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SURVEY OF ASSAY METHODS OF ANTIVENINS

Immunological Factors Influencing Antivenin Standardization

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SYNOPSIS

In view of the multiplicity of methods used at present for the preparation and assay of antivenins and as a first step towards the international standardization of antivenins, it seemed advisable to make a comparative study of the methods used in the institutes specializing in the production of these sera. With this end in view, the author circulated to the serologists of institutes concerned a detailed questionnaire on the assay methods used for the determination of the neutralization potency of the various types of antivenins prepared under their direction. The information supplied by these institutes is reproduced, in condensed form, in this report and is analysed by the author.

The author emphasizes that the great variety in the constitution of venoms necessitates: (1) the use of monovalent standard sera against homologous " test " venoms of high activity and stability; and (2) the establishment, on a regional basis, of standard antivenins corresponding to groups of snakes characterized by venoms of common or closely related antigenic constitution.

During the half-century which followed the preparation of the anti-cobra serum by Calmette, in 1894,[15, 16, 17] the assay of antivenins was the subject of numerous works. The painstaking studies made in various parts of the world where antivenins of different types are prepared reflect the complexity of the subject, as well as the diversity of the methods employed to ascertain the neutralizing and therapeutic properties of the serum of animals immunized against venoms of various antigenic compositions.

The experimental evidence, in vivo and in vitro, of the neutralizing properties of antivenins in respect of homologous venoms has led to the adoption of methods of titration based on principles and modalities similar to those used for the titration of antitoxic sera of microbial origin. Venoms and antivenins are mixed in varying proportions in vitro and injected, after allowing to stand, into test animals such as the rabbit, guinea-pig, pigeon and mouse.

In addition to the antigenic variations inherent in the composition of venoms and likely to be connected with the heterogeneous results frequently noted, a certain number of biological and technical factors related to the actual assay methods used may be considered as responsible for the differences, at times considerable, observed in these results: relationship between the homologous venom and serum components involved in the assay, as well as dosage progression in the mixtures; assay at one or several levels; species of animal used and the method of injecting the mixtures; and, finally, the mode of expressing the neutralizing potency of the antivenin, whether in terms of minimum lethal doses (MLD), or certainly lethal doses (CLD), the weight (in mg) of venom neutralized per ml of serum or in neutralizing units, chosen more or less arbitrarily.

At its session held in Geneva 1935, the Permanent Commission on Biological Standardization of the Health Organisation of the League of Nations studied the recommendation put forward by the Inter-Governmental Conference on "the possibility of standardizing anti-snake-venom sera." [55] Owing, however, to the extent of the problem, the Commission (1936 [56]) decided to limit the proposed study to antivenins for use against bites by European vipers. Following up this suggestion, the Department of Biological Standardization of the Statens Seruminstitut, Copenhagen, got into touch with the European institutes responsible for the preparation of antivenins with a view to comparing methods of titration used in respective institutes, and studying the possibility of the international standardization of these sera.

Research undertaken jointly by the Statens Seruminstitut and the Institute of Hygiene of Zagreb led to the development by Dr J. Ipsen of a method for measuring the neutralizing activity by titration at several levels of anti-*Vipera aspis* and anti-*Vipera ammodytes* serum. This technique, which is largely based on the assay method of Banic & Ljubetic (1938 [5]), of Zagreb, has the merit of permitting the determination of the relationship between the antivenin and the homologous venom, irrespective of the level chosen for titration. The proportions of the serum/venom elements in the mixtures are established by a series of points spread along a preliminary line of neutralization. The serum's neutralizing power is calculated and expressed in accordance with a formula which allows for the resistance of the test animal, namely the mouse, in the absence of an antivenin.

The mode of expression of the neutralizing potency of sera thus assayed appeared capable of being adapted to measurement in standard antitoxic units in relation to a standard serum of neutralizing potency established in respect of a standard venom of *V. aspis* and of *V. ammodytes* (Ipsen, 1938 [49]).

