VOLUME XXIX APRIL, 1929 No. 1

STUDIES IN ANTI-RABIES IMMUNISATION.

BY G. STUART AND K. S. KRIKORIAN.

(*Central Laboratories, Department of Health, Government of Palestine.*)

(With 1 Chart.)

CONTENTS.

A. THE EFFICACY OF TREATMENT WITH KILLED CARBOLISED VIRUS.

1. INTRODUCTION.

PASTEUR'S original treatment consisted of a 10 days' course, beginning with a 14 days' dried cord and finishing with one dried for 5 days. Soon it became apparent that all cases exposed to risk of infection could not be protected by this treatment, especially those bitten by wolves or those injured on face and hands. Pasteur was of opinion that even these could have been saved by an intensification of treatment, *i.e.* by the earlier exhibition of less-dried, more virulent cords. Treatment thereafter varied according to the severity of the case, and was termed "light," "medium" and "intensive" when administered over 15, 18 and 21 days, and when the most virulent cord employed during these periods was one dried for 4, 3 and 3 days respectively. Despite such modifications of the original Pasteur method, however, a mortality rate of 1 per cent. persisted, and it is asserted by Koch (1927) that there has been no improvement on this figure up till to-day.

The two main criteria of anti-rabies treatment are efficacy of the vaccine, as determined by low mortality rate, and safety following its use, as determined by freedom from harmful effects both local and general—freedom, therefore, from abscess formation, from neuro-paralytic accident and from rabies itself.

The incidence of post-vaccinal paralysis and the relation it bears to the type of vaccine employed has been discussed by many writers, and it has been shown that intensification of treatment as secured by the inoculation of cords

subjected to short periods of drying—to obviate the use of avirulent material —has resulted in by far the greatest number and relative percentage of "paralytic accidents." Further, while Hoegyes' dilution method, with its ability to effect constancy of dosage, appeared at first the logical substitute, failures of treatment, consequent on the small amount of living virus introduced, became obtrusive, and the intensified dosage demanded for serious cases was shortly followed by numerous "accidents" of a peculiarly grave type.

The question of the pathogenicity of fixed virus for man has been for many years the subject of much controversy. That rabies can follow the subcutaneous injection of fixed virus in animals is amply proved in the experimental section of this paper; whereas, however, Pasteur, Pfeiffer, Koch, Babes and others declare the subcutaneous injection of fresh fixed virus to be non-pathogenic for man, the experience of Prausnitz (1927) and of McKendrick (1927) suggests the converse. During the years 1910 and 1911 there occurred two cases of rabies due to the fixed virus contained in the protective inoculations, one at Breslau after the intensive method of Pasteur, and one at Kasauli after Hoegyes' dilution method. In both cases the degree of risk run was negligible, and the results of animal experiment incriminated fixed virus as cause of death. Moreover, the classical examples of França (1910) and of Bareggi (1889) cannot fail to influence opinion; in these cases death from rabies was caused by the injections alone. Thus França has reported on a patient who, although bitten by a healthy dog, developed symptoms of rabies on the 15th day of treatment by the dried cord method and died. Transmission experiments on rabbits proved successful. Again, Bareggi has placed on record the histories of five patients, who fell ill a few days after completion of treatment by Ferran's process, and died within a week of the onset of symptoms. In these cases experimental inoculation of rabbits intracranially produced typical paralytic rabies, with first symptoms on the 5th day and death on the 7th.

It follows, therefore, that those who practise anti-rabies treatment by the dried cord method, by the dilution method or by methods in which living virus, modified or unchanged, is employed, find themselves between the horns of a dilemma; increased dosage of living virus, together with its, for practical purposes, inseparable vehicle nerve substance, is attended by definite, if slight risk of rabies infection or of "paralytic accident," while a diminution in dosage, sufficient to guarantee absence of such risks, leads to an increase in mortality rates.

It became imperative, therefore, that a method should be devised by which efficacy and safety of treatment might be alike assured. To Fermi (1909) belongs the credit of suggesting that by the addition of phenol the virulence of fixed virus could be lessened without appreciable interference with its antigenic properties. Fermi's vaccine—a 5 per cent. suspension of Sassari virus in 0·8 per cent. N.S.S. to which 1 per cent. phenol has been added—contains a virus rendered avirulent for subcutaneous, but not for subdural infection; it is a virus, therefore, not killed but only weakened by the action of phenol.

In ordinary prophylactic vaccination against some particular disease, it is customary to use, as vaccine, killed cultures of the causal organism; a dead bacterial vaccine, in adequate and correctly interspaced dosage, can secure complete immunity to the individual without any risk of the disease itself supervening. Now, if such a wholly satisfactory immunising response can be effected by dead bacterial vaccine, then the employment of living organisms, even in "attenuated" form, to bring about this result cannot be in any way justified. At the same time it is obvious that if a killed virus is to be used with success in anti-rabies vaccination, the mode of killing the causal agent must, as in the preparation of a bacterial vaccine, be such as will produce as little alteration as possible in antigenic value and so ensure a high degree of immunising power. Semple (1908), consequently, first modified Fermi's process by using a killed carbolised vaccine in treatment; this vaccine was prepared from an 8 per cent. emulsion of fixed virus brain with an addition of 1 per cent. phenol; the basic suspension was allowed to remain in the incubator for 24 hours at 37° C., after which it was diluted to half with physiological saline solution.

The authors' method is a still greater modification; the vaccine employed throughout Palestine is a 2 per cent. emulsion of fixed virus brain, killed by the combined action over 24 hours of heat at 37° C. and of 1 per cent. phenol, prepared according to the process of Stuart and Krikorian (1925), and diluted to half with N.S.S. prior to use. The question of safety to life, then, is foreclosed by the employment of a dead carbolised virus; the adoption of such a method places anti-rabies inoculation and procedure on the simple basis of ordinary vaccine therapy; decentralisation of treatment, as practised in Palestine, is made possible; the interests of economy and utilitarianism are alike consulted. Further, the extreme rarity of paralytic accidents complicating treatment with killed carbolised virus may be gauged from the fact that up to, and including the present, there are on record only five cases, which have occurred as follows:

Institute	Period	No. of accidents	Total no. treated	Incidence per 1000 treated
Jerusalem ...	1923–1928	1	4,580	0·218
Lemberg ...	1925–1926	1	2,213	0·45
Kasauli				
(*a*) Europeans	1912–1926	2	7,296	0·27
(*b*) Natives ...	1912–1926	1	77,548	0·013

Nine other institutes, however, making use of an identical vaccine in identical dosage (0·28 grm.–0·7 grm. *in toto*), have treated 79,758 patients without ill effects. From these figures it will be seen that there have occurred five cases of paralysis among 171,395 bitten persons treated with killed carbolised virus, the incidence being thus but 0·029 per 1000.

Abscess formation, not uncommon with ordinary Pasteurian methods, is unknown.

Proof of safety having thus been advanced, there remains only to show the efficacy of the method, and this it is proposed to discuss from two standpoints, the statistical and the experimental.

2. Statistical considerations.

Ordinarily a statement of the proportion of deaths to persons attacked indicates the degree of virulence of a particular disease, and from the results thus obtained deductions may be drawn concerning the effect of a particular line of treatment.

In the case of rabies, however, once the disease has declared itself in man, the mortality rate is 100 per cent. But every one bitten by a rabid animal and remaining untreated does not contract hydrophobia, the percentage mortality varying according to different authorities from 3·8 (Kirchener) to 15 (Hoegyes, Remlinger), and being placed as a rule at from 6 to 10. As, primarily, evaluation of anti-rabies treatment as a whole must presuppose accurate knowledge of mortality rates among the untreated, such diversity of opinion, therefore, obviously tends to lessen the importance of statistical data for this purpose. An inability to procure exact figures of the total number of untreated persons exposed to risk, and a tendency only to compute statistics on the occurrence of deaths from rabies have rendered the compilation of comparable statistics impossible in practice.

The actual influence of Pasteurian treatment on the mortality rate of rabies is, therefore, incapable of accurate estimation, although it is generally credited with having reduced deaths from this disease by five-sixths to nine-tenths.

Moreover, comparison of the results of different methods of anti-rabies treatment and of institutes employing the same procedure can only be made, when various factors tending to influence statistics favourably or unfavourably have been given due consideration. Correction of mortality rates, however, must here be exceedingly complex, and could only be possible, if populations attending Pasteur Institutes were "standard," when the necessary reductions from total death rates could be made by application of correction factors. Such influences as severity of wounding, degree of exposure to risk, duration of treatment, lateness of arrival at an institute, nature of the biting animal and the constitution of attending populations must have direct bearing on statistical results. In addition, the virulence of street virus is subject to considerable local variation, and it is evident, therefore, that the virulence of the street virus peculiar to each territory must be appreciated, if an exact basis of comparison is to be provided for various institutes.

Anti-rabies Institutes commonly publish in respect of treated persons two mortality rates, viz. the total mortality, which gives the percentage of patients dying despite treatment, and the failure rate, which is limited to those dying later than 15 days after completion of treatment.

With regard to the latter rate, the soundness of the conception is open to serious criticism. Justification of the exclusion from the total death rate of all persons dying during treatment, or within 15 days after its completion, is sought for in the belief that only after 15 days, adequate immunity should be

established, and, therefore, only such cases as develop symptoms at a later date are to be regarded as failures of treatment.

But obviously delay in reporting for treatment and duration of the administration of treatment are all-important in the compilation of this "failure" rate. Institutes giving a 21 days' course might well show a zero failure rate, while those with only a 14 days' course might, *caeteris paribus*, show by no means corresponding success. If, however, treatment is delayed until perhaps a week after the injury, and the patient develops symptoms during treatment or within 15 days after its completion, it would be unfair to ascribe failure to the method employed. On the other hand, if the patient presents himself for treatment immediately after the bite, and if treatment is regularly and fully administered, early symptoms surely indicate failure of the method, as sufficient immunity has not been established to prevent onset.

Under the circumstances it would appear desirable to adopt for the present a simple death rate based on the number of persons dying, in spite of treatment, out of all those treated.

Further, in the light of the foregoing, a true comparison between methods could only be made, if at one institute various methods had been in use during different periods, and if a sufficiently large number of what must approximate to a "standard" population had been treated by each method, to reduce the limit of probable error to a minimum.

Fortunately such comparison is possible from the records of the Kasauli Institute, India. During the years 1900–1926 three methods of treatment have been in use at different periods, viz. 1900–1907 by dried cords, 1907–1912 by Hoegyes' method, 1912–1926 by killed carbolised virus.

The results may thus be tabulated:

Period	Method	No. treated	Total mortality rate %
1900–1907	Pasteur's dried cord	5,141	1·53
1907–1912	Hoegyes' dilution	8,435	1·17
1912–1926	Killed carbolised virus (0·28–0·7 grm. dosage)	84,844	0·77

From these figures it may be assumed that statistical evidence in favour of the efficacy of killed carbolised vaccine has been adduced.

The results obtained by such a method in a country wherein complete decentralisation of treatment is practised may be gauged from the following statistics collected in the anti-rabies institutes of Palestine. Dosage will be seen to have varied from a total of 0·28 grm. during the period 1923–1924 to a total of 0·7 grm. during 1924–1928.

