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SCRAPIE: THE EXPERIMENTAL DISEASE IN INBRED STRAINS OF MICE

by

H. Fraser

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SUMMARY

Factors affecting the distribution of lesions of scrapie in inbred mice have been investigated. For this purpose a quantitative system for the analysis of the intensity of vacuolation in the brain has been developed, based on the histological examination of nine defined areas of grey matter.

One of the most important conclusions of the thesis is that variants of scrapie agent can be distinguished on the basis of pathology.

However, it is shown in the thesis that other factors can influence the intensity and distribution of lesions, and therefore the need to standardise the system of agent identification has to be emphasised. Other factors that affect the pathology are: route of inoculation, genotype and sex of host, genotype of donor and organ source of infective tissue. Important interactions between inoculation route and some other sources of variation are identified, in particular with genotype of donor, genotype and sex of host and organ source of infectivity. As there is no demonstrable alteration in the pathology with passage of a particular mouse adapted agent in a homologous host strain system, the properties of an agent upon which it is identified can be regarded as stable. On this basis, by examining several sources of scrapie, four agents have so far been distinguished, one from a Suffolk sheep source, two from a Cheviot source and one from an experimental goat source.

In view of suggestions of similarities between scrapie and ageing, morphological concomitants of advancing age are identified in the brains of mice, and a comparison of their incidence in scrapie and ageing is undertaken. It is concluded that in terms of the defined parameters, there is no justification for regarding scrapie as an acceleration of any specific

manifestation of the ageing process. One feature occurring in the brains of old mice, an oedema or vacuolation of white matter, does partly resemble a lesion associated with scrapie, at least under some circumstances. Morphologically, this feature in scrapie is frequently, but not invariably, distinguishable from the condition associated with ageing. The incidence of oedema in white matter both in ageing mice and in mice infected with scrapie of various strains is examined, and it is shown that one scrapie agent exists in which white matter changes are a very prominant, but not necessarily a specific, feature.

The circumstances of a series of astrocytic gliomas in one inbred mouse strain and its crosses is studied. These neoplasms occur in a strain of mice with genetically deviant properties with regard to its response to most strains of scrapie. Based on incubation period measurements, no effect on the progress of scrapie can be associated with the presence of the glioma.

The role of the spleen in the pathogenesis of scrapie is investigated in experiments involving splenectomy at various times before or after inoculation of the agent. This results in a highly significant prolongation of incubation period in mice splenectomised at various intervals before or after intraperitoneal inoculation. However, splenectomy before or after intracerebral inoculation produces no alteration in incubation. These results suggest that replication occurs in the spleen either at a greater rate or with an overall greater yield, when compared with other peripheral tissues taken together, as far as available agent reaching the brain is concerned. They also suggest that with intracerebral inoculation, intraneural events are independant of the spleen in its role either as a possible replication site or in terms of other roles it may play in pathogenesis.

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INTRODUCTION

Scrapie is a nervous disease of sheep and rarely of goats and is characterised by neurological signs, fatal outcome and degenerative lesions of the nervous system. It is a transmissible disease in which infectivity is transferred vertically from mother to offspring and by lateral contagion. It has been transmitted experimentally to sheep, goats, mice, rats and hamsters and in both the natural and experimental diseases the incubation period extends from several months to years (Stamp, 1967).

The broad aim of this thesis is to study aspects of the pathology and pathogenesis of scrapie by analysing variation in certain manifestations of the disease in inbred mice. Basically the importance of studying scrapie in terms of variation, both of the infectivity and of the host, is to obtain a fuller understanding of factors concerning its pathogenesis and epidemiology. Conceptually the total variation of scrapie is the result of both hereditary and non-hereditary forces involving the physiology of the host, the source and specificity of the agent and the circumstances of the union between the agent and host tissues. Altogether these factors constitute the material environment of scrapie infection and it is the effects of variation among them that has been investigated in this thesis.

All studies on scrapie have to be restricted to those concerned with the intact animal. It is arguable that progress in understanding scrapie has been hampered by this and by certain of its special features. In particular the absence of a host response of an immunological or inflammatory type and the effective failure of <u>in vitro</u> cultivation, constitute tangible obstacles to progress in some types of studies. Until now, quantitative studies have been confined to the determination of levels of

infectivity by titration and to the measurement of incubation period. The major contribution of this thesis is that it provides a third quantitative criterion for investigating scrapie which is distinct from and partially independant of the other two. This criterion is based on the histopathology of the disease and consists of an assessment of the intensity and distribution of degeneration in the brain.

One of the primary endeavours of the laboratory in which this work is carried out is to investigate the properties of different sources of scrapie agent in order to establish the existence and properties of agent variants. In this thesis isolates are compared on the basis of their pathology in inbred mice and four distinct types of scrapie agent are distinguished. It is revealed that the criterion on which these distinctions are based is a very sensitive indicator of variation in the environmental framework in which the heritable properties of an agent expresses itself. In most instances, the observations are confined to a single source of variation treated separately, but on one occasion a multi-factorial experimental design is used in order to compare the effects of interaction between variables.

In scrapie the morphological lesions are confined to the nervous tissues. However, following inoculation of the agent there is an early rise in the concentration of the agent in lymphoreticular tissues, but this is not accompanied by observable structural or functional alterations. This indicates either early synthesis of scrapie in these tissues or an accumulation in them of agent which is synthesised elsewhere (Eklund, Hadlow and Kennedy, 1967; Dickinson and Fraser, 1969a). One aspect of the

pathogenesis of scrapie is therefore investigated by splenectomy which results in a prolongation of incubation period when combined with intraperitoneal inoculation.

With certain combinations of strain and dose of scrapie agent, host genotype and route of inoculation, the incubation period can be very long and can approach the life span of the host (Dickinson and Meikle, 1969). There are also similarities between scrapie and some features of ageing and this has led to the suggestion that the tissue alterations in scrapie are an exaggeration of those which occur normally in old age (Field, 1967; Adams Caspary and Field, 1969). For these reasons a critical examination is made of some morphological features of ageing and an analysis of the incidence of these features in scrapie and ageing is undertaken.

It has also been suggested that scrapie resembles a neoplastic alteration of astrocytes involving a process analagous to the 'transformation' which accompanies certain types of union between papovaviruses and some mammalian cells (Field, 1967; 1969; Field and Peat, 1969; Webb, 1967). It is partly for this reason that an account is given of a series of astrocytic gliomata occurring in an inbred mouse strain used extensively for scrapie experiments, and partly because the strain in which these tumours occur is VM which is unusual in its response to scrapie (Dickinson, Meikle and Fraser, 1968).

Scrapie attracts a considerable amount of general interest as a model for a group of so-called 'slow virus encephalopathies', of which examples are found in man and animals. Comparative studies of the human disease kuru (Hadlow, 1959), in the chimpanzee and spider monkey (Gajdusek,

Gibbs and Alpers, 1966, 1967; Gibbs, Gajdusek and Alpers, 1969; Gajdusek, Gibbs, Asher and David, 1968), of transmissible encephalopathy of mink in a wide range of animals (Marsh, Burger, Eckroade, Zu Rhein and Hanson, 1969), and recently Creutzfeld Jacob disease in the chimpanzee (Gibbs, Gajdusek, Asher, Alpers, Beck, Daniel and Matthews, 1968; Gibbs and Gajdusek, 1969), give support to the idea that scrapie is just one example of a group of infectious degenerative disorders of nervous tissue with long incubation periods, and as such it has wide biological interest. In addition, the causal agent of scrapie is the subject of speculation in view of its unusual physical and chemical properties, when compared with what are currently regarded as conventional viruses. The role of neuropathology in the investigation of these diseases has become uniquely important because the causal agents can only be identified and distinguished on the basis of their pathology. The neuropathologist, therefore, has a very special responsibility and one which is unique in studies of host parasite relationships, to construct objective analytical methods on which to base his conclusions. The particular contribution of this thesis is that it provides a method of neuropathological analysis that can reveal heritable and non-heritable variation in scrapie and which can form a basis on which the variation in other slow virus encephalopathies can be investigated.

MATERIALS AND METHODS

Mouse Strains

The mouse strains referred to in this thesis are detailed below. These designations are those officially adopted by the committee on Standardised Genetic Nomenclature (Staats, 1964, 1966):

Inbred Strain Designation	Abbreviation	
C57BL/FaBtDk	C57BL	+ ‡
BALB/cLacDkf	BALB	o
SM/RrChBtDk	SM	+
BRVR/SrDkf	BRVR	
BSVS/SrDkf	BSVS	
LG/RrChBtDk	LG	
DBA/2LacDkf	DBA	x
CBA/1LacDkf	CBA	x
C3H/LacDkf	C3H	x
A2G/LacDkf	A2G	x
NZB/LacDkf	NZB	
AKR/LacDkf	AKR	
RIII/FaDk	RIII	
VL/Dk	VL	
LM/Dk	LM	
EM/Dk	EM	
MM/Dk	MM	
VM/Dk	VM	

x Stock known to be free from mammary tumour agent

\+ Stock may carry mammary tumour agent but be free from overt signs

o Mammary tumours occasionally occur in some lines

‡ C57BL is referred to as C57 in the text.

In mice the gene sinc was first recognised by its control of the incubation period of scrapie caused by ME7 agent, and the p7 allele, which prolongs the incubation period for ME7, is only present in the VM mouse strain (Dickinson, Meikle and Fraser, 1968). All other mouse strains which have been tested carry the s7 allele, which shortens the incubation of ME7 group agents; among the s7s7 mouse strains RIII usually has marginally the shortest incubation period, whereas MM and BALB have marginally the longest incubation periods (Dickinson and Meikle, 1969). Incubation periods of all scrapie agents so far tested are controlled by the sinc gene but the effect of the two alleles varies between wide extremes according to the particular scrapie agent.

Agent Strains

The nomenclature of the sources of infectivity listed below is derived from the numbering of the initial experiment in which they were isolated. ME7 is the symbol used for an isolate obtained from the Moredun Research Institute, and in all other cases the symbols relate to isolates which were made at the Animal Breeding Research Organisation, Edinburgh.

Table 1 SOURCE OF SCRAPIE AGENTS

Agent Designation		Source and history prior to isolation in inbred mice
ME7		2nd passage brain from random bred Moredun mouse. Originated from a naturally affected scrapie Suffolk sheep spleen (Zlotnik and Rennie, 1963).
58A		Brain from a scrapie affected Suffolk sheep which had previously been inoculated intracerebrally and subcutaneously with material from a pool of 2 'acute' and 2 'chronic' natural cases of scrapie in Suffolk sheep.
79A	Σ	Brain pool from goat with 'drowsy' or 'nervous' form of experimental scrapie (Pattison and Millson, 1961) at the 8th goat passage.
80A	Σ	Brain pool from goat with 'scratching' form of experimental scrapie (Pattison and Millson, 1961) at the 5th goat passage.
22A	Σ σ	Cheviot sheep brain pool at the 21st sheep passage of SSBP/1.
22C	Σ σ	Cheviot sheep brain pool at the 21st sheep passage of SSBP/1.

Σ These agents stem from a common origin at the 16th sheep to sheep passage of the Cheviot sheep scrapie brain pool of the Moredun Institute (SSBP/1). Prior to its isolation in goats, this was further passaged twice in sheep at Compton (firstly in Cheviots, and then in the Welsh Mountain breed). The isolation in mice of the source which yielded 22A and 22C was passaged a further 5 times through Cheviot sheep at the Moredun Institute.

σ These agents were separated by subpassage in mice with different alleles of the gene, *sinc*.

Preparation of Inocula

Inocula were prepared by maceration of tissues in autoclaved glass grinding tubes made up as 10% or 1% saline suspensions, using autoclaved isotonic saline. Suspensions were spun at 500g for 10 minutes or at 200g for 15 minutes for titrations (in both cases laboratory standards were used) and the resultant supernatant fluids were either used as the inocula, or dilutions were made of them which were used as inocula. In most experiments the tissue sources had been stored for prolonged periods at -30°C.

All tissues used for inoculation were removed from mice immediately following death by decapitation or hypercapnic anoxia, by a sterile technique designed to be sufficiently rigorous as to prevent cross-contamination with different strains of scrapie agent. For this purpose only new 5ml Bijeaux bottles, which had been autoclaved, disposable gamma-irradiated petri-dishes, autoclaved surgical instruments and sterilised disposable blades were used.

Properties of the Physical Environment of Experimental Animals

Mice used in all experiments were conventionally maintained in a standard, monitored environment at a constant temperature (21°C - 22°C), and humidity (50-60% relative humidity) and partially controlled day length. They were separated into groups of 6 in plastic cages, with sexes separated. Cage cleaning, feeding and water bottle changing were carried out at the same time each week, all mice in any particular experiment being dealt with together. Two standard pelleted pasteurised diets were used, a Spillers diet and an Oxoid diet. Mice in any particular experiment were maintained throughout on a single diet.

Viral, bacterial and metazoal infections were met with occasionally. The status with regard to mammary tumour agent for each mouse strain is known and the details are given in the list of mouse strains. It is probable that the Riley lactic dehydrogenase elevating virus is present in the colony, but the status with regard to Reo III virus, polyoma, MHV or any other relatively common viruses is unknown. Various leukoses, presumably of viral origin, occur in older mice. The predominant gram-negative bowel commensal is a species of Proteus which is also found regularly in a complex of cystitis and nephritis which affects many adult males of the MM strain. The oxyurid nematode, Syphacia oblavata, became evident in the colony late in 1967, and was treated with piperazine citrate administered in the drinking water.

General Design of Experiments

It should be pointed out here that the work contained in this thesis represents a part of a multi-faceted approach to scrapie being conducted at the Animal Breeding Research Organisation, Edinburgh. Some of the experiments were designed specifically for the observations made from them in the thesis, but many observations are made from experiments designed for broad purposes. In particular much of the histopathological work is a by-product of such experiments.

Each animal is ear-punched with an autoclaved ear-punch at weaning, before it is assigned to an experiment (on the very rare occasions on which an inoculated animal is ear-punched, the ear-punch is autoclaved afterwards). At weaning each animal receives duplicates of a card describing, in coded

form, its identification. When an experiment is planned, the file-cards, which have different colours representing each sex, are used for randomising animals between the various experimental and control groups required for the experiment. This results in representatives of various groups being mixed within cages. These procedures are designed to minimise subjective and mouse behaviour biases in determining clinical end points of incubation, and to randomise any uncontrolled sources of variation between various experimental groups.

Incubation Period, Method of Measurement

The phenotypic parameter upon which agent typing is based depends upon differences in incubation period in mice carrying different alleles of the *sinc* gene. Incubation period in this context is a carefully defined parameter, (Dickinson, Meikle and Fraser, 1968). The method used is as follows. Injected mice are scored once weekly during the afternoon, on a day in which no husbandry routines are carried out. Each mouse, individually identified, is classified as 'unaffected', 'possibly affected' or 'definitely affected' with certain specified clinical signs of scrapie. The end point for calculating incubation period is defined as the day on which an animal receives a third consecutive weekly score of 'definitely affected', or the day on which a fourth such score is given within five consecutive weeks, or the day on which it is killed 'in extremis' with scrapie, if less than 3 weeks after its first definite scrapie score, or is found dead having received a 'definite scrapie' score in the previous 7 days. In most experiments, however, animals which die are excluded from experimental results unless a

histological statement concerning their scrapie status can be obtained. In all cases of animals which are killed, histological confirmation is carried out.

Lesion Distribution Profiles, Method of Assessment

The method of histological analysis is intended to obtain information concerning the distribution and intensity of lesions within the brain. Five transverse paraffin sections, 7µ thick, are prepared from each brain as described by Mould, Dawson, Slater and Zlotnik (1967), are stained with haematoxylin and eosin. Nine regions of grey matter are examined, namely, (1) the dorsal part of the medulla covering the cuneate, vestibular, or cochlear nuclei, or locus coeruleus, or the sensory nucleus of the trigeminal nerve, and associated areas, according to the exact levels available; (2) the folia of the cerebellar cortex adjacent to the fourth ventricle, particularly the lingula or nodulus; (3) the cortex of the superior colliculus; (4) the hypothalamus; (5) the thalamus, in particular the massa intermedia and associated central nuclei of the thalamus, (6) the hippocampus at the level of (4) and (5), (7) the septal nuclei of the paraterminal body, (8) an area of cerebral cortex at the level of (4), (5) and (6) lying dorsal to the corpus callosum, associated with the indusium griseum, the cingulate gyrus and cortex extending onto the dorsal surface of the brain, and (9) a similar area of cerebral cortex as in (8) associated with the section on which the septal nuclei are situated (Figure 1).

Each area is given a score from 0 to 5 depending on the degree of vacuolation which is present. All sections are examined and scored 'blind',

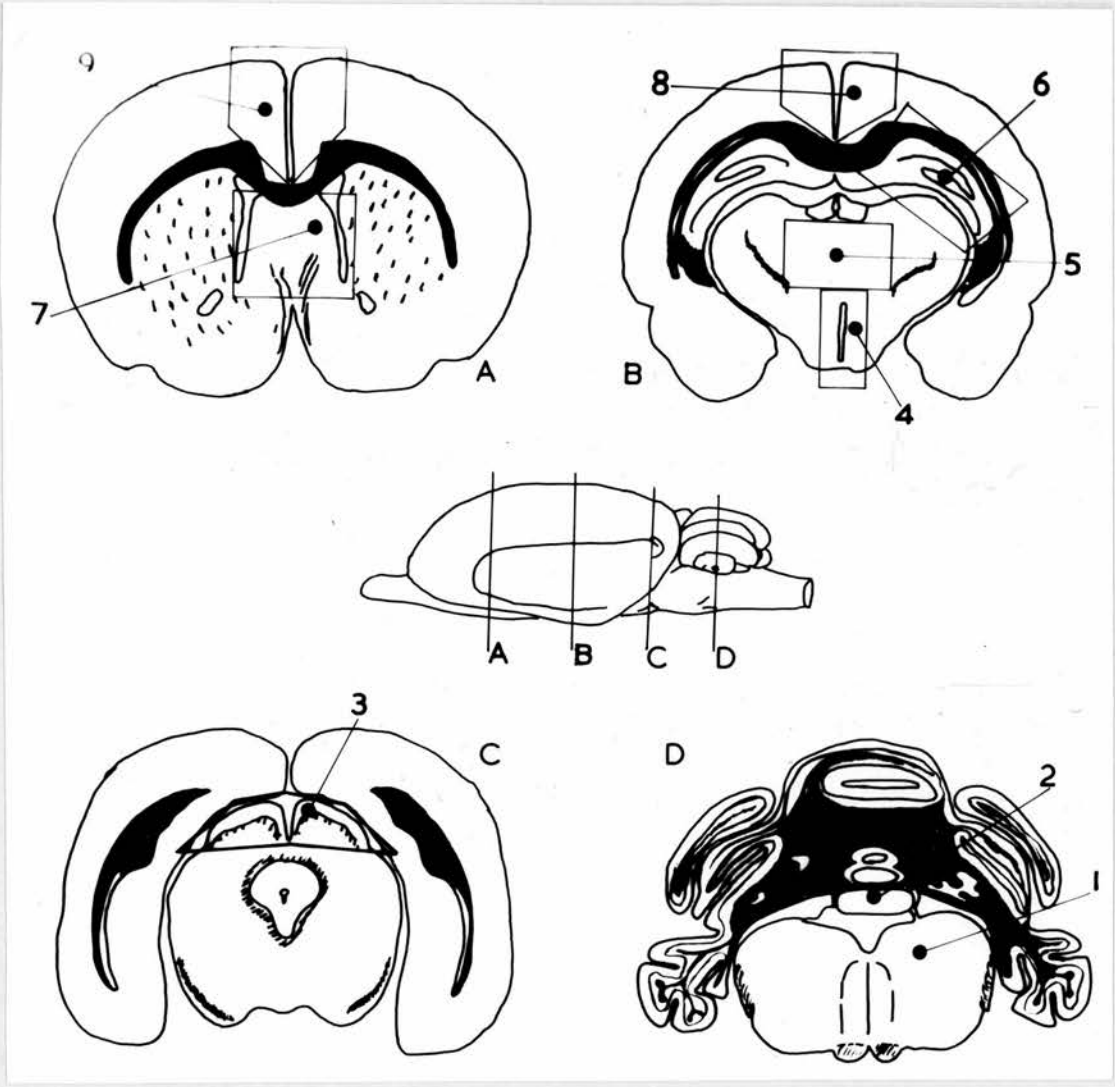


Figure 1 The 9 positions used for scoring the lesion density in scrapie in the mouse. 1: medulla; 2: cerebellum; 3: mid-brain; 4: hypothalamus; 5: thalamus; 6: hippocampus; 7: paraterminal body; 8 and 9: cerebral cortex.

with no knowledge whatsoever of experimental or injection procedures or age, strain or sex of mouse. The five degrees of brain damage have verbal definitions as follows:- (1) a few vacuoles, widely and unevenly scattered; (2) a few vacuoles evenly scattered; (3) moderate number of vacuoles evenly scattered; (4) many vacuoles with some confluence and (5) dense vacuolation with most of field confluent. Photographic representatives of these different scores are kept for reference purposes and examples for a single area, the cerebral cortex, are shown in Figures 2 and 3.

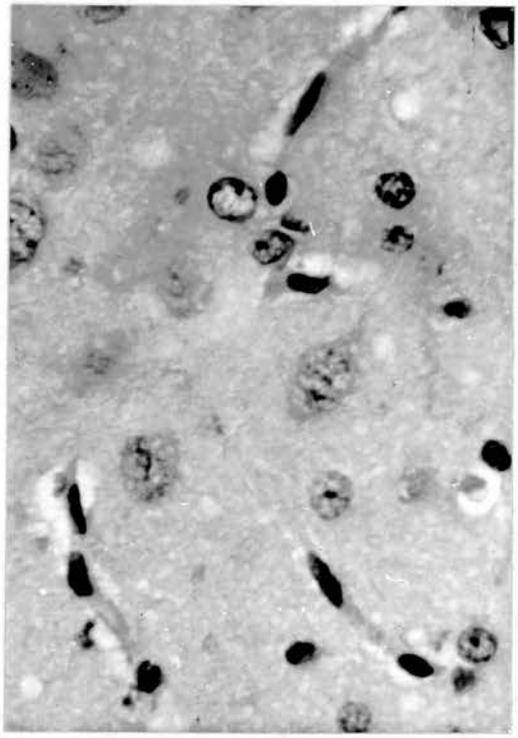
Nerve Fibre Degeneration, Method of Assessment

The criteria for scoring the degree of nerve fibre degeneration in the Nauta study were as follows:- score 0, no degeneration apparent; score 1, degenerating axons in very low numbers; score 2, degeneration fibres very frequent but fibres of normal appearance also numerous; score 3, the majority of nerve fibres showing signs of degeneration.

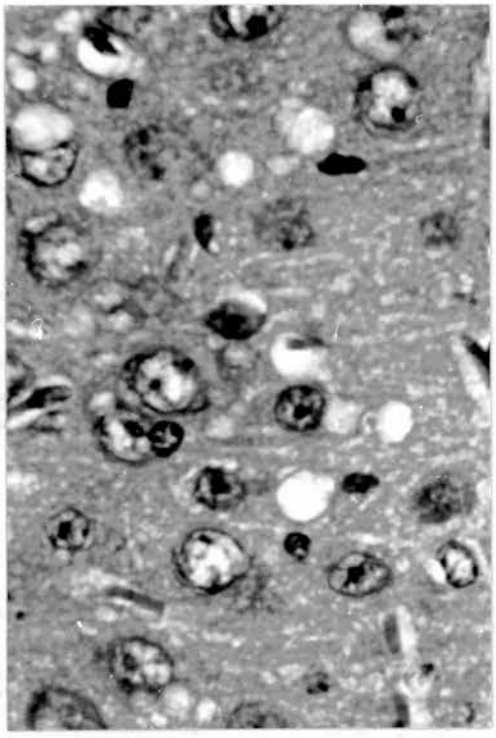
The six regions of the brain used for this assessment were:- internal capsular fibres within corpus striatum; hippocampus; junction of cerebral medulla and cortex; central mass of thalamus; tegmentum of mid-brain; dorsal tegmentum of pons and medulla.

Surgical Procedures

Splenectomy was carried out under intravenous of intraperitoneal pentobarbitone sodium anaesthesia, through a left flank incision. The splenic artery at the dorsal extremity of the organ was clamped, and sometimes ligated, and the spleen removed. A single chromic cat gut suture (00) joined the



a



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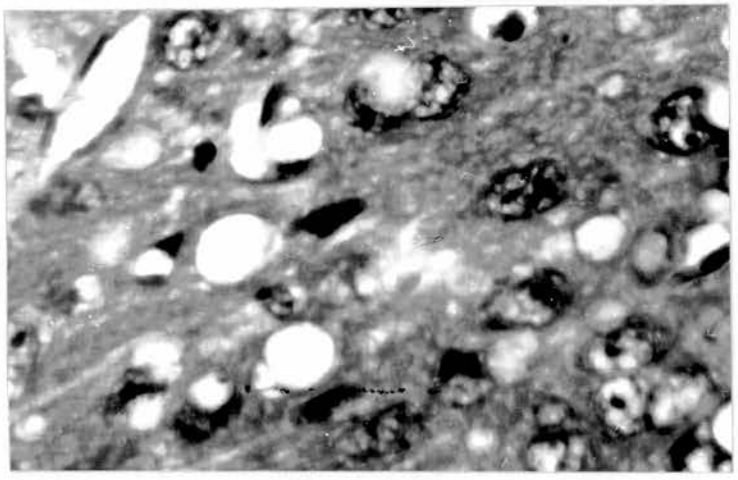
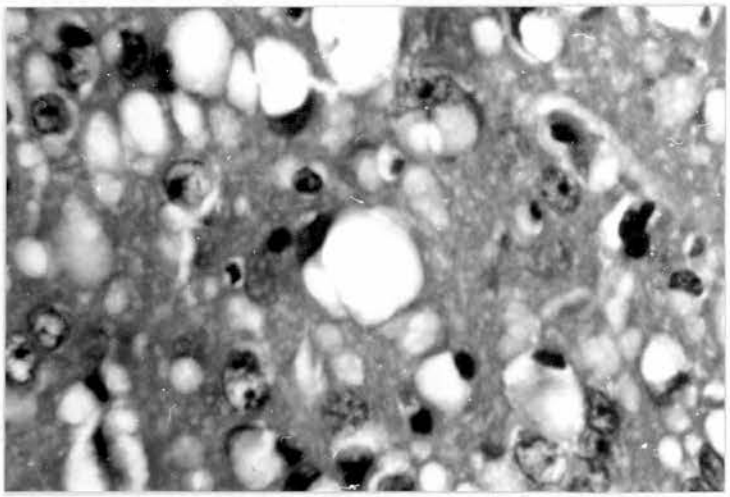


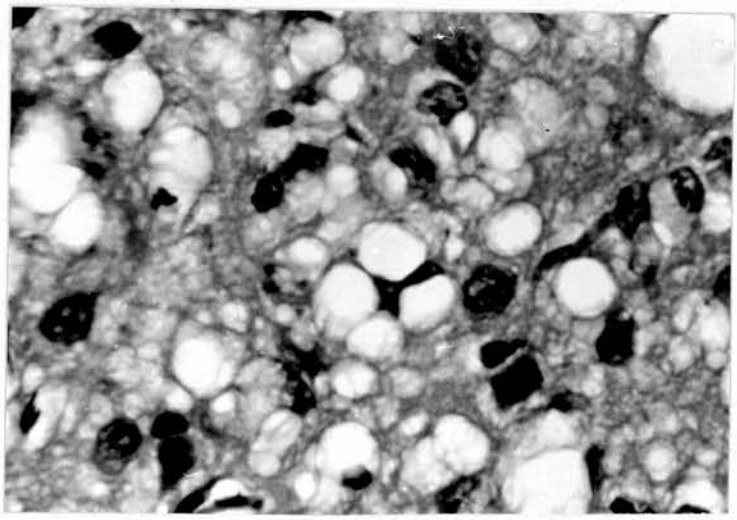
c

(Figure 2 and 3 Scrapie lesions of five degrees of severity in the cerebral cortex of mice)

Figure 2 a: score 1; b: score 2; c: score 3. H & E X600



a



b

Figure 3 a: score 4; b: score 5. H & E X600

muscle layers, and the skin was joined with stainless steel (Mitchell) clips.

Inoculations

Mice were manually constrained for all inoculations but not anaesthetised. Intracerebral inoculations were carried out with a 25 or 26 gauge needle into the right cerebral hemisphere. Intraperitoneal inoculations and those into the foot-pad were also used. Intravenous injections were made into a tail vein. Unless specifically stated to the contrary all inoculations were carried out on mice between 20 and 40 days of age, and within this range variation in body weight has no detectable effect on any of the parameters which are measured (Dickinson, personal communication).

Histological Procedures

Mice were killed by cervical fracture or hypercapnia. Brains were removed as rapidly as possible and immersed in 4% formaldehyde in saline. Other organs were fixed similarly. Fixed brains were trimmed coronally according to the method already described, and embedded in paraffin wax. Brains were occasionally cut parasagitally for frozen longitudinal sections in which case the portion which retained the mid-line was trimmed coronally and embedded. Section thickness was usually 6 microns for paraffin sections and between 15 and 22 microns for frozen sections.

Most routine histological staining was performed according to standard methods (Culling, 1963; Mallory, 1938; Lillie, 1954; Gattenby and Beams, 1950; and Drury and Wallington, 1967). Paraffin sections were routinely stained with haematoxylin and eosin.

The silver impregnation methods of Nauta and Gygax (1954) or Nauta (1957), or the modification of Heimer (Fink and Heimer, Method II, 1967) were used for the selective demonstration of degenerating nerve fibres. A modification of the Nauta and Gygax method was constructed to use with formol ammonium bromide fixed material (Appendix I). The silver technique of Glees and Marsland (Marsland, Glees and Erikson, 1954) and Holmes (Culling, 1963) were also used.

Astrocytes were revealed by the gold sublimate method of Cajal and glial fibres by the phosphotungstic acid - haematoxylin method of Mallory and by Holzer's method for fibrous glia. In addition, when gliomata were suspected the combined method of Lapham and Johnstone (1964), Heidenhain's iron haematoxylin, Hortega's silver carbonate, Weil Davenport impregnation, and Marshall's modification of the latter (Marshall, 1948), Weigert's haematoxylin and Van Giesen, and Einarsen's gallocyanin chromalum (Culling, 1963) were used. Intraneuronal inclusions were investigated by the tannic acid - ferric chloride method (Dixon, 1959), the periodic acid-Schiff method, Sudan Black B, Ziehl Neelsen, Giemsa, phloxine, methyl green-pyronin, Feulgen (Culling, 1963), paraldehyde fuchsin (Gomori, 1950) and ninhydrin-Schiff (Yasuma and Ichikawa, 1953). The microvascular lesion and associated ectopic calcification in the thalamus, in addition to some of the methods mentioned above, was investigated with alizarin red S, V. Kossa's and Perl's prussian blue methods and with congo red. The assistance of Miss Norma Robertson and Miss Patricia Clater in preparation of histological sections is gratefully acknowledged.

Statistical Methods

Standard statistical methods were carried out on lesion profile data and on incubation periods. The tests used were for the evaluation of P by t tests and chi-square tests. The significance of sex incidence differences in the case of specific lesions described in section 3 was established by the chi-square test. The repeatability of scoring of serial sections and repeat scoring of single slides was estimated by comparing the variance of scores in the nine brain sites and by calculating the coefficient of correlation at each site in the case of the repeat scoring (Section 1, subsection 1) (Fisher, 1925; Snedecor, 1946). Standard tables were used (Fisher and Yates, 1938). Analysis of variance was carried out when this was appropriate. For this purpose a computer programme for the least squares analysis of data was used and the assistance of Mr. Douglas Wilson is gratefully acknowledged.

To eliminate drift in observer bias in cases where samples were scored at widely different times, as in the comparisons of different passage states and dosages, and in the scoring of nerve fibre damage in the Nauta and Gygax preparations, slides were randomised and rescored using tables of random numbers (Snedecor, 1946).

Definition of Lesion of scrapie in the Central Nervous System of the Mouse

The lesion is identified in haematoxylin and eosin stained sections of formal fixed paraffin embedded brain tissue and is assessed using the X25 magnification of the optical microscope.

The lesion appears as a number of approximately circular holes measuring up to 20μ in diameter situated in nerve cell perikarya and neuropil. These holes are termed 'vacuoles' in this thesis, and the term 'vacuolation' is the noun used to describe the state in which vacuoles are present.

This definition includes a number of terms which have appeared in the scrapie literature. In general different terms have been used to differentiate between vacuoles in the neuropil and those in perikarya, but this distinction is not made in this thesis. The terms 'vacuole' and 'neuronal vacuole', usually refer to the holes within perikarya, and 'microvacuolation', 'microcavitation', 'extracellular vacuolation', 'status spongiosis', 'spongy degeneration', 'spongy disintegration' are all terms which have been used to describe the lesion in the neuropil.

OBSERVATIONS

SECTION 1

FACTORS INFLUENCING THE DISTRIBUTION OF LESIONS

The purpose of this section is to describe and evaluate a system of quantitative neuropathological analysis which has been applied to scrapie in mice, and with it to investigate some sources of variation. These include host genotype, sex and age, agent strain, passage status, organ passage or organ source differences of agent, route of inoculation and dosage of agent. These factors may be reflected in variations in other parameters, such as incubation period, and it is clearly important where possible to assess whether these vary independently of one another.

Subsection 1

A. Lesion density scoring system; analysis of reliability

Two main sources of bias contribute to the overall error which is inseparable from a histological scoring system of the type used in this section, these relate to the observer error and to technical variation.

The overall observer error was estimated by rescoring 50 slides chosen at random from a large number, none of which had been intended for rescoring at the time of initial examination. Table 2 shows a high correlation between the two scores for all positions except cerebellum and to a lesser extent, medulla. It is not strictly justified to compare the observer biases on the statistic of correlation coefficient because this is based on the assumption that the variability in lesion intensity is the same for each position, which is unlikely. Therefore, the variance of the

differences between the original and repeat scores for these 50 specimens, is also given in Table 2 for comparative purposes. This is a more meaningful estimate of bias because it can be compared with estimates of observer bias on the repeatability of the six scoring categories (0-5) and with the bias due to cranio-caudal variation in the level of section.

Table 2 CORRELATION COEFFICIENT (r) AND VARIANCE OF DIFFERENCE BETWEEN ORIGINAL AND REPEATED LESION DENSITY SCORE

Region	r	Variance of difference
Medulla	0.84	.18
Cerebellum	0.70	.27
Tectum of midbrain	0.93	.09
Hypothalamus	0.91	.12
Thalamus	0.94	.09
Hippocampus	0.94	.07
Paraterminal body	0.96	.06
Cerebral cortex (posterior midline)	0.95	.07
Cerebral cortex (anterior midline)	0.92	.10

There is some degree of confounding between brain region and score, because the various scores are not equally distributed in all the regions. Table 3, based on the same data of 50 x 9 repeat observations shows that the repeatability of some scores themselves show more variability than others. The higher observer bias with scores 1 and 2 will contribute to the high

repeatability variance in cerebellum (Table 2) because of the low scores in this brain region.

Table 3 VARIANCE OF REPEAT OBSERVATIONS IN GROUPS CLASSIFIED ON ORIGINAL LESION DENSITY SCORE

Original score	Variance of repeat scores
0	0.15
1	0.50
2	0.34
3	0.18
4	0.15
5	0.25

Bias due to preparative technique may arise from variation in the initial cutting of the 5 blocks of the formol fixed brain described in Material and Methods (Figure 1), and to variation in trimming and final section cutting of the embedded tissue. In some cases scoring of a particular area is prevented because of its absence in the sections.

In order to assess the effect of antero-posterior variation in the position of the cut, serial sections were prepared from fifteen randomly selected scrapie brains. Twenty four serial sections were prepared representing 1.5 mm. thickness of brain tissue and every third section was examined making eight in all. With the solitary exception of position 1 (medulla), the first sections were therefore used as the basis upon which

to judge progressive changes in scoring throughout the rest of the series: This was calculated as the variance of the difference between the scores for position y of slide 1 with that on each of the other slides in turn. The sequence of variances which emerged is shown in Table 4.

Table 4 VARIANCE OF DIFFERENCES BETWEEN SCORES ON FIRST SLIDE AND SCORES ON EVERY THIRD CONSECUTIVE SERIAL SECTION

Position	Serial section number						
	4	7	10	13	16	19	22
Medulla	.08	.15	.15	.12	.12	.08	.12
Cerebellum	.03	.54	.57	.64	.68	.71	.64
Tectum of midbrain	.11	.21	.18	.32	.25	.39	.21
Hypothalamus	.11	.11	.04	.11	.07	.11	.07
Thalamus	.14	.18	.14	.14	.14	.14	.14
Hippocampus	.21	.25	.14	.18	.14	.11	.21
Paraterminal body	.18	.11	.14	.18	.14	.18	.18
Cerebral cortex (post midline	.07	.21	.14	.14	.14	.18	.07
Cerebral cortex (ant. midline	.14	.11	.21	.18	.14	.14	.21

This analysis suggests that these technical biases are broadly similar to observer biases. Variance in the cerebellum increases quite considerably as deviation from the first slide increases, and this position also has the highest variance for repeat observations on the same slide (Table 2). The low repeatability for cerebellum can therefore be divided into two components: to observer bias both for the particular region, and for the frequent low

scoring category for the region, and for technical variability. This is in contrast to the medulla for which technical variation plays a minor role (Table 4) and the low repeatability throughout the series is evidently due to high observer error rather than spatial inconsistencies. In the tectum of the mid-brain however, spatial variability is obviously an important source of variation while the observer error component is low (Table 2). Level of section is a relatively less important source of bias in the other five regions.

To summarise the overall findings regarding error variation in the histological scoring: in the medulla the observer error is important, technical variation is more important in the mid-brain, while both sources apply to cerebellum.

B. Lesion density scoring system, a numerical valuation of the scoring in relation to the vacuolation

An estimate was made of the number of vacuoles present in each of the scoring categories. A series of standard photographs was made of a sample of regions which had previously been assigned scores and the number of vacuoles in the photographs were counted. In the case of scores 2, 3 and 4, eight randomly selected examples of each brain region were counted giving seventy-two examples in all: for score 5 only thirty-six examples, confined to five brain regions, were available and all were used. With score 1 the method was slightly different in that the seventy-two random examples often had no vacuoles within a randomly chosen field, due to wide vacuole dispersion. For score 1 the average number in seven fields was therefore used

as the basis for calculation.

The photographs were produced on a Gillett and Seibert photo-microscope using Pan F 40/50 ASA black and white film with iodine quartz illumination from which contact prints were made for counting purposes. A X40 objective, and a X5 camera eyepiece were used, and a green filter was incorporated to increase contrast. A grid was etched on to the back of a disposable petri-dish to cover the surface of the contact print and the counting was facilitated using the subdivisions of this grid and accumulating the score on a digital counter. The mean of the score in each of the fields was calculated, and the final assessment of vacuole number for each score was taken as the mean of the means for all the nine areas.

It appears that the scores from 1 to 4 bear an approximately logarithmic relation to vacuole number, and this probably also applies to score 5. The scale on which the variance of vacuole number is most homogeneous and independent of the mean for scores 2 to 5 is log(vacuole number + 3). The same scale would also appear to be appropriate for score 1 if it also were based on the individual values instead of the averages of seven observations. Using this transformed scale, smoothed distributions of equal sized samples of scores 1 to 5 are shown in Figure 4. Even though slight overlapping of vacuole number for non-adjacent scores occurs the five classes are each significantly distinct ($P < .001$) from their immediate neighbours, as estimated by tests using the transformed values.

With score 5 there was considerable difficulty in counting the large number of vacuoles which are present, using the same photographic methods as for the lower scores. The discontinuity between scores 4 and

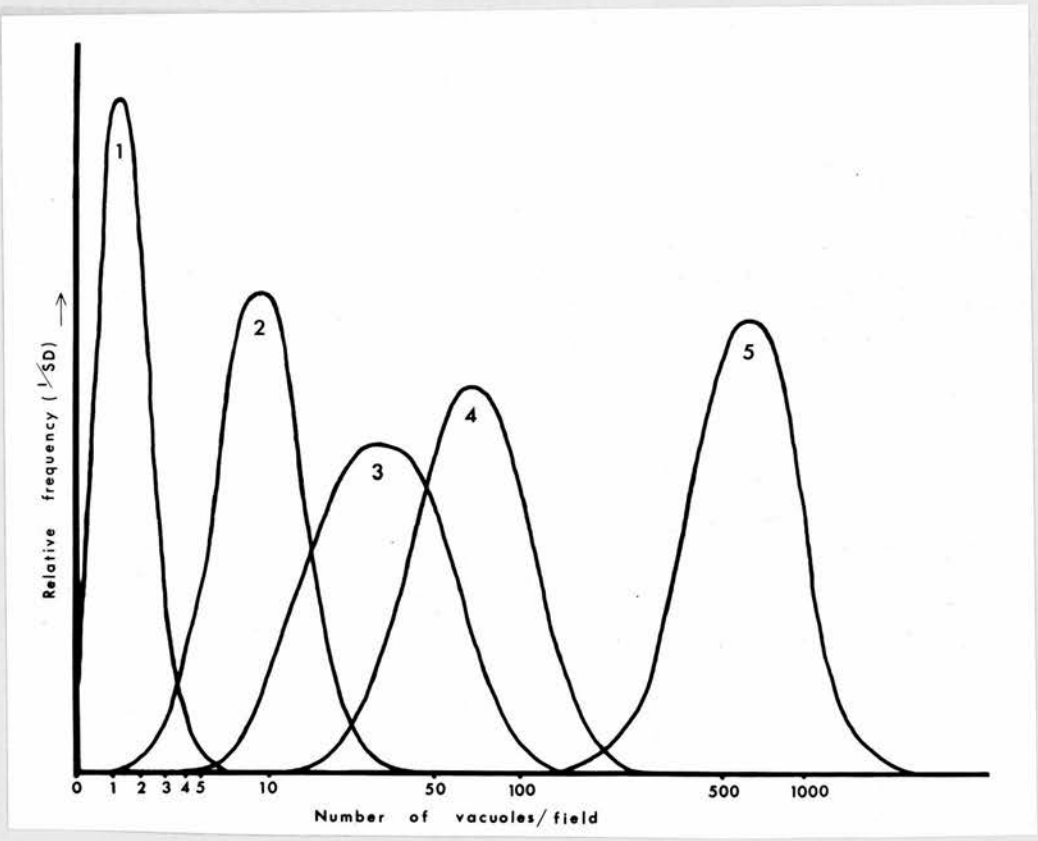


Figure 4 Distribution of numbers of vacuoles in scores 1 to 5. The distributions are calculated from the data transformed to the scale: log (vacuole number +3). The figure is based on equal sample sizes for each score, using in the case of score 1 the mean of seven observations per individual.

5 is almost certainly due to the optical problems of counting vacuoles when confluence occurs: when the vacuoles are confluent and partially superimposed the number of vacuoles tends to be overestimated.

Subsection 2

The differentiation of scrapie agents based on the parameter of lesion profile

The histological alterations in grey matter due to scrapie as revealed by routine staining are almost devoid of qualitative variation and are measurable on the single parameter of vacuolation. The properties of this parameter are described in the previous subsection, and in this, and later subsections it will be shown that many variables affect this aspect of the disease. The agent which has been studied most extensively in our laboratory is ME7 which behaves as a single agent with stable properties; the main criterion for this assertion is that serial passage through either C57 or through VM mice results in no detectable change in the agent. This is in contrast, as will be mentioned below, to some other agents. The results with ME7 do, however, prove that passage in the two genotypes does not necessarily alter an agent. ME7 agent is used to investigate a number of sources of potential variation of lesion profile and incubation period of scrapie in mice, but it cannot be assumed that the other agents, which are used in this subsection will behave as does ME7 under these various conditions.

In the comparisons which follow the donor materials for agents ME7, 22C, 58A, 79A and 80A were from mice with the s7s7 genotype (which almost always implies C57), whereas material for 22A had to be from p7p7 mice.

The reason for this requires brief explanation. Agents 79A, 22C and 22A originate from sheep sources which behave as mixtures of agents, and it has been found that these can be purified or partially purified by passage either serially through s7s7 mice or serially through p7p7 mice. At present 22A is the only agent which is not known to be available with any degree of certainty from s7s7 mice in a purified form, therefore it is necessary to use donor material for 22A from p7p7 mice. With the benefit of experience it is now the practice to inoculate a newly acquired source of scrapie into a standard panel of inbred mouse strains, but with some of the agents in this thesis this was not the case. The reason for the present practice, which is now standard, is that when the starting material is a mixture of certain types of agent these can be separated by passage in mice carrying either s7 or p7 alleles of the sinc gene, and generally this means C57 or VM mice.

For the purposes of this thesis the characterisation of agents is achieved by comparing the lesions which they induce in C57 and VM mice. The grey matter lesions are similar in type but differ in their distribution with the different agents. In addition there are profound differences between agents in the relative proportions of oedematous degeneration of white matter (section 3) which is not included in the parameter on which the agents are categorised in this section. The classification of scrapie agents based on lesion profiles has proved to be identical to that which results from a consideration of their incubation period properties, but with the probability that ME7 and 58A can be distinguished from one another by their lesion profiles but not by incubation periods, (Dickinson, 1970). In this subsection the agents to be considered are ME7, 22A, 22C, 58A, 79A and 80A.

Results

The results are confined to agents given as high dose of infected brain homogenate (10^4-10^5 infectious units) by intracerebral route, to C57BL and VM mice which are killed at the end of the defined incubation period (Dickinson, Meikle and Fraser, 1968).

The lesion profiles for the six scrapie isolates are shown in Figures 5a to 5d, and the significance of the differences between the lesions is given in Table 5. ME7, 22A, 79A and 22C differ in varying degrees from one another in overall shape. In contrast 22C and 80A are indistinguishable ($P > .05$). The position with 58A is somewhat ambivalent. On the basis of lesion profiles it bears a close resemblance to ME7, but in being significantly different in two or three brain positions, the probability that it is different from ME7 exceeds the five per cent level. Although the comparison of 58A with 80A is based on small numbers for each agent, which reduces the significance level of any differences, the lesion profiles of these two agents are dissimilar. 58A is also totally dissimilar to agents 22A, 22C and 79A.

It will be shown in subsequent sections that various procedures modify the profile and incubation period properties, but the conclusion is reached here that four of these strains of scrapie can be distinguished with confidence on the basis of their profiles. It is interesting to note that the two mouse strains are on average equally efficient in the discrimination of the different agents in terms of lesion profiles. Agent identification on the basis of incubation periods requires the use of both mouse strains. However, the same total number of mice - 10 to 15 - are required to characterise an agent on the basis of either parameter.

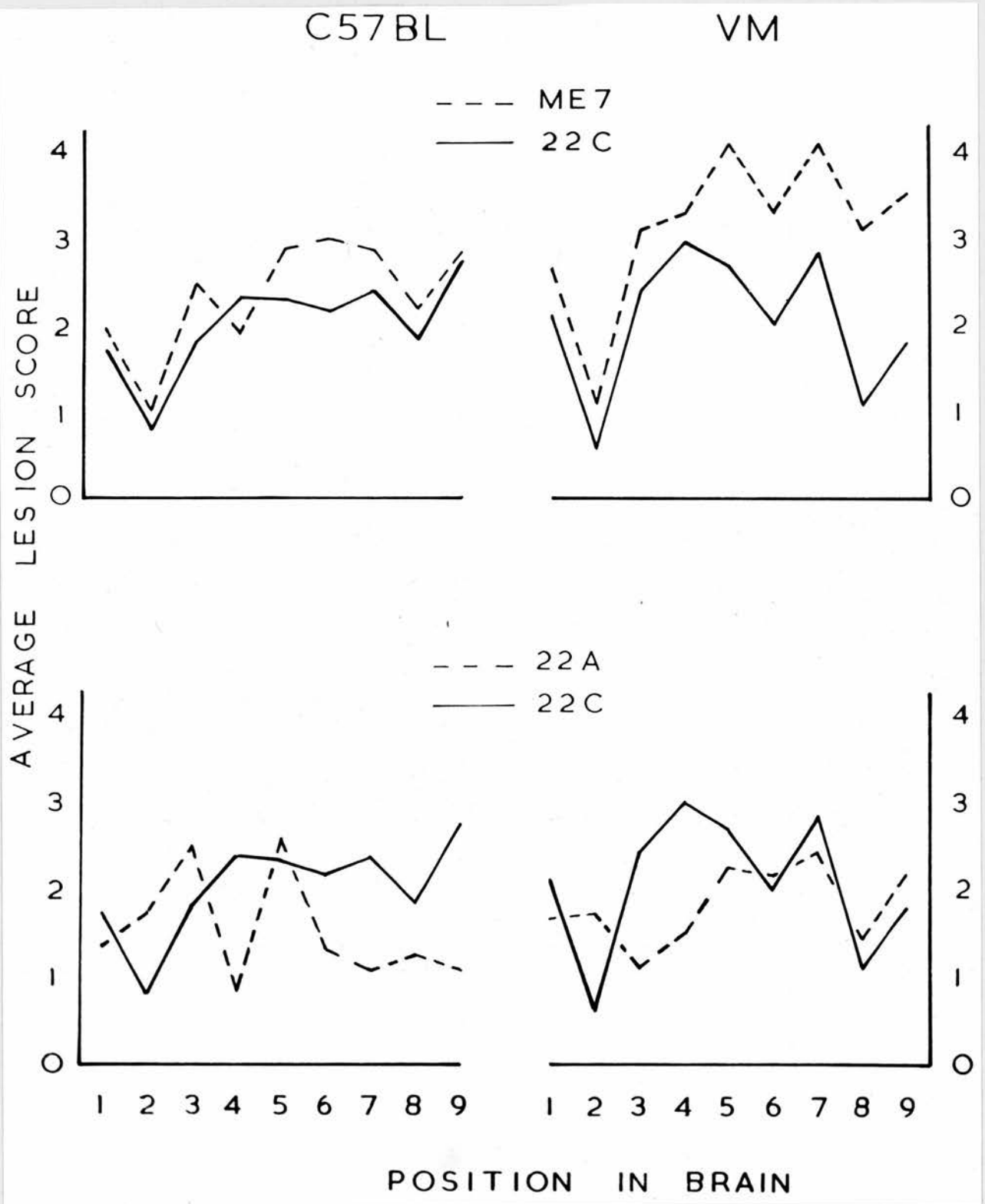


Figure 5a Comparisons of lesion profiles of ME7 and 22A, each with 22C, in two mouse strains with different genotypes at sinc: C57BL (genotype s7s7) and VM (genotype p7p7).

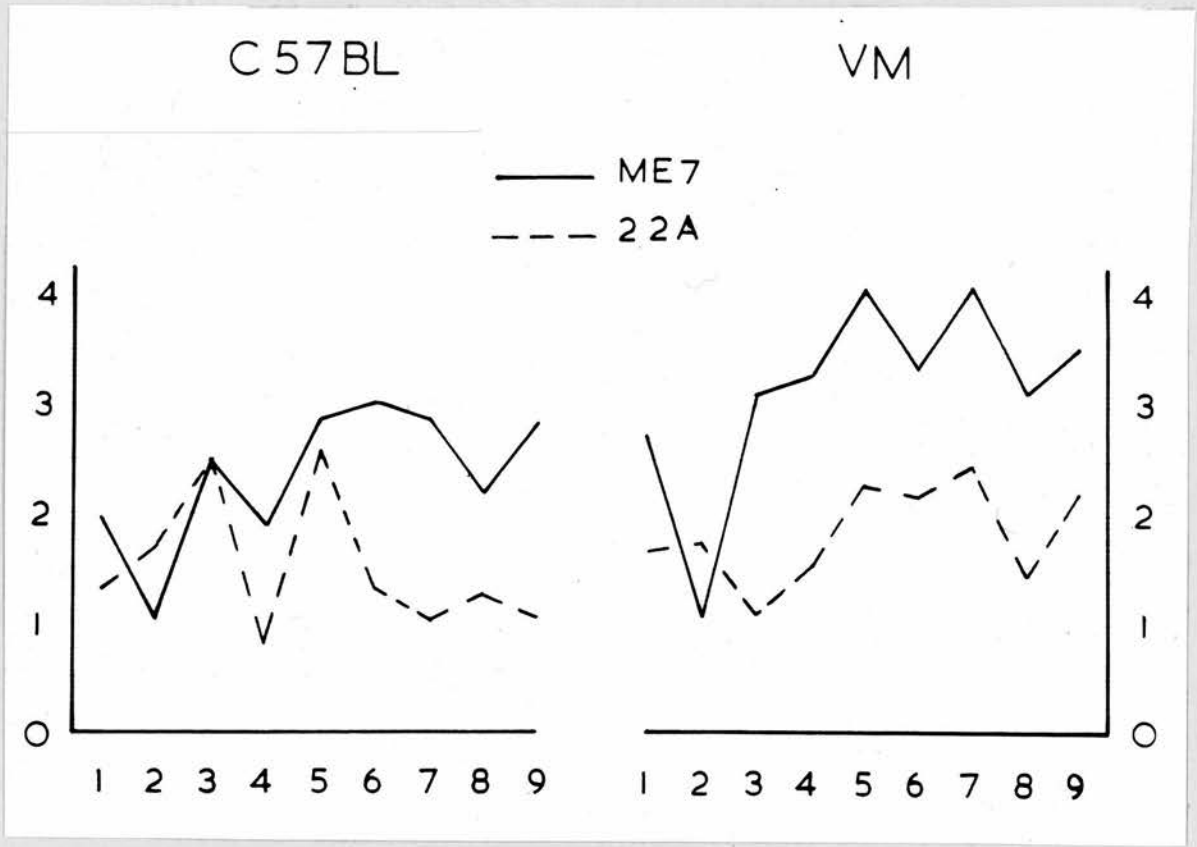


Figure 5b **Comparison of lesion profiles of 22A with ME7 in two mouse strains with different genotypes at sinc: C57BL (genotype s7s7) and VM (genotype p7p7).**

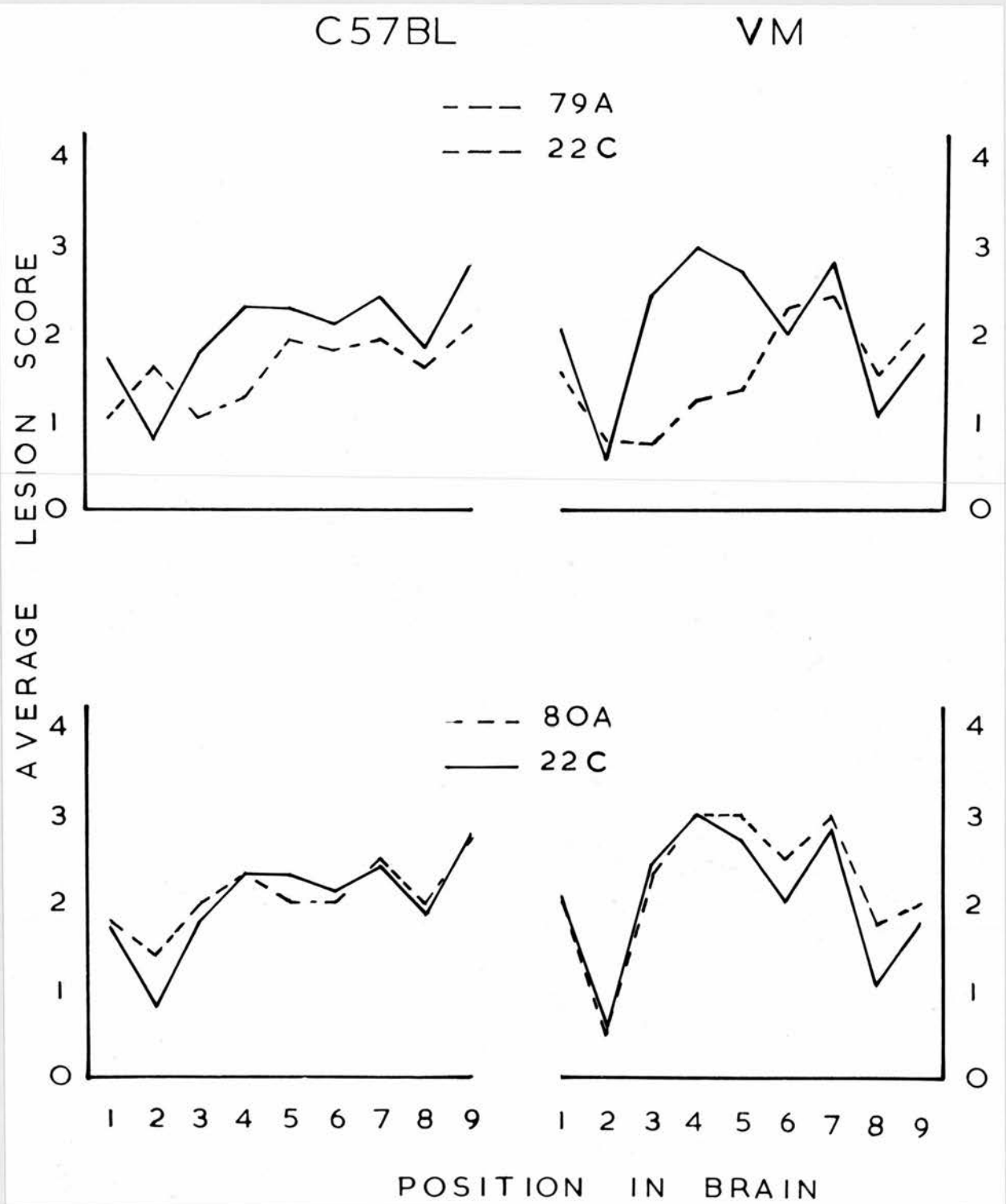


Figure 5c Comparisons of lesions profiles of 79A and 80A, each with 22C, in two mouse strains with different genotypes at sinc: C57BL (genotype s7s7) and VM (genotype p7p7).

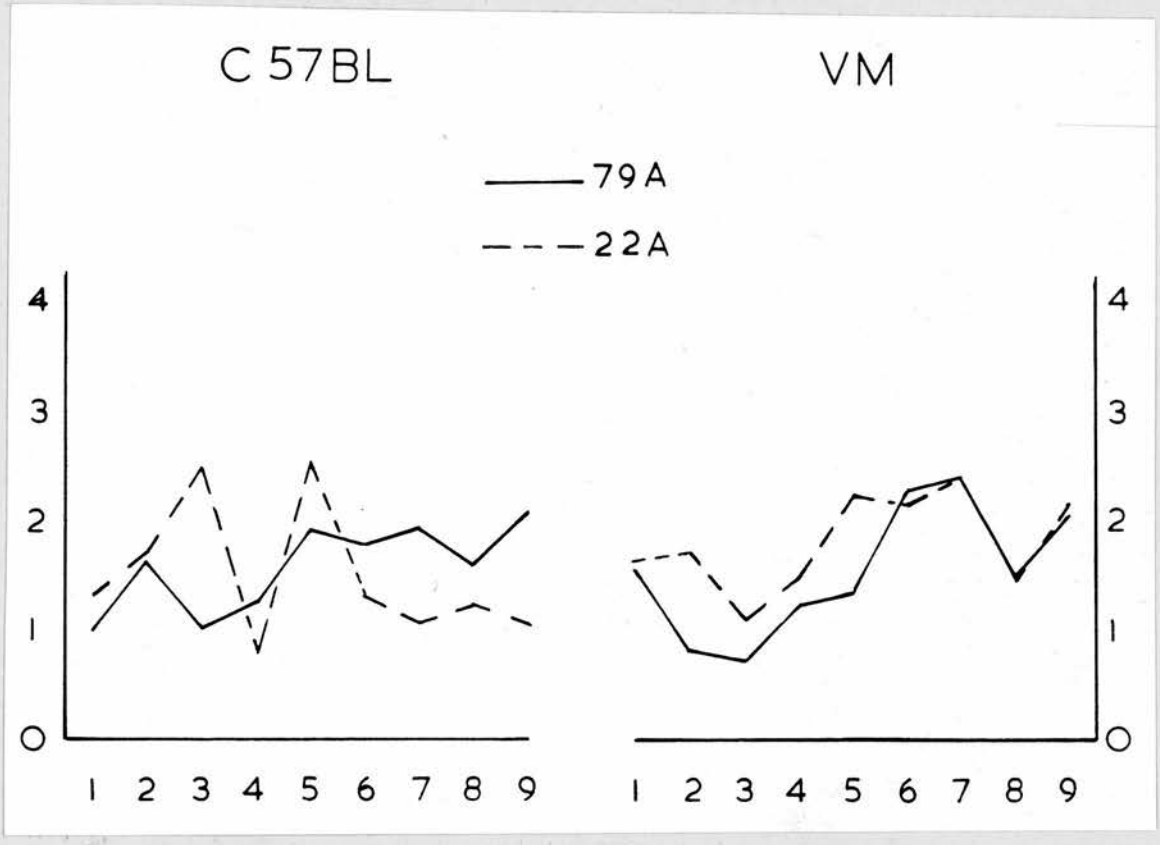


Figure 5d Comparison of lesion profiles of 22A with 79A in two mouse strains with different genotypes at sinc; C57BL (genotype s7s7) and VM (genotype p7p7).

