FURTHER STUDIES IN ANTI-RABIES IMMUNISATION. RABIES VIRUS—EXALTED AND CLASSICAL STRAINS COMPARED.

By G. STUART, O.B.E., M.A., M.D., D.P.H.
and K. S. KRIKORIAN, M.D.

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

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GENERAL INTRODUCTION.

In a previous series of studies published by us (1929) it was established that the anti-rabies immunity produced by carbolised fixed virus approximates in practice to that produced in the survivors of animal groups treated with living virus in normal saline solution. Evidence of efficacy of treatment was further sought in an estimation of antibody content in immune serum and this naturally evoked, *inter alia*, an enquiry into the rabicidal properties possessed by such serum. It was found that fresh fixed virus acts as a greater stimulus to the production of both immunity and rabicidal antibody 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 proved sufficiently high to confer protection against the street virus used during the investigations and exhibited in a dosage far exceeding that likely to be encountered in natural infections. From the results it appeared that immunity and rabicidal action are produced concomitantly, are present in greatest degree together and disappear at or about the same time. Moreover

it was inferred, although final proof was wanting, that the rabicidal properties of an animal's serum, are, in general, an indication of the immunity against rabies possessed by that animal. For obvious reasons, however, the same "standardised" strain of street virus was necessarily employed throughout our experimental work—a strain of proved constant strength in respect of virulence and infective power and one capable of producing, with certainty, in a dilution of 1:1000, symptoms in rabbits on the sixteenth day after subdural inoculation with 0·2 c.c. and death 4 days later. But street virus is derived from many sources and from many species of animals able to convey infection; it need not, therefore, be a matter for surprise that animals immunised in the ordinary way against rabies may yet be unable to survive the critical test of later inoculation with newly discovered, uninvestigated, unknown or vicarious strains of street virus. Experience has abundantly shown that in nature there exists a multiplicity of street viruses distinguishable by certain characteristics but more particularly by individual pathogenicity. Thus extreme variations in virulence are exemplified by the oulou-fato virus of West Africa on the one hand and by the Tangier B (Chabrier) strain on the other, the former, apparently innocuous to man, showing on first transfer to rabbits an incubation period of from 15 to 17 days, the latter one of 8 days only. Such difference, however, purely one of degree of virulence, would constitute no considerable problem in anti-rabies immunisation, but the existence of biological variations among various strains of street virus, the suggestion, indeed, of biological individuality, would, if proved, render of little account most modern methods of Pasteurian treatment and envisage the replacement of monovalent antigens with polyvalent and, where feasible, auto-vaccines.

The occurrence in Palestine during 1930 of a street virus of extraordinary virulence, which caused, despite treatment, the death of three bitten persons, afforded us an opportunity of investigating at first hand the basic question of plurality of viruses. To throw light upon the subject it seemed expedient first to institute enquiry into the nature of such an exalted virus and then to compare its infective, immunising and rabicidal antibody-producing powers with those of the ordinary virus in daily use here. The results of our investigations, which included cross-immunity and inoculation experiments, are given below.

I. EXALTED RABIES VIRUS (Virus de rue renforcé).

1. Occurrence.

On the same day in two neighbouring villages in Northern Palestine seven persons were bitten by a rabid jackal, and of that number, despite the administration of a full course of anti-rabies vaccine, three developed symptoms of hydrophobia. Anti-rabies procedure in Palestine includes complete decentralisation of treatment, and the vaccine used in the fifteen district

institutes and prepared for issue at the Central Laboratories consists of a 1 per cent. emulsion of carbolised fixed virus, which is exhibited over a period of 14 consecutive days in a daily dosage of 5 c.c. totalling 0·7 grm. nervous substance. Most probably because of their belonging to the ignorant peasant class all seven bitten persons delayed reporting at the nearest District Health Office until 2 days after the time of biting, thereby rendering cauterisation and other forms of local treatment inadequate.

The following is a brief synopsis of the case histories:

Case 1. Male; age 25; bitten severely on nose 2. v. 30; treated between 4. v. 30 and 18. v. 30 but absent on third day of prescribed treatment; appearance of symptoms 19. v. 30 and death 2 days later; autopsy performed and brain forwarded for laboratory examination; microscopic findings: Negri bodies numerous—and animal inoculation tests confirmed the clinical diagnosis of hydrophobia. The incubation period of the disease was, therefore, 17 days and its duration 2 days.

Case 2. Male; age 30; received two deep face wounds; attendance at treatment centre identical with that of Case 1; onset of symptoms 30. v. 30 with death next day. In this case the incubation period was 29 days.

Case 3. Male; age 4; son of Case 2; showed one deep wound on head; attended for treatment as Cases 1 and 2; date of symptoms 8. vi. 30; duration of disease 10 hours; incubation period 37 days.

Cases 4, 5, 6, 7, however, bitten with moderate severity by the same jackal are now, 6 months after the completion of treatment, alive and well. Each of the four commenced treatment 2 days after being bitten and failed to attend on the second, third and fourth days of the prescribed course. These cases may thus be summarised:

Case No.	Sex	Age	Location and nature of wounds
4	M.	60	One wound on head (scalp)
5	M.	25	Four wounds on upper arm through naked skin
6	F.	50	Two wounds on upper arm through clothing
7	M.	45	One wound on head (face)

2. RECOVERY OF VIRUS.

On 25. v. 30 the M.O.H. Safad submitted for laboratory examination part of a brain taken from a human case of suspected rabies. This, for Palestine, somewhat unusual procedure was followed in the present instance for two reasons: (*a*) the patient had been bitten less than 3 weeks before his death by a rabid jackal, and (*b*) although the symptomatology described by the deceased's relatives was suggestive of hydrophobia, the Medical Officer had had no opportunity of observing the clinical course of the disease.

Results obtained at the laboratory following direct subdural inoculation of rabbits with emulsions of the human brain indicated the atypical nature of the rabies strain—hereafter referred to as the Safad Virus—and led to an enquiry being made into its main characteristics.

