen having a molecular size of 12 mu. This fraction is obviously distinct from the viral antigen which has a molecular size of 125-150mu. Kipps et al (160) who compared the action of phenol and heat on the soluble antigen of rabies, concluded that it is composed either of two distinct factors or of two functional groups on a same substance. Based on this finding, Villemot and Provost (161) assumed that the two lines of precipitation might correspond to the

two distinct soluble antigenic fractions and pointed out that a different molecular arrangement of these two fractions could account for their respective physico-chemical properties. The plate gel diffusion precipitin test was also used by Villemot and Provost (162) as a rapid diagnostic method. Specific results were obtained in a group of 33 cases of confirmed rabies, as compared with only 30 positive histological findings. There was no precipitation in 59 non rabid brains, although non specific inclusions were found in some of them when they were examined histologically. Trypsinization of the rabid brains helped to improve the results of the test. This method was found to be very useful in the case of dogs killed at the first appearance of symptoms and could be performed in 24 hours. In addition the authors were of the opinion that this test might facilitate more extensive studies of sylvatic rabies. Results similar to those just described were obtained by Ver et al (163) who applied the test to the study of fixed rabies virus in the brain tissue of experimental guinea-pigs, mice, rabbits and sheep. The plate gel diffusion precipitin test was also carried out by Grasset and Atanasiu (164) with three strains of fixed rabies virus cultured on mouse neoplastic ependymal cells. Two lines of precipitation were observed and, in most cases, the method proved to be sensitive with material of relatively low infectious titre. The precipitin reaction was always associated with the presence of intracytoplasmic oxyphilic inclusion bodies in the tissue cultures. According to these workers, the technique offers the possibility of being used as an identification method of rabies virus.

Paper electrophoresis

Chabaud et al (165) noticed that paper electrophoresis revealed a marked increase in the alpha₂ globulins of the sera of rabid cattle, cats, dogs, donkeys and horses as compared with the sera from normal animals of these species. In dogs especially, the electrophoretic test never failed to show this increase in alpha₂ globulins in cases where rabies was subsequently confirmed by animal inoculation. These workers concluded that electrophoresis might be of value for the early diagnosis of rabies in living animals. In Ethiopia, electrophoresis, histology and inoculation of experimental animals were used by Andral and Serie (117) as routine methods for the diagnosis of 423 cases of suspected canine rabies. In 316 cases the results of these three methods were found to be in agreement. Of the remaining 107 cases, 105 gave a positive electrophoretic result although rabies could not be confirmed by the other methods. On the other hand, two dogs with a negative electrophoresis were found to be rabid by the other two methods. In an attempt to elucidate these discrepancies, the authors found that alimentary deficiencies, hepato-renal affections, myocardial infarction stress and especially canine nervous affections such as distemper and Aujeszky's disease can also increase the alpha₂ globulin content of the animals' sera. They concluded that in Ethiopia approximately 35 per cent of apparently healthy dogs exhibit a marked elevation in serum alpha₂ globulins. A few of these animals presented atypical forms of rabies or were carriers and excretors of the rabies virus. Korour and Bahmanyar (166), using paper electrophoresis, described a method whereby the antirabies fraction in immunized animals' sera may be ascertained

and the precise dilutions of such sera for titration in mice by the serum-virus neutralization test determined. These workers demonstrated that during the hyperimmunization of mules a fraction, normally absent or present in very small amounts in the serum of these animals, is markedly increased. This fraction migrating electrophoretically between the main beta and gamma-globulin regions and known as fraction T or beta₂ globulin, increased during immunization until it represented 17 to 25 per cent of the serum proteins in the hyperimmunized mule. Concurrently a marked decrease of albumin was noted as well as a slow and very slight decline in the gamma-globulin fraction. Further investigation showed a constant relationship to exist between the increase in beta₂ globulin and the increase in rabies antibodies.

Serum virus neutralization test

As mentioned by Schultz et al (152), it was reported by Babes as early as 1891 that the serum of an animal immunized against rabies possesses the property of inactivating the virus "in vitro". Similar observations were subsequently published by Kraus, Keller and Clairmont, Marie, Remlinger, Schnurer, Semple, Poor and Friedman, Schult, Bullock and Brewer, and others. In the above authors' experiments, animals of various species remained healthy following injections of a virus-antiserum mixture, whereas the controls died of rabies following injections of virus alone. In 1905, Marie (167) confirmed the finding of Kraus and Maresch that no rabicidal antibodies could be demonstrated in the sera of chickens and pigeons after intensive immunization.

Paradoxically, the rabies virus was sometimes found to be neutralized by the serum of normal chickens and pigeons. It was also noted by Stoel (168) that the addition of normal chicken serum to a tissue culture medium interfered with the "in vitro" multiplication of rabies virus.

In 1954, Koprowski and Johnson (125) described the following standard method of carrying out the serum-virus neutralization test. Healthy Swiss white mice of 21 to 35 days of age, weighing approximately 8 to 12 grams, are inoculated by the intra-cranial route. A 20 per cent suspension of the second or third laboratory passage of the rabies virus in mouse brain constitutes the infective inoculum. The source of antibodies consists of guinea-pig rabies-immune serum known to possess specific neutralizing capacity. A constant amount (0.2 ml.) of rabies-immune serum is tested against similar amounts of serial tenfold dilutions up to 10^{-5} of the suspected virus. The experimental mice are kept under observation for 21 days, and the LD_{50} dose, the amount of inoculum which kills 50 per cent of the mice, calculated from the results by the statistical method of Reed and Muench (169). If 100 LD_{50} or more of virus are neutralized by the serum, the identity of the virus is established. When the titre of the virus is already known, the test may be modified by the use of a constant dose of virus representing approximately 100 LD_{50} per mouse dose when mixed with an equal amount of serum. This method is likewise useful in determining relative titres or potencies of serum samples.

Fluorescent antibody technique

In 1958, Goldwasser and Kissling (170) reported that the fluorescent antibody technique could be utilized to stain street and fixed rabies viral antigens in the brain tissue of experimentally infected mice. When they also examined positive salivary glands from cattle, dogs, foxes, skunks and one wildcat by this method, their results proved to be encouraging. These authors hoped accordingly that the technique might prove useful for the identification of rabies antigen in brain and salivary gland tissues of animals in which Negri bodies could not be demonstrated. They found that at least some of the small acidophilic inclusion bodies seen in the brain tissue of animals infected with street rabies virus, which cannot ordinarily be considered as specific because of their similarity to inclusion bodies found in other diseases, were of rabies virus origin. At the same time it became obvious that this new more rapid method could have a definite advantage over the mouse inoculation test. Combining the speed of histological techniques and the accuracy of experimental inoculation, the fluorescent antibody technique showed promise of being of great help in deciding early whether or not exposed people should undertake a prophylactic treatment.

Goldwasser et al (171) conducted a field study in which 157 specimens were submitted to the following tests: a) examination of brain impression smears for the detection of Negri bodies; b) mouse inoculation with brain suspension; c) mouse inoculation with salivary gland suspension, and d) fluorescent antibody staining of salivary gland impression smears. Fifty-five specimens were found positive by the

inoculation of mice with salivary gland suspension, but only 48 by the fluorescent antibody staining of salivary gland impression smears. Two of the latter specimens, also positive by the mouse inoculation test, came from animals in which Negri bodies were not found. Whereas Goldwasser and Kissling (170) had investigated the indirect, complement and direct immunofluorescent staining methods, Goldwasser et al (171) utilized a modification of the direct staining method of Coons and Kaplan (172) exclusively.

McQueen et al (173), using the method of Goldwasser et al (171), found the results of the fluorescent antibody technique to be in complete agreement with those of the mouse inoculation test on a total of 325 fresh or frozen brains from rabies suspect animals. However, only 98.3 per cent of the specimens included in the study showed Negri bodies. In addition these workers found that fragments of brain tissue preserved in glycerol were not satisfactory for examination by the fluorescent antibody technique. Although in this study the fluorescent antibody technique had appeared to be more sensitive than the microscopic examination of Sellers' stained impression smears, the authors nevertheless concluded that additional studies were needed before any firm conclusions could be drawn. Several methods of diagnosis were compared by Carski et al (174) during a study on the pathogenesis of rabies in experimentally infected captive foxes and skunks. The most sensitive tests were: a) fluorescent antibody examination of brain smears by the method of Goldwasser et al (171), and b) mouse inoculation with brain suspensions. Both tests were equally sensitive but neither was completely reliable since each test provided one positive diagnosis that

was missed by the other. In studies of salivary gland specimens, both mouse inoculation and fluorescent antibody methods displayed a lower degree of sensitivity, since several specimens were diagnosed as positive in only one of the tests. From their results, the authors concluded that the mouse inoculation test could no longer be considered as the most sensitive of the rabies diagnosis tests. Furthermore, they thought it would be unwise to dismiss as false-positive any positive results obtained by the fluorescent antibody technique which did not agree with those of the mouse inoculation test.

Propagation in Tissue Culture

The first attempts to propagate the virus of rabies in tissue culture undertaken by Noguchi and other workers were not successful (175). In 1936, however, Kanazawa (175), using rabbit embryo brain and Tyrode's solution without serum, succeeded in cultivating "in vitro" the Fukuoka strain of fixed virus. The virus was subsequently propagated by various workers in cultures of mouse or rat embryo brain, brain tissue of mice up to 14 days of age, chick embryo brain or whole chick embryo to which serum or serum ultrafiltrate had been added (176). Donkey serum was used most often for this purpose. Although Veerarraghavan (177) claimed to have obtained multiplication of rabies virus in a cell-free medium containing only growth factors, Kirk et al (178), following the same procedure closely, were unable to repeat his results. In 1958, the first successful attempts to propagate the virus of rabies in non-nervous tissue culture were reported by Kissling (179), who succeeded in propagating serially in hamster kidney tissue cultures

strains of both fixed and street rabies viruses. No cytopathogenic changes were evident in these cultures. Kissling's findings were confirmed by Kaplan et al (130) who used the CVS 24 strain of fixed virus and also demonstrated that cultured rabies virus can be stained by means of the fluorescent antibody technique. Growing and staining of this particular strain by the fluorescent antibody technique were also possible in tissue culture of a continuous diploid cell line derived from normal human foetal lung. Fenje (131, 132) reproduced Kissling's findings, using the SAD strain of fixed virus, and also attempted to develop a vaccine devoid of nerve tissue. Although the initial results proved encouraging, it was deemed necessary to undertake further work before the value of such a vaccine could be assessed, particularly with respect to the measurement of the immune response in experimental animals. Furthermore, the presence in the vaccine of horse serum, an essential part of the culture medium, would still represent a danger for injection into man because his natural sensitivity to horse protein is not uncommon.

When Rossi, quoted by Dunscombe (133), attempted the propagation of street, fixed and Flury LEP 38 and 42 viruses in hamster and rabbit kidney cell cultures, he found that none of these viruses grew in rabbit kidney cell cultures whereas in hamster kidney cell cultures Flury LEP 38 and 42 grew better than fixed virus. No cytopathogenic change was observed, except for a slight pigmentation in the hamster kidney cells. On the

basis of his results, Rossi also considered the possibility of producing a vaccine free of the allergic factors present in the actual rabies vaccines. The Pasteur's strain of fixed rabies virus was grown on hamster cell kidney culture by Martos and Atanasiu (134). From the seventh day, the infected cells contained acidophilic intracytoplasmic inclusion bodies surrounded by a light halo. The presence of inclusion bodies, the virulence of the culture and the disappearance of both of these after the administration of specific immune serum suggested that these inclusions were specific for the rabies virus.

Attempts to propagate rabies virus in neoplastic cells were first reported by Stoel (163) in 1930. He obtained negative results with Rous Sarcoma and murine cancer cells when normal chicken serum was added to the culture medium. Atanasiu and Lepine (185) however succeeded in growing rabies street virus on mice ependymoma cells in tissue culture. The virus induced the formation of oxyphilic inclusion bodies resembling Negri bodies. From the fifth passage and in the course of 15 transfers, the culture fluid induced a cellular degeneration which could be prevented by a specific immune serum. From this experiment, the authors concluded that it would be possible to carry out the titration of antirabic sera on tissue cultures and that such a technique would present many advantages over the standard mouse serum-virus neutralization test.

Propagation in Embryonated Eggs

In 1938, Kligler and Bernkopf (186) reported that the virus of rabies could multiply following inoculation on the allantois of developing chick embryos. Although the allantois was not infected with regularity, the virus consistently found its way into the brain of the embryo. The development of the embryo was apparently not affected by the presence of the virus and was observed to proceed in the same manner as in the uninfected controls. These authors (187) also found that after 47 passages on the allantois of the developing chick embryo, rabies fixed virus showed no enhanced virulence for the chick embryo, as indicated by the complete development of the embryo, the relatively low virus titre in the embryo brain and the scanty pathological changes in the brain. The virulence of the chick embryo passage virus for mice and guinea-pigs remained unchanged, while that for rabbits appeared reduced considerably. The specific antigenic character of the chick embryo virus likewise remained unchanged.

Dawson (188) found the intra-cranial passage of rabies virus in the chick embryo to be associated with a profound alteration of the pathogenicity of this virus for the rabbit and the mouse. This alteration was of such magnitude that it was possible to produce a self-limited and non fatal disease in rabbits by intra-cranial injection, and in mice by sub-cutaneous or intra-muscular injection of embryo-passage virus. Furthermore, tests showed conclusively that recovery from the mild disease was followed by the development of a solid immunity to intra-cranial inoculation of rabbit fixed

virus and mouse passage virus. Koprowski and Cox (139) inoculated the Flury strain of rabies virus in the yolk-sac of seven-day-old chick embryos. By means of intra-cranial injections into mice, the virus was recovered from the chick embryo blood secured from the third to the fifteenth post-inoculation day. According to the authors, these experiments would indicate a completely different mechanism of dissemination of the virus in embryonated eggs as compared with mammals in which the virus is rarely present in the blood. Various strains of rabbit fixed rabies virus were also successfully cultivated in seven-day-old embryonated duck eggs by Powell and Culbertson (94). Eggs were infected intra-amniotically and the growths were harvested after 14 days. Apart from the embryo itself, a degree of infectivity could also be detected in the extra-embryonic fluids. After three passages, these strains seemed to have lost some of their virulence for mice. When used as a vaccine, this material protected mice against 7,700 LD₅₀, as determined by the mouse potency test.

EXPERIMENTAL STUDIES

Among the various laboratory methods available for the study of rabies, the two most commonly used in diagnostic laboratories are still the direct microscopic examination of impression smears from hippocampal tissue for the detection of Negri bodies and the experimental inoculation of laboratory animals for the detection of virus in Negri-negative brains. Swiss white mice have proved

to be the most satisfactory experimental animals for rabies virus isolation (10, 190). Unfortunately, as mentioned earlier, the first method is not 100 per cent efficient (6,7, 129, 133) and the second is too time-consuming to be used as a basis for decision regarding vaccination of contaminated individuals (170).

Recently, the fluorescent antibody technique was presented as a rabies diagnostic method which offered the possibility of combining the speed of histological techniques with the accuracy of the mouse inoculation test (170). Without giving official recognition to this new method, the Expert Committee on Rabies of the World Health Organization has encouraged rabies diagnostic laboratories to develop proficiency in carrying out the test, and to make comparative studies with other conventional diagnostic methods (107).