The importance of using standard venoms in the standardization of antivenins was emphasized by the present author in his studies on the

standardization of anti-*Bitis arietans* and anti-*Naja flava* serum, prepared at the South African Institute for Medical Research, in Johannesburg (Grasset, 1936 [37]).

After noting the satisfactory results of the work accomplished on the titration of European antiviper serum by the institutes of Copenhagen and Zagreb, the Permanent Commission on Biological Standardization recommended at a session held in Paris in 1938: [57]

" . . . that the investigations which are being carried out with a view to the standardization of the antivenom sera should be continued with the collaboration of any other Institutes interested in this problem ".

Acting on this recommendation, the present author undertook research on the standardization of African antiviper serum (*Bitis arietans*) in accordance with the assay method proposed by Ipsen but with a few modifications for the *B. arietans* antibody contained in the concentrated polyvalent African antiviper-anticobra serum.

The satisfactory results obtained in these trials encouraged the author to study the possibility of using the same technique for the titration of African anticobra *Naja flava* serum.

As concluded in the *Bulletin of the Health Organisation, League of Nations* (Grasset [37]):

" 1. By means of this method of assay at various levels, it is possible to titrate with satisfactory accuracy the neutralising power of the monovalent African viper (*Bitis arietans*) antiserum and to assess the amount of *Bitis* antibody contained in the polyvalent viper-cobra antivenene.

" 2. The method can also be applied with satisfactory accuracy to the titration of the monovalent *Naia flava* antiserum and of the amount of *Naia* antibody contained in the polyvalent viper-cobra antivenene.

" 3. Thus it is possible, by means of this method, to assess the amount of African viper and Cape cobra antibodies contained in the polyvalent antivenene prepared by the South African Institute for Medical Research."

In subsequent studies, the author defined more accurately the results obtained by the application of this method, at various levels, to the titration of a series of polyvalent antiviper sera, concentrated by fractional precipitation with sodium sulfate (Grasset,[34]) as well as of sera purified in the form of modified globulins by Pope's method [71] adapted to antiviper sera (Grasset & Christensen, 1947 [40]).

In 1949, the author emphasized the necessity of utilizing, in these titrations, standard-venoms which ensure the homogeneous nature of the homologous venoms used in these tests: [39]

" The results obtained by this method, computed by means of Ipsen's formula, showed divergencies between titres, which varied, for the same serum, depending on the various levels under consideration, from 0% to 10%, for 58% of the sera assayed, reaching a maximum of 22% in extreme cases.

" A final titre can be obtained by computing the arithmetical mean of titres obtained at various levels.

" The extent of divergencies in titration results becomes, on the whole, smaller as the interval between the increasing doses of venom contained in the various mixtures which are prepared for a given level of antivenene, is less, and the number of animals injected per mixture greater."

At the Haffkine Institute, Bombay, Hazra, Lahiri & Sokhey [47] carried out titrations by the Ipsen method on the polyvalent antivenin against the 4 most common poisonous snakes of India: *Naja naja*, *Bungarus coeruleus*, *Vipera russellii* and *Echis carinatus*. From these studies, the authors came to the conclusion that this method of titration could also be applied satisfactorily to the polyvalent Asiatic cobra-viper antivenins:

" Clear-cut results were obtained in every case even though such a small number of mice as 20 was used for each assay. The repetition of the titrations showed that the results were repeatable within very narrow limits.

" In titrations of the serum against the venoms of cobra, common krait and Russell's viper, the final neutralisation line diverged only slightly from the preliminary neutralisation line. In the case of the assay of the serum against the venom of saw-scaled viper, however, the divergence was very marked. Nevertheless, the results were as repeatable as in the other cases. This wide divergence between the preliminary and the final neutralisation lines is very probably due to the fact that a large part of the c.l.d. of this venom is neutralised by the natural defence mechanism of the mouse."