3. Characteristics of the Safad virus.

Reviewing the work of Busson (1928) and of Schweinburg (1928) on rabies virus, McKendrick (1929) observes that viruses were examined by these authors with regard to (1) infectivity (M.L.D. on subdural inoculation), (2) the incubation period which each induced (related to organ-specificity), and (3) the duration of the resulting illness (a measure of specific toxicity). "These three characteristics are fundamental," he continues, "and in comparing strains of either street or fixed virus their study is essential."

The following is an account of the Safad virus considered mainly from these standpoints.

(*a*) *Infectivity.*

Measurement of infectivity, then, is usually made by an accurate estimation of the minimal lethal dose, or M.L.D., of the substance under test, *i.e.* the smallest quantity which will, without fail, produce symptoms in a susceptible animal and cause its death in a certain time. As regards the M.L.D. of rabies virus, however, most workers in its determination pay insufficient attention, apparently, to one of its definitive criteria, viz. that the time interval between inoculation and appearance of symptoms (or death) must be a constant; indeed, in the evaluation of such viruses, they commonly regard the smallest quantity of rabid nervous tissue capable ultimately of producing symptoms as sole index of virulence. But since it is generally held that rabies virus is dependent for its activity on many unknown or unappreciated factors such as its distribution and concentration in the central nervous system, "lag," its rate of multiplication and its specific aggressiveness, the reason for the elimination from most calculations of the time factor is not immediately obvious. True it is that Schweinburg (1928) in contrasting a number of street virus strains by the effect of their subdural inoculation in varying dilution provided a notable exception; in fact he proved the non-relationship of M.L.D. to incubation period by finding that, with incubation times of 9, 9, 16, 30 and 16 days, the corresponding lethal doses were 1/2000th, 1/5000th, 1/8000th, 1/2000th and 1/7000th mg. respectively. It is submitted that in all similar investigations the definition of M.L.D. given by us (1929) should be strictly adhered to: "the highest dilution of a virus which, in rabbits of average weight (1400 grm.), will invariably produce symptoms in the same period of time after subdural infection with 0·2 c.c." Results of our determination of M.L.D. made in accordance with the requirements of this definition are now given in Table I in respect of (1) the fixed virus strain used here in vaccine preparation, (2) an ordinary Palestinian street virus, and (3) the Safad virus on the days of its second and twenty-ninth rabbit transfers.

From the results given in Table I it will be seen that the true M.L.D. of each virus under test proved to be a 1:1000 dilution.

(*b*) *Incubation time and duration of illness.*

Multiplication rate (= incubation time) of the Safad virus, together with its deleterious action (= specific toxicity), is shown in Table II, which embodies the findings consequent upon the subdural inoculation of rabbits with the virus during its first twenty subpassages.

Table I. *Estimation of the true* M.L.D. *of rabies virus.*

No. of rabbit	Dilution of virus used	Day of onset of symptoms after subdural inoculation with: Fixed virus Paris	Street virus Jaffa	Safad virus 2nd day	Safad virus 29th day	Day of death after subdural inoculation with: Fixed virus Paris	Street virus Jaffa	Safad virus 2nd day	Safad virus 29th day
1	1: 1,000	5	16	6	5	7	20	7	6
2	1: 1,000	5	16	6	5	7	20	7	6
3	1: 1,000	5	16	6	5	8	20	7	6
4	1: 1,000	5	16	6	5	7	20	7	6
5	1: 2,500	5	16	6	6	7	20	7	8
6	1: 2,500	6	18	7	6	8	22	8	8
7	1: 2,500	6	16	7	5	9	20	9	7
8	1: 2,500	5	18	6	6	7	23	7	8
9	1: 5,000	6	17	7	6	8	21	9	9
10	1: 5,000	5	16	8	7	7	21	10	10
11	1: 5,000	6	20	7	7	9	24	9	10
12	1: 5,000	6	18	9	6	8	22	11	9
13	1: 10,000	8	19	7	7	10	24	8	11
14	1: 10,000	6	18	10	9	9	22	13	12
15	1: 10,000	7	20	9	8	10	24	11	11
16	1: 10,000	22	18	11	8	24	23	14	11

Table II. *Incubation time and specific toxicity of Safad virus.*

No. of passage	Date of inoculation	Date of appearance of symptoms	Incubation period (in days)	Date of death	Duration of illness (in days)
1	26. v. 30	2. vi. 30	7	3. vi. 30	1
2	12. vi. 30	18. vi. 30	6	19. vi. 30	1
3	19. vi. 30	25. vi. 30	6	26. vi. 30	1
4	26. vi. 30	3. vii. 30	7	4. vii. 30	1
5	4. vii. 30	10. vii. 30	6	11. vii. 30	1
6	11. vii. 30	17. vii. 30	6	19. vii. 30	2
7	19. vii. 30	25. vii. 30	6	26. vii. 30	1
8	29. vii. 30	4. viii. 30	6	5. viii. 30	1
9	5. viii. 30	10. viii. 30	5	12. viii. 30	2
10	12. viii. 30	17. viii. 30	5	19. viii. 30	2
11	19. viii. 30	24. viii. 30	5	26. viii. 30	2
12	26. viii. 30	31. viii. 30	5	2. ix. 30	2
13	2. ix. 30	7. ix. 30	5	9. ix. 30	2
14	9. ix. 30	14. ix. 30	5	16. ix. 30	2
15	16. ix. 30	21. ix. 30	5	23. ix. 30	2
16	23. ix. 30	28. ix. 30	5	30. ix. 30	2
17	30. ix. 30	5. x. 30	5	7. x. 30	2
18	7. x. 30	12. x. 30	5	14. x. 30	2
19	14. x. 30	19. x. 30	5	21. x. 30	2
20	21. x. 30	26. x. 30	5	28. x. 30	2

Table II shows that the Safad virus, originally obtained from the brain of a person dead from a rabid jackal's bite and thereafter introduced subdurally into rabbit series, first caused illness in 7 days and death in 8. As a result of successive passages through rabbits symptoms appeared in 6, 6, 7, 6, 6, 6, 6, 5, 5, 5, 5, 5 days after inoculation and death occurred in 7, 7, 8, 7, 8, 7, 7, 7, 7, 7, 7, 7 days respectively, the duration of illness varying from 1 to 2 days.