(*a*) *General statistics*	1st period 1923–1924 (0·28 grm. total dosage)	2nd period 1924–1928 (0·7 grm. total dosage)
Total no. of persons treated at 10 centres ...	886	3640
No. of interrupted or unnecessary treatments	138	1228
No. of regular and complete treatments ...	748	2412
Total deaths	9	5
Mortality rate	1·2 %	0·2 %
"Failures" of treatment	4	0
"Failure" rate	0·5 %	0

(*b*) *Statistics of patients-at-risk*	1st period 1923–1924 (0·28 grm. total dosage)	2nd period 1924–1928 (0·7 grm. total dosage)
(1) Proved by laboratory examination ...	180	291
(2) Proved by veterinary officers' certificates	48	184
No. of patients presumably-at-risk*	213	1013
Total population-at-risk	441	1488
Total deaths	9	5
Death rate	2 %	0·34 %
Total "failures"	4	0
"Failure" rate	0·9 %	0

* The number of persons presumably-at-risk is strictly limited to those who have been bitten by an animal which, according to all conventions, is to be considered rabid.

Here better results have followed the employment of a dosage of 5 c.c. daily over 14 days of a 1 per cent. emulsion (0·7 grm.) than of 2 c.c. of the same emulsion over a like period (0·28 grm.). It is further of interest to note that during 1926, 1927 and 1928, with the elevated dosage, no case of rabies has occurred among a total 2105 patients treated, of whom 210 had been bitten by animals proved certainly mad at the time of biting, and 738 by animals suspected of rabies by altered behaviour.

3. Experimental evidence.

It has to be conceded that purely experimental results, however numerous and concordant, are not in themselves sufficient evidence to warrant opinion being formed as to the efficacy of any method of vaccination under normal conditions. To be admitted as sound, any conclusion must be based upon a collection of facts acquired outside the laboratory as a result of prolonged experience and observation. If, however, the evidence obtained from experimental work comes to the assistance of the statistical findings in practice and affords confirmation, the case for a particular method is doubly proved.

During the past three years, therefore, various experiments have been undertaken here to ascertain the measure and duration of immunity conferred on laboratory animals consequent on treatment with killed carbolised virus, and thus if possible to afford further proof of the efficacy of this method of anti-rabies vaccination already suggested by practical results.

(*a*) *Evidence from results following the active immunisation of animals.*

Active acquired immunity is that resistance to infection brought about by the activity of tissue cells and body fluids of an organism as a result of having experienced some particular disease or as a result of artificial inoculation with a modified or attenuated form of its causal agent. Greatest immunity is undoubtedly present after recovery from serious infections, and if this principle could be applied to rabies, immunity would be at a maximum following a non-fatal infection with street virus. Now, if this degree of immunity to rabies could be measured, it would serve as the standard with which the immunity artificially induced through inoculation with anti-rabies vaccines could be compared, and by which the efficacy of such vaccines might be estimated. It

is, however, impracticable to employ street virus in immunising experiments on account of its 100 per cent. fatality rate and the variability of its incubation period. Recourse must, therefore, be had to fixed virus to establish this "standard" immunity; fixed virus has a definite incubation period, and its M.L.D. can with accuracy be determined; moreover, from the fact that it is frequently fatal to rabbits on subcutaneous injection, it follows that the degree of immunity acquired by survivors must be as high as is reasonably possible.

In order that results from all experiments performed to estimate immunity should be strictly comparable, the same strain of fixed virus must be used throughout, and must be of proved constant strength in respect of virulence and infective power.

The maintenance of fixed virus at constant strength has been attempted in various ways by different workers. Harvey and McKendrick (1907) assert that "fixity" can be kept up by careful selection of the rabbits used to afford the material for subpassage. By the use of only those rabbits which show marked symptoms of paresis on the 6th day, it is possible to obtain a virus giving rise to paresis invariably on the 6th day. Babes (1912) recommends passage through guinea-pigs for cases where, for any reason, the fixed virus has decreased in potency for rabbits, as shown by lengthened incubation period; this method, however, of reinforcing the virus and assuring its return to previous fixity is undesirable, as it would appear that this semi-specific increase of virulence for the guinea-pig may cause a similar increase in virulence for man. Viala (1891) kept the virus at a temperature ranging between − 4° C. and + 4° C., and asserted that virulence remained unimpaired for five months. More recently Puntoni (1924) has recorded the effect of temperature on the keeping properties of rabies virus; at 0° C. street virus preserves its virulence for 13 months and fixed virus for 15 months. In these writings the reasons for considering the fixed virus to have maintained a constant potency are not clear; the first essential would appear to be an accurate estimation for the strain of its true minimal infecting dose (M.I.D.), which may be defined as the highest dilution of fixed virus which, in rabbits of average weight (1400 grm.), will invariably produce symptoms 120 hours after subdural infection.

The length of time rabies virus may be preserved without variation in its M.I.D. thus required to be determined.

The fixed virus under investigation here was derived many years ago from the Pasteur Institute in Paris, and has since been continuously passaged, first in Cairo and later in Jerusalem. A fixed virus brain, after preliminary testing, was placed in a glass vessel containing pure neutral glycerine (specific gravity = 1·265 at 15° C.), and stored in a refrigerator at − 4° C. At the end of 4, 6, 9, 12, 15, 18 and 24 months small pieces of brain were extracted.

The true M.I.D., the date on which symptoms first appeared and the date of death were then obtained from rabbits inoculated subdurally with the several pieces of fixed virus brain removed. Comparison of results were then made with those obtained with the fresh fixed virus prior to storage.

Table I shows the characteristics of the fixed virus before refrigeration (subdural inoculum 0·2 c.c.).

Table I.

No. of rabbit	Dilution of fixed virus used	Day of onset of symptoms	Day of death
1	1 : 1,000	5	7
2	1 : 1,000	5	7
3	1 : 1,000	5	8
4	1 : 1,000	5	7
5	1 : 2,500	5	7
6	1 : 2,500	6	8
7	1 : 2,500	6	9
8	1 : 2,500	5	7
9	1 : 5,000	6	8
10	1 : 5,000	5	7
11	1 : 5,000	6	9
12	1 : 5,000	6	8
13	1 : 10,000	8	10
14	1 : 10,000	6	9
15	1 : 10,000	7	10
16	1 : 10,000	22	24

Thus, fixed virus in 1 : 1000 dilution invariably produced symptoms of rabies on the 5th day with death on the 7th or 8th day; in higher dilutions, however, action was no longer certain, symptoms appearing between the 6th and 22nd days.

Table II gives the results after storage at − 4° C. over varying periods of time; at the end of each period the virus was tested by subdural inoculation into rabbits.

Storage at − 4° C. will, therefore, allow fixed virus to retain its potency unimpaired for 12 months, symptoms being invariably produced on the 5th day after subdural inoculation with 0·2 c.c. of a 1 : 1000 dilution. Beyond this period there is a tendency to lengthened incubation, although the interval between appearance of symptoms and death does not alter. Fixed virus in dilutions higher than 1 : 1000 certainly causes symptoms and death in every case, but at somewhat irregular periods after infection. It may safely be concluded, therefore, that the fixed virus used throughout the present experiments preserved its virulence completely for 1 year, and that its true M.I.D. was 0·2 c.c. of a 1 : 1000 dilution.

In parallel series with this experiment, an investigation was conducted on street virus in respect of its keeping properties at − 4° C. and of its M.I.D. As street virus was also to be employed as an infective agent, the necessity of standardising the strain will be apparent. The procedure was identical with that employed in the previous investigation, except that the longest period of storage of the virus tested was in this case 12 months—sufficient time, however, to permit completion of experiments with this form of virus.

Street virus was found to preserve its virulence unaltered for one year if kept at − 4° C. Fresh street virus and portions removed from the refrigerator at intervals during the year's storage were alike able to produce with certainty, in a dilution of 1 : 1000, symptoms on the 16th day after subdural inoculation with 0·2 c.c. and death 4 days later.

Table II. *Subdural inoculum* 0·2 *c.c. fixed virus dilution.*

No. of rabbit	Dilution of fixed virus	Duration of storage (months)	Day of onset of symptoms	Day of death
1	1 : 1,000	4	5	8
2	1 : 1,000	4	5	8
3	1 : 2,500	4	5	8
4	1 : 2,500	4	5	8
5	1 : 5,000	4	6	9
6	1 : 5,000	4	5	8
7	1 : 10,000	4	5	8
8	1 : 10,000	4	7	9
9	1 : 1,000	6	5	8
10	1 : 1,000	6	5	8
11	1 : 2,500	6	6	9
12	1 : 2,500	6	5	8
13	1 : 5,000	6	6	9
14	1 : 5,000	6	6	9
15	1 : 10,000	6	18	21
16	1 : 10,000	6	12	14
17	1 : 1,000	9	5	8
18	1 : 1,000	9	5	8
19	1 : 2,500	9	6	8
20	1 : 2,500	9	6	9
21	1 : 5,000	9	6	9
22	1 : 5,000	9	6	8
23	1 : 10,000	9	5	8
24	1 : 10,000	9	8	10
25	1 : 1,000	12	5	8
26	1 : 1,000	12	5	8
27	1 : 2,500	12	5	8
28	1 : 2,500	12	6	9
29	1 : 5,000	12	6	9
30	1 : 5,000	12	6	9
31	1 : 10,000	12	15	18
32	1 : 10,000	12	7	9
33	1 : 1,000	15	6	8
34	1 : 1,000	15	6	8
35	1 : 2,500	15	6	9
36	1 : 2,500	15	7	10
37	1 : 5,000	15	6	9
38	1 : 5,000	15	7	10
39	1 : 10,000	15	6	9
40	1 : 10,000	15	8	11
41	1 : 1,000	18	7	9
42	1 : 1,000	18	7	9
43	1 : 2,500	18	7	9
44	1 : 2,500	18	7	9
45	1 : 5,000	18	7	10
46	1 : 5,000	18	8	11
47	1 : 10,000	18	7	9
48	1 : 10,000	18	20	23
49	1 : 1,000	24	7	9
50	1 : 1,000	24	8	10
51	1 : 2,500	24	7	9
52	1 : 2,500	24	8	11
53	1 : 5,000	24	7	10
54	1 : 5,000	24	10	13
55	1 : 10,000	24	17	19
56	1 : 10,000	24	19	22

It has already been shown that fresh fixed virus cannot be used indiscriminately in anti-rabies vaccination; it becomes essential, therefore, so to deal with it as to ensure loss of infectivity without corresponding diminution in its immunising value as vaccine. Attempts to achieve this desirable end have been made in various ways, viz. by exposure to the action of physical or chemical agencies such as heat, desiccation, ether, glycerine and phenol.

Such treatment, however, may or may not interfere with the protective value of fixed virus, and it was to elucidate this point and to determine the value of the killed carbolised vaccine used in Palestine as compared with what must be unattainable perfection—vaccine prepared from fresh fixed virus—that the various experiments detailed below were instituted.

Strains of fixed virus and of street virus having been proved to remain at constant strength throughout the period of experimental work, it became possible to estimate the relative values of fresh and of carbolised fixed virus vaccines, by an observation of the resistance shown by rats and rabbits, immunised by each of these methods, to infective doses of street virus.