MATERIALS AND METHODS

Strain of Street Rabies Virus

Source

Brain material from a field case of bovine rabies was used as a source of virus for our experimental studies. A 10 per cent emulsion was prepared by triturating 0.5 gm. of brain tissue in a sterile mortar with 4.5 ml. of sterile physiological saline solution. Antibiotics were incorporated in the proportions of 1,000 I.U. of penicillin G sodium and 2.0 mg. of streptomycin per ml. of emulsion. A healthy calf, approximately three months of age, was inoculated

with 1.0 ml. of suspension into the left and right masseter muscles. The incubation period lasted for three weeks and death followed a three-day clinical course of furious rabies. The brain of the animal was removed from the cranial cavity, and the hippocampi dissected out. Microscopic examination of impression smears from hippocampal tissue, stained with William's modification of Van Gieson's stain (191), revealed the presence of numerous Negri bodies. Forty grams of brain tissue, including pieces of the cerebral hemispheres, hippocampi, medulla oblongata and cerebellum, were mixed with 40 ml. of normal rabbit serum, previously inactivated for 30 minutes at 56°C, and 120 ml. of sterile buffered saline solution (pH 7.1 phosphate buffer). The chilled mixture was homogenized in a Virtis homogenizer, and dispensed in ampoules in 2.0 ml. amounts. The ampoules were sealed with a portable gas torch, labelled and quick frozen in a mixture of absolute ethanol and carbonic ice before being stored at -70°C.

Titration of infectivity

To allow for stabilization of the virus concentration, the ampoules were stored for three weeks before titration of infectivity for Swiss white mice approximately four weeks of age. From the contents of one ampoule, serial tenfold dilutions up to 10^{-9} were prepared with sterile physiological saline solution. Antibiotics were incorporated in such proportions that each ml. of the various

dilutions contained 1,000 I.U. of penicillin G sodium and 2.0 mg. of streptomycin. One group of 10 mice each was inoculated intracranially with each dilution, each animal being given 0.03 ml. The mice were kept under observation for a period of 30 days. The brains of those that succumbed to the challenge were studied for the presence of Negri bodies through the microscopic examination of William's-stained impression smears from both hippocampi. Survivors were destroyed at the end of the 30-day observation period but not examined for the presence of Negri bodies. The 50 per cent end point, calculated by the method of Reed and Muench (169), was found to be $10^{-3.49}$.

Inoculation of Experimental Animals

Swiss white mice of both sexes and of approximately four weeks of age were obtained from the Animal Diseases Research Institute breeding colony. All animals were examined and determined to be in apparent good health before being accepted for experimental purposes. They were distributed in groups of five, housed in properly labelled glass jars and fed pelleted food and tap water.

Intra-cranial route

Mice were anaesthetized with ether and inoculated by the intra-cranial route with 0.03 ml. of the 10^{-1} dilution of this stock strain of street rabies virus. The 10^{-1} dilution was chosen because it had become obvious, while carrying out the titration, that the undiluted original 20 per cent suspension of rabid brain tissue was too concentrated to be satisfactory for intra-cranial

inoculation of laboratory animals of such a small size. Antibiotics were added in the proportion of 1,000 I.U. of penicillin G sodium and 2.0 mg. of streptomycin per ml. The various groups of experimental animals were housed and fed as described earlier. Chloroform euthanasia was performed on individual groups at daily intervals until mice finally died as a result of the infection.

Intra-muscular route

The procedures followed in this part of the experimentation were similar to the ones just described, except that mice were inoculated into the left cervical musculature with 0.05 ml. amount of the 10^{-1} dilution of the stock strain of street rabies virus.

Collection and Preservation of Tissues

The methods followed were essentially similar, irrespective of the routes of experimental inoculation. The collection of saliva preceded the euthanasia of experimental mice, whereas salivary glands and brain were dissected immediately after euthanasia or as soon as possible after death. The excess of salivary gland and brain tissue which was not needed for experimental studies was subsequently stored at -20°C for possible further reference.

Saliva

Salivation was induced by the sub-cutaneous injection of 0.25 ml. of an aqueous pilocarpine solution containing 2.0 mg. of pilocarpine hydrochloride per ml. Approximately 10 minutes after the injection, sterile swabs were introduced in the mouth of

experimental animals. The amount of saliva harvested was approximately 0.25 ml. Later, the amount of pilocarpine injected was increased to 0.5 ml. This induced salivation within a few minutes and the amount of saliva harvested was sensibly increased. However, individual reactions to pilocarpine were observed among the experimental mice; some did not salivate as much as the others, irrespective of the amount of pilocarpine injected. After collection, each swab was placed into a properly labelled sterile mortar containing 4.0 ml. of sterile physiological saline solution, 4,000 I.U. of penicillin G sodium and 8.0 mg. of streptomycin. The mixture was allowed to stand at room temperature for 30 minutes before being tested for infectivity.

Salivary glands

Both sub-maxillary salivary glands of each experimental mouse were aseptically dissected and placed in a properly labelled small plastic Petri dish.

Brain

The brain of each experimental mouse, including the cerebral hemispheres, midbrain, cerebellum and medulla oblongata, was aseptically removed from the cranial cavity and placed in a properly labelled small plastic Petri dish.

Microscopic Examination of Tissues by Standard Impression Smear Technique

Impression smears were prepared from freshly collected tissues, partially air-dried, fixed with absolute ethanol, stained with William's stain and examined for the presence of Negri bodies. An ordinary binocular microscope and 1,000 diameters magnification was used.

Salivary glands

One small piece of tissue from each sub-maxillary salivary gland was dissected, placed on blotting paper and minced with a scalpel. Impression smears were made on two frosted-end micro-slides, one being identified as left salivary gland and the other as right salivary gland.

Brain

Each hippocampus was dissected out and cut into three transversal segments which were placed side by side on a piece of blotting paper. Impression smears of the left and right hippocampi were made on two frosted-end micro-slides.

Examination of Tissues for Evidence of Infectivity for Mice

Immediately after being collected, samples of saliva and tissues were tested for infectivity by the intra-cranial injection of mice. The inoculated animals were housed and fed as described earlier, and kept under observation for a period of 30 days. Stained impression smears of both hippocampi from mice that died during the observation period were examined microscopically for

the presence of Negri bodies. At the end of the 30-day observation period, all survivors were destroyed without being examined for the presence of Negri bodies.

Saliva

Each sample of saliva was injected into a group of five mice, each mouse being given 0.03 ml. by the intra-cranial route.

Salivary glands

Approximately 0.05 gm. of tissue from each sub-maxillary salivary gland were triturated with sterile alundum in a properly labelled sterile mortar. To this were added 1.0 ml. of sterile physiological saline solution, 1,000 I.U. of penicillin G sodium and 2.0 mg. of streptomycin. The suspension was allowed to stand at room temperature for 30 minutes and 0.03 ml. amounts were injected intracranially into a group of five mice.

Brain

Approximately 0.1 gm. of tissue from the cerebellum and medulla oblongata were triturated in a properly labelled sterile mortar. To this were added 1.0 ml. of sterile physiological saline solution, 1,000 I.U. of penicillin G sodium and 2.0 mg. of streptomycin. The suspension was allowed to stand at room temperature for 30 minutes and then injected into a group of five mice, each mouse being given 0.03 ml. by the intra-cranial route.

Microscopic Examination of Tissues by Fluorescent Antibody Technique

The fluorescent antibody technique can be considered as a new way of detecting antigen-antibody reactions. According to this method, smears or sections of tissues containing antigen are stained with specific antibodies previously tagged with fluorescent material. When viewed with an appropriate microscopic equipment, the areas where the antigen-antibody reaction took place appear as small fluorescent spots. In addition to the direct antigen-antibody reaction, the method includes an inhibition test as a further proof of specificity. This inhibition procedure is based on the immunological phenomenon of blocking subsequent specific antigen-antibody reactions by first exposing the antigen to an unlabelled aliquot of homologous antibody solution. It is claimed that, in certain circumstances, the method is more rapid and as reliable as other conventional procedures.

Although direct, indirect and complement fluorescent antibody staining methods have been used to detect rabies virus antigen in infected tissues, our experimental investigations have been limited to the direct staining method of Coons and Kaplan (172), as modified by Goldwasser et al (171).

Preparation and fixation of tissues

Impression smears of salivary gland and brain tissues were prepared at the same time and from the same pieces of tissue as those intended for microscopic examination by the standard impression smear technique. Two impression smears were made from

each salivary gland and two from each hippocampus. These impression smears were air-dried for 30 minutes and then fixed by a four-hour immersion of the slides into Coplin jars containing acetone held at -20°C . After fixation, the slides were removed from the acetone, thoroughly dried at -20°C and stored at the same temperature until they were stained.

Source and concentration of globulins

The source of antibody for the preparation of conjugates was a commercial therapeutic anti-rabies serum of equine origin (Lederle). The method used for the concentration and conjugation of the immune globulins was similar to that of Coons and Kaplan (172). The globulins were precipitated from 20 ml. of serum at 2°C by mixing with an equal volume of saturated ammonium sulfate. After standing for 18 hours at 2°C , the mixture was centrifuged at 2°C and the supernatant discarded. The centrifugate was redissolved in distilled water to the original serum volume and reprecipitated with ammonium sulfate twice more. Distilled water was added to the final centrifugate to make a paste which was dialysed at 4°C for four days using many changes of physiological saline until the dialysate contained no sulfate, when tested by the barium-chloride test (192). The purified globulin concentrate was bottled in screw-cap bottles, in 5.0 ml. amounts, and stored at -20°C until further processed.

Conjugation of globulins

The protein content of the various globulin preparations was determined by a micro-Kjeldahl method (193). Dilutions of the globulins were made in saline so that each ml. contained 25 mg. of protein. A solution containing 10 ml. of physiological saline, 3.0 ml. of carbonate-bicarbonate buffer (0.5M, pH 9.0) and 2.0 ml. of acetone for each 10 ml. of globulin solution to be used, was cooled to crystal formation in a dry ice alcohol bath. While agitating, 10 ml. of the diluted globulin solution (1.0 ml. = 25 mg. of protein) and 1.5 ml. solution of fluorescein-isothiocyanate in acetone (0.05 mg. of dye per 1.0 mg. of protein) were added to the cool mixture. The solution was shaken overnight at 4°C. The conjugated material was dialyzed against phosphate buffered saline (0.01M, pH 7.2) for at least four days, until the dialysate showed no fluorescence, when viewed in the rays of a mercury lamp. It was then filtered through a pre-filter and a 0.45 micron millipore filter and stored in 2.0 ml. amounts at -20°C.

Adsorption of conjugates

All conjugates were adsorbed once with mouse liver powder and again with mouse brain powder to reduce non-specific staining (194). Chloroform euthanasia was performed on healthy Swiss white mice obtained from the Animal Diseases Research Institute breeding colony, and liver as well as brain tissue aseptically collected. To 50 gm. of liver tissue were added 50 ml. of sterile physiological saline solution. The mixture was homogenized in a Virtis homogenizer with short repeated activations of the propeller in order to avoid

heating. To the homogenate were added four volumes of acetone, while stirring. After allowing the mixture to settle, the supernatant was discarded. The tissue was packed and washed by centrifugation with several changes of sterile physiological saline solution until the supernatant was free of hemoglobin. The washed tissue was suspended in an equivalent volume of sterile physiological saline solution and four volumes of acetone added, with stirring. The suspension was allowed to settle for a few minutes, the supernatant discarded and four volumes of acetone added. The tissue was collected on a Buchner funnel using ordinary filter paper, washed with acetone and allowed to dry on the funnel. The powder obtained was finally dried overnight at 37°C and stored in small screw-cap bottles at 4°C. The procedures followed in preparing the mouse brain powder were essentially the same.

As needed, aliquots of conjugate were adsorbed with mouse tissue powders, in the proportion of 100 mg. liver powder per ml. of conjugate. The powder was moistened with sterile physiological saline solution and the required amount of conjugate added, while stirring. After standing for approximately one hour at room temperature, with occasional stirring, the supernatant was harvested following centrifugation in the cold (4°C) at 18,000 r.p.m. in an angle centrifuge. The adsorption was repeated in a similar manner with mouse brain powder, and the final product stored at -20°C in small rubber-stoppered vials, until used for staining.

Mouse brain suspensions for inhibition control of conjugates

Suspensions were prepared with normal mouse brains (NMB) and with brains of experimentally infected mice (EMB). All animals were obtained from the Animal Diseases Research Institute breeding colony. A strain of street rabies virus was used to infect mice intended for the production of the infected mouse brain tissue. The methods followed in collecting both normal and infected brains were similar to those used for the collection of tissues intended for the preparation of mouse tissue powder. One part by weight of mouse brain tissue was homogenized, in a Virtis homogenizer, in four volumes of 10 per cent egg yolk from six to seven-day-old embryonated eggs in 0.05M sodium buffered distilled water (pH 7.6-7.8). The suspensions were centrifuged at approximately 1,000 r.p.m. for 10 minutes and the supernates drawn off and stored at -20°C in properly labelled rubber-stoppered vials.

As difficulties were experienced in obtaining satisfactory inhibition on control impression smears, a titration of the infected mouse brain suspension being used was carried out in Swiss white mice. Since this titration revealed the 50 per cent end point to be as low as $10^{-1.5}$, we were prompted to obtain a more virulent strain of rabies virus from the Communicable Disease Center of Atlanta, Georgia. The CVS-56 strain of fixed rabies virus supplied to us as a 20 per cent suspension, was found to have a 50 per cent end point of $10^{-9.5}$. This material was used from then on to prepare the infected mouse brain suspensions for inhibition purposes in the staining procedures.

Staining and examination of impression smears

Slides were removed from the freezer and allowed to stand at room temperature until the condensed moisture was completely evaporated. The two separate impression smears which had been made on each slide were ringed with a Blaisdell China-Marker red pencil to prevent mixing of the conjugate. One part of conjugate, prepared as described above, was diluted in four equal parts of normal mouse brain suspension and incubated for 30 minutes at 4°C, with occasional mixing. An aliquot of the same conjugate was similarly processed with infected mouse brain suspension. One of the two impression smears on each slide was layered with the conjugate diluted 1:5 in normal mouse brain suspension, the other with the conjugate diluted 1:5 in infected mouse brain suspension. Impression smears similarly prepared with known rabid brain tissue were also stained to serve as controls for the unknowns of the experimental investigation. The slides were then incubated in a moist chamber for 30 minutes at 37°C. After draining off the excess stain, the slides were washed for 10 minutes in phosphate-buffered saline (pH 7.5), then for the same interval in distilled water to remove excess tagged antibody. After drying at room temperature, the slides were mounted with 90 per cent glycerol-saline and covered with 22x50 mm. micro cover glasses. All the examinations were carried out within a few hours of staining.

The microscope used was the Zeiss large fluorescence model fitted with a dark-field condenser and a pressure mercury vapour

lamp (OSRAM, HBO200). An excitation in the range of $5,000\text{\AA}^{\circ}$ - $3,000\text{\AA}^{\circ}$ was obtained with the BG 12 filter in the lamp holder. Examination was carried at 320 diameters magnification, using alternatively the following barrier-filter combinations in the tube-head of the microscope: OG4 and GG4, BG 23 and GG4, or GG4 alone.

