APPEARANCE AND PERSISTENCE IN RABBITS' BLOOD OF RABICIDAL ANTIBODIES PRODUCED BY VARIOUS METHODS OF ANTI-RABIES IMMUNISATION

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

(*From the Government Central Laboratories, Jerusalem, Palestine.*)

(With a Chart.)

Introduction.

Among the resolutions adopted by the Second and Third Commissions at the International Rabies Conference held in Paris during 1927 was one to the effect that comparative tests on a large scale should be carried out with anti-rabies vaccines killed by carbolic acid and by ether and that enquiries should be made into the rabicidal action of the serum of man and animals during and after immunisation. Such investigations were recommended by the conference, after due consideration of the results obtained by various recognised methods of treatment, in order that sufficient data might be made available for an accurate estimation of the comparative values of these methods. The present article deals primarily with a series of experiments performed to determine the relative content of rabicidal antibody possessed by the sera of several rabbit groups treated respectively with fresh-fixed virus, carbolised-fixed virus and etherised-fixed virus in equal quantities.

Before the main experiments could be undertaken it was obviously essential to carry out certain preliminary investigations and enquiries, the results of which have already been fully recorded by us elsewhere (1929); only brief reference, therefore, to the principal findings is necessary here.

(*a*) *The nature of the antibody content of anti-rabies immune serum.*

It is generally agreed that specific changes in the blood serum characteristic of active immunisation follow treatment with anti-rabies vaccines and that those most likely to be evoked by stimulation of the animal organism with rabies virus itself are rabicidal, specific complement-binding and precipitating antibodies. Here, however, an inability to dissociate, for practical purposes, the antigen from its necessary vehicle of administration—nervous tissue—introduces a complication not normally encountered in immunisation problems. The antibody formation, under such circumstances, might well be both specific and non-specific: specific in response to the causal agent of rabies, non-specific in response to the nervous matter in the inocula—neurolytic and precipitating in nature. The conclusion reached by Schultz, Bullock and Brewer (1928)

https://doi.org/10.1017/S0022172400010949 Published online by Cambridge University Press

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" has the support of the majority of workers including ourselves, who (1929) have shown that "degree and duration of anti-rabies immunity cannot be gauged by the Bordet-Gengou reaction." Again, Burmeister (1915) was unable to demonstrate the occurrence of specific precipitins in the sera of rabbits suffering from fixed virus rabies, and Lässer (1927) was equally unsuccessful in his efforts to adduce evidence of the formation of such precipitins in rabies-infected and in immunised animals. Broadly speaking, then, the position to-day is that, while it has not yet been finally decided whether the rabies virus *quâ* virus is wholly incapable of producing complement-fixing and precipitating antibodies in the immune organism, the occurrence of a rabicidal antibody is, from weight of experimental evidence, almost universally accepted as the sole serological effect of immunisation.

(*b*) *Rabicidal properties of immune serum.*

As considerable diversity of opinion exists in regard to the production of rabicidal antibody it became essential during our 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. After considerable experimentation it was proved conclusively that rabicidal properties are non-existent in the sera of normal untreated animals and in the sera of animals treated with non-specific inocula such as normal nerve substance, whether homologous or heterologous; they are present, however, after specific stimulation with fixed virus emulsions. Proofs of existence and of specificity of rabicidal antibody having thus been advanced, there remained then to determine what relation the serum of an animal treated with fresh-fixed virus bears to that of an animal treated with carbolised virus and to that of one treated with etherised virus—all in equal amounts.

RECORD OF RABICIDAL PROPERTIES ACQUIRED AFTER IMMUNISATION.

Owing to the very large number of rabbits envisaged by such an experiment it was considered inadvisable to undertake the complete investigation at one time. The somewhat limited accommodation ordinarily available here for purely research laboratory animals together with the difficulty of obtaining a regular supply of rabbits compelled this decision. As a result the determination of rabicidal antibody consequent on treatment with fresh-fixed virus and with carbolised-fixed virus was made during 1929 (Experiment II, Part 1, Series *A*, *B* and *C*), that after treatment with etherised-fixed virus during 1930 (Experiment II, Part 2, Series *D* and *E*). This unavoidable division of the work necessitated the use of two control series of rabbits and unfortunately precluded, in the two parts of the investigation, the withdrawal of blood at

identical intervals after completion of the several forms of treatment employed. For reasons of brevity and clearness, however, the whole investigation is described as if it had been carried out during the same period of time.