(*c*) *Occurrence of Negri bodies.*

Histological examination of the human brain submitted for diagnosis showed, with Mann's stain, Negri bodies measuring 2–6 μ in diameter to be fairly numerous in the large pyramidal cells of the hippocampus major lying close to the alveus. The brain of the first rabbit, however, inoculated with a portion of human hippocampus, revealed a total absence of Negri bodies from the pyramidal cells of the cerebral cortex in general and of Ammon's horn in particular and also from Purkinje's layer of corpuscles in the cerebellum. The same complete absence of Negri bodies was observed in the brains of rabbits examined after the second, third, fourth, fifth and sixth subpassages.

4. Characteristics of virus de rue renforcé.

Generally speaking, while there exists considerable diversity of opinion regarding the nature, if not the properties of rabies virus, the terminology "street virus" and "fixed virus" has a universal significance and the difference between these viruses is ordinarily held to be qualitative rather than quantitative. Nevertheless, although certain differences may readily be explained on quantitative grounds and/or as a result of adaptation, and although the two viruses on close study present the greatest similarity, there are five distinguishing features commonly advanced as characteristic: mode of transmission and propagation; the presence or absence of Negri bodies; the type of lesion produced in the nerve centres; the length of incubation period and the very definite reduction in pathogenicity for man of fixed virus introduced subcutaneously—an attenuation of virulence practically to the point of innocuity. Of these incubation time commonly serves to differentiate fixed virus from the street virus of its origin and as a criterion of virulence and will, therefore, first be considered.

(*a*) *Incubation time.*

Evidence of there being reinforced or exalted strains of street virus in nature is afforded by a study of available statistics giving the incubation periods of hydrophobia in man. According to Harvey and McKendrick (1930) "the period of incubation varies with the distance to be travelled (by the virus). In man, for implantation on the head it is approximately 27 days, on the arm 32 days and on the leg 64 days, but these figures are subject to wide variations." Thus, in the experience of Bauer (1886), among 510 cases hydrophobia supervened in 8·24 per cent. during the first 19 days following the bite; Alivisatos (1926) has recorded incubation periods of 10 and 13 days and Konradi (1923) periods of 12, 13 and 14 days respectively. It is the opinion of Lubinski and Prausnitz (1926) and of Alivisatos (1926) among others that these cases might well be explained by an increased virulence of rabies virus.

Further evidence of the occurrence of naturally exalted street viruses is

to be adduced from a consideration of their effects on first transmission to rabbits. Thus Babes (1912), in discussing variations in virulence of rabies virus as it occurs in nature, states that rabbits inoculated subdurally with street virus mostly succumb in about 14 days, this interval between infection and death indicating a virus of average virulence, but, he continues, "il existe en outre des virus naturellement exaltés." According to Helman (1888), ordinary incubation time is from 14 to 18 (20) days, to Lubinski and Prausnitz (1926) from 15 to 20 days, while to Harvey and Acton (1923) "this conception of an average interval of 15 days' incubation is taken for granted by practically all institutes."

Now, if from the brain of a rabbit dead from the street virus strains ordinarily encountered transference by the subdural route is effected into another rabbit and if this process is repeated in successive transfers from one rabbit series to another, it will be found that, in the course of such subpassaging, the incubation period consistently shortens until at about the fiftieth passage it achieves a constant minimum of 6 or 7 days (absolute limits are (3) 4 and 10 days). The average incubation time, therefore, for street virus may be put at 15 days and for fixed virus (6) 7.

But in nature the rare occurrence of street virus strains with, when compared with fixed virus, identical shortness of incubation period has long since been recognised. Thus Remlinger (1908), while examining cases of rabies in very young dogs, more than once obtained a strain which, on first transmission to rabbits, caused death in from 8 to 10 days. Such a strain termed "virus (de rue) renforcé" had previously been encountered and recorded by Calabrese (1896), Abba (1898) and d'Amato (1904), Calabrese having found, for example, that of 280 street viruses investigated at the Pasteur Institute in Paris twenty or 7 per cent. produced symptoms in rabbits within the first 10 days. Since then Remlinger (1924) in Morocco, Alivisatos (1926) in Servia, Koch (1929) in Germany and Harvey and Acton (1923) in India have added to the list of street viruses belonging to this category. In the case of the Moroccan virus, originally derived from a young fox, rabbits inoculated with the strain developed symptoms on the eighth day and died on the ninth; the Servian strain also, with an incubation period of 8 days following intramuscular injection, exemplifies a street virus of specially high virulence. In Koch's list are included several strains of typical hyper-virulence. Thus, for instance, a street virus obtained from a cat invariably killed experimental animals after an incubation period of 5 days, behaving from the first passage as a fixed virus. Again, from a human case of hydrophobia, in which symptoms had developed 22 days after the bite, Koch recovered a strain, which proved, on rabbit inoculation, stronger than the fixed virus he used for prophylactic vaccination, all animals dying in the second passage after an incubation period of 3 days. Other reinforced viruses gave incubation times varying from 5 to 7 days on primary rabbit transfer. Further, the records of Harvey and Acton (1923) show that 650 street viruses have been investigated at Kasauli with incuba-

tion periods varying from 5 to 61 days, and of that number onset of paralytic symptoms was observed during the first 10 days in ninety-three cases or in 14·3 per cent. Our experience in Palestine, however, in contrast to the findings of Harvey and Acton, tends to support the views expressed by Remlinger (1908, 1924) and Babes (1912) that occurrence of exalted virus is somewhat infrequent. Thus during the past 8 years the biological test, applied in the diagnosis of rabies here, has proved positive on ninety occasions, but in respect of only five viruses has the incubation period been less than 15 days, viz. 8, 11, 12, 14, 14; in the others it has varied between 15 and 28 days.