Table 5 PROBABILITIES OF DIFFERENCES BETWEEN AGENT STRAINS:

(P >0.05 -; P <0.05 x; P <0.01 xx; P <0.001 xxx)

Agent strains compared	In Mouse strain	Position in Brain								
		1	2	3	4	5	6	7	8	9
22A and ME7	C57BL	xx	xxx	-	xxx	-	xxx	xxx	xxx	xxx
	VM	xxx	xxx	xxx	xxx	xxx	xxx	xxx	-	xxx
22A and 79A	C57BL	-	-	xxx	-	xxx	x	xxx	-	xxx
	VM	-	xx	-	x	xxx	-	-	-	-
22A and 80A	C57BL	-	-	-	xx	-	-	x	-	xxx
	VM	-	xx	x	xx	xx	-	-	-	-
22A and 22C	C57BL	-	xxx	xxx	xxx	-	xxx	xxx	xx	xxx
	VM	x	xxx	xxx	xxx	x	-	x	xxx	x
ME7 and 79A	C57BL	xxx	x	xxx	xx	xxx	xxx	xxx	xx	xxx
	VM	xx	-	xxx	xxx	xxx	xxx	xxx	xxx	xxx
ME7 and 80A	C57BL	-	-	-	-	x	xxx	-	-	-
	VM	-	-	-	-	xx	xx	xxx	xxx	xxx
ME7 and 22C	C57BL	-	-	xxx	xx	xxx	xxx	xxx	x	-
	VM	x	-	x	xxx	xxx	xxx	xxx	xxx	-
79A and 80A	C57BL	x	-	x	x	-	-	x	-	x
	VM	-	-	xx	xx	xx	-	-	-	-
79A and 22C	C57BL	xx	xx	xxx	xxx	xx	-	xxx	-	xxx
	VM	-	-	xxx	xxx	xxx	-	-	x	-
80A and 22C	C57BL	-	-	-	-	-	-	-	-	-
	VM	-	-	-	-	-	-	-	-	-
58A and ME7	C57BL	-	-	xx	-	-	-	-	-	x
	VM	xx	-	x	-	-	-	-	-	xx
58A and 79A	C57BL	xx	xx	xxx	-	xxx	xxx	xxx	-	x
	VM	-	-	xxx	xxx	xxx	xxx	xxx	xxx	xxx
58A and 80A	C57BL	-	-	-	-	x	-	-	-	-
	VM	-	-	-	-	xxx	x	-	x	xx
58A and 22C	C57BL	-	-	-	xxx	x	xx	xxx	-	-
	VM	-	-	-	-	xxx	xxx	xxx	xxx	xxx
58A and 22A	C57BL	xx	xx	xxx	-	xxx	xxx	xxx	-	x
	VM	-	-	xxx	xxx	xxx	xxx	xxx	xxx	xxx

Subsection 3

The influence of host genotype, and the sequential development of the brain lesions of scrapie

Scrapie lesions in the brains of two strains of mice and their F_1 crosses, which differed genetically from one another in terms of incubation period, were examined histologically at a common clinical end-point to assess the independence of the two parameters, brain lesion distribution and incubation period. In a second experiment three strains of mice were used to determine whether differences in the distribution or rate of appearance of lesions could be demonstrated. In this experiment also, one of the strains differed from the other two in terms of the genetical control of incubation period with the particular scrapie agent used.

Experiment 1 The mouse strains were RIII and VM. The F_1's were all from RIII males mated with VM females. They were inoculated intracerebrally at between $4\frac{1}{2}$ and 9 months of age with a hundredfold dilution of third mouse passage ME7 scrapie from clinically affected male VM mice. These animals were all killed at the clinical end-point, and the incubation period for the F_1's was midway between those of the parental strains.

Experiment 2 Mice of the VM, C57 and BALB strains were inoculated intracerebrally at 4 weeks of age with a hundredfold dilution of brain from a C57 mouse clinically affected with ME7 scrapie. The experiment was carried out concurrently on the three strains which were housed under identical environmental conditions. From the 12th week after inoculation in the C57 and BALB strains and from the 20th week in the case of VM, groups of 2 or 3

mice from each strain were killed at weekly intervals. A small number of animals were allowed to reach a clinical end-point for calculation of incubation period.

Results

The results of the first experiment are illustrated Figure 6. In the positions where parental values differ markedly from one another, the values for the F_1's are nearly intermediate, and the lesion profile for RIII contrasts strikingly with that for VM and F_1's, showing relatively severe damage in positions 2 (cerebellar cortex) and 6 (hippocampus). In those positions where the lesion density is significantly different in the parental strains, the F_1 is intermediate (positions 2, 5, 7 and 8).

In the second experiment there were differences related to mouse strain in the degree of brain damage at the end of the clinical stage of the disease, in the rate at which lesions developed and in the distribution of lesions during and at the end of the incubation period. Figure 7 shows the rate of development of lesions in the strains using the sum of the individual scores for each of the nine regions as the index (total score). The relative stage of the incubation period at which the first lesions (arbitrarily defined as total score >2) occur was fairly uniform for the three strains being 59% for C57, 61% for BALB and 54% for the VM strain. The incubation periods, estimated from the small number of animals allowed to reach an end-point, was 165 ± 3 days for C57, 171 ± 0 days for BALB and 311 ± 36 days for VM. Once started, the brain damage, as estimated by total score, progressed more slowly in VM than in either of the other strains

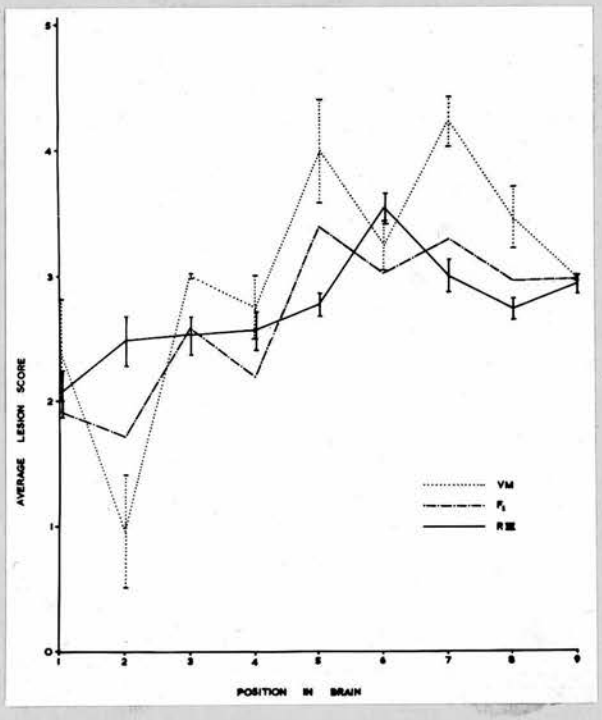


Figure 6 The lesion profiles caused by ME7 scrapie in parental and F1 generations. The vertical lines indicate standard errors for parental stocks.

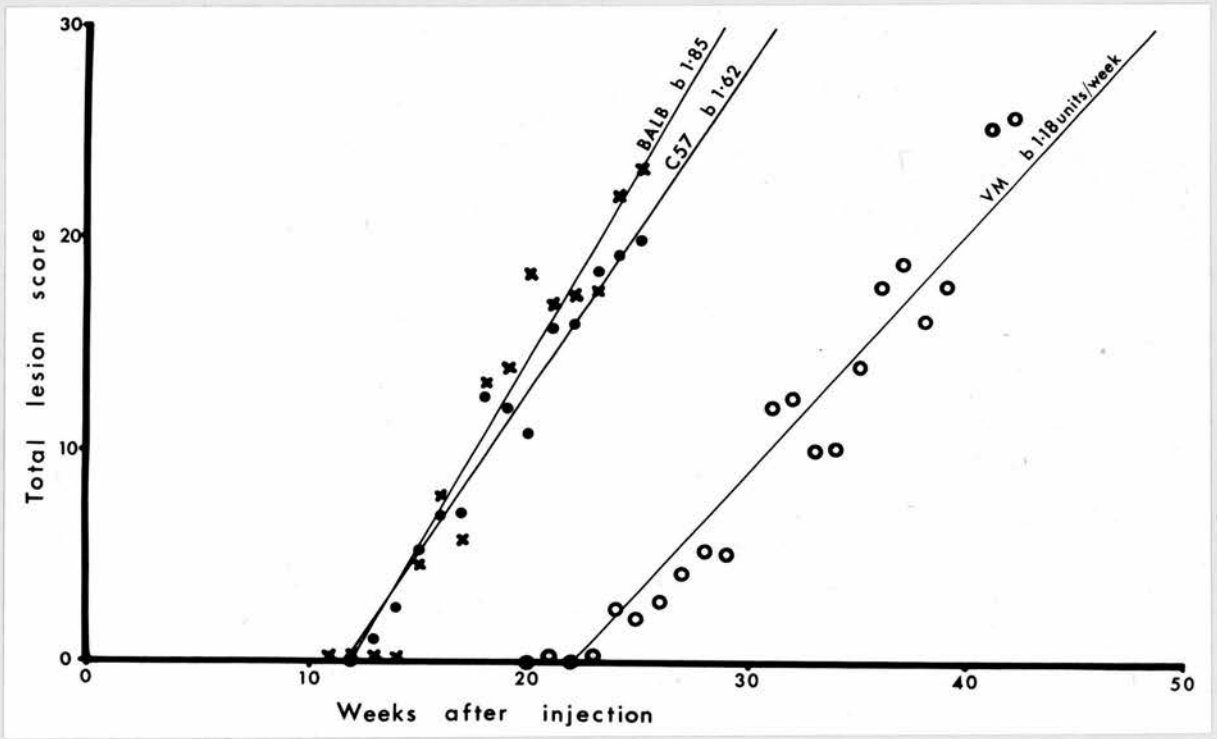


Figure 7 Rate of development of total lesion density to the end of the incubation period in three strains of mice.

x : BALB; ● : C57BL; o : VM.

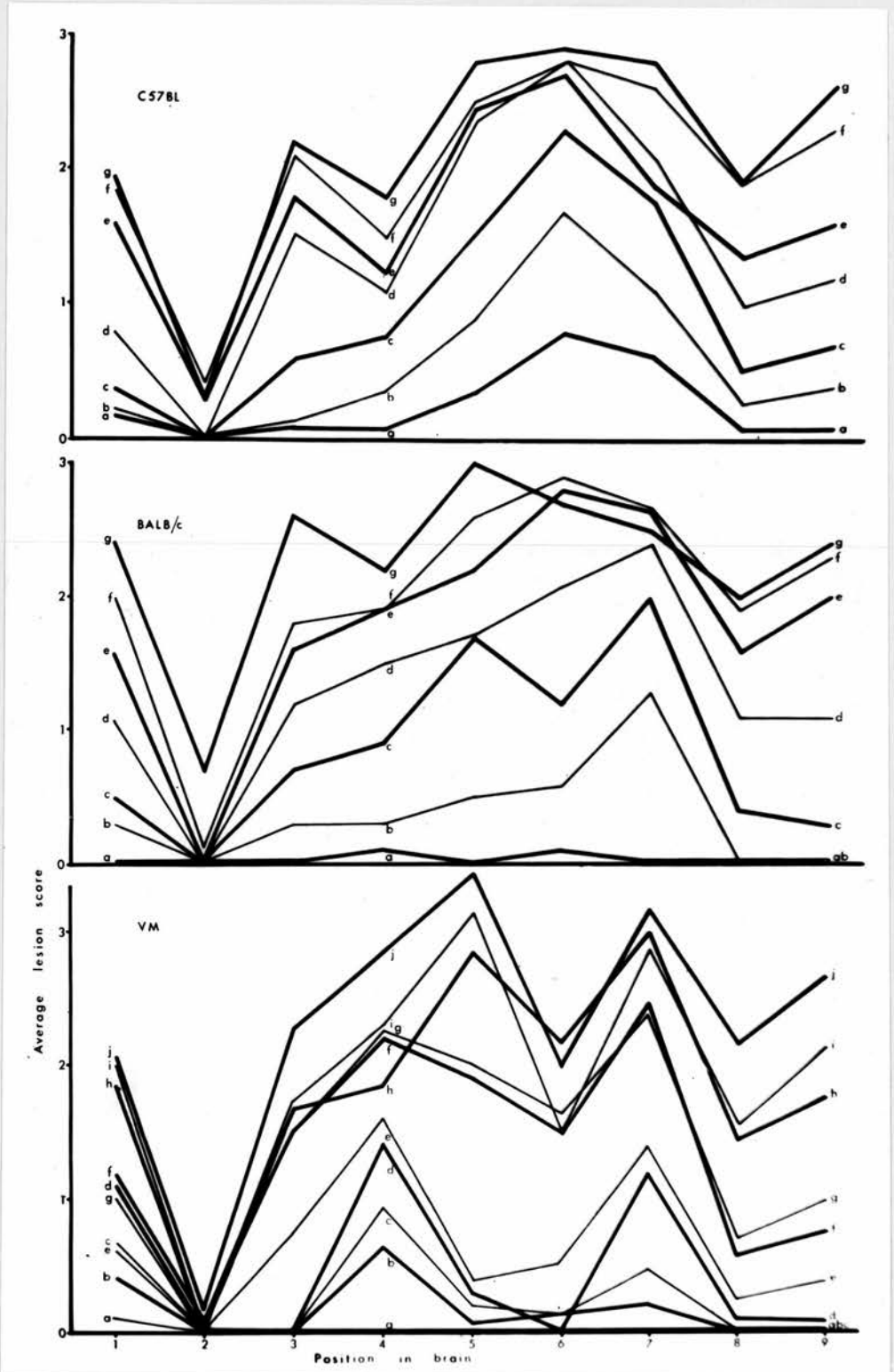
($P < .001$); BALB and C57 did not differ significantly in this respect ($P > .1$). Figure 8 shows regional development of the lesions and Figure 9 the distribution of damage at the terminal stage, with the significance of these terminal differences listed in Table 6.

Table 6 PROBABILITIES OF DIFFERENCES BETWEEN MOUSE STRAINS IN LESION DENSITY IN DIFFERENT AREAS OF THE BRAIN

Mouse strains compared	Position in brain								
	1	2	3	4	5	6	7	8	9
BALB and C57BL	>.1	<.05	<.01	<.02	>.1	>.1	>.1	<.05	>.1
C57BL and VM	<.05	>.1	<.01	<.001	<.001	>.1	<.001	<.01	>.1
VM and BALB	>.1	>.1	>.1	<.05	<.01	>.1	<.001	>.1	>.1

Three points emerge. The distribution of the terminal lesions is different between the strains, although the overall pattern in C57 and BALB is broadly more similar than either is to VM. It is also striking that the early distribution of lesions is continued throughout into the fully developed disease. Finally, although the relative stage of the incubation period at which lesions first appear is approximately the same for each of the mouse strains, the rate of development of these lesions when the whole brain is considered is less in the longer incubation period strain (VM).

Figure 8 Sequential development of lesions in the brains of three mouse strains killed at weekly intervals during the incubation period of scrapie caused by ME7 agent. Starting at 12 weeks in C57BL, 11 weeks in BALB, and 20 weeks in VM, the lines are based on four point running averages in order to minimise random fluctuations. The small letters indicate the sequence (e.g., in C57 a = 12 + 13 + 14 + 15; b = 14 + 15 + 16 + 17; c = 16 + 17 + 18 + 19; etc.)


C57BL
BALB/c
VM
Average lesion score
Position in brain

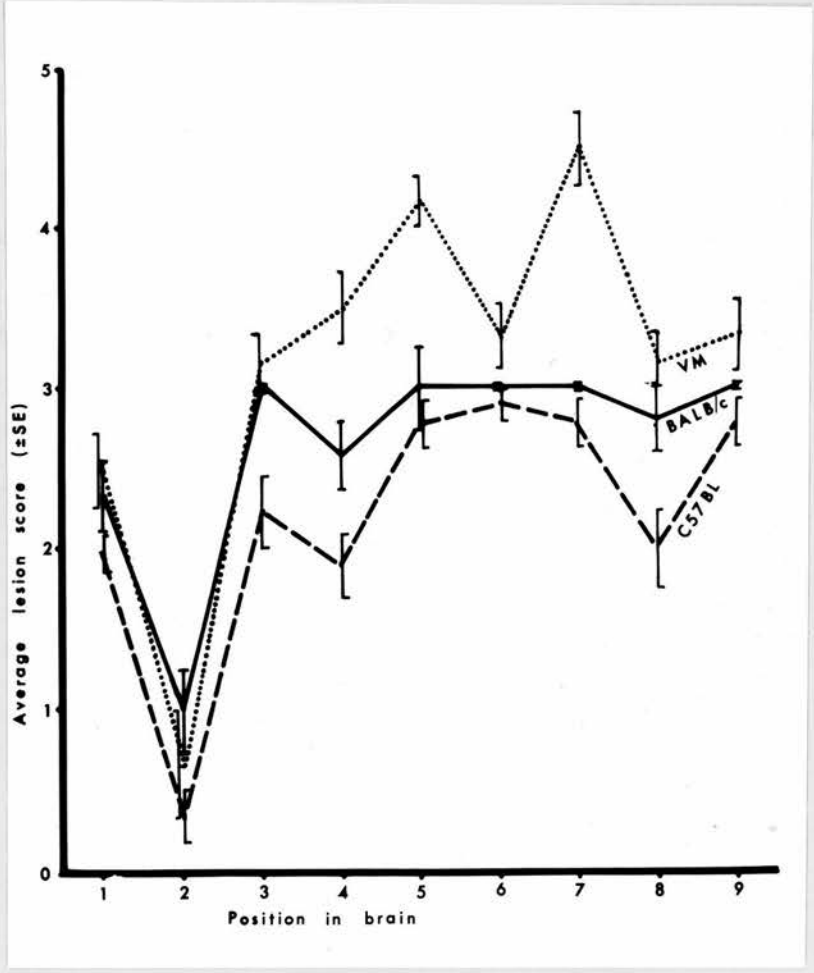


Figure 9 Distribution of brain lesions at the terminal stage of scrapie caused by ME7 agent in three mouse strains: VM; C57BL; BALB. The vertical lines indicate standard errors.

Subsection 4

The passage status of the scrapie agent, its effect on lesion profile

It was pointed out in the previous subsection that the isolation of scrapie into inbred mice with different *sinc* genotypes may result in the separation or partial separation of agents if the starting material contains a mixture of 22A-group and ME7-group agents. For this reason it is frequently impossible to identify the properties of an agent in the first passage and the categorisation in the previous subsection was confined to agents which had been passaged through mice of known *sinc* genotypes.

It has also been reported on several occasions that the lesions at the first passage into mice from goats or sheep differ from that at subsequent mouse to mouse passages: the lesions at the first mouse passage being confined to the neuraxis, whereas in subsequent mouse passages the lesions are widespread and occur in the fore-brain (Zlotnik and Rennie, 1962; 1963). This change in the distribution of lesions with mouse passage has been observed on five occasions at the Moredun Research Institute in Edinburgh (Zlotnik, 1965): twice with sheep isolates and three times with goat isolates. Two of these goat transmissions were replicates of ones carried out at the A.R.C. Institute for Research in Animal Diseases at Compton in Berkshire in which the same phenomenon was not remarked upon (Pattison and Smith, 1963; Chandler, 1963). Mouse passaged scrapie has been injected back into goats and then reintroduced into mice on two occasions. On one of these occasions an agent derived from the 'scratching syndrome' of goats was used and on the other the ME7 agent. The lesions at the first passage of this reintroduction into mice were widespread and not preponderantly in

the hind-brain. An isolate from a Southdown ewe did not cause fore-brain lesions at the second mouse passage, although there was some alteration in the lesion pattern when compared with the first passage to mice (Zlotnik, 1965).

In this subsection two different aspects of the passage of scrapie in mice are described and contrasted with previous findings. Firstly the lesion profile at the primary passage in mice of the 58A Suffolk scrapie source is shown to be indistinguishable from the pattern which has been regarded as typical for mouse to mouse passaged material, and is in fact very similar to that for the second passage of this agent in the mouse. Secondly it is shown that, for ME7, the lesion profile can remain constant for a number of passages once it has been adapted to mice, or at least to mice of specific genotypes.

Results

The lesion profiles in C57 and VM mice of the first mouse passage of 58A sheep brain source, and the profiles of the second mouse passage from the s7s7 donors in the first passage, are shown in Figure 10. The similarity between this agent and ME7 has already been remarked upon (Subsection 2). The lesion profile at the first mouse passage is therefore quite distinct from that which has sometimes been associated with the primary isolation of sheep source agents. There are good reasons for concluding that the results for 58A represent the isolation of a sheep agent and are not due to accidental contamination with mouse passaged ME7: the incubation periods in this case exceed those for limiting dilutions of ME7 and it has been

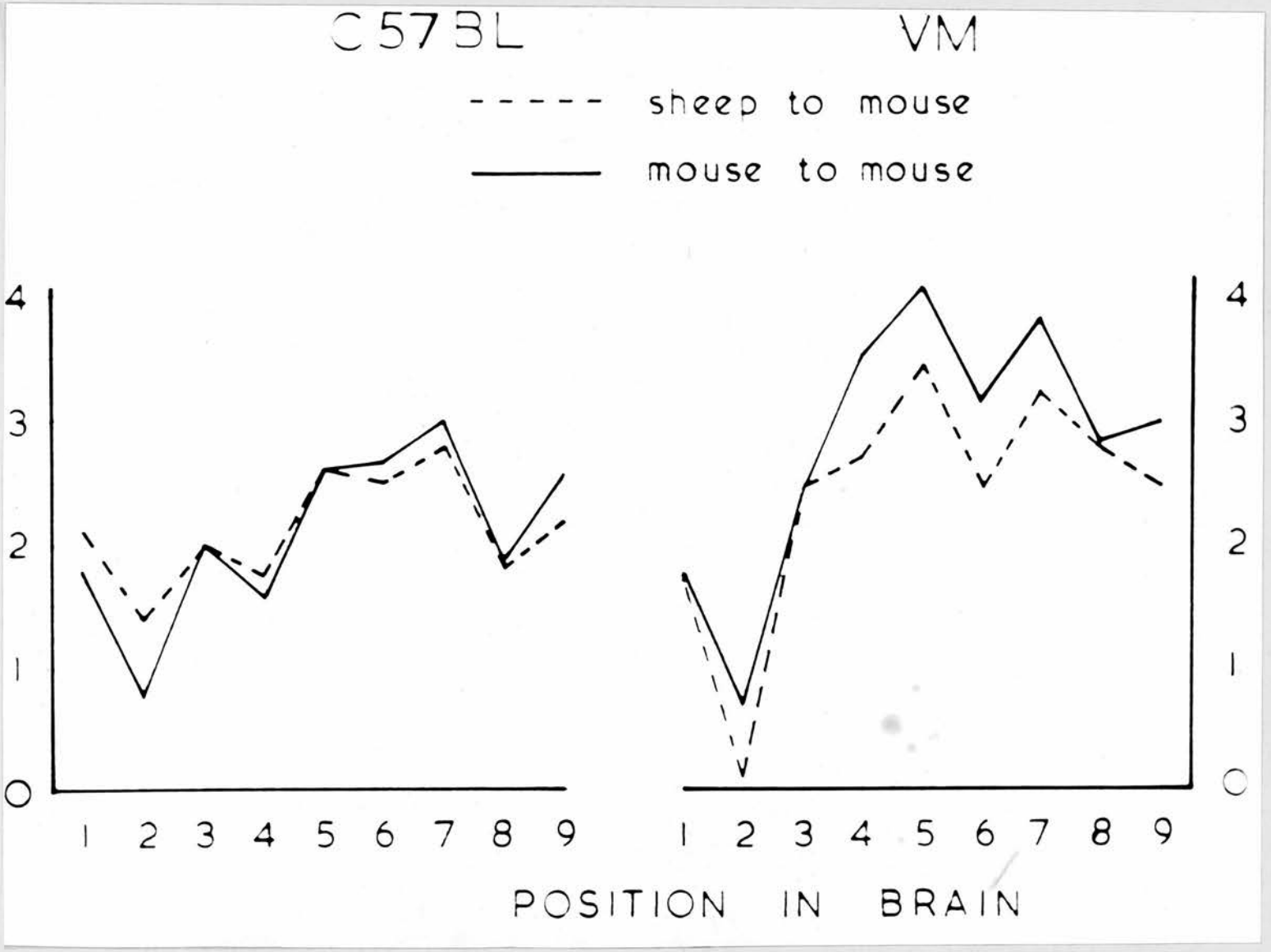


Figure 10 Lesion profiles for agent 58A at the first and second passages into two mouse strains with different genotypes at sine: C57BL (s7s7) and VM (p7p7).

shown separately that the presence of scrapie sheep brain in an ME7 inoculum does not affect the ME7 incubation period (Fraser, unpublished). Also, as pointed out in Subsection 2, the lesion profile for 58A does show some significant but limited differences from ME7, although these alone may not justify a conclusion that they are different agents.

The ME7 mouse passaged agent is examined within six combinations of organ source, inoculation route and mouse strain. The passage numbers refer to the numbers of subpassages in inbred mice. The absence of profiles for the first and sometimes the second passages is because they predated the histological methods used in this work.

The results of passage are shown in Figures 11 to 16. In each the donor organ and mouse strain for the first passage are the same as the subsequent passages. Figure 11 shows the profiles for 4 of 8 passages in which ME7 was passaged by intracerebral inoculation of brain source through C57 mice (the 6th and 8th passages are not shown both to avoid confusing the diagram and because they are very similar to the others and the 3rd and 5th passages were not examined histologically). The six passages are compared using t tests, both between individual passes, and between individual passes and the mean of the mean of them all (Table 7). There are thus 189 estimates of P. Assuming no difference between the passages and an arbitrary significance level of probable difference at 5 per cent, the number of values for P of less than 0.05, less than 0.02, less than 0.01 or less than 0.001 can be arrived at, for instance, 9 values of P of less than 0.05, and the differences between these theoretical and the actual incidences can be compared using a Chi square test. On this basis the

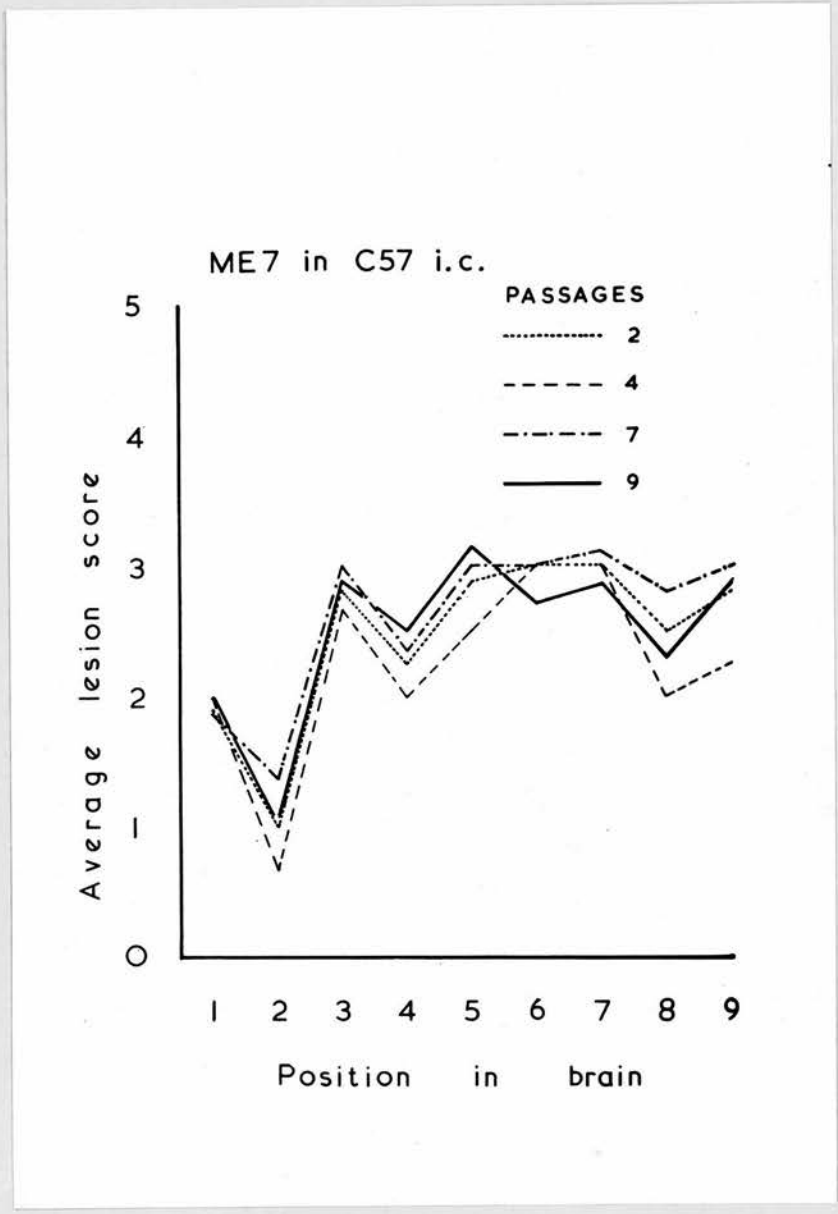


Figure 11 Lesion profiles of the 2nd, 4th, 7th and 9th passages of C57BL brain source ME7 passaged intracerebrally through C57BL mice. The probabilities of difference between these passes (and including the 6th and 8th passes) are given in Table 7.

Table 7 PROBABILITIES OF DIFFERENCE OF LESION SCORES IN NINE BRAIN REGIONS, BETWEEN SIX PASSAGES, AND THE MEAN OF THE MEANS OF SIX PASSAGES, OF ME7 AGENT FOLLOWING INTRACEREBRAL INOCULATION IN C57BL MICE, ILLUSTRATED IN FIGURE 11.
($P > 0.05$ -; $P < 0.05$ x; $P < 0.01$ xx; $P < 0.001$ xxx).

Difference between Passages	Position in Brain								
	1	2	3	4	5	6	7	8	9
2 & 4	-	-	-	-	-	-	-	-	-
2 & 6	-	-	x	-	-	-	-	-	-
2 & 7	-	-	-	-	-	-	-	-	-
2 & 8	-	-	-	-	-	-	-	-	-
2 & 9	-	-	-	-	-	-	-	-	-
4 & 6	-	-	-	-	-	-	-	-	-
4 & 7	-	-	-	-	x	-	-	x	xx
4 & 8	-	-	-	-	-	-	-	-	x
4 & 9	-	-	-	-	-	-	-	-	-
6 & 7	-	-	x	-	x	-	-	x	-
6 & 8	-	-	xxx	-	-	-	-	-	-
6 & 9	-	-	x	-	-	-	-	-	-
7 & 8	-	-	-	-	-	-	-	-	-
7 & 9	-	-	-	-	-	-	-	-	-
8 & 9	-	-	-	-	-	-	-	-	-
2 & (mean of means of all six passes)	-	-	-	-	-	-	-	-	-
4 & (mean of means of all six passes)	-	-	-	-	-	-	-	-	-
6 & (mean of means of all six passes)	-	-	x	-	-	-	-	-	-
7 & (mean of means of all six passes)	-	-	-	-	-	-	-	-	x
8 & (mean of means of all six passes)	-	-	-	-	-	-	-	-	-
9 & (mean of means of all six passes)	-	-	-	-	-	-	-	-	-

difference between the theoretical and actual incidence of P of <0.05, <0.02, <0.01, <0.001, is not significant (Chi square, P >0.5, >0.1, >0.9 and >0.05 respectively). Values of P less than 0.05 do not occur between the first two and last two passages, which might have been expected if there had been a gradual change in the properties of the agent with subpassage through this uniform system, and the overall shapes of the profiles do not suggest such differences.

Two separate lines of subpassage of ME7 as a spleen source in C57 mice inoculated intraperitoneally are shown in Figures 12 and 13. In the sequence illustrated in Figure 12 the third passage is significantly different from the 4th, 5th and 6th in several positions, but no differences are apparent between the last three passages. In the other sequence, illustrated in Figure 13, no difference can be demonstrated between the 4th, 5th and 6th passages. Similarly in a sequence of three intraperitoneal passages of ME7 spleen source in another strain (SM, sinc s7s7) no difference could be shown to occur (Figure 14) (the 1% level difference in position 1, between the 3rd and 4th passages could occur by chance in such a large number of tests, P >0.05).

Two sequences of passage of ME7 in VM mice (sinc p7p7) are shown in Figures 15 and 16. The profiles in the intracerebral passage sequence are clearly very similar in overall shape but there appears to be a gradual increasing severity over the three passages, significant at the 1% and 5% level, respectively, which is a marginally significant regression (see below). The two profiles representing intraperitoneal passages in VM mice do not differ from one another significantly at any position, and in contrast with the intracerebral series the lesion intensity means are slightly less in the

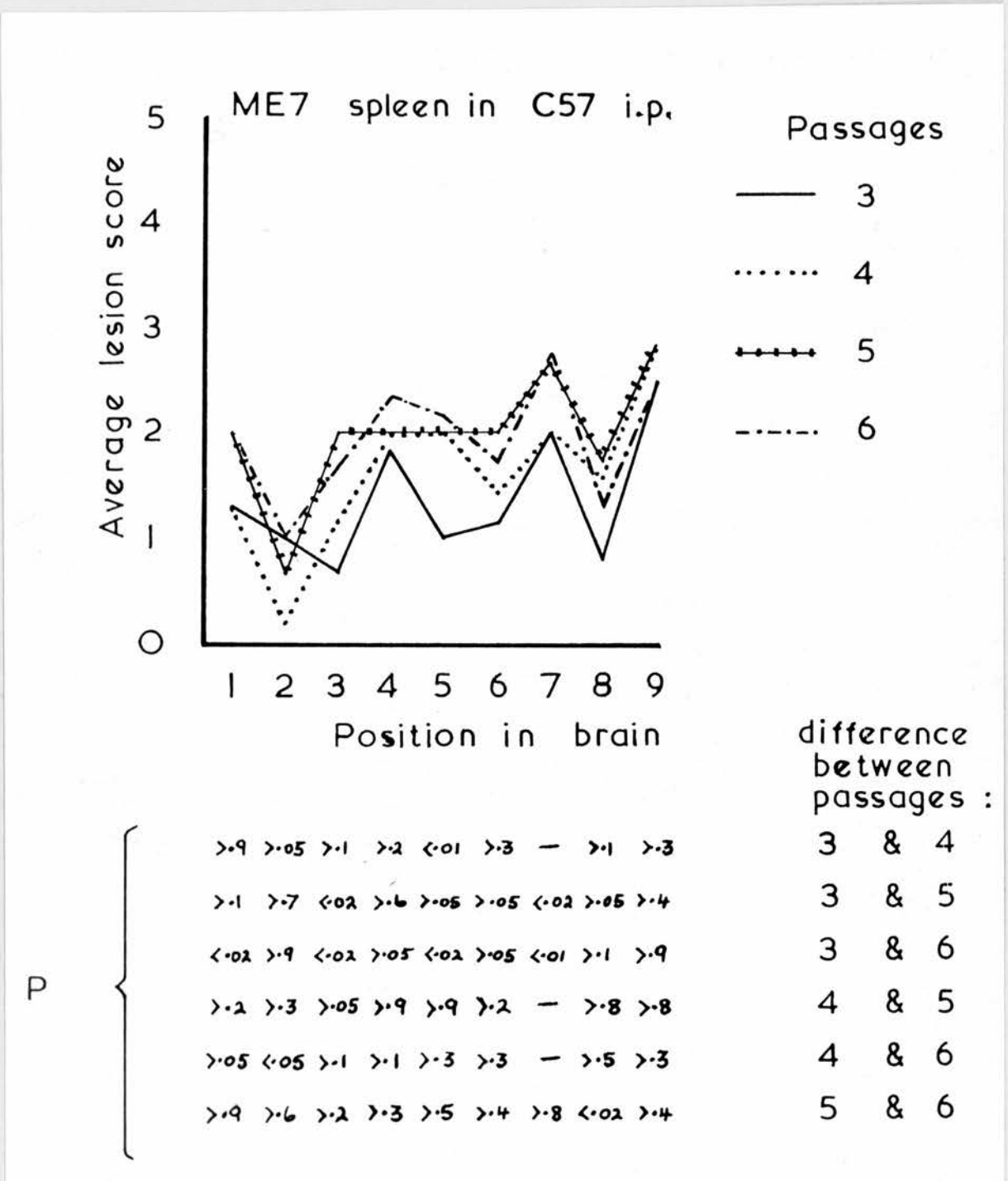


Figure 12 Passage of ME7 spleen intraperitoneally in C57BL mice.

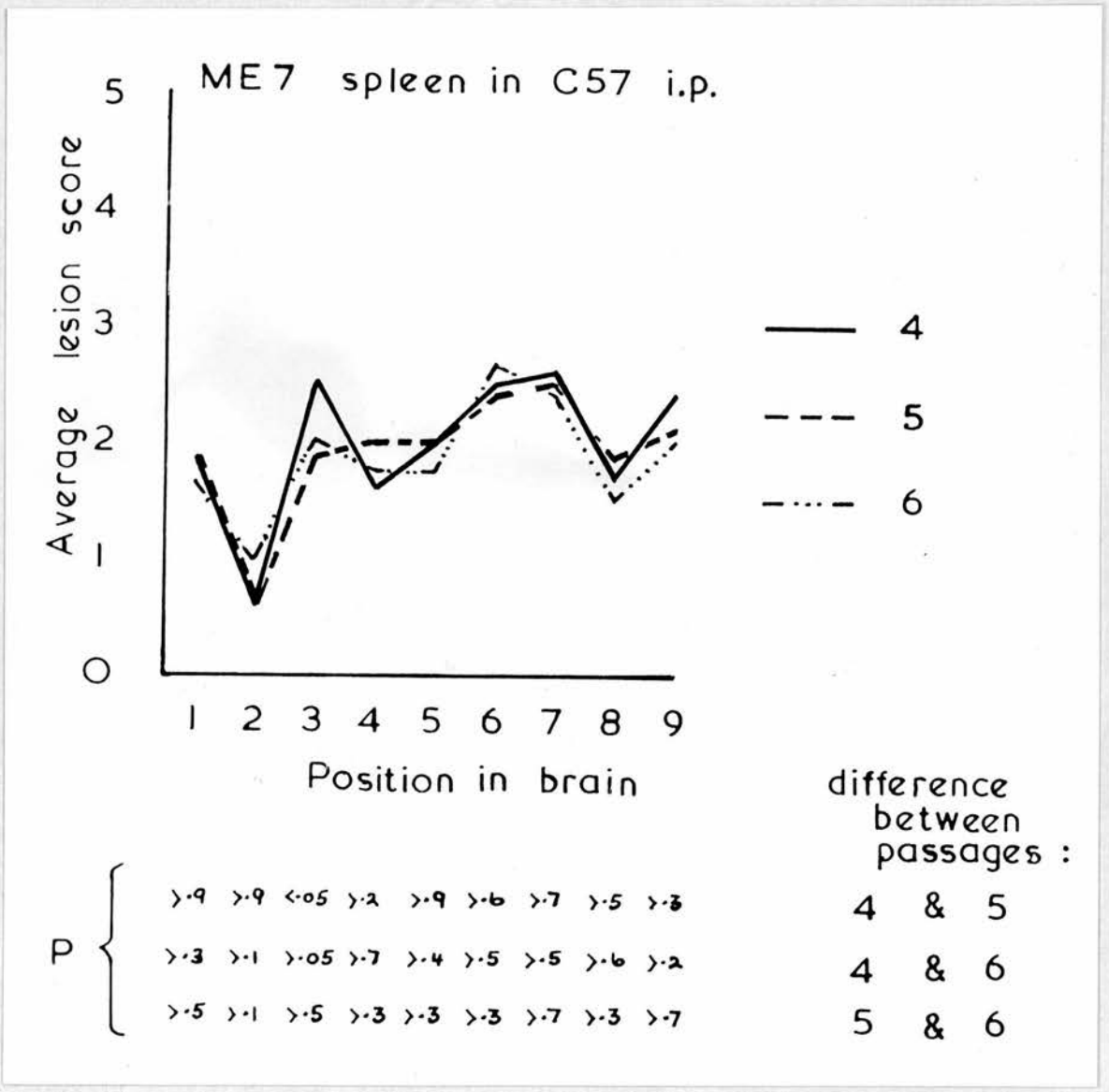


Figure 13 Passage of ME7 spleen intraperitoneally in C57BL mice.

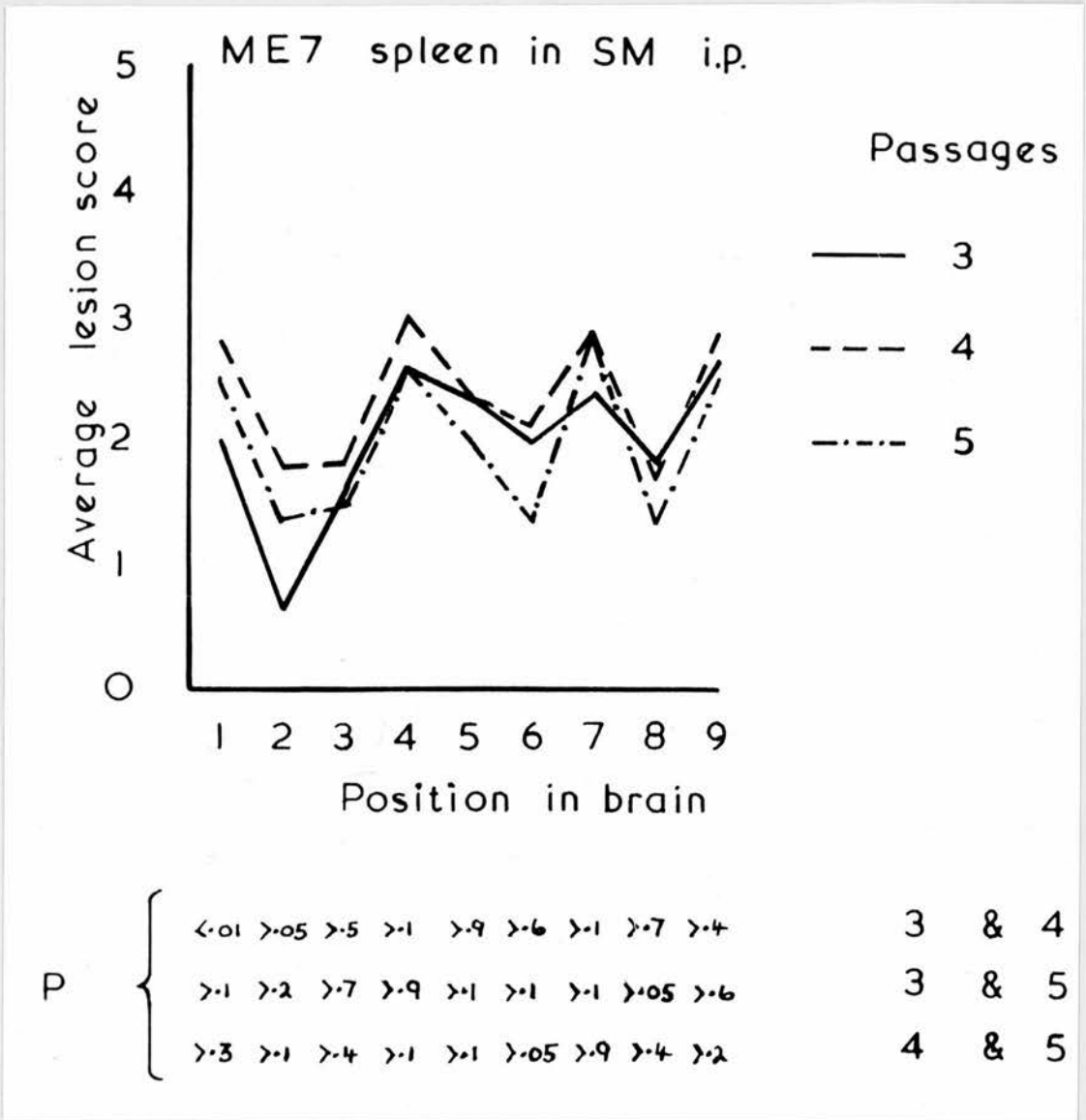


Figure 14 Passage of ME7 spleen intraperitoneally in SM mice.

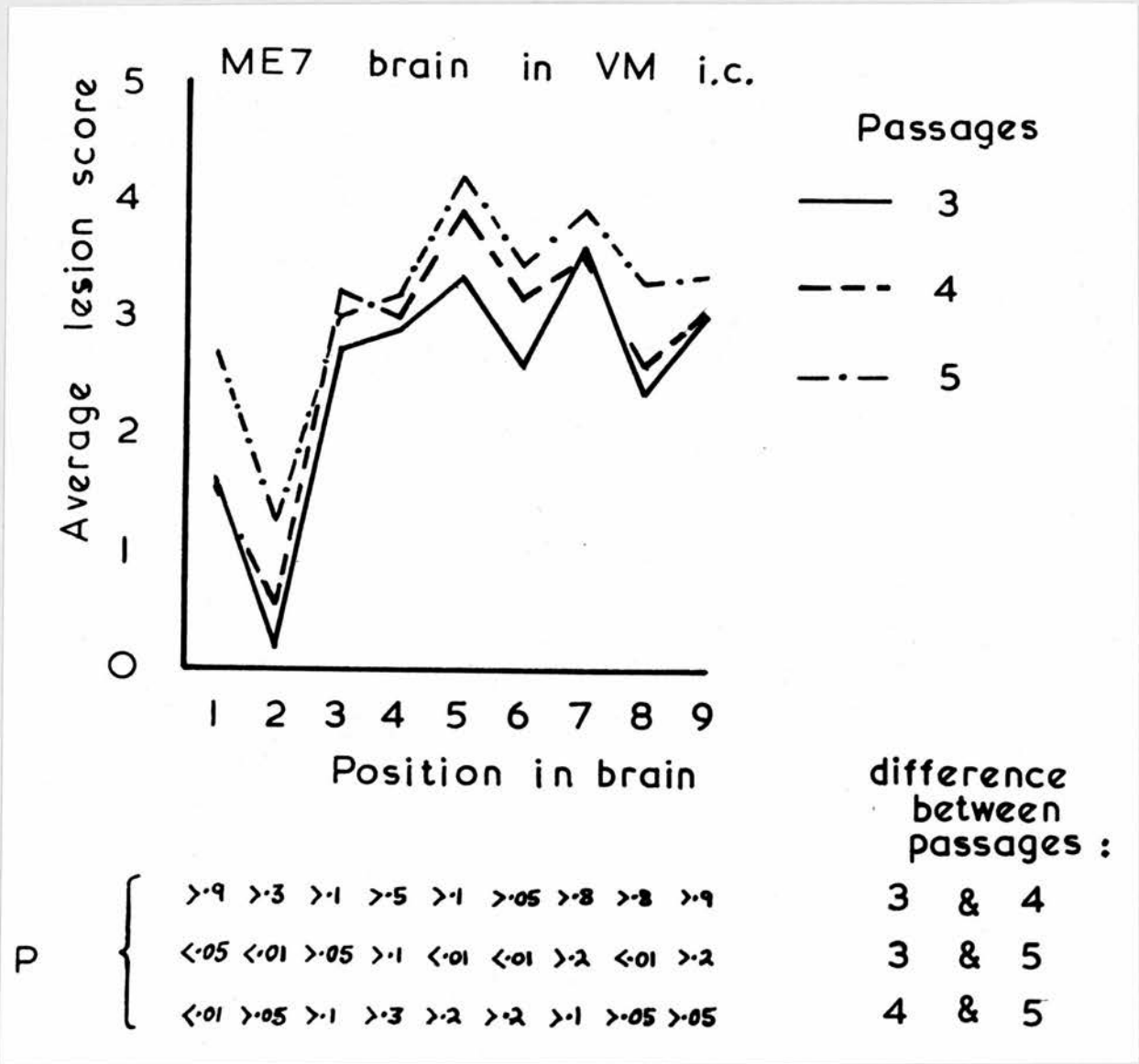


Figure 15 Passage of ME7 brain intracerebrally in VM mice.

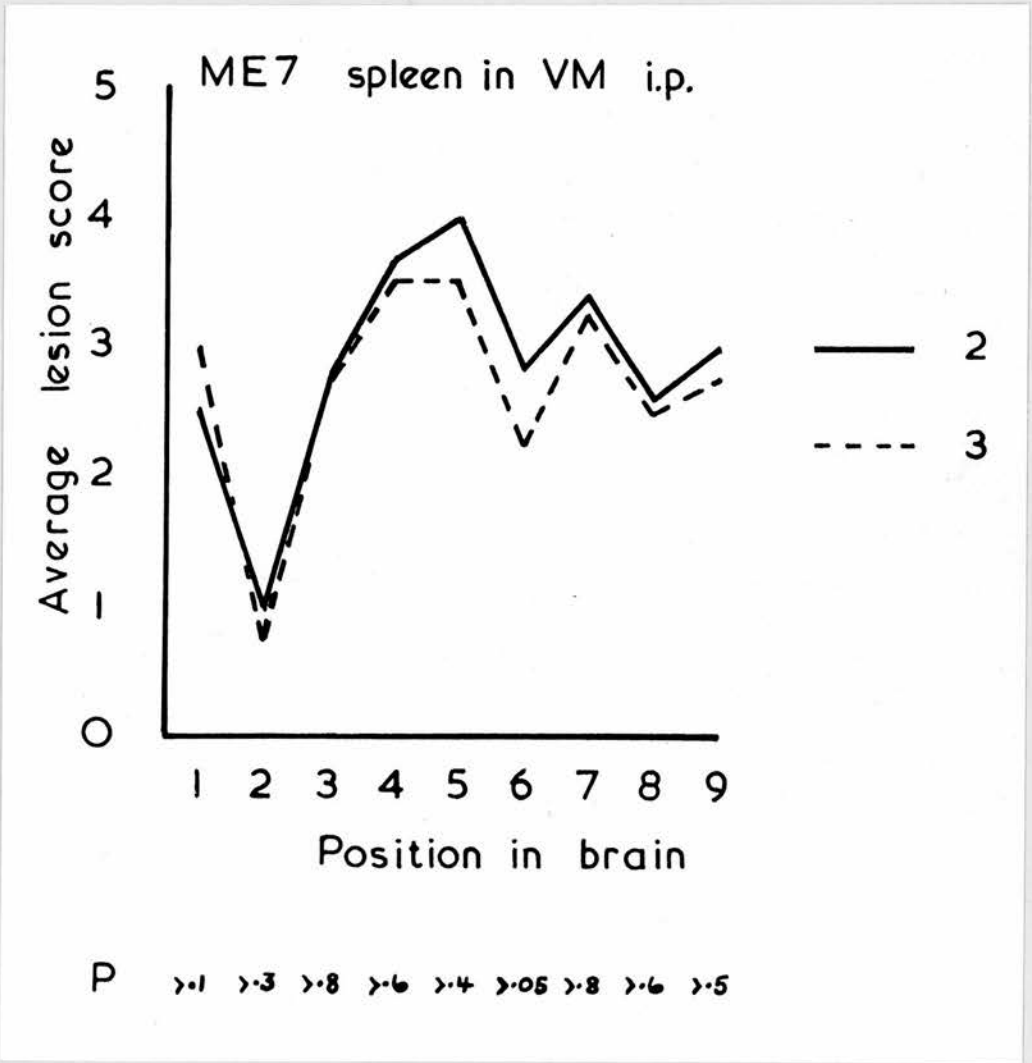


Figure 16 Two passages of ME7 spleen source intraperitoneally in VM mice.

3rd passage than in the 2nd.

In the series of eight passages of ME7 through C57 mice, referred to in Figure 11, agent from the 2nd and 8th passage was injected into two groups of VMs which resulted in indistinguishable profiles. A comparison, in C57s and VMs, between this C57 passaged ME7 and an ME7 source passaged six times in VMs is shown in Figure 17. Slight differences in the lesion profiles are apparent, which are marginally significant in the case of VMs, but as this observation is based on small numbers, without replicates, the significance levels must be treated with circumspection.

It is concluded from those analyses that the serial passage of ME7 through homologous phenotypic systems does not consistently alter the lesion profile, and this point is brought out in the analysis of regression coefficients of lesion densities in each position (Table 8).

Table 8 SIGNIFICANCE OF THE REGRESSION DUE TO PASSAGE BASED ON t TESTS ON REGRESSION COEFFICIENTS

System	In Fig.	Position in Brain								
		1	2	3	4	5	6	7	8	9
ME7 i.c. in C57	11	>.7	>.2	>.8	>.2	>.3	>.2	>.7	>.8	>.2
ME7 i.p. in C57	12	>.02	>.8	>.1	>.02	>.05	>.1	>.05	>.4	>.9
ME7 i.c. in SM	14	>.5	>.6	>.8	>.9	>.2	>.3	>.2	>.05	>.6
ME7 i.c. in VM	15	>.2	>.02	>.6	>.02	>.02	>.02	>.3	>.02	>.2

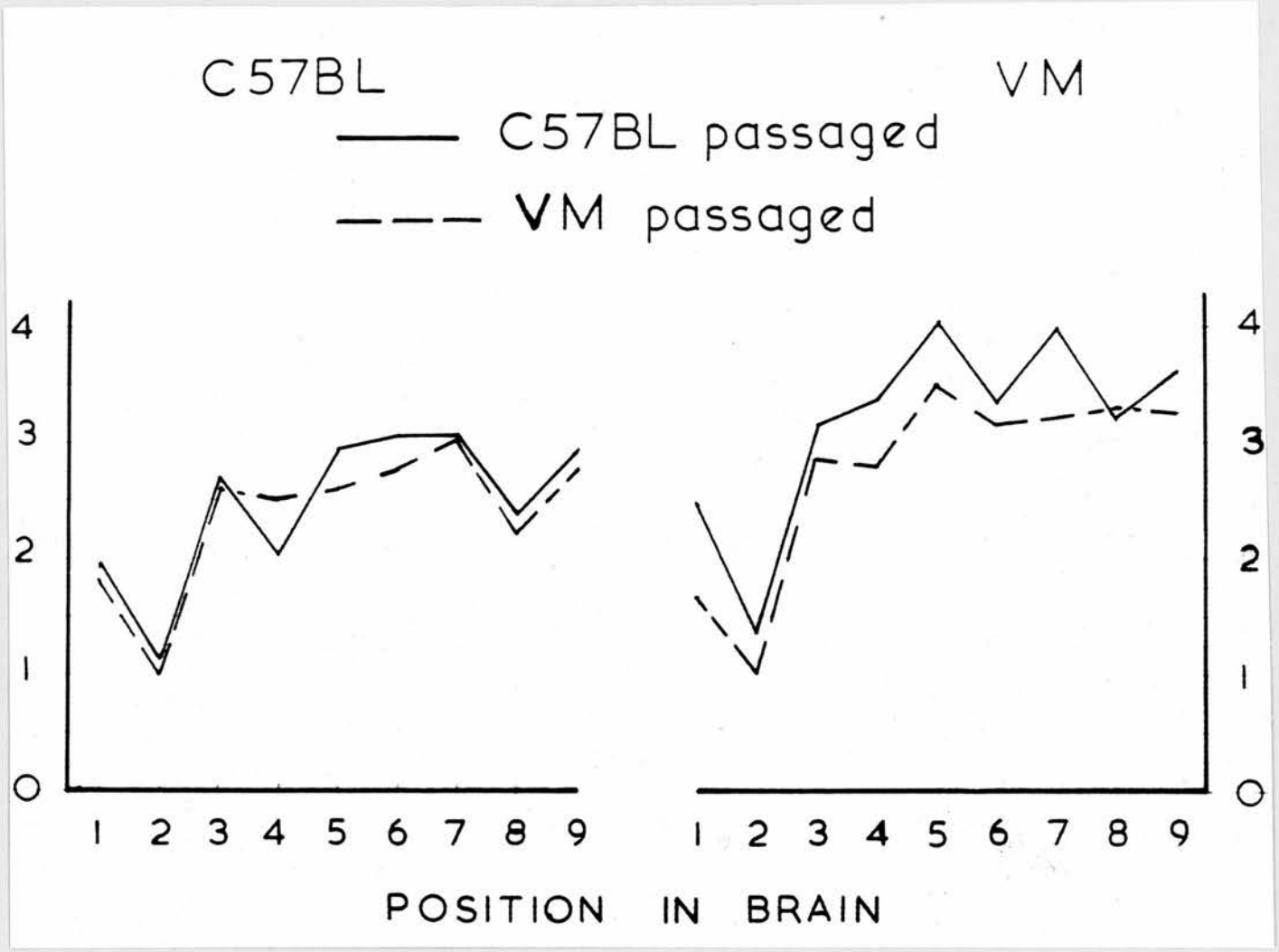


Figure 17 Comparison of lesion profiles of ME7 passaged scrapie in either VM or C57BL mice.

With the marginal exception of the intracerebral VM passages, no significant regressions occur with the passages summarised in Figures 11 to 15. There is slight evidence that ME7 passaged in VMs may possess marginally different properties from ME7 passaged in C57s, but on the present evidence it is not possible to distinguish between effects mediated by non-infectious components in the source, host induced modifications of the agent, or alterations in the hereditary properties of the agent. As pointed out earlier, a fourth possibility is the selection from a mixture of agents, but the overall stability of ME7 with multiple passage reduces the likelihood of this interpretation, but this is not the case with some other scrapie isolates.

The primary isolation of the 58A agent from sheep results in a pattern of pathology which is indistinguishable from that which occurs with mouse to mouse passage, and it needs to be emphasized that this contrasts with some previous findings (Zlotnik, 1965). A considerable amount of primary isolation work of sheep scrapie agents into inbred mice is in progress which should clarify this aspect: preliminary results suggest much more variety than had previously been supposed.

Subsection 5

Effect of organ source of agent on lesion profile

Brain has most commonly been used as the source tissue of scrapie agent, but the spleen, and other tissues have also been used. In non-inbred mice the titre varies in different organs as does the time of appearance of agent in each organ (Eklund, Hadlow and Kennedy, 1966).

A large experiment was designed to compare the rates of synthesis or accumulation of ME7 in brain and spleen, and other organs, in inbred mice which differ in their genotype with regard to sinc. Lesion profiles were analysed to assess the influence on them of sex, donor genotype, stage of incubation period of donor, and organ used as source of agent. With the particular combinations used no significant effect could be demonstrated attributable to sex, stage of incubation period of donor, or donor genotype (see also subsection 7). An effect was revealed, however, when lesion profiles from the use of different organ sources were compared.

Donors were all females of the strains C57, SM, LM and VM, the latter differing from the other three with regard to the genotype at the sinc gene locus. All the mice were inoculated by the intracerebral route with brain from a terminally affected female C57 mouse. The C57, SM and LM donors were killed at 60, 110, and 150 days after inoculation, and the VMs at 60, 110, 150 and 280 days, and their brains and spleens were made separately into inocula and injected by the intracerebral route into groups of C57 mice, in order to assay the titres of scrapie agent in the two organs (Dickinson, Meikle and Fraser, 1969).

Results

Table 9 shows that the degree and distribution of lesions is unaffected by sex. There are differences, however, when profiles from each tissue source are compared either within sex or on an overall basis (120 injected with brain compared with 153 injected with spleen), at whatever stage of the incubation period the donors are killed. The lesions are

			POSITION IN BRAIN								
			1	2	3	4	5	6	7	8	9
Stage of Disease	< 40% of incubation period	Spleen	1.58 ± 0.11	1.64 ± 0.10	2.12 ± 0.10	1.41 ± 0.05	2.41 ± 0.07	2.43 ± 0.08	2.43 ± 0.00	2.03 ± 0.08	2.33 ± 0.07
		Brain	2.04 ± 0.06	0.99 ± 0.11	2.42 ± 0.10	1.70 ± 0.11	2.65 ± 0.13	2.89 ± 0.00	2.83 ± 0.05	2.11 ± 0.08	2.78 ± 0.01
	55-75% of incubation period	Spleen	1.88 ± 0.12	1.72 ± 0.14	2.26 ± 0.11	1.58 ± 0.05	2.46 ± 0.14	2.44 ± 0.08	2.51 ± 0.04	2.09 ± 0.09	2.41 ± 0.03
		Brain	1.74 ± 0.12	1.10 ± 0.15	2.21 ± 0.11	1.67 ± 0.14	2.61 ± 0.08	2.83 ± 0.06	2.87 ± 0.05	2.11 ± 0.09	2.72 ± 0.02
	Terminal disease	Spleen	1.95 ± 0.22	1.81 ± 0.14	2.22 ± 0.14	1.64 ± 0.19	2.69 ± 0.10	2.72 ± 0.14	2.62 ± 0.15	2.11 ± 0.10	2.55 ± 0.12
		Brain	2.09 ± 0.11	1.21 ± 0.12	2.54 ± 0.09	1.71 ± 0.07	2.59 ± 0.20	3.00 ± 0.05	2.91 ± 0.04	2.14 ± 0.11	2.88 ± 0.07
Donor strain	C57BL tissue	Spleen	1.61 ± 0.10	1.92 ± 0.13	2.14 ± 0.08	1.48 ± 0.14	2.44 ± 0.10	2.67 ± 0.09	2.50 ± 0.10	2.14 ± 0.10	2.42 ± 0.09
		Brain	1.97 ± 0.14	1.07 ± 0.12	2.31 ± 0.13	1.59 ± 0.16	2.54 ± 0.16	2.93 ± 0.08	2.83 ± 0.07	2.10 ± 0.11	2.77 ± 0.08
	SK, LK and VK tissue	Spleen	1.82 ± 0.07	1.68 ± 0.08	2.22 ± 0.06	1.53 ± 0.07	2.53 ± 0.05	2.48 ± 0.05	2.50 ± 0.05	2.06 ± 0.06	2.41 ± 0.05
		Brain	1.95 ± 0.08	1.13 ± 0.08	2.45 ± 0.07	1.73 ± 0.08	2.65 ± 0.08	2.89 ± 0.04	2.92 ± 0.04	2.12 ± 0.07	2.80 ± 0.04
Sex	Female		1.91 ± 0.05	1.55 ± 0.07	2.28 ± 0.05	1.55 ± 0.07	2.55 ± 0.05	2.68 ± 0.04	2.65 ± 0.04	2.09 ± 0.04	2.53 ± 0.05
	Male		1.78 ± 0.07	1.37 ± 0.08	2.30 ± 0.06	1.65 ± 0.07	2.56 ± 0.06	2.69 ± 0.05	2.69 ± 0.05	2.10 ± 0.06	2.64 ± 0.05

Table 9 Lesion intensity in the brain of C57BL mice with advanced clinical scrapie (mean ± S.E.).