Experiment I.

Proof that rabicidal antibody did not exist naturally in the serum of rabbits used in the main experiment. Fifteen rabbits of 1400 grm. average weight were divided into three series *A*, *B* and *D*, each series including five animals. (Series *A* was later to be immunised with living fixed virus in N.S.S.[1], series *B* with carbolised virus and series *D* with etherised virus.) All the animals were bled and their sera pooled for each series. 0·5 c.c. of serum *A*, 0·5 c.c. of serum *B* and 0·5 c.c. of serum *D* were then mixed respectively with quantities ranging from 0·125 to 0·5 c.c. of a 1:100 dilution of fixed virus and the serum-fixed virus 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: 0·5 c.c. of a 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 three rabbits. Table I shows the results obtained:

Table I.

Pooled sera of rabbit series	Test inoculum after 2 hours at 37° C. (0·2 c.c. subdurally)	Effects on rabbits of subdural inoculation	Rabicidal content of sera
A	0·5 c.c. serum + 0·125 c.c. of 1 : 100 F.V.	All died in 8 days	Nil
	0·5 „ + 0·25 „		
	0·5 „ + 0·5 „		
B	0·5 „ + 0·125 „	All died in 8 days	Nil
	0·5 „ + 0·25 „		
	0·5 „ + 0·5 „		
D	0·5 „ + 0·125 „	All died in 8 days	Nil
	0·5 „ + 0·25 „		
	0·5 „ + 0·5 „		
Control series	0·5 c.c. of 1 : 100 F.V.* in N.S.S.†	All died in 8 days	—
	0·5 c.c. of 1 : 100 F.V. in N.S.S.		
	0·5 c.c. of 1 : 100 F.V. in N.S.S.		

* Fixed virus. † Normal salt solution.

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 the incubation period, the total absence of rabicidal antibodies in the sera of all three series of rabbits selected for later immunisation—first symptoms were invariably observed on the 5th day after subdural infection. Further, the experiment also shows from the control series that the fixed virus employed 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 used in all experiments here. (Our fixed virus in a 1:1000 dilution produces without exception symptoms of rabies on the 5th day with death on the 7th or 8th day.) The fixed virus emulsions referred to throughout this investigation were

[1] Normal salt solution.

in all cases prepared from the bulb of a freshly-extracted F.V. brain and the procedure of employing in all comparative tests the same anatomical part of a fresh brain is regarded by us as of first importance. The bulb is weighed and then thoroughly ground up in a sterile mortar; enough N.S.S. is gradually added to make an emulsion of 1:100 by weight. The emulsion is now filtered through four layers of gauze and the filtrate utilised to make up the various serum—F.V. mixtures used for rabbit inoculations. The mixtures are then incubated for 2 hours at 37° C. to permit of adequate union and 0·2 c.c. of each is inoculated subdurally into rabbits of 1400 grm. weight. This technique differs considerably from that advised by the International Rabies Conference of 1927, but appears to us equally satisfactory. A note describing the alternative procedure, however, is appended (see p. 422).

Experiment II.

To guard against possible breakdown from fatalities among the animals undergoing immunisation, this, the main experiment, required the original employment of 15 rabbits, whose sera had been proved naturally free from rabicidal properties by the results detailed in Table I.