As will be seen later in this paper, this method of titration has, during the last ten years, been studied in a number of other institutes responsible for the preparation of antivenins of different types and in which it has been applied with varying modifications. These institutes, however, form only a minority and great diversity in the methods used for the titration of antivenins (against vipers and cobras), prepared in the different continents, is still evident.

In view of the multiplicity of the methods in use at present and the diversity of modes of expressing the properties of antivenins, the World Health Organization decided in 1954 to include this question among those to be discussed at the ninth session of the WHO Expert Committee on Biological Standardization held in Geneva in 1955.[82]

In order to ensure that the study of the complex question of the standardization of antivenins on the international level would be carried out on rational lines, it seemed advisable, in the first place, to make a comparative study of the various assay methods used in the institutes specializing in the production of antivenins and to compare the results obtained by the respective techniques. In view of the diversity of these techniques and of the methods of expressing the results obtained, it was thought useful to classify more definitely the various aspects of the problem.

With that end in view, the author circulated to the directors of the various institutes concerned, a detailed questionnaire on the working methods used for the determination of the neutralizing potency of the various types of antivenins prepared under their direction.

In view of the close immunological relationship between the nature of the venomous antigens used in the hyperimmunization of horses producing antivenins and in the titration of the activity of the latter, it was decided to incorporate in the questionnaire the various points connected with biological aspects and techniques concerning the question as a whole. These included:

1. Venoms used in the immunization of horses for the preparation of monovalent, as well as polyvalent, antiviper and anticobra sera.

2. Methods of titration used in vivo and in vitro: titration at one or several levels, test animal used, weight, method of injection, etc.

3. Method of expressing the neutralizing potency of sera with respect to homologous venoms; group neutralization, in terms of minimum lethal dose or certainly lethal dose, or alternatively, of weight of dried venom (in mg) of neutralizing units.

4. Utilization of standard serum or reference serum.

5. Legislative aspects of supervision of antivenins and minimum requirements.

6. Nature of antivenins prepared for therapeutic use: natural, concentrated or refined, in liquid or freeze-dried form.

7. Recommended doses for therapeutic use in volume (ml) or neutralizing units.

8. Period of validity of serum and other factors of special interest.

Thanks to the collaboration shown by our colleagues and by the competent public health authorities, and to the detailed information which they kindly supplied, it has been possible to gather a quantity of information of great interest concerning the preparation and assay of antivenins against viper and cobra venom as well as against that of scorpions and spiders; this information was prepared by 22 different institutes, including 6 in Europe, 4 in Africa, 6 in the Americas, 5 in Asia and one in Australia.

With this documentation, we have been able to make a comparative study of the various methods used in the titration of monovalent and polyvalent antivenins, and to analyse the reasons which, in special cases, have determined the adoption of given methods complying with certain regional desiderata or requirements.

The methods, techniques and information contained in this report were supplied by the directors of the institutes mentioned above in reply to the questionnaire and are reproduced with their kind permission.

For various reasons, this report will be confined to the discussion of certain aspects of the problem of antiviperine and anti-elapine sera, and the study of antiscorpion and antispider sera will be the subject of another paper.

ASSAY METHODS AND STANDARDIZATION OF EUROPEAN ANTIVIPER SERA

France

According to information provided by Professor J. Trefouël, director of the *Institut Pasteur, Paris*, on the preparation of antiviper serum in this institute, serum is obtained from horses immunized by means of *Vipera aspis* (Phisalix [67, 68]) (yellow venom) and *Vipera berus*. The serum is titrated against these two venoms, as well as against the white venom of *V. aspis*, by using standard venoms consisting of a homogeneous mixture of venom from a large number of vipers collected over a period of 3 consecutive years. Titration is carried out using rabbits weighing 2250-2300 g; the neutralizing power of the serum is expressed in terms of a multiple of the CLD of the homologous venom injected intravenously and calculated per kg body-weight of rabbit (Cesari & Boquet [22, 23, 24]).