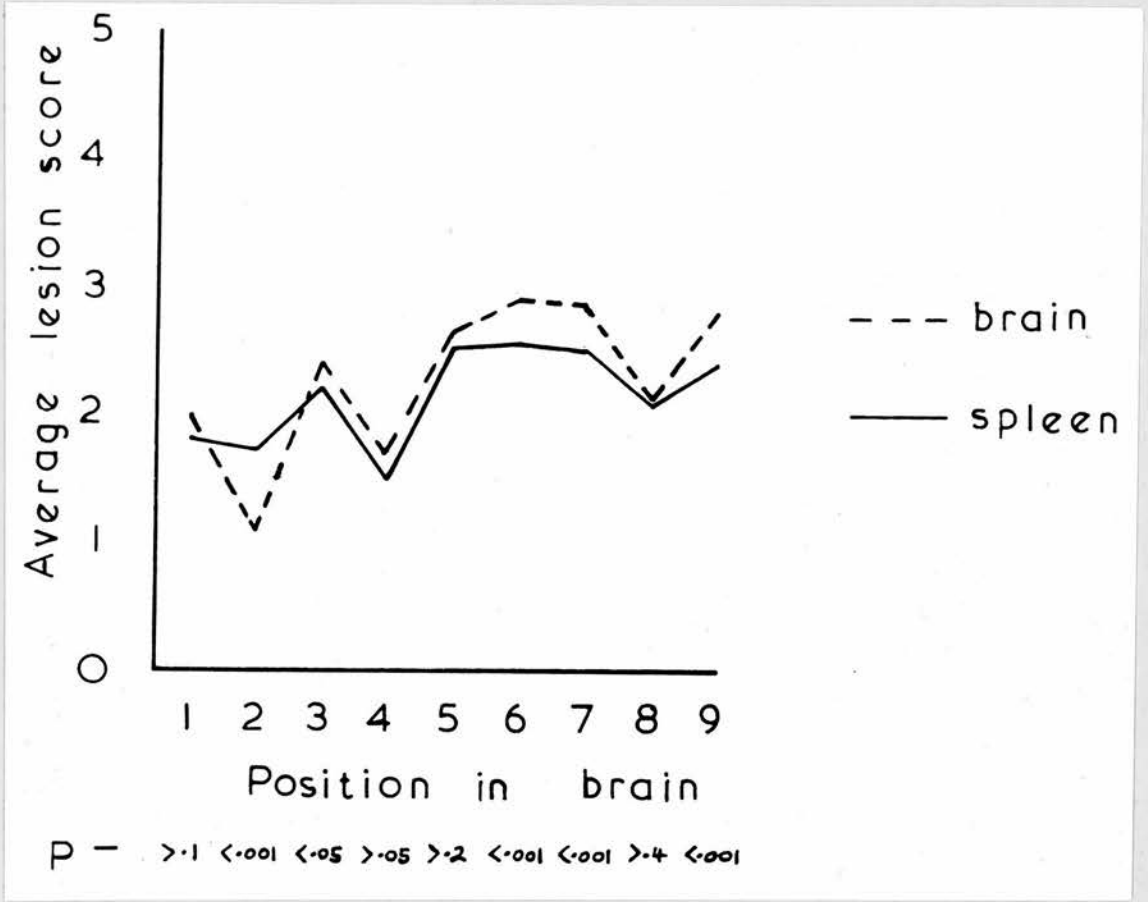


Figure 18 Scrapie lesion distribution in nine brain regions resulting from intracerebral inoculation with scrapie brain or spleen.

more severe in the anterior midline cerebral cortex, hippocampus and septal area, and less advanced in the tonsil of the cerebellum when brain is used as the source than when spleen is used (Figure 18). These differences are independent of the presence or absence of isogeny between donor and recipient, although in a separate experiment which involved the same agent but different strains of mice, genetic differences between donor and recipients are shown to influence the lesion profile (Subsection 7). In the present experiment lesion profile was also independent of the scrapie titre in the donor tissue because the stages of incubation at which the mice were killed represent from 10,000 to 100,000-fold increase in titre in the brain. The spleen levels show no increase through these stages, and the final titres reached in brain exceed those in spleen (Dickinson, Meikle and Fraser, 1969).

In conclusion, the lesion profile of ME7 is different when brain or spleen is used as the source and these results are consistent throughout all the subgroups in this experiment. There are three main possibilities which might give rise to these results; either the agent is modified by the organ in which it is synthesised or accumulated; or some organ specific component in the crude inoculum, which is not integrated into the agent itself, can exert an effect; or there are different agents in the starting material which have different affinities for brain and spleen.

Subsection 6

The Influence of Route of Inoculation

By comparing the intensity of astrocytic reaction in rat scrapie,

Pattison and Jones (1967) showed differences which they attributed to route of inoculation. The astrocytic reaction was more severe and widespread in those inoculated by the intracerebral route compared with the intraperitoneal route. However, the mean incubation periods were the same for both routes which suggests that histological differences may reflect different stages in the progress of the infective process, because the incubation periods following extraneural inoculation usually exceed that for intraneural by a considerable interval (Chandler, 1963; Gibbs, Gajdusek and Morris, 1965; Dickinson and Fraser, 1969b). In this subsection lesion profiles are compared for intracerebral and extraneural routes in experiments terminated at a common clinical end-point. Some relevant results concerning effects of route difference and its interactions with other variables are given in subsection 7.

In this subsection the prime aim is to determine the effect of injection route on lesion profile. In most experiments the comparison also involves a difference in incubation period but in others incubation period was intentionally arranged to be similar for different routes in order to discount the possibility that the profile differences were due to differences in incubation.

The various combinations of agent and host which were used to compare effects of route are given in Table 10. In the experiment in which intravenous inoculation was used the intracerebral needle damage was carried out in the different groups at 24, 6, 3 and $1\frac{1}{2}$ hours before inoculation; immediately after inoculation; at $1\frac{1}{2}$, 3, 6, 10, 24 hours, 3, 10, 29 and 35 days after inoculation. However, as there was no difference between these

Table 10 SUMMARY OF EXPERIMENTS ON THE INFLUENCE OF ROUTE OF INOCULATION

Agent	Host strain	Route combinations compared
ME7	C57BL, BALB, LM, MM, A2G	1. intracerebral 2. intraperitoneal
22A	VM	1. intracerebral 2. intraperitoneal
ME7	C57BL	1. intracerebral low dose 2. intraperitoneal standard dose) both groups culled to a common incubation period
ME7	C57BL	1. intraperitoneal 2. intraperitoneal with an intracerebral saline injection (0.02 ml) between 24 hours before and after inoculation
ME7	C57BL	1. intracerebral 2. intraperitoneal (0.02 or 0.1 ml) 3. hind footpad (0.02 or 0.1 ml)
ME7	RIII, LM	1. intracerebral, septal area 2. " , hippocampus and dorsal thalamus 3. " , cerebellum and tegmentum 4. " , posterior medulla
ME7	A2G, MM, BRVR, BALB	1. intracerebral * 2. intraperitoneal * 3. intravenous 4. intravenous with an intracerebral needle damage between 1 day before and 35 days after inoculation

* in A2G and MM only

groups, either between themselves, or between them and the intravenous control group, in terms either of incubation period or lesion profile, in the results these subgroups have been combined.

In the experiment in which four sites of intracerebral inoculation were chosen the injection sites were identified according to the plan in Figure 19, and the direction of the needle was such that it passed through the skull at right angles to it. The depth of the injection was standardised by using a Gillette 26 gauge x 3/8 needle with a plastic guard-stop on the needle to standardise the depth of insertion to 0.44 cm.

By dissection it was found that this technique resulted in the needle passing into confined areas of the brain: injection sites 1, into fore-brain near septal nuclei; 2, through mid-cerebral cortex into hippocampus and dorsal thalamus; 3, through cerebellum and into the tegmentum; 4, into medulla behind cerebellum.

Results

There is a profound difference in lesion distribution between the intracerebral and intraperitoneal inoculation routes (Figures 20 to 25). This is a phenomenon which is invariably found when this route difference is compared with either ME7 or 22A agent, although there is some preliminary evidence that the profiles are relatively similar by both routes with 79A agent. However, these results may not be a direct outcome of inoculation route, rather they could reflect the different incubation periods which are a direct consequence of route of inoculation. That this is not the case is indicated by Figure 26 in which the profile for C57 mice inoculated by the

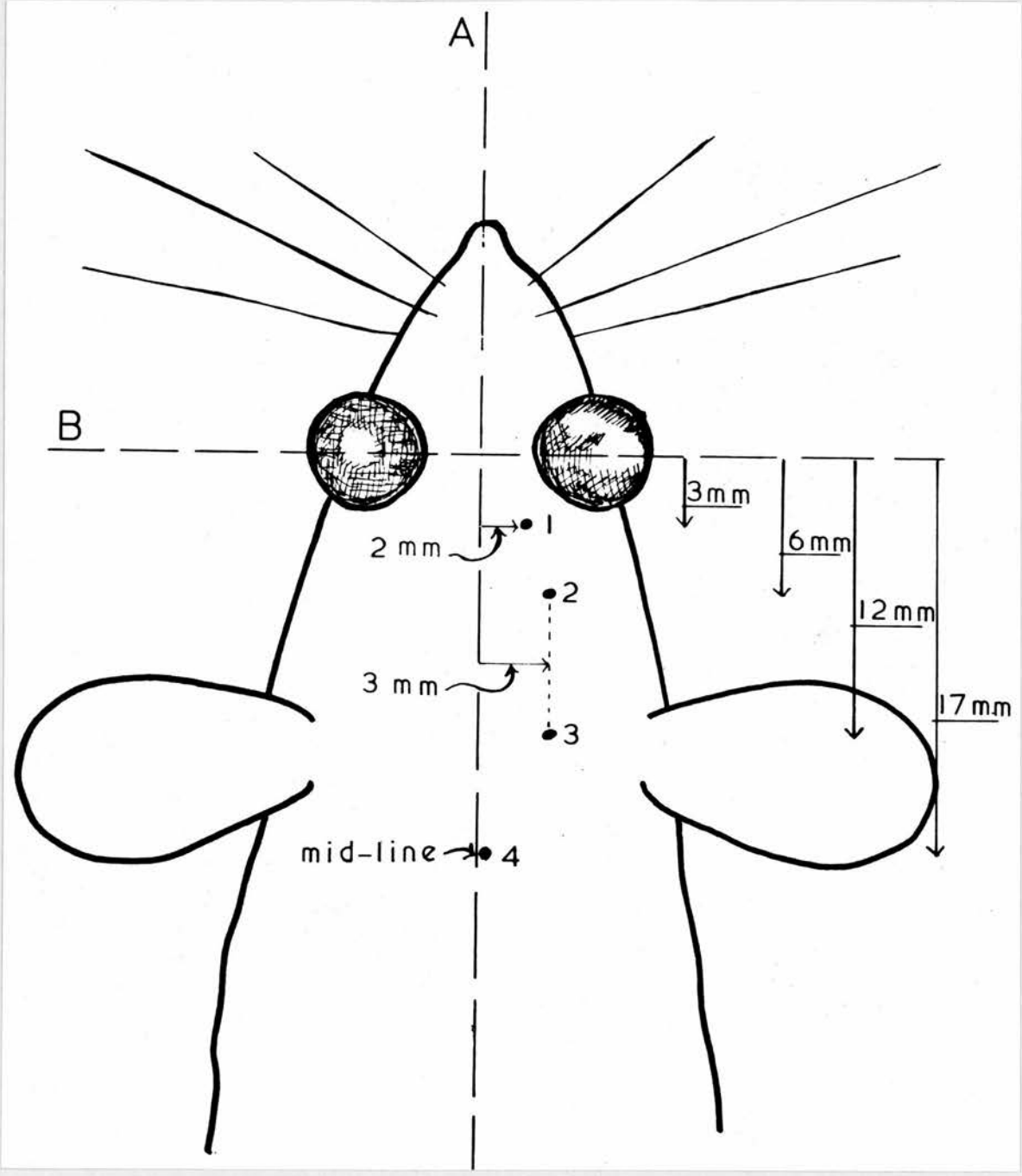


Figure 19 Diagram of four positions of intracerebral injection, in relation to reference lines: A, the midline; B, through medial canthus of each eye.

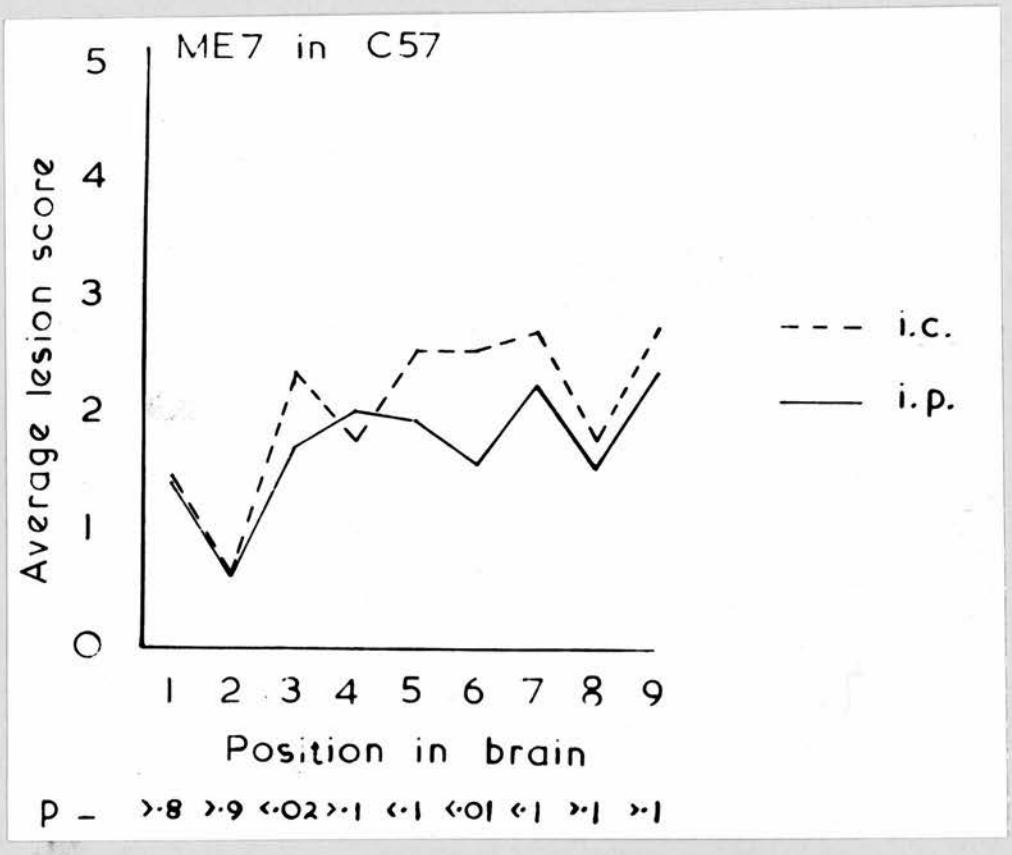


Figure 20 **Lesion profiles for ME7 scrapie in C57BL mice inoculated by the intracerebral or intraperitoneal routes.**

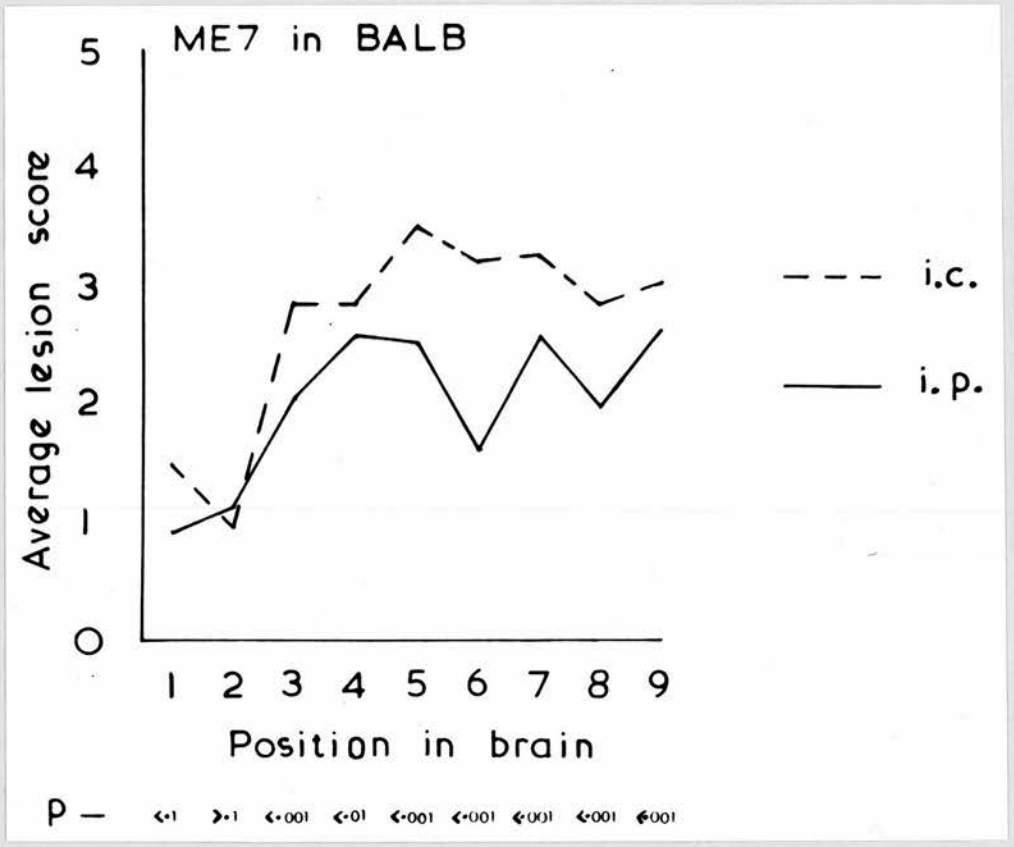


Figure 21 Lesion profiles for ME7 scrapie in BALB mice inoculated by the intraperitoneal or intracerebral routes.

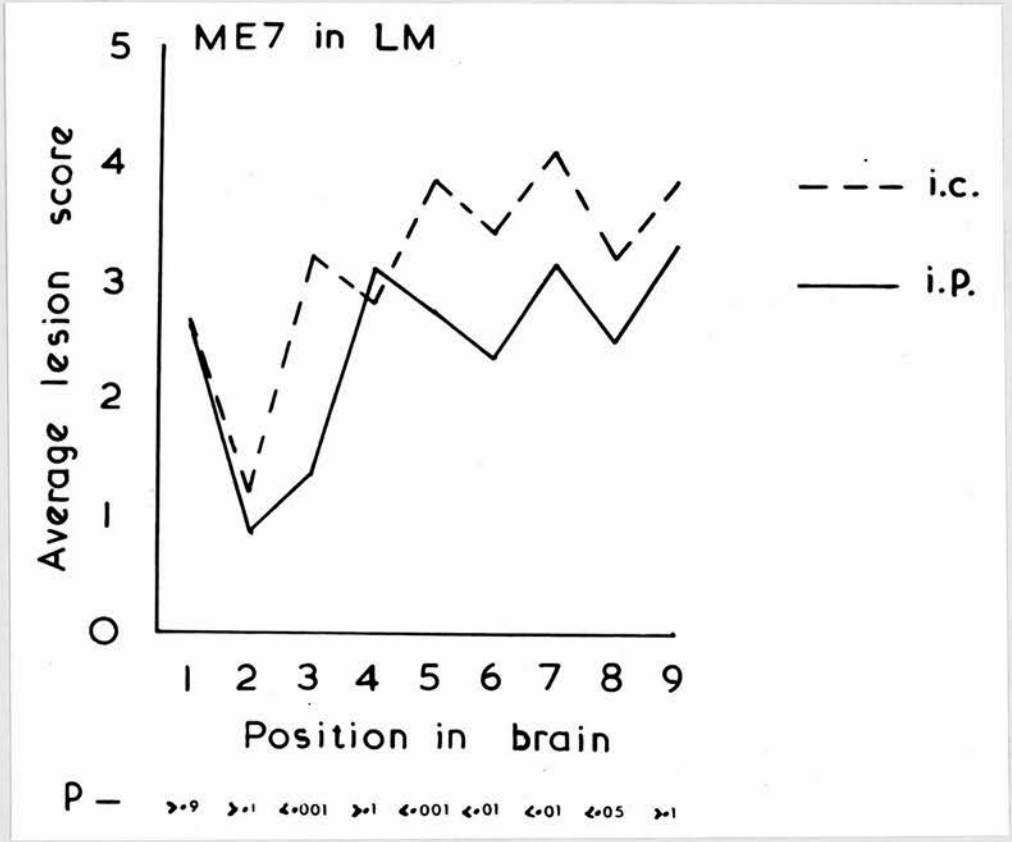


Figure 22 Lesion profiles for ME7 scrapie in LM mice inoculated by the intracerebral or intraperitoneal routes.

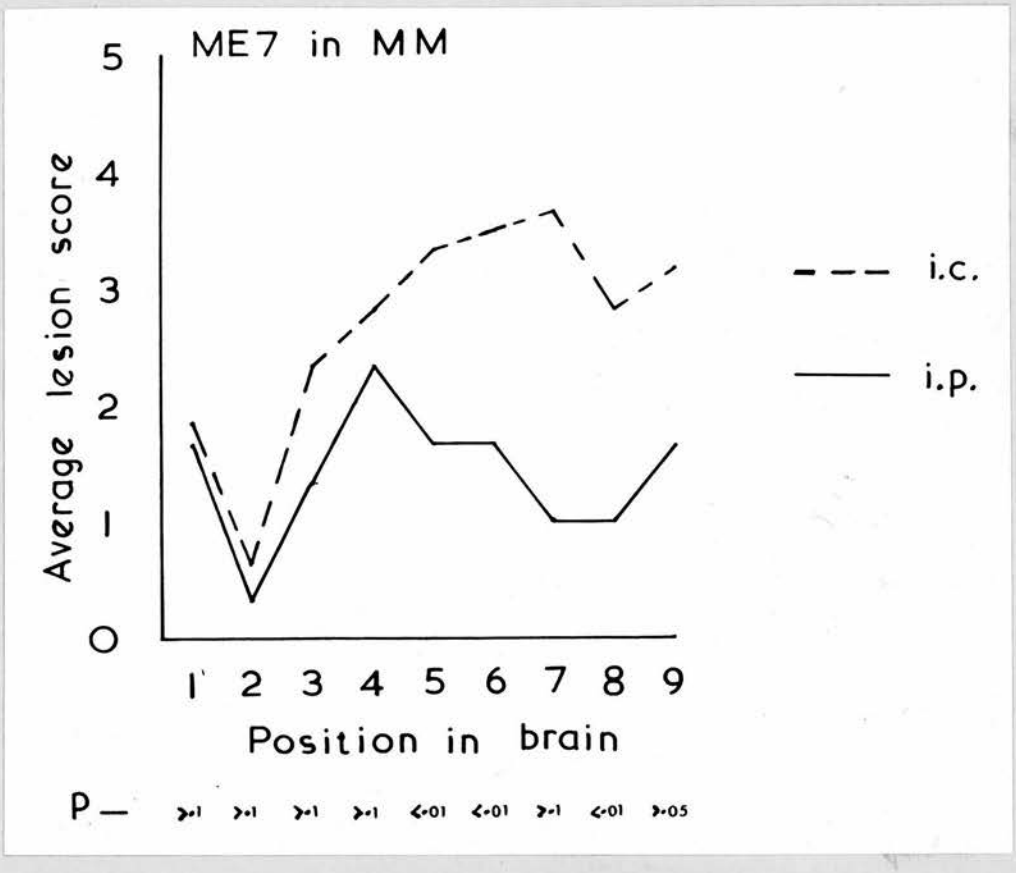


Figure 23 **Lesion profiles for ME7 scrapie in MM mice inoculated by the intracerebral or intraperitoneal routes.**

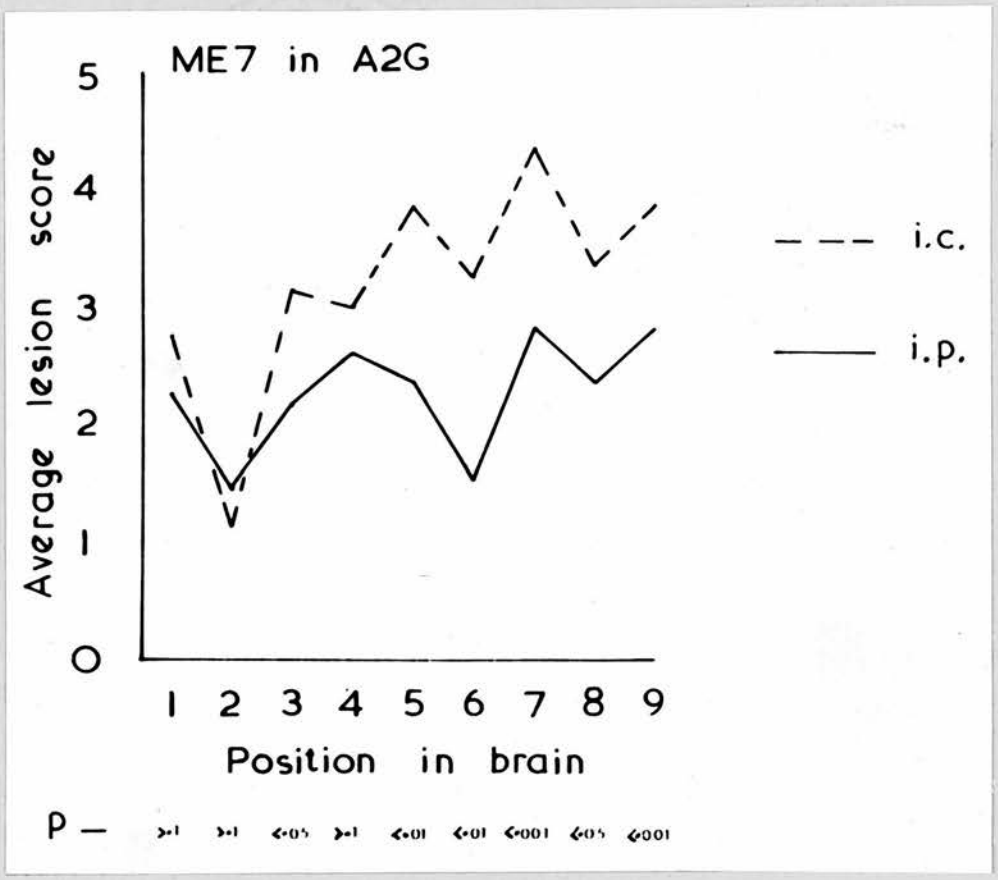


Figure 24 **Lesion profiles for ME7 scrapie in A2G mice inoculated by the intracerebral or intraperitoneal routes.**

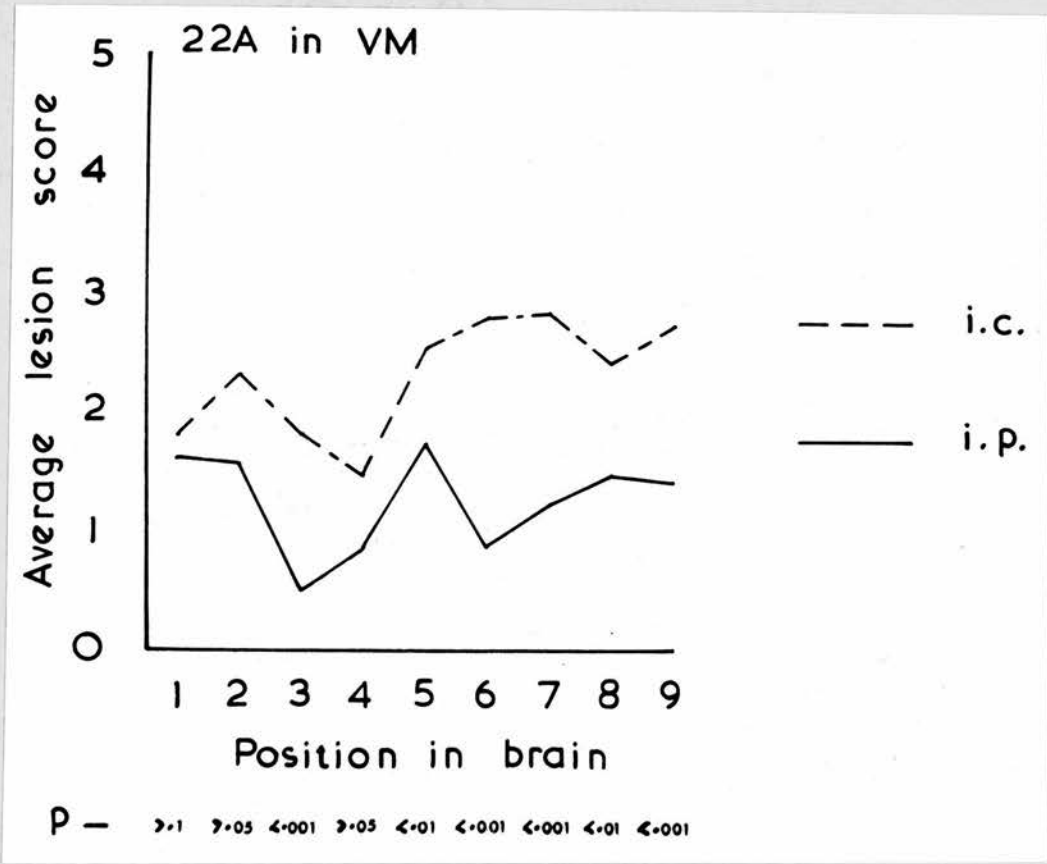


Figure 25 Lesion profiles for 22A scrapie in VM mice inoculated by the intracerebral and intraperitoneal routes.

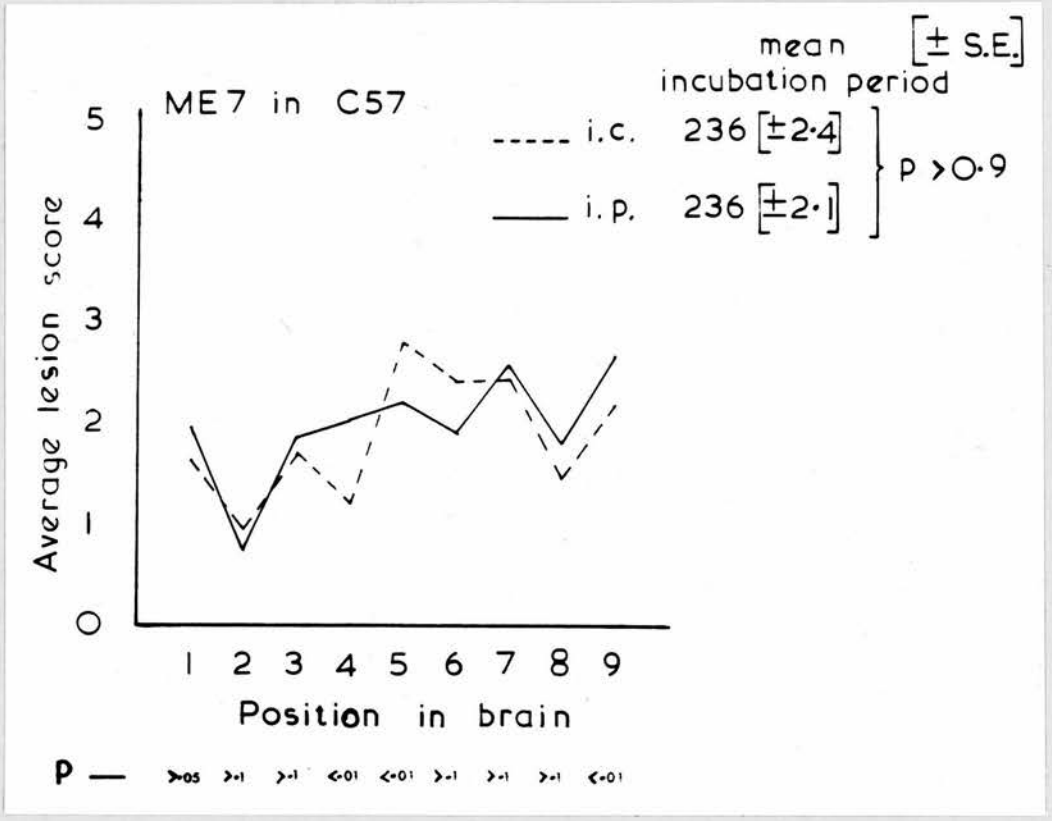


Figure 26 **Lesion profiles for ME7 scrapie in C57BL mice inoculated intracerebrally with low dose or intraperitoneally with high dose (to allow comparison at equal incubation periods).**

intracerebral route with a low number of infectious units is compared with that for those inoculated by the intraperitoneal route with a high level of infectivity, the doses being chosen to equalise the incubation periods (The effect of dose is analysed in subsection 7). That the superimposition of brain trauma by the needle and injection of a 0.02 ml. volume of fluid into the brain is not the cause is indicated in Figure 27 in which an intraperitoneal injection is not demonstrably different if an intracerebral injection of physiological saline is given at the same time or within 24 hours before or after the inoculation of ME7 scrapie. The introduction of brain trauma also makes no significant difference to the incubation period for ME7 following intraperitoneal inoculation (245 ± 13 days following intraperitoneal alone; 248 ± 17 days with a superimposed intracerebral saline injection).

The lesion profile for the intraperitoneal route is indistinguishable from that following injection into the hind footpad (Figure 28), and there is no significant difference between the incubation periods for the two routes, either between comparable doses ($P > 0.8$) or overall ($P > 0.5$). Shorter incubation results from using the larger volume of inoculum but the effect is hardly significant ($P < 0.02$ for intraperitoneal route, $P < 0.1$ for footpad route) (Table 11).

Intracerebral inoculation of either ME7 or 22A thus produces characteristically different lesion profiles compared with peripheral inoculation as represented by injection into the footpad or peritoneal cavity, and this is not caused by the different incubation period or by the injection damage in the brain. As the intracerebral route has such wide application

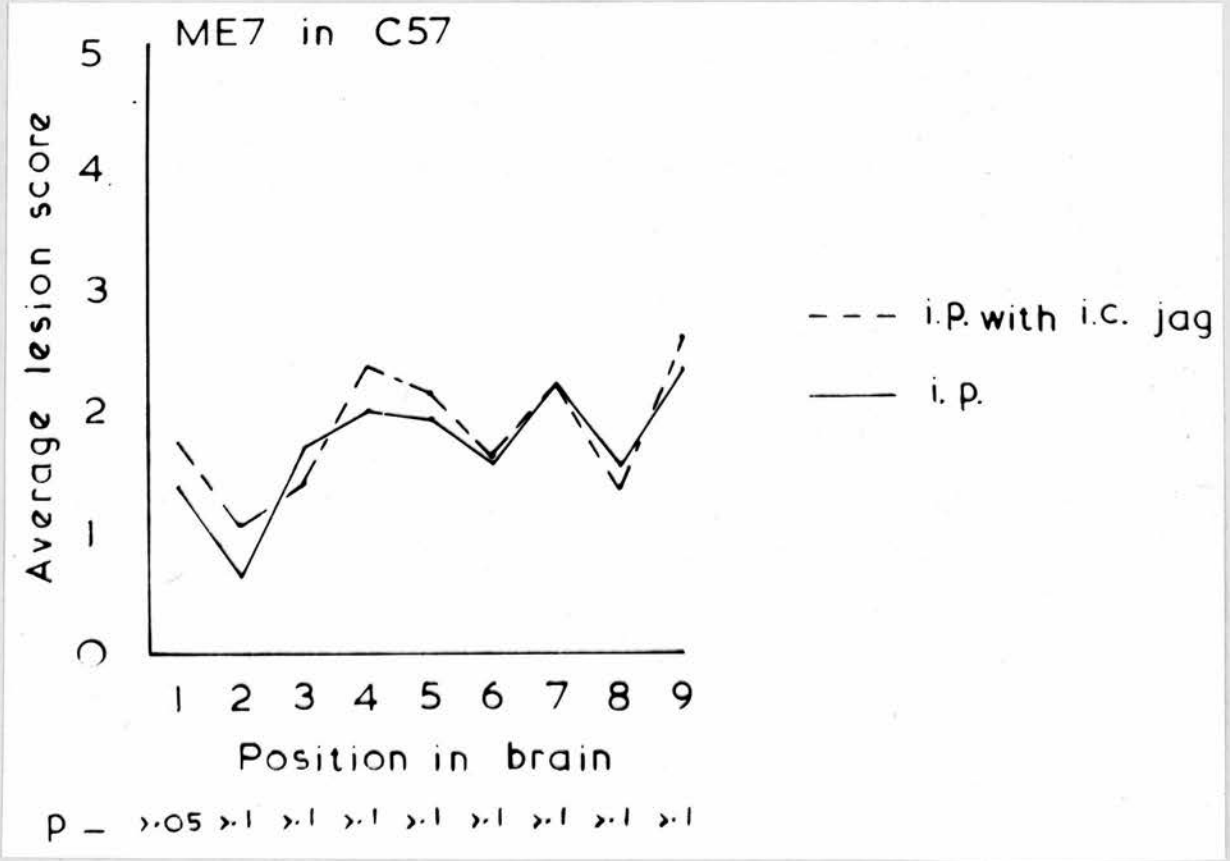


Figure 27 **Lesion profiles for ME7 scrapie in C57BL mice inoculated by the intraperitoneal route with or without an intracerebral injection of saline between 24 hours before or after inoculation.**

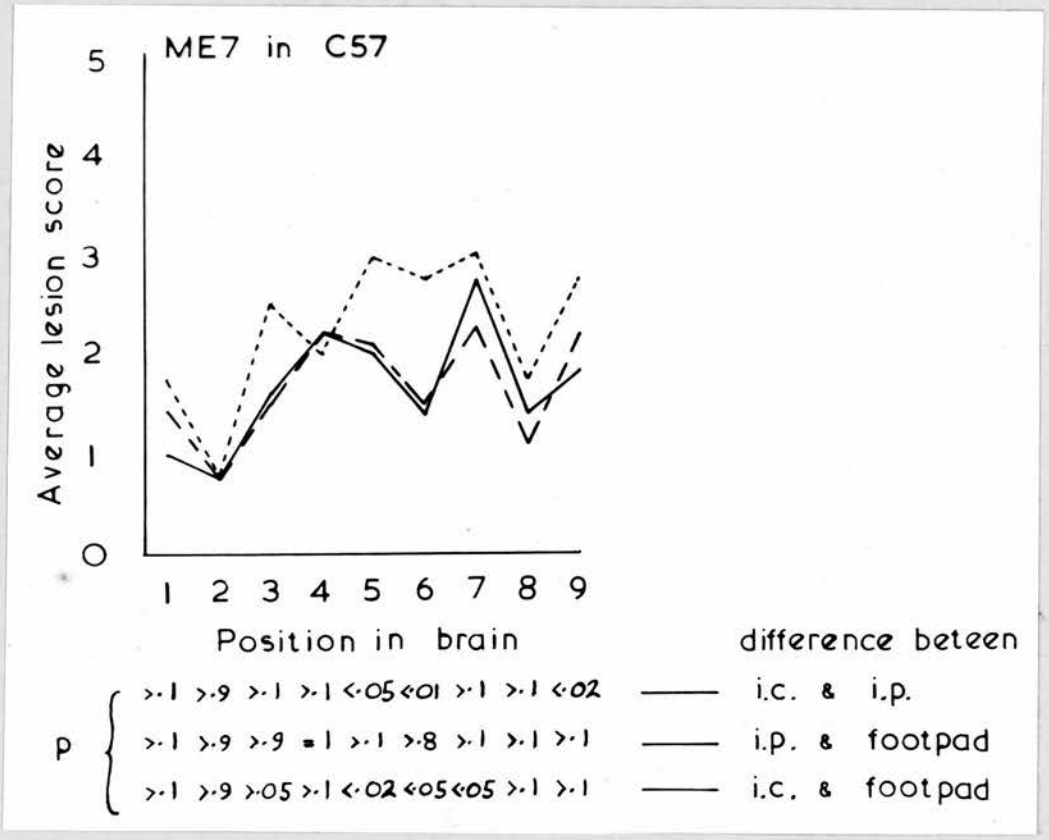


Figure 28 **Lesion profiles for ME7 scrapie in C57BL mice inoculated by the intracerebral, intraperitoneal and footpad routes, ----- i.c.; —— i.p.; — — — footpad.**

Table 11 COMPARISON OF INCUBATION PERIODS (DAYS ± S.E.) FOLLOWING FOOTPAD OR INTRAPERITONEAL INOCULATION OF TWO DIFFERENT VOLUMES OF INOCULUM OF ME7 SCRAPIE INTO C57BL MICE

Volume inoculated (ml)	Route	
	Intraperitoneal	Footpad
0.02	274 ± 7	279 ± 9
0.1	238 ± 4	250 ± 9

Table 12 INCUBATION PERIOD DATA (DAYS ± S.E.) FOR FOUR DIFFERENT INTRACEREBRAL INJECTION SITES IN TWO MOUSE STRAINS (RIII AND LM) WITH ME7 SCRAPIE

Mouse Strain	Site of Injection			
	Forebrain & septal area	Hippocampus & thalamus	Cerebellum	Medulla
RIII	159 ± 2	157 ± 1	158 ± 3	159 ± 2
LM	163 ± 7	171 ± 5	173 ± 5	172 ± 4

Table 13 INCUBATION PERIODS (DAYS ± S.E.) IN FOUR MOUSE STRAINS INOCULATED INTRAVENOUSLY AND WITH SUPERIMPOSED CEREBRAL TRAUMA

Mouse strain		Intravenous	Intravenous with cerebral trauma	Probability of difference
A2G	Σ	207 ± 5	207 ± 2	>0.8
MM	Σ	221 ± 10	208 ± 2	>0.05
MM	σ	225 ± 7	203 ± 8	>0.05
BRVR	σ	208 ± 1	183 ± 7	<0.02
BALB	σ	221 ± 2	225 ± 2	>0.5

Σ Timing of cerebral damage varied between 24 hours before to 35 days after inoculation. Frozen brain source of ME7 used.

σ Cerebral damage administered simultaneously with inoculation. Fresh brain source of ME7 used.

Table 14 INCUBATION PERIODS (DAYS ± S.E.) IN FOUR MOUSE STRAINS FOLLOWING INTRACEREBRAL, INTRAVENOUS AND INTRAPERITONEAL INOCULATIONS

Mouse strain	Intracerebral	Intravenous	Intraperitoneal
A2G	181 ± 2	207 ± 5	227 ± 2
MM	170 ± 3	215 ± 3	258 ± 3
BRVR	168 ± 3	195 ± 5	250 ± 6
BALB	165 ± 4	224 ± 3	250 ± 5

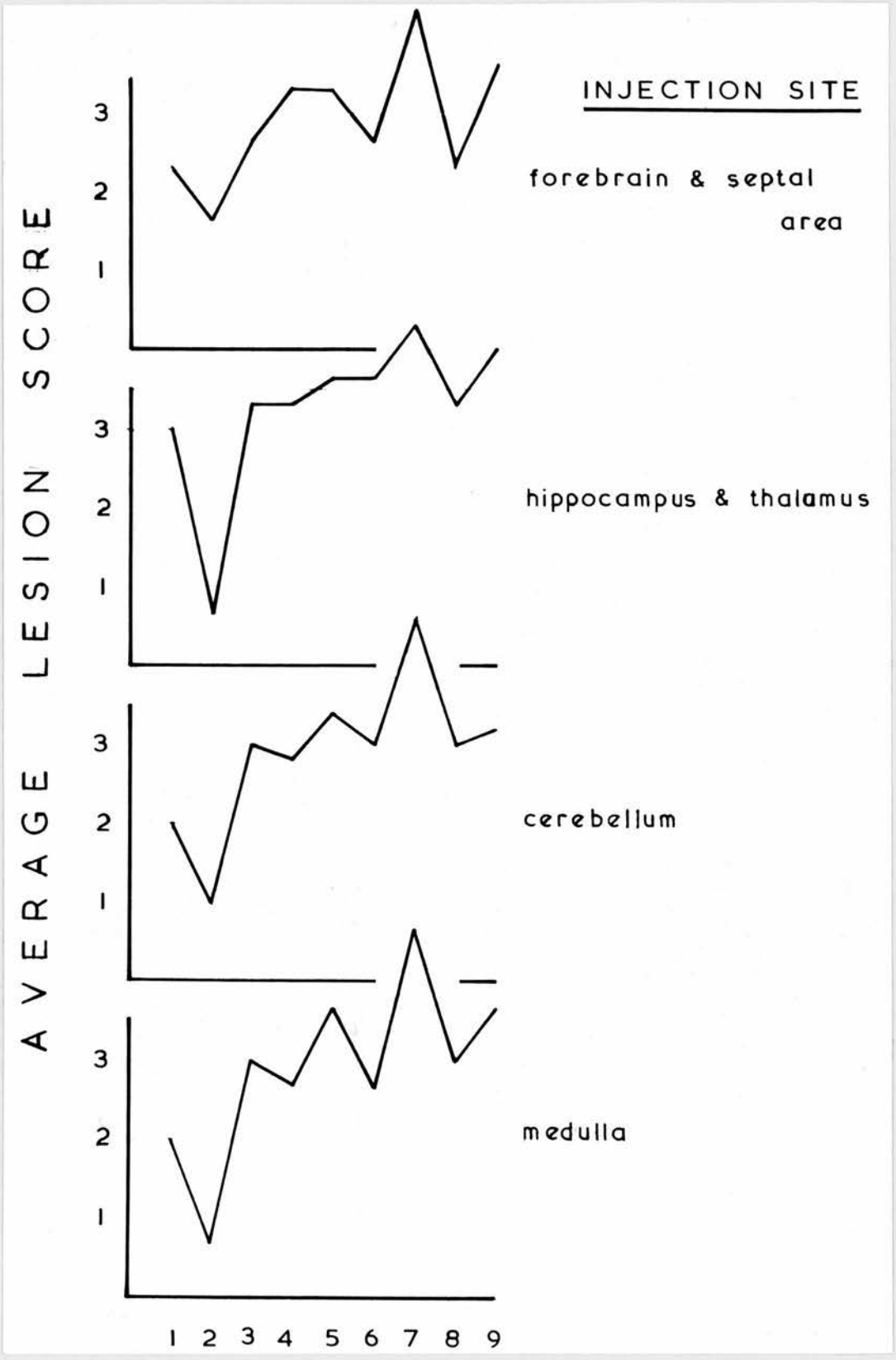


Figure 29 Lesion profiles in RIII mice following inoculation of ME7 scrapie into one of four brain sites as shown in Figure 19.

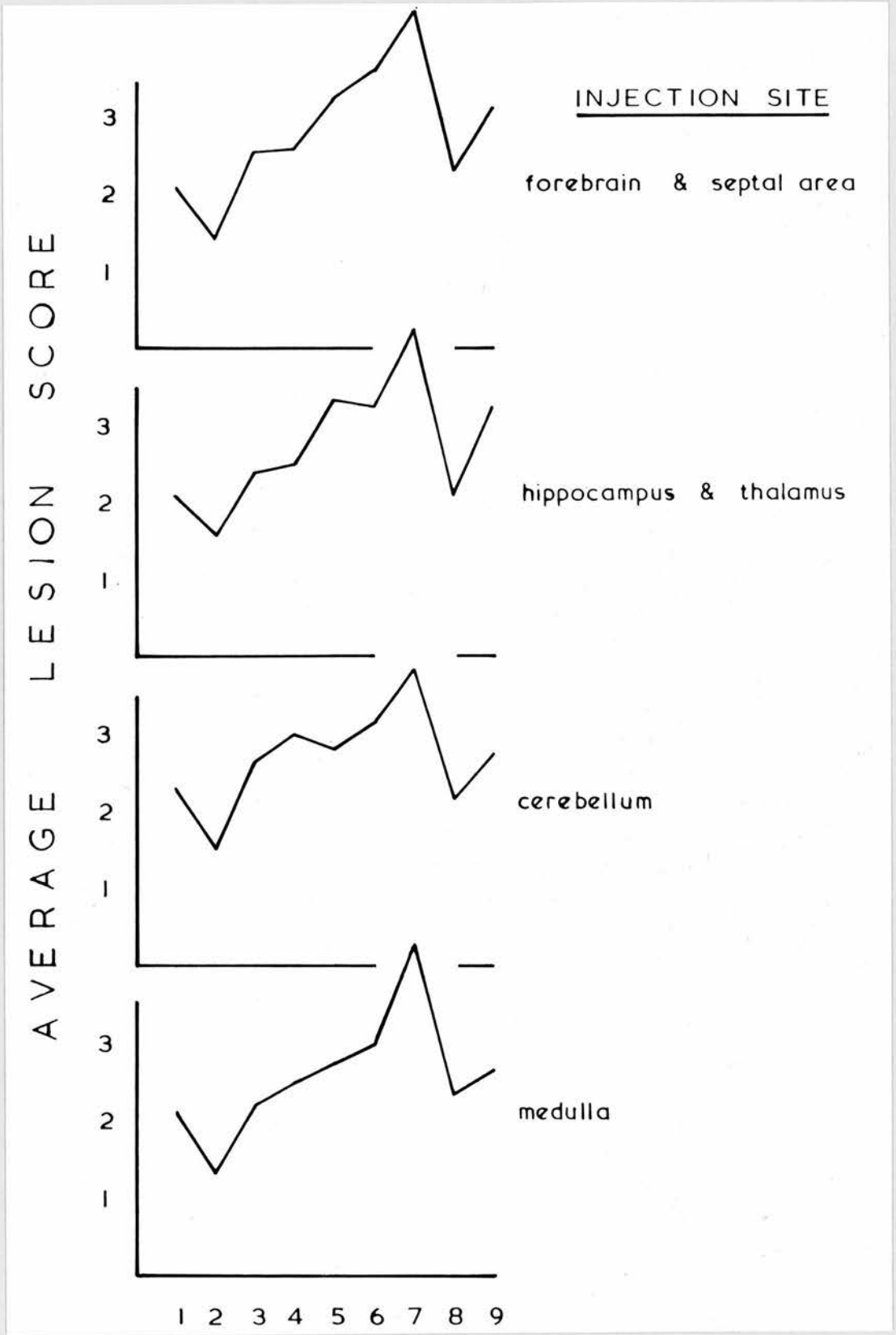


Figure 30 Lesion profiles in LM mice following inoculation of ME7 scrapie into one of four brain sites as shown in Figure 19.

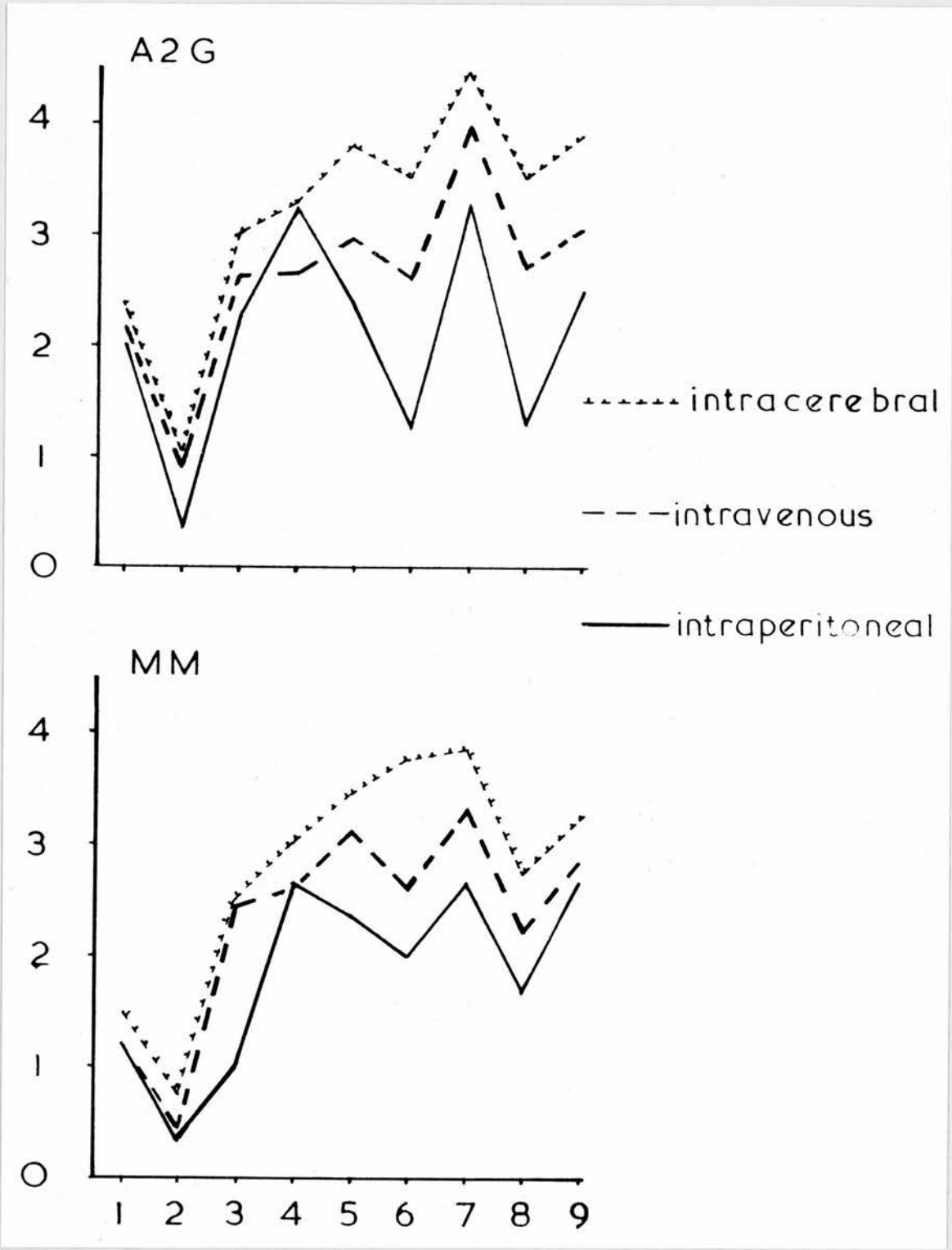


Figure 31 Lesion profiles of ME7 scrapie in A2G and MM mice following injection by the intracerebral, intravenous and intraperitoneal routes.

in experiments with mouse scrapie, any effect of variation in the technique of injection might be important if it resulted in variation in measured parameters. Intracerebral inoculation of two mouse strains, RIII and LM, with standard dose and dilution of ME7 brain source (from RIII donor), but using four very distinct brain sites for injection (Figure 19) resulted in virtually indistinguishable lesion profiles (Figures 29 and 30) and incubation periods which were not significantly different (Table 12).

The failure of cerebral trauma consistently to reduce the incubation period following intravenous administration is shown in Table 13. As no differences can be attributed to variation in the time of administration of cerebral trauma relative to inoculation, the groups have been combined. The incubation periods following intravenous administration are almost midway between the values following intraperitoneal and intracerebral inoculations (Table 14). Similarly, the lesion profiles following intravenous inoculation fall approximately midway between the intracerebral and intraperitoneal route profiles (Figure 31).

Subsection 7

Dose of Agent

The effect of variation in dose of scrapie agent was assessed in C57, SM and VM mice. The dosage variation was achieved by serial tenfold dilutions of ME7 scrapie brain source derived from terminally affected female C57 mice. All inoculations for this subsection were intracerebral, with a volume of 0.02 ml.

Figures 32, 33 and 34 illustrate the lesion profiles of brain

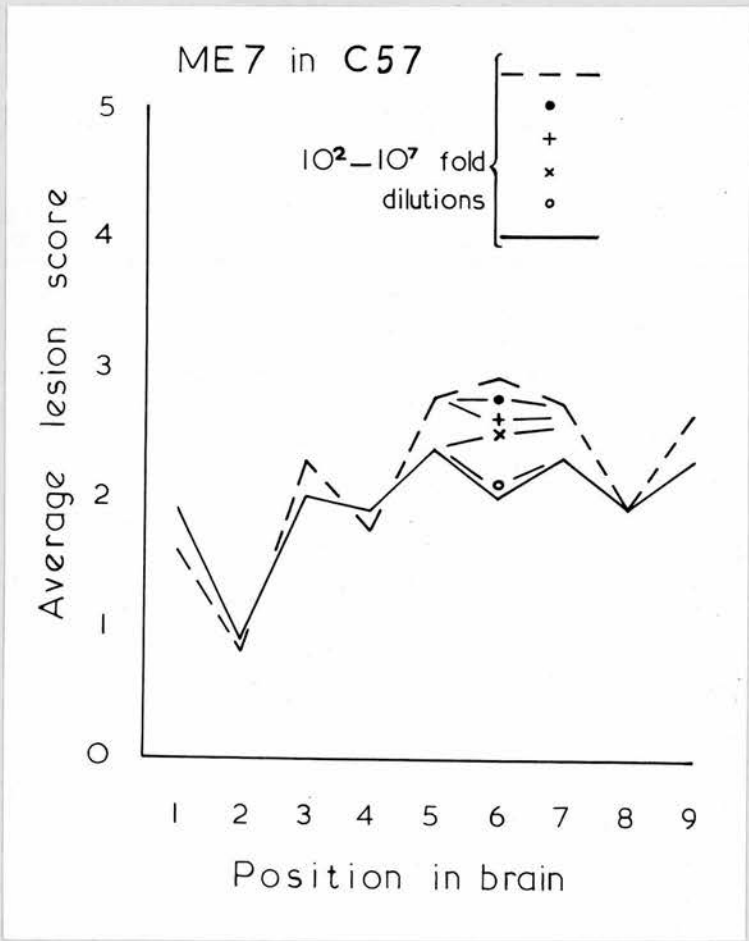


Figure 32 **Lesion profiles for ME7 scrapie agent in C57BL mice inoculated intracerebrally with serial tenfold dilutions of infected brain source. The four intermediate dilutions are not shown except in position 6 for clarity of presentation.**

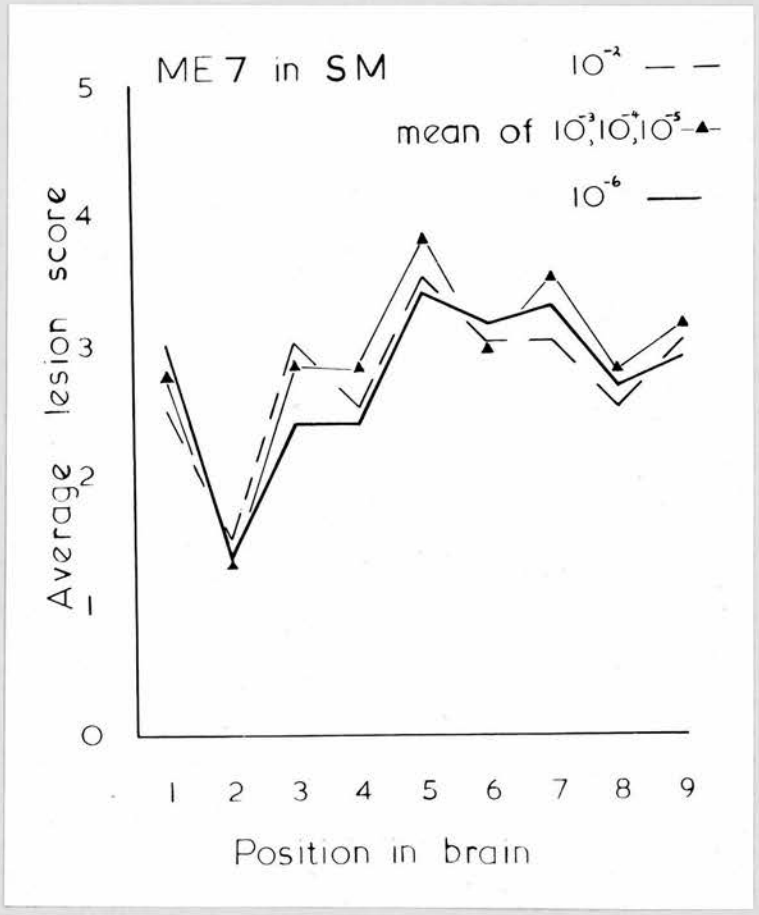


Figure 33 **Lesion profiles for ME7 scrapie agent in SM mice inoculated intracerebrally with serial tenfold dilutions of infected brain source. The three intermediate dilutions are pooled on a single plot for clarity of presentation.**

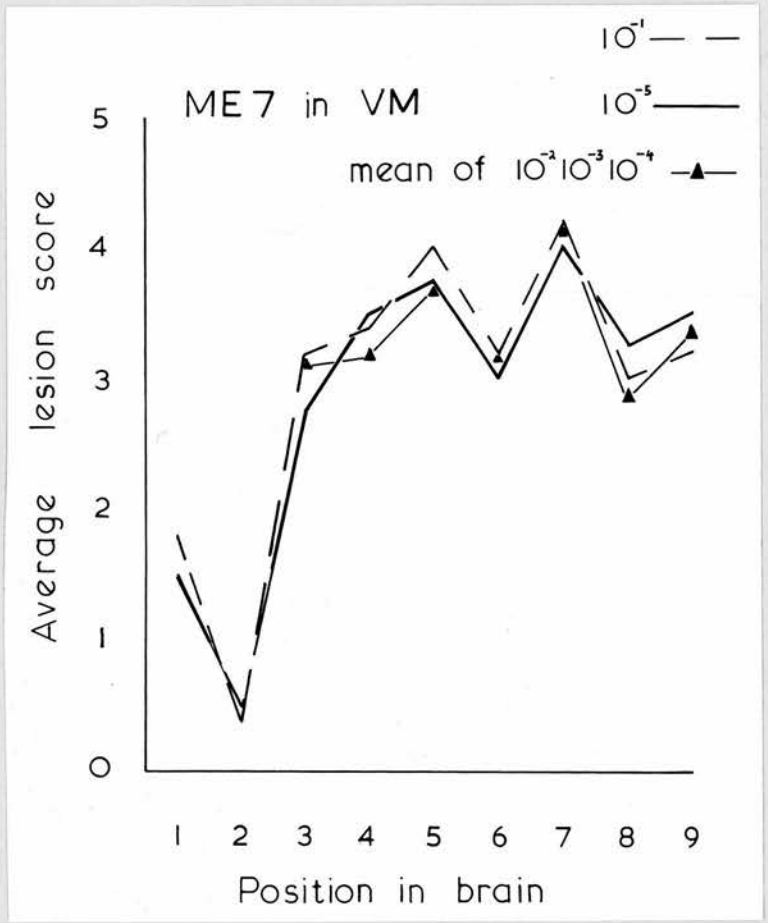


Figure 34 **Lesion profiles for ME7 scrapie agent in VM mice inoculated intracerebrally with serial tenfold dilutions of infected brain source. The three intermediate dilutions are pooled on a single plot for clarity of presentation.**

damage in the three mouse strains. The significance of the regressions of mean lesion scores associated with dosage variation, based on t tests on their regression coefficients is given in Table 15. No consistent variation in brain damage can be attributed to dose in the VM mice, and this is probably the case with the SM system, the exception in the latter case being in position 3. In the C57 mice, on the other hand, significant regressions of means occurred in several positions, and this is most clearly shown in position 6.