These rabbits were divided into three series *A*, *B* and *D*, each series consisting of five rabbits. Each rabbit in series *A* received on 14 consecutive days 5 c.c. daily of a 2 per cent. emulsion of fixed virus in N.S.S., each rabbit in series *B* received an identical dosage of killed carbolised virus and each rabbit in series *D* an identical dosage of killed etherised virus administered over the same period. In order that an accurate determination might be made of the true relative degree of rabicidal power possessed by the sera of rabbits immunised by the three methods, it was essential that one rabbit should be selected from each series and its blood taken at intervals after commencement and completion of treatment. The selection of the three representative rabbits entailed the laborious task of comparing the rabicidal power of the serum of each rabbit in each series during the earlier bleedings with that of the pooled serum of each rabbit group. The rabbit selected in each case was necessarily one whose serum showed identical rabicidal antibody content with the pooled serum of its own series. Fortunately all three rabbits ultimately chosen survived throughout the entire period of investigation.

The rabbit representing series *A* was bled 7, 14, 18, 22, 26, 34, 42, 67, 111, 129, 149 and 164 days after commencement of treatment, that representing series *B* 7, 14, 18, 22, 26, 34, 42, 67, 111, 129, 149, 164, 197, 218, 240 and 250 days after commencement, and that representing series *D* 7, 14, 18, 22, 46, 57, 70, 118, 144, 174, 204, 222, 240 and 256 days after commencement. One unit volume of undiluted serum was mixed with a varying number of unit volumes of a 1:100 suspension 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 control series were

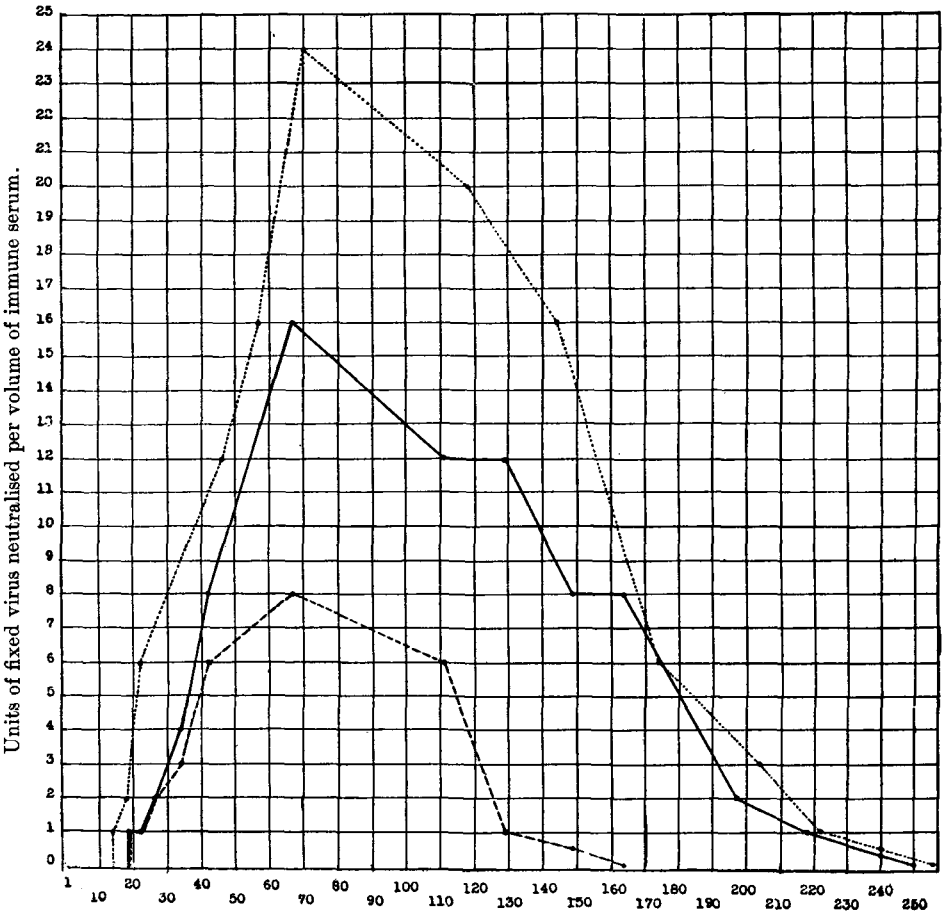
carried out with the serum of non-immunised rabbit groups, viz.: series *C* in respect of series *A* and *B*, series *E* in respect of series *D*.