The French pharmacopoeia lays down the assay methods for antivenins as follows:

" The antitoxic activity of an antivenin is established by determining the dose of serum which, when mixed with venom, can neutralize several lethal doses and make them atoxic.

" This test is carried out on the rabbit or the mouse. After leaving for 30 minutes in the oven at 37° C, the serum-venom mixture is injected intravenously and must not cause any form of poisoning.

" It is generally admitted that the potency of an antivenin is adequate for therapeutic use when 1 ml of serum is capable of neutralizing, in the conditions described above, at least 1 mg of dried venom." (Translated from *Codex Medicamentarius Gallicus* [27])

In addition to these requirements, according to additional information supplied by Dr N. Lamy, who is responsible for the production of this serum at the Institut Pasteur, in order to increase the accuracy of the assay, ten times more venom and a tenfold dose of serum is used for the test. The result is that if, for each lethal dose of venom, 1/10th of the serum is not neutralized, a total surplus of one lethal dose will remain; in this way the accuracy of the assay is considerably increased.

The number of animals injected per dose is not fixed. According to Lamy, as many animals are injected with the same dose as are considered necessary for the accuracy of the titration.

The period of validity of this antiviper serum is 3 years. The therapeutic dose recommended is 10 ml, to be injected subcutaneously, preferably close to the bite. This dose may be repeated; in serious cases, the serum should be injected intravenously.

In addition to the European antiviper serum ER (*V. aspis* and *V. berus*), the Institut Pasteur of Paris and its various branches prepare AN serum

neutralizing the venoms of the North African snakes (*Cerates cornutus, Naja haja*), AO serum, neutralizing the venoms of the west and equatorial African snakes (*Bitis gabonica, B. arietans, Sepedon haemachates, Naja*), and C serum neutralizing the venom of *N. tripudians* found in Indochina and India.

Boquet & Lehoult [10] applied Ipsen's method for titrating several lots of the sera of anti-*B. arietans*, of anti-*V. aspis* and of anti-*N. naja* as compared with Calmette's classical technique for the titration for antivenins. Commenting on the results obtained, these authors concluded that of the various methods for titrating antibody in sera prepared against snake venoms, Calmette's classical technique, which consists of injecting into rabbits mixtures of a fixed quantity of antigens and variable doses of specific antitoxin, makes possible a simple and rapid assessment of the therapeutic value of antivenins; however, this method is expensive. The method proposed by Ipsen has the advantage of being economical and of expressing the results obtained by a neutralizing line. Ipsen's formula does not, however, appear to be applicable when small quantities of venom and antivenin are used. The authors observed that the neutralization line for certain sera is slightly incurved in its inferior part. In this connexion, they recall the observations of Vital Brazil [12, 13] who, as early as in 1907, had noted that certain immune sera used in low concentrations, neutralized proportionally more venom than when used in high concentrations. Similar reports were made in 1921 by Houssay & Negrete [48] and in 1947 by Eichbaum.[30]

Christensen,[26] in recent studies on South African snake venoms, comes to the same conclusions as those reached by Boquet & Lehoult concerning the inferior curve of the neutralization line.

In previous publications on the standardization of anti-*N. flava* serum by means of Ipsen's method, the present author showed that the neutralization results obtained at low levels, corresponding to smaller doses of serum and to a limited number of lethal doses of venom, do not give the same regularity as those observed at higher levels.

Similarly, comparative studies on the titrations of the neutralization activity of *N. flava* antibodies by means of Ipsen's method of several levels and that applied at a single but selected level, show, on the whole, a comparable degree of accuracy, provided that the amount of venom used as test dose in the venom-antivenin mixtures corresponds to a minimum of 5 lethal doses of venom for the mouse (Grasset [39]).