Table 15 SIGNIFICANCE OF REGRESSIONS DUE TO DOSE VARIATION IN THREE STRAINS OF MICE INOCULATED INTRACEREBRALLY WITH ME7 SCRAPIE

Mouse Strain	Profile in Fig.	Position in Brain								
		1	2	3	4	5	6	7	8	9
C57	32	>.5	>.5	<.01	>.6	<.02	<.001	<.01	>.2	>.02
SM	33	>.1	>.3	<.01	>.3	>.8	>.2	>.5	>.9	>.3
VM	34	>.8	>.9	>.05	>.5	>.7	>.1	>.1	>.4	>.3

Dilution of the scrapie source results in a consistent prolongation of incubation period (Dickinson and Meikle, 1969), resulting in a difference in the age at which clinical disease develops. However, no difference in lesion distribution occurs in experiments where variation in age of intracerebral inoculation of ME7 takes place between 9 and 112 days of

age and this also has only a slight effect on incubation period (Dickinson, personal communication). The inoculation by the intracerebral route of very old C57 mice (>18 months) with ME7 scrapie has resulted in lesion profiles which are indistinguishable from those of C57 weanlings similarly injected.

It is difficult on the available evidence to draw a simple conclusion concerning the effect of dose of ME7 scrapie on the lesion profile in view of the inconsistent results between the different mouse strains. The effects in the C57 mice are evidently not due to age difference. It cannot be due to the lower dilutions being equivalent to peripheral inoculation because inoculations of the lowest dilutions which are infective by intracerebral inoculation are not infective by intraperitoneal inoculation. (Dickinson, personal communication; Kimberlin, personal communication).

Subsection 8

Variation of Donor and Host Genotype, Host Sex and Organ Source
An Assessment of Multiple Effects in a Single Multifactorial Design

In this subsection combinations of variables with ME7 scrapie in the mouse are determined simultaneously. The agent source is varied by passage into two inbred mouse strains, BALB and BRVR, from which brain source and spleen source are compared. The assay hosts are males and females of the same genotypes as the donors, and are inoculated by intracerebral or intraperitoneal routes. These combinations are summarised in Table 16.

Donors were injected by intracerebral injection of a single brain source of ME7 scrapie from a female C57 mouse. Donors and recipients

(assay hosts) were killed when clinically affected with advanced scrapie.

Table 16 COMBINATIONS OF DONOR, RECIPIENT, ROUTE AND ORGAN SOURCE (NUMBERS OF MICE IN BRACKETS)

Scrapie Source (all female)	Inoculation Route	Recipient (Male and Female)
BRVR brain (67) BRVR spleen (50)	intracerebral (128)	BRVR (M 62; F 39)
BALB brain (56) BALB spleen (51)	intraperitoneal (96)	BALB (M 57; F 66)

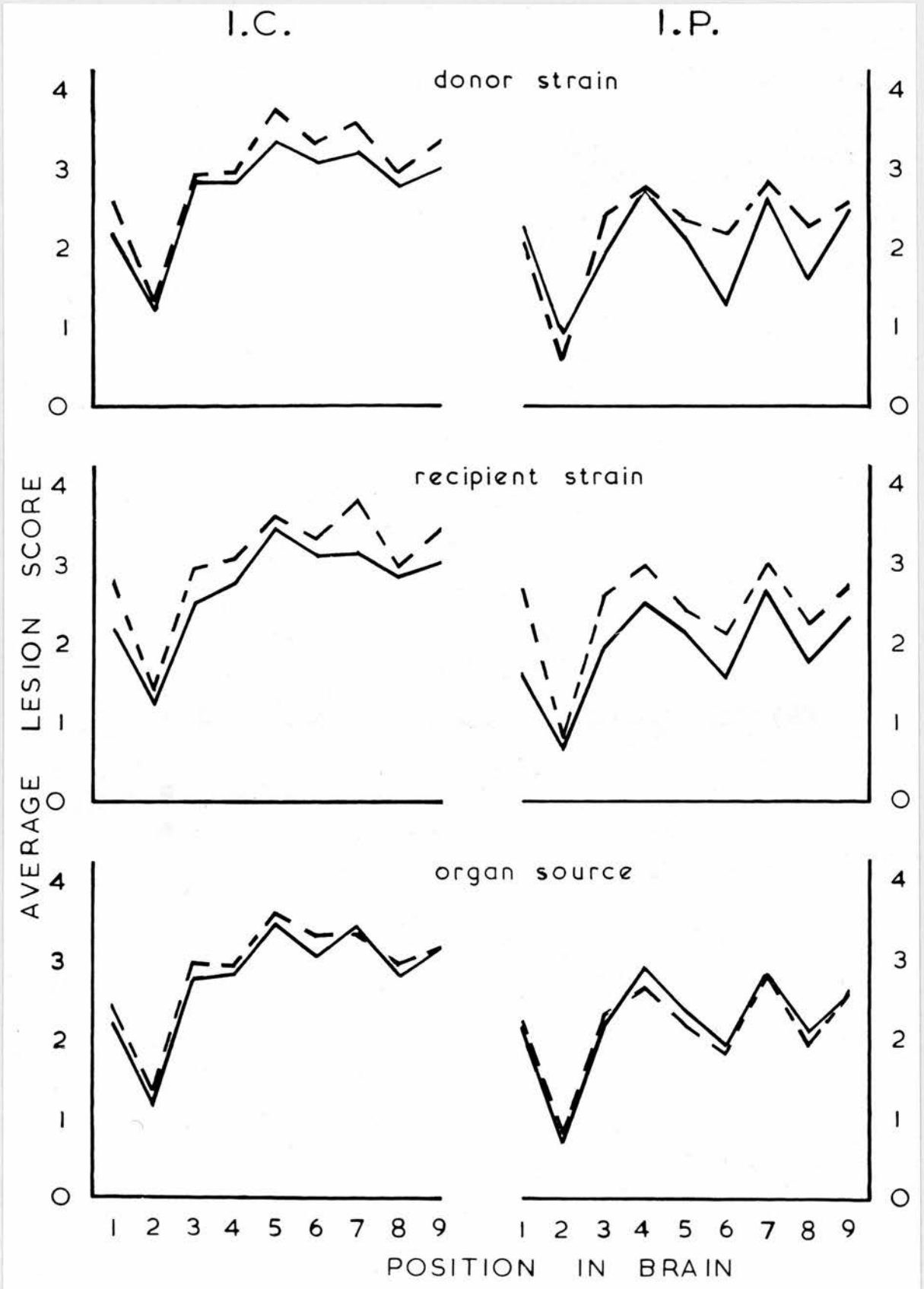
Results

Lesion profiles associated with the four major sources of variation are shown in Figure 35. There are the anticipated considerable differences between the two inoculation routes ($P < .001$) and less extensive differences arising from donor ($P < .001$) and recipient ($P < .001$) variation are also shown. Significance tests for individual combinations of donor strain, recipient strain and organ source indicate an influence due to organ source which is revealed only with the intracerebral route ($P < .05$) particularly in the combination with BALB as donor and recipient ($P < .01$). The results of this experiment have been analysed in two ways: in one the nine brain positions are considered separately, in the other the pattern of damage in the nine positions is treated jointly in a multi-variate analysis (Table 17). These both reveal an additional source of variation due to sex

Figure 35 The mutual influence of variation in donor and recipient in scrapie.

- - - BALB donor; BALB recipient; spleen

——— BRVR donor; BRVR recipient; brain


I.C.
I.P.
donor strain
recipient strain
organ source
AVERAGE LESION SCORE
0
1
2
3
4
1 2 3 4 5 6 7 8 9
POSITION IN BRAIN

of the recipient ($P < 0.05$).

Variation due to inoculation route is the most profound single effect of all the five sources of variation, but highly significant interaction terms with the other four variables occur which can be more readily interpreted when the data for the two routes is taken separately. The highly significant difference between BALB and ERVR as recipients occurs in both route combinations but is slightly more marked with intracerebral inoculation. The difference due to donor genotype has almost as great an effect as recipient genotype, and this is revealed in both route combinations. The interaction term of route with recipient sex is particularly striking because no differences occur between males or females inoculated by the intracerebral route whereas with the intraperitoneal route this difference is highly significant ($P < .01$), and this is in contradistinction to the effect of recipient genotype where the intracerebral route showed the greater effect. The use of brain or spleen as the source of ME7 scrapie produced no significant effect when the data is considered overall, but there is a significant interaction term of organ source with route: the difference due to brain or spleen is only significant in mice inoculated by the intracerebral route ($P < .05$).

In summary, the patterns of brain damage of scrapie caused by ME7 agent in inbred mice are dependent upon their genotype, and to a lesser extent upon their sex. The patterns are also influenced quite considerably by the genotype of the donor, and to a minor extent by the organ used as the source of infectivity. The extent of these influences depends on the precise combinations of the test system, particularly the route of inoculation.

Table 17 SUMMARY OF ANALYSIS OF VARIANCE AND MULTIVARIATE ANALYSIS. (P >0.05 -; P <0.05 x; P <0.01 xx; P <0.001 xxx)

Source of Variation	Position in Brain									Multivariate
	1	2	3	4	5	6	7	8	9	
Route	xx	xxx	xxx	-	xxx	xxx	xxx	xxx	xxx	xxx
Recipient	xxx	-	xxx	xxx	-	-	xxx	-	xx	xxx
Donor	-	-	xx	-	xx	xxx	-	xxx	-	x
Sex	-	-	x	-	x	-	-	x	xx	x
Organ	-	-	-	-	-	-	-	-	-	-
First Order Interactions										
Route x recipient	-	-	xx	-	-	-	x	x	-	-
Route x donor	x	-	xx	-	-	xxx	-	xx	-	xx
Route x sex	-	-	x	-	xx	-	-	x	-	-
Route x organ	-	-	-	-	x	xx	-	x	-	x
Recipient x donor	-	x	-	-	-	-	-	-	-	-
Recipient x sex	-	-	-	-	-	-	-	-	-	-
Recipient x organ	-	-	-	-	-	xx	-	-	-	-
Donor x sex	-	-	-	-	x	-	x	-	-	-
Donor x organ	-	-	x	-	-	-	-	-	-	-
Sex x organ	-	xx	-	-	-	-	xx	-	-	-
Intracerebral route										
Recipient	xxx	-	-	x	-	-	xxx	-	xxx	xxx
Donor	xx	-	-	-	xxx	x	-	x	xx	xxx
Sex	-	-	-	-	-	-	-	-	-	-
Organ	-	-	-	x	-	x	-	-	-	x
Intraperitoneal route										
Recipient	xxx	-	xx	-	-	-	-	-	x	xx
Donor	-	-	x	x	-	xx	-	xx	-	x
Sex	-	-	xx	x	xx	-	-	x	x	xx
Organ	-	-	-	-	-	-	-	-	-	-

SECTION 2

NERVE FIBRE DEGENERATION IN SCRAPIE

The occurrence of nerve fibre degeneration in the brains of scrapie infected mice is described in this section. This demonstration is facilitated by the use of silver impregnation methods for the selective staining of degenerating axons.

Mice of three inbred strains, C57, BALB and VM inoculated intracerebrally with ME7 scrapie agent, were killed at weekly intervals throughout the second half of the incubation period. A silver impregnation method (Nauta and Gygax, 1954; Nauta, 1957) was used to stain degenerating nerve fibres selectively. Uninoculated control mice of the same three strains were treated similarly. Some mice of other strains, inoculated with scrapie either intracerebrally or intraperitoneally were similarly examined including some inoculated with 22A agent. Some examinations were made using a modification of the Nauta method (Fink and Heimer, Method 2, 1967). A further small series were examined at the Rocky Mountain Laboratory, Hamilton, Montana, U.S.A. Appendix I describes a modification of the Nauta method which was devised for use with the material fixed in formol ammonium bromide, which was the fixative routinely in use at the Rocky Mountain Laboratory.

A degree of degeneration greater than in the contemporary controls or in mice inoculated intracerebrally with control material appears soon after halfway through the incubation period (which differs between the three strains). The sum of a three grade scoring system as applied to six

regions of the brain is adopted as an arbitrary measurement of the intensity of the nerve fibre lesion. Fitures 36a and b show that the fibre lesion develops earlier and more rapidly in the C57 and BALB strains, and later and slightly more slowly in the VM strain which has a longer incubation period with this agent. Figure 37 shows that a direct relationship exists between the development of spongy degeneration and the nerve fibre lesion. In addition to the six areas used for scoring the nerve fibre lesion is widespread throughout the brain, and does not involve particular systems. The lesions are most clearly seen in the bundles of parallel running fibres of the internal capsule within the corpus striatum, where the beaded and granular disintegrating fibres contrast with those in this site in the control brains (Figures 38-45). The lesions are also seen in the cerebro cortico-medullary junction, in the thalamus, the tectum of the mid-brain in the nucleated and reticular parts of the brain stem and occasionally in the hippocampus. In the cerebellar cortex it is rarely possible to identify degenerating nerve fibres although they are frequent in the vicinty of the roof nuclei.

Qualitatively similar lesions are demonstrable in other mouse strains affected with terminal scrapie, inoculated intraperitoneally or intracerebrally with ME7 or 22A agents. These findings were confirmed at the Rocky Mountain Laboratory in Montana, U.S.A., where the distribution of the lesion was compared with that of astrocytic hypertrophy. (A modified Nauta method, for FAB fixed material, was devised which was only suitable if fixation did not exceed 8 days, Appendix I). The mouse brains examined at the Rocky Mountain Laboratory were for routine scrapie diagnosis from random

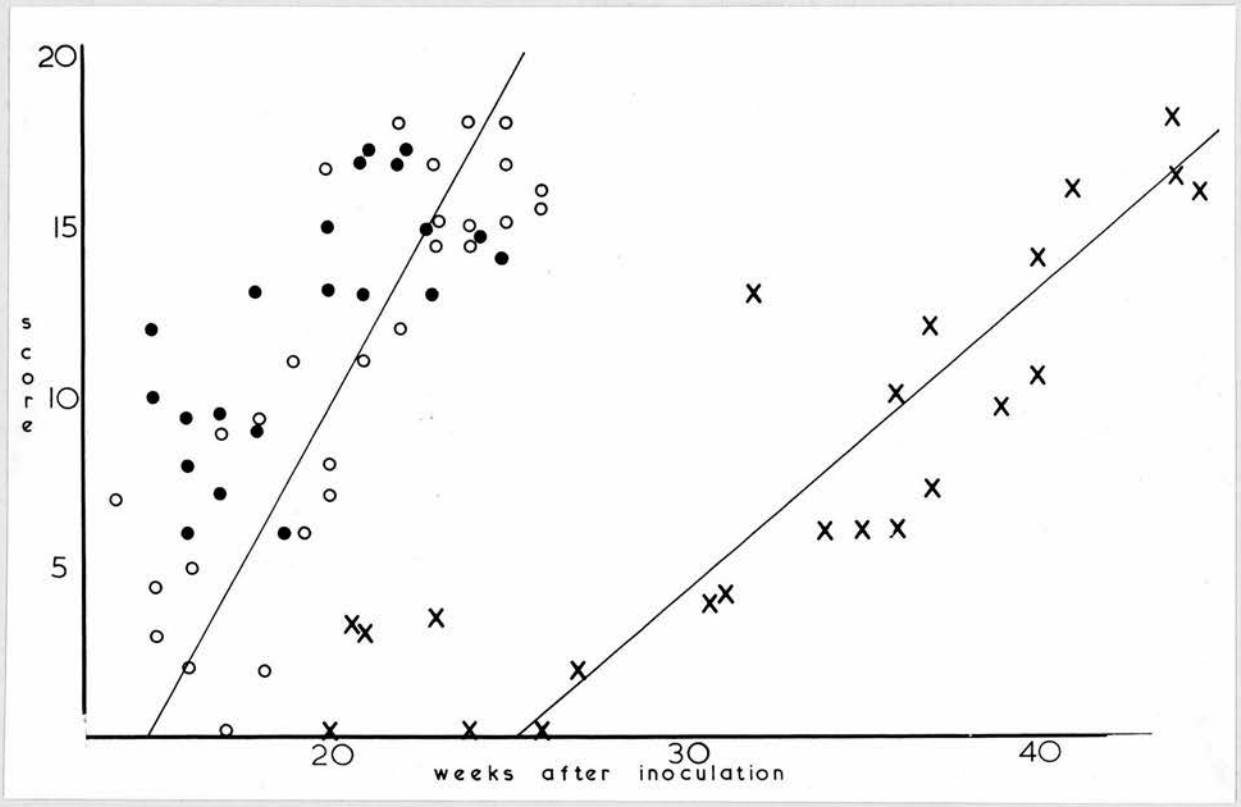


Figure 36a Development of nerve fibre degeneration during the pre-clinical and clinical phases of scrapie in 3 strains of mice.

o = BALB; ● ▲ C57BL; X = VM.

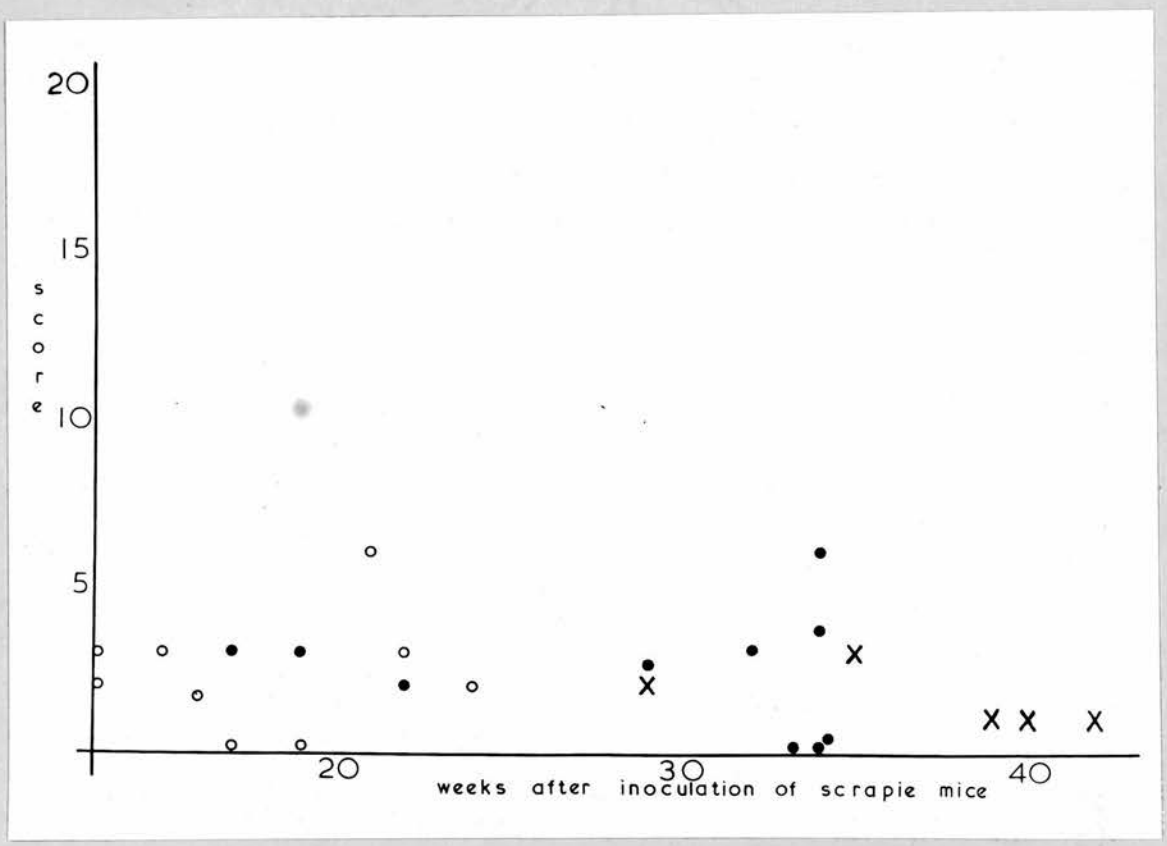


Figure 36b Estimate of integrity of nerve fibres of uninoculated control mice, the same strains as in Figure 36a, and killed at corresponding ages.

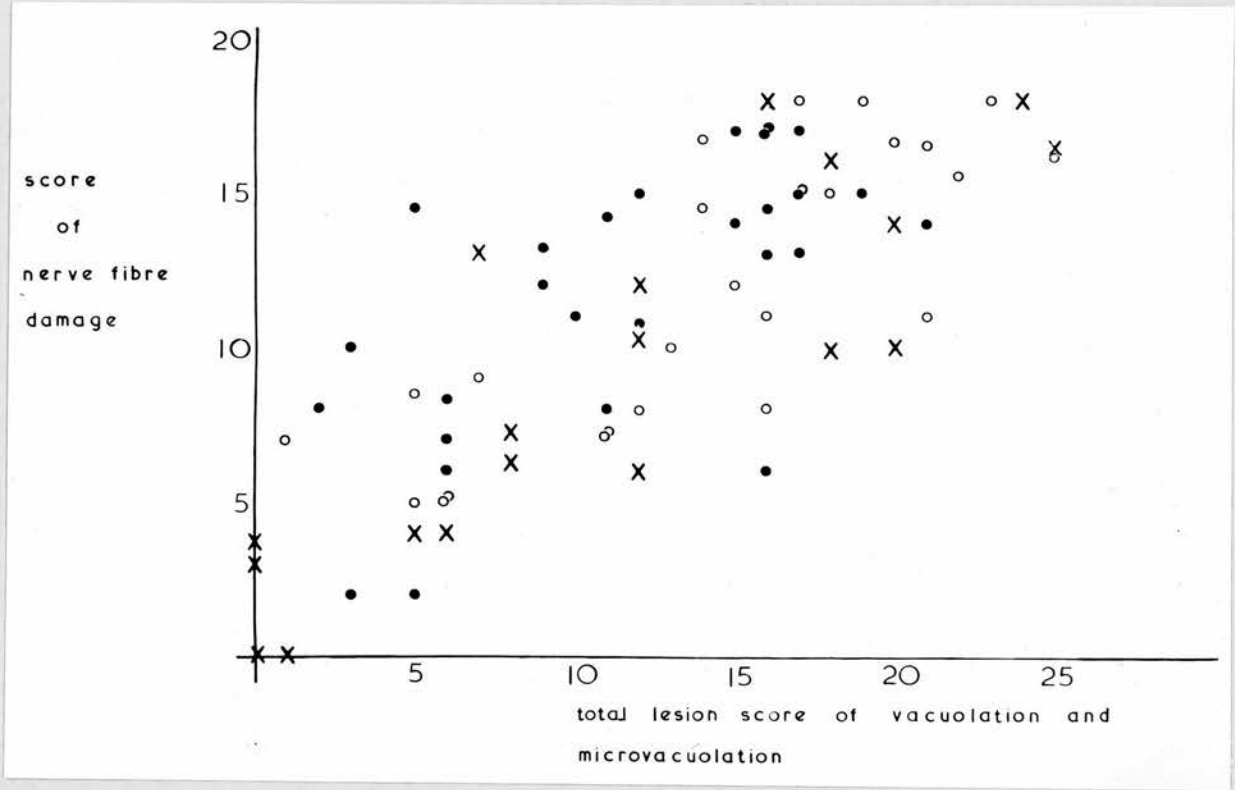


Figure 37 **A direct relationship between the development of degenerative changes in nerve fibres and vacuolation and microvacuolation in the 3 strains of mice as in Figure 36a.**

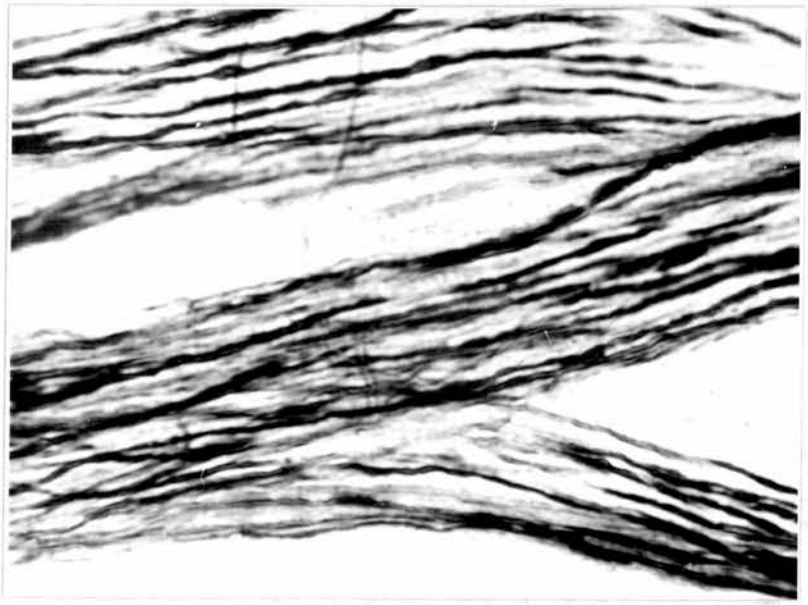


Figure 38 Corpus striatum in normal mouse. Nauta and Gygax X900.

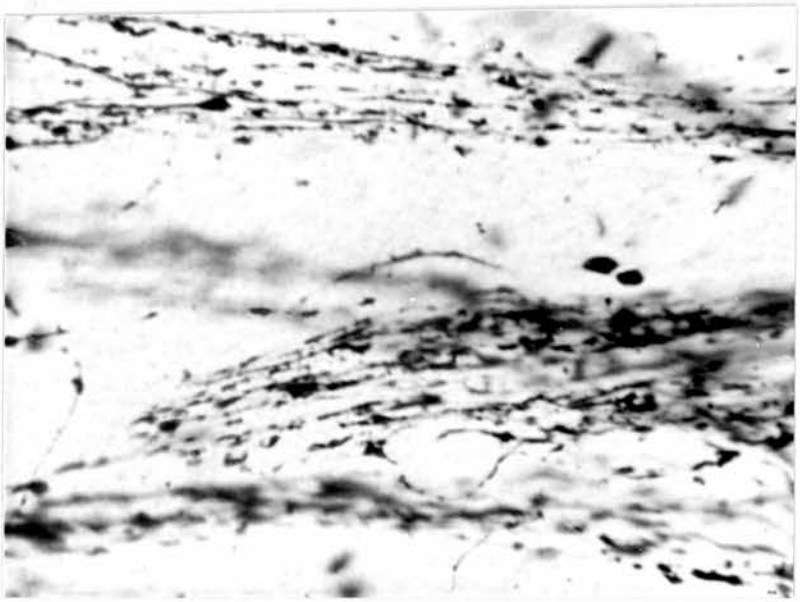


Figure 39 Corpus striatum in scrapie mouse. Nauta and Gygax X900.

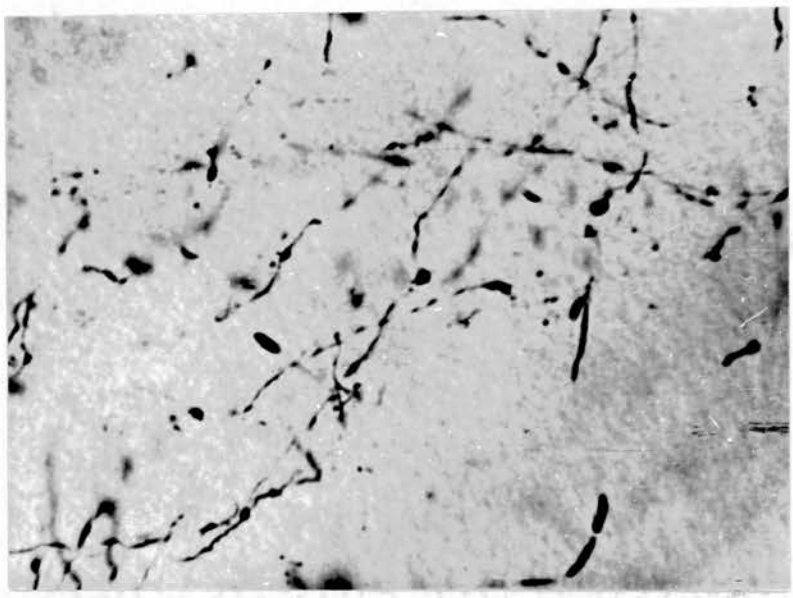


Figure 40 Hippocampus from scrapie mouse. Nauta and Gygax X900.

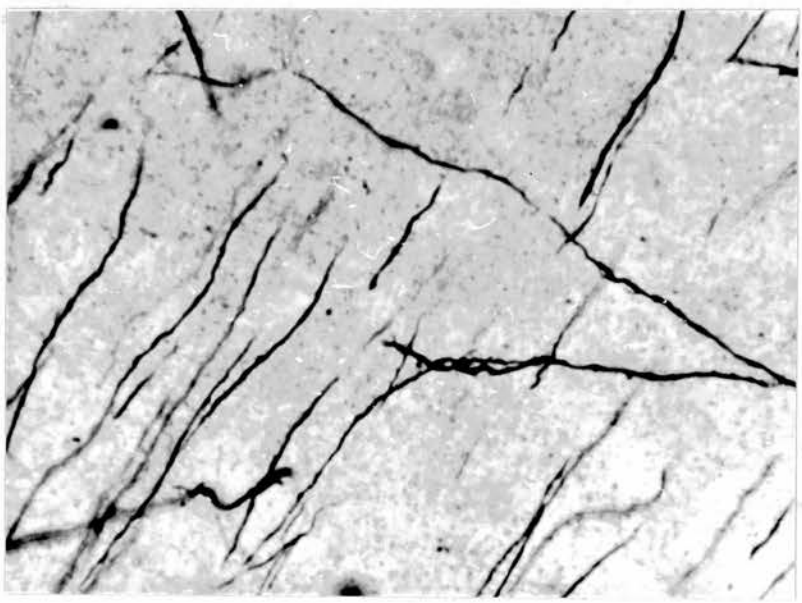


Figure 41 Cerebral cortex at cortico-medullary junction in normal mouse. Nauta and Gygax X900.

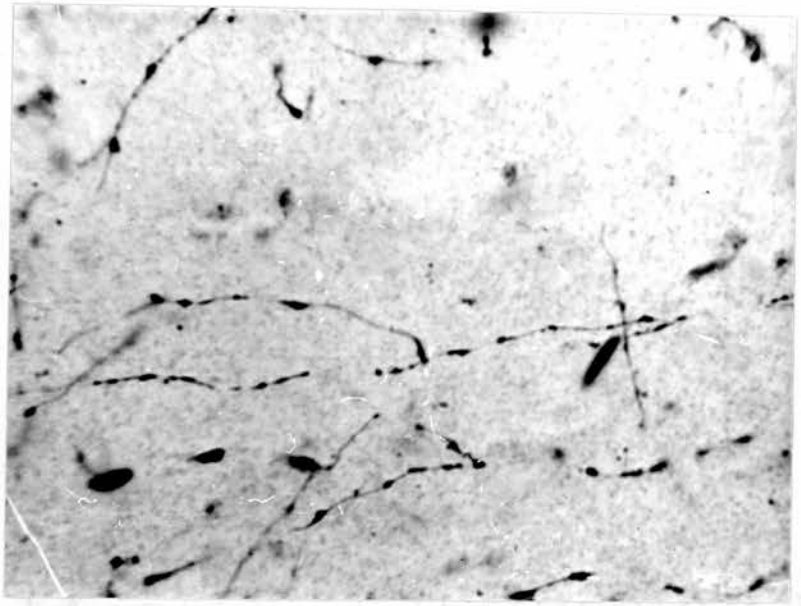


Figure 42 Cerebral cortex at cortico-medullary junction in scrapie mouse.
Nauta and Gygax X900.

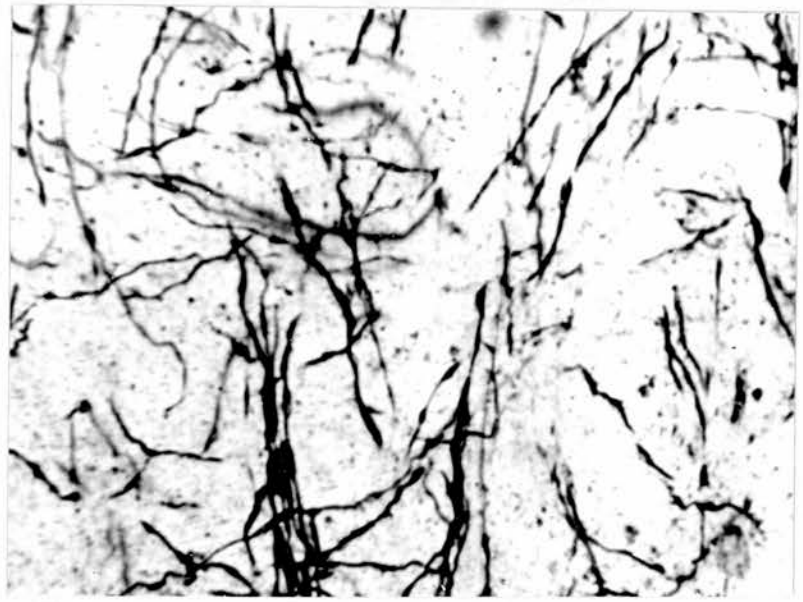


Figure 43 Central mass of thalamus in normal mouse.
Nauta and Gygax X900.

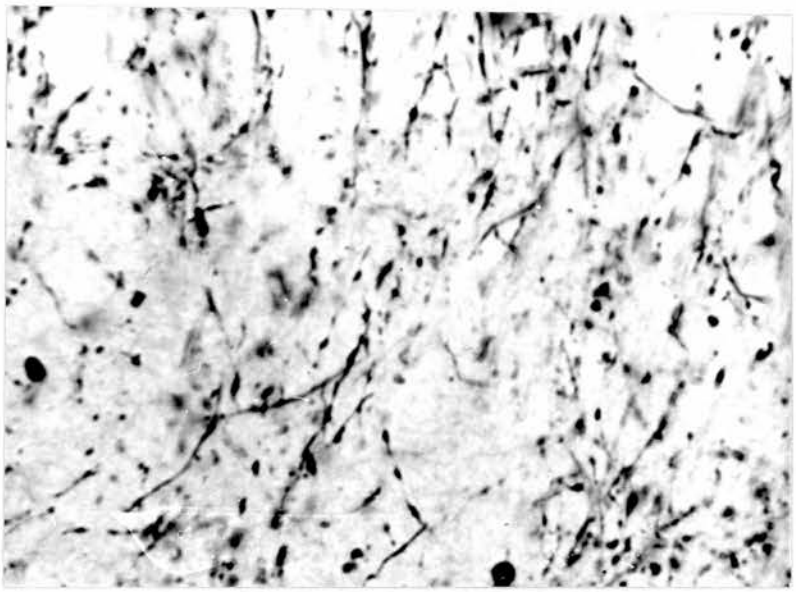


Figure 44 Central mass of thalamus in scrapie mouse. Nauta and Gygax X900.

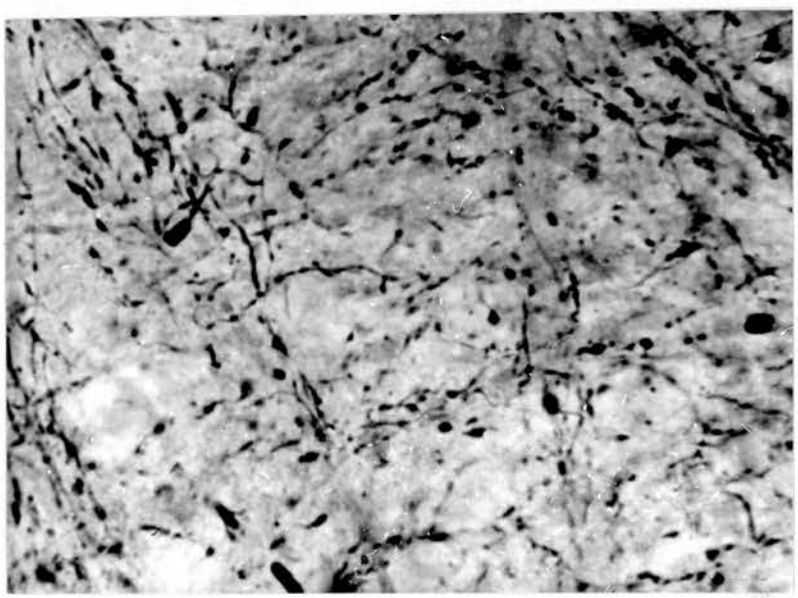


Figure 45 Dorsal medulla in scrapie mouse. Nauta and Gygax X900.

bred mice inoculated subcutaneously or intracerebrally with scrapie, and the astrocytic hypertrophy and nerve fibre degeneration coincided spatially with one another.

It is concluded that the nerve fibre lesion is a secondary phenomenon in view of its contemporaneous development and topistic coincidence with the vacuolar and microvacuolar lesions.

SECTION 3

AGE ASSOCIATED CHANGES, A COMPARISON BETWEEN SCRAPIE AND NON-SCRAPIE AFFECTED MICE

Three morphological features occurring concomitantly with advancing age have been observed. These findings are relevant to this thesis for two reasons. It has been suggested (Field, 1967; Adams, Caspary and Field, 1969) that similar mechanisms underly the alterations in nervous tissues associated with both ageing and scrapie and other degenerative C.N.S. disorders, and so it is important to attempt to compare any features which may occur in each. Also, although scrapie can be induced in relatively young mice with some combinations of agent strain and host genotype, with other combinations the disease can only coincide with old age. It is, therefore, important in the latter, to be able to distinguish ageing changes from scrapie lesions.

The three features are: bilateral thalamic calcification; eosinophilic inclusions in thalamic neurons; oedema in white matter. These are described from the morphological standpoint, and on the basis of their incidence with age in scrapie affected and non-scrapie affected mice.

Bilateral Thalamic Calcification in Ageing Mice

A preliminary report of this condition has been published and many more cases have been seen subsequently (Fraser, 1968; Appendix II). The lesions can measure up to 100μ and several deposits of different size and

shape usually occur together. In haematoxylin and eosin sections they appear as deep blue-black irregular deposits in the dorso-medial parts of the ventral thalamic nuclei. Developmental stages of this bilateral thalamic calcification are shown in Figures 46-49. The early lesion (Figure 47) in which calcification is not readily demonstrable, is clearly associated with small arterioles in the lateral thalamus, but in more advanced lesions (Figure 49) large areas of the thalamus are affected which obscures their relationship with blood vessels. Occasionally concretions occupy the lumen of the blood vessel (Figure 46). In ten cases in which Cajal gold chloride preparations were performed a reactive astrocytosis was not associated with them. If a relationship between protein exudation and astrocytic reaction (Blakemore, 1969; Kristensson & Sourander, 1969) is widely true, this may signify that these lesions are initiated by some mechanism other than by vascular leakage of protein, and their early intravascular occurrence would support this.

Severe cases show a microscopic similarity to idiopathic non-arteriosclerotic cerebral calcification in man (Adachi, Wellmann and Volk, 1968). In the mouse similar structures to those described in this section have been described in the cerebral cortex and called calcified 'psammoma' bodies (Tucker and Baker, 1967). They have never occurred in the cerebral cortex in the extensive series described in this thesis. The amyloid bodies described in rats with scrapie (Field, Raine and Joyce, 1967) bear no resemblance to the calcified lesions, or the developmental stages of them, described here. Also, the classical descriptions of corpora amylacea (Wagner, Damodoran and Feigin, 1957) differ from the bilateral thalamic calcification. It is concluded that this lesion is a senile change and is

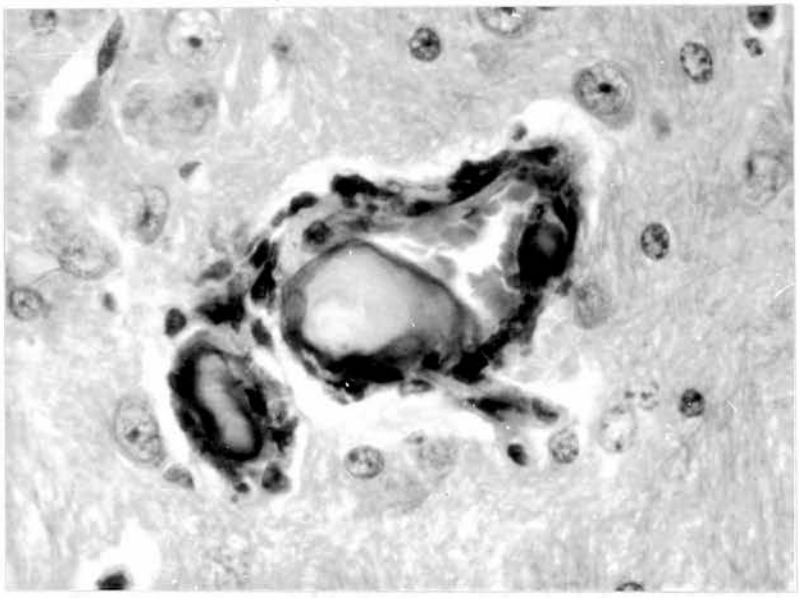


Figure 46 Early intraluminal lesion in the development of the massive calcified lesion shown in Figure 49. H & E X400.

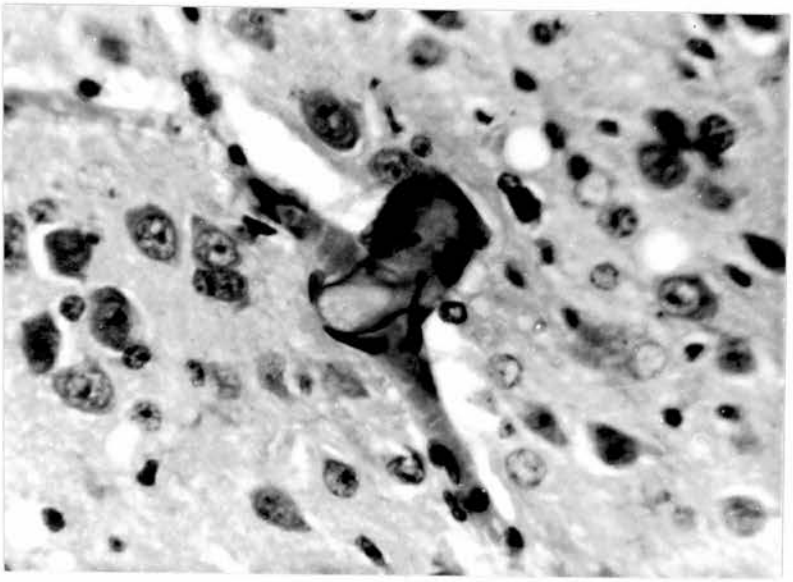


Figure 47 Early lesion in the development of the massive calcified lesion shown in Figure 49. H & E X400.

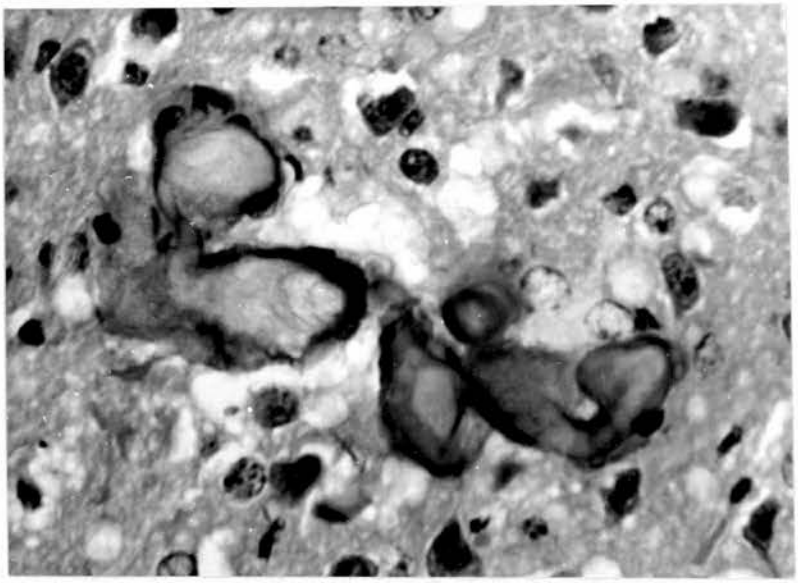


Figure 48 More advanced stage in the development of thalamic calcification. The origin from blood vessels is still apparent. H & E X400.

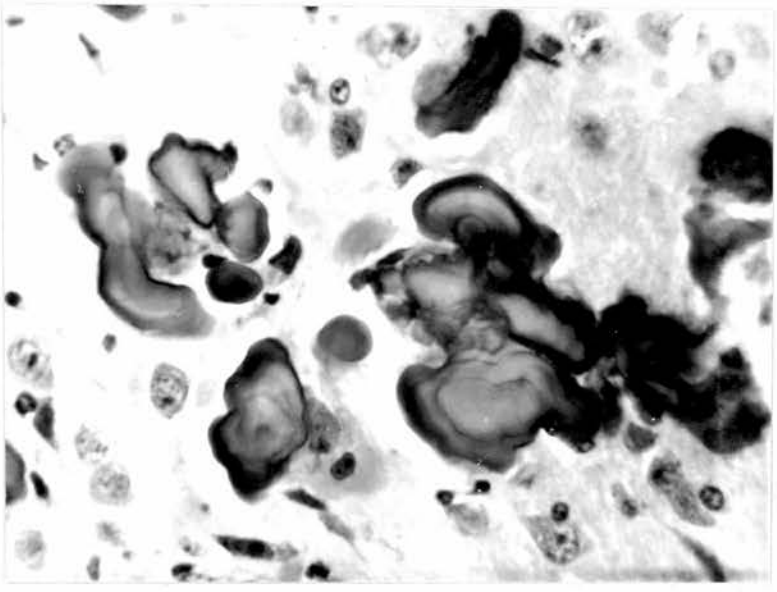


Figure 49 Advanced lesion of thalamic calcification. The relationship to blood vessels is obscured. H & E X400.

confined to the region of the ventral thalamic nuclei (Zeman and Innes, 1963) where they appear to arise from or within the vicinity of small blood vessels. There is no lesion associated with scrapie in younger mice which is similar to this ageing calcification.

Eosinophilic Inclusions in Thalamic Neurons

In a similar but more extensive area of the thalamus another ageing lesion has been observed. Characteristic eosinophilic bodies are regularly seen within the cytoplasm of neurons in ageing mice. A report of this has been published (Fraser, 1969; Appendix II) and numerous cases have been seen since, and it is concluded that their incidence increases with age. Based on histological assessment, the number of affected neurons in individual mice also increases with age. These bodies vary from about 5μ to 20μ in diameter and occur singly within cells. They stain vividly with eosin and occasionally feature a central basophilic spot or strand (Figures 50-53). They are usually round but sometimes are elongated or fusiform in which case a longitudinally striated substructure is faintly distinguishable (Figures 54a and b). Preparative shrinkage sometimes results in the presence of a clear halo around the inclusion which thus appears to occupy a vacuole. They bear no resemblance to the eosinophilic structures or granules seen within vacuoles of scrapie sheep (Stockman, 1926; Palmer, 1957). The staining properties of these inclusions are summarised in Table 18. Their properties contrast with those of lipofuscin which invariably occurs in thalamic neurons in old mice, despite statements to the contrary, (Strehler, 1967) and both frequently

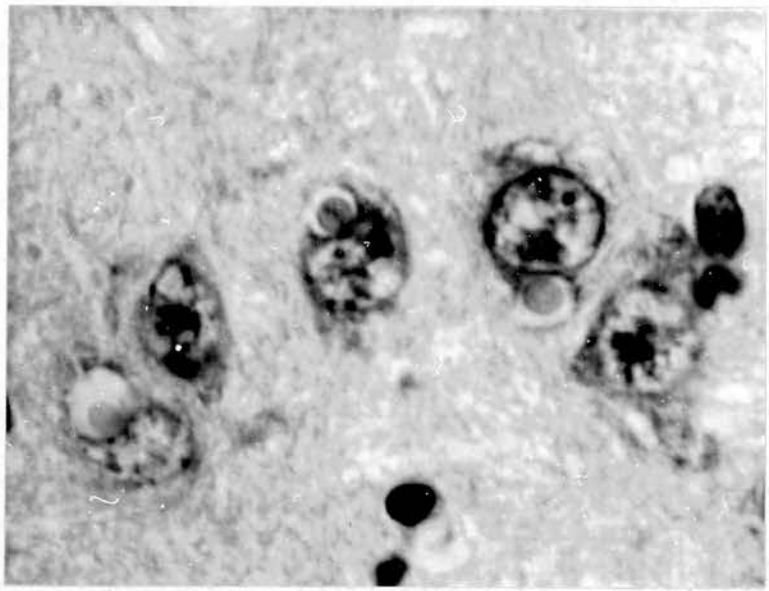


Figure 50 Eosinophilic inclusions within thalamic neurons of aged mouse one of which shows a basophilic strand. H & E X11,000.

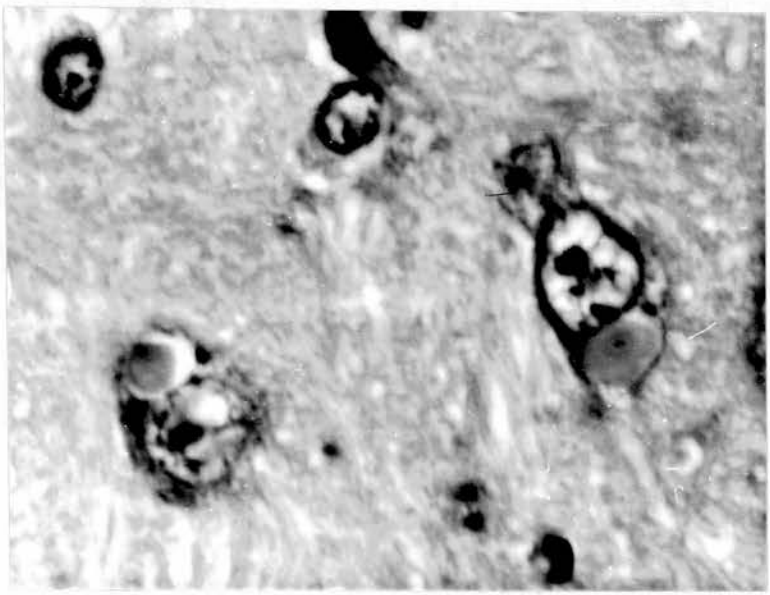


Figure 51 Eosinophilic inclusions in thalamic neurons. H & E X11,000.

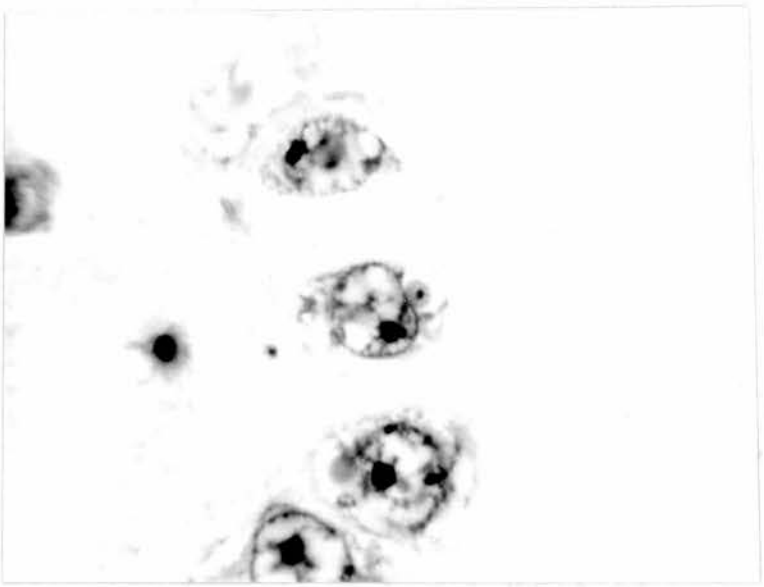


Figure 52

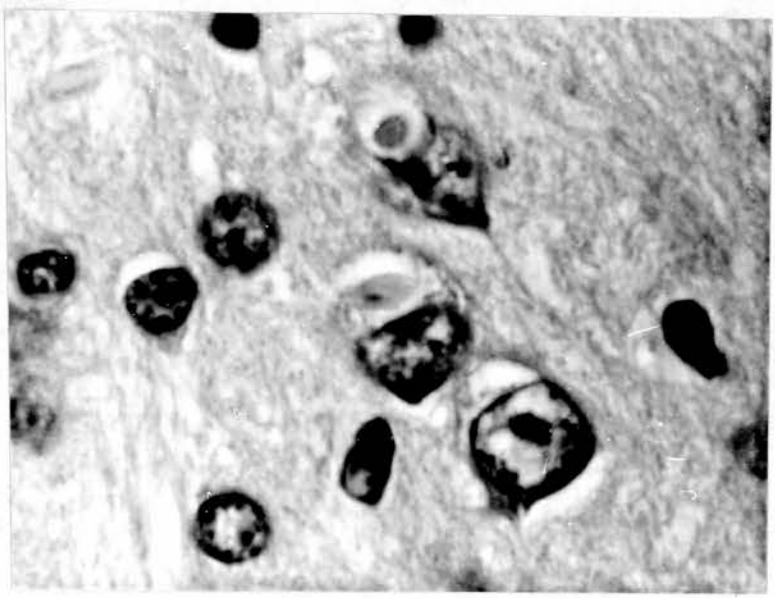


Figure 53

Figures 52 and 53 Neurons in the thalamus of ageing mice, showing eosinophilic bodies in the cytoplasm. A basophilic spot is sometimes apparent within them. H & E X11,000.

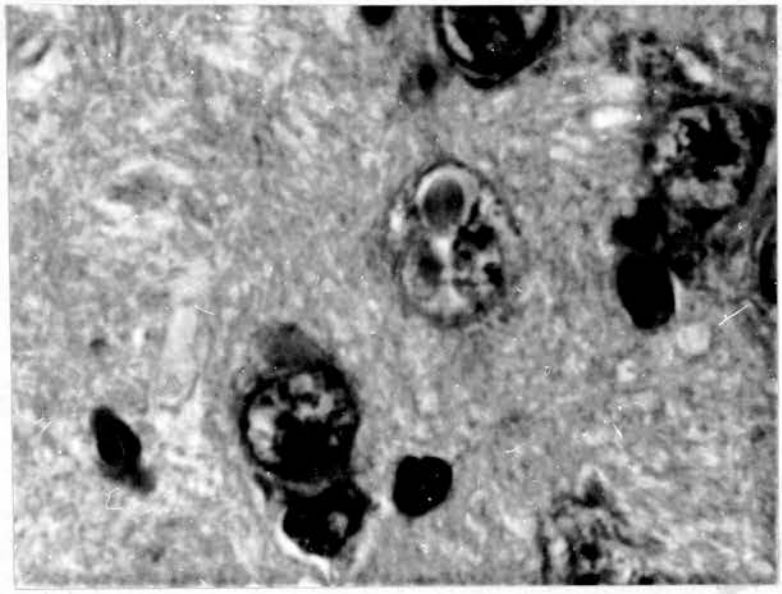


Figure 54a

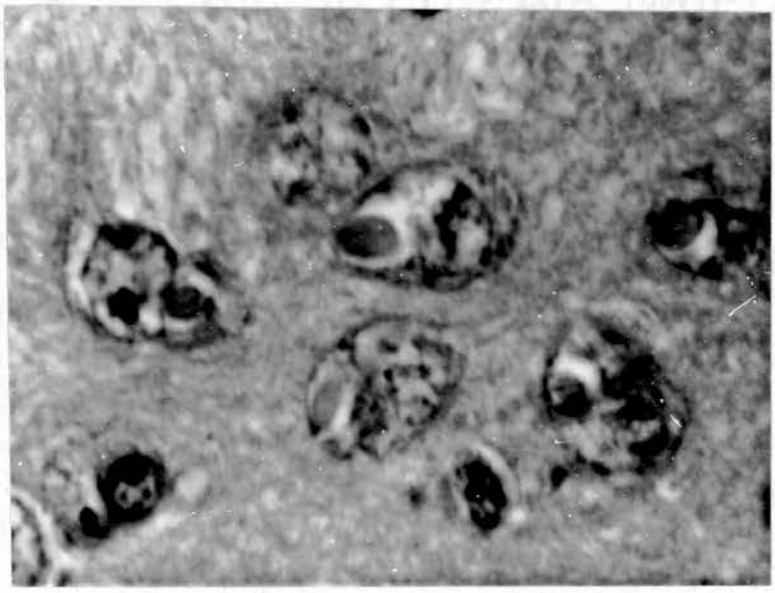


Figure 54b

Figures 54a and b Thalamic neurons showing eosinophilic inclusions which possess a faintly distinguishable longitudinally striated appearance. H & E X11,000.

co-exist within the same cell (Figure 56). The eosinophilic inclusions appear to be made up largely of protein. The central basophilic core seen in haemotoxylin and eosin preparations has not so far been identified in any histochemical preparations.

Ultrastructurally these bodies are arrays of parallel fibrillar crystalline material with a well defined substructure, consisting of longitudinal and transverse striations (Figures 56 and 57). The longitudinal

Table 18 STAINING AND OPTICAL PROPERTIES OF THE EOSINOPHILIC BODIES FOUND IN THE THALAMIC NEURONS OF MICE

Method	Results	Conclusion
Periodic acid-Schiff	Negative	Not carbohydrate or lipofuscin
Gomori's paraldehyde fuchsin	Negative	
Sudan black B	Negative	Not lipid or lipofuscin
Fluorescent microscopy	Not fluorescent	Not lipofuscin
Ziehl Neelsen's	Not acid fast	Not lipoid
Giemsa's	Pink with purplish tinge	Slightly acidophilic
Lendrum's phloxine tartrazine	Rich yellow orange colour	Acidophilic possibly protein
Ninhydrin-Schiff	Strong positive	Protein
Dixon's tannin iron	Strong positive	Protein
Methyl green-pyronin	Unstained	Not DNA or RNA
Feulgen's	Unstained	Not DNA
Glees and Marsland's	Argyrophilic	Acidophilic protein

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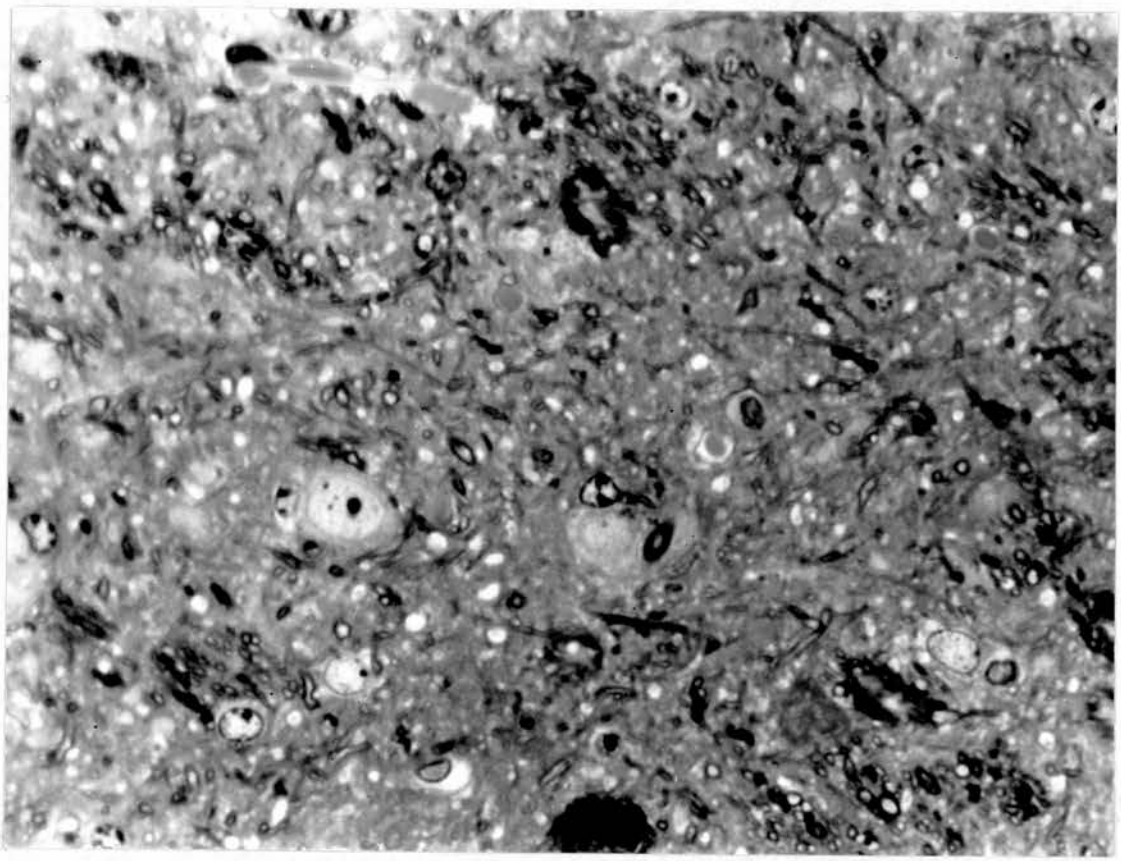

Figure 55 Optical microscopic appearance of inclusion within cytoplasm of thalamic neuron in araldite embedded material. Toluidine blue X800.