Eighty white rats were divided into two main groups of 40 rats each, and each main group was further divided into four subgroups. Rats in Group I were inoculated subcutaneously with carbolised fixed virus in a dosage varying in the subgroups of from 1 c.c. to 2·5 c.c. daily for 5 days. Group II was treated with living fixed virus in identical dosage.

Forty days after the last injection all rats were tested with a 2 per cent. suspension of standardised street virus, of which 2 c.c. were inoculated deeply into the muscles of the neck.

The results are given in Table III.

Certain conclusions may be drawn from this experiment:

(1) 2 per cent. fixed virus in N.S.S. given to rats of 200 grm. in doses of from 100 to 250 mg. is a prophylactic of undoubted value against infection with street virus rabies.

(2) The highest degree of immunity is obtained when 750 mg. per kilo body weight is given. Larger doses do not confer greater immunity.

(3) 2 per cent. fixed virus in 1 per cent. phenol given in identical dosage immunises in practically equal degree.

(4) With carbolised fixed virus immunity is greatest following administration of 750 mg. per kilo body weight.

It will be appreciated that the actual value of these prophylactics is not indicated by the results obtained, as it is improbable that in nature an infection equal in intensity to that given in the experiment would be encountered.

The necessity for previous specific treatment to ensure the development of anti-rabies immunity in animals has been denied by Babes (1892, 1898) and by Fermi (1908), who maintain that a similar result may be achieved by non-specific means. Babes (1892, 1898) had conducted experiments on dogs to discover what protective action, if any, the subcutaneous injection of normal

nerve substance could confer against infection with street virus (by natural transmission) and with fixed virus (by subdural inoculation). He found that with sheep's normal brain used as immunising agent, dogs could resist infection, whether treatment had been pre- or post-infectional. Fermi (1909) found normal nerve substance to be as effective in immunisation as fixed

Table III.

(*a*) *The immunising power in rats of 2 per cent. carbolised fixed virus.*

Main group I.

No. of rat	Nature of treatment, using 2 % carbolised fixed virus daily for 5 days (c.c.)	Ill-effects of treatment. 0 = no effect. D. = died	Test dose of street virus, 1 % suspension, injected into muscles of neck after 40 days (c.c.)	Result. L. = lived. D. = died	% of immunity
Sub-group 1					
1	1	0	2	L.	50
2	1	0	2	D.	
3	1	0	2	D.	
4	1	0	2	D.	
5	1	0	2	D.	
6	1	0	2	D.	
7	1	0	2	L.	
8	1	0	2	L.	
9	1	0	2	L.	
10	1	0	2	L.	
Sub-group 2					
1	1·5	0	2	D.	80
2	1·5	0	2	L.	
3	1·5	0	2	L.	
4	1·5	0	2	D.	
5	1·5	0	2	L.	
6	1·5	0	2	L.	
7	1·5	0	2	L.	
8	1·5	0	2	L.	
9	1·5	0	2	L.	
10	1·5	0	2	L.	
Sub-group 3					
1	2	0	2	D.	60
2	2	0	2	L.	
3	2	0	2	D.	
4	2	0	2	D.	
5	2	0	2	L.	
6	2	0	2	L.	
7	2	0	2	D.	
8	2	0	2	L.	
9	2	0	2	L.	
10	2	0	2	L.	
Sub-group 4					
1	2·5	D.	2	—	37·5
2	2·5	D.	2	—	
3	2·5	0	2	L.	
4	2·5	0	2	D.	
5	2·5	0	2	D.	
6	2·5	0	2	L.	
7	2·5	0	2	D.	
8	2·5	0	2	L.	
9	2·5	0	2	D.	
10	2·5	0	2	D.	

(*b*) *The immunising power in rats of* 2 *per cent. fixed virus in* N.S.S.

Main group II

No. of rat	Nature of treatment, using 2 % fixed virus in N.S.S. daily for 5 days (c.c.)	Ill-effects of treatment. 0 = no effect. D. = died	Test dose of street virus, 1 % suspension, injected into muscles of neck after 40 days (c.c.)	Result. L. = lived. D. = died	% of immunity
Sub-group 1					
1	1	D.*	2	—	66
2	1	0	2	D.	
3	1	0	2	L.	
4	1	0	2	L.	
5	1	0	2	D.	
6	1	0	2	D.	
7	1	0	2	L.	
8	1	0	2	L.	
9	1	0	2	L.	
10	1	0	2	L	
Sub-group 2					
1	1·5	0	2	D.	80
2	1·5	0	2	L.	
3	1·5	0	2	L.	
4	1·5	0	2	D.	
5	1·5	0	2	L.	
6	1·5	0	2	L.	
7	1·5	0	2	L.	
8	1·5	0	2	L.	
9	1·5	0	2	L.	
10	1·5	0	2	L.	
Sub-group 3					
1	2	0	2	D.	60
2	2	0	2	L.	
3	2	0	2	L.	
4	2	0	2	D.	
5	2	0	2	L.	
6	2	0	2	D.	
7	2	0	2	D.	
8	2	0	2	L.	
9	2	0	2	L.	
10	2	0	2	L.	
Sub-group 4					
1	2·5	D.*	2	—	62
2	2·5	0	2	L.	
3	2·5	0	2	D.	
4	2·5	0	2	L.	
5	2·5	D.*	2	—	
6	2·5	0	2	D.	
7	2·5	0	2	D.	
8	2·5	0	2	L.	
9	2·5	0	2	L.	
10	2·5	0	2	L.	

* Died of fixed virus.

virus brain, and in certain cases it proved even more effective. Thus the normal nerve substance of lamb saved 97·7 per cent. of rats infected subcutaneously with street virus, whereas rabies nerve substance saved 86 per cent. Fresh normal nerve substance of the lamb also protected 86·6 per cent. of rats infected subcutaneously with street virus as compared with the 70 per cent. protected by Pasteurian treatment.

To combat the criticism caused by the publication of Fermi's results, Babes and Simici (1910) carried out further experiments on dogs, rabbits, guinea-pigs and mice which might serve as controls for Fermi's work. Although unable to secure for these animals a degree of protection equal to that achieved by Fermi, they, nevertheless, felt convinced from their results that normal nerve substance was material of undoubted immunising value, and

Table IV.

(*a*) *Anti-rabies treatment of rats with subcutaneous injections of carbolised normal brain.*

Group I

Rat no.	Nature of treatment, using 2 % carbolised normal brain daily for 5 days (c.c.)	Ill-effects of treatment	Test dose of street virus, 1 % suspension, injected into muscles of neck after 40 days (c.c.)	Result
Sub-group 1				
1	1	0	2	All rats died of rabies. Percentage of immunity nil
2	1	0	2	
3	1	0	2	
4	1	0	2	
5	1	0	2	
6	1	0	2	
7	1	0	2	
8	1	0	2	
9	1	0	2	
10	1	0	2	
Sub-group 2				
1	1·5	0	2	All rats died of rabies. Percentage of immunity nil
2	1·5	0	2	
3	1·5	0	2	
4	1·5	0	2	
5	1·5	0	2	
6	1·5	0	2	
7	1·5	0	2	
8	1·5	0	2	
9	1·5	0	2	
10	1·5	0	2	
Sub-group 3				
1	2	0	2	All rats died of rabies. Percentage of immunity nil
2	2	0	2	
3	2	0	2	
4	2	0	2	
5	2	0	2	
6	2	0	2	
7	2	0	2	
8	2	0	2	
9	2	0	2	
10	2	0	2	
Sub-group 4				
1	2·5	0	2	All rats died of rabies. Percentage of immunity nil
2	2·5	0	2	
3	2·5	0	2	
4	2·5	0	2	
5	2·5	0	2	
6	2·5	0	2	
7	2·5	0	2	
8	2·5	0	2	
9	2·5	0	2	
10	2·5	0	2	

(*b*) *Anti-rabies treatment of rats with subcutaneous injections of normal brain in* N.S.S.

Group II

Rat no.	Nature of treatment, using 2 % of normal brain in N.S.S. daily for 5 days (c.c.)	Ill-effects of treatment	Test dose of street virus, 1 % suspension into muscles of neck after 40 days (c.c.)	Result
Sub-group 1				
1	1	0	2	All rats died of rabies. Percentage of immunity nil
2	1	0	2	
3	1	0	2	
4	1	0	2	
5	1	0	2	
6	1	0	2	
7	1	0	2	
8	1	0	2	
9	1	0	2	
10	1	0	2	
Sub-group 2				
1	1·5	0	2	All rats died of rabies. Percentage of immunity nil
2	1·5	0	2	
3	1·5	0	2	
4	1·5	0	2	
5	1·5	0	2	
6	1·5	0	2	
7	1·5	0	2	
8	1·5	0	2	
9	1·5	0	2	
10	1·5	0	2	
Sub-group 3				
1	2	0	2	All rats died of rabies. Percentage of immunity nil
2	2	0	2	
3	2	0	2	
4	2	0	2	
5	2	0	2	
6	2	0	2	
7	2	0	2	
8	2	0	2	
9	2	0	2	
10	2	0	2	
Sub-group 4				
1	2·5	0	2	All rats died of rabies. Percentage of immunity nil
2	2·5	0	2	
3	2·5	0	2	
4	2·5	0	2	
5	2·5	0	2	
6	2·5	0	2	
7	2·5	0	2	
8	2·5	0	2	
9	2·5	0	2	
10	2·5	0	2	

thereby confirmed the opinion first advanced to that effect by Babes in 1892. Harvey and Acton (1923), however, hold the converse view, and believe such non-specific stimulation to be followed by greater susceptibility to infection. It seemed desirable that experiments to test the value of Fermi's assertion should be carried out; the effect on rats of treatment with suspensions of normal brain was, therefore, studied with a view to deciding as to the possible establishment of anti-rabies immunity by non-specific means.

Rats were treated daily over 5 days with a dosage varying from 1 c.c. to 2·5 c.c. of 2 per cent. normal brain in N.S.S. and in 1 per cent. phenol. Forty days after completion of treatment, tests of resistance were made by the injection of 2 c.c. of a 1 per cent. emulsion of standardised street virus deep into the muscles of the neck. The results obtained are shown in Table IV (*a*) and (*b*).

Thus treatment of rats with carbolised normal brain and with normal brain in N.S.S. has been proved to confer no anti-rabies immunity. The results of the experiments tabulated argue against a non-specific immunisation.

Confirmation of the results obtained in rats from the several methods of treatment above described was next sought in a number of rabbit experiments. Forty rabbits, therefore, received subcutaneous injections with two different dosages of fixed virus in N.S.S., of fixed virus in 1 per cent. phenol, of normal brain in N.S.S. and of normal brain in 1 per cent. phenol. Forty days after completion of treatment, each rabbit was tested for anti-rabies immunity by an injection of street virus deep into the muscles of the neck. The results are given in Table V.

It follows from the above experiments that the immunity produced by carbolised fixed virus approximates in practice to that produced in the survivors of animal groups treated with living virus in physiological saline solution.

Further, there is no evidence in rats and rabbits of immunity following treatment with normal brain in normal saline or in 1 per cent. phenol solution, when resistance is tested by injection of street virus into the neck muscles.