Two rabbits were inoculated subdurally with each proportion of serum—F.V. mixture and in every case where the incubation period was unduly pro-

GRAPH COMPARING RABICIDAL PROPERTIES ACQUIRED BY RABBITS' SERUM AFTER IMMUNISATION BY VARIOUS METHODS

(*a*) With fresh-fixed virus —— continuous line.
(*b*) With carbolised-fixed virus ----- interrupted line.
(*c*) With etherised-fixed virus ..... dotted line.

NOTE: One unit of fixed virus = 0·5 c.c. of a 1 % emulsion of F.V. in N.S.S.
One volume of serum = 0·5 c c. of undiluted immune serum.



longed or the symptoms in any way atypical, two rabbits were subpassaged with the brain in question to prove that death had been due to rabies.

As on the completion of the main experiment the findings proved somewhat unexpected, it was resolved to repeat on two further occasions the

https://doi.org/10.1017/S0022172400010949 Published online by Cambridge University Press

TABLE II. *Experiments to show the relative content of rabicidal antibody possessed by the sera of various rabbit series treated with fresh-fixed virus, carbolised-fixed virus and etherised-fixed virus respectively.*

Rabbit series *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 series *B*: immunised with a 2 % emulsion of killed carbolised virus in a dosage of 5 c.c. daily on 14 consecutive days.
Rabbit series *C*: not immunised and serving as controls for series *A* and *B*.
Rabbit series *D*: immunised with a 2 % emulsion of killed etherised virus in a dosage of 5 c.c. daily on 14 consecutive days.
Rabbit series *E*: not immunised and serving as controls for series *D*.

PART 1. Series *A*, *B* and *C*.

Serum tested	Serum and varying amounts of living fixed virus mixed and tested after 2 hours' incubation at 37° C. Amount of serum in c.c.	Amount of 1 : 100 F.V. in c.c.	Columns showing number of days after commencement 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. (− = rabbit lived; + = rabbit died) 7	14	18	22	26	34	42	67	111	129	149	164	197	218	240	250
Rabbit Series *A*	0·5	0·125	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
	0·5	0·250	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+
	0·5	0·5	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+
	0·5	1·0	+	+	+	+	−	−	−	−	−	−	−	−	+*	+	+	+
	0·5	1·5	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+
	0·5	2·0	+	+	+	+	+	−	−	−	−	−	−	+	+	.	.	.
	0·5	3·0	+	+	+	+	+	+	−	−	−	−	−	+	.	.	.	.
	0·5	4·0	+	+	+	+	+	+	−	−	−	−	−	+	.	.	.	.
	0·5	6·0	+	+	+	+	+	+	+	−	−	−	+	+	.	.	.	.
	0·5	8·0	+	+	+	+	+	+	+	−	+	+	+	+	.	.	.	.
	0·5	10·0	+	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.
Rabbit Series *B*	0·5	0·125	+	+	−	−	−	−	−	−	−	−	−	+†	.	.	.	.
	0·5	0·250	+	+	−	−	−	−	−	−	−	−	−	+	.	.	.	.
	0·5	0·5	+	+	−	−	−	−	−	−	−	−	+*	+	.	.	.	.
	0·5	1·0	+	+	+	+	−	−	−	−	−	+	+	+	.	.	.	.
	0·5	1·5	+	+	+	+	+	−	−	−	−	+	+	+	.	.	.	.
	0·5	2·0	+	+	+	+	+	+	−	−	−	+	.	.	.	.	.	.
	0·5	3·0	+	+	+	+	+	+	−	−	−	+	.	.	.	.	.	.
	0·5	4·0	+	+	+	+	+	+	+	−	+	+	.	.	.	.	.	.
	0·5	6·0	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.
	0·5	8·0	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.
	0·5	10·0	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.
Rabbit Series *C*	0·5	0·125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0·5	0·250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0·5	0·5	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.

* Incubation period 10 days. † Incubation period normal.