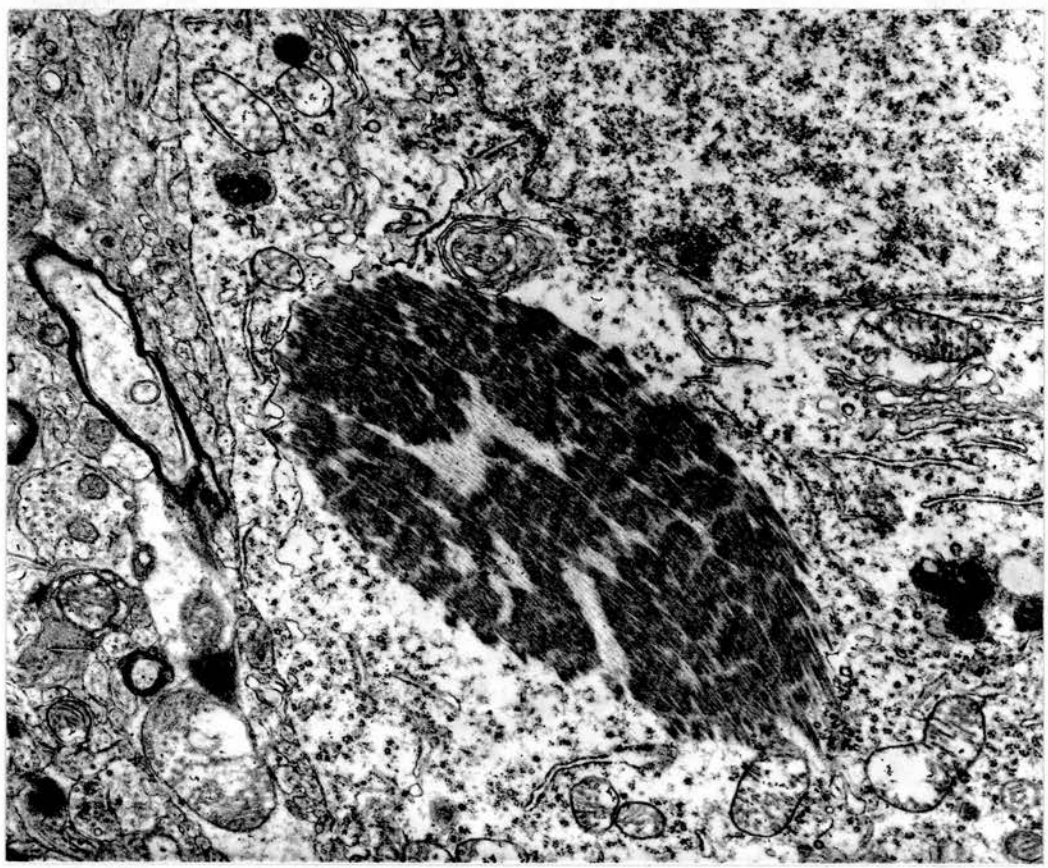

Figure 56 Electron-microscopic appearance of same inclusion as Figure 55. X15,000.

Figure 57 Electron-microscopic appearance of inclusion showing crystalline material arranged in bundles. X45,000.

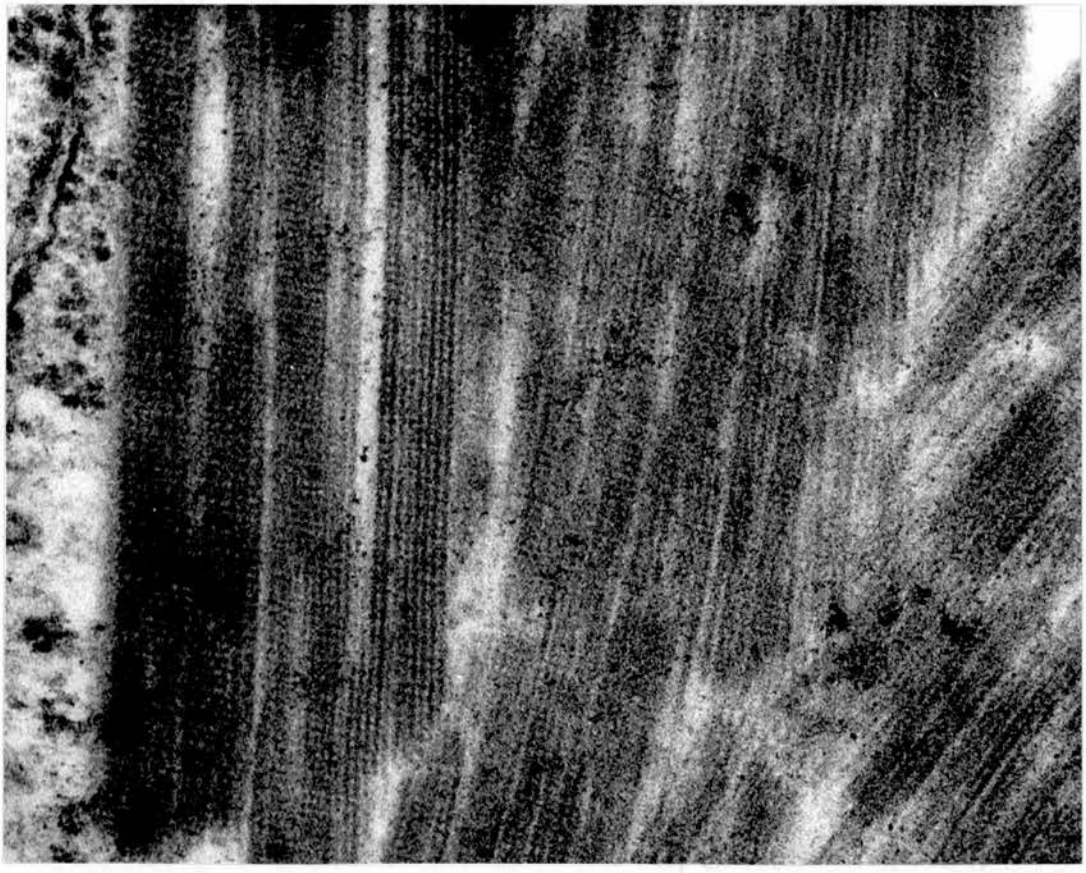

Figure 58 Electron micrograph showing transverse and longitudinal striations. X105,000.

striations consist of alternating electron dense and electron transparent lines, the distance between the centre of one electron dense line and the next being 15nm. The transverse striations are less obvious and appear to result from an alternate narrowing and widening of the longitudinal lines, the periodicity of which is in phase across the thickness of the fibre (Figure 58).

Where bundles of fibres are separated from one another faint lines can be discerned which frequently appear to be continuous with the longitudinal striations of the individual fibres (Figures 56 and 57). Preparations for ultrastructural microscopy were made by Messrs. W. Smith and E. Gray.

Discussion of the nature and significance of these inclusions has been published (Fraser, 1969; Fraser, Smith and Gray, 1970, Appendix II).

Oedema in White Matter

In Section 1 variations in host and scrapie agent and the circumstances of their union are assessed by comparing the effects on the lesions occurring in the grey matter. It is widely accepted that an oedema can occur spontaneously in white matter in ageing mice (Innes, 1967; Rosenblum, Donnenfeld and Aleu, 1966) and a similar lesion albeit more severe, has been described or illustrated in scrapie (Pattison and Smith, 1963; Pattison and Jones, 1968; Gordon, 1966).

During the course of the examination of mouse brains which was required for analysis of Section 1, oedema in the white matter of the cerebellum and tegmentum in scrapie affected and non-scrapie mice was frequently

noted. Sometimes no qualitative distinction was recorded between the oedema occurring in the two instances although it was usually more severe when accompanying scrapie. Viewed subjectively, in addition to this difference in severity, the lesions do sometimes differ qualitatively: in mice with scrapie the oedema manifests itself as clefts or fusiform vacuoles lying between the parallel to fibres (Figure 59), whereas in aged mice a more 'punched out' appearance is seen (Figure 60). In a further important respect the white matter oedema occurring in scrapie sometimes differs from that seen in old mice. In scrapie a distinct oedema and degeneration in ascending and descending long tracts sometimes occurs which is never seen in non-scrapie mice of any age. Typically this involves some or all of the following: the cortico-bulbar and cortico-spinal tract system, the medial lemniscus, the superior, middle and inferior cerebellar peduncles, the spinal tract of the Vth nerve, the intracerebral portions of the VIIth and VIIIth nerves and occasionally unidentified structures in the vicinity of the medial raphe, the medial longitudinal fasciculus and fibre systems in the general reticular substance such as arcuate fibres. Signs of Walleroid changes, typified by digestion vacuoles of Cajal are occasionally seen in association with this type of long tract degeneration.

In scrapie there are important agent differences in respect of the occurrence and incidence of oedematous changes in white matter, and this is the subject of the analysis below. In the data recording system of oedematous changes were categorised under two main headings: 1. an oedema of white matter (no separation being made in the analysis between cerebellar or tegmental white matter), 2. an oedema and degeneration in long tracts.

Because of the qualitative similarity between the lesion in aged and scrapie mice and because of the similar age incidence of the lesion in scrapie with some agents and mice without scrapie there could be an area of uncertainty concerning the lesion. With 79A agent a white matter component is a very distinct feature of the pathology and as this occurs at a high incidence at an age when these changes are not recorded in non-scrapie mice, and as it is frequently associated with the oedematous lesion in long tracts, it is concluded that changes in white matter are a characteristic feature of 79A scrapie. On the other hand the incidence of the lesion with 22A is no greater than occurs in non-scrapie mice of the same age but long tract degeneration is seen with scrapie caused by this agent.

Analysis of the Incidence of Thalamic Calcification, Eosinophilic Inclusions and Oedema of White Matter

The incidence of these phenomena was assessed in a population of approximately 7000 consecutive samples which had been examined for brain histology. This population was made up of both non-scrapie and scrapie affected mice. The non-scrapie mice came from various experiments as well as from sacrificed breeding stock. The scrapie mice came from a large number of scrapie experiments and represented different strains of scrapie, different host strains, incubation periods and killing ages. The object of the analysis was to discover the incidence of these phenomena in terms of agent strain, and host sex and age. The age range of the population was to represent the adult life span of the mouse. The age and sex distribution of the populations was estimated on the basis of a 12% random sample and the scrapie group was subdivided according to the type of agent. The sex

ratio of the overall population was 1 : 1.1 (M : F) and ratios within age groups (50 days intervals) of the sample did not differ significantly from 1 : 1 except in two age groups (151-200 days of age, ratio = 1 : 1.57, $P < 0.001$; 401-450 days of age, ratio = 1 : 4, $P < 0.01$). The overall sex ratio of the samples was 1 : 1.07 ($P > 0.2$), while the ratio in experiments in which cases of white matter oedema or long tract oedema occurred is 1 : 1.5. The sex ratios of the various lesions were estimated on the assumption that they occurred in a population with a sex ratio of 1 : 1.1

Table 19 SEX RATIO ESTIMATES OF FOUR PHENOMENA SEEN IN AGED MICE AND SCRAPIE, M : F

White Matter Oedema		Degeneration and oedema in long tracts	Thalamic calcification	Eosinophilic inclusions
Scrapie	Non-Scrapie			
1 : 2.6 ($P < 0.001$)	1 : 2.4 ($P < 0.001$)	1 : 4.5 ($P < 0.001$)	1 : 0.68 ($P < 0.05$)	1 : 1.1 ($P > 0.5$)

Table 19 illustrates the sex incidence of four features in which significantly higher incidence of the white matter change can be seen to occur in females. Figures 61-63 show the age distribution of oedema in white matter, thalamic calcification, and eosinophilic inclusions. There is no significant difference in the incidence of thalamic calcification between scrapie and non-scrapie mice although it does occur marginally more frequently in non-scrapie mice. This suggests a retardation in development of a degenerative entity. Such a conclusion is not justified in the case

of the eosinophilic inclusions because in scrapie a destructive lesion occurs in the thalamus which obscures the visualisation of these structures.

The white matter oedema occurs with increasing incidence with advancing age, and, with the exception of 79A and ME7 agents, does not differ in its incidence in age groups between scrapie and non-scrapie mice. For instance, the relatively high incidence with 22A agent is no greater than in non-scrapie mice of the same age. An exception to this can be seen with agent 79A, in which this lesion is frequently seen, and at an incidence greatly in excess of non-scrapie mice, or mice with scrapie caused by other agents.

The analysis of the cases of the degeneration and oedema occurring in long tracts represents a distinct finding. Although this is frequently indistinguishable from white matter oedema on histological grounds, the incidence in terms of agent strain and age does not reflect that of the white matter oedema, particularly in regard to 22A agent. The number of cases recorded were insufficient to justify a complete age analysis, but its absence from aged non-scrapie mice suggests that this is a specific scrapie lesion for the two agents with which it occurs. It is therefore concluded that some form of alteration in white matter is a specific accompaniment of scrapie caused by 79A and 22A agents. This is in striking contrast to agent 80A in which white matter changes are not observed.

In the case of the long tract lesion this is completely distinguishable from age associated phenomena, whereas oedema in white matter is a lesion of some forms of scrapie which is not clearly distinguishable from an occasional manifestation of ageing. Its incidence is higher and its severity

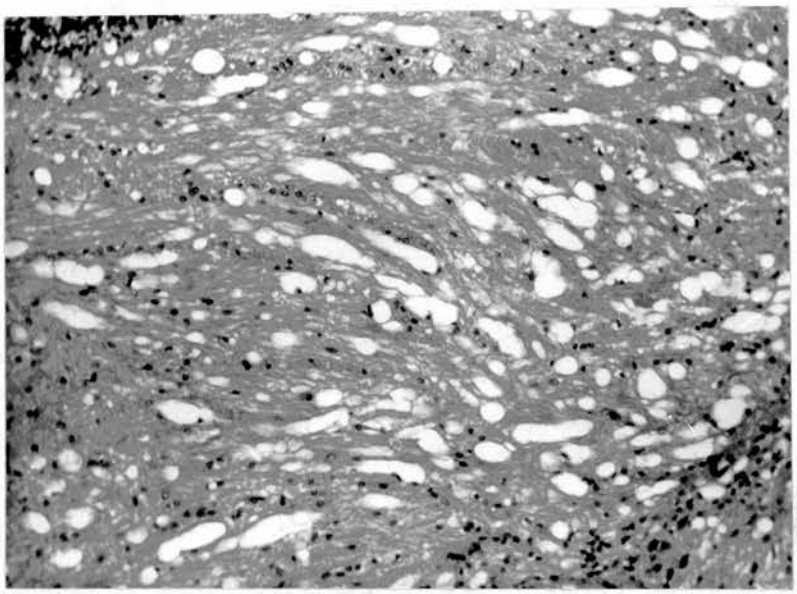

Figure 59 Oedema in white matter of the cerebellum of young (160 day old) mouse clinically affected with scrapie caused by 79A agent. H & E X62.

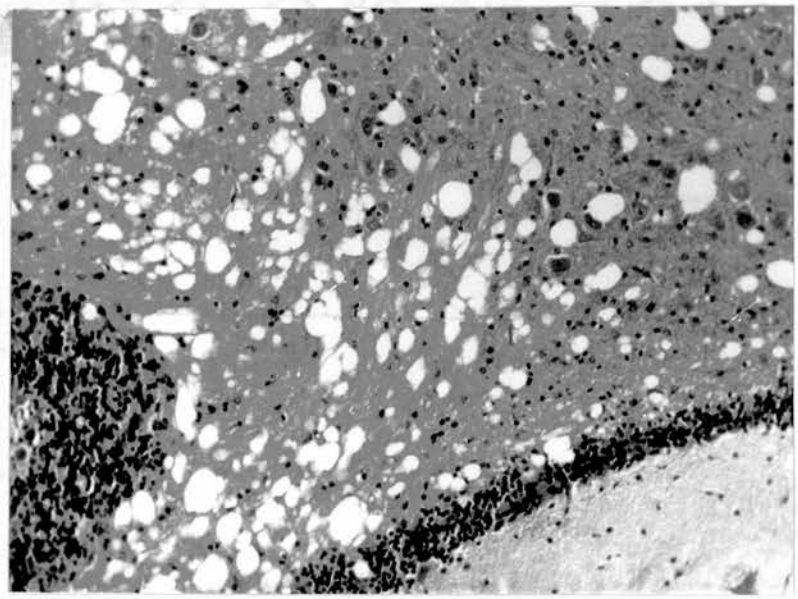

Figure 60 Oedema in white matter of the cerebellum of aged (770 day old) mouse. H & E X62.

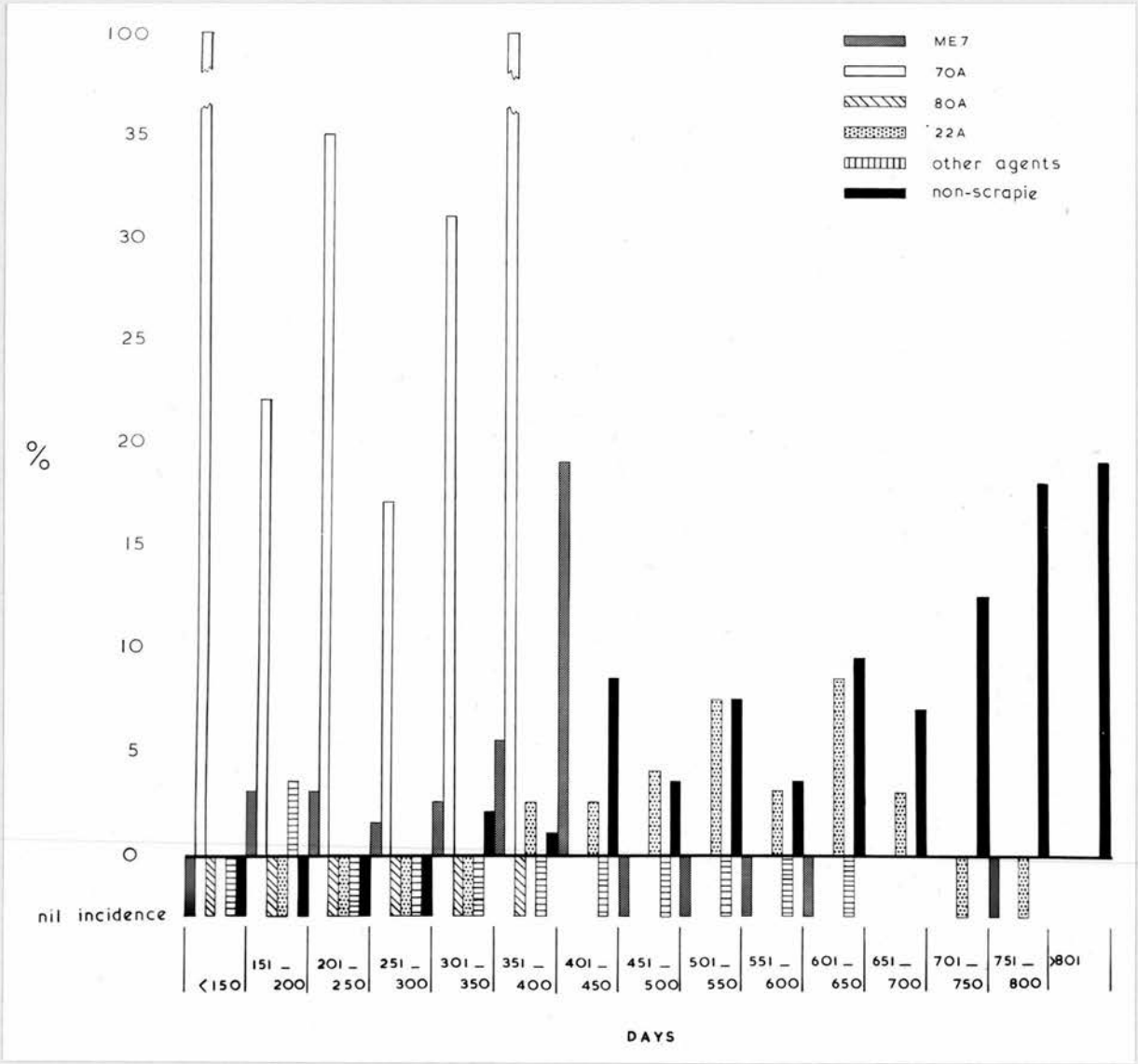

Figure 61 Age incidence in non-scrapie and scrapie (with various agents) mice of white matter oedema.

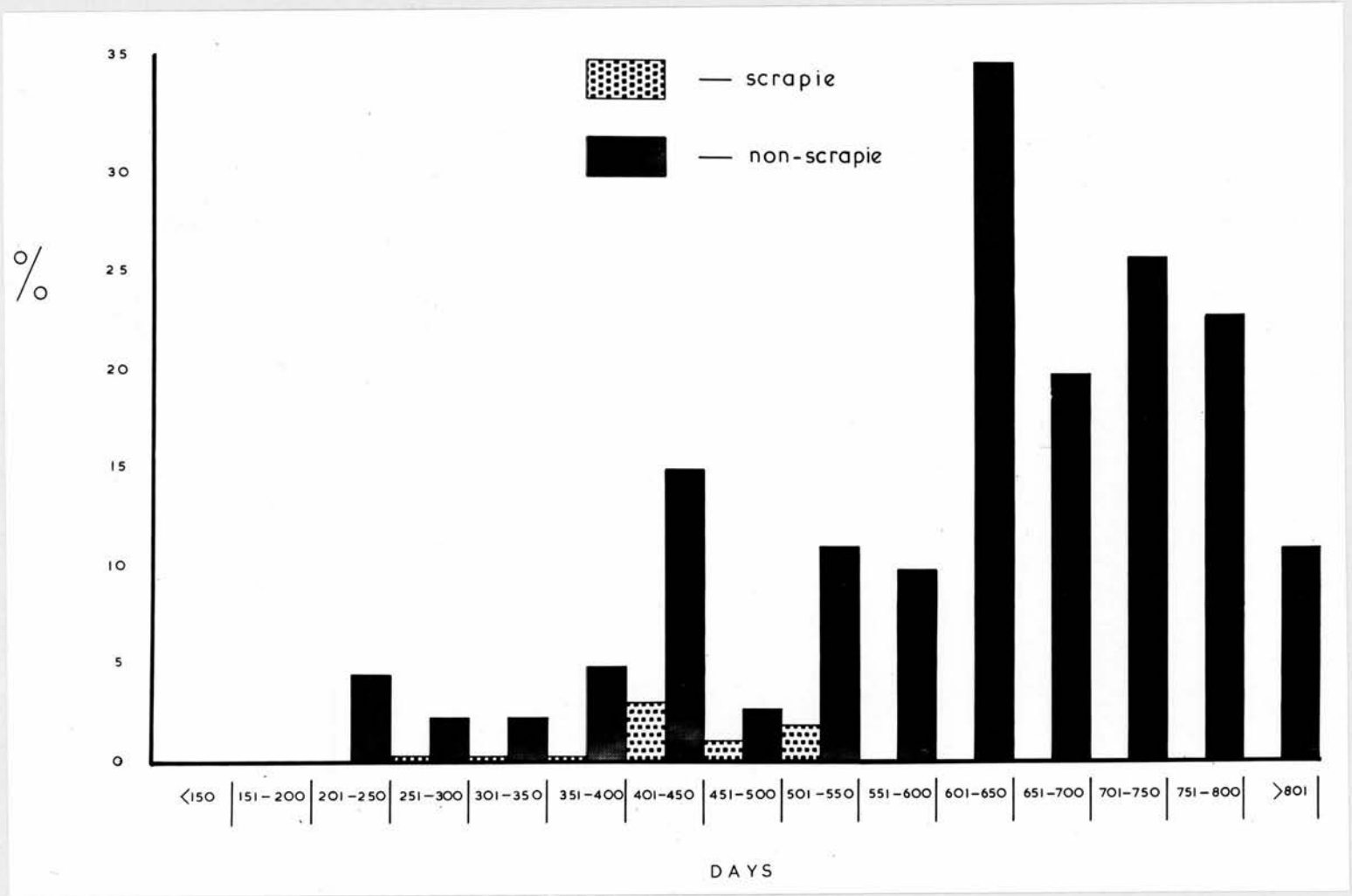

Figure 62 Age incidence of eosinophilic inclusions in thalamic neurons in non-scrapie and scrapie mice.

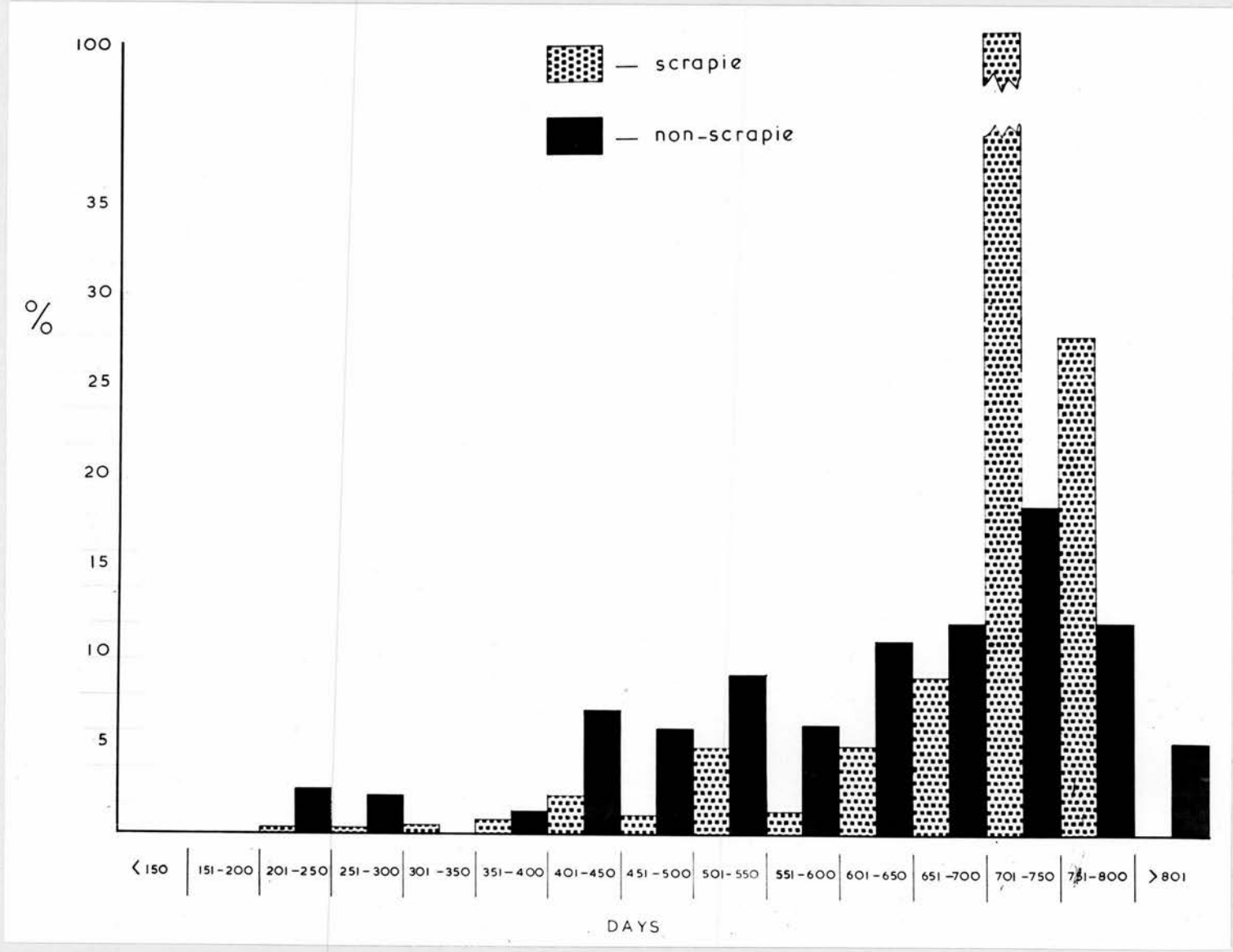

Figure 63 Age incidence of thalamic calcification in non-scrapie and scrapie affected mice.

greater in scrapie caused by 79A agent, than in aged mice, and a qualitative difference is sometimes discernible (Figures 59 and 60). As pointed out above, a marginally higher incidence in ME7 scrapie, compared with non-scrapie mice, is associated with a difference in histological appearance. It can be concluded that there are distinct differences between scrapie agents in the extent and nature of pathological changes in white matter.

SECTION 4

THE OCCURRENCE OF A SERIES OF ASTROCYTOMAS IN AN INBRED MOUSE STRAIN

The mouse is a species in which spontaneous tumours of the central nervous system have rarely been recorded. In a survey of such tumours in animals, (Luginbuhl, 1963a and b), only two neuro-ectodermal tumours in the mouse, an oligodendroglioma and glioblastoma, were listed. A cerebral tumour which was identified as an astrocytoma occurred in one mouse which, together with over eight hundred others, had been injected with material from a slow-virus degenerative encephalopathy of mink (Marsh, Burger, Eckroade, Zu Rhein and Hanson, 1969).

A series of astrocytomata have been identified on twenty-nine occasions in one inbred mouse strain, on two occasions in VM crosses and on one occasion in another mouse strain. This was an incidental finding made during the examination of over 10,000 mouse brains from experiments involving various aspects of scrapie research. This finding is relevant because the astrocytic changes in scrapie have been said to resemble a benign neoplastic change (Field, 1967, 1969; Field and Peat, 1969), and similarities have been suggested between astrocytic changes in scrapie and those neoplastic alterations or transformations which occur in some cell systems with oncogenic viruses, (Webb, 1967), but the astrocytomata have not been found to influence in any way the progression the lesions of scrapie.

In the four year period when these tumours have been seen, the VM

strain has progressed from the sixth to the twentieth inbreeding generation and during this time approximately four thousand VM brains have been examined histologically, ninety five per cent of which received various types of scrapie injections, whereas the remainder received no experimental treatment.

With the single exception of a case in a BRVR mouse, astrocytomata have not been seen in any other strains, even though they constitute the majority.

Results

Table 20 summarises the circumstances in which astrocytic gliomata have been seen. The tumour has been identified in all but three of the 14 inbreeding generations of the VM strain in which extensive neurohistological examinations have been performed and no increase in the mean incidence of cases of 1.5 per cent per generation has occurred during this time.

Table 20 DETAILS OF THIRTY-TWO MICE WITH ASTROCYTOMA

No.of Cases	Mouse strain (sinc / (genotype))	Sex Ratio	Inoculations	Clinical Assessment at time of death
32	VM (p7p7) - 29	$\frac{M}{F} = \frac{20}{12}$	Brain i.c. - 23	Scrapie - 19
	F1 (p7s7) - 1		Spleen i.c.- 1	
	F3 (p7s7) - 1		Brain i.p. - 4	Focal neurological signs - 11
	BRVR (s7s7)- 1		Brain i.c. & i.p. - 2	Severe mammary tumour - 1
			Uninjected - 2	I11 - 1

These gliomata have appeared in both scrapie and non-scrapie mice, and in the nineteen cases which developed terminal scrapie the incubation

Table 21 INCUBATION PERIODS OF SCRAPIE IN 19 VM MICE WITH ASTROCYTIC GLIOMA AND THEIR IDENTICAL CONTEMPORARIES ± S.E.

Incubation period of individual with astrocytic glioma (days)	Mean incubation period of identical contemporaries (days)	Per cent increase (+) or decrease (-) of incubation period
294	294 ± 1.7	0
304	309 ± 13	+ 1.66
186	186 ± 2.6	0
335	330 ± 3.3	- 1.5
441	445 ± 8.3	+ 0.9
213	215 ± 1.2	+ 0.93
259	259 ± 0	0
208	200 ± 4.3	- 4.0
411	432 ± 6.4	+ 4.8
328	294 ± 10.7	- 11.0
195	177 ± 4.6	- 10.2
195	195 ± 0	0
216	217 ± 1	+ 0.49
230	225 ± 3	- 4.4
245	239 ± 3.4	- 2.5
229	219 ± 4.7	- 4.5
257	259 ± 4.6	+ 0.77
252	216 ± 9.7	- 12.8
270	310 ± 34.0	+ 14.8
	Mean deviation in incubation period	- 1.21

periods did not deviate significantly from the means of non-tumour bearing identical contemporaries ($P > 0.4$; Table 21). The mean age of death of the 10 non-scrapie affected VM mice which were killed with focal neurological signs attributable to the tumour was 250 days, ranging from 138 to 520 days.

The age of death (571 days) of the solitary non-scrapie BRVR case was beyond the range for all the cases in the VM mice. Two cases have occurred in crosses involving VM mice (one F1; one F3): both of these cases were heterozygous at the sinc locus. This suggests that if the manifestation of the glioma in VM has any genetic component this could not be mediated through a recessive gene.

The majority of cases of the astrocytoma, have occurred in males (62 per cent), but this preponderance in males is not significant ($P > 0.1$). The ten mice which had focal neurological signs were incoordinated, held their heads to one side and walked in circles. In one case paralysis of the hind limbs developed. The duration of illness varied between 2 and 55 days.

The sites of tumour growth within the brains of the 32 cases are detailed in Table 22. The tumour confines itself to white matter, and appears to spread along fibre tracts. It is frequently seen in the pyramidal tract at all levels and to follow the decussation into the spinal cord (Figure 64). Similarly it is identified in individual cases at different levels of the medial longitudinal fasciculus and medial lemniscus.

The cerebellar white matter is extensively involved in over half the cases together with the cerebellar peduncles and the spino-cerebellar tracts (Figure 65). The tumour can be identified at various levels of the internal capsule, or extending within the corpus callosum, the fimbria, fornix or cerebral white matter (Figure 66). Frequently the tegmental areas of the thalamus and brain stem are severely involved, and often unilaterally. The tumour sometimes invades the seventh and eighth cranial nerves. The

hippocampus is affected in a few cases and when it is, it usually appears to have been invaded from the fimbria. Cases in which focal neurological signs occur are represented mainly by those in which lesions are present in the brain stem (Table 22).

The cellular morphology of the tumours is consistent with that for moderately anaplastic astrocytoma. Within a single case very little pleomorphism is apparent although some variation in cellular morphology is apparent between cases. The nuclei of the neoplastic cells are sometimes small and hyperchromic (Figure 67), but are sometimes larger with visible chromatin structure and mitotic figures are occasionally seen (Figure 68). Scattered between these tumour cells are occasional very large vesicular nuclei in cells with watery cytoplasm (Figure 69). These large nuclei sometimes have eosinophilic inclusions occupying their centre. Pilocytic metaplasia is seen in situations where the fibre tracts are out longitudinally (Figure 70) and microcystic degeneration often occurs in the tumour foci, particularly in the central medulla (Figure 71). There is no evidence of any mesenchymal or reticular components in these tumours. In no case has reticulin or collagen been identified, and the tumour cells do not behave like microglia in silver carbonate treated sections in which microglia are readily identified in other parts of the brain.

The distribution of the lesions within the brain where the sites of tumour growth in individual cases are in either the forebrain or brain stem, suggests that the astrocytoma arises in a confined region of the brain, and does not have a multifocal origin.

Although a similar tumour has been seen only once in the other mouse

Table 22 SITES OF ASTROCYTOMATA IN BRAINS OF 32 MICE

Site	VM mice																												BRVR mice	Totals
Dorsal columns																							X				X			2
Medial Longitudinal fasciculus																			X		X			X		X				4
Medial lemniscus																	X		X			X		X		X	X			6
Medullary reticular formation																		X	X		X		X			X	X	X	X	8
VIIth nerve																			X	X									X	3
VIIIth nerve																						X							X	2
Cerebellar peduncles																						X	X				X		X	4
Cerebellar white matter																		X	X	X	X	X					X		X	7
Tract of spinal nucleus of Vth nerve																						X								1
Cortico-spinal (bulbar) tract												X					X	X	X	X	X	X	X				X		X	10
Mid-brain tegmentum											X				X	X	X	X	X	X									X	9
Decussation of cerebellar peduncle																		X												1
Cerebral peduncle									X	X																				2
Medial geniculate area								X																						1
Mamillary body and tracts																X														1
Thalamus							X	X		X	X	X	X	X	X	X	X													10
Habenula area										X																				1
Hippocampus										X																				1
Corpus striatum					X	X	X	X		X	X	X	X			X														9
Fornix	X			X							X	X																		4
Septal area	X		X																											2
Fimoria		X						X	X	X		X																		5
Anterior Commissure												X																		1
Corpus callosum	X				X			X		X	X	X																		6
Cerebral white matter									X																					1
											†				†			†	†	†	†	†		†		†	†		†	

† Cases exhibiting focal neurological signs.

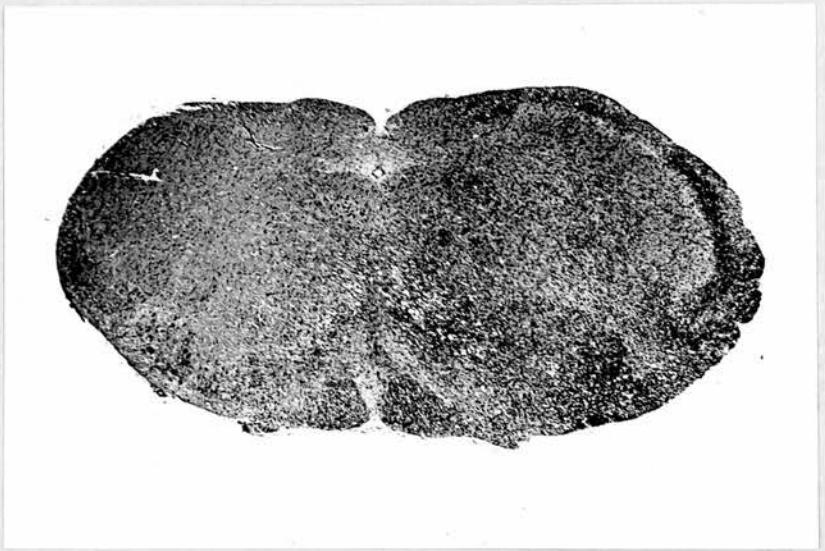

Figure 64 Cross section at junction of medulla and spinal cord with astrocytoma in spino-cerebellar and pyramidal tract H & E X20.

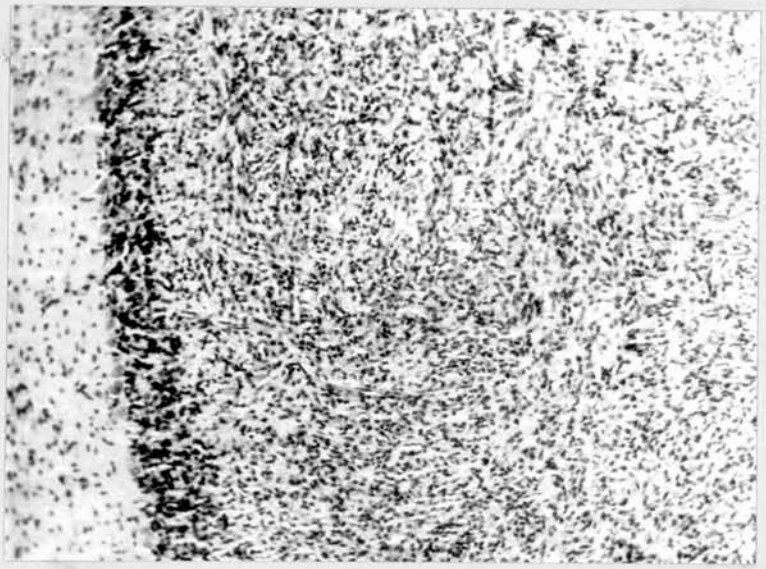

Figure 65 Cerebellum with astrocytoma in white matter. H & E X62.

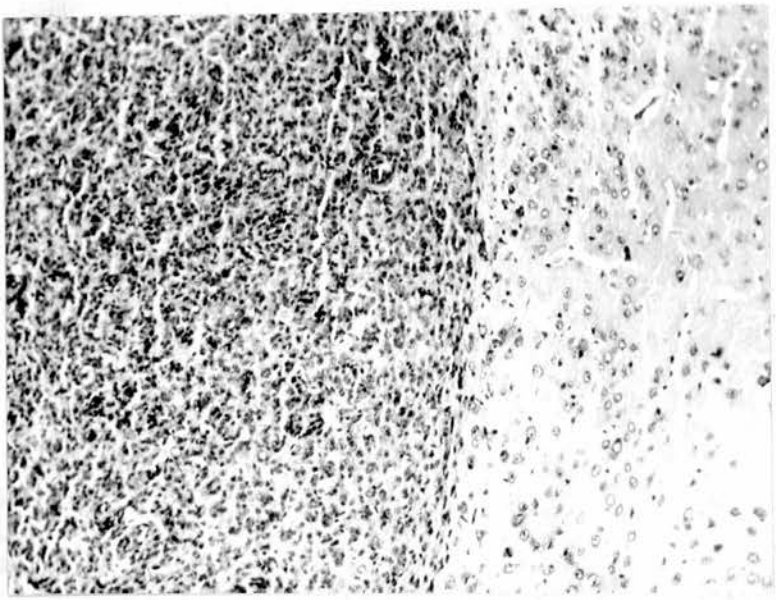

Figure 66 Astrocytoma confined to cerebral medulla. H & E X140.

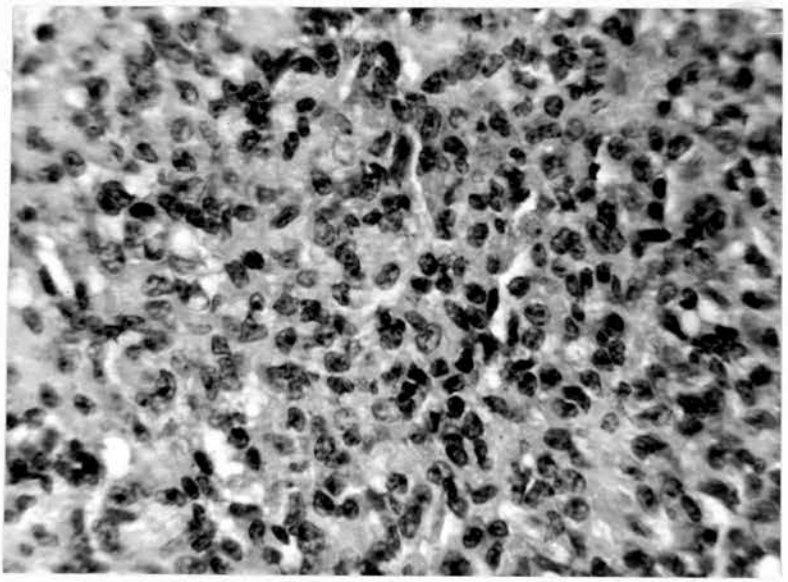

Figure 67 Small hyperchromic nuclei of cells in astrocytoma. H & E X580.

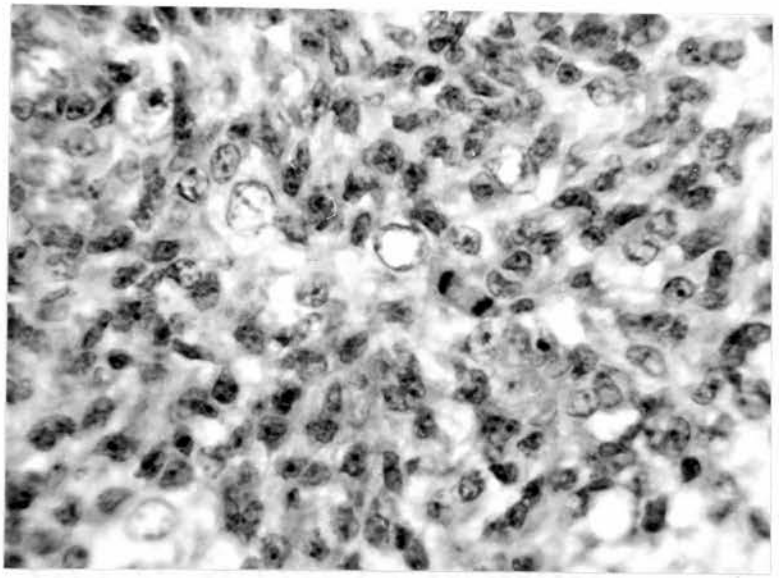

Figure 68 Cells in astrocytoma with larger nuclei and a single mitosis. H & E X580.

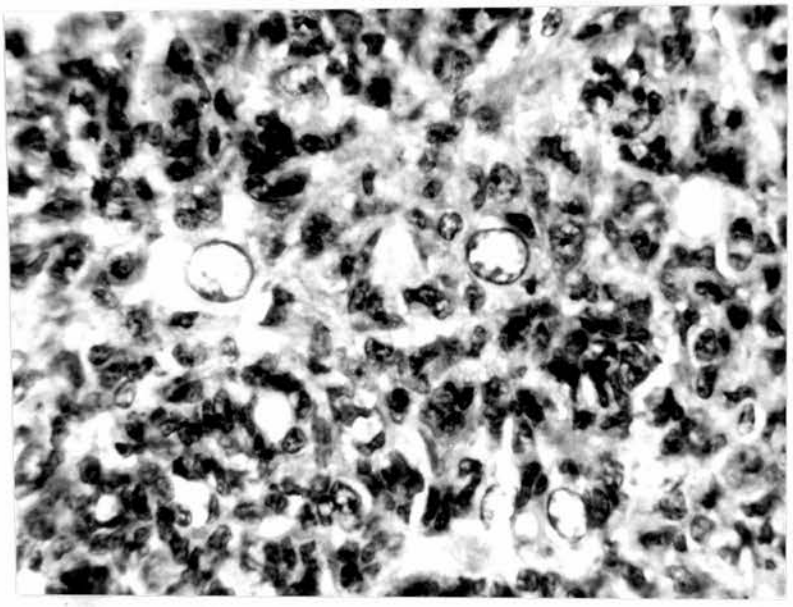

Figure 69 Large watery nuclei in astrocytoma. H & E X580.

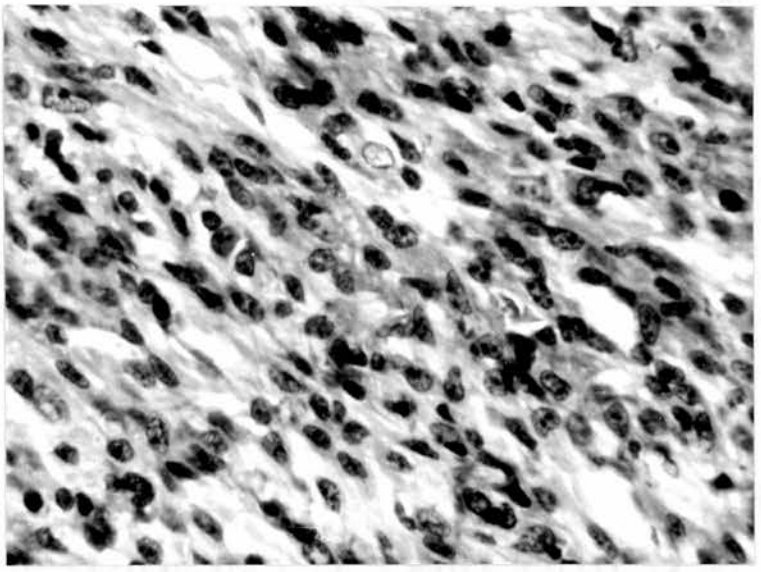

Figure 70 Pilocytic metaplasia of astrocytoma. H & E X580.

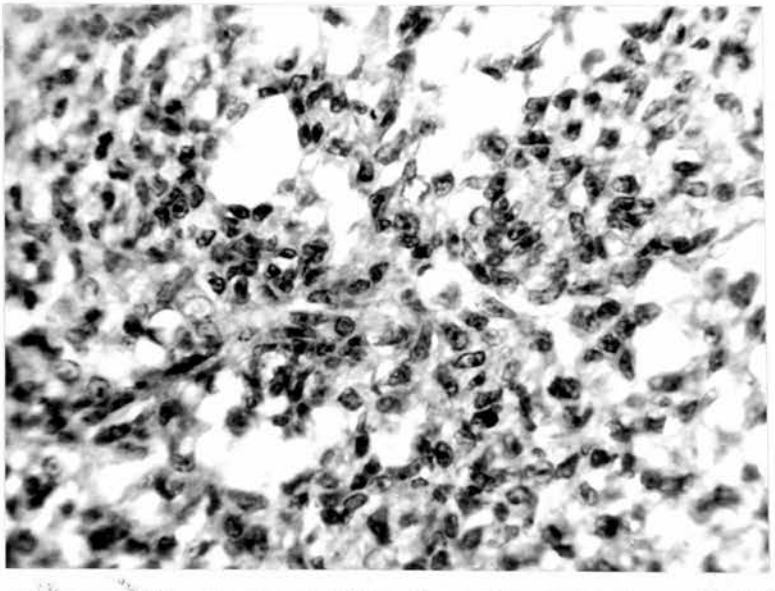

Figure 71 Microcystic degeneration in astrocytoma. H & E X580.

strains in the same colony, other types of intracranial neoplasma do occasionally occur in them. Lipomata and pituitary adenomata are most frequent, and have been seen in C57, BALB, BRVR, SM and VM, while single cases of fibroplastic meningioma and meningeal myxosarcoma have been identified in a C57 and a VM respectively. Two neurofibromata have occurred: one was in a C3H and the other in an outbred mouse homozygous for the gene, reeler.

SECTION 5

THE ROLE OF THE SPLEEN IN THE PATHOGENESIS OF SCRAPIE

This aspect was investigated by splenectomy and the measured parameter was incubation period. In mice inoculated with scrapie the titre in the spleen and other lymphoreticular tissues increases earlier than in other organs (Eklund, Kennedy and Hadlow, 1966, 1967; Dickinson and Fraser, 1969a). This increase in the spleen is unaccompanied by any observed tissue damage or physiological alteration, and indicates either active synthesis within the spleen itself or accumulation of agent being synthesised elsewhere. The time when the agent can first be demonstrated to have increased in amount in the spleen is dependent upon host genotype (Dickinson and Fraser, 1969a) and probably also upon other factors such as dose and strain of agent, which are known to influence the overall incubation period (Dickinson, Meikle and Fraser, 1969; Dickinson and Meikle, 1969). The incubation period following intracerebral inoculation of ME7 scrapie agent is approximately two thirds of that for an equivalent dose by the intraperitoneal route (Dickinson and Fraser, 1969b). Also, following intracerebral inoculation, levels in spleen rise marginally earlier than they do after intraperitoneal inoculation.

If the spleen is an important site for early agent synthesis its removal prior to inoculation might, by reducing the number of sites for synthesis, delay the later lethal events in the nervous tissue. Alternatively, splenectomy after inoculation would deplete the host both of

synthesis sites as well as some of the inoculum.

BALB mice were inoculated intraperitoneally or intracerebrally at 28 days of age in the main series of experiments, and splenectomised at various times before of after inoculation. In addition some BALB mice were splenectomised at 7 to 9 days of age and inoculated intraperitoneally 7 days later. In the main experiments C57 mice were inoculated from 7 to 58 days after splenectomy at 35 days of age. In addition some C57 mice were splenectomised at 5 to 7 days of age and inoculated 28 to 56 days later.

Splenectomised and control mice were mixed within cages for assessment of incubation period. All scrapie inoculated mice developed scrapie which was confirmed histologically.

Results

Splenectomy both before (7 to 58 days) and after (4 to 36 days) intraperitoneal inoculation prolonged the incubation period by an average of 12 per cent ($P < 0.001$), in both BALB mice inoculated at 28 days of age and in C57 mice splenectomised at 35 days of age (Table 23). In contrast, when intracerebral inoculation was used splenectomy had no effect on incubation period in BALB mice in any of the combinations tested (splenectomy 7 days before; 7 to 28 days after inoculation) (Table 24). The average marginal shortening of 1.36 per cent was not significant ($P > 0.1$). In other experiments in which age of splenectomy and intraperitoneal inoculation differed from those in the main experiments, increases in incubation period over shams were also demonstrated (8.5 per cent longer in BALB mice splenectomised at 7 to 9 days of age and inoculated 7 days later, $P < 0.01$;

Table 23 Incubation periods in BALB and C57BL mice splenectomised before or after intraperitoneal inoculation with scrapie (mean ± S.E.).

Number of mice		Age of inoculation (days)	Splenectomy or sham before (-) or after(+) inoculation (days)	Age of splenectomy or sham (days)	Incubation period (days)			Increase in mean incubation period by splenectomy (%)+	Probability of difference between controls and splenectomised	
Splenectomised	Controls				Splenectomised	Controls				
		BALB								
-	19	28	-	unoperated -	-	264 ± 1		-		
7	9	28	-20	8	277 ± 12	271 ± 3		4.13	<0.001	
8	1	28	-7	21	311 ± 3	263		16.91	<0.001	
3	1	28	+4	32	294 ± 2	263		10.53	<0.001	
3	-	28	+7	35	301 ± 6	-	266 ± 1*	13.16	<0.001	<0.001
3	-	28	+14	42	292 ± 16	-		9.77	>0.1	
3	1	28	+20	48	280 ± 15	270		5.26	>0.1	
3	1	28	+28	56	299 ± 11	264		12.41	<0.01	
2	1	28	+36	64	281 ± 17	257		5.64	>0.1	
		C57BL								
-	16	54-150	-	unoperated -	-	261 ± 4		-		
3	1	42	-7	35	305 ± 4	260		17.3	>0.1	<0.001
6	2	56	-21	35	293 ±11	243 ± 13	260 ± 1.5*	12.8	<0.01	
7	3	93	-58	35	307 ± 16	257 ± 12		18	<0.01	

+ For calculation of increase in incubation period and probabilities of difference 266 ± 1 days used in the case of the BALB mice and 260 ± 1.5 in the case of C57BL mice.

* No significant difference between shams and unoperated controls (p>0.1).

Table 24 Incubation period in BALB mice splenectomised before or after intracerebral inoculation with scrapie (mean ± S.E.).

Number of mice		Age of inoculation (days)	Splenectomy or Sham before (-) or after (+) inoculation (days)	Age of splenectomy or sham (days)	Incubation period (days)		Increase in mean incubation period by splenectomy (%)+	Probability of difference between controls and splenectomised
Splenectomised	Controls				Splenectomised	Controls		
-	18	28	- unoperated	-	-	183 ± 3)	-	-
8	1	28	-7	21	179 ± 1	176)	1.6	>0.1
4	-	28	+4	32	176 ± 2	-)	-3.3	>0.1
2	1	28	+7	35	186 ± 6	181) 182 ± 2*	2.2	>0.1
3	1	28	+14	42	178 ± 4	185)	-2.2	>0.1
3	-	28	+20	48	175 ± 1	)	-3.8	<0.02
3	1	28	+28	56	177 ± 2	180)	-2.7	<0.1
3	-	28	+36	64	174 ± 4	-)	-4.4	<0.1

+ 182 ± 2 days used for calculation of increase in incubation period and probabilities of differences.

* No significant difference between shams and unoperated controls (p>0.1).

10 per cent longer in C57 mice splenectomised at 5 to 7 days of age and inoculated 28 or 56 days later, P >0.1). There was no significant correlation of the interval between splenectomy and prior or subsequent inoculation with the degree of prolongation of incubation period in BALB mice (P >0.1), although there was a decrease in the degree of prolongation when the interval between splenectomy at less than 10 days of age and subsequent intraperitoneal inoculation was extended from 7 to 20 days in BALB mice. This decrease was not observed in C57 mice or mice splenectomised at over 10 days of age when this interval was extended in this way. Although this decrease in prolongation was not significant, it may indicate that, in terms of scrapie agent synthesis, some recovery of splenic function occurs in young mice.

These results support the view that scrapie is synthesised within the spleen. However, the similar prolongation of incubation period with splenectomy before or after inoculation suggests that other tissues are present which have marginally slower overall rates of synthesis. Following intracerebral inoculation it would seem that intraneural events are initiated immediately at the time of inoculation leading to agent synthesis and nervous tissue degeneration, irrespective of extraneural synthesis.

DISCUSSION

It is now possible to study scrapie quantitatively with parameters based on histopathology, incubation period and titre of the infectious agent. The histopathological parameter is independent of incubation period, and on the basis of histopathology alone different strains of scrapie agent can be distinguished from one another.

These criteria are needed particularly because other quantitative laboratory methods are unavailable, for scrapie or related diseases. It has not been possible to induce or demonstrate specific immunological responses to scrapie agent or infection. Because of the preliminary nature of the findings, in vitro cultivation (Clarke and Haig, 1970) is not yet suitable for analysing or detecting variations in scrapie, and electron microscopic studies have failed to identify a causal virus in any of the slow virus encephalopathies. It is in view of these deficiencies that the system of study developed in this thesis, based on a quantitative pathological criterion, becomes tactically important, quite apart from the intrinsic interest of the questions raised by the findings reported here.

The points for discussion concern: the identification of strains of scrapie agent and effects of other variables originating in the host or agent source, such as genotype and sex, organ source, route of inoculation and interactions between variables; some implications concerning pathogenesis arising from experiments involving the function of the spleen in scrapie; a comparison of some morphological features associated with scrapie and ageing mice; the circumstances of a glioma in one mouse strain with a deviant scrapie incubation period.

The broad objective of the thesis is to investigate the extent and

nature of variation in scrapie. Sources of variation are situated in the agent and its source, in the host and in the circumstances of the union between them. A diagram summarising the sources of potential variation in scrapie and methods for investigation is shown in figure 72.

Discussion of the causes of variation in the manifestations of an infectious disease can logically commence with that proceeding from the infectious agent itself. This can result from stable or genetic differences between variants or from acquired variation which an agent incorporates into its phenotype from the host environment in which it has been synthesised. When, as is at present the case with scrapie, the agent is inseparable from the products of tissue destruction or other host components, these by-products may themselves influence the expression of the agent. The complete failure to isolate a scrapie agent in a purified form means that the properties of any source reflect, to an unknown extent, both heritable and acquired properties of the infectivity as well as effects of other replicating or non-replicating components in the source.

In scrapie the probability of agent strain variation reflecting stable variants arose from differences in the transmissibility of the disease from different breeds of sheep (Dickinson and Stamp, 1969), and from the emergence in scrapie affected goats of two distinct clinical syndromes. The latter were stable on subpassage between goats, but were virtually indistinguishable when tested in other susceptible species, including mice, except for marginal differences in incubation period and pathology (Pattison and Millson, 1961; Pattison, 1966). However, the identification of agent variation of the extent and nature suggested and undertaken in this

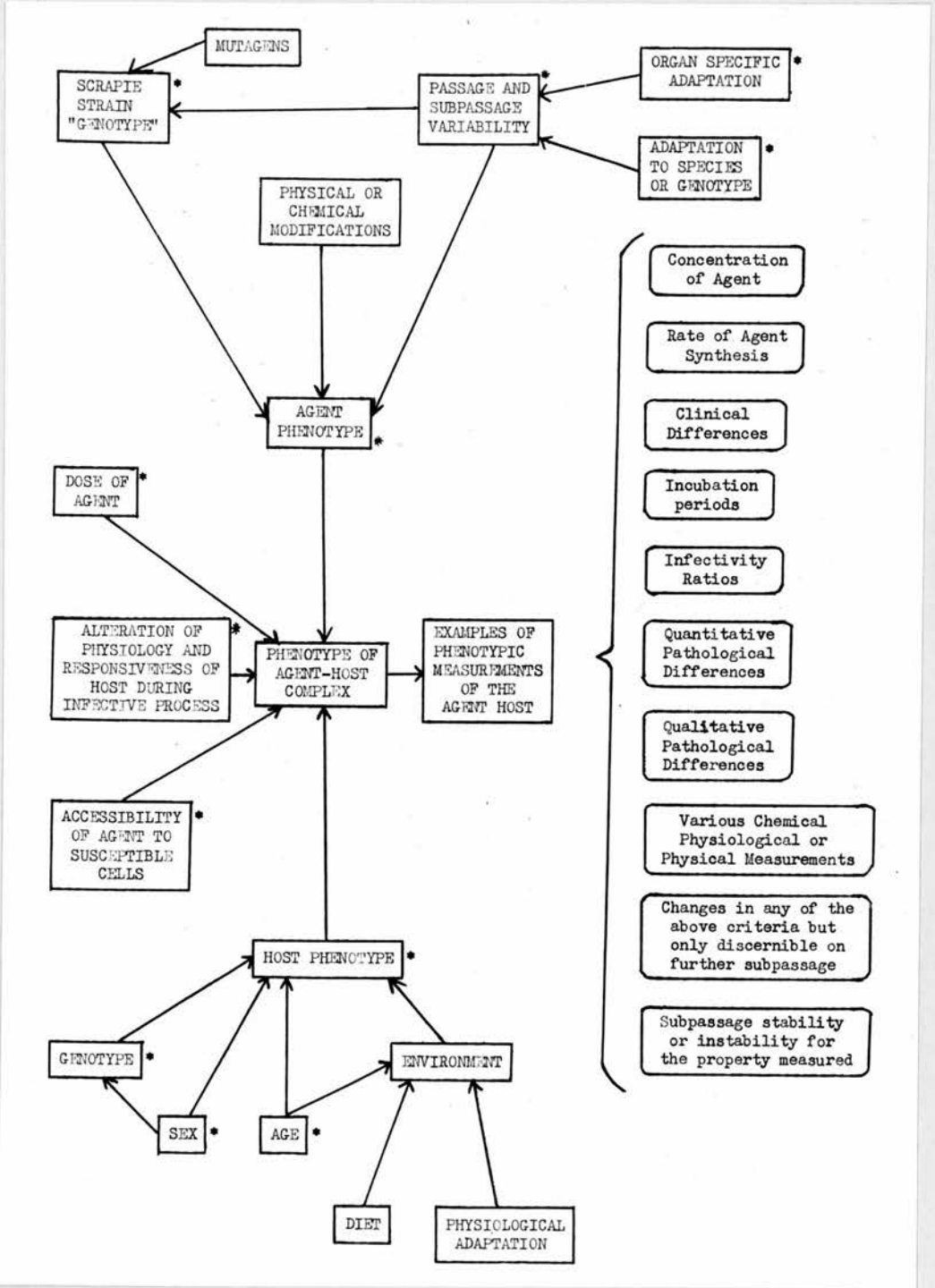

Figure 72 Summary of variables in scrapie in inbred mice. (* - factors investigated in thesis)

thesis would not be either possible or practicable on the basis of clinical syndromes in goats.