Commercial reagents

In the course of our experimental studies, a commercial fluorescein-labelled anti-rabies globulin became available from the Baltimore Biological Laboratory. The product could be obtained in rubber-stoppered vials containing 5.0 ml. amounts of concentrated desiccated fluorescein-labelled anti-rabies globulin, already adsorbed with mouse brain and liver tissue powders and ready to be diluted with distilled water and used for staining. After successful staining trials of known rabid and non-rabid brain tissue with the 1:25 dilution of the product, it was decided to refer to this commercial anti-rabies conjugate for economic and time-saving reasons.

Field Specimens

As a supplement to our experimental studies on mice, brain material from a wide variety of domestic and wild animals submitted to this laboratory for rabies diagnosis was also examined by the three methods just described. The purpose of this comparative investigation was to obtain information on the possibility of replacing the time-consuming mouse inoculation test by the fluorescent antibody technique for the examination of

field specimens implicated with human exposure, but found negative when examined by the standard impression smear technique.

RESULTS

Infection by the Intra-Cranial Route

Brain

The results of examinations of brains from mice inoculated by the intra-cranial route are shown in Table I. It can be seen that Negri bodies were found in the hippocampi of only 35 or 58.3 per cent of the 60 experimental animals, on microscopic examination by the standard impression smear technique. Positive results were first obtained on the fifth post-inoculation day, when Negriogenesis was demonstrated in the hippocampi of one experimental animal. On the sixth post-inoculation day, four of the five experimental animals disclosed Negriogenesis. From the seventh to the twelfth and last post-inoculation day, Negri bodies were found in the hippocampal tissue of every experimental animal. Clinical symptoms of rabies were not observed in any of the experimental animals, however, until the tenth post-inoculation day. By that time, one of the five mice in this group exhibited posterior paralysis, whereas the other four appeared thin with a rough coat.

Rabies infection was confirmed in 43 or 70.1 per cent of the experimental animals by examination for evidence of infectivity for mice. Moreover, evidence of infection was obtained earlier by this method than by microscopic examination of William's stained

impression smears. By the fourth and fifth post-inoculation days respectively, four of the five animals examined were found to harbour detectable amounts of rabies virus in the brain tissue. From the sixth to the twelfth and last post-inoculation day, rabies virus could be demonstrated in the brain tissue of every experimental animal.

The greatest number of positive findings were obtained through the use of the microscopic examination by fluorescent antibody technique. The presence of rabies viral antigen was demonstrated in 52 or 86.6 per cent of the experimental animals. As early as the second post-inoculation day, antigenic rabies virus material was shown in the brain tissue of every experimental animal. Of the five animals sacrificed on the third post-inoculation day, however, only two appeared positive by the fluorescent antibody technique. From the fourth to the twelfth and last post-inoculation day, rabies viral antigen was detected in the brain tissue of every experimental animal. In summary, rabies viral antigen was demonstrated by the fluorescent antibody technique in the brain tissue of all animals shown to be infected by the other two methods. Furthermore, it was shown in 17 Negri-negative brains, 9 of which also failed to yield rabies virus by the mouse infectivity test.

TABLE I

RESULTS OF EXAMINATIONS OF BRAINS FROM MICE
INOCULATED BY THE INTRA-CRANIAL ROUTE

Animal number	Days after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
Microscopic examination by standard impression smear technique												
1	-	-	-	-	-	-	+	+	+	+	+	+
2	-	-	-	-	-	+	+	+	+	+	+	+
3	-	-	-	-	-	+	+	+	+	+	+	+
4	-	-	-	-	-	+	+	+	+	+	+	+
5	-	-	-	-	+	+	+	+	+	+	+	+
Examination for evidence of infectivity for mice												
1	-	-	-	+	+	+	+	+	+	+	+	+
2	-	-	-	+	-	+	+	+	+	+	+	+
3	-	-	-	-	+	+	+	+	+	+	+	+
4	-	-	-	+	+	+	+	+	+	+	+	+
5	-	-	-	+	+	+	+	+	+	+	+	+
Microscopic examination by fluorescent antibody technique												
1	-	+	+	+	+	+	+	+	+	+	+	+
2	-	+	+	+	+	+	+	+	+	+	+	+
3	-	+	-	+	+	+	+	+	+	+	+	+
4	-	+	-	+	+	+	+	+	+	+	+	+
5	-	+	-	+	+	+	+	+	+	+	+	+

- = Negative
+ = Positive

Salivary glands

As indicated in Table II, evidence of rabies infection was seldom established in salivary gland tissue of experimental animals. Negri bodies were not found in any of the glands following microscopic examination by standard impression smear technique. Only three, that is 5.0 per cent of the experimental animals proved to be excreting detectable amounts of rabies virus in salivary glands following examination for infectivity for mice. Invariably, negative results were also obtained following microscopic examination of salivary gland tissue by fluorescent antibody technique.

Saliva

Examination for evidence of infectivity for mice was the only method investigated in this case, as shown in Table III. Since saliva could not be collected from moribund and dead experimental animals on the 11th and 12th post-inoculation days, only 50 samples were tested. Of these, one, that is 2.0 per cent, proved to contain detectable amounts of rabies virus. It is noteworthy that this was also the only instance in which both saliva and salivary glands of an experimental animal were found to contain detectable amounts of rabies virus.

TABLE II

RESULTS OF EXAMINATIONS OF SALIVARY GLANDS FROM
MICE INOCULATED BY THE INTRA-CRANIAL ROUTE

Animal number	Days after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
Microscopic examination by standard impression smear technique												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
Examination for evidence of infectivity for mice												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	+	-	-	-
3	-	-	-	-	-	-	-	-	-	+	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	+	-	-	-	-
Microscopic examination by fluorescent antibody technique												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-

- = Negative
+ = Positive

TABLE III
RESULTS OF EXAMINATION OF SALIVA FROM MICE INOCULATED BY THE INTRA-CRANIAL ROUTE

Animal number	Days after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
1	-	-	-	-	-	-	-	-	-	-	ND	ND
2	-	-	-	-	-	-	-	-	+	-	ND	ND
3	-	-	-	-	-	-	-	-	-	-	ND	ND
4	-	-	-	-	-	-	-	-	-	-	ND	ND
5	-	-	-	-	-	-	-	-	-	-	ND	ND

Examination for evidence of infectivity for mice

- = Negative
+ = Positive
ND = Not Done

Infection by the Intra-muscular Route

Brain

Table IV shows that infection developed more slowly in animals inoculated by this route; 100 per cent mortality was not obtained until the 25th post-inoculation day. By contrast, all of the intracranially injected mice had died by the 12th post-inoculation day. Not only the late appearance but the marked irregularity of positive findings are also quite obvious. As noted for the intra-cranial route of infection, the microscopic examination by standard impression smear technique appeared to be the least sensitive of the three methods of detecting evidence of rabies infection. In fact only 19 or 15.2 per cent of the 125 experimental animals developed a Negri-genesis. Negative findings were obtained in the course of the first nine post-inoculation days as well as on the 12th, 16th and 20th post-inoculation days. When Negri-genesis could be confirmed, it was limited to one or two animals of individual experimental groups. Negri bodies were found in the hippocampal tissue of only one experimental animal on the 10th, 15th, 17th, 18th, 21st, 22nd and 23rd post-inoculation days. On the other hand, two experimental animals disclosed Negri-genesis on the 11th, 13th, 14th, 19th, 24th and 25th post-inoculation days.

Rabies infection was confirmed by examination for evidence of infectivity for mice in 33 or 26.4 per cent of the experimental animals. This represents a lower rate of infection than the one obtained by the intra-cranial route where it reached 70.1 per cent.

TABLE IV

RESULTS OF EXAMINATIONS OF UNGUES FROM MICE
INOCULATED BY THE INTRA-MUSCULAR ROUTE

Animal number	Days after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
Microscopic examination by standard impression smear technique												
1	-	-	-	-	-	-	-	-	-	+	+	-
2	-	-	-	-	-	-	-	-	-	-	+	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
Examination for evidence of infectivity for mice												
1	-	-	-	-	-	-	-	-	-	+	+	-
2	-	-	-	-	-	-	-	-	-	-	+	-
3	-	-	-	-	-	-	-	-	-	+	+	-
4	-	-	-	-	-	-	-	-	-	-	+	-
5	-	-	-	-	-	-	-	-	-	+	-	-
Microscopic examination by fluorescent antibody technique												
1	-	-	-	-	-	-	-	-	-	+	-	-
2	-	-	-	-	-	-	-	-	-	-	+	-
3	-	-	-	-	-	-	-	-	-	+	+	-
4	-	-	-	-	-	-	-	-	-	-	+	-
5	-	-	-	-	-	-	-	-	-	+	-	-

(continued)

TABLE IV.- RESULTS OF EXAMINATIONS OF BRAINS FROM MICE
INOCULATED BY THE INTRA-MUSCULAR ROUTE (continued)

Animal number	Days after inoculation												
	13	14	15	16	17	18	19	20	21	22	23	24	25
Microscopic examination by standard impression smear technique													
1	+	+	-	-	-	+	+	-	+	-	-	-	-
2	-	-	+	-	-	-	+	-	-	+	+	+	-
3	-	+	-	-	-	-	-	-	-	-	-	+	+
4	+	-	-	-	+	-	-	-	-	-	-	-	+
5	-	-	-	-	-	-	-	-	-	-	-	-	-
Examination for evidence of infectivity for mice													
1	+	+	-	-	-	+	+	+	+	+	+	+	-
2	-	-	+	-	-	-	+	-	+	+	+	+	+
3	-	+	-	-	-	-	-	-	-	-	+	+	+
4	+	-	-	-	+	-	-	-	-	-	+	+	+
5	-	+	-	-	-	-	-	-	-	-	-	-	-
Microscopic examination by fluorescent antibody technique													
1	+	+	-	-	-	+	+	+	+	+	+	+	+
2	-	-	-	-	-	+	+	+	+	+	+	+	+
3	-	-	-	+	-	-	+	-	-	-	+	+	+
4	+	-	-	+	+	-	-	-	-	-	+	+	+
5	-	-	-	-	-	-	-	-	-	-	-	-	+

- = Negative
+ = Positive

Furthermore, positive findings were not obtained earlier than by microscopic examination by standard impression smear technique. Evidence of rabies infection could not be detected in the course of the first nine post-inoculation days. Negative results were also obtained on the 12th and 16th post-inoculation days. Detectable amounts of rabies virus were uncovered in the brain tissue of only one experimental animal on the 15th, 17th, 18th and 20th post-inoculation days; in that of two experimental animals on the 13th, 19th, 21st and 22nd post-inoculation days; in that of three experimental animals on the 10th, 14th and 25th post-inoculation days; in that of four experimental animals on the 11th, 23rd and 24th post-inoculation days. It is noteworthy that every one of the 19 brains in which Negri bodies were found following microscopic examination by standard impression smear technique also yielded rabies virus when examined for evidence of infectivity for mice.

The presence of rabies viral antigen was demonstrated by the fluorescent antibody technique in the brain tissue of 36 or 23.3 per cent of the experimental animals inoculated by the intra-muscular route. Such results, however, are quite inferior to those obtained by the intra-cranial route of infection where they reached 86.6 per cent. Evidence of rabies infection could not be established earlier by this method than by microscopic examination by standard impression smear technique or by examination for evidence of infectivity for mice. Negative results were obtained in the course of the first nine post-inoculation days as

well as on the 12th and 15th post-inoculation days. Rabies viral antigen was demonstrated in the brain tissue of only one experimental animal on the 14th and 17th post-inoculation days respectively; in that of two experimental animals on the 13th, 16th, 18th, 20th, 21st and 22nd post-inoculation days; in that of three experimental animals on the 10th, 11th and 19th post-inoculation days; in that of four experimental animals on the 23rd and 24th post-inoculation days and in that of every experimental animal on the 25th post-inoculation day. In summary, rabies viral antigen was demonstrated by the fluorescent antibody technique in 20 Negri-negative brains, 7 of which also failed to yield rabies virus by the mouse infectivity test. On the other hand, fluorescent rabies viral antigen could not be demonstrated in four brains found positive by the mouse inoculation test. Three of these brains had also disclosed Negri bodies in Williams' stained impression smears.

Salivary glands

Table V shows that Negri bodies were not found in any of the salivary glands following microscopic examination by standard impression smear technique. Four, that is 3.2 per cent of 124 experimental animals proved to be excreting detectable amounts of rabies virus in salivary gland tissue, as shown by the examination for evidence of infectivity for mice. These results are in close agreement with the ones obtained by the intra-cranial route of infection in which 5.0 per cent of the salivary glands

TABLE V

RESULTS OF EXAMINATIONS OF SALIVARY GLANDS FROM
MICE INOCULATED BY THE INTRA-MUSCULAR ROUTE

Animal number	Days after inoculation											
	1	2	3	4	5	6	7	8	9	10	11	12
Microscopic examination by standard impression smear technique												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
Examination for evidence of infectivity for mice												
1	-	-	-	-	-	-	-	-	-	+	-	-
2	-	-	-	-	-	-	-	-	-	-	+	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
Microscopic examination by fluorescent antibody technique												
1	-	-	-	-	-	-	-	-	-	+	-	-
2	-	-	-	-	-	-	-	-	-	-	+	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-

(continued)

TABLE V.- RESULTS OF EXAMINATIONS OF SALIVARY GLANDS FROM MICE INOCULATED BY THE INTRA-MUSCULAR ROUTE (continued)

Animal number	Days after inoculation												
	13	14	15	16	17	18	19	20	21	22	23	24	25
Microscopic examination by standard impression smear technique													
1	-	-	-	-	-	-	-	-	-	-	-	ND	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-
Examination for evidence of infectivity for mice													
1	-	-	-	-	-	-	-	-	+	-	-	ND	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-
Microscopic examination by fluorescent antibody technique													
1	-	-	-	-	-	-	-	-	+	-	-	ND	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-

- = Negative
 + = Positive
 ND = Not Done due to cannibalism

yielded rabies virus when tested for evidence of infectivity for mice. In contrast to what was experienced by the intra-cranial route of infection, rabies viral antigen could be demonstrated, although in small amounts, in those salivary glands which were found to excrete detectable amounts of rabies virus by the mouse infectivity test. As noted in mice infected by the intra-cranial route, only one excreted virus both in salivary glands and saliva.

Saliva

As shown in Table VI, examination for evidence of infectivity for mice was the only method investigated in this case. Since saliva could not be obtained from 19 moribund or dead experimental animals in the course of the last six post-inoculation days as well as from one that died on the 17th post-inoculation day, only 105 samples were tested. Of this number, two, that is 1.9 per cent, proved to contain detectable amounts of rabies virus. Such findings are in close agreement with those obtained by the intra-cranial route of infection where 2.0 per cent of the experimental animals were found to excrete rabies virus in saliva. It might be pointed out that virus was isolated from the saliva of a mouse, but not from its salivary glands, on the 11th post-inoculation day.