(*b*) *Evidence from an estimation of antibody content in immune serum.*

It is commonly accepted that specific changes in the blood serum characteristic of all active immunisation must follow treatment with anti-rabies vaccine. Rabicidal, specific complement-binding and precipitating antibodies are those most likely to be evoked by stimulation with rabies virus itself, but in this case an inability to dissociate the antigen from its vehicle of administration—nerve tissue—introduces a complicating factor not ordinarily encountered in immunization. Antibody formation might, as a result, be both specific and non-specific: specific in response to the causal agent of rabies, non-specific in response to the nerve substance in the inocula-neurolytic and precipitating in nature.

In rabies, it has not been decided whether the virus quâ virus is capable of producing specific complement-fixing and precipitating antibodies in the animal organism, although the majority agrees to the occurrence of a rabicidal antibody. Recently Schultz, Bullock and Brewer (1928) have conclusively demonstrated that specific precipitating antibody formation does not follow immunisation by the method of Stuart and Krikorian, or by that of Kraus and Takaki. Sera were tested in accordance with the technique of Tomarkin and Suarez (1917), and in none was any evidence of specific precipitins elicited. Such negative results are in complete agreement with the findings of Burmeister

Table V. *Anti-rabies treatment of rabbits with fixed virus and normal brain substance.*

No. of rabbit	Nature of treatment	Ill-effects of treatment	Test dose of 1 % street virus into muscles of neck after 40 days (c.c.)	Result. L. = lived. D. = died of rabies	% of immunity
Group I. Sub-group 1					
1	5 c.c. daily for 5	0	2	L.	60
2	days of 2 % car-	0	2	D.	
3	bolised fixed virus	0	2	L.	
4		0	2	L.	
5		0	2	D.	
Group I. Sub-group 2					
1	5 c.c. daily for 10	0	2	L.	80
2	days of 2 % car-	0	2	D.	
3	bolised fixed virus	0	2	L.	
4		0	2	L.	
5		0	2	L.	
Group II. Sub-group 3					
1	5 c.c. daily for 5 days	Died of F.V.	2	—	100
2	of 2 % fixed virus in	Died of F.V.	2	—	
3	N.S.S.	Died of F.V.	2	—	
4		0	2	L.	
5		0	2	L.	
Group II. Sub-group 4					
1	5 c.c. daily for 10 days	0	2	L.	80
2	of 2 % fixed virus in	0	2	L.	
3	N.S.S.	0	2	L.	
4		0	2	D.	
5		0	2	L.	
Group III. Sub-group 5					
1	5 c.c. daily for 5 days	0	2	All died of	0
2	of 2 % carbolised	0	2	rabies	
3	normal brain	0	2		
4		0	2		
5		0	2		
Group III. Sub-group 6					
1	5 c.c. daily for 10 days	0	2	All died of	0
2	of 2 % carbolised	0	2	rabies	
3	normal brain	0	2		
4		0	2		
5		0	2		
Group IV. Sub-group 7					
1	5 c.c. daily for 5 days	0	2	All died of	0
2	of 2 % normal brain	0	2	rabies	
3	in N.S.S.	0	2		
4		0	2		
5		0	2		
Group IV. Sub-group 8					
1	5 c.c. daily for 10 days	0	2	All died of	0
2	of 2 % normal brain	0	2	rabies	
3	in N.S.S.	0	2		
4		0	2		
5		0	2		

(1915), who was unable to demonstrate the occurrence of specific precipitins in the sera of rabbits suffering from fixed virus rabies, and of Lässer (1927), who was equally unsuccessful in his efforts to adduce evidence of the formation of such precipitins in rabies-infected and in immunised animals.

An enquiry into the rabicidal and complement-fixing properties of immune serum has formed the subject of investigations here.

1. *Rabicidal properties of immune serum.*

Nearly 40 years ago Babes and Lepp (1889) first succeeded to some extent in protecting susceptible animals with the serum of dogs immunised against rabies. Shortly afterwards Babes (1891) demonstrated that the serum of an immunised animal had the power to inactivate rabies virus *in vitro*, and this observation has since been confirmed by Kraus, Kellner and Clairmont (1902), Marie (1904), Remlinger (1905), Schnürer (1905), Semple (1908), Stuart and Krikorian (1925) and Pereira da Silva (1926). That a similar rabicidal property is possessed by the serum of immunised human beings has been shown by Kraus and Kreissl (1902), Semple (1908), Kostrezewski (1920), Nikolajewa (1925) and Pereira da Silva (1926). The results obtained, however, have also gone to prove that, while rabicidal antibodies probably do occur in the blood of all immunised mammals, it is ordinarily in a concentration insufficient to confer passive immunity or to exert definite therapeutic effect.

In this connection it is noteworthy that in pigeons and chickens rabicidal antibody has been shown by Kraus and Maresch (1902) and by Marie (1905) to be incapable of production, even after intensive methods of immunisation.

The actual nature of the rabicidal antibody remains undecided, however, and the mechanism of inactivation of rabies virus *in vitro* by immune serum is still far from clear.

According to the majority of observers the rabicidal antibody is specific, anti-rabies immunisation being dependent on previous specific treatment with fixed virus nerve substance. Babes and Fermi, however, are in opposition to this view for reasons already stated.

The thermostability of this antibody has been demonstrated by Kondo (1922) and by Kraus, Gerlach and Schweinburg (1926); in the experience of Kondo (1922) it is unaffected by temperatures below 70° C. but is destroyed after half an hour's exposure to 80° C.

Neutralisation of the virus in *vitro* by immune serum would seem to depend on the combined action of time and temperature, 2 hours at 37° C. being ideal for adequate union (Kondo). Marie (1905), however, disputes this view, asserting union to be immediate and uninfluenced by conditions of time and temperature. Further, opinion as to the mechanism of the inactivation differ widely: thus, according to Lubinski and Prausnitz (1926) it is bacteriolytic action or neutralisation of toxin by antitoxin; again, while Marie (1908) contends that union is loose and readily broken by washing, Nikolajewa (1925) claims that union is stable, asserting that, even when the serum has been

removed by washing, the virus remains avirulent; it is considered by Friedberger and v. Eisler (1907) that the activation does not conform to the law of definite proportion.

Finally Marie (1912), having found it possible to extract from brain substance an albuminoid possessing *in vitro* very distinct rabicidal properties, has concluded that the destruction of the rabies virus *in vivo* is brought about by the action of the albuminoid substances in brain matter.

Not only, therefore, is there lack of uniformity regarding the mechanism of neutralisation; agreement has not been reached as to the specificity of the rabicidal antibody, indeed its very existence has been questioned by Isabolinsky and Zeitlina (1927), who assert an inability to demonstrate rabicidal action even with the sera of rabbits immunised subcutaneously with fixed virus.

If the rabicidal action of the sera of animals treated with anti-rabies vaccines is, indeed, as suggested by Semple (1911), one of the factors which indicate immunity, a second means of determining the efficacy of carbolised virus would appear to be readily available. The true value of such treatment would then be estimated by an accurate comparison being made of the rabicidal power possessed by the sera of animals immunised by carbolised vaccine with that possessed by the "standard" immune sera attained by treatment with fresh fixed virus in physiological saline solution.

In view, however, of the diversity of opinion expressed above in regard to rabicidal antibody, it seemed essential, before any practical application could be made of this theory of evaluating immunity, to conduct certain preliminary investigations to establish, if possible, proof of the existence and specificity of the antibody in immune serum, and of its absence in the sera of untreated animals and of animals treated with fresh normal brain.

(*a*) *Proof of the existence of rabicidal antibody in immune serum.* In 1925 experiments carried out here demonstrated the presence of rabicidal substances in the serum of rabbits immunised by means of the writers' carbolised virus method. Four rabbits were treated during 14 days with 2 c.c. daily of a 1 per cent. emulsion of carbolised fixed virus. Fifteen days after completion of treatment each animal was bled, and 1 c.c. of its serum, mixed with 1 c.c. of a 1 per cent. emulsion of fresh fixed virus was, to allow full contact, placed for 2 hours in the incubator at 37° C.; four rabbits were then inoculated subdurally with 0·2 c.c. of the mixture. At the same time a control experiment was carried out in which 1 c.c. of serum taken from each of four non-immunised rabbits was mixed with 1 c.c. of a 1 per cent. emulsion of fresh fixed virus in N.S.S. and exposed, after admixture, to a temperature of 37° C. for 2 hours. Of the mixtures 0·2 c.c. was inoculated subdurally into four rabbits. The results are shown in Table VI.

Results show that animals which had received virus-immune serum mixtures remained well, while control animals died from rabies in the usual time, 8 days. The experiment also suggests that rabicidal properties do not

Table VI.

Animals immunised	Method of immunisation	Time after completion of treatment when serum was tested	Proportion of serum and 1 % F.V. emulsion used in test after 2 hours' incubation at 37° C.	Result in rabbits after subdural inoculation with mixture
4 rabbits	2 c.c. daily over 14 days of a 1 % carbolised fixed virus vaccine	15 days	Serum 1 c.c. + F.V. 1 c.c.	All lived
4 rabbits	Not immunised	—	Serum 1 c.c. + F.V. 1 c.c.	All died in 8 days

exist to an appreciable extent in normal rabbits' serum. Recently Schultz, Bullock and Brewer (1928) have confirmed the occurrence of rabicidal antibody in the sera of animals immunised by the method of Stuart and Krikorian, but these workers made no attempt to estimate the degree of rabicidal power possessed by such sera.

(*b*) *Proof that rabicidal antibody did not exist naturally in the serum of rabbits used in the main experiment.* Ten rabbits of 1400 grm. average weight were divided into two series *A* and *B*, each series consisting of five animals. (Series *A* was later to be immunised with living fixed virus in N.S.S., series *B* with carbolised virus.) All the animals were bled and their sera pooled for each series. 1 c.c. of *A* serum and 1 c.c. of *B* serum were then mixed with quantities of a 1 : 100 dilution of fixed virus, varying in each case from 0·1 c.c. to 1 c.c., and the mixtures exposed to a temperature of 37° C. for 2 hours.

At the end of this time 0·2 c.c. of each mixture was inoculated subdurally into rabbits. A control series was also performed: 1 c.c. of 1 : 100 fixed virus in N.S.S. was placed for 2 hours in the incubator at 37° C. and thereafter 0·2 c.c. was introduced subdurally into each of four rabbits. Table VII shows the results obtained:

Table VII.

Pooled sera of rabbit series	Test inoculum after 2 hours at 37° C. (0·2 c.c. subdurally)	Effect on rabbits of subdural inoculation	Rabicidal content of serum
A	1 c.c. serum + 1 c.c. of 1 : 100 F.V.	All died in 8 days	0
	1 „ + 0·5 „		
	1 „ + 0·25 „		
	1 „ + 0·1 „		
B	1 „ + 1 „	All died in 8 days	0
	1 „ + 0·5 „		
	1 „ + 0·25 „		
	1 „ + 0·1 „		
Control series	1 c.c. of 1 : 100 F.V. in N.S.S.	All died in 8 days	—
	1 „ „		
	1 „ „		
	1 „ „		

The death in 8 days of all rabbits inoculated with mixtures of normal serum + fixed virus in varying amounts proves, from the non-prolongation of incubation period, the total absence of rabicidal antibodies in the sera of both series of rabbits selected for later immunisation—first symptoms were invariably observed on the 5th day after subdural infection.