PART 2. Series *D* and *E*.

Serum tested	Serum and varying amounts of living fixed virus mixed and tested after 2 hours' incubation at 37° C. Amount of serum in c.c.	Amount of 1 : 100 F.V. in c.c.	Columns showing number of days after commencement 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. (− = rabbit lived; + = rabbit died) 7	14	18	22	46	57	70	118	144	174	204	222	240	256
Rabbit Series *D*	0·5	0·125	+	−	−	−	−	−	−	−	−	−	−	−	−	+
	0·5	0·250	+	−	−	−	−	−	−	−	−	−	−	−	−	+
	0·5	0·5	+	−	−	−	−	−	−	−	−	−	−	−	+	+
	0·5	1·0	+	+	−*	−	−	−	−	−	−	−	−	+‡	+	+
	0·5	1·5	+	+	+	−	−	−	−	−	−	−	−	+	+	+
	0·5	2·0	+	+	+	−	−	−	−	−	−	−	+	+	+	+
	0·5	3·0	.	+	+	−	−	−	−	−	−	−	+	+	+	.
	0·5	4·0	.	.	+	+	−	−	−	−	−	+†	+	.	.	.
	0·5	6·0	.	.	+	+	−	−	−	−	−	+	+	.	.	.
	0·5	8·0	.	.	.	+	+	−	−	−	−	+	.	.	.	.
	0·5	10·0	.	.	.	+	+	+	−	−	+	+	.	.	.	.
	0·5	12·0	.	.	.	.	+	+	−	+	+	+	.	.	.	.
Rabbit Series *E*	0·5	0·125	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0·5	0·250	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0·5	0·5	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* One rabbit died after 18 days' incubation.
† Death occurred after 4 days' symptoms—subpassage proved rabies.
‡ Symptoms on 10th day: subpassage produced symptoms on 6th day and death on 10th.

https://doi.org/10.1017/S0022172400010949 Published online by Cambridge University Press

process of immunisation with the three kinds of virus and of serum testing 7, 14, 18 and 22 days after commencement of treatment. The results in each case proved practically identical with those first obtained, and we feel justified, therefore, in our conclusions regarding the relative antigenic power of the viruses under test.

Preparation of the etherised virus was effected as follows: a fresh-fixed virus brain after having been weighed and cut into four pieces was placed in ether; after 90 hours' immersion it was removed and the ether allowed to evaporate under aseptic conditions; the nervous substance was then triturated in a sterile mortar, N.S.S. being gradually added till a 1:50 proportion was reached; finally the resultant emulsion was filtered through sterile gauze.

The results of our final investigations are shown in Table II, but the relative power of fresh-fixed virus, of carbolised-fixed virus and of etherised-fixed virus to evoke rabicidal antibody formation in the sera of immunised rabbits is more readily appreciated by reference to the Chart on p. 418.

Summary.

A. Results following the subcutaneous inoculation of 1400 grm. rabbits with a 2 per cent. suspension of living fixed virus in a dosage of 5 c.c. daily on 14 consecutive days (1·4 grm.):

(1) Fresh living fixed virus introduced into rabbits subcutaneously produces in the sera of these animals so high a degree of rabicidal power that one unit volume of undiluted serum is capable of neutralising, when antibody formation is at a maximum, 16 unit volumes of a 1:100 suspension of fresh-fixed virus in N.S.S.

(2) Rabicidal properties in the serum are demonstrable 18 days after the commencement of treatment, one unit volume of serum then neutralising one volume of a 1:100 F.V. suspension in N.S.S.

(3) Rabicidal antibody content reaches a maximum between 50 and 60 days after completion of treatment, and thereafter diminishes somewhat gradually.

(4) Immune sera retain their rabicidal properties for a period extending over 222 days, viz. from the 4th to the 226th day after completion of treatment.