TABLE VI

RESULTS OF EXAMINATION OF SALIVA FROM MICE INOCULATED BY THE INTRA-MUSCULAR ROUTE

Animal number	Days after inoculation																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
	Examination for evidence of infectivity for mice																									
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = Negative
 + = Positive
 ND = Not Done

Field Specimens

Domestic animals

The results of examinations of field brain specimens from domestic animals are shown in Table VII. A total of 73 brains collected from 20 bovines, 15 cats, 20 dogs, 6 horses, 8 pigs and 4 sheep were examined by the three methods used in the course of our experimental studies on mice.

Bovines - Negri bodies were found in the hippocampal tissue of only two bovine brains, following microscopic examination by standard impression smear technique. Examination for evidence of infectivity for mice, however, resulted in the isolation of rabies virus not only from the two brains in which Negri bodies were found, but also from six others. Rabies viral antigen could be demonstrated by the fluorescent antibody technique from only seven brains, since negative results were obtained from one that otherwise yielded rabies virus when inoculated into mice. From these results, it can be concluded that the examination for evidence of infectivity for mice was the most sensitive method of examination. It is also noteworthy that every rabid bovine died of the dumb or paralytic form of rabies.

Cats - Of the 15 cat brains studied, none disclosed Negri bodies when examined microscopically by the standard impression smear technique. However, examination for evidence of infectivity for mice resulted in the isolation of rabies virus

TABLE VII

RESULTS OF EXAMINATIONS OF FIELD BRAIN
SPECIMENS FROM DOMESTIC ANIMALS

Animals	Methods of examination			Remarks
	Impression smears	mouse inoculation	fluorescent staining	
Bovines # 1	-	-	-	
2	N.D.	-	-	
3	-	-	-	
4	-	+	-	D.D.R.
5	+	+	+	D.D.R.
6	-	-	-	
7	-	-	-	
8	-	-	-	
9	-	-	-	
10	N.D.	-	-	
11	-	-	-	
12	-	-	-	
13	-	+	+	D.D.R.
14	-	-	-	
15	?	-	-	
16	-	+	+	D.D.R.
17	+	+	+	D.D.R.
18	-	+	+	D.D.R.
19	-	+	+	D.D.R.
20	-	+	+	D.D.R.

(continued)

TABLE VII. - RESULTS OF EXAMINATIONS OF FIELD BRAIN SPECIMENS FROM DOMESTIC ANIMALS (continued)

Animals	Methods of examination			Remarks
	Impression smears	mouse inoculation	fluorescent staining	
Cats # 1	-	+	+	Killed
2	-	-	-	
3	-	-	-	
4	-	-	-	
5	-	-	-	
6	-	+	+	K.F.R.
7	-	+	+	K.F.R.
8	-	-	-	
9	-	+	+	D.F.R.
10	-	+	+	K.F.R.
11	-	+	+	K.F.R.
12	-	-	-	
13	?	-	-	
14	-	-	-	
15	-	-	-	

(continued)

TABLE VII. - RESULTS OF EXAMINATIONS OF FIELD BRAIN SPECIMENS FROM DOMESTIC ANIMALS (continued)

Animals	Methods of examination			Remarks
	Impression smears	mouse inoculation	fluorescent staining	
Dogs # 1	-	-	-	
2	-	+	+	D.F.R.
3	-	-	-	
4	-	+	-	K.F.R.
5	-	-	-	
6	-	-	-	
7	-	-	-	
8	-	-	-	
9	-	+	+	K.F.R.
10	-	+	-	D.D.R.
11	-	+	+	D.F.R.
12	-	+	+	K.D.R.
13	-	+	-	D.D.R.
14	?	-	-	
15	-	+	+	D.F.R.
16	-	+	+	D.D.R.
17	-	+	+	
18	-	+	+	D.D.R.
19	-	+	+	K.D.R.
20	+	+	+	D.D.R.

(continued)

TABLE VII. - RESULTS OF EXAMINATIONS OF FIELD BRAIN SPECIMENS FROM DOMESTIC ANIMALS (continued)

Animals	Methods of examination			Remarks
	Impression smears	mouse inoculation	fluorescent staining	
Horses # 1	-	+	+	D.F.R.
2	-	+	+	K.D.R.
3	-	-	-	
4	-	+	+	D.D.R.
5	-	+	+	D.D.R.
6	-	+	+	D.F.R.
<hr/>				
Pigs # 1	-	-	-	
2	-	+	+	D.D.R.
3	-	+	+	D.D.R.
4	-	+	+	D.D.R.
5	-	+	+	D.D.R.
6	+	+	+	D.F.R.
7	-	+	+	D.F.R.
8	-	+	+	D.F.R.
<hr/>				
Sheep # 1	-	+	+	D.D.R.
2	-	-	-	
3	-	+	+	
4	-	-	-	
<hr/>				
D.D.R.	Died Dumb Rabies			
K.D.R.	Killed Dumb Rabies			
D.F.R.	Died Furious Rabies			
K.F.R.	Killed Furious Rabies			
N.D.	Not Done due to putrefaction			
?	Inconclusive results			

from six of these brains. Rabies viral antigen was also demonstrated by the fluorescent antibody technique from the same brains. Whereas the first method of examination completely failed to detect evidence of rabies infection in the cat brains studied, the second and third methods appeared to be equally sensitive. In contrast to bovines, the rabid cats for which adequate clinical information was available had been suffering from furious rabies.

Dogs - Following microscopic examination by standard impression smear technique, Negri bodies were found in the brain tissue of dog #20 and so-called distemper inclusions, in that of dog #14. It may be pointed out that the latter type of inclusions were very difficult to distinguish from Negri bodies, as it often occurs when distemper susceptible animals submitted to the laboratory for rabies diagnosis are examined by the standard impression smear technique. Examination for evidence of infectivity for mice resulted in the isolation of rabies virus from the brain in which Negri bodies were found and also from 12 additional ones. On the other hand, rabies viral antigen could be demonstrated by the fluorescent antibody technique in only 10 of the 13 brains that yielded rabies virus by the mouse infectivity test. It is noteworthy that the three brains missed by the fluorescent antibody technique had not shown Negri bodies when examined by the standard impression smear technique. From the above results, it is obvious that the mouse inoculation test proved to be the most sensitive method of examination. The

information available for 12 rabid dogs indicated that seven were suffering from dumb or paralytic rabies and five from furious rabies.

Horses - Negri bodies were not found in any of the horse brains, following microscopic examination by standard impression smear technique. However, rabies virus was detected in five brains by the mouse infectivity test. Rabies viral antigen could also be demonstrated by the fluorescent antibody technique in the same five brains. Whereas microscopic examination by standard impression smear technique failed to reveal the presence of Negri bodies, the other two methods of examination proved to be equally sensitive. Three horses were affected with dumb or paralytic rabies and two with furious rabies.

Pigs - Negriogenesis was disclosed in only one out of eight pig brains when they were examined microscopically by the standard impression smear technique. Nevertheless, examination for evidence of infectivity for mice resulted in the isolation of rabies virus from the brain in which Negri bodies were found as well as from six others. Rabies viral antigen was demonstrated by the fluorescent antibody technique in the seven brains that yielded rabies virus. Such results indicate that the last two methods were of equal sensitivity and more reliable than the microscopic examination of Williams' stained impression smears. Four pigs had died of dumb or paralytic rabies and three of furious rabies.

Sheep - Microscopic examination by standard impression smear technique failed to reveal the presence of Negri bodies in any one of the four sheep brains studied. However, examination for evidence of infectivity for mice resulted in the isolation of rabies virus from two brains. These brains also showed rabies viral antigen when examined by the fluorescent antibody technique. Whereas the microscopic examination by standard impression smear technique failed to detect the two rabid sheep, both of the other two methods did so. One sheep had died of dumb rabies, but no clinical information was available for the other animal.

Wild animals

The results of examinations of field brain specimens from wild animals are shown in Table VIII. A total of 27 brains collected from 11 bats, 1 coyote, 3 foxes, 2 raccoons and 5 skunks were examined by the three methods used in the course of our experimental studies on mice.

Bats - Only one out of 11 bat brains exhibited Negri-genesis when examined microscopically by the standard impression smear technique. However, examination for evidence of infectivity for mice resulted in the isolation of rabies virus not only from the brain in which Negri bodies were found, but also from two others. Rabies viral antigen was demonstrated by the fluorescent antibody technique in the three brains that yielded rabies virus. These results indicate that the last two methods of examination were equally sensitive and more so than the microscopic examination by standard impression smear technique. Clinical information was

TABLE VIII

RESULTS OF EXAMINATIONS OF FIELD BRAIN
SPECIMENS FROM WILD ANIMALS

Animals		Methods of examination			Remarks
		impression smears	mouse inoculation	fluorescent staining	
Bats	# 1	-	+	+	
	2	-	-	-	
	3	+	+	+	K.F.R.
	4	-	-	-	
	5	-	-	-	
	6	-	-	-	
	7	-	-	-	
	8	-	-	-	
	9	-	+	+	
	10	-	-	-	
	11	-	-	-	
Coyote	# 1	-	-	-	
Foxes	# 1	+	+	+	Killed
	2	+	+	+	
	3	-	+	+	Killed
	4	-	+	+	Killed
	5	-	+	+	Killed
	6	-	-	-	
	7	-	-	-	
	8	-	+	+	Killed
Raccoons	# 1	-	-	-	
	2	-	-	-	
Skunks	# 1	+	+	+	K.F.R.
	2	+	+	+	K.F.R.
	3	+	+	+	
	4	-	-	-	
	5	+	+	+	Found Dead

K.F.R. Killed Furious Rabies

available in one case and indicated that the bat had been killed when exhibiting symptoms of furious rabies.

Coyote - Only one coyote brain was studied and negative results were obtained by the three different methods of examination.

Foxes - Negri bodies were found in two out of eight fox brains examined microscopically by the standard impression smear technique. However, the mouse infectivity test resulted in the isolation of rabies virus from the two Negri-positive brains and also from four additional ones. Rabies viral antigen was demonstrated by the fluorescent antibody technique in every brain that yielded rabies virus. These results bring out the higher sensitivity of the last two methods as compared with the microscopic examination by standard impression smear technique. No information was available as to whether the foxes found rabid had exhibited symptoms of furious or dumb rabies.

Raccoons - Two raccoon brains were studied by the three different methods of examination with negative results.

Skunks - Out of five skunk brains studied, four showed evidence of rabies infection regardless of the method of examination employed. Therefore, skunks were the only animal species in which the three different methods of examination proved to be equally sensitive. Two rabid skunks were reported as having been killed when showing symptoms of furious rabies; one was found dead, whereas adequate information was missing for the other.

DISCUSSION

Mice have been experimentally infected with a strain of street rabies virus: some by the intra-cranial route and others by the intra-muscular route. The results obtained from this comparative study are listed in Tables I to VI and indicate beyond doubt that the first route of infection is more sensitive than the second. In this respect, our findings closely agree with the ones previously obtained in other experimental laboratory animals (57). They also substantiate the opinion that rabies virus is strictly neurotropic (36, 196).

From this study, it is also obvious that samples of brain tissue from experimental mice yielded rabies virus earlier and much more regularly than those of salivary glands or saliva. Moreover, the presence of rabies virus was never confirmed in salivary glands and/or saliva of a mouse without being also demonstrated in its brain. It is noteworthy that so-called "closed rabies" has been found by Thiery (133) to be of frequent occurrence in mice infected with street rabies virus. It has not been determined, however, whether the virus fails to reach the salivary glands or does so and is later destroyed as a result of an auto-sterilization process. Furthermore, it has already been demonstrated that in rabid animals other than mice, rabies virus is not always excreted in salivary glands (64, 65, 66). Our failure to demonstrate detectable amounts of rabies virus in the saliva of certain mice whose salivary glands had given positive results when examined by the mouse infectivity test parallels the findings obtained by Sikes (66) in experimentally infected foxes and skunks.

Of the three methods employed for the examination of tissues from experimental mice, the standard impression smear technique appeared to be the less sensitive. In fact, Negri bodies were found in only 29.2 per cent of the brains and in none of the salivary glands. It must be born in mind, however, that Negri bodies must first reach a certain stage of development before they can be identified properly. Otherwise they could easily be mistaken for the so-called "mouse confusion bodies" which are often normally present in mouse brain tissue (149). It is true that in previous studies Negri bodies have been found relatively early in the brain tissue of mice experimentally infected with street rabies virus, but they could not be demonstrated earlier than by the sixth post-inoculation day (10, 190). Our complete failure to find Negri bodies in impression smears from salivary glands may be due to the fact that these inclusions are said to occur only in the cytoplasm of the nervous cells of the ganglia normally present in the interstitial tissue of salivary glands (139). Furthermore, mouse salivary glands are rather small and a good portion of each had to be used for the examination for evidence of infectivity for mice.

Mouse infectivity tests detected the presence of rabies virus in brain and salivary gland samples in the following proportions: 41.5 per cent and 3.8 per cent. These results confirm the greater sensitivity of the mouse test in comparison with the

microscopic examination by standard impression smear technique where the corresponding percentages were 29.2 and 0.

Rabies viral antigen was demonstrated by the fluorescent antibody technique in 47.5 per cent of the brains and in 2.2 per cent of the salivary glands from experimental mice. Therefore, this method of examination appeared to be somewhat more sensitive than either of the others for the detection of rabies in the brain tissue. For the examination of salivary glands, however, the method appeared to be slightly less sensitive than mouse inoculation, but more so than the standard microscopic examination. The greatest advantage of the fluorescent antibody technique over the standard impression smear technique lies in the fact that specifically stained minute rabies virus aggregates, or so-called "dust particles", as well as the Negri bodies can be visualized by the former, whereas only Negri bodies can be seen by the latter. The possibility of demonstrating dust particles by the fluorescent antibody technique was particularly evident in specimens taken during the first post-inoculation days before Negri bodies had yet appeared in the brain tissue of mice infected by the intra-cranial route. As Negri bodies became demonstrable by the standard impression smear technique, they could also be shown by the fluorescent antibody technique along with more numerous dust particles. Rabies viral antigen could also be demonstrated by the fluorescent antibody technique in some of the infected salivary glands, but always in small amounts and only in the form of dust particles.

It should be pointed out that certain initial difficulties had to be overcome when the examination of tissues by the fluorescent antibody technique was undertaken. In order to obtain a satisfactory inhibition on control impression smears, fixed rabies virus (strain CVS-56) had to be used instead of street rabies virus for the infection of mice intended for the preparation of rabid mouse brain suspensions. It was also discovered that impression smears stained with fluorescein tagged antibody could not be examined satisfactorily with a combination of exciter filter BG 12 and barrier filters OG4-OG4. With this kind of illumination, fluorescent rabies viral antigen could be visualized as green spots against a black background, but it was soon suspected that certain artefacts were also reacting to the stain in such a way as to be confused with specific lesions. Fortunately, a shift to barrier filters BG23 and OG4, or to OG4 alone, made it possible to differentiate specific lesions from these misleading artefacts. As a result of the modifications in filters, specific lesions containing rabies virus antigen combined with fluorescent rabies antibody retained their greenish coloration, whereas confusing artefacts appeared as bright reddish-blue spots against a blue background.