(*b*) *Fixation time.*

Mutation of street virus into fixed virus is normally accompanied by characteristic changes due to its progressive adaptation to the rabbit's nervous system. Reference has already been made to the fact that fixed virus is ordinarily obtained from the street virus of its origin only after some fifty passages through the rabbit, but Hoegyes (1900), for example, found that, when young rabbits were employed, a fixed virus could be achieved after about fifteen passages. Beham (1915), also, working in this country, effected fixation in respect of three street viruses after the fourth and sixth passages and reports have been made by many other workers concerning strains varying widely from the mean as regards rate of fixation. Indeed, if the suggestion of Levaditi and his colleagues (1924 *a*) were followed this difference in fixation-rate would constitute a satisfactory basis for classification of all street viruses and permit their separation into three main groups, viz.:

Group I. Highly virulent strains of street virus which show, *even at the first passage*, exceptionally short incubation periods and which, throughout all subsequent passages, retain their properties intact. Such viruses are termed "souches spontanément mutées" or "virus naturellement renforcé de Remlinger."

Group II. Less virulent strains in which mutation from street to fixed virus is easily effected but only after a certain number of passages in the rabbit. These viruses, referred to as "souches facilement mutables," cannot, despite ease of mutation, be included in the category of truly exalted strains.

Group III. Strains of low virulence in which change from street to fixed virus does not take place even after prolonged rabbit passage. These "souches non mutables" do, it is true, convey rabies to laboratory animals but only after an incubation period of irregular duration and of greater length than is experienced with any virus belonging to the first two groups.

In connection with this classification it appears to us desirable further to divide Group II into two sub-groups, viz.:

Sub-group II (*a*). Strains of abnormally high virulence in which complete fixation follows after but few passages.

Sub-group II (*b*). Strains of ordinary virulence in which fixation is completed after the average number of passages, *i.e.* about fifty.

We feel it all the more necessary to emphasise distinctions between street virus strains by such division and subdivision, as it is our considered opinion that a definite ratio exists between the degree of virulence and the fixation rate of rabies strains.

It is not proposed to discuss here Groups II (*b*) and III, which have little bearing on the subject under review; brief reference, however, must be made to certain typical strains in Groups I and II (*a*) if the use of fixation rate as a means of group differentiation is to be fully appreciated. Well-known viruses to be described under Group I are Tangier B (Chabrier), Puymyrol, Soucy and Koritschoner, under Group II (*a*) Gibraltar (Gréen), Tétuan, Tangier D (Martin) and Teodorascu. Of the Group II (*a*) strains, Gibraltar, Tétuan and Tangier D were first examined by Remlinger (1926) and then sent in glycerine to Levaditi for further investigation.

Group I.

(1) Moroccan strain—Tangier B (Chabrier) was originally derived from a young fox; rabbits first inoculated with the virus subdurally developed symptoms on the eighth day and died on the ninth, while in succeeding passages death occurred 7, 8, 7, 7, 8, 6, 6, 7, 7, 6, 7, 7, 7 days after inoculation. (Strain examined by Remlinger.)

(2) Paris strain—Puymyrol was recovered from a dog which had bitten his master on the day preceding its death; inoculated intramuscularly into a guinea-pig this virus caused death in 23 days; subsequent passages in rabbits showed periods between inoculation and death to be 8, 8, 8, 8, 9, 9, 8, 8, 8 days. (Strain examined by Levaditi.)

(3) Paris strain—Soucy was obtained from the brain of a dog diagnosed clinically rabid; guinea-pig infected intramuscularly died in 15 days; subpassages in rabbits produced death 8, 8, 8, 8, 8, 8, 8, 9, 9 days after subdural inoculation. (Strain examined by Levaditi.)

(4) Koritschoner virus was first found in the brain of a man bitten by a rabid dog and dead of hydrophobia 24 days after completion of anti-rabies treatment (incubation period = 36 days); rabbits inoculated subdurally developed symptoms after 3 or 4 days' incubation and subsequent passages proved this interval between infection and appearance of symptoms to be constant. (Strain examined by Schweinburg (1925).)

Group II (a).

(1) Gibraltar strain (Gréen) was isolated from a dog which had, while showing signs of furious rabies, bitten three persons and a number of animals; first rabbit passage showed symptoms to follow subdural inoculation after 12 days, and death occurred in 14; death followed further rabbit subpassaging in 20, 11, 9, 10, 10, 8, 7, 8, 8, 8, 8 days and fixation was thus effected after the seventh passage.

(2) Moroccan strain—Tétuan was obtained from a dog dead of furious rabies; brain emulsions introduced into the anterior chamber of a rabbit's

eye caused the animal's death in 13 days; later research proved the intervals between subdural infection of rabbits and their death to be successively 10, 10, 11, 9, 12, 8, 10, 9, 8, 7 days; fixation took place here between the sixth and tenth passages.

(3) Moroccan strain—Tangier D (Martin) was derived from a basset hound, which had bitten many human beings and animals; subdural inoculation of a rabbit with bulb suspensions caused symptoms in 12 days and death in 14; subsequent passages caused death in 14, 11, 10, 9, 9, 10, 9, 9, 8, 9, 9, 9, 6 days; mutation of this virus began about the fourth passage and was complete at the eleventh.

(4) Teodorascu's virus was found in the brains of four people who, despite intensive anti-rabies treatment, had died of hydrophobia during the first 13 days following completion of the course (Puscariu III and Hoegyes I). Emulsions of brain matter taken from two of the cases were introduced into laboratory animals, killing guinea-pigs between the seventh and twentieth days and rabbits between the fourteenth and seventeenth days after subdural inoculation but producing no symptoms after subcutaneous infection. After the fifth intracranial passage through guinea-pigs, however, the virus became fixed with a constant incubation period of from 5 to 6 days, and it has since preserved a uniform virulence for rabbits, killing invariably between the seventh and tenth days.

Study of these strains amply shows that certain street viruses (Group I) behave from their first subpassages as fixed viruses and that thereby they can be sharply distinguished from a less exalted type—Group II (*a*), in which mutation is complete only after several passages in the rabbit.