The *Institut Merieux, Lyons*, prepares a European antiviper serum. According to information received from Dr C. Merieux, director of the institute, horses producing this serum undergo joint immunization against *V. aspis* and *V. berus*. The neutralizing power of this serum against these two venoms is determined according to assay methods of the French pharmacopoeia and meets the requirements of the *Codex Medicamentarius Gallicus*.[27]

Germany (Federal Republic)

According to information supplied by Professor A. Demnitz, antivenins are prepared under his direction at the *Behringwerke, Marburg*, against the venom of the European vipers, *V. aspis* and *V. ammodytes*, the venom of *V. russellii* of India, and against a number of African vipers, including *B. arietans*, *B. gabonica*, and *Echis carinatus*, the latter being found both in Africa and Asia.

On the basis of these monovalent sera, polyvalent antiviper and antiviper-anticobra sera are prepared, according to requirements, by mixture with anticobra sera, for the Asiatic and African continents.

Assays are made of the specific neutralizing power of each of the monovalent sera against the respective venoms. The neutralizing potency of these antivenins is determined by using mice weighing 14-16 g. The titration of sera is affected at a single level. The dose of venom employed usually corresponds to 2.5 CLD for the mouse. After allowing to stand for 30 minutes at 37°C, the mixtures are injected into the mouse intravenously. Two mice are used for each mixture. The neutralizing power is expressed in mg of the homologous tried venom per ml of the respective sera.

According to research carried out by Bieling et al.,[7] the anti-*V. ammodytes* serum exercises a more or less pronounced group neutralizing power on the venoms of *V. aspis*, *V. berus*, *V. lebetina* and *B. gabonica*.

Furthermore, in the case of polyvalent sera obtained by mixing monovalent sera, tests are made to determine the group neutralization action on the various venoms employed in the preparation of each of the monovalent sera making up the polyvalent sera.

A similar neutralizing action is also exercised by anti-*Echis carinatus* serum towards the venom of *B. arietans*. The neutralizing power is determined in relation to a quantity of venom corresponding to 2.5 CLD of the respective venoms for the mouse, allowance being made for the quantity of venom tolerated by the mouse in the absence of antivenin.

After leaving for 30 minutes at 37°C, mixtures with a fixed level of toxin are injected intravenously into white mice weighing 14-16 g, 2 animals being used per mixture.

According to the standards approved by the federal serum-control authorities (Paul-Ehrlich-Institut, Frankfurt-on-Main), 1 ml of antiviper sera must neutralize at least 100 CLD. Sera intended for therapeutic use is purified by means of Pope's method.

Italy

The *Istituto sieroterapico milanese Serafino Belfanti, Milan*, according to information received from its director, Professor A. de Barbieri, prepares an antiviper serum against the venom of *V. aspis* and of *V. ammodytes* (Belfanti ;[6] Pepen [65, 66]).

The neutralizing potency of this serum is determined against these two venoms. Assays are carried out by mixing 3 volumes of serum (0.5 ml, 0.25 ml and 0.1 ml) with a fixed concentration of venom. After allowing to stand for 1 hour at laboratory temperature, guinea-pigs are injected subcutaneously, 3 animals being used for each dose.

The neutralizing potency of this natural serum is expressed in terms of the CLD of the respective standard venom, killing the guinea-pigs in 4-6 hours. No account is taken of the animals' natural power of resistance to the venoms used which is, according to Ipsen, in the neighbourhood of $^4/_5$ of a lethal dose. This serum is used therapeutically in its natural form. The recommended dose is a minimum of 20 ml. The period of validity is 3 years.

An antiviper serum is also prepared by the *Istituto seroterapico e vaccinogeno toscano* " *Sclavo* ", *Sienna.* According to information provided by the institute's director, Professor D. d'Antona, the venoms of *V. aspis*, *V. ammodytes* and *V. berus* are used as antigens for injection into the horses producing this serum. The neutralizing activity of the natural serum thus obtained is determined by titration against these three venoms. This is done at a single level, increasing volumes of serum being added to 0.01 mg of venom from the above-mentioned vipers. After standing for 5 minutes at laboratory temperature, the mixtures are injected intravenously into mice weighing 15-20 g. Five animals are used per mixture. One ml of the serum must neutralize 1 mg of venom, corresponding to 50 CLD for the mouse.