As has been shown in Table I, positive findings were obtained earlier by the fluorescent antibody technique than by the examination for evidence of infectivity for mice. This discrepancy may be explained by the fact that shortly after the intra-cranial infection of mice, the rabies virus has been found

to penetrate into the susceptible cells and undergo an eclipse phase during which infectious particles break down to non-infectious smaller units (196). It is presumed that the dust particles observed in the brain tissue of experimental mice sacrificed within a few days after infection would represent such small non-infectious units of virus.

The failure of the fluorescent antibody technique to demonstrate rabies viral antigen in certain proven infected brains and salivary glands, Tables II and IV, may stem from a lack of experience on the part of the operator at the time the examination was made. However, it may also have been due to an irregular distribution of rabies virus in infected brains and salivary glands or to the auto-sterilization of these tissues. The last two possibilities have been reported as occurring in naturally infected animals and humans (195, 171, 87). Furthermore, although the impression smears intended for microscopic examination by the standard impression smear technique and by the fluorescent antibody technique were prepared from the same piece of brain or salivary gland tissue, a piece from a different area of each tissue was used for the mouse infectivity test.

The results of examinations of 100 field brain specimens from domestic and wild animals indicate that microscopic examination by the standard impression smear technique was the least sensitive by far of the conventional methods of diagnosing rabies. Whereas evidence of infection could be detected in only 11 field specimens by this method, it was demonstrated in 54 by the examination for

evidence of infectivity for mice, and in 50 by the fluorescent antibody technique. Many factors are known to account for the absence of Negri bodies in the brain tissue of naturally infected animals. For example, it has been reported that approximately 5 to 15 per cent of rabid animals fail to develop a Negriogenesis (6, 7, 8, 9). It is also known that animals suffering from dumb or paralytic rabies are not likely to show Negri bodies in the brain tissue (134). Animals killed early in the clinical course of the disease as well as those sacrificed or dying of rabies following immunization against the disease do not usually disclose a Negriogenesis (141, 7). Of the 100 fixed specimens examined in the course of this study, 20 had died of dumb rabies and three had been killed when exhibiting symptoms of dumb rabies. Nine had died of furious rabies and eight had been killed when exhibiting symptoms of furious rabies. It is noteworthy that Negri bodies were found in the brain of only one animal having died of furious rabies. Unfortunately, adequate clinical information was missing for the remaining 14 rabid animals.

Difficulties were also encountered because of the presence of non-specific inclusions in Gilliam's stained brain impression smears from certain field specimens. For example, such inclusions were seen in the ones from bovine No. 15. Whether these inclusions had developed as a result of other viral infections such as mucosal disease or malignant catarrhal fever could not be ascertained (143). Misleading inclusions were also seen in impression smears from the brain tissue of cat No. 13 and dog No. 14. Because of the failure to demonstrate rabies virus in the two brains by mouse inoculation

or by the fluorescent antibody technique, it was surmised that these inclusions were non-specific in the case of the cat and attributable to distemper in the case of the dog.

The microscopic examination for evidence of infectivity for mice proved to be the most sensitive of the three methods employed for the detection of rabies infection in field specimens. However, it had to be repeated with additional brain material from dog No. 12 and pig No. 5 in order to confirm positive results obtained by the fluorescent antibody technique. This finding emphasizes the importance of including in the inoculum pieces of tissue from various regions of the brain on account of the possible irregular distribution of the virus in the brain of rabid animals.

Rabies viral antigen could not be demonstrated by the fluorescent antibody technique in the brain tissue of bovine No. 4 or dogs Nos. 4, 10 and 13. On the other hand, examination for evidence of infectivity for mice revealed the presence of rabies virus in each of these four specimens. As suggested earlier in the discussion of the results of comparative examinations of the brains of experimentally infected animals, this discrepancy may have been traceable to my inexperience with the fluorescent antibody technique at the time or to an irregular distribution of virus in the material. Unfortunately, additional brain tissue was not available from these animals except for dog No. 13 from which negative results were obtained following an additional examination.

In spite of these discrepancies, we are of the opinion that the fluorescent antibody technique presented certain advantages over the other two methods of examination. Of particular importance was the fact that rabies viral antigen could be demonstrated almost exclusively in the form of fluorescent dust particles in the brain tissue of 39 animals which had failed to show Negri bodies when examined by the standard impression smear technique. It is noteworthy that both dust particles and Negri bodies could be demonstrated in larger numbers in the brain tissue of the 11 animals found Negri-positive following microscopic examination by the standard impression smear technique. Furthermore, the inclusions seen in William's stained impression smears from the brain tissue of bovine No. 4, cat No. 13 and dog No. 14 could be definitely considered as non-specific since they were not stained by fluorescein tagged rabies antibody. It is true that four out of 54 field brain specimens that yielded rabies virus on mouse inoculation were missed when examined by the fluorescent antibody technique. Nevertheless, a diagnosis of rabies could be confirmed in 50 field specimens within 24 hours of their submission to the laboratory. This represents an obvious advantage over the mouse inoculation test that requires an average time of 10 to 15 days. As we are aiming at a specific and rapid diagnosis on field specimens, particularly those implicated with human contamination, it seems that the use of the fluorescent antibody technique offers encouraging possibilities. Where there is a history of potential human infection, it will still be necessary, however, to inoculate mice with brain material from specimens

that prove to be negative by the fluorescent antibody technique.

SUMMARY

A study was undertaken to determine the route of inoculation, the tissue and the method of laboratory examination which would be most satisfactory for the early demonstration of infection following inoculation of laboratory animals with street rabies virus.

A strain of virus was obtained from a field case of bovine rabies and re-passaged in a calf. A suspension prepared from brain tissue of this animal was titrated in mice and used as challenge material.

Swiss white mice were selected as experimental animals. In the first part of the investigation, individual groups of five mice were inoculated intracranially and then sacrificed at daily intervals until they died of the infection. The second part differed from the first only in the route of infection, mice being inoculated into the cervical musculature. The sensitivity of each route of inoculation was compared as to the time of appearance of Negri bodies and/or infectivity in the brain and salivary glands. When obtainable, saliva samples were also examined by the mouse infectivity test.

Three laboratory methods were employed and their sensitivity compared: a) microscopic examination by standard impression smear technique, b) examination for evidence of infectivity for mice, and c) microscopic examination by fluorescent antibody technique.

The relative sensitivity of these three laboratory methods was also evaluated in the examination of 100 field brain specimens from domestic and wild animals.

REFERENCES

1. MacCALLUM, F.O. Virus and rickettsial diseases. Second ed. Edward Arnold Ltd., London, 296-304, 1955.
2. PASTEUR, L., CHAMBERLAND, ROUX et TRUILLIER. Sur la rage. C.R. Acad. Sci. 92: 1259-1260, 1881.
3. PASTEUR, L. Méthode pour prévenir la rage après morsure. C.R. Acad. Sci. 101: 765-774, 1885.
4. REMLINGER et RIFAT-BEY. Le virus rabique traverse la bougie Berkefeld. C.R. Soc. Biol. 55: 730-731, 1903.
5. NEGRI, A. Beitrag zum studium der aetiologie der tollwuth. Zeitschr. Hyg. Infektkr. 43: 507-528, 1903.
6. NEGRI-LUZZIANI, L. Le diagnostic de la rage par la démonstration du parasite spécifique. (Résultats de dix ans d'expérience). Ann. Inst. Pasteur, 27: 907-923, 1039-1062, 1913.
7. LEACH, C.W. Comparative methods of diagnosis of rabies in animals. Amer. J. Pub. Hlth. 28: 162-166, 1938.
8. DAWSON, S.E. and SELLERS, T.F. A note on the probability of error in the diagnosis of rabies by microscopic search for Negri bodies. J. Lab. Clin. Med. 27: 71-74, 1941.
9. JOHNSON, H.N. The significance of the Negri body in the diagnosis and epidemiology of rabies. Ill. Med. J. 81: 382-388, 1942.
10. WEBSTER, L.T. and DAWSON, J.H. Early diagnosis of rabies by mouse inoculation. Measurement of humoral immunity to rabies by mouse protection test. Proc. Soc. Exper. Biol. Med. 32: 570-573, 1935.
11. HOARE, E.W. A system of veterinary medicine. Bailliere, Tindall and Cox, London, Vol. I, 599-630, 1913.
12. HULL, T.G. Diseases transmitted from animals to man. Fourth ed. Charles C. Thomas, Springfield, Ill., U.S.A., 250-280, 1955.
13. RIVERS, T.M. and HORSFALL, F.L. Viral and rickettsial infections of man. Third ed. J.B. Lippincott Company, Philadelphia, Montreal, 405-431, 1959.
14. HUTYMA, P., MAREK, J. and MANNINGER, R. Special pathology and therapeutics of the diseases of domestic animals. Fourth ed. Bailliere, Tindall and Cox, London, Vol. I, 525-555, 1938.

15. MCKENDRICK, A.G. Rabies. A review of recent articles. Trop. Dis. Bull. 30: 575-587, 1933.
16. DODLEY, F.W. The prevalence of hydrophobia in the Philippine Islands. J. Amer. Med. Assoc. 51: 2143-2149, 1903.
17. NICOLAU, S., MATHIS, C. et CONSTANTINESCO, V. La rage autochtone (maladie du chien fou) en Afrique Occidentale Francaise. (étude critique et expérimentale). Ann. Inst. Pasteur, 50: 778-839, 1933.
18. PLUMMER, P.J.G. Rabies in Canada, with special reference to wildlife reservoirs. Bull. Wld. Hlth. Org. 10: 767-774, 1954.
19. LASSEN, H.C.A. Paralytic human rabies in Greenland. The Lancet. i: 247-249, 1962.
20. CHADWICK, V.D. Report of the committee on rabies. Proc. 53th Ann. Meet. U.S. Livestock Sanit. Assoc., 357-362, 1954.
21. GARINI, A. Sur une grande epizootie de rage. Ann. Inst. Pasteur, 25: 343-346, 1911.
22. PAVAN, J.L. The transmission of paralytic rabies in Trinidad by the vampire bat (*Desmodus rotundus murinus*, Wagner 1840). Ann. Trop. Med. Parasitol. 30: 101-130, 1936.
23. METVIER, H.V.M. Paralytic rabies in livestock. J. Comp. Path. and Therap. 48: 245-260, 1935.
24. HURST, H.W. and PAVAN, J.L. An outbreak of rabies in Trinidad (without history of bites, and with the symptoms of acute ascending myelitis). The Lancet, ii: 622-623, 1931.
25. WILLIAMS, H.S. Bat transmitted paralytic rabies in Trinidad. Can. Vet. J. 1: 20-24, 45-50, 1960.
26. SMIGHT, J.S. Bats and their relation to rabies. Ann. Rev. Microbiol. 10: 369-392, 1956.
27. TIEMBL, E.S. Advances in Vet. Sci. Vol. V, Academic Press Inc., New York and London, 183-227, 1959.
28. WENTERS, H.D., ROFFERT, W.R., SCOTT-DUNAY, J.W. and HARDY, A.V. Rabies in bats in Florida. Amer. J. Pub. Hlth. 44: 182-189, 1954.
29. C.D.C. Veterinary Public Health News Letter, Atlanta, Georgia, April 1963.
30. C.D.C. Veterinary Public Health News Letter, Atlanta, Georgia, August 1963.

31. Anon. Bat rabies. J. Amer. Vet. Med. Assoc. 142: 750, 1963.
32. ENRIGHT, J.B. Geographical distribution of bat rabies in the United States, 1953-1960. Amer. J. Pub. Hlth. 52: 484-488, 1962.
33. BUNKE-GAFFNEY, R.J. O'D. Rabies. Trop. Dis. Bull. 52: 764, 1955.
34. NIKOLIC, M. and JELISIC, Z. Isolation of rabies virus from insectivorous bats in Yugoslavia. A preliminary report. Bull. Wld. Hlth. Org. 14: 301-304, 1956.
35. AVSEY, R.J. and TAILLOUR, J.M. The isolation of the rabies virus from insectivorous bats in British Columbia. Can. J. Comp. Med. 24: 143-146, 1960.
36. BEAUREGARD, M. and STEWART, R.C. Bat rabies in Ontario. Can. J. Comp. Med. 28: 43-45, 1964.
37. NICOLAU, S., DEIANCESCO-NICOLAU, O. et GALLOWAY, I.A. Etude sur les septinévrites à ultravirus neurotropes. Ann. Inst. Pasteur, 43: 1-88, 1929.
38. PAGAN, R. Rabies, an epidemiological analysis. Proc. Book 89th Ann. Meet. Amer. Vet. Med. Assoc. 394-407, 1952.
39. LEPINE, P. Advances in the control of zoonoses. Wld. Hlth. Org. Mono. Ser. No. 19, Geneva, 215-225, 1953.
40. PASTEUR, CHAMBERLAND et ROUX. Nouvelle communication sur la rage. C.R. Acad. Sci. 98: 457-463, 1884.
41. PASTEUR, CHAMBERLAND et ROUX. Sur la rage. C.R. Acad. Sci. 98: 1229-1231, 1884.
42. HAYENS, L.C. and MAYFIELD, C.R. Antigenic properties of rabies virus. III. Composition of serologic variants and nature of fixed virus. J. Infect. Dis. 52: 364-373, 1933.
43. MOHLER, J.R. Rabies or Hydrophobia. Farmers' Bull. 449, U.S. Dept. of Agriculture, May 12, 1911.
44. REMLINGER, P. Les travaux récents sur la rage. Bull. Inst. Pasteur, 2: 793-806, 1902.
45. REMLINGER, P. Les travaux sur la rage pendant ces dix dernières années. Arch. Inst. Past. Algérie, 13: 310-350, 1924.
46. KONFADI, D. Hérité de la rage. Ann. Inst. Pasteur, 30: 33-48, 1916.

47. MIMS, R.A., ALLEN, R. and SULKIN, S.E. Studies on the pathogenesis of rabies in insectivorous bats. III. Influence of the gravid state. *J. Infect. Dis.* 112: 17-27, 1963.
48. CONSTANTINE, D.G. Rabies transmission by nonbite route. *Pub. Hlth. Repts.* 77: 287-289, 1962.
49. BELL, J.F., BUNGDORFER, W. and MOORE, G.J. The behavior of rabies virus in ticks. *J. Infect. Dis.* 100: 278-283, 1957.
50. STIMSON, A.M. Facts and Problems about rabies. *Bull. No. 65*, Hyg. Lab. Washington, U.S.A., June, 1910.
51. CANALEIA. Etude sur la rage paralytique chez l'homme. *Ann. Inst. Pasteur*, 1: 63-83, 1887.
52. GIBBONS, W.J. Diseases of the nervous system. *Vet. Med.* 43: 139-142, 1948.
53. HAGAN, W.A. and BRUNER, D.W. The infectious diseases of domestic animals. Fourth ed. Comstock Publishing Company, Inc. Ithaca, New York, 769-790, 1961.
54. RAYMOND, F. Some notes on rabies. *J. Trop. Vet. Sci.* 4: 275-285, 1909.
55. REMLINGER, P. et BAILLY, J. La rage du coq. *Ann. Inst. Pasteur*, 43: 153-167, 1929.
56. REMLINGER, P. Revue. Les travaux récents sur la rage. *Bull. Inst. Pasteur*, 2: 753-764, 1904.
57. PASTEUR, L., CHAMBERLAND, ROUX et THUILLEB. Nouveaux faits pour servir à la connaissance de la rage. *C.R. Acad. Sci.* 95: 1187-1192, 1882.
58. REMLINGER, P. et EFFENDI, M. Deux cas de guérison de la rage expérimentale chez le chien. *Ann. Inst. Pasteur*, 18: 241-244, 1904.
59. ROUX et NOCARD. A quel moment le virus de la rage apparaît-il dans la bave des animaux enragés? *Ann. Inst. Pasteur*, 4: 163-171, 1890.
60. REMLINGER, P. Persistence du virus dans la salive du chien guéri de la rage. *Bull. Inst. Pasteur*, 5: 586, 1907.