(*c*) *Absence of Negri bodies.*

Mutation of street virus is normally accompanied by considerable modifications not only of its biological properties but also of its morphological characteristics. Negri bodies, for example, found regularly in the hippocampi of all creatures dead of street virus rabies, are hardly ever present in fixed virus infections. Absence of Negri bodies is, therefore, an important distinguishing feature between fixed virus and the street virus from which it is derived. The extent, then, to which Negri bodies proved to be present in the brains of rabbits infected with the strains just considered as typifying Groups I and II (*a*) is obviously germane to the present discussion and will now, therefore, be considered in brief. In Group I viruses Tangier B (Chabrier), Puymyrol and Soucy showed alike, from the first rabbit transfer, a complete absence of Negri bodies in the usual sites or at most a very rare occurrence of atypical forms. The Koritschoner virus, also, was wholly unable to effect Negri body formation in the brain of man or of experimental animals in successive passages, although, in the hippocampus of the dog, from which it was originally obtained, these bodies were present in exceptionally large numbers. Not only, therefore, in degree of infectivity and in brevity of incu-

bation period do naturally reinforced street viruses approximate to fixed virus but also in their loss of Negri body-producing power.

In Group II (*a*) the Gibraltar strain, as has already been shown, required for fixation seven passages in the rabbit; at the third passage Negri bodies appeared in the hippocampus and cerebral cortex to the extent of 80 per cent.[1], at the fourth this figure had dropped to 10, at the sixth to 3 and at the seventh to zero; complete fixation and absence of Negri bodies were thus achieved at precisely the same passage. Tétuan virus, which proves fixation to have taken place between the sixth and tenth passages, showed an 80 per cent. incidence of Negri bodies between the second and fifth passages, but, from the sixth passage onwards the time between rabbit inoculation and death became shorter and more regular (7–9 days) and Negri bodies were altogether absent or only very occasionally present in an undeveloped form. In the Tangier D (Martin) strain fixation was finally secured at the eleventh passage, at a time when Negri bodies, previously present to the extent of 70–80 per cent., disappeared entirely from the brains of inoculated rabbits and when the interval between subdural infection and death had decreased to 7–9 days. As regards Teodorascu's virus, no information about Negri bodies is, unfortunately, available.

It is clear from an examination of Group II (*a*) strains that the virulent street viruses in this category do not become fixed at the first rabbit passage (as is the case with Group I strains) but give evidence of rapid mutation by a shortening of incubation period, by a greater regularity in the interval between inoculation and death and by a progressive decrease in the number and size of Negri bodies towards final disappearance; first total absence of Negri bodies synchronises, be it noted, with complete fixation of virus.

With regard to this subject it is unhappily the case that, while the research work of Williams and Lowden (1906), Negri (1909), J. Koch and Rissling (1910), Manouélian (1912), Watson (1913), Manouélian and Viala (1924) and Levaditi, Nicolau and Schoen (1924 *a*, 1924 *b*, 1926) has thrown considerable light on the nature and mode of transmission of rabies virus as well as on the significance of the Negri body, the International Rabies Conference of 1927 was unable from available evidence to decide whether the virus was a protozoon or a bacterium or whether Negri bodies represent a stage in the evolution of a living microsporidium or are produced by cellular degeneration. A plausible explanation, however, of the differences in Negri body content of brains infected with fixed and street viruses in general is to be found in the theory supported by many authors, that the rabies virus is protozoal in nature, belonging to the *Microsporidiida*, sp. *Glugea lyssae*. According to this conception the microparasite has a two-phase life history, one a phase of filtrable spores (only just, if at all, within the limits of visibility = Koch's

[1] The method adopted by Levaditi in his percentage estimation of Negri bodies was to note their occurrence in thirty-five pyramidal cells in the right Ammon's horn, thirty-five in the left horn and thirty in the cerebral cortex.

granulations) and of ultramicroscopic germs, the other a phase of intracellular sporoblastic encystment (Negri bodies). Fixed virus, on the one hand, consists solely of elementary and ultramicroscopic organisms in which reproduction occurs so rapidly that death of the infected animal is caused before the microsporidium has had time to complete its entire complex cycle of development and to arrive at the formation of the spherical uninucleate cells, pansporoblasts or sporonts considered to be Negri bodies. In street virus, on the other hand, where ordinarily development proceeds much more slowly, the survival of the infected animal together with the resistance offered to the virus by certain neurons, which have preserved their anatomical and physiological integrity, will permit the completion of the entire evolutionary cycle and consequently result in the appearance of the characteristic pansporoblasts. But in certain strains of street virus, in those "fixed at once," *i.e.* virus de rue renforcé, this distinction no longer holds, for, on account of the extraordinary virulence of these viruses, incubation period and interval to death are so short that, just as in fixed virus, there is no time for the pansporoblastic stage to be reached. (It has to be understood that this explanation is "based only on external analogies, that up to the present the whole developmental cycle (of the parasite) has not been sufficiently demonstrated and that, therefore, this theory, however attractive it may be, cannot claim to be anything more than a working hypothesis" (Lubinski and Prausnitz, 1926).)

5. Definition.

From a consideration of the foregoing we suggest that virus de rue renforcé should be defined as a naturally exalted street virus, characterised by high degree of infectivity, by abnormally short incubation period and duration of illness, by complete mutation or fixation at the first passage in rabbits and by a behaviour thereafter in all respects akin to fixed virus, including an inability to effect Negri body formation.

The Safad virus, therefore, subscribing as it does to all these criteria, must be included in the category of such viruses.