In this thesis several scrapie sources have been compared on the basis of their pathology in mice. In order to distinguish heritable from acquired differences the studies have been confined to agents passaged through standard inbred mouse strains, using standardised techniques. The completely objective or 'blind' examination and scoring of brain sections has so far revealed four distinct pathology profiles in each of two inbred assay mouse strains. Thus ME7, 22A and 79A are identifiable as separate agents, distinct from 22C and 80A which cannot be distinguished from one another. It has been shown that these agents preserve their characteristics even when boiled or subjected to long-term formaldehyde treatment, thus discounting the possibility that the differences derive from an accompanying unidentified conventional virus (Dickinson, personal communication). The agent variation is interpreted as having an heritable basis. The identity of agents on the histological criterion accords with a classification based on the ratios of incubation periods in mice with different alleles of the gene sinc (Dickinson and Meikle, 1969; Dickinson, 1970).

On the basis of a small number of published reports, when scrapie was introduced into mice from sheep or goats it was found that the lesions were confined to subcortical centres, particularly along the neuraxis, whereas in subsequent mouse passages the lesions became widely distributed in the brain, including the cerebral cortex (Zlotnik, 1965, 1966; Pattison and Smith, 1963). However, one report shows that, on two occasions when

scrapie from goats, which have themselves received 'mouse passaged scrapie' from two quite different origins, is reintroduced into mice by the intraperitoneal route, the lesions resemble closely those which are seen in mouse to mouse passage (Zlotnik, 1965), but whether this will prove to be a general phenomenon is uncertain. With the agent designated 58A, however, an exceptional situation appears in which a 'mouse adapted' type of lesion pattern occurs in mice inoculated by intracerebral injection with sheep brain source material. Both at this first mouse passage and at the subsequent mouse to mouse passage the lesions are almost the same as those of ME7. As exemplified by agent ME7, the lesion distribution of a 'mouse adapted' agent is a stable property through serial passage in a single mouse genotype. 58A and ME7 are agents which have arisen from the Suffolk breed of sheep, and are quite unlike the three agents, 79A, 22A and 22C (indistinguishable from 80A), which are individually distinct even though they have arisen either directly, or indirectly via goats, from the Cheviot breed of sheep.

Previous claims of stable agent differences must be treated with caution because of the failure to control several of the more important variables shown in this thesis to affect the incubation period and distribution of lesions. Thus Zlotnik (1965) failed to control the host genotype, or the route of inoculation. Even if, as is likely, the differences which Zlotnik described were partly the result of heritable properties of the agents, it is not possible to distinguish the extent to which other variables influence these observations. As these observations are based on phenotypic expressions, unless contributions of the environment are

controlled, differences in phenotype cannot be interpreted as being due to variation in agent genotype. In this discussion the "environment" is defined as the circumstances which interact with and influence the expression of the agent genotype, and includes all the variables on the left hand side of the diagram on Figure 72 except "agent genotype".

The modification of an agent or the acquisition of properties from the environment in which it is synthesised or recovered has been investigated by introducing variables singly or in combination. Scrapie from different organ sources and donor genotypes and at different doses were compared. A multi-factorial experiment (Subsection 8) was designed to measure the mutual influences of donor and organ source simultaneously with effects of host sex and genotype and route of inoculation.

An indication that the consequences of scrapie infection differ according to the environment from which the agent is isolated came from comparing in C57 mice, the lesion profiles of ME7 scrapie from brain or spleen sources. It was found that significant differences occurred in the cerebral and cerebellar cortices, the hippocampus and the paraterminal body. If an immunological process was involved it could only have arisen from antigenic differences between brain (or damaged brain) and spleen. This possibility indicates the need for experiments in which preimmunisation with non-scrapie donor tissue is used: this is currently being attempted. Alternatively, the scrapie agent may have been modified by a process analagous to host induced modification (Hoskins, 1959; Fenner, 1968) or the tissue source might conceivably mediate some influence with specific enzymes or other substances, not necessarily through antigenic effects,

although no biochemical differences have been found between scrapie activity isolated from brain or spleen (Adams, Caspary and Field, 1969). There was no comparable effect which could be correlated with the presence or absence of isogeny between donor and host, although histocompatibility antigens have been identified in brain (Gervais, 1970), and the effect was also present when the titres of agent varied. Further possibilities should be considered, that the different organ passage environments were selective either for possible mutants or for different agents in a mixed starting material, though deliberate attempts to check this possibility have not yielded any positive results (Dickinson, personal communication).

It is important to point out that this effect of organ source followed intracerebral inoculation. There is good evidence from the splenectomy experiments that some intracerebrally inoculated agent remains in the brain and initiates agent synthesis and cerebral damage regardless of a far greater proportion which leaves the brain. In the experiment in which the mutual influences of organ source, donor, recipient, recipient sex and route were treated in a single multi-factorial design, (the multifactorial experiment), a marginal organ source effect (brain and spleen) occurred following intracerebral but not intraperitoneal inoculation. This was to a large extent dependant upon the particular combinations used, being significant in this case when there was isogeny between donor and recipient, and the combination of BALB donor and recipient revealing the difference more significantly. However, in the experiment in which C57 recipients were used, the presence or absence of isogeny between donor and recipients had no bearing on the effect due to organ source difference, and the extent to which

organ-induced modifications can be revealed may be genotype specific.

The splenectomy experiments suggest that agent which initiates cerebral synthesis and damage following intraperitoneal inoculation is a product of extraneural tissues, and the complete identity of the brain and spleen lesion profiles for this route, both within individual combinations and on an overall basis, indicates that the organ source differences following intracerebral inoculation reflect unstable properties of the agent or are due to organ specific components in the source.

ME7 scrapie passed in distinct donor genotypes (BALB and BRVR) underwent either selection or host-induced modification which was revealed in assay mice inoculated intracerebrally or intraperitoneally. This donor effect exceeded the effect due to difference in organ source, and this, together with its occurrence in recipients inoculated by both routes, indicates either stable variants or the stimulation of mechanisms of host reactivity by donor constituents which differ qualitatively from the organ components. With the present data it is not possible to exclude the first possibility but it can be shown that reactivity to a donor constituent is involved (Outram, personal communciation).

The distribution and intensity of lesions of ME7 scrapie are also under the influence of the genetic machinery of the host. In the serial killing experiment, with C57, BALB and VM mice, in which host genotype was the imposed variable, and in the multi-factorial experiment, significant differences of a high order were found in the intensity of lesions in the brain, accompanied by differences in the shapes of the profile. The profile shapes were also analysed by a multi-variate analysis for the multi-factorial

experiment and a highly significant difference associated with host genotype difference was revealed. These findings emphasise the usefulness of conducting experiments in a number of host genotypes and underlines the weakness, as far as drawing generalised conclusions is concerned, of work restricted to one arbitrarily chosen genotype.

The earliest lesions develop in those regions in which severe damage occurs in the fully developed disease, and the rate of development of lesions is more gradual in the VM strain which has the long incubation period with ME7 scrapie agent. VM also has the most distinctive profile shape. The rate of increase in the titre of ME7 infectivity in the brain following intracerebral inoculation (Dickinson, Meikle and Fraser, 1969) is directly correlated with the development of brain lesions and as there is an approximately tenfold increase in vacuole number for each unit increase in score it is suggested that there is a direct numerical relationship between the number of infective units in the brain and the number of vacuoles. Such a relationship contradicts the hypothesis that the vacuolar lesion is a non-specific alteration, secondary to the suggested primary disorder of experimental and natural scrapie as a specific system degeneration (Beck, Daniel and Parry, 1964; Beck and Daniel, 1965 a and b). Titrations of infectivity in defined brain locations are required to establish a more precise relationship between vacuole number, or any other lesion, and titre.

The direct relationships between the development of the vacuolar lesion and, the stage of the disease, or brain titre of infectivity, also apply to another lesion which occurs in the brains of mice with scrapie: a degeneration of nerve fibres. In scrapie in the mouse the demonstration

of this degeneration requires methods which stain degenerating nerve fibres selectively or preferentially. With the standard methods for staining nerve fibres, such as Holmes, or Glees and Marsland, it is not possible to distinguish between mice which are or are not affected or infected with scrapie, although it is possible to identify structures which may be interpreted as axonal or nerve fibre degeneration in any of these four situations. Such changes have been described in the cerebellar cortex (Beck and Daniel, 1965b). The use of the selective silver impregnation method of Nauta reveals an extensive and widespread nerve fibre change in scrapie affected mice, which does not occur in controls. It was the occurrence of specific nerve fibre degeneration together with Holzer-positive gliosis and neuronal loss in natural sheep scrapie which led to the suggestion that the natural disease is a system degeneration (Beck, Daniel and Parry, 1964; Beck and Daniel, 1965a), a conclusion which was later extended to include the experimental disease in mice (Beck and Daniel, 1965b), although the authors later admit that this was not based on an extensive experience of the experimental disease. (Beck, Daniel, Gajdusek and Gibbs, 1969). A system degeneration implies a concomitant dying-back process (Greenfield, 1954; Cavanagh, 1964), which is not demonstrable in the Nauta preparations of murine scrapie. If a system degenerative is partially responsible for the nerve fibre lesion as well as the vacuolation, then the sites for dying back in the early stages would have a different distribution to the vacuolar degeneration and the former would precede the latter by an interval of time. As the two lesions develop in parallel both temporarily (in both s7s7 and p7p7 genotype mice, compare Figures 7 and 36a) and spatially, the concept of

a system degeneration cannot be substantiated. This, for instance, contrasts with hereditary ataxia in the Ax rabbit: this is caused by an autosomal recessive gene and bears morphological similarities to scrapie, particularly in the brain stem lesions of neuronal vacuolation, and the initial neuropathological lesion is a Nauta type degeneration in certain preterminal endings (O'Leary, Smith, Harris and Fox, 1968). If such an observation had been made in scrapie the suggestion of a system degeneration might be justified, although such a conclusion has not, in fact, been reached for the Ax rabbit disease.

However, in scrapie two processes might co-exist, each with a different pathogenesis, one a dying back phenomenon and the other a nerve fibre lesion associated spatially with the vacuolar degeneration. If these processes occurred simultaneously the systemic pattern would be obscured. With this reservation, it is not possible to identify a dying back component within the haphazard fibre degeneration revealed by the Nauta method.

In view of the profound differences in the patterns of lesion distribution in grey matter which occur both with the various strains of scrapie agent and with variation in the environment of the host/agent interaction, it would not be justified to extrapolate these findings with ME7 agent in the mouse to include the lesions of scrapie generally. A dying back process has been suggested in natural sheep scrapie, although not demonstrated (Palmer, 1968), and it is likely that the agent inoculated by Drs. Gibbs and Gajdusek into the mice examined by Mrs. Beck and Professor Daniel (Beck and Daniel, 1965b) originated from the Chandler agent which arose from the same source as the agent designated 79A which is very different in pathological

terms from ME7. In 79A induced murine scrapie oedematous alterations in various white matter areas are typical (see below). In view of the pathological variation in scrapie, it would clearly be important to use a Nauta technique in other scrapie situations such as natural sheep scrapie, first passage in mice, or with other agents such as 79A and 22A.

In kuru, a human neurological analogue of scrapie, experimentally transmitted to the chimpanzee (Gajdusek, Gibbs and Alpers, 1966, 1967) nerve fibre degeneration with Nauta staining occurs which closely resembles the changes seen in mouse scrapie (Klatzo, personal communication).

The distinctive lesion patterns which characterise certain strains of mice, even though they share the s7 allele, indicate that there are other genes, in addition to sinc, controlling the expression of the disease. Any conclusion on whether sinc exerts control over the expression of lesions must be speculative, although the characteristic lesion pattern of VM suggest this as a possibility. Interpretation of any property linked to the VM genotype, if it is involved in the particular properties of VM in the pathogenesis of scrapie, must be understood in terms of the incubation period reversal phenomenon of sinc with the different groups of scrapie agents (Dickinson and Meikle, 1969). It must be anticipated that genetic variability of structure or chemical specificity relating to brain metabolism or function will be present between mice of different genotypes, quite unrelated to sinc, as indeed such variability has been demonstrated in particular instances (Miller, Cox and Maickel, 1968; Wimer, Wimer and Roderick, 1969). The spontaneous astrocytoma in VM and its crosses is of interest, occurring as it does in a strain which is genetically deviant with

regard to scrapie. A uniform incidence of any single type of spontaneous glioma through many generations of one inbred mouse strain is an unrecorded occurrence. The fact that this strain is VM justifies its fullest investigation. If the manifestation of the tumour is in any way gene dependant, the single case of the same tumour in a first cross with VM in the parental generation, suggests that this gene cannot be a recessive. The failure to demonstrate any significant difference in the incubation periods of scrapie between mice with or without the tumour is noteworthy in view of the assertion that scrapie may represent a benign neoplastic transformation of astrocytes (Field, 1967, 1969; Field and Peat, 1969) analagous to the transformation of cells in tissue culture infected the papova viruses (Webb, 1967). The identity of this glioma as originating from the astrocytic cell series has been confirmed independently (Zimmerman, personal communication).

In addition to the grey matter lesion upon which much of this work is based, an oedematous degeneration is frequently seen in white matter. An analysis of the incidence of this change reveals that it occurs predominantly under two sets of conditions, in scrapie in mice affected with agent 79A and in non-scrapie aged mice. In contrast it is less frequent with ME7 infection, and is completely absent with 80A. Its incidence with 22A infection is similar to the incidence in non-scrapie mice of the same age.

The 79A and Chandler strains of scrapie have a common origin from the drowsy goat agent. The Chandler agent is the most widely studied, being used for most scrapie work in Britain and the United States. In a recent isolation of the Chandler agent into mice at the Animal Breeding

Research Organisation an oedematous white matter degeneration, indistinguishable from that associated with 79A is a consistent feature (Fraser, unpublished). This atypical lesion of scrapie, associated with the Chandler and 79A strains emphasises the variability in scrapie and underlines a need for caution in generalising assertions about 'scrapie' by those who only have experience of one agent. This white matter lesion is present in 79A induced scrapie heated to over 100°C and in scrapie induced by limiting dilutions of 79A (Fraser, unpublished). However, in another agent, 79V, derived from the same "drowsy" goat source as "Chandler agent" and 79A, but by passage in p7p7 genotype hosts, (Dickinson, personal communication) a white matter lesion does not occur.

A resemblance between ageing and the slow virus encephalopathies has given rise to a so-called unitary hypothesis in which scrapie, kuru, multiple sclerosis and ageing all constitute the same basic process: a primary astrocytic disease (Field, 1967, 1970). This hypothesis is based on similarities between these conditions at the ultrastructural level, on the astrocytic hypertrophy which occurs in scrapie and to a lesser extent in old animals, and on the occurrence in young adult scrapie affected rats of tubular arrays in cerebellar axons that were also seen, to a lesser extent, in normal old rats (Field, Raine and Joyce, 1967). These structures have not been seen in mice. On a more general level it has been conceived that other viral agents may produce biochemical or molecular lesions which might result in changes akin to premature ageing, and that ageing itself might be regarded as a 'slow virus disease' (Hotchin and Sikora, 1970; Gajdusek, 1970).

An analysis of two other alterations which develop with age in mice, the calcified vascular lesions in the thalamus, and the intracytoplasmic inclusions within thalamic nerve cells, does not support the hypothesis that scrapie is analagous to ageing processes because their incidence is not increased in scrapie affected mice. Although it may be possible to conceive of degenerative neurological diseases as analoges of some components of the tissue alterations which accompany ageing, the suggestion that the pathogenesis of the cellular changes of ageing, multiple sclerosis and scrapie are essentially the same is unjustified because it depends upon unrepresentative data. As this is based largely upon astrocytic hypertrophy and possible proliferation, in view of the non-specific nature of astrocytic change as a reaction to brain injury and oedema, and to alterations in the osmolarity of the blood, and in view of the suggested function of the astrocyte as a monitor of the extracellular space (Davson, 1967; Klatzo, 1967; Rosenbluth, 1968; De Robertis, Alberici and Arnaiz, 1969; Zlotnik, 1968; Blakemore 1969; Kristensson and Sourander, 1969; Gonatas, 1970), any unitary hypothesis based on astrocytosis is extremely presumptive.

According to whether scrapie is administered by an intracerebral or extraneural route, differences in pathogenesis occur which are reflected in the rates of increase of infectivity in brain and spleen, in incubation periods (Dickinson, Meikle and Fraser, 1969; Dickinson and Fraser, 1969a and b), and in lesion distribution. Also, with a single exception (Gibbs, Gajdusek and Morris, 1965; Gibbs, 1967) assay mice inoculated by the intracerebral route are more sensitive indicators of infectivity than mice

inoculated intraperitoneally (Dickinson and Fraser, 1969b; Kimberlin, personal communication). Differences in ratios of titres obtained between intracerebral and peripheral inoculations determine the neuroinvasiveness of encephaloviruses (Albrecht, 1968), and it is conceivable that such differences may apply to different scrapie agents.

The splenectomy experiments reveal a further route difference in which mice inoculated by an intraperitoneal injection have a longer incubation period if their spleens are removed before or after inoculation, whereas in mice inoculated intracerebrally splenectomy is without this effect. From these findings it is suggested that, although only a small portion of an inoculum is retained in the brain parenchyma after intracerebral inoculation (Mims, 1960; Cairns, 1950), in the case of scrapie this initiates cerebral synthesis of agent and encephalopathy, which proceed independently of and more slowly than, synthesis of agent in extraneural tissues. The prolongation of incubation period which follows splenectomy with intraperitoneal inoculation indicates that splenic synthesis of agent is not independent of subsequent cerebral events, although synthesis is only marginally more active in the spleen than in other unidentified tissues, On the other hand it has been shown that following intravenous administration of tick-borne encephalitis (T.B.E.) virus to mice the primary site for virus synthesis is the spleen (Malkova and Smetana, 1966). As in the case of scrapie, needle puncture into the brain, during the viraemic phase with encephalitis viruses have much smaller effects than would be expected from the breaking down of an efficient barrier between blood-borne virus and susceptible brain tissue (King, 1940; Imam and Hammon, 1957; Berge, Gleiser,

Gochenour, Miesse and Tigertt, 1961). Estimates of the size of these encephalitis viruses (Andrews and Pereira, 1967; Filshie and Rehacek, 1968; Murphy, Harrison, Gary, Whitfield and Forrester, 1968; Oyanagi, Ikuta and Ross, 1969) exceed those for the lower limits of the scrapie agent (Gibbs, 1967; Alper, Haig and Clarke, 1966; Field, 1969) although the physical association of scrapie agent with tissue components in the inoculum will determine its penetration through cerebral capillaries following intravenous inoculation. In view of the enhanced passage of blood-borne virus to the brain parenchyma by CO_2 inhalation (Sellers, 1969), the effect of hypercapnia on scrapie following intravenous inoculation should be investigated. The higher blood levels of scrapie agent following intravenous and intracerebral inoculation conceivably makes more agent available to other tissues than following intraperitoneal injection, as in the case of the spleen (Dickinson and Fraser, 1969a). However, an alternative explanation to that given above, concerning the pathogenesis following intracerebral inoculation based on the involvement of some tissue other than brain, must account for the difference between the intravenous and intracerebral routes. It is conceivable that with intravenous inoculation, but not with intracerebral, the events in the brain are dependent on spleen, as in the case of T.B.E. virus cited above, or some other extraneural tissue, which obtain higher levels of scrapie earlier than following intraperitoneal injection. Splenectomy must be combined with intravenous and intracerebral inoculation of serial tenfold dilutions of agent to delineate the role of this organ in more detail, but the involvement of other lymphoreticular tissues also need investigations using techniques for reticulo-endothelial blockade and

lympholysis. With different peripheral inoculation routes the role of particular tissues as sources of scrapie for subsequent cerebral events may depend on levels of agent within their vicinity (e.g. in blood), on the effectiveness of tissue barriers (e.g. to blood compartment), as well as on the balance between agent destruction and synthesis in different cells and tissues. The last point would appear particularly relevant in view of the lower sensitivity of intraperitoneal compared with intracerebral inoculation to detect low levels of infectivity. If strains of scrapie agent possess different degrees of neuroinvasiveness, as in the case of encephaloviruses (Albrecht, 1968), the extent to which splenectomy influences scrapie following peripheral inoculation may differ from one agent to another.

Alternatively, or additionally, it might be considered that the spleen functions in the pathogenesis of scrapie following intraperitoneal inoculations in some way other than as a site for agent synthesis. Splenectomy for example, can alter tumour growth in mice by depriving the host of a source of enhancing antibodies (Ferrer, 1968a and b), but the apparent lack of an immune response in scrapie (Gardiner, 1966; Clarke and Haig, 1966; Katz and Koprowski, 1968) as well as the apparent normal immunological responsiveness of scrapie infected mice (Gardiner and Marucci, 1969) makes it unlikely that the results of splenectomy are explicable in immunological terms.

The studies contained in this thesis show that several factors can influence the distribution of lesions in scrapie, and the mechanism of these effects is the single biggest question posed in it. That both agent and

host genotype properties can exert profound effects suggests that there are intrinsic properties of susceptibility of various brain regions that differ according to precise host agent combinations, although the mechanisms of this regional susceptibility remains unknown. That both the donor genotype of the scrapie source and the genotype of the host effect the pathology is an indication that host components are intricately involved in the scrapie process, and similar mechanisms may also apply to effects of organ source. The examples of the regional differences in lesion intensity arising from different routes of inoculation suggest that factors other than intrinsic regional susceptibility, such as accessibility to regions, may apply in some instances. In the cases of donor genotype and organ source effects it is tempting to suggest some immunological basis, but effects due to agent difference and host genotype would appear to involve unknown chemical factors. Although this thesis leaves these many questions unanswered it makes important contributions by providing a workable system for distinguishing different scrapie agents and giving some insight into the pathogenesis and biology of the disease.

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PERSONAL COMMUNICATIONS

A.G. Dickinson	A.R.C. Animal Breeding Research Organisation, Edinburgh, U.K.
R.H. Kimberlin	A.R.C. Institute for Research in Animal Diseases, Compton, Berkshire, U.K.
I. Klatzo	National Institutes of Health, Bethesda, Maryland, U.S.A.
G. Outram	A.R.C. Animal Breeding Research Organisation, Edinburgh, U.K.
H.M. Zimmerman	Chief Laboratory Division, Montefiore Hospital and Medical Centre. New York 10467 U.S.A.

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APPENDIX I

A Modification of the Silver Impregnation Method of Nauta and Gygax

(Devised at the Rocky Mountain Laboratory, Hamilton, Montana, U.S.A., for Mouse Brain Material Fixed in Formol Ammonium Bromide).

By

H. Fraser

The selective methods of Nauta (1954) and Nauta and Gygax (1957) were modified in order to stain material fixed in FAB. To do this it was necessary to omit the oxidation stage in 0.05% potassium permanganate and the subsequent decolouration in the mixture of equal parts of 1% oxalic acid and 1% hydroquinone. Having made this modification, the resulting silver impregnation was less selective than these methods on formol fixed material.

The fixation time is critical. In no case in which fixation in FAB exceeded 2 weeks was impregnation in the least successful.

Finally, it appeared that either the original Nauta (1954) ammonical silver solution or the Laidlaws silver carbonate solution was successful. A critical comparison between these two was not carried out.

Method

Don't attempt to stain more than 4 or 5 specimens at one time.

1. Freshly cut 20 nm sections of FAB fixed material. Fixation time critical, and not more than about a week. Use about 4-5 sections for each specimen.
2. Wash sections in distilled water.
3. Soak sections in 0.5% phosphomoybdic acid for 30 minutes.

4. Wash two times in distilled water. It is useful to do one wash in a 500ml. beaker and then the second wash can be in the collecting petri dish.

5. Soak sections for 30-45 minutes in 1.5% silver nitrate solution.

6. Wash two times in distilled water as at 4.

Now stain sections one at a time. Have 4 petri dishes prepared.

a) ammoniacal silver solution

b) reducing fluid

c) distilled water wash

d) 1% sodium thiosulphate

7. Soak for 45-60 seconds in the ammoniacal silver.

8. Transfer immediately to the reducer and remove when darkened suitably. The result can be influenced by the time allowed in reducer and is usually about 30-45 seconds. The sections should darken appreciably. The impregnation can be modified by adding either strong ammonia or 2.5% sodium hydroxide solution to the ammoniacal silver. In general, addition of ammonia will lighten the sections, and may increase the specificity for staining of degenerating nerve fibres, whereas addition of 2.5% caustic soda solution will have the reverse effect and darken the section and may make interpretation easier or more difficult. It is recommended that, with the 4 or 5 sections from each specimen, that these variations are tried.

9. Wash in distilled water.

10. Fix in 1% sodium thiosulphate for 1 minute.

11. Collect in water and mount. Pass mounted sections through alcohols,

xylol and Canada balsam. The gelatin formaldehyde mounting technique may prove useful.

Solutions required

Use either ammoniacal silver solutions:

1. Laidlaw's Silver Carbonate

12gms. silver nitrate

20ml. distilled water

230ml. saturated lithium carbonate

Dissolve

Wash the silver carbonate (yellow/black precipitate) 3 times by allowing it to settle, and pouring off the supernatant fluid and adding distilled water to the 250ml. level and shaking vigorously. The precipitate should fall to around the 70ml. mark on the measuring cylinder after each washing. After the last washing add strong ammonia slowly, with constant shaking until a nearly clear solution is obtained (about 9.5ml.). This will have some black specks in it. These should be filtered out, and the resultant solution made up to 120ml. with distilled water. The solution should be sealed in a clear glass bottle and ripened in the sun for 2 weeks. Then store in a dark bottle in the cold. It is only moderately stable - it may deteriorate.

2. Ammoniacal Silver Nitrate Solution

This must be used freshly prepared.

1.8 grams silver nitrate

40ml. distilled water

Dissolve and add:

20ml. absolute ethanol

3.6ml. strong ammonia

3.0ml. 2.5% sodium hydroxide (stock solution).

3. Reducing fluid:

95% ethanol	45 vols.
1% citric acid	13.5 vols.
10% formalin	13.5 vols.

Add tap water to 400 vols.

Note:

For the original Nauta methods interpose a 5-12 minute oxidation in 0.05% potassium permanganate followed without washing with a one minute decolouration in an equal part mixture of 1% oxalic acid and 1% hydroquinone. The time allowed for this oxidation can be extremely critical.

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APPENDIX II

ABSTRACT OF THESIS

Name of Candidate Hugh Fraser

Address 7 Horsburgh Gardens, Balerno, Midlothian.

Degree Ph.D. *Date* 18th December, 1970

Title of Thesis Scrapie: The experimental disease in inbred strains of mice.

Factors affecting the distribution of lesions of scrapie in inbred mice have been investigated. For this purpose a quantitative system for the analysis of the intensity of vacuolation in the brain has been developed, based on the histological examination of nine defined areas of grey matter.

One of the most important conclusions of the thesis is that variants of scrapie agent can be distinguished on the basis of pathology.

However, it is shown in the thesis that other factors can influence the intensity and distribution of lesions, and therefore the need to standardise the system of agent identification has to be emphasised. Other factors that affect the pathology are: route of inoculation, genotype and sex of host, genotype of donor and organ source of infective tissue. Important interactions between inoculation route and some other sources of variation are identified, in particular with genotype of donor, genotype and sex of host and organ source of infectivity. As there is no demonstrable alteration in the pathology with passage of a particular mouse adapted agent in a homologous host strain system, the properties of an agent upon which it is identified can be regarded as stable. On this basis, by examining several sources of scrapie, four agents have so far been distinguished, one from a Suffolk sheep source, two from a Cheviot source and one from an experimental goat source.

In view of suggestions of similarities between scrapie and ageing, morphological concomitants of advancing age are identified in the brains of mice, and a comparison of their incidence in scrapie and ageing is undertaken. It is concluded that in terms of the defined parameters, there is no justification for regarding scrapie as an acceleration of any specific manifestation of the ageing process. One feature occurring in the brains of old mice, an oedema or vacuolation of white matter, does partly resemble a lesion associated with scrapie,

Use other side if necessary.

at least under some circumstances. Morphologically, this feature in scrapie is frequently, but not invariably, distinguishable from the condition associated with ageing. The incidence of oedema in white matter both in ageing mice and in mice infected with scrapie of various strains is examined, and it is shown that one scrapie agent exists in which white matter changes are a very prominant, but not necessarily a specific, feature.

The circumstances of a series of astrocytic gliomas in one inbred mouse strain and its crosses is studied. These neoplasms occur in a strain of mice with genetically deviant properties with regard to its response to most strains of scrapie. Based on incubation period measurements, no effect on the progress of scrapie can be associated with the presence of the glioma.

The role of the spleen in the pathogenesis of scrapie is investigated in experiments involving splenectomy at various times before or after inoculation of the agent. This results in a highly significant prolongation of incubation period in mice splenectomised at various intervals before or after intraperitoneal inoculation. However, splenectomy before or after intracerebral inoculation produces no alteration in incubation. These results suggest that replication occurs in the spleen either at a greater rate or with an overall greater yield, when compared with other peripheral tissues taken together, as far as available agent reaching the brain is concerned. They also suggest that with intracerebral inoculation, intraneural events are independant of the spleen in its role either as a possible replication site or in terms of other roles it may play in pathogenesis.

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Distribution of Experimentally Induced Scrapie Lesions in the Brain

LESIONS of scrapie are confined to the central nervous system. Differences in the distribution of these lesions have been described for the two strains of scrapie agent used in goats[1] and for the lesions which arise as a result of injecting mice with boiled and unboiled material[2]. We have also collected unpublished data showing wide variation in the distribution of lesions which result from using different strains of the agent in mice or by inoculating different genotypes of mice with the same agent.

In order to estimate the concentration of scrapie agent in various tissues of four strains of mice, the tissues were removed from animals which had been killed at intervals between the time of challenge with the *ME*7 strain of agent and development of the clinical disease. One of the four mouse strains, *C57BL*, also served as the test strain in bioassay of the scrapie titre. As a result of the routine use of a system which we have developed[3] for measuring the degree and spatial distribution of scrapie lesions in the brain, it has been possible to examine a number of potential sources of variation in these lesions, such as sex of the test animal, titre of scrapie in the inoculum, strain differences between donor and host, and the finer specificities associated with the particular donor tissue which forms an integral part of the inoculum. So far we have data for scrapie agent in the brain and in the spleen.

All the donors which we used were females of the strains *C57BL*, *SM*, *LM* and *VM*; the latter differed notably from the others in the alleles of the gene *sinc*, which controls *ME*7-scrapie incubation period[4]. Rigorous aseptic precautions were used when tissues were removed, to prevent cross contamination between different organs. For injection, the tissues were ground and made up in a saline suspension to give an overall concentration of 10^{-2}. This was then spun at 300*g* for 10 min and 0·02 ml. of the supernatant was administered intracerebrally into mice. An elaborate bioassay design involving 273 *C57BL* mice was used to minimize random or fluctuating sources of error, both at the time of preparation of the inoculum and during measurement of the incubation period. The brains were removed for histological examination at the same stage in the progression of the clinical disease. The degree of vacuolation in the various regions of the brain was scored on a scale ranging from 0 (no vacuolation) to 5 (vacuoles confluent throughout the defined brain location)[3].

As seen in Table 1, the degree and distribution of lesions are unaffected by sex. When, however, the degree and distribution of lesions from each tissue source are compared, either within the same sex or on an overall basis as in Fig. 1 and Table 1 (col. 3), differences can be seen. Thus the lesions are more advanced in the anterior midline cerebral cortex, hippocampus and paraterminal body and less advanced in the cerebellum when scrapie brain is used as the inoculum than when scrapie spleen is used as the source. These differences are present in tissues taken from donors in all stages of the disease (Table 1, columns 4–9). It has been established that the scrapie titre in spleen is uniformly high in all strains at

Table 1. DEGREE OF VACUOLATION OF GROUND SUBSTANCE AND NEURONES IN NINE REGIONS OF THE BRAIN OF *C57* MICE WITH ADVANCED CLINICAL SCRAPIE (MEAN ± STANDARD ERROR)

Location	Sex		Tissue used for inoculum	Stage of disease				Donor strain—host strain difference			
	Female	Male	Prob. of difference between spleen and brain (see Fig. 1) (%)	≤40% of incubation period		55–75% of incubation period		Terminal scrapie phase		*C57* tissues into *C57* recipients		*SM*, *LM* and *VM* tissues into *C57* recipients	
				Spleen	Brain	Spleen	Brain	Spleen	Brain	Spleen	Brain	Spleen	Brain
Medulla	1·91 ± 0·05	1·78 ± 0·07	> 10	1·58 ± 0·11	2·04 ± 0·06	1·88 ± 0·12	1·74 ± 0·12	1·95 ± 0·22	2·09 ± 0·11	1·61 ± 0·10	1·97 ± 0·14	1·82 ± 0·07	1·95 ± 0·08
Cerebellum	1·55 ± 0·07	1·37 ± 0·08	< 0·1	1·64 ± 0·10	0·99 ± 0·11	1·72 ± 0·14	1·10 ± 0·15	1·81 ± 0·14	1·21 ± 0·12	1·92 ± 0·13	1·07 ± 0·12	1·68 ± 0·08	1·13 ± 0·08
Tectum of midbrain	2·28 ± 0·05	2·30 ± 0·06	2–5	2·12 ± 0·10	2·42 ± 0·10	2·26 ± 0·11	2·21 ± 0·11	2·22 ± 0·14	2·54 ± 0·09	2·14 ± 0·08	2·31 ± 0·13	2·22 ± 0·06	2·45 ± 0·07
Hypothalamus	1·55 ± 0·07	1·65 ± 0·07	5–10	1·41 ± 0·05	1·70 ± 0·11	1·58 ± 0·05	1·67 ± 0·14	1·64 ± 0·19	1·71 ± 0·07	1·48 ± 0·14	1·59 ± 0·16	1·53 ± 0·07	1·73 ± 0·08
Thalamus	2·55 ± 0·05	2·56 ± 0·06	> 20	2·41 ± 0·07	2·65 ± 0·13	2·46 ± 0·14	2·61 ± 0·08	2·69 ± 0·10	2·59 ± 0·20	2·44 ± 0·10	2·54 ± 0·16	2·53 ± 0·05	2·65 ± 0·08
Hippocampus	2·63 ± 0·04	2·69 ± 0·05	< 0·1	2·43 ± 0·08	2·89 ± 0·00	2·44 ± 0·08	2·83 ± 0·06	2·72 ± 0·14	3·00 ± 0·05	2·67 ± 0·09	2·93 ± 0·08	2·48 ± 0·05	2·89 ± 0·04
Paraterminal body	2·65 ± 0·04	2·69 ± 0·05	< 0·1	2·43 ± 0·00	2·83 ± 0·05	2·51 ± 0·04	2·87 ± 0·05	2·62 ± 0·15	2·91 ± 0·04	2·50 ± 0·10	2·83 ± 0·07	2·50 ± 0·05	2·92 ± 0·04
Cerebral cortex (posterior midline)	2·09 ± 0·04	2·10 ± 0·06	> 40	2·03 ± 0·08	2·11 ± 0·08	2·09 ± 0·09	2·11 ± 0·09	2·11 ± 0·10	2·14 ± 0·11	2·14 ± 0·10	2·10 ± 0·11	2·06 ± 0·06	2·12 ± 0·07
Cerebral cortex (anterior midline)	2·53 ± 0·05	2·64 ± 0·05	< 0·1	2·33 ± 0·07	2·78 ± 0·01	2·41 ± 0·03	2·72 ± 0·02	2·55 ± 0·12	2·88 ± 0·07	2·42 ± 0·09	2·77 ± 0·08	2·41 ± 0·05	2·80 ± 0·04

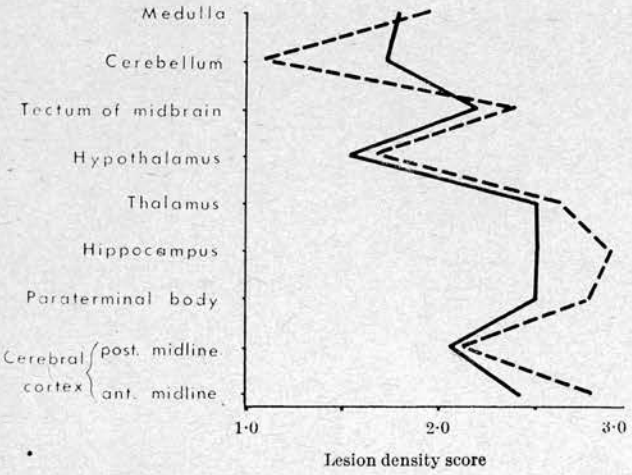

Fig. 1. Scrapie lesion density in nine regions of the brain resulting from intracerebral challenge with inoculum of scrapie spleen (solid line) or scrapie brain (broken line). The order of brain regions is arbitrary.

these stages of incubation; the stages do, however, represent an increasing titre in the brain, over a 10,000 to 100,000-fold range, as incubation advances. Final levels in the brain and spleen are about the same (ref. 5 and our unpublished results). The general pattern of differences shown in Fig. 1 is independent of the presence or absence of isogeny between donor and host (Table 1, cols. 10–13).

These findings are the unexpected results of an experiment which was designed with an entirely different aim in view and we had no initial intention of analysing the lesion distributions in the assay animals. The results seem to be entirely consistent throughout the data. Other experiments are now needed to discriminate between two chief possibilities, either that the scrapie agent may have been modified in the different donor tissues, or the described effect stems from accompanying organ-specific constituents which could involve some immunological process.

H. Fraser
A. G. Dickinson

ARC Animal Breeding Research Organization,
Edinburgh.

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THE SEQUENTIAL DEVELOPMENT OF THE BRAIN LESIONS OF SCRAPIE IN THREE STRAINS OF MICE

By

H. Fraser and A. G. Dickinson

A.R.C. Animal Breeding Research Organisation, Edinburgh

INTRODUCTION

Four types of criteria have been used to describe different aspects of the disease process in scrapie. Of these, two are applicable to the living host, namely the type of symptoms which appear in the clinical phase and the interval between challenge and death; the other two are estimation of the concentration of scrapie agent by some form of bio-assay and description of the distribution and intensity of central nervous system lesions. Studies of the neuropathology have included comparisons of the intensity of medullary lesions in natural cases of scrapie in different sheep breeds (Zlotnik and Rennie, 1958), of differences in distribution of lesions in goats inoculated by different routes (Zlotnik, 1962), of the experimental production of the disease using agents of different sources or passage histories (Zlotnik, 1965) and of differences following heat treatment of the agent (Zlotnik and Rennie, 1967).

Clinical syndrome, incubation period, agent concentration and neuropathology are obviously interrelated, but it is important to know the extent of their independence and the efficiency with which they can be measured. Refinement and application of the criteria should facilitate the identification of differences between scrapie agents from different sources and lead to a fuller understanding of the pathogenesis of the disease; in particular, the nature of the response of different host strains to different strains of scrapie agent.

The purpose of this paper is to describe and evaluate a system of quantitative, neuropathological analysis which has been applied to scrapie in mice. The system was used to investigate the development of the lesions in several specific regions of the brain. Three strains of mice were used to determine whether they showed differences in the distribution or rate of appearance of the lesions and one was selected for use because it differed markedly in incubation period from the other two. In a related paper (Dickinson, Meikle and Fraser 1968) it is concluded that differences in brain lesion distribution and incubation period are not necessarily determined by the same genes.

MATERIALS AND METHODS

Mice. The mice used were of three strains, C57BL and BALB/c, both fully inbred, and VM, referred to as 5M in some previous publications, which was at the 12th inbred generation at the time of the experiment. All three strains have been maintained conventionally in a single closed environment at the Animal Breeding Research Organisation mouse colony for at least two years and there has been no evidence of either latent or overt infection with standard mouse pathogens. The VM strain is known to carry the mouse mammary tumour agent, which is absent from the other

two strains. During the experiment there was no evidence of infectious disease, tumours or ectoparasites.

The experiment was carried out concurrently on the three strains, which were housed adjacently on the same rack under identical environmental conditions (21 to 22°C., 50 to 60 per cent. relative humidity). Strains and sexes were separated in groups of 6 in plastic mouse cages and the groups were fed identically on a standard pasteurised pelleted diet. Cage cleaning, feeding and water bottle changing were carried out at the same time each week, all animals in this experiment being dealt with together.

The inoculum was prepared from the brain of a C57BL mouse affected with ME7 scrapie. Brain material, stored at −30°C. for several months, was homogenised with isotonic saline in a ground glass tissue grinder at 1 per cent. concentration. The suspension was spun at 500 *g.* for 10 minutes and 0·02 ml. of the supernatant fluid used for intracerebral inoculation.

The mice were inoculated at 4 weeks of age. From the 12th week after inoculation in the case of the C57 and BALB strains, and from the 20th week in the case of VM, groups of 2 to 3 mice from each strain were killed weekly. The brains were fixed immediately in 10 per cent. formol-saline and subjected to histological examination. From the 15th week (C57 and BALB) or the 25th week (VM) clinical signs of scrapie in the remaining live animals were scored according to the method described by Dickinson *et al.* (1968).

Histological analysis. The method of histological analysis was designed to extract information concerning both the distribution and intensity of the lesions within the brain. Five transverse paraffin sections, 7 μ thick, were prepared from each brain as described by Mould, Dawson, Slater and Zlotnik (1967) and were stained with haematoxylin and eosin. Nine regions of grey matter were examined, namely, (1) the dorsal half of the medulla, including the cuneate, vestibular and cochlear nuclei, or the locus coeruleus, or the sensory nucleus of the trigeminal nerve, and associated areas, depending on the exact levels available, (2) the cerebellar cortex of the folia adjacent to the fourth ventricle, particularly the lingula or nodulus, (3) the cortex of the superior colliculus, (4) the hypothalamus, (5) the thalmus, in particular the massa intermedia and central nuclei of the thalamus, (6) the hippocampus at the same level as (4) and (5), (7) the septal nuclei of the paraterminal body, (8) an area of the cerebral cortex at the level of (4) and (5) lying dorsal to the corpus callosum, associated with the indusium griseum, the cingulate gyrus and cortex extending onto the dorsal surface of the brain, and (9) a similar area of cerebral cortex as in (8) associated with the section on which the septal nuclei are situated (Fig. 1).

Each of the 9 areas was given a score from 0 to 5 depending on the density of the vacuolation and status spongiosus. Five degrees of brain lesion intensity were distinguished, which, with the exception of score 1, had similar verbal definitions to those given previously (Mould *et al.*, 1967). The verbal definitions used were 1, a few vacuoles, widely and unevenly scattered, 2, a few vacuoles evenly scattered, 3, moderate numbers of vacuoles, evenly scattered, 4, many vacuoles with some confluence and 5, dense vacuolation with most of field confluent. Photographs representative of these different scores were kept for reference purposes and examples for the cerebral cortex are shown in Fig. 2.

RESULTS

Lesion Density Scoring System; Analysis of Properties

Before describing the results of the experiments, a detailed analysis of the scoring system will be given as a basis for assessing its reliability, the extent of subjective and technical bias, and the relationship of score to vacuole number.

Observer errors can take two forms: one is due to inaccuracy in locating the intended region of the brain on the prepared slides, and the accuracy of this is

presumably specific to each region; the other is due to subjective variation in assigning a score to a chosen microscope field. The combined effect of these biases was estimated by rescoring 50 slides chosen at random from a large number, none of which had been intended for rescoring at the time of initial examination. Table 1 shows a high correlation between the two scores for all positions except cerebellum and, to a lesser extent, medulla. Since the variance between individual brains differs from position to position in the brain, the correlations are not strictly comparable with one another and variance between repeat observations is also given in Table 1 for comparative purposes: this is a more appropriate variate and is used again below in a slightly different form. There is some degree of confounding between brain region and score, because the various scores are not equally distributed in all the regions. Table 2, based on data from the same series of 50×9 repeated observations, shows that some scores are more repeatable than others.

TABLE 1

CORRELATION COEFFICIENT (r) AND VARIANCE OF DIFFERENCE BETWEEN ORIGINAL AND REPEATED LESION DENSITY SCORE

Region	*r*	*Variance of difference*
Medulla	0·84	·18
Cerebellum	0·70	·27
Tectum of midbrain	0·93	·09
Hypothalamus	0·91	·12
Thalamus	0·94	·09
Hippocampus	0·94	·07
Paraterminal body	0·96	·06
Cerebral cortex (posterior midline)	0·95	·07
Cerebral cortex (anterior midline)	0·92	·10

TABLE 2

VARIANCE OF REPEAT OBSERVATIONS IN GROUPS CLASSIFIED ON ORIGINAL LESION DENSITY SCORE

Original score	*Variance of repeat scores*
0	0·15
1	0·50
2	0·34
3	0·18
4	0·15
5	0·25

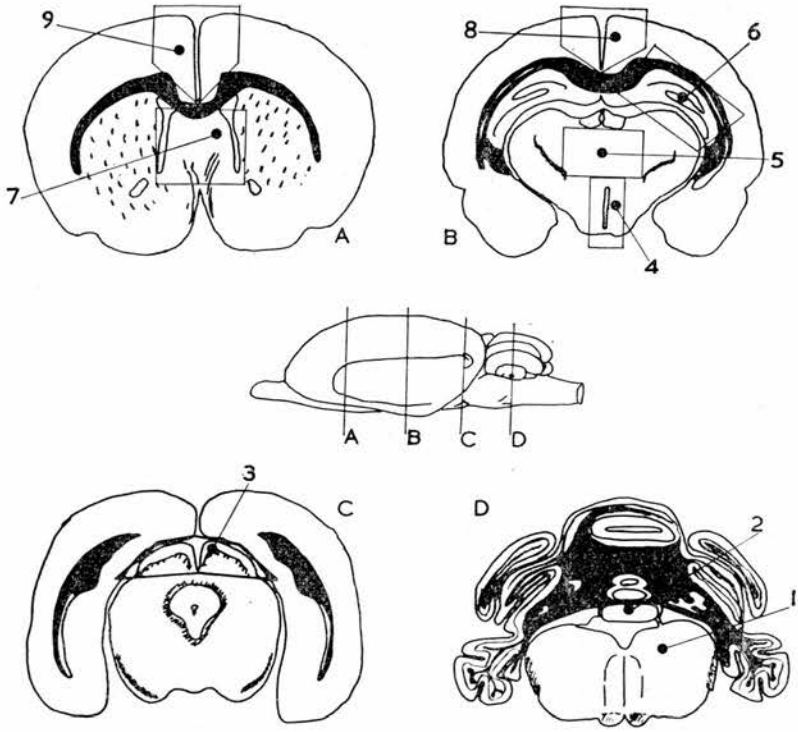

Fig. 1. The 9 positions used for scoring the lesion density in scrapie in the mouse. 1: medulla; 2: cerebellum; 3: mid-brain; 4: hypothalamus; 5: thalamus; 6: hippocampus; 7: para terminal body; 8 and 9: cerebral cortex.

Bias due to preparative technique may arise from variation in the primary cutting of the fixed brain into five pieces in the positions shown in Fig. 1. Slight variation in the position of the cuts and angular deviations from the intended line of cut occur, and additional sources of variation may arise in trimming the embedded tissues. In some cases scoring is prevented because these sources of error result in the absence of the intended regions from any of the prepared sections.

In order to assess the effect of antero-posterior variation in the position of the cut, serial sections were prepared from fifteen randomly selected scrapie brains. Twenty-four serial sections were prepared, representing 1·5 mm. thickness of brain tissue, and every third section was examined making eight in all With a solitary exception in position 1 (medulla), the first sections were suitable for scoring in the nine regions The first sections were therefore used as the basis from which to judge progressive changes in scoring throughout the series: this was calculated as the variance of the difference between the score for position y of slide 1 with that on each of the other seven slides in turn. The sequence of

TABLE 3

VARIANCE OF DIFFERENCES BETWEEN SCORES ON FIRST SLIDE AND SCORES ON EVERY THIRD CONSECUTIVE SERIAL SECTION

Position	*Serial section number*						
	4	*7*	*10*	*13*	*16*	*19*	*22*
Medulla	·08	·15	·15	·12	·12	·08	·12
Cerebellum	·03	·54	·57	·64	·68	·71	·64
Tectum of midbrain	·11	·21	·18	·32	·25	·39	·21
Hypothalamus	·11	·11	·04	·11	·07	·11	·07
Thalamus	·14	·18	·14	·14	·14	·14	·14
Hippocampus	·21	·25	·14	·18	·14	·11	·21
Paraterminal body	·18	·11	·14	·18	·14	·18	·18
Cerebral cortex { post. midline	·07	·21	·14	·14	·14	·18	·07
Cerebral cortex { ant. midline	·14	·11	·21	·18	·14	·14	·21

variances which emerged is shown in Table 3. The inferences which can be drawn from this analysis are broadly similar to the conclusions from the repeat observations on a single slide discussed above. Cerebellum shows a relatively large increase in variance as deviation from the first slide increases: this position has the lowest correlation and highest variance for repeat observations on the same slide as was discussed in connection with Table 1. The lower repeatability of observations on medulla (Table 1) is evidently more a function of observer error than of spatial inconsistencies in that part of the brain. For midbrain tectum the observer error component is low (Table 1), but spatial variability within the tectum is relatively important as a source of variation in score (Table 3). Scores for the other five positions are relatively unaffected by small changes in the level from which the section is taken.

The other property of the scoring system which is of interest is the relationship between score and vacuole number. This was estimated by taking standard photographs of a sample of regions which had previously been assigned scores and counting vacuole number from these photographs. In the case of scores 2, 3 and 4, eight randomly selected examples for each brain region were counted giving seventy-two examples in all: for score 5 only thirty-six examples, confined to five brain regions, were available and all were used. With score 1, the method was slightly different in that seventy-two random examples often had no vacuoles within a randomly chosen microscope field, due to wide vacuole dispersion. For score 1 the average number in seven fields was therefore used as the basis for calculation.

As it happens the first four consecutive scores have approximately equal intervals when a logarithmic transformation of vacuole number is used and, for reasons given below, score 5 is probably not exceptional in this. The scale on which the variance of vacuole number is most homogeneous and independent of the mean for scores 2 to 5 is log (vacuole number+3) and the same scale would also be the most appropriate for score 1 if it were based on the individual values instead of on averages of seven observations: the variance of the averages is of course lower in these data, being half that for individual observations. Using this transformed scale, smoothed distributions of equal sized samples of scores 1 to 5 are

shown in Fig. 3. Even though slight overlapping of vacuole number for non-adjacent scores occurs, the five classes of each significantly distinct ($P < \cdot 001$) from their immediate neighbours, as estimated by tests using the transformed values.

With score 5 there is considerable difficulty in counting the large number of vacuoles which are present, using the same photographic methods as for the lower scores. The problem arises from confluence of vacuoles and overlapping of vacuoles at different layers in the 7μ thickness of the section. Fig. 3 shows a discontinuity in vacuole number with an apparent shortage of fields containing about 150 to 300 vacuoles. This is almost certainly due to counting difficulties, with overestimation of number starting to occur when the true number exceeds about 150. If this is so, the average number of vacuoles per field in score 5 should be in the order of 400 rather than 630 as indicated in Fig. 3.

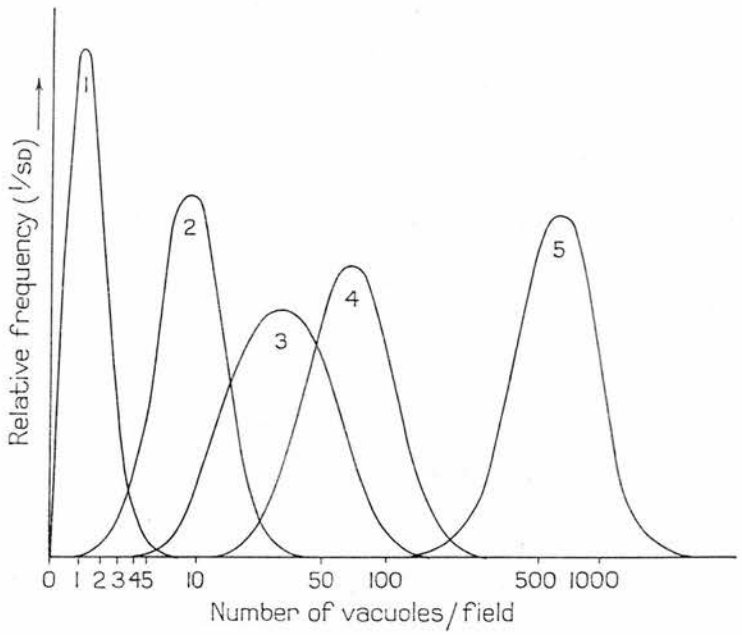

Fig. 3. Distribution of numbers of vacuoles in scores 1 to 5. The distributions are calculated from the data transformed to the scale log (vacuole number + 3). The figure is based on equal sample sizes for each score, using in the case of score 1 the mean of seven observations per individual.

Development of Brain Lesions

In the histological assessment of the lesions of experimental scrapie there were differences related to mouse strain in the degree of brain damage during the clinical stage of the disease, in the rate at which the lesions developed and in

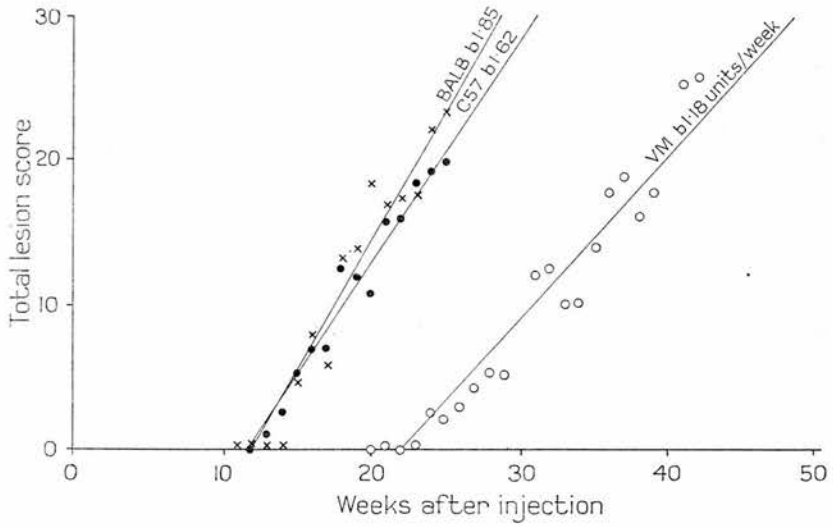

Fig. 4. Rate of development of total lesion density to the end of the incubation period in three strains of mice.

the distribution of lesions during and at the end of the incubation period. Fig. 4 shows the rate of development of lesions in the three strains, using as the index total score, i.e. the sum of the individual scores for each of the nine regions. The relative stage of the incubation period at which the first lesions (defined as total score $\geqslant 2$) occur was fairly uniform for the three strains, being 59 per cent. for C57BL, 61 per cent. for BALB, and 54 per cent. for the VM strain. The incubation period, estimated from the small number of inoculated animals allowed to reach an end point, was $23{\cdot}6 \pm 0{\cdot}4$ weeks for C57BL, $24{\cdot}4 \pm 0{\cdot}0$ weeks for BALB/c and $44{\cdot}4 \pm 5{\cdot}1$ weeks for VM. Once started, the brain damage, as estimated by total score, progressed more slowly in VM than in either of the other strains (P, $<{\cdot}001$); BALB and C57 did not differ significantly in this respect (P, $>{\cdot}1$). Fig. 5 shows the regional development of the lesions, and Fig. 6 the distribution of damage at the terminal stage, with the significance of these terminal differences listed in Table 4.

TABLE 4

PROBABILITIES OF DIFFERENCES BETWEEN STRAINS IN LESION DENSITY IN DIFFERENT AREAS OF THE BRAIN

Strains compared	*Position in brain*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
BALB and C57	$>{\cdot}1$	$<{\cdot}05$	$<{\cdot}01$	$<{\cdot}02$	$>{\cdot}1$	$>{\cdot}1$	$>{\cdot}1$	$<{\cdot}05$	$>{\cdot}1$
C57 and VM	$<{\cdot}05$	$>{\cdot}1$	$<{\cdot}01$	$<{\cdot}001$	$<{\cdot}001$	$>{\cdot}1$	$<{\cdot}001$	$<{\cdot}01$	$>{\cdot}1$
VM and BALB	$>{\cdot}1$	$>{\cdot}1$	$>{\cdot}1$	$<{\cdot}05$	$<{\cdot}01$	$>{\cdot}1$	$<{\cdot}001$	$>{\cdot}1$	$>{\cdot}1$

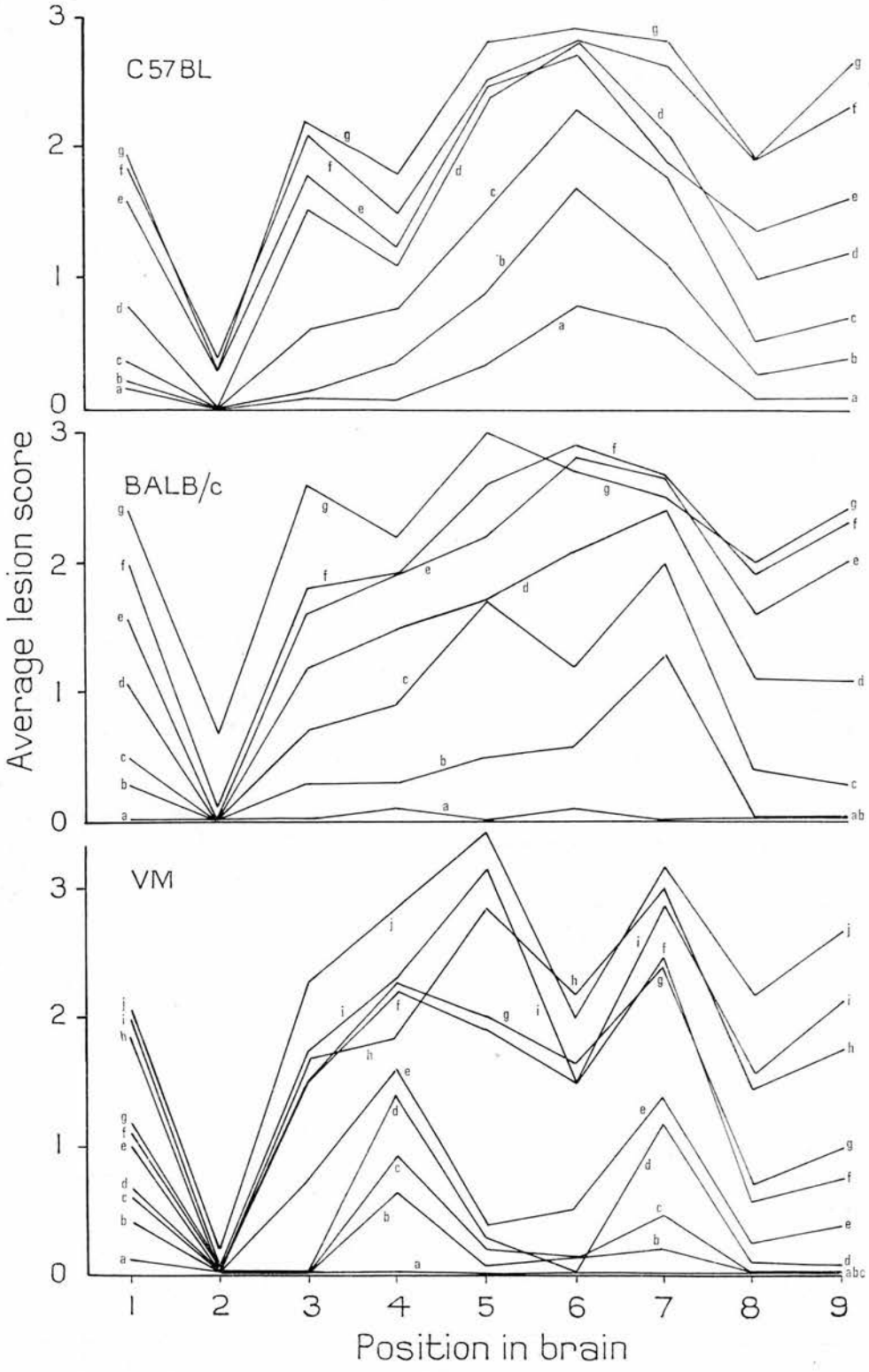

Fig. 5. Sequential increase in lesion density in different regions of the brain during the incubation period. Starting at 12 weeks in C57, 11 weeks in BALB and 20 weeks in VM, the lines are based on four-point running averages in order to minimise random fluctuations. The small letters indicate the sequence (e.g. in C57 a=12+13+14+15; b=14+15+16+17; c=16+17+18+19; etc.)

Three points therefore emerge. The distribution of terminal lesions is different between the strains, although the overall pattern in C57 and BALB is broadly more similar than either is to VM. It is also striking that the early distribution of lesions is continued throughout the incubaton perod into the fully developed disease, since those areas where lesions first appear have the highest terminal density. Finally, although the relative stage of the incubation period at which lesions first appear is approximately the same for each of the three strains, the rate of development of these lesions when the whole brain is considered is less for the longer incubation period.

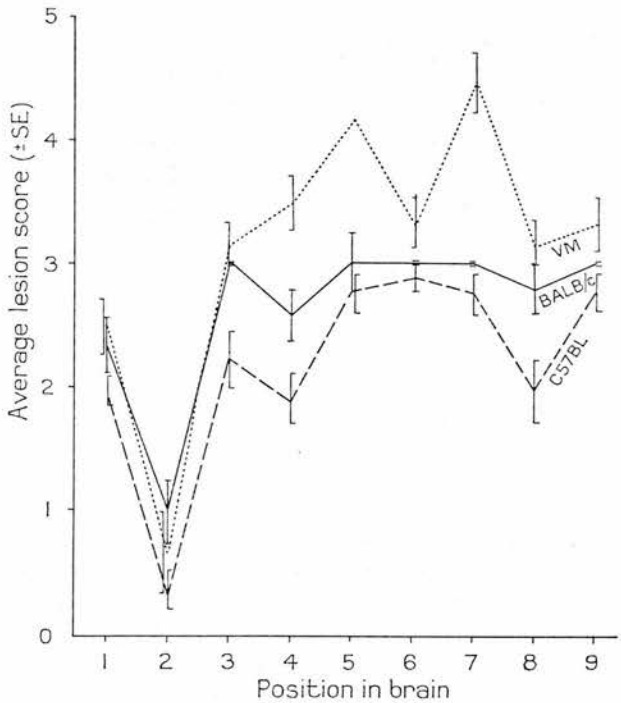

Fig. 6 Distribution of brain lesions at the terminal clinical stage of scrapie in three mouse strains.