61. BEQUIGNON, R., LAMY, R. et VIALAT, C. Contribution à l'étude de la rage. I. De la virulence de la rage fixe par voie sous-cutanée après addition de bave de chien normal. Ann. Inst. Pasteur, 76: 283-285, 1949.
62. BEQUIGNON, R., SUSSARD, A. et VIALAT, C. Contribution à l'étude de la rage. II. De la virulence de la rage fixe par voie sous-cutanée après addition de hyaluronidase. Ann. Inst. Pasteur, 76: 285-286, 1949.
63. HAROUJIAN, Y. Le mécanisme de l'infection de la salive au cours de la rage. Ann. Inst. Pasteur, Suppl.: Numéro commémoratif sur la rage. 97-107, 1935.
64. MCKENDRICK, A.G. Rabies: A review of recent articles. Trop. Dis. Bull. 21: 703-711, 1924.
65. VEERARACHAVAN, N., BALASUBRAMANIAN, A. and KANGASWAMI, N. Virus content of brains and submaxillary glands and occurrence of Negri bodies in animals suspected of having died of natural rabies. Bull. Wld. Hlth. Org. 18: 469-471, 1958.
66. SIKES, R.K. Pathogenesis of rabies in wildlife. I. Comparative effect of varying doses of rabies virus inoculated into foxes and skunks. Amer. J. Vet. Res. 23: 1041-1047, 1962.
67. DAVIES, B. Ottawa: portrait of a capital. McGraw-Hill Company of Canada Limited, 26-29, 1954.
68. WELLS, K.P. The rabies menace in Canada. Can. J. Pub. Hlth. 48: 239-243, 1957.
69. McLEAN, D.M., KRAUSE, V.H., WILSON, W.M. and DAWKIN, L.A. Rabies following skunk bite. Can. Med. Assoc. J. 82: 315-317, 1960.
70. ROYNERAN, W.A. Personal communication.
71. HUMPHREY, G.L., KEMP, G.E. and GOOD, D.G. A fatal case of rabies in a woman bitten by an insectivorous bat. Pub. Hlth. Repts. 75: 317-326, 1960.
72. REYER, F.K. Man contracting rabies from man. J. Amer. Med. Assoc. 165: 158-159, 1957.
73. JARIS, A. Hygiène, prophylaxie, désinfection. Bull. Inst. Pasteur, 2: 405, 1907.
74. REMLINGER, P. et BAILLY, J. Contribution à l'étude du passage du virus rabique dans le lait. C.R. Soc. Biol. 110: 239-241, 1932.
75. JOHNSON, N.W. Rabies (Oxford loose-leaf medicine). New York, Oxford University Press, 1943.

76. NICOLAS, J. De la non-virulence du lait des animaux rabiques ou soumis à des injections intra-veineuses de virus rabique. *J. Méd. Vét. Zootech.* 56: 721-726, 1905.
77. SCHOSNING, H.S. *The Yearbook of Agriculture*, Washington, D.C., 195-202, 1956.
78. COURMONT, J. et PANISSKY, L. Précis de microbiologie des maladies infectieuses des animaux. Octave Doin et Fils, Ed., 8, Place de l'Odeon, Paris, 860-893, 1914.
79. LEFINI, P. et CRUVEILLIER, L. Cinquante années de vaccinations antirabiques à l'Institut Pasteur. *Ann. Inst. Pasteur, Suppl.*: Numéro commémoratif sur la rage, 18-32, 1935.
80. HABEL, K. Rabies prophylaxis in man. *Pub. Hlth. Repts.* 72: 667-673, 1957.
81. REMLINGER, P. Accidents paralytiques au cours du traitement antirabique. *Ann. Inst. Pasteur*, 19: 625-646, 1905.
82. REMLINGER, P. La rage dite de laboratoire. *Ann. Inst. Pasteur, Suppl.*: Numéro commémoratif sur la rage. 35-63, 1935.
83. STUART, G. and KRIBORIAN, K.S. The neuroparalytic accidents of anti-rabies treatment. *Ann. Trop. Med. Parasitol.* 22: 327-377, 1928.
84. OLITSKI, P.K. and TAL, O. Acute disseminated encephalomyelitis produced in mice by brain proteolipide (Volch - Lees). *Proc. Soc. Exper. Biol. Med.* 79: 50-53, 1952.
85. VOLCH, J. and LEES, M. Proteolipides, a new type of tissue lipoproteins. Their isolation from brain. *J. Biol. Chem.* 191: 807-817, 1951.
86. REMLINGER, P. Contribution à l'étude de la "rage de laboratoire". Episodes de Fortaleza et de Beyrouth. *Arch. Inst. Pasteur d'Algérie*, 39: 254-260, 1961.
87. LEVADITI, C., BANCHIS - BAYARRI, V. et SCHONN, R. Neuro-infections auto-stérilisables. (Encéphalite, herpès, rage). *C. R. Soc. Biol.* 98: 911-914, 1928.
88. REMLINGER, P. et BAILLY, J. La rage et les neuro-infections auto-stérilisables. *C. R. Soc. Biol.* 102: 296-299, 1929.
89. KOPROWSKI, H. and COX, H.S. Studies on chick-embryo adapted virus. I. Culture characteristics and pathogenicity. *J. Immunol.* 60: 533-554, 1948.

90. KOPROWSKI, H. and BLACK, J. Studies on chick-embryo adapted rabies virus. II. Pathogenicity for dogs and use of egg-adapted strains for vaccination purposes. *J. Immunol.* 64: 185-196, 1950.
91. FOX, J.P., CONWELL, D.P. and GERHARDT, P. Immunization of man with living avianized rabies virus. (Flury strain). *California Vet.* 3: 20-24, 1955.
92. HURSEBORN, J.M., BLACK, J. and SHARPISS, G.R. Primary anti-rabies immunization of man with H. E. P. Flury virus vaccine. *Amer. J. Pub. Hlth.* 51: 706-716, 1961.
93. KOMAROV, A. and HORNSTEIN, K. Studies on the pathogenicity of an avianized street rabies virus. *Cornell Vet.* 43: 344-361, 1953.
94. POWELL, H.M. and CULBERTSON, C.G. Cultivation of fixed rabies virus in embryonated duck eggs. *Pub. Hlth. Repts.* 65: 400-401, 1950.
95. POWELL, H.M. and CULBERTSON, C.G. Recent advances in the preparation of antirabies vaccines containing inactivated virus. *Bull. Wld. Hlth. Org.* 10: 815-822, 1954.
96. MACFARLANE, J.C. and CULBERTSON, C.G. Attempted production of allergic encephalomyelitis with duck-embryo suspensions and vaccines. *Can. J. Pub. Hlth.* 45: 28-29, 1954.
97. BETTER, L.W., COHN, M. and DEUTSCH, H.F. Immunological studies of egg white proteins of various species. V. The cross reactions of egg white proteins of various species. *J. Immunol.* 70: 507-513, 1953.
98. RAINE, B. and UNTRACHT, S. Allergy to virus and rickettsial vaccines. I. Allergy to influenza A and B vaccines in children. *J. Amer. Med. Assoc.* 132: 899-905, 1946.
99. SCHNURLENBERGER, P.R., ANDERSON, G.R., RUSSELL, J.H. and WEMKARTH, F.H. Avian embryo rabies immunization. II. A comparison of the antigenicity of high egg-passage and duck-embryo vaccines administered intradermally in man. *Amer. J. Hyg.* 74: 1-6, 1961.
100. GREENBERG, M. and CHILDRESS, J. Vaccination against rabies with duck-embryo and Semple vaccines. *J. Amer. Med. Assoc.* 173: 333-337, 1960.
101. HABEL, K. Seroprophylaxis in experimental rabies. *Pub. Hlth. Repts.* 60: 545-560, 1945.

102. BALTAZARD, M. and GHOESSI, M. Prevention of human rabies. Treatment of persons bitten by rabid wolves in Iran. Bull. Wld. Hlth. Org. 10: 797-803, 1954.
103. BALTAZARD, M. et BAHMANIAR, M. Essai pratique du sérum antirabique chez les mordus par loups enragés. Bull. Wld. Hlth. Org. 13: 747-772, 1955.
104. Expert Committee on Rabies. Third Report. Wld. Hlth. Org. Tech. Rep. Ser. No. 121, Geneva, 1957.
105. SOLOVIEV, V.D. and KOBINSKI, G.D. Local application of antirabies gamma-globulin in dried form for the prevention of rabies. Bull. Wld. Hlth. Org. 26: 777-782, 1962.
106. BURNS, K.F., SHELTON, D.F., LUKEMAN, J.M. and GROCAN, E.W. Cortisone and ACTH impairment of response to rabies vaccine. Pub. Hlth. Repts. 75: 441-445, 1960.
107. Expert Committee on Rabies, Fourth Report. Wld. Hlth. Org. Tech. Rep. Ser. No. 201, Geneva, 1960.
108. BEAUREGARD, M. Unpublished data.
109. SIKES, R.K. Unpublished data.
110. FINCHER, M.C., GIBBONS, W.J., MAYER, K. and PARK, S.E. Diseases of Cattle. American Veterinary Publications, Inc., Evanston, Ill., 646-653, 1956.
111. THIERY, G. La rage en Afrique Occidentale. Ses particularités. Sa contagiosité. Rev. Elev. Méd. Vét. Pays Tropicaux, 12: 27-42, 1959.
112. GEBLINSKA, P. et BAILLY, J. La grenouille est réfractaire à la rage. C. R. Soc. Biol. 105: 189-190, 1930.
113. PAMAN, J.L. Rabies in the vampire bat of Trinidad, with special reference to the clinical course and the latency of infection. Ann. Trop. Med. Parasitol. 30: 401-422, 1936.
114. LANNETTE, E.H., SOAVE, O.A., NARAMURA, K. and KELLOGG, C.H. A fatal human case of rabies following the bite of a rabid bat (*Lasiurus noctivagans*). J. Lab. Clin. Med. 55: 89-93, 1960.
115. SULKIN, S.E. Bat rabies: Experimental demonstration of the "reservoiring mechanism". Amer. J. Pub. Hlth. 52: 489-498, 1962.

116. BROZ, O. and PHAN-TRINH. Contribution au problème du chien "porteur sain" du virus rabique. J. Hyg. Epidemiol. Microbiol. Immunol. 5: 403-408, 1961.
117. AMERICAL, A. et SERIE, C. Etudes expérimentales sur la rage en Ethiopie. Ann. Inst. Pasteur, 93: 475-488, 1957.
118. YURKOVSKY, A.M. Hydrophobia following the bite of apparently healthy dogs. J. Hyg. Epidemiol. Microbiol. Immunol. 6: 73-78, 1962.
119. KANTOROVICH, S.A. The etiology of "madness" in polar animals. Acta Virologica, (English Ed.), 1: 220-228, 1957.
120. RESSLINGER, P. Une courte épidémie de rage chez des rats d'égout. Bull. Acad. Méd. 136: 317-318, 1952.
121. SVET - KOLDAVSKAYA, I.A. Experimental study of the infectivity of salivary glands of laboratory animals in the latent form of street rabies. Acta Virologica, (English Ed.), 2: 228-234, 1958.
122. BABES, V. Sur certains caractères des lésions histologiques de la rage. Ann. Inst. Pasteur, 6: 209-223, 1892.
123. VAN GEBUCHTEN, A. et NELLE, C. Les lésions histologiques de la rage chez les animaux et chez l'homme. Bull. Acad. Roy. Méd. Belgique, 14: 31-66, 1900.
124. GALTIER, V. Etudes sur la rage. C. R. Acad. Sci. 89: 444-446, 1879.
125. Laboratory Techniques in Rabies. Wld. Hlth. Org. Mon. Ser. No. 23, Geneva, 1954.
126. HARRIS, W.A. and EVANS, W.M. The diagnosis of rabies. Rep. XIVth Inter. Vet. Cong., London. Vol.II: 457-464, 1948.
127. PROTHINGHAM, L. Something about glanders and rabies. Cornell Vet. 10: 163-175, 1920.
128. HENZOO, E. Histologic diagnosis of rabies. Arch. Path. 39: 279-280, 1945.
129. LAPI, A., DAVIS, C.L. and ANDERSON, S.A. The Gasserian ganglion in animals dead of rabies. J. Amer. Vet. Med. Assoc. 120: 379-384, 1952.
130. BOIRON, H. et CARAIN, R. Quelques considérations sur un cas de rage humaine observé à Dakar. Bull. Soc. Path. Exot. 46: 178-180, 1953.

131. TUNCHMAN, A.M. and AKERL, I.S. On a histopathologically atypical case of rabies. *Rabies*, 1: 91, 1961.
132. NISHIHA, S. Histopathological studies on the salivary glands of rabid dogs, with special reference to the parotid and mandibular glands. (Appendix on cervical lymph nodes). *Gunma J. Med. Sci.* 4: 117-127, 1955.
133. BERRY, G. Particularites de la rage dans l'Ouest Africain. *Bull. Epizoot. Dis. Afrique*, 7: 265-286, 1959.
134. SERGENT, E. Corps de Negri et rage. *Bull. Inst. Pasteur*, 3: 198-199, 1905.
135. HUNST, E.W. and PANAS, J.L. A further account of the Trinidad outbreak of acute rabic myelitis. *Histology of the experimental disease. J. Path. Bact.* 35: 301-321, 1932.
136. GOODPASTORH, E.W. A study of rabies, with reference to a neural transmission of the virus in rabbits, and the structure and significance of Negri bodies. *Amer. J. Path.* 1: 547-582, 1925.
137. POOR, D.W. Pathological studies in rabies. *Proc. N.Y. Path. Soc.* 4: 101-109, 1904.
138. STEFANESCU, E. La presence des corpuscules de Negri dans les glandes salivaires des chiens enrages. *C. R. Soc. Biol.* 62: 886-888, 1907.
139. MARQUELIAN, Y. Recherches histologiques sur les glandes salivaires dans la rage. De l'existence des corpuscules de Negri dans les ganglions nerveux des glandes salivaires. *Ann. Inst. Pasteur*, 28: 233-237, 1914.
140. LEVADITI, C. et SCHÖEN, R. Les corps de Negri dans le cytoplasme des epitheliums de la cornee. *Ann. Inst. Pasteur*, Suppl: numero commemoratif sur la rage, 69-96, 1935.
141. SERGENT, E. Corps de Negri et rage. *Bull. Inst. Pasteur*, 3: 196, 1905.
142. SALIMONI, A. Actions pathogenes exercees par les microbes. *Bull. Inst. Pasteur*, 4: 220, 1906.
143. MARQUELIAN, Y. Contribution a l'histologie pathologique de la rage a virus fixe. *C. R. Soc. Biol.* 53: 374-376, 1906.
144. NIGLAU, M.S. et KOPCJOWSKA, L. Zone elective pour les corps de Negri chez les lapins morts de rage experimentale a virus fixe. *C. R. Acad. Sci.* 194: 1865-1867, 1932.