II. CROSS-IMMUNITY EXPERIMENTS.

1. Introduction.

Among the resolutions adopted at the International Rabies Conference held in Paris during 1927 was one to the effect that, *inter alia*, "enquiries should be made into the plurality of street virus strains." The need for such systematic investigation was apparent in view of the conflicting opinions expressed by leading authorities on the causes underlying abnormal behaviour of certain strains; thus by some, differences in "power of aggression" were explained on purely biological grounds, by others solely by specific variations in degree of virulence. That street viruses vary in individual power of attack is unquestionable; the observations of Bouffard (1912) and Heckenroth (1918), for example, on the occurrence in Senegal of a disease known as mad-dog

sickness or oulou-fato, which is in all respects similar to the rabies of Europe but which appears to be non-transmissible to man, instance in strains the nadir of virulence, while at the zenith are the naturally exalted viruses just considered. But at first sight this problem of plurality of strains appears capable of easy solution; theoretically submission of "atypical" viruses to the test of cross-immunity and to the action of the sera of immunised animals should place the matter beyond all doubt; in practice, however, results have been widely divergent. Thus Puntoni (1923) found that rabbits did not develop rabies if, during the period of ordinary incubation, they were given by the intravenous route carbolised suspensions of the street virus used for their subdural infection; in fact the only survivors proved to be those inoculated with homologous viruses and vaccines. Further, in a second set of experiments he tried the effect on fixed virus and on street virus of the serum of a human case treated with carbolised fixed virus and mixtures were prepared containing the viruses under test in 1 per cent. suspensions and 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:5000 dilutions of immune serum. For fixed virus a 1:500 dilution of serum was found to be rabicidal, but in the case of street virus 1:200 was required. These results led Puntoni to assume the biological individuality of certain strains of street virus and to raise the question of the expediency of polyvalent anti-rabies vaccines in treatment. Additional arguments in favour of the plurality of strains have since then been advanced by Calderini (1928) and by Teodorascu (1929). The former, in his examination of many street viruses, discovered one particular bovine strain, which differed markedly in its degree of virulence from all others examined. Normally even after many subpassages Calderini's strains were unable to cause the death of guinea-pigs in less than 5 days, but the bovine virus after thirty-five passages exhibited a degree of virulence sufficient to kill without exception on the fourth day. This hyper-virulence or exaltation of strain remained constant throughout twenty-six further passages through guinea-pigs and was not, in the author's opinion, the result of continued subpassage but was entirely dependent on some inherent character originally present while in the cow. Teodorascu makes use of the hypothesis of plurality of strains to account for four failures of treatment with monovalent vaccines. In support of this contention he refers to the results of his experimental work, viz. that treatment with ordinary fixed virus developed no immunity against his strains of street virus and, conversely, that vaccination with his strains afforded no protection against ordinary fixed virus when tested on rabbits. Again, the occurrence in Equatorial Africa of the practically avirulent viruses described by Bouffard and Heckenroth is held by some to constitute reason for the employment of polyvalent vaccines in the treatment of man. In direct opposition to these views, are the opinions expressed by Remlinger and his co-workers. Thus in discussing the viruses of Senegal, Remlinger (1928), while admitting an appreciable difference between the symptomatology of natural rabies in tropical countries and that encountered in more temperate zones—

the paralytic form largely predominates in the former—considers such distinction to indicate a geographical but not a radical difference in the strains. Further, by means of cross immunity and inoculation tests, Remlinger and Curasson (1924) claim to have established the identity of the oulou-fato virus with the naturally exalted Tangier B (Chabrier) strain and therefore with the virus of rabies itself. Again, Remlinger and Bailly (1930) investigated on similar lines a street virus, which appeared to be atypical in having, despite treatment, caused the death of two bitten persons; in as much as, according to their findings, the strain was indistinguishable by serological and immunity tests from a classical rabies virus, they concluded that the apparent difference was due to the specific power of activity and aggression possessed by the strains under review and to that power alone. According to this conception, therefore, the strains were biologically identical and the authors felt justified in pronouncing that "from the practical standpoint, whatever the virus may be, whether attenuated or exalted, polyvalent and auto-vaccines constitute a needless complication of anti-rabies treatment."

Under the circumstances, then, it seemed to us desirable to institute enquiry into the behaviour of Safad virus in cross-immunity experiments and thereby to throw light, if possible, upon this vexed question, which is of paramount importance in anti-rabies immunisation.

2. Experimental evidence of cross immunity following treatment with carbolised suspensions of Paris (standard) and Safad (atypical) strains of rabies virus.

Degree of anti-rabies immunity possessed by rabbits actively immunised against rabies virus is ordinarily measured by their acquired resistance to infection with rabies strains and by an estimation of the rabicidal antibody content of their immune sera; should, then, any apparently atypical strain be encountered, its actions and reactions must surely be investigated along similar lines and compared with those of a classical virus. Moreover, this method of research obviously permits the following two arguments to be advanced regarding the question of "unity" or "plurality" of rabies virus, viz. (*a*) if, when tested by later subdural infections, the immunity following the exhibition of a classical virus in vaccine form is sufficient to confer on rabbits protection against homologous strain and atypical virus alike—and *vice versa*—proof of relationship between the two viruses is at once established; (*b*) if the classical strain is neutralised *in vitro* by the serum of rabbits immunised with the atypical virus and, conversely, if the atypical virus is neutralised under similar conditions by the serum of rabbits immunised with the classical strain, proof of actual identity now exists and the two viruses, whatever their differences in original virulence, cannot be separated on biological grounds.

The Safad virus at its twentieth subpassage in rabbits has, therefore, been compared in these respects with the Paris strain of fixed virus, it being

remembered that the Safad virus was "fixed" from the first rabbit transfer and that, in any case, fixed viruses, as far as crossed serum reactions are concerned, "act as the street viruses from which they are derived" (Marie, 1927).

This investigation required the employment of twenty rabbits whose sera had been proved naturally free from rabicidal properties. These rabbits were divided into four series, *A*, *B*, *C* and *D*, each series consisting of five rabbits. Each rabbit in Series *A* and *B* was treated for 14 days with 5 c.c. daily of a 2 per cent. suspension of killed carbolised Paris virus, and each rabbit in Series *C* and *D* received an identical dosage of killed carbolised Safad virus during the same period. The following experiments were then performed:

(*a*) Absolute immunity was tested in each rabbit series 52 days after completion of treatment, at a time when our previously recorded work (1929) has shown immunity to be at a maximum. Cross-immunity tests were carried out by the subdural infection of rabbits in each series with 0·2 c.c. of a 1:1000 suspension of fixed virus, viz. Series *A* and *C* were inoculated with Paris F.V., Series *B* and *D* with Safad F.V. The results are shown in Table III.