From the control series the experiment also shows that the fixed virus used is not in any way affected by exposure to a temperature of 37° C. for 2 hours, 8 days being the normal killing time of the standard fixed virus employed in all experiments here.

(*c*) *Proof that rabicidal properties are not possessed by the sera of rabbits treated with subcutaneous injections of normal nerve substance, whether homologous or heterologous.* Ten rabbits, whose sera had been tested for the presence of rabicidal antibody with negative results, were divided into series *A* and *B*. In series *A*, five rabbits received daily for 14 days 5 c.c. of a 1 per cent. suspension of normal rabbit's brain in N.S.S., in series *B*, five rabbits received identical treatment but with normal sheep's brain.

Rabbits were bled on the 12th, 20th and 30th day after completion of treatment and the sera pooled for each series. Serum *A* and serum *B* were then tested quantitatively for the presence of rabicidal antibody after each bleeding. 1 c.c. of the serum of each series was mixed with quantities of 1 per cent. fixed virus in N.S.S. varying from 0·1 c.c. to 1 c.c., and thereafter exposed to the action of 37° C. for 2 hours. Of the mixtures so prepared 0·2 c.c. was injected subdurally into a second lot of rabbits. The rabbits used throughout this experiment were of an average weight of 1400 grm. It will be obviously impossible, for consideration of space, to tabulate the results of more than one complete experiment in this investigation. Table VIII shows the tests applied to the sera of series *A* and *B* 30 days after completion of treatment.

Table VIII.

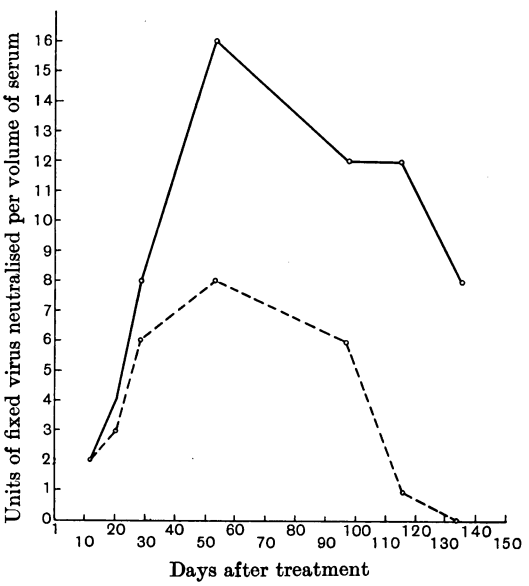
Pooled sera of rabbit series	Test inoculum after 2 hours at 37° C. (0·2 c.c. subdurally)	Effect on rabbits of subdural inoculation of test mixture	Rabicidal content of serum
A (homologous)	1 c.c. serum + 1 c.c. of 1 : 100 F.V. 1 „ + 0·5 „ 1 „ + 0·25 „ 1 „ + 0·1 „	All died in 8 days	0
B (heterologous)	1 cc. serum. + 1 c.c. of 1 : 100 F.V. 1 „ + 0·5 „ 1 „ + 0·25 „ 1 „ + 0·1 „	All died in 8 days	0

Precisely similar results were obtained with the pooled sera of series *A* and *B* tested on the 12th and on the 20th day after completion of treatment with normal brain. It therefore follows that non-specific stimulation evoked in rabbits no rabicidal antibody formation.

Rabicidal properties have now been shown to be non-existent both in the sera of normal untreated animals and in the sera of animals treated with non-specific inocula such as normal brain; they are present, however, after specific stimulation with fixed virus emulsions.

Proofs of existence and of specificity of rabicidal antibody having thus been established, there remains only to determine what relation the serum of an animal treated with carbolised virus bears to that of an animal treated with fresh fixed virus in respect of rabicidal antibody content.

This investigation, to ensure against possible breakdown from fatalities among the experimental animals during the process of immunisation, required the employment of 10 rabbits, whose sera had been proved naturally free from rabicidal properties by the results shown in Table VII. These rabbits were divided into two series, *A* and *B*, each series consisting of five rabbits. Each rabbit in Series *A* was treated for 14 days with 5 c.c. daily of a 2 per cent. emulsion of fixed virus in N.S.S., and each rabbit in Series *B* received an identical dosage of killed carbolised virus during the same period. In order that the true relative degree of rabicidal power possessed by the sera of rabbits immunised by the two methods could be determined, it was necessary for one rabbit to be selected from each series and its blood taken at intervals after completion of treatment. Fortunately the two rabbits originally chosen survived throughout the entire period of investigation.



Graph comparing rabicidal properties acquired by rabbits after immunisation:
(*a*) With fresh fixed virus ———— continuous line.
(*b*) With carbolised fixed virus – – – – – interrupted line.
One unit of F.V. = 0·5 c.c. of 1 % emulsion of F.V. in N.S.S.
One volume of serum = 0·5 c.c. of undiluted immune serum.

Each rabbit was bled 12, 20, 28, 53, 97, 115, 135 and 150 days after completion of treatment. One unit-volume of undiluted serum was mixed with a varying number of unit volumes of a 1 : 100 dilution of fixed virus in N.S.S., and of these mixtures, after their exposure for 2 hours to a temperature of 37° C., 0·2 c.c. was introduced subdurally into rabbits. At the same time a control series of experiments was carried out with the serum of a non-immunised rabbit. The results are shown in Table IX. The relative power of fresh and of carbolised fixed virus vaccines to evoke, in the sera of treated rabbits, rabicidal antibody formation is best appreciated by reference to the chart inserted.

Table IX. *Experiments to show the relative content of rabicidal antibody possessed by the sera of rabbits treated with fresh fixed virus, and of rabbits treated with carbolised fixed virus in equal amount.*

Rabbit *A*: immunised with a 2 % emulsion of living fixed virus in N.S.S. in a dosage of 5 c.c. daily on 14 consecutive days.

Rabbit *B*: immunised with a 2 % emulsion of killed carbolised virus in a dosage of 5 c.c. daily on 14 consecutive days.

Rabbit *C*: control rabbit, not immunised.

Serum of	Serum and varying amounts living fixed virus, mixed and tested after 2 hours' incubation at 37° C.		Columns showing number of days after completion of treatment when serum was tested, and results following subdural inoculation of rabbits with 0·2 c.c. of F.V. serum mixtures prepared on these days. (L. = rabbit lived; D. = rabbit died)							
	Amount of serum in c.c.	Amount of 1 : 100 F.V. in c.c.	12 days	20 days	28 days	53 days	97 days	115 days	135 days	150 days
Rabbit *A*	0·5	0·5	L.	L.	L.	L.	L.	L.	L.	L.
	0·5	1	L.	L.	L.	L.	L.	L.	L.	L.
	0·5	1·5	D.	L.	L.	L.	L.	L.	L.	L.
	0·5	2	D.	L.	L.	L.	L.	L.	L.	L.
	0·5	3	D.	D.	L.	L.	L.	L.	L.	L.
	0·5	4	D.	D.	L.	L.	L.	L.	L.	L.
	0·5	6	D.	D.	D.	L.	L.	L.	D.	D.
	0·5	8	D.	D.	D.	L.	D.	D.	D.	D.
	0·5	10	D.	D.	D.	D.	D.	D.	D.	—
Rabbit *B*	0·5	0·5	L.	L.	L.	L.	L.	L.	D.*	D.†
	0·5	1	L.	L.	L.	L.	L.	D.	D.	D.
	0·5	1·5	D.	L.	L.	L.	L.	D.	D.	—
	0·5	2	D.	D.	L.	L.	L.	D.	D.	—
	0·5	3	D.	D.	L.	L.	L.	D.	D.	—
	0·5	4	D.	D.	D.	L.	D.	D.	D.	—
	0·5	6	D.	D.	D.	D.	D.	D.	D.	—
	0·5	8	D.	D.	D.	D.	D.	D.	D.	—
Rabbit *C*	0·5	0·5	D.	D.	D.	D.	D.	D.	D.	—
	0·5	1	D.	D.	D.	D.	D.	D.	D.	—
	0·5	1·5	D.	D.	D.	D.	D.	D.	D.	D.

* Incubation period = 10 days.

† Incubation period = normal.

The results obtained from this investigation may now be summarised as follows:

(*a*) Fresh fixed virus inoculated into rabbits subcutaneously produces in the sera of these animals a high degree of rabicidal power. One unit volume of such serum (undiluted) is capable of neutralising, when antibody formation has reached a maximum, as many as 16 volumes of a 1 : 100 dilution of fresh fixed virus in N.S.S.

(*b*) Rabicidal antibody content is definitely demonstrable 12 days after completion of treatment; it reaches a maximum some 38 days later and then subsides fairly gradually.

(*c*) Rabbits immunised with fresh fixed virus retain in their sera rabicidal properties in high degree for a period extending over more than 150 days. An accurate estimate of the duration of rabicidal power unfortunately proved impossible in this case on account of increasing difficulties experienced in technique during the last bleedings.

(*d*) Carbolised (killed) virus inoculated into rabbits subcutaneously also produces in the sera of these animals a high degree of rabicidal power. The

maximum reached here, however, falls considerably short of that attained by immunisation with fresh fixed virus, one unit volume of serum being capable of neutralising only 8 unit volumes of a 1 : 100 dilution of fixed virus in N.S.S.

(*e*) Rabbits immunised with carbolised fixed virus retain in their sera rabicidal properties up to a period of 135 days.

It is obviously impossible from these experiments to make any unqualified pronouncement as to the actual relationship between immunity and the rabicidal power possessed by immune sera.

Certain suggestions, however, may be brought forward:

(*a*) Fresh fixed virus acts as a greater stimulus to the production of both immunity and rabicidal action than killed carbolised virus. The degree of immunity and rabicidal action, however, possessed by the sera of rabbits and rats treated with killed carbolised virus has proved sufficient to confer protection against street virus infections (Tables III and V).

(*b*) Immunity and rabicidal action are produced concomitantly; they appear together, are present in greatest degree together, and disappear together.

(*c*) It may be concluded with reasonable certainty that the rabicidal properties of the serum of an animal are an indication of the immunity against rabies possessed by that animal.

2. *Specific complement-binding antibody.*

The principle of complement fixation—the Bordet-Gengou phenomenon—has been extensively employed in the sero-diagnosis of various infectious diseases, for by this method the presence of specific antibodies in immune sera can, as a rule, be determined with certainty on exhibition of the homologous antigen. As the test is reputed to be very many times more delicate than the precipitin reaction, it is not surprising that it has frequently been used in an attempt to detect the occurrence and to estimate the quantity of specific antibody in the sera of human beings and animals highly immunised against rabies infection. It is obvious, however, that if qualitative and quantitative tests of specific complement fixation were available, they would necessarily be limited in practical application. In the sera of human beings exposed to definite risk of infection, for example, a positive reaction would fail to differentiate between response to treatment and response to street virus attack. Such tests could be usefully employed only to evaluate treatment in non-infected animals by different immunising methods. With the majority of aqueous and alcoholic extracts variously prepared from brains of rabid animals to act as antigen, however, serum reactions in such cases have proved non-specific, similar results having been obtained with identical preparations of normal brain. Such non-specific complement fixation has been recorded successively by Heller and Tomarkin (1907), Friedberger (1907), Centanni (1908), Donati and Satta (1908), Baroni, Ciuca and Jonescu (1908), Berry and Mann (1910), Kozewaloff (1910), Moses (1910) and Glusmann and Solowjowa (1927).