DISCUSSION

Previous studies concerning the genesis of the lesions of scrapie during the incubation period have been made by Hadlow (1961), who made a purely qualitative assessment in three goats killed while clinically normal, and Pattison and Smith (1963). The latter employed mice and, using a three class scoring system, showed that the time at which the lesions first became detectable depended upon the dilution of the agent and could be related to incubation period; they detected

lesions in the hippocampus and adjacent cerebral cortex 56 days after inoculation of the largest dose, considerably earlier than in the present study.

It is difficult to make direct comparisons with the observations recorded by Pattison and Smith (1963) who used longitudinal sections in a different strain of mice, and a different scrapie agent. However, these authors noted that the earliest lesions developed in the hippocampus and adjacent cortex, while the cerebellum was the last structure to become affected. In the present study similar results were obtained with the C57 strain, which showed early lesions in the hippocampus. This was in striking contrast to the other two mouse strains used: in VM, the hippocampus was one of the last regions to be affected, while the hypothalamus and paraterminal body were affected early; in the BALB strain the paraterminal body showed more severe lesions than elsewhere in the early stages. In all three strains involvement of the cerebellar cortex was a late feature, although it became more severe in BALB than in VM or C57. It might be safe to conclude, therefore, that within the narrow limits of the experiment, the degree of brain damage bears a direct relationship to the clinical stage of the disease, other factors such as genotype, agent, dose and route of challenge being constant. Such qualifications are necessary because it is apparent from other observations that either the degree or distribution of brain damage can sometimes be affected by length of incubation period, route of inoculation, and by strain and passage history of the agent used (Fraser, unpublished observations). With a single agent and identical challenge, the distribution of lesions during and at the end of incubation period is under the influence of the host genotype.

SUMMARY

The genesis of the lesions of scrapie in 3 strains of mice under comparable circumstances was investigated, using a system of scoring the brain pathology which depended on an assessment of the intensity of neuronal vacuolation and status spongiosus in paraffin sections stained with haematoxylin and eosin. By applying this method to narrowly defined areas of the brain a quantitative analysis of the distribution of lesions and total brain damage could be arrived at, allowing precise comparisons to be made.

There were differences between mouse strains in the rate of development of the lesions, the distribution and intensity of which was characteristic of each strain. Within a single strain, however, the distribution of lesions during the incubation period was closely similar to the distribution at the end point, differing only in intensity.

ACKNOWLEDGMENTS

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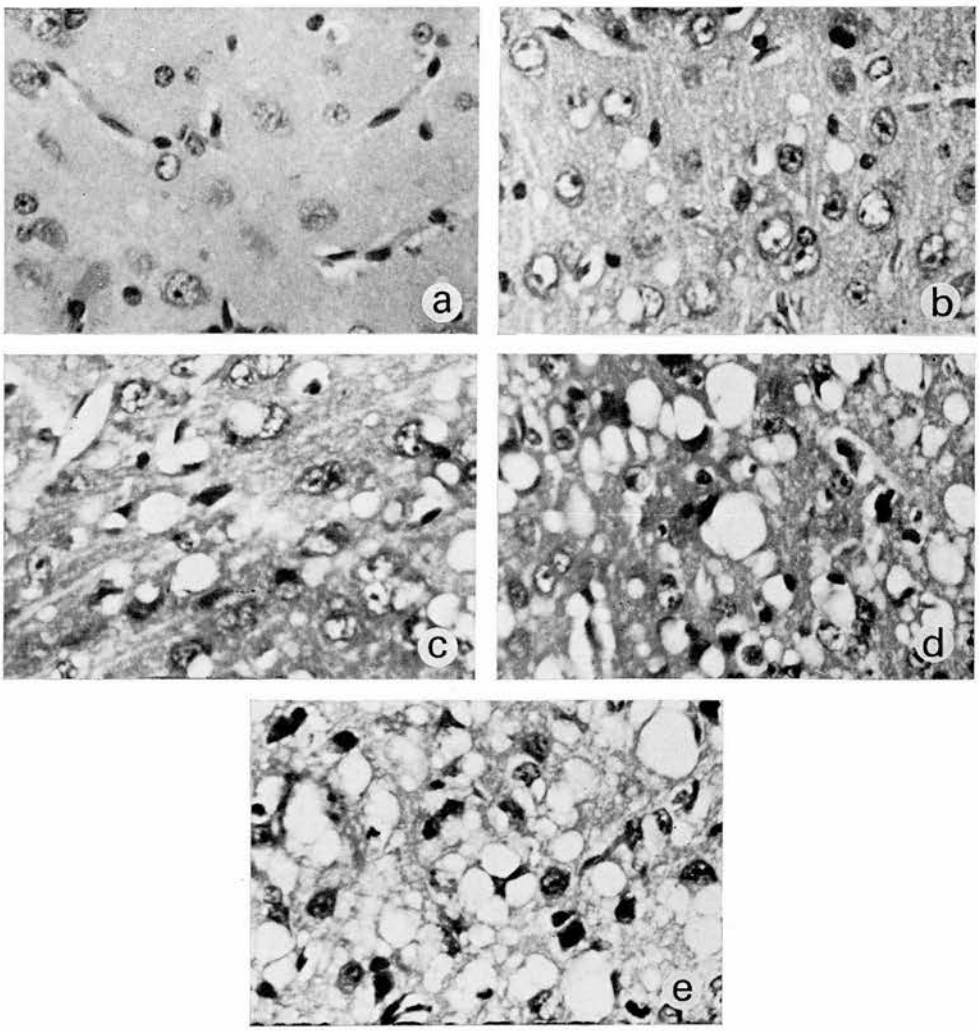

Fig. 2. Scrapie lesions of five degrees of severity in the cerebral cortex of mice. a: score 1; b: score 2; c: score 3; d: score 4; e: score 5. Haematoxylin and eosin, × 375.

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THE OCCURRENCE OF NERVE FIBRE DEGENERATION IN BRAINS OF MICE INOCULATED WITH SCRAPIE

BY

H. FRASER

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The Occurrence of Nerve Fibre Degeneration in Brains of Mice Inoculated with Scrapie

H. FRASER
A.R.C. Animal Breeding Research Organisation and Moredun Institute, Gilmerton, Edinburgh

SUMMARY. Mice of 3 inbred strains were inoculated intracerebrally with a standard scrapie agent. At weekly intervals, from about halfway through the incubation period, groups of each strain were killed and their brains examined histologically. A silver impregnation method was used to demonstrate degenerating nerve fibres selectively. Uninoculated mice of the same 3 strains were treated similarly. A number of mice of other strains inoculated both intracerebrally and intraperitoneally were similarly examined. During the development of the disease and at the clinical stage, when nerve fibre degeneration became apparent, this was much more severe in the scrapie inoculated mice than in the controls.

THE MOST STRIKING histological lesions which occur in the brains of mice affected with experimentally induced scrapie are neuronal cytoplasmic vacuolation and degeneration, vacuolation of the ground substance, and hypertrophy of astrocytes (Chandler, 1961; Zlotnik & Rennie, 1962; Eklund, *et al.*, 1963). In addition, the accumulation of neutral fat in the grey matter has been described (Mackenzie & Wilson, 1966), and slight destruction of myelin in the cerebrum and cerebral penduncles has been observed in the terminal phase of the disease (Eklund *et al.*, 1963).

The purpose of this paper is to describe the occurrence of nerve fibre degeneration in the brains of scrapie infected mice. This demonstration has been facilitated by the use of silver impregnation methods for the differential staining of degenerating axons. A method of scoring the severity of axonal damage is used to distinguish different degrees of severity of the lesion.

MATERIAL AND METHODS

The details of the mice used, the scrapie agent, inoculation, and the procedure of serial killing were as given previously (Fraser & Dickinson, 1968). Mice of 3 strains (C57/Bl, BALB/c and VM) were inoculated intracerebrally with 0·02 ml. of a hundredfold dilution of ME7 scrapie infected mouse brain agent. At weekly intervals from the 11th week in the cases of the C57 and BALB strains, and from the 20th week in the case of the VM strain, small groups of mice were killed and their brains examined histologically.

The brains from uninoculated control mice of the same 3 strains killed at corresponding ages were similarly examined. Miscellaneous mice of several strains terminally affected with ME7 scrapie, as well as inoculated and uninoculated controls of various ages, were also examined.

Paraffin sections of half brains, cut transversely, were stained by haematoxylin and eosin (H&E), Glees and Marsland, and Holmes silver methods. Frozen longitudinal sections were stained by the Nauta and Gigax techniques (Nauta & Gigax, 1954; Nauta, 1957). The Nauta and Gigax technique, using Laidlaw's silver carbonate solution, was employed most frequently (Chambers *et al.*, 1956) although occasionally the earlier method using a freshly prepared ammoniacal silver nitrate solution was substituted (Nauta & Gigax, 1954).

An essential prerequisite for the assessment of the proportion of normal and degenerating fibres was to vary the concentration of ammonia in the silver solution to control the degree of impregnation of nerve fibres of normal appearance.

The distribution and severity of vacuolation and status spongiosus were assessed in H&E, transverse paraffin sections (Fraser & Dickinson, 1968). To assess the degree of nerve fibre degeneration in

longitudinal frozen sections stained by the Nauta and Gigax technique 6 areas of the brain were scored on a scale from 0 to 3. As the criteria used to assess the degree of fibre degeneration were open to subjective bias, the slides were examined randomly and in such a way that the control and experimental groups could not be distinguished.

The criteria of scoring were as follows:—score 0, no nerve fibre degeneration apparent; score 1, degenerating axons in very low numbers; score 2, degenerating fibres very frequent but fibres of normal appearance also numerous; score 3, the majority of nerve fibres showing signs of degeneration.

Six regions of the brain were used for this assessment:—fibres of the internal capsule in the corpus striatum; the hippocampus; the junction of the cerebral cortex and cerebral medulla; the thalamus; the tegmentum of the mid-brain and the dorsal tegmentum of the medulla and pons.

RESULTS

The total number of mice examined by the staining method of Nauta and Gigax was 132. The results of the main part of the experiment are given in Figures 1a and 1b. Pre-terminal nerve fibre degeneration developed increasingly from about half-way through the incubation period of scrapie in all 3 strains, the lesions developing earlier and more rapidly in the C57 and BALB strains, which have a short incubation period with the ME7 scrapie agent, and later and slightly more slowly in the VM strain, which has a long incubation period with this particular agent. Fig. 2 shows that a direct relationship exists between the development of the nerve fibre lesions and the development of vacuolation and microvacuolation in grey matter areas of the brain, described as total lesion score and calculated according to the method previously reported (Fraser & Dickinson, 1968).

The distribution of the nerve fibre lesion was haphazard and did not involve any particular systems. It was widespread in all grey matter areas of the brain, in addition to the 6 areas chosen for scoring. The lesions were most readily appreciated in the fibre bundles of the corpus striatum where the parallel fibres of the internal capsule of the normal mouse contrasted strikingly with the beaded and granular

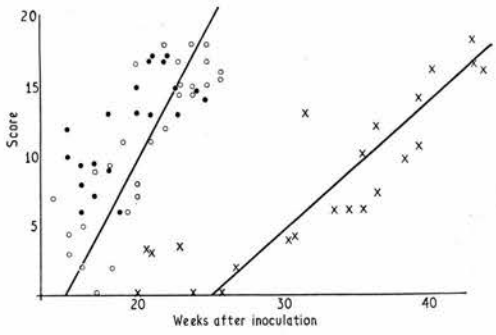

FIG. 1a. Development of nerve fibre degeneration during the pre-clinical and clinical phases of scrapie in 3 strains of mice.
○ = BALB/c ● = C57/B1 × = VM

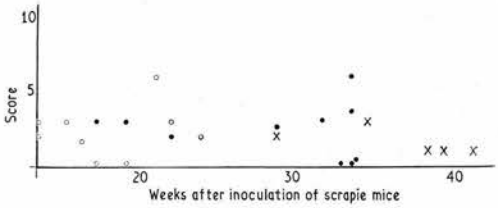

FIG. 1b. Estimate of integrity of nerve fibres in uninoculated control mice. The same strains as Fig. 1a and killed at corresponding ages.

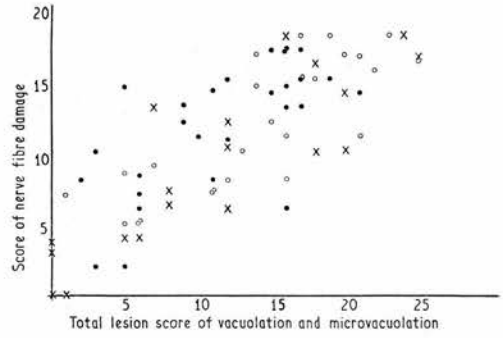

FIG. 2. A direct relationship between the development of degenerative changes in nerve fibres and vacuolation and microvacuolation in 3 strains of mice as in Figure 1a.

appearance of the degenerating fibres of the affected brain (Figs. 3 & 4). Although more difficult to demonstrate, the degeneration which occurred in the hippocampus was a useful feature because it had not proved possible to stain fibres of normal appearance in this position (Fig. 5). The lesion was clearly seen also in the cerebro-cortico-medullary junction, in the central mass of the thalamus, in the tectum and tegmentum of the mid-brain and in the nucleated and reticular parts of the brain stem (Figs. 6, 7, 8, 9 & 10). In the cerebellar cortex it was rarely possible to identify degenerating nerve fibres, although they were frequent in the vicinity of the root nuclei.

In the early stages of the disease, when lesions were beginning to become apparent, in the cerebro-cortico-medullary junction there was sometimes a suggestion of a greater degree of fragmentation in those fibres which pass parallel to the surface of the cortex than in those which cross the former at right angles and which pass either into or out of the cortex. In the white matter as a whole, and as represented by the main fibre tracts in the brain stem, large diameter fibres never appeared to display any evidence of damage, even when the other scrapie associated lesions were well advanced. In paraffin sections of the brains of scrapie affected mice it was not possible to distinguish any axonal degeneration with the conventional silver techniques of Glees and Marsland, or Holmes, in which all nerve fibres were impregnated.

In a miscellaneous group of 15 mice in the terminal stages of scrapie, identical changes in the nerve fibres were seen. In 26 controls, which consisted of uninoculated mice and mice inoculated intracerebrally and intraperitoneally with control material, including animals up to 130 weeks of age, no comparable lesions were seen, although a small degree of nerve fibre degeneration was occasionally found in some older mice.

DISCUSSION

The advantage of selective over non-selective silver impregnation methods for staining the central nervous system lies in the easier identification of pre-terminal axonal degeneration when this is present to a limited extent. Their disadvantage is that they fail to stain nerve terminals and terminal degeneration. The comparative aspects of the silver impregnation techniques have been reviewed by Bowsher *et al.* (1960) and Heimer (1967). Although the Nauta and Gigax methods have been used extensively in neuroanatomical studies, where they have largely replaced the Marchi technique, their use in pathology has been very limited (Cavanagh & Patangia, 1965; Illis *et al.*, 1966).

Nerve fibre degeneration in scrapie has been described in the sheep (Wight, 1960) and the goat (Hadlow, 1961). The report of myelin breakdown in scrapie mice (Eklund *et al.*, 1963) does not imply concomitant axonal degeneration, although the chemical basis of the earlier Nauta and Gigax (1954) method (Giolli, 1965) and other selective and non-selective silver impregnations may involve the staining of myelin breakdown products (Cottle & Mitchell, 1966). Any account of the presence or absence of axonal degeneration must consider the fact that stainable breakdown products may have a limited survival time. The optimal staining time following secondary degeneration (Wallerian) in the central nervous system is thought to vary between 5 and 14 days, although some axonal by-products may remain demonstrable for several months (Nauta, 1957). The kinetics of the turnover

FIG. 3. Corpus striatum in normal mouse. Nauta and Gigax. ×900.

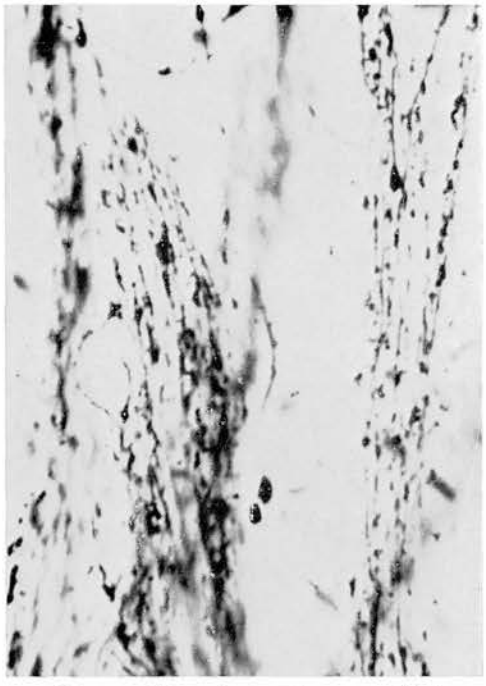

FIG. 4. Corpus striatum in scrapie mouse. Nauta and Gigax ×900.

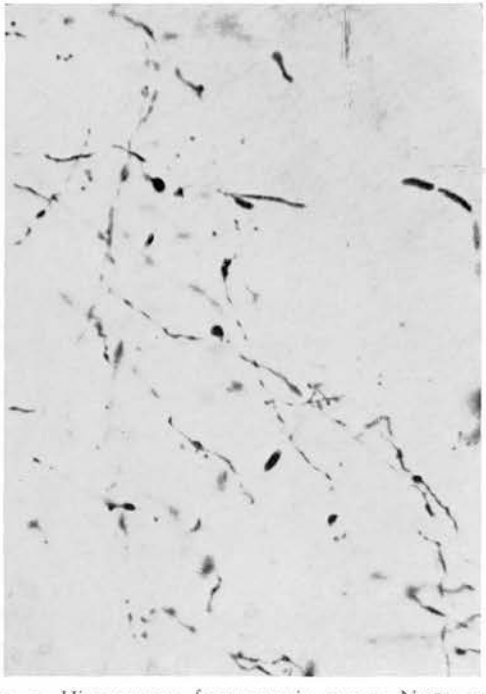

FIG. 5. Hippocampus from scrapie mouse. Nauta and Gigax ×900.

FIG. 6. Cerebral cortex at cortico-medullary junction in normal mouse. Nauta and Gigax ×900.

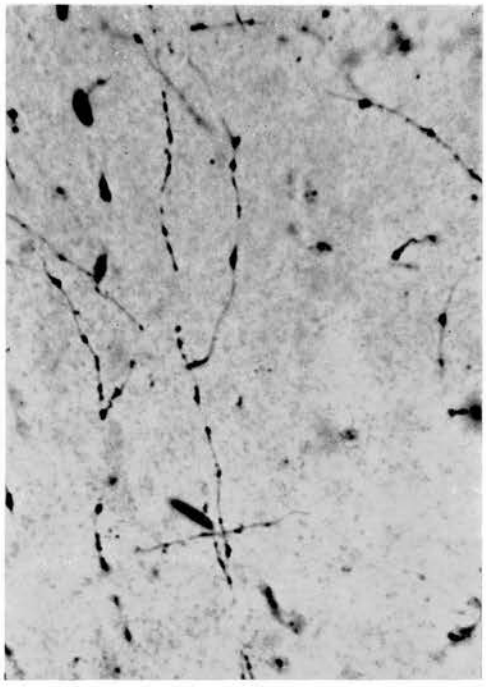

FIG. 7. Cerebral cortex at cortico-medullary junction in scrapie mouse. Nauta and Gigax ×900.

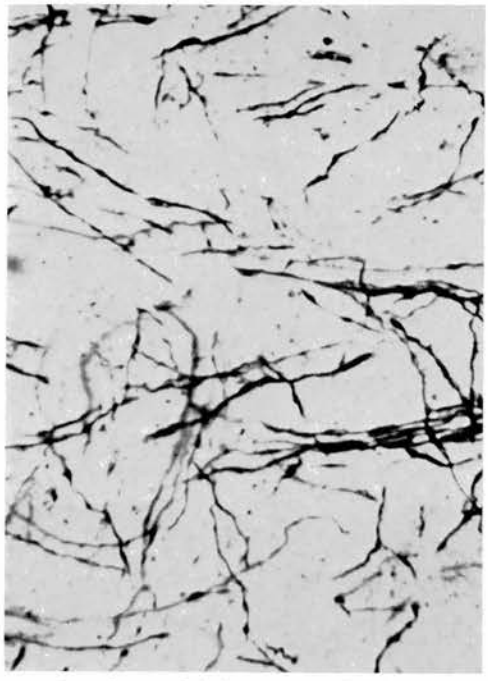

FIG. 8. Central mass of thalamus in normal mouse. Nauta and Gigax ×900.

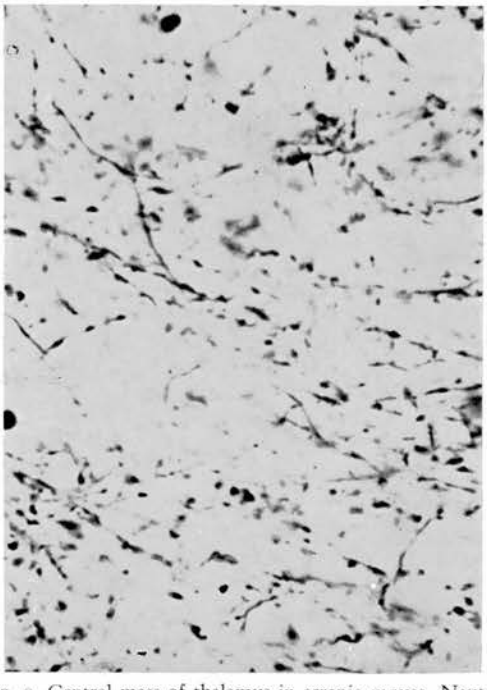

FIG. 9. Central mass of thalamus in scrapie mouse. Nauta and Gigax ×900.

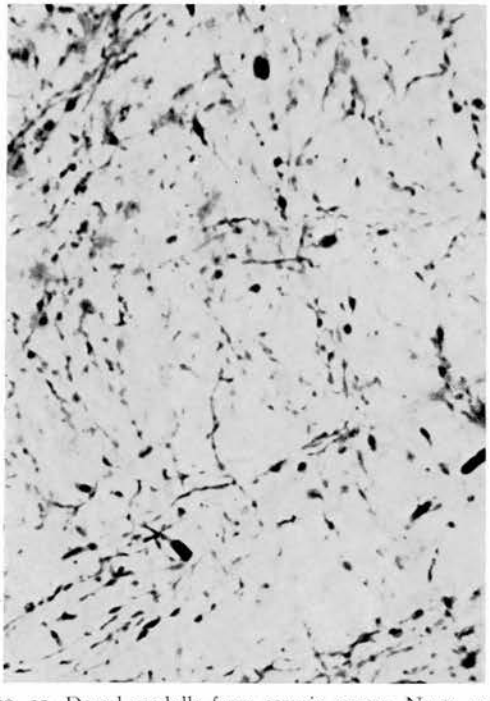

FIG. 10. Dorsal medulla from scrapie mouse. Nauta and Gigax ×900.

of these stainable degradation products in pathological conditions may differ fundamentally from that following secondary degeneration. In an electron-microscopic examination of scrapie in the rat, structures which were described as degenerating myelinated fibres were identified in the thalamus and hippocampus (Chandler, 1967). The occurrence of degeneration both of mossy fibre afferents of the spinocerebellar system, and of terminals in the pontine nuclei, have been described as some of the earliest lesions of scrapie in the sheep by Beck *et al.* (1964), who concluded that natural scrapie in the sheep bore features of a system degeneration, a conclusion which they based largely on the findings of Holzer-positive gliosis, neuronal loss, myelin breakdown products and nerve fibre loss in specific locations.

A system degeneration implies a concomitant dying-back process (Greenfield, 1954). The present investigation has failed to demonstrate such a process in experimental scrapie in the mouse. If this were partially responsible for the nerve fibre lesion in mouse scrapie as well as the classical lesion of vacuolation and microvacuolation, then the sites of the dying-back process in its earliest stages would be distributed in a manner distinct from the vacuolation type of lesion. Also, the former lesion would precede the latter by an interval of time. In neither case was this found to be so in the present investigation (Fig. 2).

However, 2 processes might co-exist, each with a fundamentally different underlying pathogenesis, one being a dying-back process and the other a different type of fibre degeneration. The latter could result from and be associated spatially with the vacuolation type of lesion. The processes could conceivably occur simultaneously with the result that the systematic pattern would be obscured. With the one reservation implicit in this last statement, considering the haphazard distribution of the lesion, it is not necessary to attribute it to a dying-back process, although such a process in natural scrapie in the sheep has recently been suggested (Palmer, 1968). It is concluded, therefore, that the lesion described in this paper is a non-specific change and is secondary to the primary oedematous encephalopathy.

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IDENTIFICATION OF A GENE WHICH CONTROLS THE INCUBATION PERIOD OF SOME STRAINS OF SCRAPIE AGENT IN MICE

By

A. G. Dickinson, Veronica M. H. Meikle and H. Fraser

A.R.C. Animal Breeding Research Organisation, Edinburgh

INTRODUCTION

In a previous paper (Dickinson and Mackay, 1964) it was shown that one partially inbred strain of mice, when injected intracerebrally with the mouse-passaged ME7 scrapie agent had an incubation period of 40 weeks in contrast with three other partially inbred strains and five standard inbred strains which had average incubation periods between 21 and 26 weeks.

At that time, because of the early stage of the inbreeding, segregation was likely to be occurring in the long incubation period strain which would be liable to confuse interpretation of the genetical basis of incubation period. Since then this strain has been further inbred with selection against sublines with average incubation periods below 37 weeks and the stock is now showing no evidence of segregation affecting this character. The purpose of this paper is to describe the investigation of the genetical basis of the long incubation period, as revealed by crossing with an inbred strain which has a short incubation period when challenged with the ME7 scrapie agent.

MATERIALS AND METHODS

Mouse stocks. The RIII mice, which have been inbred for over 40 generations, were derived from the Falconer substrain which is free from mammary tumours; this inbred strain was used as the short ME7 incubation period strain. For the long incubation the strain VM, referred to as 5M in some previous publications, was used and were from the 7th and 8th inbred generations. Neither stock has been caesarian derived and all the mice are kept under conventional conditions.

Crossing generations. (a) The F_1 generation were all from RIII males mated with five VM females. The reciprocal cross was not made. (b) The F_2 generation were produced from full sib matings of randomly selected individuals from all the F_1 families. The number of males was restricted to eight. (c) The backcross of F_1 to RIII (Bx_1) was made by mating the same eight F_1 males used for producing F_2's, with RIII females. (d) The backcross of F_1 to VM (Bx_2) was made by mating each of the original five VM females with one of her F_1 progeny (i.e. with five of eight F_1 males used for F_2 and Bx_1).

Age at injection, route and dose. Since many of the mice had to be used for breeding before they could be injected a late challenge age was used for all individuals. Most of the mice were injected at 6 months old but a few were challenged as early as 4½ months and a few as late as 9 months. All injections were administered intracerebrally with a 26G needle and 0·02 ml. of inoculum was given.

Inoculum. The scrapie agent was from the third mouse passage of the ME7 strain and the brains used were from males of the VM stock killed with advanced clinical scrapie, which had been stored at −30°C. for over two years. The inoculum was

made up from this pool on five separate occasions when mice of appropriate ages were available. On each occasion the tissue was macerated in a glass grinding tube, made up as one per cent. saline suspension and spun at 150 g for 5 minutes to remove the larger tissue particles. The supernatant was used as inoculum.

Clinical criteria used to define incubation period. Injected mice were scored once weekly during the afternoon. On the day of scoring no husbandry routine such as cage cleaning was undertaken before each animal was scored. By this means the normal behaviour of the mice was not disturbed until the time of scoring at which each mouse was classified as either "unaffected", "possibly affected" with the clinical signs of scrapie or "definite scrapie". These clinical signs normally progress through a jerky, overexcited stage which is not easy to score and is never classified as "definite scrapie", to a stage where there is a transient or, later, a semipermanent inactivation in which the mouse "freezes" in an awkward posture and vibrissae movements are absent and the righting reflex is largely abolished. These later stages with unequivocal inactivation are scored as "definite scrapie". Occasional mice do not become inactive in this way, instead they develop either extreme incoordination of gait or flaccid paralysis of the hind limbs : these symptoms would have been classified as "definite scrapie", but they did not appear in the particular experiment. Urinary incontinence occurs sporadically during the clinical phase of scrapie at least with the ME7 agent, and is frequent in animals within a week or two of the time at which they would die from the disease. Although this leaking is noted—it affected 44 per cent. of animals in this experiment—it is not used as a criterion of incubation period.

The end point for calculating incubation period is defined as the day on which either (a) an animal receives a third consecutive weekly score of "definite scrapie" and was 79 per cent. of animals in this experiment or (b) the day on which a fourth "definite scrapie" score is given within five consecutive weeks, in the case of animals having a transient remission to a lesser score and was 7 per cent. in this experiment or (c) as the day on which the animal has to be killed in extremis with scrapie or is found dead after having received at least one "definite scrapie" score in the proceeding week and was 14 per cent. in this experiment. Incubation period is the interval in days between injection and the end point as defined.

The scoring system has been found to be efficient in a large number of experimental circumstances, the only complication coming from the differences between the many inbred strains with which it has been used, which vary widely in their normal behaviour. Most of the mouse strains have a short incubation period with the ME7 agent. Of these strains, some are very active and others relatively lethargic, with the RIII strain being of intermediate activity. However, of all the inbred strains with which we have experience, the VM strain which differs from the rest in incubation period is also the least active and this leads to some complications in scoring. Therefore, for general purposes, the scoring criterion for each stock has to be judged as a deviation from the normal type of activity for that stock. However, where crossbreeding is used, as in this experiment, this simple standard for comparison is not applicable because we have no relevant experience with uninoculated crossbreds.

Although the difficulties liable to be encountered in scoring have been dealt with at some length, bias or random error from this source should not be over-emphasised as the system has been designed to be buffered against subjective error and to provide an end point as near to death from scrapie as is tactically compatible with the common requirement to remove uncontaminated brains as agent source or for histological checking.

Histological examination. All brains of purebred and F_1 mice which were killed at an appropriate end point, together with samples of those from the F_2, Bx_1 and Bx_2 generations have been examined histologically for confirmation that the clinical symptoms were due to scrapie. For each animal, nine regions of the brain were examined and the clinical diagnosis was confirmed in every case. The density of characteristic scrapie vacuoles in neurones or ground substance in each of the nine

regions was scored according to the system described by Fraser and Dickinson (1968).

RESULTS

Scrapie has occurred in every one of the 222 animals which were injected and which did not die from some unrelated cause before an appropriate age for scrapie to develop. Only 10 animals died before developing any signs of clinical scrapie and 14 died during the very early clinical phase: all of these are excluded from the subsequent data. The distribution of incubation period is shown for the different generations in Fig. 1. It will be seen that the F_1 distribution is unimodal and intermediate between the two parental nodes, that each backcross distribution is in the range from the F_1 position to that of the parent stock to which it was backcrossed, being clearly bimodal in the case of Bx_2 though less

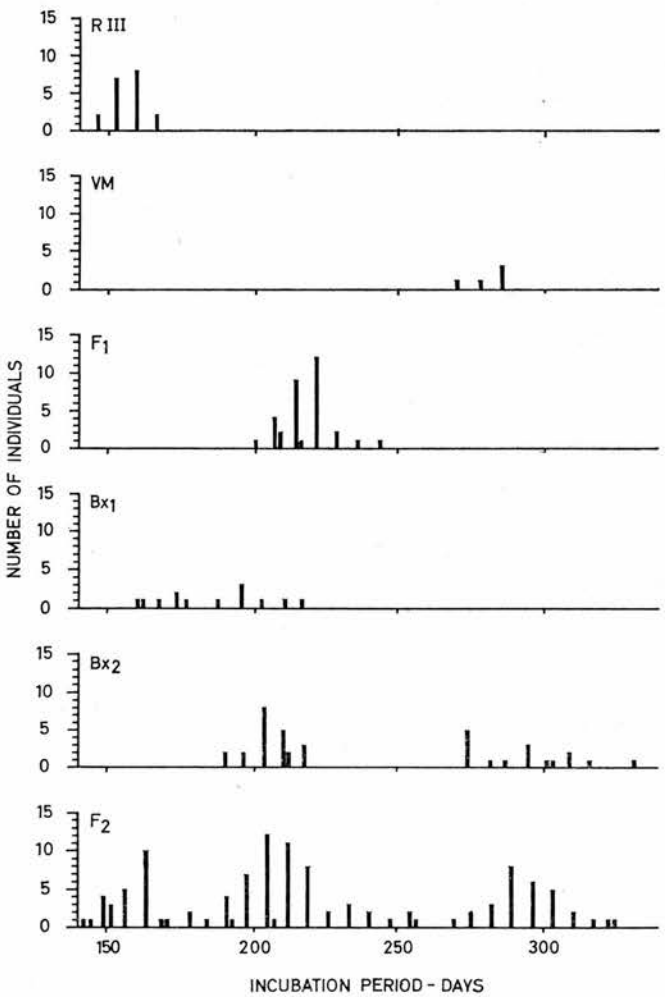

Fig. 1. Distribution of incubation periods in parental, F_1, F_2 and backcross generations.

clearly so and covering a shorter range than expected in the case of Bx_1, whereas in the F_2 generation there are three distinct modes corresponding to the F_1 and two parental distributions. There are no differences in incubation period between the sexes in any of the generations.

All these results correspond closely with the expectations for an autosomal gene with a single pair of alleles, without dominance on the scale used to measure incubation period, and with very little evidence that any other genes in the two stocks involved have any effect at all on this character. The gene has been designated "sinc" to denote scrapie incubation period and the two alleles are denoted s7 for the one which shortens incubation of the ME7 agent and p7 for that which prolongs it.

In terms of the pathological scoring system for the brain lesions, the results do not necessarily indicate that the two sinc alleles have any effect on the lesion density and distribution because the F_2 generation was unfortunately not examined histologically. The average lesion density is similar in these two mouse strains with this scrapie agent and the F_1 average lesion density in each brain region is close to the parental values and generally within the range of their standard errors. The details of this are shown in Figure 2.

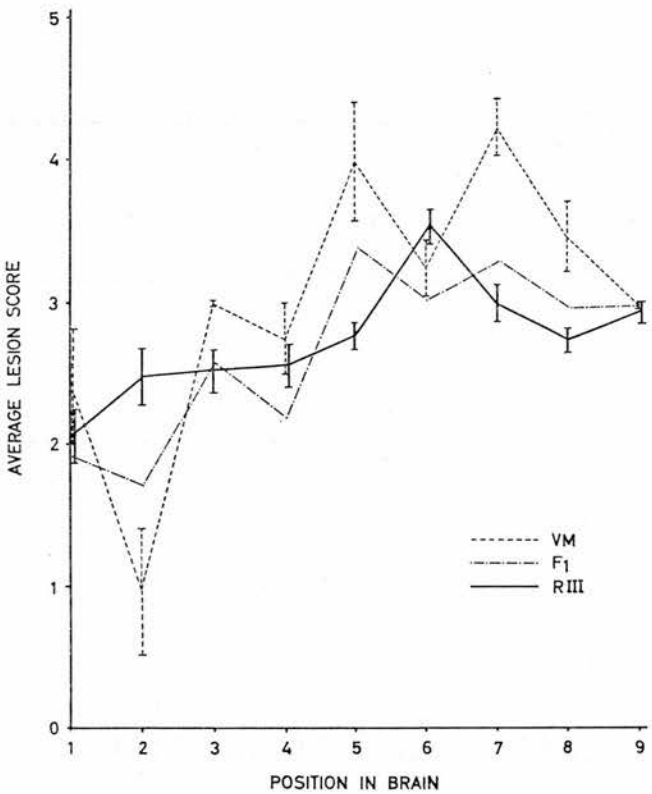

Fig. 2. The intensity of the ME7 scrapie lesion density in nine regions of the brain in parental and F_1 generations. Vertical lines indicate standard errors for parental stocks. (The pathological scoring method is that of Fraser and Dickinson, 1968).

DISCUSSION

A few minor points require some comment before the general implications are discussed.

There are some instances of small average incubation period differences between the same genotype for the sinc alleles in different generations. Thus the heterozygote, s7 p7, takes about two weeks longer in the F_1 generation than in either of the other three generations where it occurs. Similarly the p7 p7 homozygote takes about two weeks less in the VM parent than in either the F_2 or Bx_2. Variation in the average age at injection between the different groups is unlikely to have caused these differences because any effect of age at intracerebral inoculation of scrapie on incubation period is very small (Gibbs, Gajdusek and Morris, 1965; Dickinson, unpublished). A more likely source of part of these differences comes from the variation in titre of the agent which was made up on five different occasions, the different inocula being used on unequal proportions of each generation as they became available for injection. However, from other unpublished work which we have done, incubation period differences of this order would correspond to hundred fold differences in the concentration of the inoculum, which seems unlikely, and it is possible that there is some other source of variation of which we are unaware.

Another minor point for comment is the Bx_1 generation where the bimodality is hard to discern. This may be due to sampling biases associated with small numbers, but it should be pointed out that no allowance for any maternal effects has been made in the design of this experiment—as could have been done by making reciprocal crosses in each case. The Bx_1 generation, apart from the RIII parents are the only ones to have purebred RIII mothers. But any maternal effect would have to be extremely persistent to affect scrapie developing at eleven months old and no such effects have been observed on occasions when reciprocal crosses were included (Dickinson and Mackay, 1964).

It should be mentioned that in a previous publication (Dickinson and Mackay, 1964) a moderate degree of partial dominance was reported in F_1's from VM × RIII and this has not been observed in this experiment. It seems probable that the earlier observation was due to segregation occurring in the VM stock which was then at a very early stage of inbreeding, but during the intervening inbreeding and selection between VM sublines it would appear, from distinct subline differences which occurred, that other genes or alleles than those now retained were present in this stock. No strict comparisons should be drawn between the current genetic constitution of this stock and that at an early inbreeding stage.

In the intensity of the brain lesions the two parent stocks differ relatively little from one another or from the F_1. In the three positions where the two parental stocks differ most in average score, namely positions 2, 5 and 7, the F_1 mean is in these cases in an intermediate position. However, it is probably of greater significance that in general the pattern of the distribution of lesions in the F_1 cross more closely resembles that for the VM stock than the pattern for the RIII's. If these features are real, they may indicate that the sinc alleles have a slight effect on the lesion pattern, but they could equally well be due to other genes differing between the parent strains. That other such genes exist is likely because

some stocks not used here and having short ME7 incubation periods (presumably of the constitution, sinc s7 s7) show differences between their lesion distribution patterns with this agent and differ from the RIII pattern given here (Fraser and Dickinson, 1968; Fraser, unpublished).

From unpublished observations the distribution and intensity of brain lesions have shown consistent results and host strain/agent strain combinations have been found which show very large differences compared with those presented in this paper. For instance the differences between the mouse stocks C57 and SM with ME7 scrapie agent are highly significant and quite independent of the very marginal differences in incubation period. In the case of VM very substantial differences occur, when ME7 and 22A scrapie agents are compared (Fraser, unpublished observations), and the incubation periods are also different. These examples contrast with the present data where the lesion distribution is similar despite large differences in incubation period. In general the relationship between incubation period and "brain lesion intensity" seems complex, but it is apparent that the two criteria are more or less independent.

The only other example of a single gene controlling response to scrapie is in the case of Cheviot sheep challenged with the SSBP/1 scrapie agent (Dickinson, Stamp, Renwick and Rennie, 1968). In these sheep the gene appears to have a fully dominant allele which confers susceptibility to either subcutaneous or intracerebral challenge within about 18 months by the former route or about 9 months by the latter route. Although this dominant gene evidently controls absolute susceptibility rather than acting on the length of incubation, the situation in the recessive homozygote is not simple, because with intracranial challenge some of them can develop the disease but not within eighteen months of challenge.

Returning to the question of the sinc gene in mice, it is of considerable interest to find that the two alleles show no dominance, as this leads to certain deductions about the type of mechanism by which the gene acts in controlling the incubation period. If there had been complete dominance, as is often found where a single gene controls susceptibility or resistance, it would have been possible to conceive a model in which one allele determined the presence of a particular component, such as specific cell receptors. However, in the absence of dominance, when each allele has a quantitative effect, one is forced to adopt some model where the mechanism is quantitative rather than an all-or-none effect. Thus, if cell receptors are involved, then the mechanism must work in terms of the number of receptors per cell or their relative efficiency. These are presumably only acceptable models, in view of the very long time scales involved in the disease, if there is a very small number of such receptors per cell. Alternatively, the model must conceive of some biochemical intermediary such as an enzyme present either in varying dose according to which alleles are present or of two broadly similar enzymes with different efficiencies, one determined by each allele.

We have found that the two alleles do not affect the level of ME7 scrapie titre in the spleen, at least from 60 days after injection and subsequently, but appear to act by altering its access to multiplication or storage sites in brain tissue (Dickinson, unpublished): effective access to these sites is, of course, distinct from the mere physical presence of agent within the brain as a result of intracerebral

injection. These findings might suggest that attention should be directed towards discovering some biochemical differences in the brains of animals with the different alleles. However, before such an attempt the following should be taken into account. Over 20 strains of mice have now been challenged with the ME7 and 22A agents and it is an unexpected coincidence that the only stock which has a very deviant incubation period with both agents is VM: the surprising point being that this stock has the longest incubation period with ME7 agent but the shortest one with 22A agent (Dickinson and Meikle, unpublished). We are investigating the genetic basis which determines the response to the 22A agent and any relationships which it may have to the sinc gene and no purpose would be served by speculating about the outcome of this. Even so, the diametrically opposite response of the VM stock to these two strains of scrapie suggests that the control mechanism occurs at a very fine cellular level rather than at a more gross biochemical level.

SUMMARY

A gene in mice which controls the incubation period of the ME7 scrapie agent has been identified. The name "sinc", to denote scrapie incubation period, is proposed for it and the two alleles which show no dominance are designated s7 for the one which shortens the incubation period and p7 for the one which prolongs it. The distribution and intensity of brain lesion are shown to be very similar in the two homozygotes, as represented by two mouse strains, and the heterozygote, as represented by the F_1 cross. The possible modes of action of the two alleles of this gene are discussed.

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Pathogenesis of Scrapie in the Mouse : the Role of the Spleen

In mice experimentally inoculated with scrapie agent, some sites for the early increase in titre lie within lymphoreticular tissues, particularly the spleen[1,2]. The time at which the agent can first be demonstrated to have increased in amount in the spleen depends on the host genotype[2] and also, probably, on other factors such as dose and strain of agent, which influence the overall time course of the disease[3,4]. The incubation period after intracerebral inoculation of ME7 scrapie agent prepared as a saline homogenate of infected brain is about two-thirds of that for an equivalent dose by the intraperitoneal route[5]. Further, after intracerebral inoculation, levels in the spleen rise marginally earlier than they do following intraperitoneal inoculation[2].

It is widely accepted that the clinical signs of scrapie result from a progressive degeneration of nervous tissues associated with an increase in titre within the brain[1,4,6]. The earlier events in the spleen, on the other hand, are unaccompanied by any observed tissue damage or physiological alterations, and seem to indicate an early synthesis of agent somewhere in the body—either by active synthesis within the spleen or by accumulation of agent being synthesized elsewhere. If the spleen is an important site for early synthesis, then removal of this organ before inoculation might, by reducing the number of sites for synthesis, delay the later lethal events in the nervous tissue. Alternatively, removal of the spleen after inoculation would deplete the host both of important synthesis sites and a proportion of the inoculum as well.

Supernatant (500*g*, 10 min) of 1 per cent saline homogenate of brain from C57Bl mice clinically affected with ME7 scrapie was used as inoculum (intracerebrally 0·02 ml. or intraperitoneally 0·1 ml.). Splenectomy and sham-splenectomy were carried out through a left flank incision under nembutal anaesthesia. Splenectomized and control mice were mixed within cages bearing coded labels so that bias in scoring[3] incubation periods was avoided. The incidence of scrapie was 100 per cent and the clinical diagnosis was confirmed histologically. The two strains of mice used (C57Bl, BALB/c) have in common the s7 allele of the *sinc* gene, which exerts a major effect on incubation period of scrapie with ME7 agent[3].

Table 1. INCUBATION PERIODS IN BALB/c AND C57Bl MICE SPLENECTOMIZED BEFORE OR AFTER INTRAPERITONEAL INOCULATION WITH SCRAPIE (MEAN ± STANDARD ERROR)

Number of mice		Age of inoculation (days)	Splenectomy or sham before (−) or after (+) inoculation (days)	Age of splenectomy or sham (days)	Incubation period (days)			Increase in mean incubation period by splenectomy (per cent)*	Probability of difference between controls and splenectomized	
Splenectomized	Controls				Splenectomized	Controls				
		BALB/c								
—	19	28	—	Unoperated —	—	264 ± 1		—		
7	9	28	−20	8	277 ± 12	271 ± 3		4·13	< 0·001	
8	1	28	−7	21	311 ± 3	263		16·91	< 0·01	
3	1	28	+4	32	294 ± 2	263		10·53	< 0·001	
3	—	28	+7	35	301 ± 6	—	266 ± 1†	13·16	< 0·001	< 0·001
3	—	28	+14	42	292 ± 16	—		9·77	> 0·1	
3	1	28	+20	48	280 ± 15	270		5·26	> 0·1	
3	1	28	+28	56	299 ± 11	264		12·41	< 0·01	
2	1	28	+36	64	281 ± 17	257		5·64	> 0·1	
		C57Bl								
—	16	54–150	—	Unoperated —	—	261 ± 4		—		
3	1	42	−7	35	305 ± 4	260		17·3	> 0·1	
6	2	56	−21	35	293 ± 11	243 ± 13	260 ± 1·5†	12·8	< 0·01	< 0·001
7	3	93	−58	35	307 ± 16	257 ± 12		18	< 0·01	

* For calculation of increase in incubation period and probabilities of difference 266 ± 1 days used in the case of the BALB/c mice and 260 ± 1·5 in the case of C57Bl mice.

† No significant difference between shams and unoperated controls ($P > 0·1$).

Table 2. INCUBATION PERIODS IN BALB/c MICE SPLENECTOMIZED BEFORE OR AFTER INTRACEREBRAL INOCULATION WITH SCRAPIE (MEAN ± STANDARD ERROR)

Number of mice		Age of inoculation (days)	Splenectomy or sham before (−) or after (+) inoculation (days)	Age of splenectomy or sham (days)	Incubation period (days)			Increase in mean incubation period by splenectomy (per cent)*	Probability of difference between controls and splenectomized
Splenectomized	Controls				Splenectomized	Controls			
—	18	28	—	Unoperated —	—	183 ± 3		—	—
8	1	28	−7	21	179 ± 1	176		1·6	> 0·1
4	—	28	+4	32	176 ± 2	—		−3·3	> 0·1
2	1	28	+7	35	186 ± 6	181		2·2	> 0·1
3	1	28	+14	42	178 ± 4	185	182 ± 2†	−2·2	> 0·1
3	—	28	+20	48	175 ± 1	—		−3·8	< 0·02
3	1	28	+28	56	177 ± 2	180		−2·7	< 0·1
3	—	28	+36	64	174 ± 4	—		−4·4	< 0·1

* No significant difference between shams and unoperated controls ($P > 0·1$).

† 182 ± 2 days used for calculation of increase in incubation period and probabilities of difference.

Experiments consisted of various combinations of splenectomy (from 5 to 64 days of age) before or after inoculation of scrapie by the intracerebral or intraperitoneal routes. In the main experiments (Table 1), with BALB/c mice age at injection was constant, and with C57Bl mice age at splenectomy was constant.

Splenectomy both before (7 to 58 days) and after (4 to 36 days) intraperitoneal injection prolonged the incubation period by an average of 12 per cent. The prolongation occurred in every age combination in both BALB/c and C57Bl mice, but there was no significant correlation of the interval between splenectomy and injection with the degree of prolongation of incubation ($P > 0{\cdot}1$). In contrast, when an intracerebral scrapie injection was used, splenectomy had no effect on incubation period in BALB/c mice in any of the combinations tested (splenectomy 7 days before, and 7 to 28 days after inoculation) (Table 2). In subsidiary experiments in which ages of splenectomy and intraperitoneal inoculation differed from those in the main experiment, increases in incubation period over shams were also demonstrated (8·5 per cent longer in BALB/c mice splenectomized at 7 to 9 days old and inoculated 7 days later ($P < 0{\cdot}01$); 10 per cent longer in C57Bl mice splenectomized at 5 to 7 days old and inoculated 28 or 56 days later ($P > 0{\cdot}1$)).

These results support the view that scrapie agent is synthesized within the spleen. But the failure of splenectomy after inoculation to prolong incubation more than splenectomy before inoculation indicates that a functionally insignificant proportion of the intraperitoneal inoculum is retained in the spleen and that other tissues are present which have marginally slower overall rates of synthesis.

An alternative or additional explanation which might be considered is that the spleen functions in the pathogenesis of scrapie following extraneural inoculation in some way other than as a site of synthesis. Splenectomy, for example, can alter tumour growth in mice by depriving the host of a source of enhancing antibodies[7], but the apparent absence of an immune response in scrapie[8–10] makes it unlikely that the present results are explicable in any such immunological terms. There is some evidence that a spleen scrapie source differs from a brain source when histopathological criteria are compared[11]. If the agent includes some tissue specific component, removal of the spleen could conceivably alter the balance of properties of agent which eventually reaches the central nervous system, with a consequent modification of incubation period.

The suggestion that the spleen functions only as a site of agent synthesis is reinforced by the failure of splenectomy either before or after intracerebral inoculation to alter the course of the disease. Although most of an

inoculum leaves the brain within minutes of intracerebral inoculation[12], it would seem that intraneural events are initiated immediately at the time of inoculation leading to agent synthesis and nervous system degeneration, irrespective of extraneural synthesis.

H. Fraser

Moredun Research Institute,
Gilmerton, Edinburgh.

A. G. Dickinson

ARC Animal Breeding Research Organization,
Edinburgh.

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Modification of the Pathogenesis of Scrapie in Mice by Treatment of the Agent

Two major areas in scrapie research depend on estimates of the concentration of the agent: investigation of the nature of the agent by the effect of various chemical or physical treatments and of the pathogenesis of the disease. Two methods have been used for these estimates. One is conventional serial dilution, with the end-point measured either by appearance of clinical disease[1-6] or by the occurrence of the brain lesions at a chosen time after injection[7]. The second method relies on the finding that the incubation period is very closely related to dose if other variables are constant[8,9]; when this relationship has been found for a given combination of strain of agent, tissue source, route and dose of injection and genotype of recipient, the concentration of agent can be estimated from the incubation period of a single dilution group.

Because of the very long incubation period, ranging in mice from 4·5 months to 2 yr, a number of workers have chosen to terminate experiments after a fixed interval[7,8,10]. This would be fully justified if it could be assumed that the dose/time relationship for a given scrapie agent injected intracerebrally into a specified mouse strain was constant irrespective of the physical or chemical treatment of the inoculum. If, however, a treatment were to delay the normal course of pathogenesis for some reason, then arbitrary termination of experiments after a fixed interval could lead to an overestimate of loss of titre. One purpose of this report is to show that this type of phenomenon can occur.

It is generally agreed that incubation in mice is shorter after intracerebral injection of a saline homogenate of scrapie-infected mouse tissue than after peripheral injection[2]. Presumably the intracerebral route enables the agent to bypass early steps in pathogenesis, permitting earlier replication in the brain. Our experiments show that the agent can be modified so that the incubation period is similar after intracerebral or intraperitoneal injection.

Supernatant (500*g*, 10 min) of saline homogenate of brain from C57BL mice clinically affected with ME7 scrapie was prepared and used either directly as inoculum (intracerebrally 0·02 ml. or intraperitoneally 0·1 ml.) or after boiling. The various experiments differed in several respects: the number of mouse passages of the agent varied (4 to 9) as did concentration of brain in the saline

homogenate (1 per cent or 10 per cent); the period of immersion in a boiling water bath ranged from 5 to 30 min with the inoculum reaching a temperature of 98·5–99·5° C; boiling was usually followed by homogenization and centrifuging (500*g*, 10 min), with the supernatant being used as inoculum. Only an inoculum prepared without being rehomogenized after boiling failed to affect some mice; it is likely that much of the activity had been removed in the flocculated sediment during centrifugation.

Table 1. AVERAGE INCUBATION PERIODS (DAYS ± *S.E.*) IN MICE INJECTED EITHER INTRACEREBRALLY OR INTRAPERITONEALLY WITH UNHEATED ME7 SCRAPIE AGENT OR BOILED ME7 SCRAPIE AGENT

Route	Exps. using only unheated inocula	Exps. using both unheated and boiled inocula	
		Unheated inoculum	Boiled inoculum
Intracerebral	160 ± 1 (98/98)*	163 ± 2 (17/17)	315 ± 8 (38/42)†
Intraperitoneal	236 ± 2 (116/116)	233 ± 4 (15/15)	331 ± 6 (39/45)†

* Number affected/number injected.
† Survivors were confined to one of the five subgroups (see text).

Incubation period end-points were measured by the method of A. G. D., Meikle and H. F.[11] and the diagnosis was confirmed histologically; survivors were observed until senile. Six strains of mice were used in a balanced design (C57BL, SM, A2G, RIII, BRVR and BSVS). These strains have in common the s7 allele of the gene *sinc*, which exerts a major effect on incubation of the ME7 agent[11]. The results do not differ significantly between these mouse strains ($P > 0{\cdot}05$).

In the five chief experiments, the four combinations of treatment of inoculum and route of injection were used: analysis of variance showed that there were no incubation period differences between experiments or between genotypes of mice within experiments ($P > 0{\cdot}1$). On a number of other occasions only unheated inoculum was used in these six mouse strains and the results did not differ between strains ($P > 0{\cdot}05$). Table 1 shows the composite results of all the relevant experiments which we have conducted. The results for the unheated inocula used in the boiling experiments are clearly consistent with those for unheated inocula used in the other experiments. The two groups given boiled inocula do not differ significantly ($P > 0{\cdot}1$); apart from this, all groups in the chief experiments differ from one another ($P < 0{\cdot}001$).

When the ME7 agent is injected intracerebrally the average incubation period for boiled agent is 40 days longer than the average for unheated agent at the 50 per cent end-point in C57BL mice (Fig. 1). The excessive delay for the boiled inoculum is, however, much greater than may appear from this comparison because in most of the groups given boiled inoculum the incidence was 100 per cent. The additional delay due to boiling is probably in the order of 3 months, which is similar to the delay following boiling reported by Zlotnik and Rennie[6]. Fig. 1 also shows incubation periods for different doses of unheated ME7 agent injected intraperitoneally; the incubation periods for boiled inocula are within the

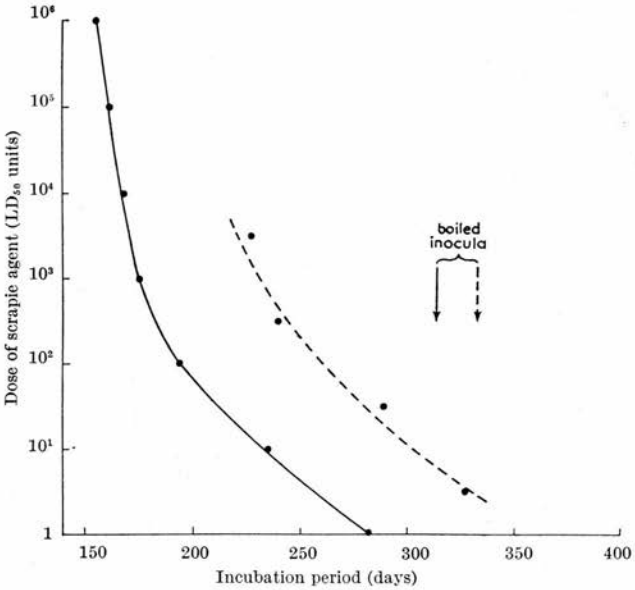

Fig. 1. Relationship between dose and incubation period following intracerebral injection[9] (———) or intraperitoneal injection (- - -) of C57BL mice with unheated ME7 scrapie agent from C57BL mouse brain. For comparison the average incubation periods for boiled inocula given by each route are shown.

range for small doses of unheated agent administered intraperitoneally. Although the curves are based only on C57BL results, no differences are evident from the limited results available for the other five strains.

Our results show that the quantitative relationship between intracerebral dose and incubation period for inoculum prepared with minimum interference does not apply where boiled inoculum is used—the course of pathogenesis must be different in some way. Because this could also apply to treatments other than boiling, these results urge caution in the interpretation of data which seem to indicate partial or total loss of activity following chemical or physical abuse of the agent, where experiments were terminated after an arbitrary interval of 9 months or even less, chosen on the basis of the dose time curve for untreated agent.

We suggest that the equality of incubation periods by the two routes for boiled inoculum may be due to the inability of boiled agent to replicate in the brain following intracerebral injection. The disease in this case would result from a primary multiplication of agent in other parts of the body, starting with that part of the inoculum which reaches other tissues almost immediately after intracerebral injection[12]. This contrasts with the course when untreated inoculum is used, when early replication presumably takes place in the brain.

The failure of agent in the boiled inoculum to replicate in the brain could be explained if the agent present in scrapie brain material has a heat-labile host component of some type which facilitates access to replication sites in brain cells.

A. G. DICKINSON
ARC Animal Breeding Research Organization,
Edinburgh.

H. FRASER
Moredun Research Institute,
Gilmerton,
Edinburgh.

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GENETICAL CONTROL OF THE CONCENTRATION OF ME7 SCRAPIE AGENT IN THE BRAIN OF MICE

By

A. G. Dickinson and Veronica M. H. Meikle

A.R.C. Animal Breeding Research Organisation, Edinburgh

and

H. Fraser

Moredun Research Institute, Gilmerton, Edinburgh

INTRODUCTION

Eklund, Kennedy and Hadlow (1965) have investigated the increase in titre of scrapie agent in a variety of organs from mice of a random-bred stock injected subcutaneously with the Chandler strain of scrapie agent. They found the earliest increases in titre to be associated with spleen and lymph nodes, the build-up commencing before four weeks after injection, with the titre reaching a plateau at a concentration in the 10^{-5} to 10^{-7} range from about eight weeks, which was roughly a quarter of the way through the incubation period. In the brain, scrapie was first detected after sixteen weeks and with a similar plateau concentration to that for spleen. The plateau was reached in brain after about twenty-four weeks. Some organs yielded only low sporadic concentrations of scrapie late in incubation: uterus and liver were of this type. Other tissues, of which serum was one, failed to give any evidence of the presence of scrapie agent.

The experiment reported here aims at determining the concentration of scrapie in mouse brain, spleen, and blood at three or four stages of the incubation period following injection of the ME7 scrapie agent. The principal difference from the earlier work described above is that four inbred strains of mice are considered, one of which (VM) has an incubation period double that in the other three strains. The control of the incubation period of this scrapie agent is due to an autosomal gene, designated sinc, neither allele of which shows dominance (Dickinson, Meikle and Fraser, 1968). Our basic purpose was to investigate possible modes of action of different alleles of the sinc gene.

MATERIALS AND METHODS

General outline of experiments. In the main experiments either brain, spleen or whole blood from one female mouse of each of four strains removed approximately 60, 110 and 150 days after challenge, and also at 280 days in the case of the long-incubation VM stock, were used. These tissues were stored frozen for several months until being made up as standard saline suspensions for intracerebral injection into C57 mice. The concentration of scrapie in the inoculum was estimated from the incubation period in these C57 mice. The procedure for estimating titre using only one concentration of inoculum has been found to be satisfactory if the experimental design and management are carefully planned. Subsidiary serial dilution experiments have been carried out in which conventional titration with serial log dilutions of brain or spleen were used. The results from these have been used as quantitative standards for the interpretation of results in the main experiments.

Mouse strains. Two fully inbred strains, C57BL and SM, together with two strains, LM and VM, at the seventh inbred generation were used. The VM strain is homozygous for the p7 allele of the sinc gene which results in a long incubation period with the ME7 scrapie agent; the three other strains are homozygous for the s7 allele, which gives a short incubation period (Dickinson, Meikle and Fraser, 1968).

The mice were maintained, up to six to a cage, throughout the experiments at 21 to 22°C. and at 50 to 60 per cent. R.H.

Inoculum for mice used as source of tissues. ME7 scrapie agent at the fourth passage in mice was used, the last two intracerebral passages having been in C57BL mice. The inoculum for the mice which were killed to collect tissues, was prepared from two freshly removed brains of female C57 mice with advanced clinical scrapie. It consisted of the supernatant of a 1 per cent. saline suspension of brain spun at 50 *g* for 5 minutes. This was administered to each of four 3-week-old female mice of each strain as a 0·02 ml. intracerebral injection and a 0·1 ml. intraperitoneal injection using a 25 G needle. In each of the strains with short incubation periods only three of the four mice contributed tissues for assay; the fourth mouse was used solely for clinical and histological observations. For the assays, C57BL mice were used to determine the concentration of scrapie in tissues from mice of all four donor strains.

Removal of infected tissues. The results presented in this paper are part of a larger series in which many tissues were taken for assay and which included embryos. The procedure necessitated killing pregnant donors and led to slight variations between strains in the exact date of killing. The mice were mated with uninoculated males of their own strain and attempts were made to synchronise litters in the different strains. The intention was to kill one female at about the 15th day of pregnancy, as near to 60, 110, 150 and 280 days as possible: small deviations from these intervals resulted from mating delays. In the event, only those animals in the preclinical phase of scrapie became pregnant. The animals were killed by ether anaesthesia.