(*b*) Rabicidal antibody content was estimated in the immune sera of the treated rabbits. All rabbits were bled on the thirtieth day after completion of treatment and the sera of Series *A* and *B* and of Series *C* and *D* pooled separately. One unit volume of immune serum (0·5 c.c.) was then mixed with quantities of a 1:100 fixed virus, varying in each case from 0·5 c.c. to 3 c.c., and each mixture exposed to a temperature of 37° C. for 2 hours. At the end of this time 0·2 c.c. of each mixture was introduced subdurally into rabbits. By these means the rabicidal properties of Series *A* and *C* immune sera were examined in respect of Paris fixed virus, those of Series *B* and *D* immune sera in respect of Safad fixed virus.

From the results given in Table III it follows that Paris fixed virus, when used as immunising agent, was able to protect five out of five rabbits infected with living Paris fixed virus (100 per cent.) and three out of five rabbits infected with living Safad fixed virus (60 per cent.). Safad fixed virus, on the other hand, when used as immunising agent, was able to protect only one out of five rabbits infected with living Paris fixed virus (20 per cent.) and two out of five rabbits infected with living Safad fixed virus (40 per cent.).

Study of Table IV will show that: (*a*) One unit volume of the undiluted serum of rabbits immunised with killed carbolised Paris fixed virus and tested 30 days after completion of treatment was capable of neutralising 6 volumes of a 1:100 dilution of fresh living Paris fixed virus (homologous strain) and 4 volumes of fresh living Safad fixed virus (heterologous strain) in equal dilution. (*b*) One unit volume of the undiluted serum of rabbits immunised with killed carbolised Safad fixed virus and tested under identical conditions was capable of neutralising 2 volumes of a 1:100 dilution of fresh living Safad fixed virus (homologous strain) and 1 volume of fresh living Paris fixed virus (heterologous strain) in equal dilution.

Table III. *Immunity tests.*

The immunising power in rabbits of Paris and Safad fixed virus compared.

Rabbit series	Rabbit No.	Method of immunisation	Method, dose and time of infection with test dose	Result	Rabbit series	Rabbit No.	Method of immunisation	Method, dose and time of infection with test dose	Result
A	1	2 % suspension of Paris F.V.: 5 c.c. daily for 14 days	0·2 c.c. of a 1 : 1000 suspension of Paris F.V. subdurally, 52 days after end of treatment	Lived	*B*	1	2 % suspension of Paris F.V.: 5 c.c. daily for 14 days	0·2 c.c. of a 1 : 1000 suspension of Safad F.V. subdurally, 52 days after end of treatment	Lived
	2			Lived		2			Died*
	3			Lived		3			Died
	4			Lived		4			Lived
	5			Lived		5			Died*
C	1	2 % suspension of Safad F.V.: 5 c.c daily for 14 days	0·2 c.c. of a 1 : 1000 suspension of Paris F.V. subdurally, 52 days after end of treatment	Died	*D*	1	2 % suspension of Safad F.V.: 5 c.c. daily for 14 days	0·2 c.c. of a 1 : 1000 suspension of Safad F.V. subdurally, 52 days after end of treatment	Lived
	2			Lived		2			Died
	3			Died		3			Died
	4			Died		4			Lived
	5			Died		5			Died*
Control	1	Nil	0·2 c.c. of a 1 : 1000 suspension of Paris F.V. subdurally	Died	Control	1	Nil	0·2 c.c. of a 1 : 1000 suspension of Safad F.V. subdurally	Died
	2			Died		2			Died
	3			Died		3			Died

* Incubation period prolonged.

Table IV. *Estimation of rabicidal antibody content in immune sera.*

Rabicidal properties possessed by the sera of rabbits immunised with Paris and with Safad fixed virus respectively.
Rabbit Series *A* and *B*: immunised with a 2 % emulsion of killed carbolised Paris virus in a dosage of 5 c.c. daily on 14 consecutive days.
Rabbit Series *C* and *D*: immunised with a 2 % emulsion of killed carbolised Safad virus in a dosage of 5 c.c. daily on 14 consecutive days.

Immune serum	Rabbit No.	Mixtures of immune serum and varying amounts of living fixed virus: Amount of serum in c.c.	Amount of 1 : 100 Paris F.V. in c.c.	Result of subdural inoculation of rabbits with 0·2 c.c.	Immune serum	Rabbit No.	Mixtures of immune serum and varying amounts of living fixed virus: Amount of serum in c.c.	Amount of 1 : 100 Safad F.V. in c.c.	Result of subdural inoculation of rabbits with 0·2 c.c.
Series *A* (Paris)	1	0·5	0·5	Lived	Series *B* (Paris)	1	0·5	0·5	Lived
	2	0·5	0·5	Lived		2	0·5	0·5	Lived
	3	0·5	1·0	Lived		3	0·5	1·0	Lived
	4	0·5	1·0	Lived		4	0·5	1·0	Lived
	5	0·5	2·0	Lived		5	0·5	2·0	Lived
	6	0·5	2·0	Lived		6	0·5	2·0	Died
	7	0·5	3·0	Lived		7	0·5	3·0	Died
	8	0·5	3·0	Lived		8	0·5	3·0	Died
Series *C* (Safad)	1	0·5	0·5	Lived	Series *D* (Safad)	1	0·5	0·5	Lived
	2	0·5	0·5	Lived		2	0·5	0·5	Lived
	3	0·5	1·0	Died		3	0·5	1·0	Lived
	4	0·5	1·0	Died		4	0·5	1·0	Lived
	5	0·5	2·0	Died		5	0·5	2·0	Died
	6	0·5	2·0	Died		6	0·5	2·0	Died
	7	0·5	3·0	Died		7	0·5	3·0	Died
	8	0·5	3·0	Died		8	0·5	3·0	Died
Controls not immunised	1	0·5	0·5	Died	Controls not immunised	1	0·5	0·5	Died
	2	0·5	1·0	Died		2	0·5	1·0	Died
	3	0·5	1·5	Died		3	0·5	1·5	Died

3. Discussion.

A combination of the results given in Tables III and IV permits an interesting comparison to be made between Paris (classical) fixed virus and Safad (atypical) fixed virus in respect of their immunising and rabicidal antibody producing powers—a comparison which has a very definite bearing on the question of plurality of rabies strains.