The recommended therapeutic dose is 10-20 ml injected by the subcutaneous, intramuscular or intravenous routes. The period of validity is 1-2 years, according to how the serum is stored.

Yugoslavia

A monovalent anti-*Vipera ammodytes* serum is prepared by Dr M. Stanić at the *Central Institute of Hygiene, Zagreb*, under the direction of Dr I. Brodarec. The serum of horses immunized by means of this venom is concentrated by means of Pope's method. Its neutralizing power is titrated against the venom of *V. ammodytes.*

Titration is effected at a single serum level and either rabbits weighing 1500 g or mice weighing 18 g are used. After allowing to stand for 30 minutes, the mixtures are injected intravenously. The neutralizing power of the serum is expressed in multiples of the lethal dose of venom, i.e., 0.02 mg for the mouse.

According to the requirements of Yugoslav legislation, 1/350th of a ml of purified serum thus prepared must be able to neutralize one lethal dose

of 0.02 mg of venom in the case of an 18-g mouse, or alternatively, one lethal dose of 0.05 mg for a rabbit of 1.5 kg.

The institute also determines the group neutralizing potency of anti-*V. ammodytes* serum with respect to the venoms of *V. aspis* and *V. berus*. According to information supplied by Dr Stanić, this antivenin, in its natural form, exercises a group protective action against the venom of *V. aspis*, such that one lethal dose of the venom of this viper requires 1/13th of the volume of serum needed to neutralize one lethal dose of *V. ammodytes* venom. Thus, 1/1100th of a ml of serum protects the mouse against 0.0125 mg (1 CLD) of the venom of *V. aspis*, whereas 1/80th of a ml of the same serum is required to neutralize 1 CLD of the venom of *V. ammodytes*.

On the other hand, five times more anti-*V. ammodytes* serum is necessary to protect the mouse against one lethal dose of the venom of *V. berus* than is required against 1 CLD of *V. ammodytes*.

The therapeutic dose of the concentrated anti-*V. ammodytes* serum is 3-5 ml. In serious cases, this dose is repeated and administered intramuscularly.

In addition to these in vivo tests, a test of the antivenomous power of the serum is carried out by the method employing the inhibition of the haemolytic action exercised by the venom of *V. ammodytes* on red blood corpuscles in vitro.

This institute also prepares an anti-*Latrodectus tredecimguttatus* serum against poisoning caused by this spider, which is common in Yugoslavia.

* * *

The above analysis of the biological and technical elements of the methods used for the assessment and titration of the neutralizing potency of the European antiviper sera used in the various European institutes brings out the diversity of the methods employed. These divergencies concern a variety of factors: the kind of animal adopted for the assays, the time of contact, and the temperature at which the serum-venom mixtures are kept before being injected into the animals. The same variety is found in the techniques adopted for the establishment of the dilution series of venoms and serums either at one or several levels. Finally, the same is true of the methods and terms used for expressing the neutralizing potency of the antivenins assayed, which may be in MLD or in CLD for a given animal species or in units of weight (mg) of dried venom neutralized per ml of antivenin, or finally, but more rarely, in terms of an arbitrarily chosen " neutralizing unit ".

A final remark may be added concerning the sex of the animals utilized, which is generally not specified. Studies carried out by Dossena [29] at the Institut d'Hygiène, Geneva, emphasize the importance of the sex of the animals in the response to venom poisoning, in particular by the venom

of *Naja flava*, and the reservations which such facts impose in the choice of the sex of the animals utilized for the titration of antivenins. These facts were confirmed by Schöttler,[77] who called attention to the definitely greater resistance of the female mouse to poisoning by the venoms of the South American *Bothrops*.