145. LEFEBVRE, P. et SAUTER, V. Sur les lésions spécifiques des neurones dans la rage à virus fixe. C. R. Soc. Biol. 119: 805-807, 1935.
146. LEFEBVRE, P. Méthode de coloration histologique du névraxe pour l'étude cytologique de la rage et des maladies à virus (inclusions nucléaires et inclusions protoplasmiques). C. R. Soc. Biol. 119: 804-805, 1935.
147. MATHIS, A. Actions pathogènes exercées par les microbes. Bull. Inst. Pasteur, 7: 202-203, 1909.
148. SCHIFFER, I.A., WHELETT, D.F., SHUMWAY, R.F. and RICHARDS, E.H. Mucosal disease of cattle. Vet. Med. 50: 431-434, 450, 1955.
149. NICOLAU, S., KOPCZYNSKA, L. et BALAB, G. Inclusions cytoplasmiques simulants les corps de Negri, dans le cerveau de la souris normale. C. R. Soc. Biol. 113: 851-855, 1933.
150. NICOLAU, S. Etude sur les inclusions qui caractérisent la maladie de Carre (maladie du jeune âge du chien). C. R. Soc. Biol. 119: 269-272, 1935.
151. ARAI, K., ISHII, K., OSA, Y., IZUMAWA, J., SHIMADA, K. and KITA, I. Studies on the immunological diagnosis of the animals suspected of rabies. Jap. J. Med. Sci. Biol. 6: 221-245, 1953.
152. SCHULZ, E.W., BULLOCK, L.T. and HENNER, H.V. Studies on the antigenic properties of the ultraviruses. III. The antigenic properties of rabies virus. J. Immunol. 15: 265-281, 1928.
153. HAYNES, L.C. and PAYFIELD, C.R. The antigenic properties of rabies virus. J. Infect. Dis. 50: 367-376, 1932.
154. CARLIS, J. and PALACIOS, J. The complement-fixation test in the diagnosis of virus infections of the central nervous system. J. Exper. Med. 74: 409-426, 1941.
155. ARAI, K., ISHII, K., OSA, Y., IZUMAWA, J., SHIMADA, K., KITA, I. and MURAKAMI, H. Studies on the immunological diagnosis of the animals suspected of rabies (second report). Jap. J. Med. Sci. Biol. 6: 659-666, 1953.
156. DEBOUX, R. et MARTELLE, P. Sur la valeur de la réaction de déviation du complément dans le diagnostic de la rage. Ann. Inst. Pasteur, 90: 182-186, 1956.
157. SUDANA, Y.I. and SOEK, D.S. On the value of the complement-fixation test in the diagnosis of rabies. Ind. J. Pub. Hlth. 2: 258-264, 1958.

158. VILLENOT, J.M. et PROUST, A. Précipitation en milieu gélifié du virus rabique par le serum rabique hyperimmun. *Rev. Elev.* 11: 387-397, 1958.
159. FOLBERG, A. and ROSSIGN, P. Particle size of soluble antigen of rabies. *Proc. Soc. Exper. Biol. Med.* 84: 317-320, 1953.
160. WOLSTENHOLME, G.E.W. and MILHAM, E.C.P. Siba symposium on the nature of viruses. J. and A. Churchill Ltd., London, 224-248, 1957.
161. VILLENOT, J.M. et PROUST, A. Etude sur l'antigène soluble du virus rabique. *Ann. Inst. Pasteur*, 96: 712-722, 1959.
162. VILLENOT, J.M. et PROUST, A. Diagnostic rapide de la rage par le test de précipitation en milieu gélifié. *Proc. 15th Inter. Vet. Congress, Madrid, Vol. II*: 629-631, 1959.
163. VER, B.A., SAO, S.S. and JHALA, N.I. Antigenic analysis of rabies fixed virus by gel diffusion technique. *Ind. J. Path. Bact.* 5: 131-136, 1962.
164. GRASSET, N. et ATANASIU, P. Etude de la diffusion en gélose d'antigènes de la rage fixe obtenus sur culture de tissus. *Ann. Inst. Pasteur*, 101: 639-647, 1961.
165. CHABAUD, M.A., SERIE, C. et ARNEAL, L. Electrophorèse et diagnostic de la rage. *Ann. Inst. Pasteur*, 83: 420-434, 1955.
166. KOEJUR, A. and BANERJEE, N. Electrophoretic study of antirabies serum. Practical application. *Arch. Inst. d'Hessarek*, 12: 81-84, 1960.
167. MANIE, A. Recherches sur le sérum antirabique. *Ann. Inst. Pasteur*, 19: 1-8, 1905.
168. STOLL, C. Symbiose du virus de la rage avec les cultures cellulaires. *C. R. Soc. Biol.* 104: 851-852, 1930.
169. REED, L.J. and KUMMEL, H.A. A simple method of estimating fifty per cent end points. *Amer. J. Hyg.* 27: 493-497, 1933.
170. GERSHASSER, A.A. and KISLING, R.S. Fluorescent antibody staining of street and fixed rabies virus antigens. *Proc. Soc. Exper. Biol. Med.* 98: 219-223, 1958.
171. GERSHASSER, A.A., KISLING, R.S., GRESKI, T.A. and RUSY, F.S. Fluorescent antibody staining of rabies virus antigens in the salivary glands of rabid animals. *Bull. Wild. Dis. Org.* 20: 579-582, 1959.

172. COOME, A.B. and KAPLAN, M.A. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exper. Med.* 91: 1-13, 1950.
173. McQUEEN, J.L., LEVINE, A.L. and SCHMIDT, N.J. Rabies diagnosis by fluorescent antibody. I. Its evaluation in a public health laboratory. *Amer. J. Pub. Hlth.* 50: 1743-1752, 1960.
174. GATSKI, T.R., WILSNACK, W.H. and SIKES, R.K. Pathogenesis of rabies in wildlife. II. Fluorescent antibody studies. *Amer. J. Vet. Res.* 32: 1048-1052, 1962.
175. KANAZAWA, K. Sur la culture "in vitro" du virus de la rage. *Jap. J. Exper. Med.* 14: 519-522, 1936.
176. SANDERS, M., KIEM, I. and LAGUNOFF, B. Cultivation of viruses. A critical review. *Arch. Path.* 56: 148-225, 1953.
177. VEENARASHAVAN, N. Studies on the cultivation of the aetiological agent of rabies "in vitro" and its nature. *Ind. J. Med. Res.* 34: 207-224, 1946.
178. KIRK, R., HASHEEB, M.A. and DAVIS, A.T. Attempts to cultivate rabies in a cell-free medium. *J. Trop. Med. Hyg.* 53: 167-170, 1950.
179. KESLING, R.E. Growth of rabies virus in non-nervous tissue culture. *Proc. Soc. Exper. Biol. Med.* 98: 223-225, 1958.
180. KAPLAN, M.M., FORSEK, Z. and KOPROWSKI, H. Demonstration of rabies virus in tissue culture with fluorescent antibody technique. *Bull. Wld. Hlth. Org.* 22: 434-435, 1960.
181. FENJE, P. Propagation of rabies virus in cultures of hamster kidney cells. *Can. J. Microbiol.* 6: 479-484, 1960.
182. FENJE, P. A rabies vaccine from hamster kidney tissue cultures: preparation and evaluation in animals. *Can. J. Microbiol.* 6: 605-609, 1960.
183. DUNSTON, J.K. Rabies. *Trop. Dis. Bull.* 59: 1147-1148, 1962.
184. BARTOS, L. et ATANASIU, P. Presence d'inclusions spécifiques dans les cellules de rein de hamster en culture de tissu infectée par le virus de la rage fixe (bouche Louis Pasteur). Etude cinétique du virus. *Ann. Inst. Pasteur*, 101: 428-452, 1961.

185. AIAI-SIU, P. et LEVINE, P. Multiplication du virus rabique des rues sur la tumeur ependymaire de la souris en culture de tissus. Effet cytolytique. Ann. Inst. Pasteur, 26: 72-78, 1959.
186. KLICKER, I.J. and WALKOFF, R. Cultivation of rabies virus in the allantois of the developing chick embryo. Proc. Soc. Exper. Biol. Med. 32: 212-214, 1938.
187. BLANKFELT, H. and KLICKER, I.J. Characteristics of a fixed rabies virus cultivated on developing chick embryos. Proc. Soc. Exper. Biol. Med. 45: 332-335, 1940.
188. DAWSON, J.A. A study of chick-embryo-adapted rabies virus. Amer. J. Path. 17: 177-187, 1941.
189. KOPROWSKI, H. and COX, H.H. Occurrence of rabies virus in the blood of developing chick embryo. Proc. Soc. Exper. Biol. Med. 63: 612-615, 1943.
190. UEKI, H., KATO, T., OISHI, J., MURAHASHI, H. and SHIMADA, K. Observations of the time of appearance of Negri bodies, complement-fixing antigen and virulence in mouse brains inoculated with street rabies virus. Amer. J. Vet. Res. 12: 216-218, 1957.
191. MALLORY, F.B. Pathological technique. W. B. Saunders Co., Philadelphia and London, 235, 1933.
192. HARK, P.B. and BERGHEIM, O. Practical physiological chemistry. The Blackinton Co., Philadelphia, 11th Ed., 758-759, 1942.
193. HARK, P.B. and BERGHEIM, O. Practical physiological chemistry. The Blackinton Co., Philadelphia, 11th Ed., 453-455, 1942.
194. COONS, A.H., LEBUC, E.S. and CONNALLY, J.M. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to the study of the hyperimmune rabbit. J. Exper. Med. 102: 49-59, 1955.
195. ANGLADE, P. et SAHLY, J. Sur l'existence de regions avirulentes dans le systeme nerveux central de chiens morts de rage. C. R. Soc. Biol. 106: 1201-1203, 1931.
196. SCHINDLER, I. Studies on the pathogenesis of rabies. Dtsch. Zts. Bakt. Orig. 35: 119-126, 1941.

ACKNOWLEDGMENTS

The writer desires to express his gratitude to Professor H.J. Gibbons, Head, and to Professor C.A. Mitchell of the Department of Bacteriology under whose supervision these investigations were made.

He wishes to acknowledge his indebtedness to Drs. Christine E. Rice and P. Boulanger of the Department of Immunology and Serology at the Animal Diseases Research Institute for their encouragement and guidance.

The author is also thankful to Dr. P.J.G. Plummer, Director and to Dr. J.F. Frank, Assistant Director, of the Animal Diseases Research Institute for provision of facilities to carry out this work.

He also wishes to acknowledge the assistance of Mr. W.A. Boyd of the Animal Diseases Research Institute and that of the personnel of the Bio-Graphic Unit, Research Branch, Canada Department of Agriculture, in the preparation of the illustrations.

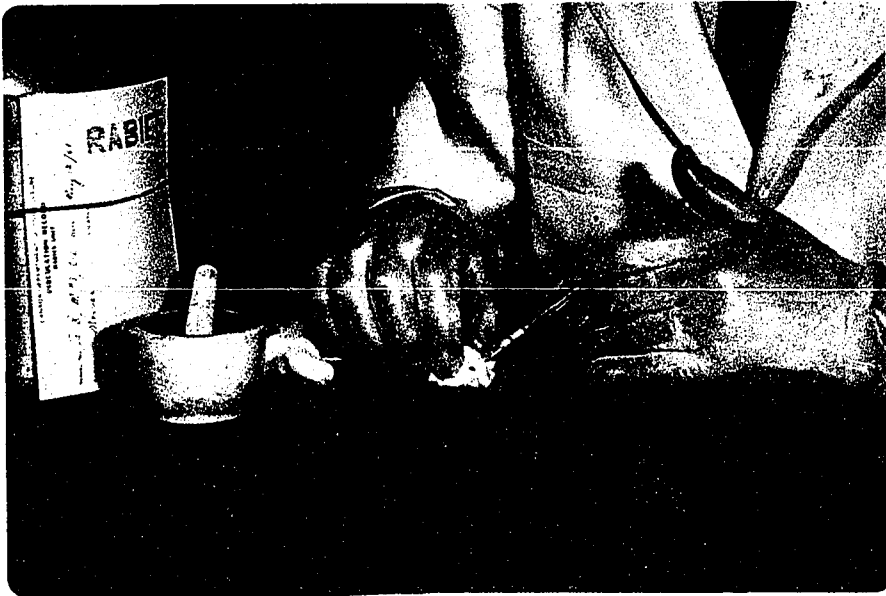


Plate 1.- Intra-cranial inoculation of an experimental mouse.



Plate 2.- Experimental mice one day after intracranial inoculation.

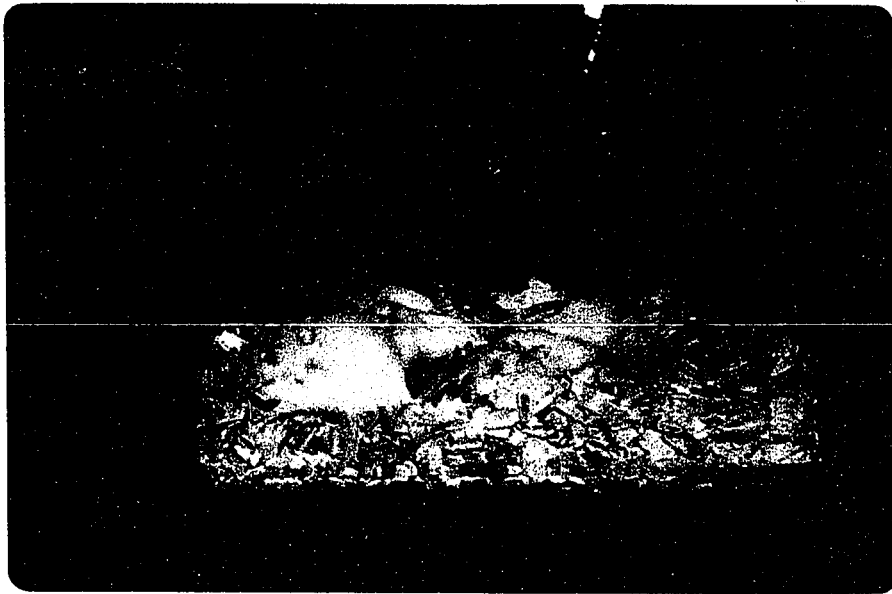


Plate 3.- Experimental mice, 11 days after intracranial inoculation.