In order to minimise cross-contamination of tissues at all stages between the handling and removal of the different tissues maximum aseptic precautions were taken either by intense flaming of instruments or by exchanging them for clean sterile instruments. Tissues were removed in the following order, care being taken to minimise oozing of blood on to abdominal organs which were to be removed subsequently: blood (by cardiac puncture with a sharpened pasteur pipette), gravid uterus, part of liver, ovaries, spleen, kidney, lung, heart, thymus, salivary gland and brain. Each tissue was stored in a separate bottle and kept in dry ice for several months until all the mice had been killed. The containers with tissues from the thirteen donors were then transferred to a freezer at −30°C.

Preparation of inocula for assay of scrapie concentration. Brain and spleen were prepared in glass grinding tubes as 10^{-2} saline suspensions and whole blood as 10^{-1} saline suspensions. They were centrifuged at 300 *g* for 10 minutes and 0·02 ml. of the supernatant fluid was inoculated intracerebrally into 3 to 4-week-old C57BL mice.

Design of assay experiments. Inoculum was prepared from each individual tissue on two occasions and on each occasion half of the recipients were from each sex. Each tissue was assayed using a total of 11 to 15 C57 weanling mice. In any given cage the animals were never all of the same inoculum group: either half were injected with inoculum from one donor and the rest from another or a saline-injected control animal was present. The inoculation groups which shared cages were chosen at random and cage labels were in a coded form. The design was intended to minimise inaccuracies in preparation of the inocula, any fluctuating environmental effects on incubation period and any subjective bias in determination of the clinical end-point of incubation, the method for which has been described (Dickinson, Meikle and Fraser, 1968). All brains of assay animals were examined pathologically for confirmation of scrapie.

One of the spleens used in the above assay experiments was also titrated by conventional serial dilutions to provide a standard for the interpretation of incubation periods in terms of the concentration of agent. For brain, results from unpublished work are used as standard criteria. No attempt has been made to carry out a serial dilution titration using blood. The spleen used for conventional titration was from the C57 female tissue donor in the main experiment which was killed 105 days after challenge : a 10 per cent. saline suspension was spun at 2,000 *g* for 15 minutes and the supernatant diluted for the series 10^{-1} to 10^{-8}. Two to four mice of each sex were injected intracerebrally (0·02 ml.) in each dilution group and the mice in each group were randomly allocated, within sex, among the cages. Observation of the survivors was continued for 18 months.

RESULTS

The brains of all donor mice were examined histologically. The characteristic vacuolation was absent from all at 60 days; at 110 days no such lesions were present in the VM, early lesions, consistent with this stage of scrapie, were found in the SM and C57 while those in the LM were rather more advanced; at 150 days advanced lesions were present in SM, C57 and LM but none had developed in the VM, while in the 280 day VM the lesions were in an advanced condition.

The incubation periods for mice injected with different dilutions of scrapie spleen are given in Table 1. This information can be interpreted in terms of the incubation period which results from challenge with different doses of scrapie agent in spleen by calculation back from the LD_{50} end-point (Fig. 1). This figure includes information on results for ME7 scrapie agent in the brain (Dickinson and Meikle, unpublished). The different titrations on which these curves are based demonstrate the high degree of repeatability of the relationship between incubation period and the concentration of the ME7 agent within a given genotype of assay animal.

The average incubation periods which resulted from injection of spleen from the thirteen mice in the main experiment are shown in Fig. 2 and those for injection of brain in Fig. 3. In both these figures the scale is also given for the interpretation of the results in terms of concentration of scrapie agent in the assayed tissue, using the information from Fig. 1 as the basis of estimation.

TABLE 1

INCUBATION PERIOD (WEEKS) AND INCIDENCE OF SCRAPIE FOR SERIAL DILUTIONS OF ME7 SCRAPIE SPLEEN INJECTED INTRACEREBRALLY IN C57 MICE

Dilution	*Incubation period (weeks)*	*Incidence %*
10^{-1}	24·4 ± 0·7	100
10^{-2}	24·5 ± 0·2	100
10^{-3}	27·3 ± 0·5	100
10^{-4}	36·0 ± 4·3	100
10^{-5}	45·9 ± 5·9	83
10^{-6}	—	0
10^{-7}	—	0
10^{-8}	—	0
Titre (Reed-Muench)		$10^{-5\cdot4}$

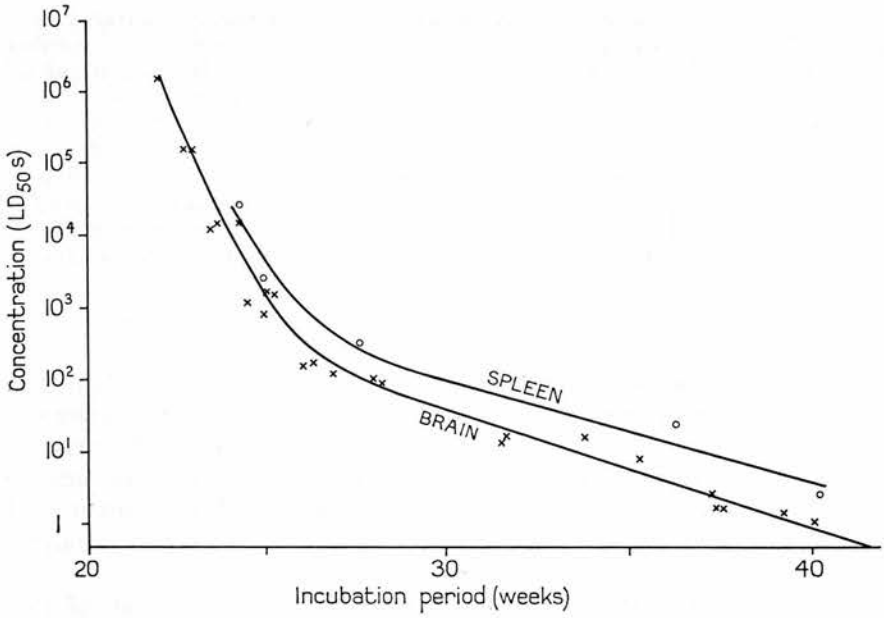

Fig. 1. The relationships between incubation period and concentration of ME7 scrapie agent injected intracerebrally into C57 mice.

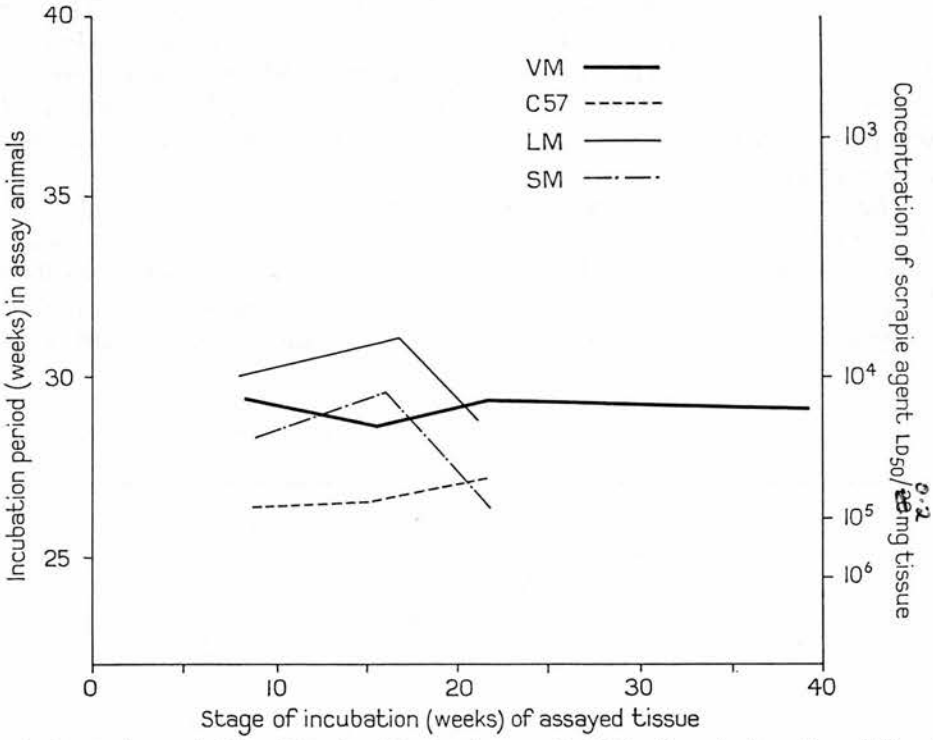

Fig. 2. Incubation period in C57 mice following intracerebral injection of spleen from different strains of mice at different stages of incubation. The right-hand scale gives the estimated concentration of scrapie in each spleen.

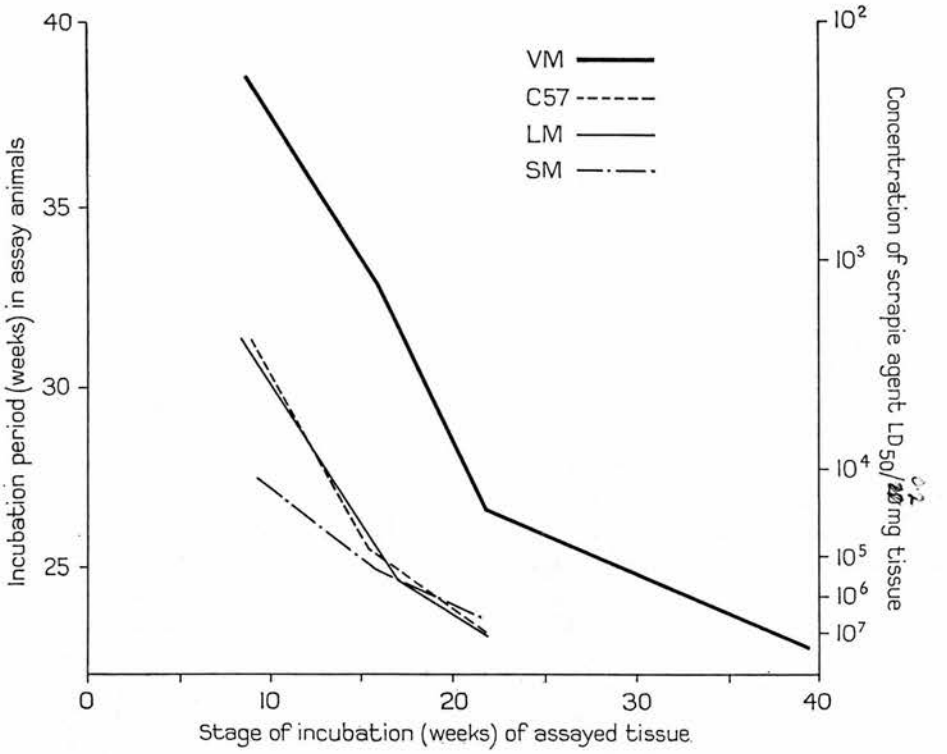

Fig. 3. Incubation period in C57 mice following intracerebral injection of brain from different strains of mice at different stages of incubation. The right-hand scale gives the estimated concentration of scrapie in each brain.

TABLE 2

INCIDENCE (%) OF SCRAPIE IN C57 MICE INJECTED INTRACEREBRALLY WITH BRAIN, SPLEEN OR BLOOD, FROM SCRAPIE-INJECTED MICE OF FOUR STRAINS, AT VARIOUS STAGES OF INCUBATION

Tissue	*Mouse strain*	*Stage of incubation in days*			
		56–63	*105–117*	*146–152*	273
Spleen	C57	100	100	100	—
	SM	100	100	100	—
	LM	100	82	100	—
	VM	100*	100	100	100
Brain	C57	100	100	100	—
	SM	100	100	100	—
	LM	100	100	100	—
	VM	20	100	100	100
Blood	C57	0	17(†287, 294)	8 (535)	—
	SM	0	0	0	—
	LM	0	0	0	—
	VM	0	0	8 (387)	0

*One with long incubation deviating by 10 standard deviations from the rest of the group is excluded from results in Fig. 2.

†Individual incubation periods (days) — = not applicable

With the mice which were injected with whole blood, there have been only occasional cases of scrapie and these are given in Table 2 which also shows the incidence of scrapie when the corresponding spleen or brain was used.

DISCUSSION

On the basis of the type of experiment using blood injections reported in this paper it is impossible to exclude the occurrence of a brief "viraemic" phase during incubation of scrapie. The purpose of these experiments was, therefore, to cover the possibility that the estimates of scrapie concentration for the other organs might be seriously biased by their blood content. In failing to demonstrate any consistent or high levels of scrapie in blood, these results show that blood content need not be taken into account for the interpretation of the rest of the data.

There have been previous reports of scrapie occurring after injection of serum from an affected sheep (Gibbs, Gajdusek and Morris, 1965) and from affected mice (Clarke and Haig, 1967) into mice. In the latter report the long incubation periods suggested a low concentration of scrapie and the incidence was reduced when blood was collected by cardiac puncture instead of using blood flowing from severed neck vessels. These authors admit the possibility of tissue contamination as the source of scrapie and we would suggest that this is the most likely explanation for our cases. It is relevant to note that fibroblasts can regularly be grown from human blood collected by venepuncture : the possibility that these came from surrounding tissues carried in by the needle could not be excluded (Paul, 1958). In the experiment of Eklund *et al.* (1965) where tissues were assayed from mice at eleven stages of incubation, scrapie agent was not detected in either whole blood or serum at any stage. Pattison and Millson (1962) also failed to find scrapie agent in blood in their experiments with goats.

It is possible that some systematic bias may be introduced into the estimation of agent concentration on the basis of incubation period, due to antigenic differences between tissue donor and assay strains. Although no direct estimate of this possibility is available there are two reasons to expect that such bias would not be large. We have used twelve strains of inbred mice in an attempt to find any which vary widely in the estimate of scrapie titre in C57 brain and the maximum differences of sensitivity are of a ten-fold order (Dickinson, unpublished). Secondly, the brain lesion distribution, although it is affected by the tissue used as a source of agent, is unaffected by mouse strain differences where the same tissue is used from each strain (Fraser and Dickinson, 1967), and, as can be seen from Fig. 1, the dose-incubation period relationship is almost the same for the different tissues.

It is possible that there are small differences between strains in the plateau concentration of scrapie in the spleen, but these are only in the order of a ten-fold difference. What is much more striking is that there is a prolonged concentration plateau from at least 60 days and this is so for the short and long incubation period strains. The VM strain, though radically different from the rest in the gene controlling incubation period, does not differ in the concentration of scrapie in spleen. On the basis of interim results in current experiments, it is probable that the very early multiplication rate in the spleen is lower in the VM strain and independent of route of injection.

The major effect of the two alleles of the sinc gene is to control the concentration of scrapie in the brain—and presumably also in other parts of the central nervous system. This difference between the genotypes could be due to differences in the replication of scrapie in the brain or to differences in accumulation, within the brain, of the scrapie agent replicating elsewhere. The latter possibility may be less likely in view of the probable absence of scrapie from the circulation, at least for most of the time, and, pending clear evidence to the contrary, it seems simpler to accept the former alternative. A feature of the results is that the "long" and "short" genotypes share a remarkable property, that at a common proportionate stage of incubation they have the same concentration of scrapie in the brain.

The rate of multiplication in the brain is similar for both alleles once multiplication has started, but the difference appears to relate to the relative delay in the onset of multiplication. This could be interpreted as the p7 alleles in the VM strain delaying access of the agent into the central nervous system. If this is so, the type of access must be in terms of the effective entry of the agent into susceptible cells in the brain, as distinct from the crude presence of the agent within the brain, because all the mice received an intracerebral injection. Given the very long time scale involved it is difficult to understand what is involved in the distinction which has just been made, because it implies that an essential part of the inoculum has persisted within the brain, but has failed to replicate over many weeks. Because both genotypes have much longer incubation periods when challenged intraperitoneally than intracerebrally it seems evident that some agent, or an essential component of it, remains in the brain after intracerebral injection. The efficiency of clearance of inoculum from the brain immediately after injection may differ among genotypes, but this is not really acceptable as a full explanation for the mode of action for the alleles for two reasons. One is that the relative lengths of incubation are similar following injection by alternate routes in the two genotypes using ME7 agent, e.g. C57; i.c. (0·02 ml.) 23·5 weeks, i.p. (0·01 ml.) 36·6 weeks: VM; i.c. (0·02 ml.) 36·4 weeks, i.p. (0·1 ml.) 61·7 weeks (Dickinson, unpublished). The second reason has already been mentioned, namely, the lower rate of very early increase of the scrapie titre in VM spleens. It is unknown whether the agent needs to be modified in some way before it can effectively enter susceptible brain cells, but this is one process in which differences due to the sinc alleles could potentially arise. Alternatively cells which are susceptible to the agent in the form in which it occurs in the inoculum may be less frequent in the VM genotype.

In a previous publication (Fraser and Dickinson, 1968) the stage at which lesions first appeared, and the subsequent rate of their overall development was shown to relate directly to the incubation period. After intracerebral inoculation of the ME7 agent, the lesions were first demonstrable early in the second half of incubation for both long and short incubation period genotypes. There appears to be, therefore, a consistent correlation between the development of brain lesions and the increase in titre of agent within the brain. It was also pointed out that the vacuole number bore an approximately logarithmic relationship to the score which makes it tempting to suggest that there exists a direct numerical relation-

ship between the number of infective units in the brain and the number of vacuoles when the ME7 agent is used in these strains of mice.

SUMMARY

Genetical variation in the incubation period of the ME7 scrapie agent in mice is controlled by the gene, sinc. The strain with the p7 alleles for long incubation period does not differ in the titre of scrapie in the spleen from strains carrying the short incubation period s7 alleles, for the period subsequent to 60 days after intracerebral injection. The difference between the alleles is shown in the titre of the agent in the brain where there is a long initial delay in the increase of the titre with the p7 alleles although the subsequent rate of increase and terminal titre are similar for both alleles.

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GENETICAL CONTROL OF THE CONCENTRATION OF ME7 SCRAPIE AGENT IN MOUSE SPLEEN

By

A. G. Dickinson

A.R.C. Animal Breeding Research Organisation, Edinburgh

and

H. Fraser

Moredun Research Institute, Gilmerton, Edinburgh

INTRODUCTION

We have recently shown that the *sinc* gene in mice, which controls the incubation period of the ME7 group of scrapie agents, has a major effect on the increase of scrapie titre in the brain throughout the period which was studied, starting at 60 days after intracerebral injection and continuing up to the terminal phase (Dickinson, Meikle and Fraser, 1969). During this period, the mouse strains with the s7 alleles, of which LM was one, have a progressive increase in titre in the brain from the earliest stage, whereas the long incubation period VM strain, with the p7 alleles, showed a long delay in the initiation of titre build-up in the brain but, once started, the rate of increase was broadly similar to that in the other strains. In contrast, the titre in the spleen was at a similar high level in both genotypes at all the stages from 60 days onwards.

These findings raised the question whether stocks carrying the different *sinc* alleles would be found to differ in spleen titre at a very early stage of incubation or whether the action of this gene might be confined to scrapie in the brain—either in terms of the access of the agent to the brain or delay in its replication there.

This paper considers changes in titre in the spleen during the four weeks after inoculation in two mouse stocks carrying different *sinc* alleles. Intraperitoneal and intracerebral routes of injection were compared because these are well known to exert a large effect on the incubation period—the peripheral route takes 60 to 70 per cent. longer—and the role of the spleen, as reflected in titre changes, may differ according to route of challenge.

MATERIALS AND METHODS

Scrapie agent, doses and routes. The mice whose spleens were to be assayed were inoculated with fifth mouse-passaged ME7 strain of scrapie agent from a clinically affected female C57BL mouse, using brain as the source of agent. The brain was made up as a 10^{-2} saline suspension centrifuged at 500 g for 10 minutes and the supernatant stored at −30°C. until required. For injection this was thawed, reground in a glass tissue grinder and recentrifuged as before and this supernatant used for injection, either as a 0·02 ml. intracerebral (i.c.) dose or a 0·1 ml. intraperitoneal (i.p.) dose. The dose of scrapie which each mouse received was between 10^4 and 10^5 LD_{50} as estimated in C57BL mice.

Mouse strains. The stock LM (14 to 15 generations inbred), homozygous for the s7 allele, was used as the strain having a short incubation period. With the present

dose of this agent these periods are about 24 weeks i.c. and 38 weeks i.p. The VM stock (12 to 13 generations inbred), homozygous for the p7 allele, has with this agent a long incubation period of about 48 weeks i.c. and 77 weeks i.p. The age at injection was 3 to 6 weeks; the age composition of the different experimental groups was balanced and each litter was split between groups.

Design of the experiment. The animals from which spleens were to be assayed for scrapie titre were killed by cervical fracture and the spleen removed with sterile precautions, weighed immediately and each stored in a separate bottle at −30°C. Spleens were removed from two animals of each sex in each mouse strain at four days after injection and at each subsequent five-day interval up to 29 days. The concentration of scrapie was estimated by intracerebral inoculation of C57BL mice as given previously (Dickinson *et al.*, 1969), but with the modification that the inocula were from pooled spleen tissue from the four mice of each stock on each occasion. Each inoculum was the supernatant of a 5×10^{-2} spleen suspension in saline which had been spun at 500 g for 10 minutes : each mouse received 0·02 ml.

Some details of the pooling and sampling procedure are necessary so as to validate the comparisons which will be made between stocks and between stages of incubation. There was, of course, variation in weight between individual spleens (45 to 156 mg.) mainly due to age and preweaning litter size, but the average weight of the groups of four spleens showed no trend with the stage of incubation and was not significantly different between strains. Only part of each spleen was used in making the pools, but the proportion of each was made as close to one quarter of the whole as was visually possible. So as to give a basis for comparison with previous data the supernatant of a tissue homogenate was used as inoculum rather than the whole homogenate; because of this the estimates are probably approaching the order of a hundred-fold less than the full concentration of scrapie which the spleen tissue could yield.

RESULTS

The incidence of scrapie in the assay animals given in Table 1 shows the average incubation periods in the affected animals, together with the estimate of scrapie concentration in the spleens being assayed. The C57 assay mice were kept under observation for 18 months, which is adequately in excess of the duration which we have previously shown to be necessary with this strain of scrapie agent.

A progressively increasing amount of scrapie was found in the LM spleens during the four-week period studied. However, for the VM stock, there was no consistent evidence of any increase in concentration in the spleen during the same period, as it was only erratically possible to detect the presence of agent and then only at very low levels. With regard to any differences between the two routes of challenge the VM data are largely uninformative, because of the marginal quantities involved, and detection of the agent in proportionately more of the intraperitoneally injected VM mice is probably of no significance. However, in the LM stock, once the amount of agent is clearly increasing, the concentration is somewhat higher in the intracerebrally injected mice, even though the dose by this route is only one-fifth of that given intraperitoneally.

DISCUSSION

With regard to the low level of scrapie in the VM mice, the inoculum which the LM and VM agent received would be diluted due to spread from the site of injection and again during preparation of the spleen homogenates, but there

TABLE 1

INCIDENCE OF SCRAPIE AND AVERAGE INCUBATION PERIODS

C57 mice injected intracerebrally with spleen from mice of two strains which had been injected with ME7 scrapie agent

Stage of incubation in spleen (days)	*Spleen donor type*											
	Intracerebrally injected LM			*Intraperitoneally injected LM*			*Intracerebrally injected VM*			*Intraperitoneally injected injected VM*		
	*Incidence**	*Incubation period (± SE) (days)*	*Conc. of scrapie†*	*Incidence*	*Incubation period (± SE) (days)*	*Conc. of scrapie*	*Incidence*	*Incubation period (± SE) (days)*	*Conc. of scrapie*	*Incidence*	*Incubation period (± SE) (days)*	*Conc. of scrapie*
4	6/6	264 ± 20	5–10	6/6	307 ± 21	1–5	0/6	—	0	0/6	—	0
9	6/6	255 ± 20	10	4/5	262 ± 18	<5–10‡	0/6	—	0	0/6	—	0
14	8/8	246 ± 17	10–20	7/7	241 ± 17	20	0/6	—	0	3/6	256 ± 9	<10
19	6/6	224 ± 6	30	6/6	240 ± 11	20	0/6	—	0	3/6	270 ± 14	<5
24	6/6	206 ± 2	100	6/6	214 ± 4	70	1/6	255	<10	1/5	261	<5–10
29	6/6	203 ± 4	100	6/6	214 ± 3	70	2/6	250 ± 14	<10	3/6	295 ± 33	<5

* Number of cases of scrapie / number injected.

† Number of LD_{50} units of scrapie per unit dose of inoculum, estimated from incubation period by method of Dickinson *et al.* (1969).

‡ Where the incidence is below 100 per cent the estimated concentration, on the basis of average incubation period, is necessarily a maximum.

is no evidence that this original inoculum would not have remained infective. If the dilution factor is taken into account, the levels of infectivity detected in all the VM spleens and in the 4-day LM spleens could be attributable to carry-over of the original inoculum. The results provide no indication either that the agent has replicated in the spleen in these cases, or that the agent which might have replicated elsewhere has been concentrated in these spleens.

At 60 days after intracerebral injection the concentration in the spleen, as estimated by the same methods as used here, is about 5×10^2 LD_{50}/unit dose of 5 per cent. inoculum of spleen tissue in both VM and LM strains of mice (Dickinson *et al.*, 1969). The present results show that the concentration in LM spleens at 29 days is almost up to the plateau value previously found at 60 days. In contrast, only traces of scrapie were detected in any of the VM spleens. Although we have no data on the rate of change in VM spleens between 29 and 50 days it is reasonable to infer that once effective increase of titre has started it must proceed at a rate which cannot be very much less than that found during the first four weeks in LMs. The major action of the two *sinc* alleles on scrapie concentration in the spleen is, therefore, similar to that which was found in brain at a much later stage of incubation—that is, in terms of initial delay with the p7 allele, rather than a consistent difference in the rate of increase of titre throughout incubation.

SUMMARY

The titre of ME7 scrapie agent in the spleen was estimated at various intervals up to 29 days after inoculation in two strains of mice. The mice differed in alleles of the *sinc* gene which controls the incubation period of the agent used. The long incubation period strains were found to have a four-week delay in the initiation of titre increase in the spleen, whereas the increase starts almost immediately in the short incubation period stock.

ACKNOWLEDGMENT

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BILATERAL THALAMIC CALCIFICATION IN AGEING MICE

H. FRASER
ARC Animal Breeding Research Organisation, Edinburgh

PLATE LI

DURING the histological examination of the brains of many strains of mice to confirm a diagnosis of scrapie in a research programme, bilateral v. Kóssa-positive deposits were found in the ventral thalamic nuclei.

MATERIALS AND METHODS

Mice were killed by either hypercapnic anoxia or decapitation, and the brains were removed immediately and fixed in 4 per cent. formaldehyde in saline. Transverse paraffin-wax-embedded sections were prepared and stained with haematoxylin and eosin (HE). If possible, sections were stained by the alizarin red S, v. Kóssa's, the periodic acid-Schiff, Ziehl-Neelsen's, Perls' prussian blue and the congo red methods whenever thalamic calcification was suspected.

RESULTS

On microscopic examination of HE sections the lesions appear as deep blue-black irregular deposits distributed symmetrically in the dorso-medial parts of the ventral thalamic nuclei. The lesions measure up to 100 μ and several deposits of different shape and size are usually present together (fig. 1). In two cases in which less extensive lesions occur, the calcium deposits appear to be related to small blood vessels. The cranial or caudal extent of the incrustations is very limited, because in some cases, when spare sections were required for differential staining, the lesions were lost during re-trimming. Of all the additional staining methods used, the only positive results were obtained with the v. Kóssa method (fig. 2).

Calcification within the thalamus was identified during the examination of over 2000 brains from mice of many strains whose ages ranged from less than 100 days up to over 2 yr, the majority being less than 250 days when killed.

The condition was identified 34 times in 4 mouse strains, in two F_1 crosses and in both sexes. A variety of experimental situations was involved, and of the 34 cases 3 also had cerebral lesions of scrapie. With one exception all were very old mice at the time of death, their ages ranging from 279 to 827 days, the exception being a 185-day-old C57 male in which less extensive lesions were found. Twenty-eight animals had received intracerebral inoculations. The details of each affected animal are given in the table.

DISCUSSION

Ectopic calcification can result from a number of nutritional or infective causes or as a secondary phenomenon and, in general, results either from a local tissue change (dystrophic calcification) or from an alteration in the normal homeostatic mechanisms that control calcium metabolism (metastatic calcification).

It does not appear that local tissue damage is the starting stimulus for the symmetrical thalamic calcification, and the absence of information about metastatic calcification in visceral organs makes it unconvincing to postulate disturbed homeostasis. Although there is no information about the state of the parathyroids in the affected mice, it is worth recalling that bilateral symmetrical calcification of the basal ganglia can occur in idiopathic hypoparathyroidism in man. It is perhaps significant that all but one of the 34 mice were very old, in view of the increased cellular concentration of calcium with age in many types of cells (Cameron, 1952).

TABLE

Details of 34 mice with thalamic calcification

Case no.	Strain	Sex	Age at death in days	Inoculation details	Other brain lesions
1	MM	M	563	I/C and I/P	...
2	MM	F	583	I/C and I/P	lymphosarcoma including CNS
3	VL	M	584	I/C and I/P	...
4	VM	F	559	I/P	...
5	VM×RIII	M	407	I/C	scrapie
6	VM	M	523	...	...
7	C57	M	185	I/C	scrapie
8	VM	F	598	...	...
9	VM	M	304	...	...
10	VM	M	641	...	...
11	C57	M	279	I/C and I/P	...
12	VL	M	827	I/P	...
13	VM×RIII	F	708	I/C and I/P	...
14	C57	M	804	I/C	...
15	C57	M	807	I/C	...
16	C57	M	589	I/C	...
17	C57	M	589	I/C	...
18	C57	M	589	I/C	...
19	C57	M	589	I/C	...
20	C57	M	731	I/C	...
21	VM	M	416	I/C	scrapie
22	RIII	M	709	I/C	...
23	RIII	M	709	I/C	...
24	RIII	F	709	I/C	...
25	MM	M	628	I/C	...
26	MM	F	628	I/C	...
27	C57	M	793	I/C	...
28	C57	F	752	I/C	...
29	C57	M	733	I/C	...
30	C57	M	752	I/C	...
31	C57	M	755	I/C	...
32	C57	M	764	I/C	...
33	C57	M	752	I/C	...
34	C57	F	752	I/C	...

I/C = Intracerebral; I/P = intraperitoneal.

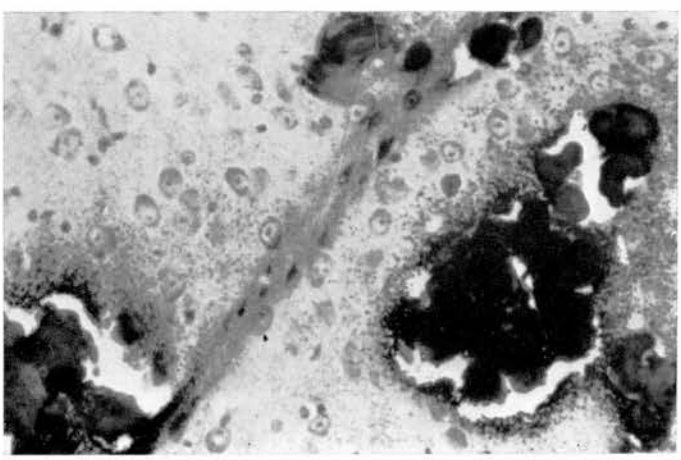
Calcium, together with iron, has been identified in the brains of old horses and cattle, and in man (Hurst, 1934; Strassmann, 1949) and was related to blood vessels. The lesions described in this paper did not contain demonstrable iron, although an association with small vessels was suspected in two apparently early cases.

SUMMARY

Bilateral symmetrical calcified deposits in the thalamus, occurring in 34 ageing mice, did not contain demonstrable iron and probably arose in relation to blood

THALAMIC CALCIFICATION IN AGEING MICE

FIG. 1.—Deposits of calcium in the thalamus. Haematoxylin and eosin. ×30.



2.—Positive v. Kóssa aining of mineral de- sits. ×500.

vessels. The lesions did not follow any recognisable disease process and may be related to similar lesions found in senile human and other animal brains.

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EOSINOPHILIC BODIES IN SOME NEURONES IN THE THALAMUS OF AGEING MICE

H. FRASER
ARC Animal Breeding Research Organisation, and
Moredun Institute, Gilmerton, Edinburgh

PLATE LXXVIII

THE histological changes that occur with normal ageing in laboratory animals become important when experimental investigations requiring observations over a considerable period of their life-span are undertaken. Experimental scrapie in the mouse is an example of this, because the very long incubation periods with some agents in some mouse strains can approximate to their life-span (Dickinson and Meikle, 1969).

The observations reported here were made on the brains of ageing mice of many strains, from a colony that is used almost exclusively for investigations of various aspects of scrapie infection. During the histological examination of brains of many of these mice eosinophilic bodies were found in the cytoplasm of neurones in the thalamus. Their topographical distribution and staining properties resemble closely those of inclusions described previously in normal mice by Nicolau, Kopciowska and Balmus (1933), who did not comment on an association with ageing.

MATERIALS AND METHODS

Mice were killed by hypercapnic anoxia or decapitation, and the brains were removed immediately and fixed in either 4 per cent. formaldehyde in saline or Carnoy's fixative. Transverse and occasionally longitudinal paraffin-embedded sections were prepared and stained with haematoxylin and eosin (HE). In addition some sections were stained by the following methods: periodic acid-Schiff (PAS); Feulgen's; ninhydrin-Schiff (Yasuma and Ichikawa, 1953); Lendrum's phloxine tartrazine; Gomori's paraldehyde fuchsin; methyl green-pyronin; Giemsa's; Glees and Marsland's for axons; sudan black B; Ziehl Neelsen's; and the tannin iron method for protein (Dixon, 1959). Cleared unstained sections were also examined by fluorescent microscopy prior to differential staining.

RESULTS

On microscopic examination of HE sections the eosinophilic bodies are seen within a proportion of neurones in the thalamus (figs. 1–3). They are most frequently round, but are sometimes oval or rod-shaped and occasionally appear disc- or saucer-shaped with a pale centre. A striking feature in some is a minute central basophilic particle *c.* 2 μm in diameter. Rarely a thin strand appears to connect this particle to the chromatin of the nucleus (fig. 1). Frequently the eosinophilic bodies are contained within a clear space, and although they almost invariably occur in the cytoplasm of the neurone, they are rarely found in a neuronal nucleus and only occasionally within astrocytes. Their size varies between 5 and 20 μm diameter when round or in length when elongated. They are confined to neurones within the dorsal and ventral thalamic nuclei. The staining and optical properties of these bodies are given in table I.

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The inclusion bodies were identified during the examination of over 4000 brains from mice of many strains whose ages ranged from less than 100 days to over 2 yr, the majority being less than 250 days old when killed. They were seen in 80 brains from 6 strains of mice and in 2 F1 crosses, and in both sexes. A variety of experimental situations was involved and 4 of the 80 cases have lesions of scrapie. With the exception of 2 mice—232 and 286 days of age—that did not have scrapie and in which only occasional neurones are affected, all the mice were over 1 yr old, their ages ranging from 405 to 1126 days. A large proportion had received intracranial injections, usually of normal or of suspect scrapie-infected material, and with the 4 exceptions were mice that failed to develop the disease. Some animals had received intraperitoneal or multiple mixed inoculations, and a small proportion were uninoculated. Other age-associated neuropathological features are frequently seen. An oedema

TABLE I

Staining and optical properties of the eosinophilic bodies found in the thalamic neurones of mice

Method	Result	Conclusion
Periodic acid-Schiff	Negative	Not carbohydrate or lipofuscin
Gomori's paraldehyde fuchsin	Negative	
Sudan black B	Negative	Not lipid or lipofuscin
Fluorescent microscopy	Not fluorescent	Not lipofuscin
Ziehl Neelsen's	Not acid-fast	Not lipid
Giemsa's	Pink with a purplish tinge	Slightly acidophilic
Lendrum's phloxine-tartrazine	Rich yellow orange colour	Acidophilic, possibly protein
Ninhydrin-Schiff	Strong positive	Protein
Dixon's tannin iron	Strong positive	Protein
Methyl green-pyronin	Unstained	Not DNA or RNA
Feulgen's	Unstained	Not DNA
Glees and Marsland's	Argyrophilic	Acidophilic protein

of cerebellar white matter and of the tegmentum of the brain-stem is very common and occurs less commonly elsewhere; and likewise, lipofuscin in many neurones in the brain including the thalamus is an almost universal feature. The latter contrasts with the eosinophilic bodies in its optical and tinctorial properties, although they are both found together in some neurones in the thalamus. In a high proportion of cases in which the level of section through the brain was optimal, calcified deposits in the thalamus, as described previously, are seen (Fraser, 1968). Neuro-axonal dystrophy in the gracile nuclei is also observed in some mice.

Details of the 80 mice with the inclusions are given in table II.

DISCUSSION

Eosinophilic bodies in some thalamic neurones in mice from a single colony were associated with old age. The specific correlation of this feature with age must await its demonstration in mice from different environments. It is unlikely that the finding is related to the presence of experimentally induced scrapie in the colony. According to current criteria only 5 per cent. of the affected mice had lesions of scrapie. The presence of these inclusions in uninoculated animals makes any causal connexion with scrapie even more tenuous when the rare occurrence of contact transmission even within cages is considered (Dickinson, Mackay and Zlotnik, 1964; personal observations). It might be postulated that these are inclusions of virus origin caused by a latent infectious agent, although the absence of any inflammatory reaction when immunological competence is intact makes this unlikely. Although differing

NEURONAL INCLUSIONS IN MOUSE THALAMUS

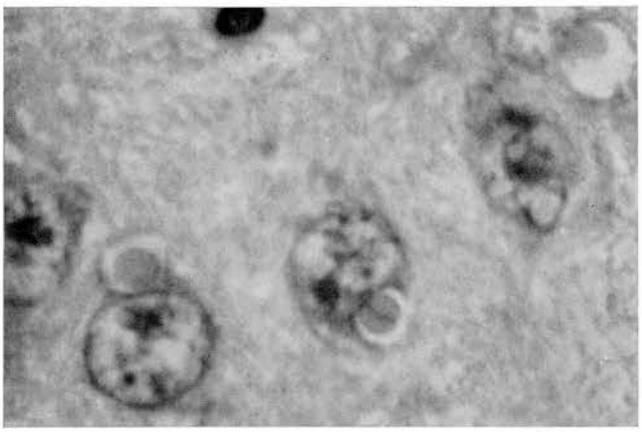

FIG. 1.—Neurones in the thalamus of aged mouse, showing eosinophilic bodies in the cytoplasm. A basophilic strand is apparent in the centre nucleus. Haematoxylin and eosin. ×1100.

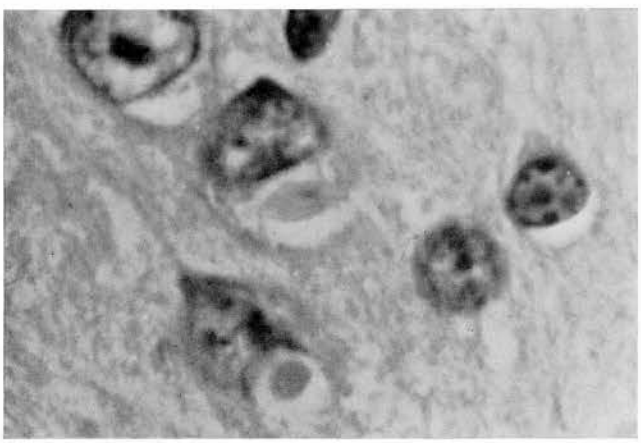

FIG. 2.—Neurones in the thalamus of aged mouse, showing intracytoplasmic bodies. One body is elongated with a basophilic centre. HE. ×1100.

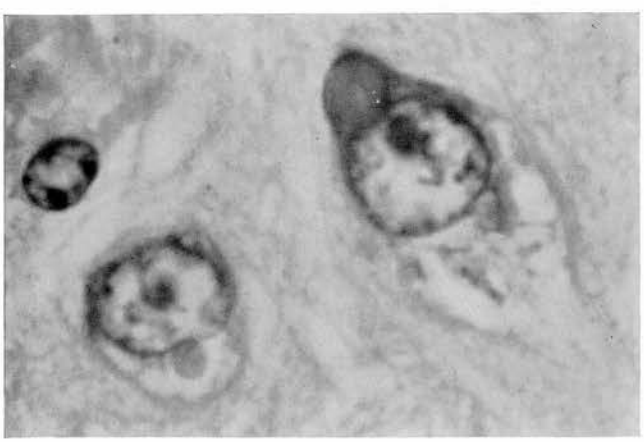

FIG. 3.—Intracytoplasmic inclusions in thalamic neurones of aged mouse. HE. ×1100.

TABLE II

Details of 80 mice with eosinophilic bodies in thalamic neurones

Number of mice with eosinophilic bodies in thalamic neurones	Mouse strains affected	Sex ratio M/F	Age distribution (days)		Number of inoculated and uninoculated mice		Other lesions found	
80	C57BL SM LG 129 MM VM LM×SM C57BL×LM	38/42	<400 400–600 600–800 800–1000 >1000	2 7 60 6 5	Intracerebral inoculations only Intraperitoneal inoculations only Mixed inoculation Uninoculated	66 1 4 9	Calcified deposits in thalamus Neuro-axonal dystrophy in gracile nucleus Hydrocephalus Lymphosarcomatous infiltration into brain Scrapie Non-CNS histological lesions Cases in which no histological lesions occurred	17 8 1 2 4 9 47

in distribution, they resemble to some extent intracytoplasmic eosinophilic bodies within neurones in experimental louping-ill in the mouse (Hurst, 1931). The occurrence of astrocytic hypertrophy has indeed suggested to Field (1967) that transmissible agents may be obtainable from the brains of ageing animals and man that might have an aetiological significance both in ageing and in some chronic degenerative diseases of the nervous system. However, astrocytic hypertrophy frequently occurs secondarily to brain oedema (Klatzo, 1967) and therefore astrocytic hypertrophy of ageing may be a non-specific reactive change in response to the oedema of white matter frequently seen in old mice (personal observations). In addition other histological features are commonly seen in aged mice. Calcification associated with blood vessels in the thalamus is frequent (Fraser, 1968) and similar lesions have been described in the cerebral cortex (Tucker and Baker, 1967). Neuroaxonal dystrophy in the gracile nuclei (Sung, 1966) is also frequently seen in this colony.

As the mouse is used in the diagnosis of rabies it should be mentioned that these bodies bear little resemblance to Negri bodies, which most frequently occur in the hippocampus and have characteristic morphological and tinctorial properties.

The nature of the intraneuronal bodies described in this paper must await further investigation. Their positive staining reaction with tannin iron and with ninhydrin-Schiff strongly suggests a protein component, which is supported by their eosinophilia and phloxinophilia. They would appear to lack either carbohydrate or lipid. Although the main mass of the acidophilic material does not appear to contain nucleic acid, the occasional deeply basophilic centre, sometimes with a thin basophilic strand connecting it to the chromatin of the nucleus, suggests that a nucleic acid component might sometimes be present.

Summary

Eosinophilic bodies in the neurones in the thalamus are described. They occur in aged mice, and appear to consist predominantly of protein.

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Ultrastructural Morphology of Cytoplasmic Inclusions within Neurons of Ageing Mice

H. FRASER, W. SMITH AND E. W. GRAY

A.R.C. Animal Breeding Research Organisation (H.F.) and Moredun Research Institute, Gilmerton, Edinburgh (Great Britain)

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INTRODUCTION

Eosinophilic inclusions within the cytoplasm of neurons in the thalamus of ageing mice have been described. These are round, oval or disc-shaped bodies ranging in size up to 25 μm in diameter, sometimes having a pale centre or containing within them a basophilic strand or spot. They are confined to the dorsal and ventral thalamic nuclei and are most frequently seen in mice over 1 year of age. They contain protein, and are seen in both sexes of many strains of mice (FRASER 1969).

The present report is a result of an investigation of the ultra-structure of these eosinophilic, protein-containing bodies.

MATERIALS AND METHODS

Two 2-year-old female C57BL mice were killed by decapitation and blocks from the thalamus were placed into fixative within 1 min of death. Fixation was in 3% gluteraldehyde followed by post-fixation in 1% osmium tetroxide. Blocks from the thalamic region were embedded in araldite, and 0.5–1 μm thick sections were stained with toluidine blue and examined with the optical microscope. When the neuronal inclusions were identified the blocks were trimmed down to the relevant area and sections prepared for electron microscopy.

RESULTS

The inclusions which were identified in the thick sections with the optical microscope were sometimes homogeneous, or contained a pale centre, and lay within the cytoplasm of neurons and stained greenish blue with toluidine blue (Fig. 1).

Ultrastructurally these bodies consist of arrays of parallel fibrillar crystalline

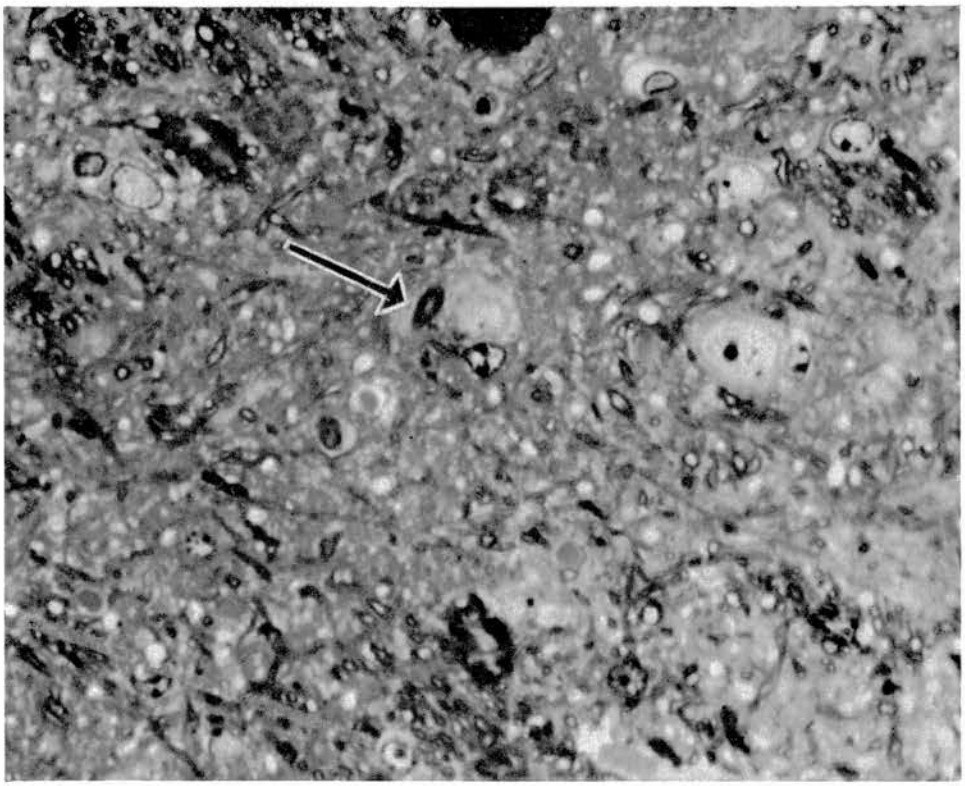

Fig. 1. Optical microscopic appearance of inclusion within cytoplasm of neuron. Toluidine blue; × 800.

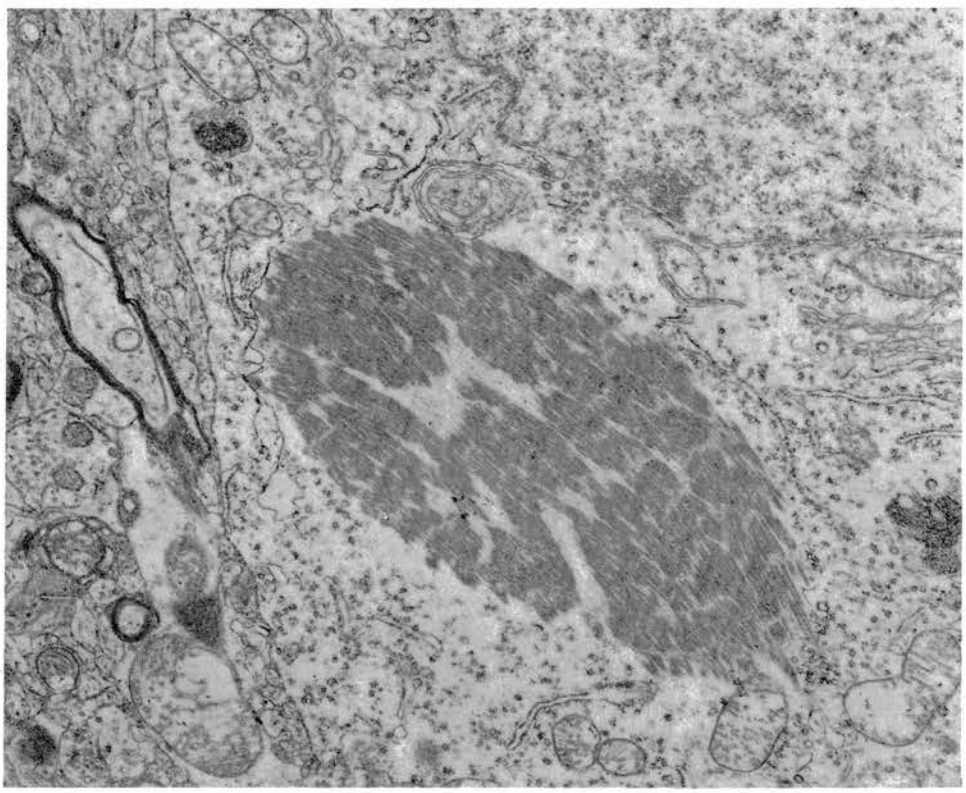

Fig. 2. Electron-microscopic appearance of same inclusion as in Fig. 1. × 15,000.

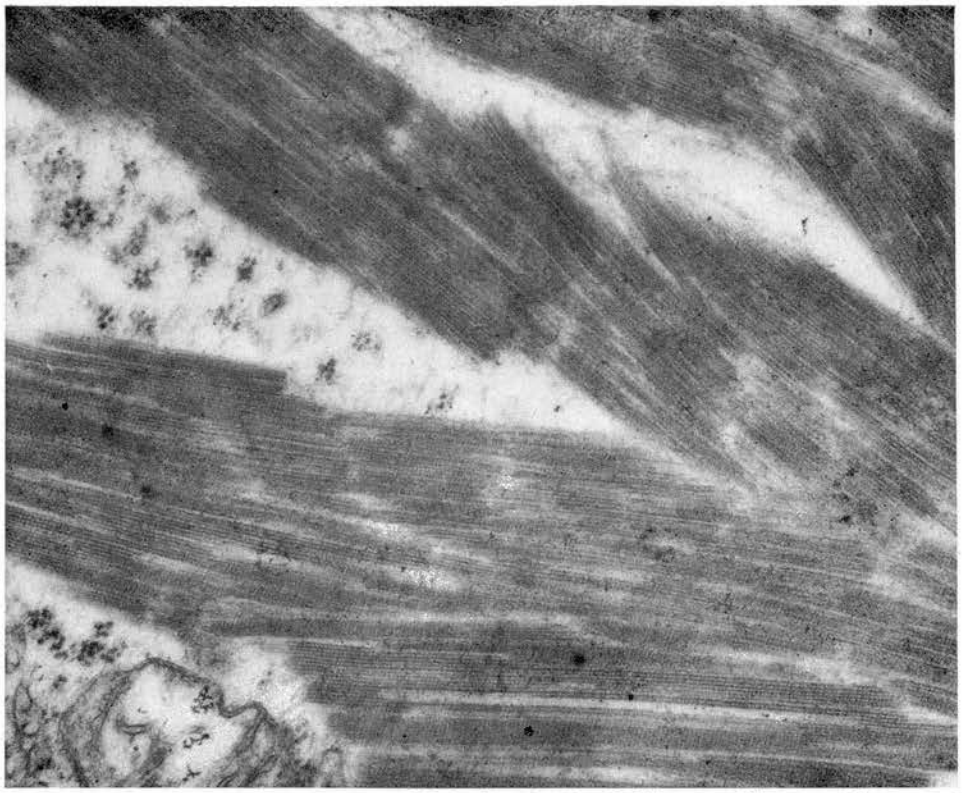

Fig. 3. Electron-microscopic appearance of inclusion showing fibrillar crystalline material arranged in bundles. × 45,000.

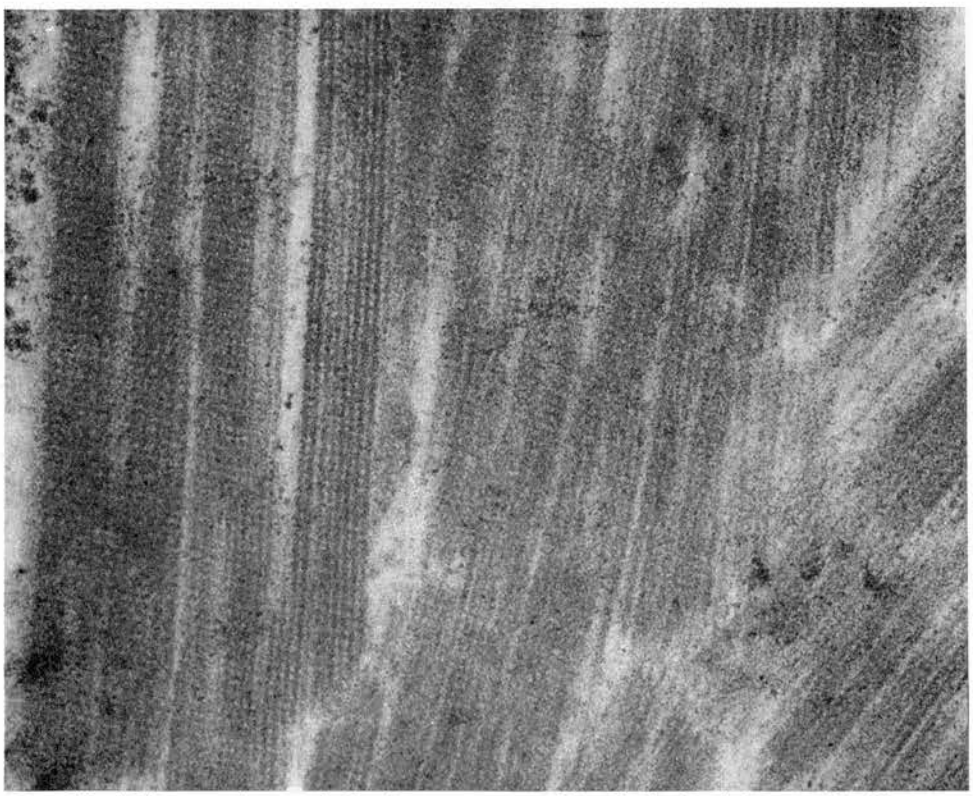

Fig. 4. Electron micrograph showing transverse and longitudinal striations. × 105,000.

material with a well defined substructure, consisting of longitudinal and transverse striations (Fig. 2). The longitudinal striations consist of alternating electron-dense and electron-transparent lines, the distance between the centre of one electron-dense line and the next being 15 nm. The transverse striations are less obvious and appear to result from an alternate narrowing and widening of the longitudinal lines, the periodicity of which is in phase across the thickness of the fibre (Fig. 3 and 4).

Where bundles of fibres are separated from one another faint lines can be discerned which frequently appear to be continuous with the longitudinal striations of the individual fibres (Fig. 2).

DISCUSSION

The ultrastructure of these inclusions supports the conclusion that they consist of protein (FRASER 1969). They are distinguishable both from the laminated bodies described within neurons in the cerebellum and lateral geniculate body of the cat (MORALES AND DUNCAN 1966; MORALES *et al.* 1964) and from the cytoplasmic laminar bodies in the visual cortex of the monkey (KRUGER AND MAXWELL 1969). They can also be distinguished from various crystalline and developmental forms of lipofuscin described in ageing mice (SAMORAJSKI *et al.* 1965).

It appears from their structure that these bodies form from the union of many identical macromolecules. Their similarity between different cells and their homogeneity between and within various host genotypes (FRASER 1969) strongly suggests that they represent an expression of some normal cell activity. Why, then, do they occur in association with ageing? They may represent the accumulation with the passage of time of a normal metabolite; or there may be a reduction, with age, in the homeostatic mechanisms which catalyse their removal. Alternatively they may reflect a reduced cell function with age unaccompanied by a commensurate reduction in the synthesis of the particular macromolecule. It is unlikely that these protein masses represent the morphological equivalent of those abnormal proteins which, it has been suggested, should be biochemically demonstrable in ageing cells (HOLLIDAY 1969), because this hypothesis assumes an accumulation of errors in the specificity of the enzymes responsible for gene translation, resulting in the synthesis of various proteins which are surplus or irrelevant to the functional requirements of the cell and predicts the presence of many different types of protein.

Alternatively it might be postulated that these inclusions represent a protein of virus origin caused by an unidentified latent agent, similar to the intranuclear crystalline protein seen in adenovirus infection (MORGAN *et al.* 1960). If the protein of these inclusions is in all cases identical this would support the concept of an endosymbiotic relationship with a specific latent virus, but if variation occurs this would suggest that it represents a morphological example of the molecular chaos which occurs with ageing (MEDVEDEV 1966, 1967).

ACKNOWLEDGEMENT

The authors wish to thank Mrs. P. M. Scarborough for the photographs.

SUMMARY

Eosinophilic inclusions occur within the cytoplasm of thalamic neurons of aged mice. The ultrastructure of these inclusions as seen in two 2-year-old C57BL mice, is described. Their structure, arrays of parallel fibrillar material with repeating longitudinal and transverse striations, confirms a protein consistency, and suggests that they form from the union of many identical macromolecules. The identical nature of the bodies between different cells and hosts suggests that they are the result of normal cell activity, and represent the accumulation with time of a normal metabolite or a retardation of homeostatic mechanisms which catalyse their removal.

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Sitzungsberichte

Vereinigung Deutscher Neuropathologen und Neuroanatomen.
15. Jahrestagung

Die 15. wissenschaftliche Jahrestagung der Vereinigung Deutscher Neuropathologen und Neuroanatomen hat auf Einladung der British Neuropathological Society als eine gemeinsame deutsch-britische Tagung unter dem Vorsitz von I. A. N. Corsellis, Wickford, und G. Kersting, Bonn, in der Zeit vom 10.—12. Juli 1969 in Oxford stattgefunden.

H. Fraser und A. G. Dickinson (Edinburgh): Eine Serie von wahrscheinlich astrocytären Gliomen in einem genetisch definierten Mäusestamm.

Ein bestimmter Hirntumor wurde 16mal im VA-Mäusestamm identifiziert, das entspricht einer Häufigkeit von 0,85%. Hierbei kann es sich um eine Unterschätzung des wirklichen Vorkommens handeln, da die Untersuchung auf einer Analyse von standardisierten Routinemethoden am Gehirn beruht, die für quantitative Analysen der Hirnpathologie bei der Scrapie bestimmt ist (Fraser und Dickinson, 1968). Diese Beobachtungen konnte man während der Untersuchung in einer einzigen Kolonie von über 10 000 Mäusen aus 13 Stämmen machen. Allein vom VA-Stamm weiß man, daß er das p7 allele Gen sinc trägt, über das die Inkubationszeit der Scrapie bei der Maus festgesetzt wird. Aber es ist unbekannt, ob dieses Gen in einem ursächlichen Zusammenhang mit der Tumorentstehungsbereitschaft steht. Der Tumor wurde bei der Maus vom 138.—535. Lebenstag beobachtet; eine Geschlechtsprädilektion bestand nicht. Fokale Zeichen gab es etwa in der Hälfte der Fälle. Die Mehrzahl der Fälle war ordnungsgemäß inoculiert, aber 8 Fälle entwickelten klinisch eine Scrapie, und zwar nach einem Inkubationsintervall, welches im normalen Irrtumsbereich ähnlicher Betrachtungsweisen liegt und die Vermutung zuläßt, daß der Tumor sich nicht auf die Pathogenese oder den zeitlichen Ablauf der Hirnscrapie auswirkt. Der Tumor wurde in anderen Mäusestämmen derselben Kolonie nicht gesehen, aber verschiedene andere intrakranielle Tumoren fand man in mehreren Mäusestämmen. Am häufigsten fand man Lipome und Hypophysenadenome, und zwar in den Stämmen C57BL, BALB/c, VM, SM und BRVR, während ein einzelner Fall eines fibroplastischen Meningioms und eines meningealen Myxosarkoms im C57/BL und resp. im VM-Stamm diagnostiziert wurde. 2 Neurofibrome traten auch auf, eines im C3H/f Stamm und eines bei einer in bezug auf das Gen reeler homozygot geborenen Maus. Morphologisch handelte es sich bei dem Tumor um ein Gliom, möglicherweise astrocytären Ursprunges. Die einzelnen Schnitte zeigten eigentlich keine Pleomorphie, aber verschiedengradige Anaplasie. In den meisten Fällen tendierten die anaplastischen Zellen zu spongioblastenartiger Morphologie. Die Mehrzahl der Fälle zeigte geringe kleincystische Degeneration, Palisadenformen oder Rosetten fanden sich dagegen nicht. Allgemein bot sich das Bild einer gewissen pilocytischen Metaplasie, die aber hauptsächlich wohl von der Ortsbeziehung zur weißen Substanz herrührte. Der Tumor kam an verschiedenen Stellen vor: Fasciculus medialis longitudinalis (2), Lemniscus medialis (3), Formatio reticularis der Medulla oder Pons (5), intracerebrale Anteile des Nervus VII (1), Pedunculus cerebellaris superior (1), weiße Kleinhirnsubstanz (4), Tractus nuclei spinalis nervi V (1), Tractus cortico-spino-bulbaris (5), Tegmentum (3), Thalamus (4), Hippocampus (1), Capsula interna (4), Fornix (3), Septum (2), Fimbria (2), Corpus callosum (3), weiße Substanz des Großhirns (1).

Apology

It is regretted that a clerical error resulted in the duplication of page numbers of pages 15, 52 and 56.

The duplicate page numbers have been changed to 15a, 52a and 56a respectively.