In the present author's opinion this difference of sex may be especially significant if the test dose of venom is limited to a small number of LD, such as 2 CLD, particularly when bearing in mind the appreciable quantity, corresponding to about 4/5th of a CLD of venom, which, according to Ipsen, can be tolerated by the animal in the absence of antivenin.

Official control measures

As to the official control measures regulating the neutralizing power of antiviper sera in countries where such sera are prepared or imported, there are three alternatives to be considered:

(1) absence of legislation;

(2) minimum requirements concerning the nature of the neutralizing properties of the serum;

(3) legislative provisions concerning methods of control and the neutralizing power of antiviper serum, defined in terms of the serum, by weight, lethal doses of dried venom, or in antitoxic units.

As indicated above, in France, the pharmacopoeia requires that 1 ml of serum ER neutralizes 1 mg of venom. In Germany, the minimum requirements call for the neutralizing activity of manufactured antiviper serum to be assayed against a reference serum with a defined neutralizing unit corresponding to 100 CLD of venom per ml of serum. This power is expressed in terms of neutralizing units per ml, one unit representing the quantity of serum capable of neutralizing 1 CLD in the mouse.

In countries not producing antivenins but which import them, such legislation as exists is, in the large majority of cases, confined to general supervisory measures referring to sterility and other general requirements applying to all sera intended for clinical use. In a limited number of countries, however, special legislation has been introduced which lays down certain requirements for imported antivenins.

The disparity of the measures taken for the supervision and titration of European antiviper sera clearly shows both the complexity of the whole question, and the existing need for adopting a standard serum which would enable the specific neutralizing potency of sera manufactured in the various countries to be compared with corresponding venom, as well as an assessment of the group properties of these sera in respect of other types of vipers found in the same countries, although not necessarily used in the preparation of the antivenin under consideration.

Immunological aspects

From the immunological standpoint, the problem of the assay of European antiviper sera is comparatively simple because of the relative homogeneity of the common or related antigenic components of the venoms of the various European vipers.[a]

On the other hand, the problem becomes considerably more complicated when one considers antivenomous serotherapy against the venoms of snakes of other continents, in particular those of Africa, Asia and America.

Indeed, in the family of Viperidae alone, the toxic principles of the venoms of the venomous species belonging to the same zoological group and having a common geographical habitat, differ in certain cases to such a degree that one is obliged to prepare specific sera for the different species.

Moreover, the existence of Elapidae in the same area calls for the preparation of sera for use against the bite of the main types of elapines. As, however, in practice, it is impossible to foresee by what snake one may be bitten, it is essential to prepare sera which are as polyvalent as possible, and are effective against the venoms of the different snakes encountered.

ANTIVENINS AGAINST THE BITE OF VIPERS AND COBRAS OF THE AFRICAN CONTINENT

African Antiviper Sera

In north Africa, the antivenin prepared by the *Institut Pasteur, Algeria,* is obtained, according to information supplied by Dr E. Sergent, from horses immunized by means of the venoms of *C. cornutus* and of *V. lebetina.* The neutralizing potency of this serum is determined for these two venoms respectively. After one hour's contact in a water-bath at 37°C, the mixtures are injected intra-muscularly into mice weighing 18-20 g. As a rule, 4 animals are used per mixture; each assay includes 24-36 mice.

The neutralizing potency of the serum is expressed in terms of mg of the respective dried venoms, independently of the corresponding number of CLD neutralized. The neutralizing potency of 1 ml of the natural liquid antivenins used for therapeutic purposes must be sufficient to neutralize 5 mg of *C. cornutus* venom or 2.5 mg of *V. lebetina* venom.