In the first place it will be observed that the effect on rabbits of vaccination with Paris fixed virus was (*a*) to afford 100 per cent. protection against the homologous strain and 60 per cent. against the exalted or atypical strain,

and (*b*) to confer on the sera of rabbits so treated rabicidal properties capable of neutralising 6 unit volumes of a 1 per cent. suspension of the homologous virus and 4 volumes of the atypical strain. Secondly it is shown that the effect on rabbits of vaccination with Safad fixed virus was (*a*) to afford 40 per cent. protection against the homologous strain and 20 per cent. against the classical Paris strain, and (*b*) to confer on the sera of rabbits so treated rabicidal properties capable of neutralising 2 unit volumes of a 1 per cent. suspension of the homologous virus and 1 volume only of the classical strain. The results of the immunising experiments, then, clearly show that, whereas Paris fixed virus in the form of carbolised vaccine—the ordinary immunising agent used here in anti-rabies treatment—protected rabbits in a highly satisfactory way against subsequent infections with homologous and heterologous strains alike, the Safad fixed virus as vaccine compared unfavourably in its power of protecting rabbits against either virus used in the subdural test. Indeed, the resistance to fresh Safad virus offered by rabbits previously treated with Safad fixed virus vaccine fell considerably short of that shown by rabbits treated with ordinary Paris fixed virus vaccine, while the protection conferred by Safad fixed virus vaccine against subsequent infection with living Paris fixed virus was decidedly unsatisfactory. As an immunising agent, therefore, both in general and in particular, the Paris strain of fixed virus proved unquestionably far superior to the Safad strain. Treatment of the Safad cases with an autogenous vaccine would obviously then have served no useful purpose and it is further logical to assume that, if strain *X* be a virus endowed with adequate immunising power and strain *Y* be deficient in this respect, the use in anti-rabies treatment of strain *X* vaccine alone would be of greater service than a vaccine in which strain *X* and *Y* were combined. An argument is thus advanced in support of the continued use of a monovalent anti-rabies vaccine of proved immunising ability and against the need for preparation of polyvalent or autogenous vaccines.

Next with regard to the serum-neutralisation experiments, it is immediately apparent that anti-viral bodies were evoked in rabbits to a much greater extent by treatment with Paris virus than with Safad virus, quantitative estimation being based on the power possessed by immune sera of neutralising, volume by volume, centesimal fixed virus emulsions. Moreover, as a result of this investigation, a very definite, indeed an almost mathematical relationship was established in treated rabbits between their degree of acquired immunity and the rabicidal antibody content of their blood; thus when the percentage of acquired immunity was 100 (homologous), 60 (heterologous), 40 (homologous) and 20 (heterologous) the rabicidal power of the corresponding immune sera was respectively 6-, 4-, 2- and 1-fold. It is our belief that this remarkable concurrence is unlikely to arise from random causes or be accounted for by coincidence alone; on the contrary, despite the small number of rabbits employed, it would appear to be of certain significance: that, with the notable exception of those cases wherein solid immunity has

been recorded without corresponding anti-viral formation in the serum, rabicidal antibody content is ordinarily, if not identical with, at least indicative of anti-rabies immunity. If, now, this conclusion is allowed, it follows that a further reason has been advanced against the necessity of employing autogenous or polyvalent vaccines in anti-rabies treatment.

To turn finally to the question of relationship between the two viruses under review. "To-day it is generally recognised that modifications in the activity of rabies virus strains can only be explained by the presence of a micro-organism" (Marie, 1927). The same criteria, therefore, must, where possible, be applied in the classification of strains of rabies virus and of bacteria. Now, of the several means available for determining the identity or non-identity of two bacteria, the power possessed by the bacterial body of calling forth in the blood of living animals substances or properties of an antagonistic nature is unquestionably the most delicate. The protein, protein-lipoid complex or other compound capable of acting as an antigen gives rise to its own special antibody and the reaction between antibody and antigen is of a highly specific character. Upon the degree of specificity, therefore, in immunological reactions will the establishment of intimate phylogenetic relationship between two bacteria or two rabies strains ultimately depend, and in the case of rabies virus such will be achieved by means of protective tests and serum-neutralisation experiments. A glance at Tables III and IV will show that, from the point of view both of cross-protection tests and crossed serological reactions, the classical (Paris) strain and the Safad (exalted) strain may, despite apparent differences, be safely included within the same category of rabies virus. True, a higher degree of protection was afforded to rabbits and the rabicidal antibody content in their sera was greater after treatment with Paris virus than with the Safad strain, but this can readily be explained by different immunising properties of the two viruses; moreover, that rabies viruses differ in this respect need occasion no more surprise than that certain strains of *Bact. typhosum* should be preferred in anti-enterica prophylaxis.

From a consideration of the above and in the light of our experience with these two strains, we are of opinion that exalted virus and classical virus are not to be differentiated by immunological tests and are, indeed, identical. Our views, therefore, support the conclusions of Remlinger and his co-workers and are in opposition to those expressed by Puntoni, Calderini and Teodorascu.

SUMMARY.

1. The occurrence of an exalted strain of rabies virus has been recorded and a definition given of virus de rue renforcé.

2. The characteristics of exalted virus have been examined in respect of incubation time, fixation rate and absence of Negri bodies.

3. An adequate classification of street virus strains has been suggested.

4. Cross-immunity experiments have been carried out and, as a result of

cross-protection tests and of crossed serum reactions, identity of exalted virus with the classical strain has been established.

5. The employment of autogenous and polyvalent vaccines has been shown to be unnecessary in anti-rabies immunisation.

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