Reasoning that the occurrence of non-specific complement fixation was to be explained by the antibody formation consequent upon the unavoidable introduction, during protective inoculations, of foreign nerve protein—inseparable vehicle of fixed virus—Nedrigailoff and Sawtschenko (1911) sought to overcome the difficulty by using extracts of the salivary glands of rabid dogs and human beings as antigen, by which means they claimed to have achieved specific fixation. The extracts of normal salivary glands or of the salivary glands of rabbits dead of fixed virus proved insensitive in the latter because fixed virus is supposed rarely to reach these glands. Zell (1913), employing extracts of salivary glands of rabid animals as antigen, also succeeded in obtaining, with the sera of animals infected with street virus, complement fixation before the appearance of clinical symptoms. Kostrezewski (1920) found that fresh extracts of fixed virus cords brought into contact with the sera of treated patients showed a much higher complement fixation following the intensive method of Pasteur than the Hoegyes' dilution process. As a result, he contended that Hoegyes' method was of less value in the production of anti-rabies immunity, despite the fact that non-specific reactions during his experiments were by no means infrequent. Lubinski and Prausnitz (1926) rightly insist that "it is inadmissible to draw any inference as to the value of the immunisation process from the strength of the serum thus estimated," for, in this instance, the quantity of nerve protein introduced probably played a much greater part in the reaction than the virus itself. Horowitz (1926) asserted that positive results could be obtained either with the serum of rabbits treated intravenously with extracts of salivary glands taken from rabid animals + an extract of fixed virus brain as antigen, or with the serum of rabbits infected subdurally with fixed virus or immunised intravenously + the extract of salivary glands of rabid animals as antigen. In his experimental work, however, he would not appear to have used control antigens of normal salivary gland extracts. He concluded, nevertheless, that subcutaneous injection of anti-rabies vaccine is insufficient to evoke detectable complement-fixing antibody, and thus explained why the sera of immunised animals and human beings invariably give negative results.

Kraus and Takaki (1926), by the subcutaneous injection of fixed virus in increasing virulence, recorded successful production in the rabbit of an immune serum with which a cocto-antigen, made from fixed virus brains, showed considerable complement-binding properties. The reaction, however, was soon shown to be non-specific, as cocto-antigens prepared from the brains of dogs suffering from distemper also fixed complement in the presence of rabies immune sera. Kraus and Michalka (1926) then claimed to have obviated the occurrence of non-specific reactions by substituting glycerine extracts for cocto-antigens. From the foregoing it will be apparent that no uniformity of opinion has been reached in respect of the value of sero-diagnosis in rabies or of quantitative estimation of immunity response, and the main difficulty would seem to lie in an inability to prepare a sufficiently sensitive antigen.

Experiments here were carried out with a view to determining, if possible, in the sera of rabbits immunised respectively with fresh fixed virus and carbolised virus in equal quantities, the relative proportion of specific complement-binding antibodies present, so that a comparison of the immunity-producing capacities of each method might be arrived at. Four antigens were employed in each test—two prepared from fixed virus brains, and two from normal brains to serve as controls. The antigens were prepared as follows: 1 grm. of normal or of fixed virus brain was ground for several minutes with 9 c.c. of absolute alcohol in a mortar with clean sand. The mixture was then stored in an incubator at 37° C. for 1 week, but during this time it was removed daily and placed for 1 hour in a mechanical shaker, screened from light. The suspensions were then centrifugalised at 2000 r.p.m. for 15 minutes; the supernatant fluids, mixed in the proportion of five parts of extract to four parts of a 1 per cent. alcoholic solution of cholesterin immediately prior to serum testing, served as antigen. All four antigens were titrated for anti-complementary and haemolytic action, and the strength of extract used in the tests was half the quantity which just failed to deviate 3 M.H.D. of complement when in contact with normal serum. The tests were performed in accordance with the Rochester Row technique, and for each serum in each test 3, 4, 5 and 6 M.H.D. of complement were employed.

Rabbits were bled and sera tested at various intervals after completion of treatment ranging from 5 days to 2 months; in this way transitory, early or late occurrence of antibody could not be overlooked.

The results of the investigation are shown in Table X.

It will thus be seen that uniformly negative results have been obtained, even with the sera of animals injected subcutaneously with a total dosage of 1200 mg. or of 860 mg. per kilo body weight. Such dosage in man would be equivalent to an administration of 56 grm. *in toto*, a quantity largely in excess of that exhibited in any known method of treatment.

Recently Schultz, Bullock and Brewer (1928) have confirmed these negative findings for sera immunised by the present authors' method. The antigens employed in the numerous tests performed were those of Kostrezewski (10 per cent. suspension of fresh fixed virus, preserved 18–20 hours in an ice chest, and centrifugalised at high speed for 10 minutes), of Kozewaloff (10 per cent. fixed virus in 95 per cent. alcohol, placed in a shaking machine and thereafter centrifugalised for 15 minutes)—in each case the supernatant fluid was used—of Kraus and Takaki and of Kraus and Michalka. The conclusion reached was that "no evidence exists for the presence of specific complement-fixing antibodies against the virus of rabies in the serum of rabbits immunised in various ways with fixed virus brains." Degree and duration of anti-rabies immunity is not, therefore, to be gauged by the Bordet-Gengou reaction.

B. EXAMINATION INTO THE POSSIBILITY OF ANTI-RABIES IMMUNISATION BY SHORT COURSES OF TREATMENT.

To secure post-infectional immunisation in the case of dogs, Gamaléia was advised by Pasteur (1886) to complete treatment in the shortest possible time by the subcutaneous injection of fixed virus cords, varying in virulence from the 10-days' dried to the fresh. The course consisted in administering within 24 hours 1 cm. from each strength of cord, and this treatment was repeated on the following day.

Table X.

No. of rabbits employed	Immunising extract administered	Duration of treatment in days	No. of days after treatment when tests performed	Nature of alcoholic brain extract employed as antigen	Result of complement fixation tests
2	5 c.c. of 2 % F.V. in N.S.S.	1	5, 17, 29, 41	F.V.	Nil
2	,, ,,	1	5, 17, 29, 41	Normal	,,
2	,, ,,	2	5, 15, 25, 34, 46, 58	F.V.	,,
2	,, ,,	2	5, 15, 25, 34, 46, 58	Normal	,,
2	,, ,,	3	5, 14, 20, 29, 46, 58	F.V.	,,
2	,, ,,	3	5, 14, 20, 29, 46, 58	Normal	,,
2	,, ,,	4	13, 19, 27, 39, 51	F.V.	,,
2	,, ,,	4	13, 19, 27, 39, 51	Normal	,,
2	,, ,,	6	11, 17, 25, 39, 51, 60	F.V.	,,
2	,, ,,	6	11, 17, 25, 39, 51, 60	Normal	,,
2	,, ,,	8	9, 15, 24, 30, 44, 58	F.V.	,,
2	,, ,,	8	9, 15, 24, 30, 44, 58	Normal	,,
2	,, ,,	9	9, 14, 22, 29, 14, 58	F.V.	,,
2	,, ,,	9	9, 14, 22, 29, 14, 58	Normal	,,
2	,, ,,	12	6, 11, 27, 39, 51, 60	F.V.	,,
2	,, ,,	12	6, 11, 27, 39, 51, 60	Normal	,,
2	5 c.c. of 2 % F.V. in 1 % phenol	3	5, 14, 20, 29, 46, 58	F.V.	,,
2	,, ,,	3	5, 14, 20, 29, 46, 58	Normal	,,
2	,, ,,	6	11, 17, 25, 39, 51, 60	F.V.	,,
2	,, ,,	6	11, 17, 25, 39, 51, 60	Normal	,,
2	,, ,,	9	9, 14, 22, 29, 44, 58	F.V.	,,
2	,, ,,	9	9, 14, 22, 29, 44, 58	Normal	,,
2	,, ,,	12	6, 11, 27, 39, 51, 60	F.V.	,,
2	,, ,,	12	6, 11, 27, 39, 51, 60	Normal	,,
2	No treatment	—	—	F.V.	,,
2	,,	—	—	Normal	,,

It was found that such a method of rapid immunisation protected animals against subcutaneous and intra-muscular infection with street virus, but the results of subdural and intra-ocular injections were inconstant.

In pre-infectional immunisation, *i.e.* in purely prophylactic treatment, various rapid methods have been employed, but almost exclusively in the protection of dogs, so that the human population may thereby be safeguarded against rabies.

Thus Schnürer's process (1905) consists of the subcutaneous injection in 4 days of a fresh suspension of fixed virus filtered through gauze, the total dosage amounting to 0·75 grm. of virulent material. In Miessner's prophylactic (1912), suspensions of fresh fixed virus or of virus dried for 24 hours at 37° C. and thereafter subjected to pulverization with chalk are employed. One subcutaneous injection of this product "lyssin" totalling 0·75 grm. is the treatment recommended. Carbolised virus is employed in the protective

vaccines of Umeno and Doi (1921) and of Kondo (1922) to secure the same result. In the former 1 part of fixed virus (rabbit's) brain is emulsified in 4 parts of the following mixture: 40 volumes of a 0·5 per cent. solution of phenol in distilled water + 60 volumes of pure neutral glycerine. Of this suspension, exposed before use to a temperature of 20° C. for 14 days or preserved in an ice chest for 1 month, a single subcutaneous injection of 5 c.c. is considered sufficient to confer immunity on a dog weighing 15 kgm. In Kondo's method a 20 per cent. suspension of canine fixed virus is kept for 3 days at 37° C. in a 0·5 per cent. solution of phenol to which 50 per cent. glycerine has been added. Each animal receives a single dose of 5 c.c. administered subcutaneously.

For purely prophylactic treatment, however, the method employed must obviously be free from the possibility of itself conveying infection, and this pre-requisite cannot be fulfilled either by Schnürer's or Miessner's method. Confidence, therefore, has largely been reposed in carbolised virus preparations, particularly in the prophylactic of Umeno and Doi. The virus contained in this vaccine, however, is not dead; in a dosage of 0·0001 grm. it is still capable, on subdural inoculation, of producing rabies in rabbits of 1400 grm. weight.

During 1927 experiments were carried out in this laboratory to determine the time of exposure to 37° C. required by a 20 per cent. suspension of fixed virus brain in 1 per cent. phenol before infectivity is wholly lost. Seventy rabbits of average size and weight were divided into 7 series, 10 in each. Of the suspension under test 0·2 c.c. was introduced subdurally into each of the rabbit series, but after periods of exposure to 37° C. varying from 24 to 168 hours: thus series 1 received as inocula the suspension after 24 hours' exposure, series 2 after 48 hours', series 3 after 72 hours', series 4 after 96 hours', series 5 after 120 hours', series 6 after 144 hours' and series 7 after 168 hours'. The results may be summarised as follows :

In series 1. All 10 rabbits died from fixed virus rabies; symptoms on 5th day.