B. Results following the subcutaneous inoculation of 1400 grm. rabbits with a 2 per cent. suspension of killed carbolised-fixed virus in a dosage of 5 c.c. daily on 14 consecutive days (1·4 grm.):

(1) Killed carbolised virus administered to rabbits subcutaneously produces a very considerable degree of rabicidal power. The maximum reached, however, falls much short of that attained by immunisation with fresh-fixed virus and is only one-third of that following the use of etherised vaccine, one unit volume of serum neutralising 8 unit volumes of a 1:100 F.V. suspension in N.S.S.

(2) Rabicidal properties in the serum are demonstrable 18 days after the commencement of treatment, one unit volume of serum neutralising one unit volume of a 1:100 F.V. suspension in N.S.S.

(3) Rabicidal antibody content reaches a maximum some 50–60 days after completion of treatment, and thereafter subsides fairly rapidly.

(4) Sera of rabbits immunised with carbolised-fixed virus retain their rabicidal properties for a period of 131 days, viz. from the 4th to the 135th day after completion of treatment.

C. Results following the subcutaneous inoculation of 1400 grm. rabbits with a 2 per cent. suspension of killed etherised virus in a dosage of 5 c.c. daily on 14 consecutive days (1·4 grm.):

(1) Killed etherised virus introduced into rabbits subcutaneously produces in these animals' sera such an extraordinary degree of rabicidal power that, when antibody formation has reached a maximum, one unit volume of undiluted serum is capable of neutralising as many as 24 volumes of a 1:100 F.V. suspension in N.S.S.

(2) Rabicidal properties in the serum are demonstrable on the last day of treatment (14 days after commencement), one unit volume of serum neutralising one unit volume of a 1:100 F.V. suspension in N.S.S.

(3) Rabicidal antibody content reaches a maximum between 50 and 60 days after completion of treatment, and thereafter diminishes fairly gradually.

(4) Sera of rabbits immunised with etherised-fixed virus retain their rabicidal properties for a period of 226 days, viz. from the 14th to the 240th day after commencement of treatment.

CONCLUSION.

In the immune sera of rabbits treated with killed etherised-fixed virus, rabicidal antibodies make an earlier appearance, are present in greater degree and persist a longer time than in the immune sera of rabbits treated with equal quantities by weight of fresh-fixed virus or of killed carbolised virus.

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NOTE (REFERRED TO ON P. 417).

Technique recommended by the International Rabies Conference, 1927. "Et pour permettre de mieux comparer les résultats obtenus par les différentes méthodes de traitement employées dans les Instituts antirabiques, elle recommande la technique suivante, non parce qu'elle soit la meilleure, mais parce qu'elle est la plus simple et en conséquence elle peut être mise en pratique par tous les Instituts:

A partir du 4ème et 5ème jour de traitement antirabique, tous les jours ou un jour sur deux ou trois, prélever par ponction dans une veine du pli du coude, 10 cent. cube de sang de façon à avoir 5 cent. cube de sérum. Faire d'autre part une émulsion à 1/100 de virus fixe, prélèvement au niveau du plancher du 4ème ventricule, passer celle-ci à travers un papier buvard et dans des flacons à hémolyse stérilisée, opérer des mélanges d'émulsion et de sérum en commençant par 5 parties de sérum pour 1 d'émulsion et en s'élevant jusqu'à 1 partie pour 2, 5, 10, 15, 20 parties d'émulsion, ces derniers taux (supposant un pouvoir antirabique déjà très élevé) n'ayant du reste besoin d'être atteints qu'à la fin du traitement ou quelque temps après lui.

Laisser en contact 24 heures à la glacière les mélanges de sérum et d'émulsion.

Après 24 heures, agiter, aspirer dans une seringue et inoculer 1/4 cent. cube du mélange sous la dure-mère du lapin ou du cobaye. Après avoir déterminé quand les substances rabicides apparaissent dans le sang au cours du traitement et quel taux elles atteignent, poursuivre ces recherches au moyen d'examens répétés les plus souvent possible pendan le mois qui suit le traitement. Essayer ensuite de déterminer quand elles disparaissent au moyen de prélèvement effectués chaque mois."

(*MS. received for publication* 7. II. 1931.—Ed.)