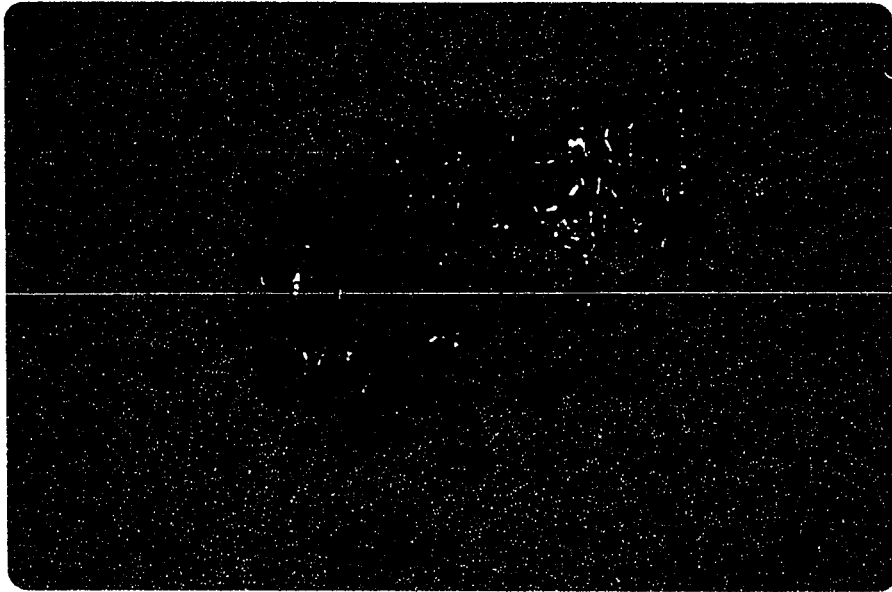


Plate 4.- Brain of an experimental mouse from which the right hippocampus has been dissected and is shown in the upper right quarter.



Plate 5.- Experimental mouse whose sub-maxillary salivary glands have been exposed for dissection.



Plate 6.- Preparation of impression smears from the salivary gland of an experimental mouse.

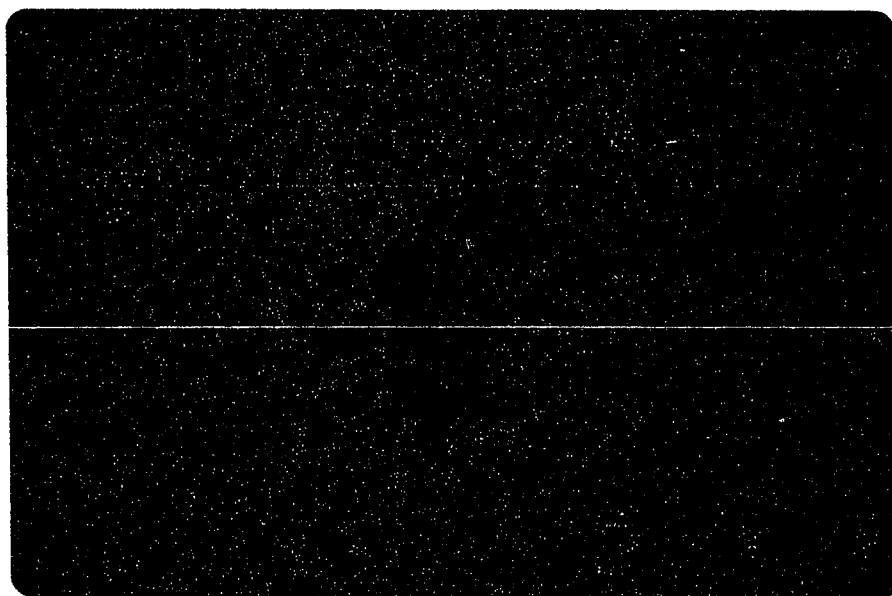


Plate 7.- Williams' stained impression smear of a
Negri-negative salivary gland from an
experimental mouse. 700X.

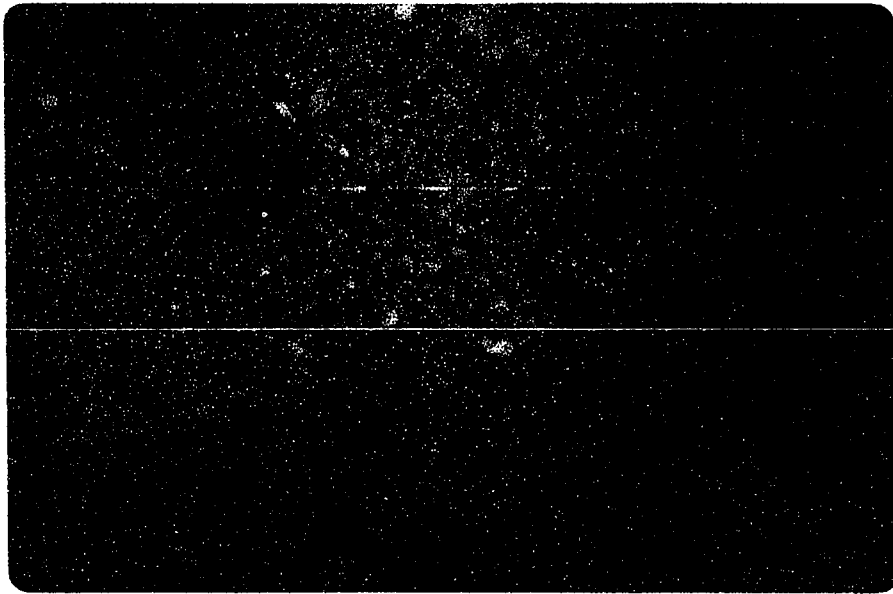


Plate 8.- Williams' stained impression smear of a
Negri-negative hippocampus from an
experimental mouse. 700X.

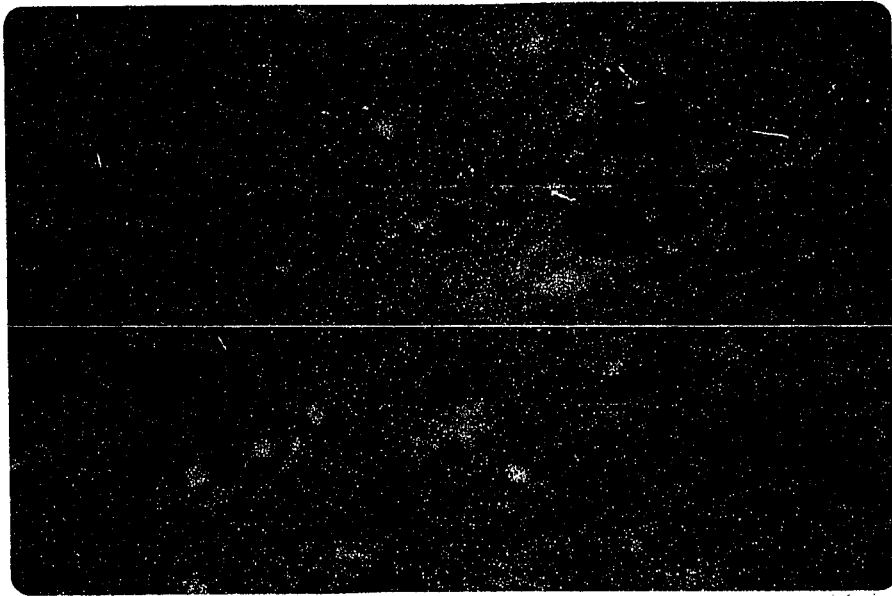


Plate 9.- Williams' stained impression smear of the hippocampus from a rabid experimental mouse. Negri bodies are indicated by arrows. 700X.

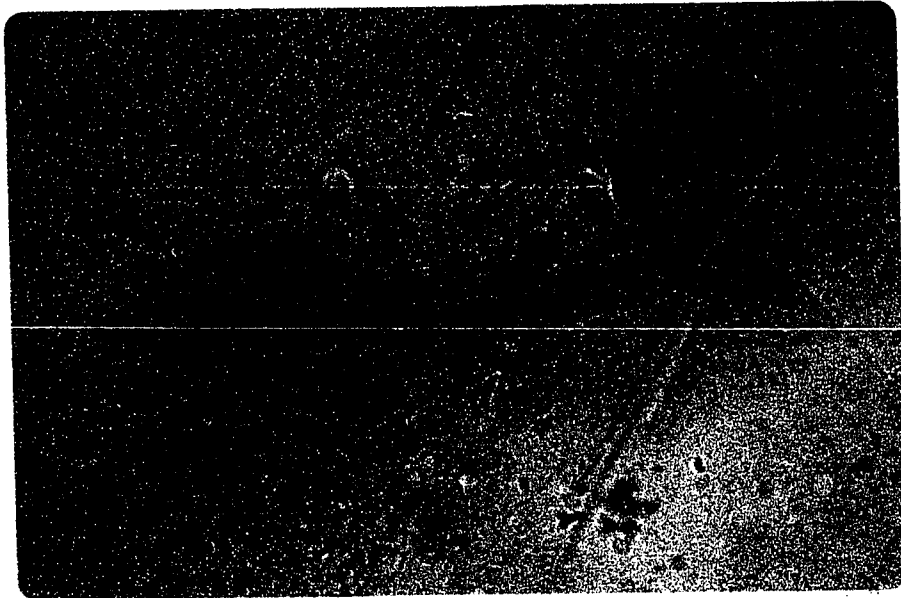


Plate 10.- Williams' stained impression smear of the hippocampus from a rabid bovine field specimen. Negri bodies are indicated by arrows. 700X.

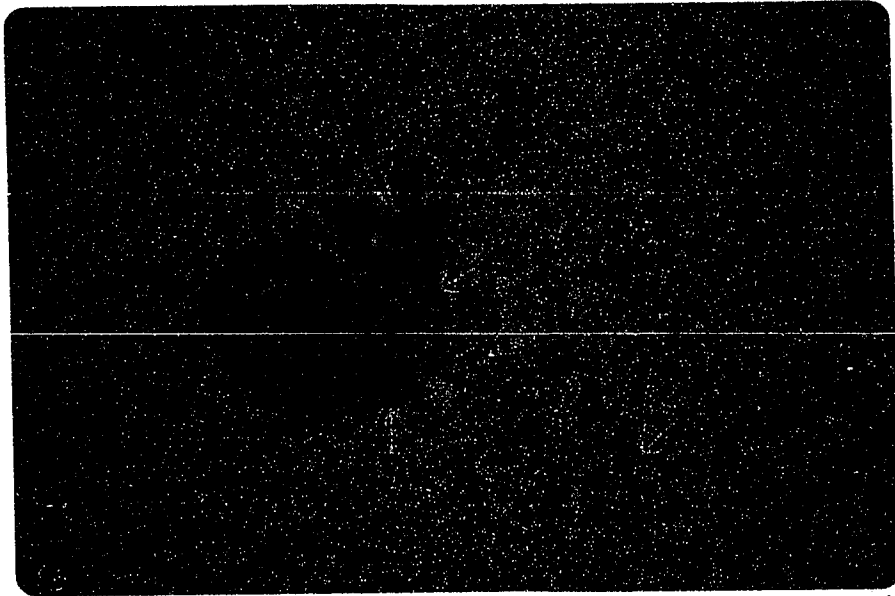


Plate 11.- Williams' stained impression smear of the hippocampus from a dog field specimen showing distemper inclusions, as indicated by arrows. 700X.

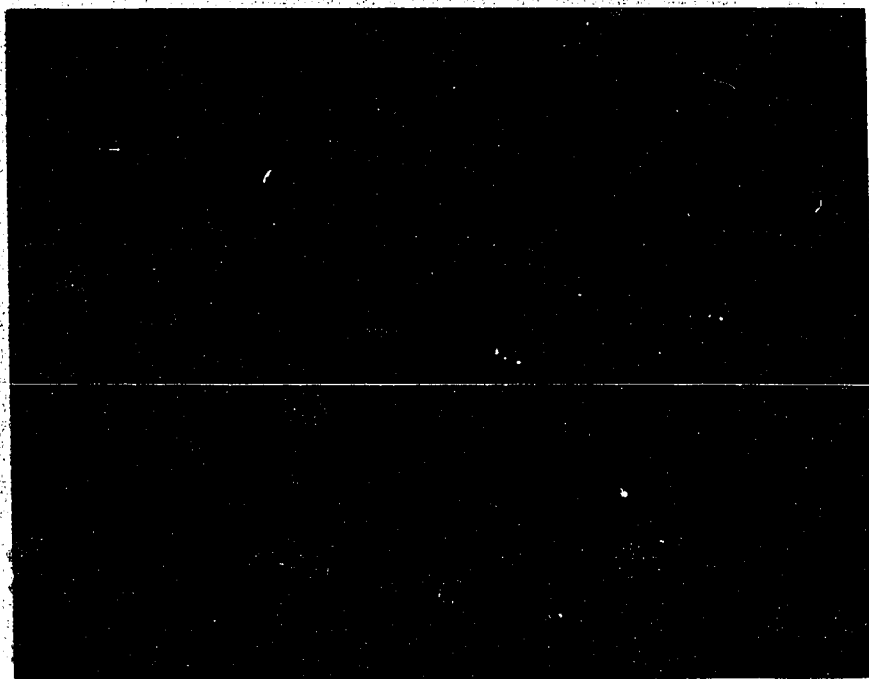


Plate 12.- Negri bodies and dust particles as shown by the fluorescent antibody technique in the hippocampal tissue of a rabid experimental mouse. 400X.

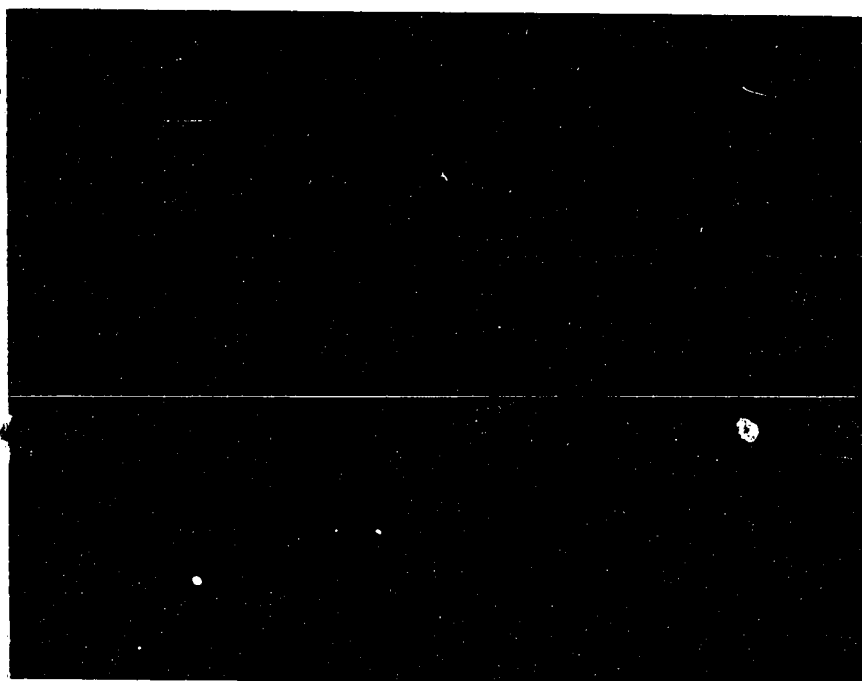


Plate 13.- Negri bodies and dust particles as shown
by the fluorescent antibody technique
in the brain tissue of a rabid skunk
field specimen. 400X.