In series 2. All 10 succumbed; 5 showed a lengthening of incubation period to 7 days.

In series 3. All 10 succumbed; 3 showed a lengthening of incubation period to 9 days.

In series 4. All 10 succumbed; no noticeable increase in incubation period.

In series 5. All 10 succumbed; period of incubation normal.

In series 6. All 10 survived.

In series 7. All 10 survived.

It was concluded, therefore, that a 20 per cent. emulsion of fixed virus in 1 per cent. phenol can be rendered avirulent by exposure to 37° C. for 144 hours.

From available records, however, the administration of 5 c.c. of a carbolised vaccine, prepared according to the process of Umeno and Doi, would appear to be sufficiently innocuous to have justified its extensive adoption in the prophylactic treatment of dogs, which tolerate such dosage with ease.

Whether the attempt to confer full immunity by means of a single injection has been as successful as if several successive doses of less amount had been administered is open to question; according to Kitt (1925) one injection of carbolised vaccine gives no lasting result, whereas with three inoculations (5 c.c. on the 1st, 8th and 15th day of treatment) effective protection can be secured.

According to Vallée (1927), however, it is uniformly agreed that the quantity of vaccine injected should be increased as much as two-fold for some species, according to the weight of the individual animal treated. The dose should be appropriate according to the nature of each of the animal species.

To throw light on the subject of rapid pre-infectional immunisation, the following experiment was devised:

Twenty rabbits of 1400 grm. weight were separated into five lots, four rabbits in each series. As, in the opinion of Umeno and Doi, dogs of 15 kg. could be rendered immune by a single injection of 1 grm. of fixed virus or by 66 mg. per k.b.w. it might be argued that rabbits of 1400 grm. should be protected against subsequent infection by a total dosage of 93 mg. administered on one occasion. To parry criticism, however, regarding the relative immunity response of dogs and rabbits, a much higher dosage per k.b.w. was given to each of the experimental series. Of a 1 per cent. emulsion of carbolised fixed virus four rabbits were treated, therefore, with 2 c.c. daily for 14 days (200 mg. per k.b.w.), four with 4 c.c. daily for 14 days (400 mg. per k.b.w.), four with 6 c.c. daily for 10 days (430 mg. per k.b.w.), four with 15 c.c. daily for 3 days (320 mg. per k.b.w.) and four with one injection of 30 c.c. (214 mg.).

The results obtained from the several methods of treatment are shown in Table XI, and, as will be seen, better results would appear to follow the giving of small doses over a number of days (14) than that of larger doses over a shorter period; further, it would appear impossible to reduce below a certain limit the time over which the total quantity of vaccine, ordinarily sufficient for complete immunisation, can be usefully administered.

The suggestion, therefore, advanced by numerous authorities that it might be of advantage so to alter the method of anti-rabies treatment as to administer larger quantities of fixed virus nerve substance in shorter periods and thus save the patient the necessity of attending at an anti-rabies institute for from 14 to 21 days, is not supported by experimental evidence. The number of observations, however, is probably too few to permit of generalisation.

C. EXPERIMENTAL ENQUIRY INTO THE APPLICABILITY OF FRESH FIXED VIRUS IN ANTI-RABIES TREATMENT.

Evidence that the survivors among rabbits subjected to pre-infectional treatment with fresh fixed virus are afterwards immune to subcutaneous injections with street virus in large dosage has already been given in preceding pages. Such a method of vaccination would, if practicable, be ideal, but, on account of the risk attendant upon its administration in ordinary Pasteurian dosage, it has generally been considered unsafe to use. From personal observa-

NOTICE TO BINDER.

This page should be inserted in *The Journal of Hygiene*, Vol. 29, so as to face page 28.

The omission of Table XI from this paper was notified to the Editors in a letter from Dr Stuart, dated 11 Jan. 1930, who overlooked the omission when he read the proof. We regret that it was left to a reviewer in *The Tropical Diseases Bulletin*, Vol. 26, p. 733 (September, 1929) to note the oversight.—Ed.

Table XI. *Twenty rabbits were used for these experiments.*

Rabbit No.	Dose and period for immunisation: 2 c.c. daily for 14 days	4 c.c. daily for 14 days	6 c.c. daily for 10 days	15 c.c. daily for 3 days	One injection of 30 c.c.	Whether or not animal survived treatment	Subdural test 2 weeks after treatment tested with 0·2 c.c. of 1 % emulsion of fixed virus
1	1	.	.	.	.	Survived	Survived
2	1	.	.	.	.	,,	Died
3	1	.	.	.	.	,,	,,
4	1	.	.	.	.	,,	Survived
5	.	1	.	.	.	,,	,,
6	.	1	.	.	.	,,	Died
7	.	1	.	.	.	Died	.
8	.	1	.	.	.	Survived	Died
9	.	.	1	.	.	,,	Survived
10	.	.	1	.	.	,,	Died
11	.	.	1	.	.	,,	,,
12	.	.	1	.	.	,,	,,
13	.	.	.	1	.	,,	,,
14	.	.	.	1	.	,,	,,
15	.	.	.	1	.	,,	,,
16	.	.	.	1	.	,,	,,
17	.	.	.	.	1	,,	,,
18	.	.	.	.	1	,,	,,
19	.	.	.	.	1	Died	.
20	.	.	.	.	1	Survived	Died

tions, however, on the effect of fresh-fixed virus on human beings and on dogs, Ferran (1888) has concluded that whereas small doses are dangerous and infect, larger doses are harmless and immunise. Moreover, on what seem at first sight to be paradoxical findings, Ferran has based his particular method of treatment.

It appeared, therefore, of immunological interest and importance to ascertain the results in rabbits following on the exhibition of fresh-fixed virus in varying dosage over varying periods. Experiments conducted towards this end would obviously throw light on the rate of immunity response; further, they would be self-controlled, for, if a short intensive course of treatment were to prove unable to effect immunity, the fixed virus itself must have acted as infective agent, whereas, if immunity were wholly established, the virus must have been rendered inert.

Here, then, the inocula were two-fold in nature, infective and immunising, and protection against rabies could only be assured if and when the infectivity of the virus was nullified by its own immunising power.

Table XII.

NOTE. All rabbits were treated with 5 c.c. of a 2 % suspension of fixed virus in N.S.S. administered daily.

No. of exp.	Rabbit series	No. of rabbit	Period of administration (days)	Results of treatment
I	1	1	5	Died in 11 days of F.V. rabies*
		2	5	Lived
		3	5	Died in 11 days of F.V. rabies*
		4	5	" "
		5	5	Lived
	2	6	10	Lived
		7	10	"
		8	10	"
		9	10	"
		10	10	"
II	3	1	5	Died in 11 days of F.V. rabies*
		2	5	Lived
		3	5	"
		4	5	Died in 11 days of F.V. rabies*
		5	5	Died in 12 days of F.V. rabies*
	4	6	10	Lived
		7	10	"
		8	10	"
		9	10	"
		10	10	"
III	5	1	7	Died in 11 days of F.V. rabies
		2	7	Lived
		3	7	Died in 11 days of F.V. rabies
		4	7	Lived
		5	7	"
	6	6	14	Lived
		7	14	"
		8	14	"
		9	14	"
		10	14	"

* Fixed virus rabies confirmed by "passage" experiments in each case. The use of fresh fixed virus as vaccine was thus shown to be unsafe for rabbits in a total dosage over 5 and 7 days of from 500 mg. to 700 mg. (*i.e.* from 350 mg. to 500 mg. per k.b.w.); the administration during 10 and 14 days of from 1000 mg. to 1400 mg. (*i.e.* 700 mg. to 100 mg. per k.b.w.) proved harmless, and capable of conferring immunity.

The first experiment in the series consisted of the subcutaneous injection of 10 rabbits with 5 c.c. daily of a 2 per cent. suspension of fixed virus in N.S.S. Five rabbits received this dosage on 5 consecutive days, and 5 on 10 consecutive days.

The results approximated so closely to Ferran's that it was felt necessary to repeat the experiment. The findings in both series proved identical. The third experiment was conducted on 10 more rabbits, the same daily dosage being administered to two lots of 5 rabbits over 7 days and 14 days respectively.

The results are shown in Table XII.

As an inoculum of 5 c.c. of 2 per cent. fixed virus administered on 5 and on 7 consecutive days had been followed in rabbits by a 60 and a 40 incidence percentage respectively of fixed virus rabies, and as the same inoculum had proved innocuous and, indeed, immunising when given for 10 days and 14 days consecutively, it was resolved to determine the smallest number of days' treatment with this inoculum capable of producing with certainty fixed virus rabies in rabbits, and the smallest number capable of establishing complete immunity.

Table XIII.

NOTE. All rabbits were treated with 5 c.c. of a 2 % suspension of fixed virus in N.S.S. administered daily.

Series	Rabbit no.	Period of administration (days)	Result of treatment	% of deaths from F.V. rabies
1	1	1	Lived	0
	2	1	,,	
	3	1	,,	
	4	1	,,	
	5	1	,,	
2	1	2	Died	80
	2	2	Lived	
	3	2	Died	
	4	2	,,	
	5	2	,,	
3	1	3	Died	40
	2	3	Lived	
	3	3	Died	
	4	3	Lived	
	5	3	,,	
4	1	8	Lived	0
	2	8	,,	
	3	8	,,	
	4	8	,,	
	5	8	,,	
5	1	9	Lived	0
	2	9	,,	
	3	9	,,	
	4	9	,,	
	5	9	,,	
6	1	10	Lived	0
	2	10	,,	
	3	10	,,	
	4	10	,,	
	5	10	,,	

NOTE. All 6 rabbits dying as a result of treatment died in 11 days of fixed virus rabies. This was proved in each case by "passage" experiments.

Thirty rabbits, therefore, were divided into six series of five each. To series 1, 2 and 3 was given a suspension of 2 per cent. fresh-fixed virus in N.S.S. for 1 day, 2 days and 3 days respectively; to series 3, 4 and 5 the same inoculum was administered for 8, 9 and 10 days respectively.

The results are shown in Table XIII.

It will be seen from Tables XII and XIII that, in respect of death rates, markedly different results have been obtained among rabbits treated with fresh-fixed virus over short periods from those following on treatment over longer periods.

Such differences cannot be ascribed (as has been suggested) to accidental wounding of muscle during inoculation, as results are too uniform, viz. death rate is considerable after small dosage administered over short periods, but absent after larger and more prolonged dosage.

It now appears reasonable to make certain deductions:

(*a*) Fresh-fixed virus may be fatal to rabbits on subcutaneous inoculation.

(*b*) Fresh-fixed virus on repeated inoculation produces immunity in rabbits.

(*c*) The life or death of a rabbit treated with fresh-fixed virus is determined by the quantity of virus introduced and by the length of period of its administration.

Few inoculations are more likely to cause death than several; repeated inoculations generally produce immunity.

(*d*) Death from fixed virus injected subcutaneously occurs in rabbits in 11 or 12 days after the commencement of treatment.

Immunity in survivors must therefore have been completely established within that period.

SUMMARY.

1. Statistical evidence and animal experiment have alike proved carbolised killed anti-rabies vaccine to be of undoubted efficacy in both pre-infectional and post-infectional treatment. Statistical evidence is furnished by the results of treatment of 90,000 patients, while experimental proof rests partly on the resistance shown by immunised animals to artificial infections with street virus, and partly on an estimation of degree and duration of rabicidal properties in their sera.

2. The Bordet-Gengou reaction is of no value as a qualitative or quantitative test for the estimation of anti-rabies immunity.

3. The efficacy of killed carbolised vaccine and probably of other anti-rabies vaccines is largely dependent on the concentration of the dosage and the period within which it is administered. Better results follow the giving of small doses over a number of days than the giving of larger doses over a shorter period.

4. It is impossible to reduce below a certain limit the time over which the total quantity of vaccine ordinarily sufficient for complete immunisation can be usefully administered.

5. The effect on rabbits of the subcutaneous injection of fresh-fixed virus is dependent on the quantity of virus introduced and on the duration of its period of administration. Few inoculations are more likely to cause death than several; repeated inoculations ordinarily confer immunity. Immunity thus acquired must have been produced within 11 or 12 days.

REFERENCES.

BABES (1891). Bakteriologisches vom VII internationalen Kongress für Hygiene und Demographie zu London. *Centralbl. f. Bakt.* **10**, 767.

—— (1892). [Communication made to the Académie de medecine] cited in *Traité de la rage*, 1912, 540–541.

—— (1898). Sur le traitement de la rage par l'injection de substance nerveuse normale. *C. R. Acad. des Sci.* **116**, 986.

BABES and LEPP (1889). Recherches sur la vaccination antirabique. *Ann. Inst. Pasteur*, **3**, 384.

BABES and SIMICI (1910). Sur l'action de la substance nerveuse normale dans la rage. *C. R. Soc. Biol.* **68**, 71.

BAREGGI (1889). Sui cinque casi di rabbia paralitica da laboratorio nell' uomo. *Gazz. med. Lombard.* **48**, 217.

BARONI, CIUCA and JONESCU (1908). Recherches sur la présence d'anticorps spécifiques dans le sérum et les extraits d'organes d'animaux vaccinés contre la rage. *C. R. Soc. Biol.* **65**, 96.

BERRY and MANN (1910). The complement binding test in rabies. *J. Exper. Med.* **12**, 338.

BURMEISTER (1915). The absence of demonstrable specific antibodies in rabies caused by fixed virus. *J. Infect. Dis.* **17**, 423.

CENTANNI (1908). Sulla diagnosi della rabbia per mezzo della sotrazione del complemento. [1]*Centralbl. f. Bakt.* **41**, 706.

CONTANI (1908). Sulla diagnosi della rabbia per mezzo della sotrazione della del complemento. *Atti della Regia Accademia dei Fisiocratici in Siena.* Serie IV, **18**. Cited in *Centralbl. f. Bakt.* **41**, 706.

DONATI and SATTA (1908). Sulla deviazione del complemento nella rabbia. *Pathologica*, **1**. 1.

FERMI (1908). Ueber die immunisierende Kraft der normalen Nervensubstanz vergleichen mit der Wutnervensubstanz der Wut gegenüber. *Centralbl. f. Bakt.* **48**, 168.

—— (1909). Nuovi contributi allo Studio della Rabia. *Ann. Inst. d'Igiene Sper.* **19**, 325.

FERRAN (1888). Sur la vaccination antirabique de l'homme. *Ann. Inst. Pasteur*, **2**, 97.

FRANÇA (1910). Du danger de l'emploi des moëlles plus virulentes dans le traitement de la rage. *Centralbl. f. Bakt.* **55**, 154.

FRIEDBERGER (1907). Hat die Methode der Komplementablenkung eine Bedeutung für die Diagnose der Lyssa? *Wien. klin. Wochenschr.* **20**, 879.

FRIEDBERGER and V. EISLER (1907). Ueber das Bindungsvermögen des Lyssavirus für rabicides Serum und die Natur der rabiciden Substanz. *Centralbl. f. Bakt.* **44**, 695.

GLUSMANN and SOLOWJOWA (1927). Ueber den diagnostischen Wert der Komplementbindungsreaktion bei Lyssa. *Zeitschr. f. Immunitäts.* **54**, 199.

HARVEY and ACTON (1923). The degree of efficacy of antirabic treatment. *J. Ind. Med. Res.* **10**, 1039.

HARVEY and MCKENDRICK (1907). Theory and Practice of Antirabic Immunization. *Sci. Memoirs, Gov. of India*, No. 30.

[1] Unless otherwise stated, references throughout relate to *Centralb. f. Bakt.* Abt. I. Orig.

HELLER and TOMARKIN (1907). Ist die Methode der Komplementbindung beim Nachweis spezifischer Stoffe für Hundewut und Vaccine brauchbar? *Deutsche med. Wochenschr.* **33**, 795.

HOROWITZ (1926). Ueber die Komplementablenkungsreaktion bei der Tollwut. *Centralbl. f. Bakt.* **98**, 216.

ISABOLINSKY and ZEITLINA (1927). Zur Frage über die biologischen Eigenschaften des Virus fixe. *Zeitschr. f. Immunitätsforschung.* **49**, 67.

KITT (1925). [Diskussion zu Schnürer.] *Centralbl. f. Bakt.* **78**, 160.

KOCH (1927). Ueber paralytische atypische abortive und die sogenannte Impfwut. *Ibid.* **104**, 381–409.

KONDO (1922). On the antirabic vaccination of the dog; on the rabicidal property of antirabies serum. *J. of Japan. Soc. Veter. Sci.* **1**, 279.

KOSTREZEWSKI (1920). Untersuchungen über die Blutserumeigenschaften bei den tollwutschutzgeimpften Menschen. *Centralbl. f. Bakt.* **84**, 106.

KOZEWALOFF (1910). Ueber komplementbindende und rabizide Substanzen im Blute wutkranker Kaninchen. *Ibid.* **54**, 564.

KRAUS, GERLACH and SCHWEINBURG (1926). *Lyssa bei Mensch und Tier.* (Berlin and Vienna: Urban and Schwarzenberg.)

KRAUS, KELLNER and CLAIRMONT (1902). Ueber das Verhalten des Lyssavirus im Zentralnervensystem empfänglicher, natürlich immuner und immunisierter Tiere. *Zeitschr. f. Hyg. u. Infektionskr.* **41**, 486.

KRAUS and KREISSL (1902). Ueber den Nachweis von Schutzstoffen gegen Hundswut beim Menschen. *Centralbl. f. Bakt.* **32**, 810.

KRAUS and MARESCH (1902). Ueber die Bildung von Immunsubstanz gegen das Lyssavirus bei natürlich empfänglichen Tieren. *Zeitschr. f. Hyg. und Infektionskr.* **41**, 527.

KRAUS and MICHALKA (1926). Die Diagnose des Lyssavirus mittels Komplementablenkung mit Koktoimmunogen und Glycerinextrakt. *Zeitschr. für Immunitätsf.* **47**, 504.

KRAUS and TAKAKI (1926). Der Nachweis der neurotropen Virusarten mittels Komplementablenkung mit Koktoantigen. *Wien. klin. Wochenschr.* **39**, 624.

LÄSSER (1927). Zur Diagnose der Lyssa durch Präzipitation. *Zeitschr. f. Immunitätsf.* **53**, 1.

LUBINSKI and PRAUSNITZ (1926). Lyssa. *Ergebnisse der Hygiene, Bakteriologie, Immunitätsforschung und Experimentellen Therapie*, **8**, 1.

MARIE (1904). De quelques propriétés du sérum antirabique. *C. R. Soc. Biol.* **56**, 1030.

—— (1905). Recherches sur le sérum antirabique. *Ann. Inst. Pasteur*, **19**, 1.

—— (1908). Recherches sur le sérum antirabique. *Ibid.* **22**, 271.

—— (1912). Propriétés biologiques de substances albuminoïdes extraites du cerveau. *Ibid.* **26**, 441.

MCKENDRICK (1927). Rabies: A Review of recent articles, VIII. *Trop. Dis. Bull.* March 1928, p. 188.

MIESSNER (1912). Ueber Tollwutschutzimpfung bei Tieren. *Centralbl. f. Bakt.* **54**, 73.

MOSES (1910). Ueber den Nachweis von Antigen und Antikörper durch Komplementablenkung. *Mem. Inst. Oswaldo Cruz*, **1**, 109.

NEDRIGAILOFF and SAWTSCHENKO (1911). Ueber die Anwendung der Komplementbindungsmethode für die Diagnose der Tollwut. *Zeitschr. f. Immunitätsf.* **8**, 353.

NIKOLAJEWA (1925). Antiwutimpfung mittels Carbolvaccine nach der Methode von Prof. Fermi. *Centralbl. f. Bakt.* **95**, 423.

PASTEUR (1886). Cited in Babes (1912). *Traité de la rage*, 613.

PEREIRA DA SILVA (1926). Persistance des substances rabicides dans le sang des individus traités par le virus rabique fixe étherisé. *C. R. Soc. Biol.* **95**, 323.

PRAUSNITZ (1927). Ergebnisse der Internationalen Tollwutkonferenz in Paris. *Centralbl. f. Bakt.* **87**, 529–548.

PUNTONI (1924). Effetti della glicerina e del freddo sulla soprovivenza sul perioda di incubazione del virus rabico. *Ann. d'Igiene.* **34**, 716.

REMLINGER (1905). Contribution à l'étude des mélanges de sérum antirabique et de virus fixé. *Ibid.* **57**, 637.

SCHNÜRER (1905). Zur praeinfektionellen Immunisierung der Hunde gegen Lyssa. *Zeitschr. f. Hyg. u. Infektionskr.* **51**, 46.

SCHULTZ, BULLOCK and BREWER (1928). The antigenic properties of rabies virus. *J. of Immunology*, **15**, 265–281.

SEMPLE (1908). On the preparation and use of antirabic serum and on the rabicidal properties of the serum of patients after undergoing antirabic treatment. *Lancet*, i, 1611.

—— (1911). On the preparation of a safe and efficient antirabic vaccine. *Scientific Memoirs by Off. of Med. and San. Dep. of Gov. of India.*

STUART and KRIKORIAN (1925). Antirabic procedure in Palestine with special reference to decentralisation of treatment. *Ann. of Trop. Med. and Parasit.* **19**, 393–418.

TOMARKIN and SUAREZ (1917). Präzipitation und Thermopräzipitation bei Vaccine. *Zeitschr. f. Immunitätsf.* **26**, 385.

UMENO and DOI (1921). A study on the antirabic inoculation of dogs, and the results of its practical application. *Kitasato Arch. of Exper. Med.* **4**, 89.

VALLÉE (1927). Anti-Rabies vaccination of animals. *Report to International Rabies Conference*, 137.

VIALA (1891). Sur les causes de l'atténuation des moëlles rabiques. *Ann. Inst. Pasteur*, **5**, 695.

ZELL (1913). Serodiagnosis of rabies. *Amer. J. Veter. Med.* **8**, 637.

(*MS. received for publication* 5. XI. 1928.—Ed.)