The nature of *C. cornutus* venom differs so widely from that of the venom of the European vipers that it cannot be neutralized within usable thera-

[a] The various aspects of the question connected with the antigenic and biological characteristics and the pathogenic potency of the different types of European viper venoms have been discussed in a large number of works which cannot all be referred to in this paper. For information on the specific neutralizing potency of the European antiviper sera with respect to the homologous venoms of *V. aspis, V. berus, V. ammodytes,* and the group neutralizing power of a given antivenin against the two other types as well as against the venoms of *V. lebetina* and the vipers of Africa, Asia and America, the reader is referred to the works of Krauss [51, 58] Grasset,[36] Schlossberger, Bieling & Demnitz [74] and Cesari & Boquet.[23, 24]

peutic limits by any of these European antiviper sera, and a specific serum has therefore to be prepared against this venom. The same is true of another species of viper found in the northern part of tropical Africa, namely, *Echis carinatus*, whose habitat also extends across Asia to India.

Poisoning resulting from the bite of the north African cobra (*Naja naja*) is treated by antivenin C (anticobra) prepared by the Institut Pasteur, Paris. Furthermore, the Institut Pasteur of Algeria prepares a serum against the bite of North African scorpions (Sergent [a]). One ml of this serum neutralizes 1 mg of the venom of *Androctonus australis*.

In addition to these north African reptiles, the African Viperidae are represented throughout the continent by various species of the large *Bitis* family of which the most common, *B. arietans*, is found in Liberia and Ethiopia (Scortecci [78]) and in central and southern Africa.

The composition of the venom of most of the members of the *Bitis* group is homogeneous enough to allow the venom of a great number of this group to be neutralized by serum prepared with the venom of *B. arietans* which, moreover, is one of the most widely distributed species on the African continent.

Certain exceptions must, however, be made, in particular for the venom *B. gabonica*—one of the largest of the African vipers—which is characterized, as may be seen from the studies of Grasset,[39] by a coagulating fraction absent in the venom of *B. arietans*. In the same equatorial zones, another member of the same group, *B. nasicornis* (rhinoceros-adder), which is as large as the *B. gabonica*, is also characterized by venom containing toxic constituents differing to such a degree from those of *B. gabonica* and *B. arietans*, that it cannot be neutralized by the specific serum used against the bites of these two species. Moreover, the anti-*B. nasicornis* serum prepared by the author neutralizes the venom of *B. gabonica* only very partially (Grasset [36a]).

These facts imply the necessity of including *B. gabonica* venom and, as far as possible, *B. nasicornis* venom, among the antigens used in the immunization of horses producing antivenins for equatorial Africa. The protection tests show that the anti-*B. gabonica* serum neutralizes the *B. arietans* venom as well as that of the *Causus rhombeatus* (night-adder) of southern Africa (Grasset [38]).

The correlation of these immunological findings with antivenin therapy therefore suggests the necessity, for a given geographical area, of selecting from the various venomous antigens used in the immunization of horses those characterized by the widest possible antigenic spectrum and covering most effectively the toxic and antigenic components of the venoms of other snakes of the geographical zone considered. These results should be confirmed by establishing, on the one hand, the neutralizing potency of the

[a] In view of the size of Dr E. Sergent and co-workers' bibliography on scorpions, the reader is referred to the numerous articles published in *Archives de l'Institut Pasteur d'Algérie* from 1933 onwards.

antivenins in respect of the corresponding venom and by supplementing this test by group neutralization tests in respect of the venoms of other reptiles of the geographical zone in question.

As can be seen, the problem of standardization, in the biological sense of the word, is complicated by various immunological elements, a judicious interpretation of which ensures the effectiveness and safety, which, in practice, is to be expected from all monovalent and polyvalent antivenins, whether of an antiviper or an anticobra nature.

By way of example, Table I shows the neutralizing potency of the concentrated polyvalent antivenin (*B. arietans, B. gabonica, N. flava*) prepared by the South African Institute of Medical Research, Johannesburg, against the venom of the main vipers and elapines of southern and equatorial Africa, with the exception of *B. nasicornis*. The table also shows, for each of the venoms, the MLD for the rabbit of the particular sample of venom used in the test (Grasset & Christensen [40]).

TABLE 1. NEUTRALIZING POTENCY OF POLYVALENT ANTIVENINS *

Species	Weight of venom (mg) neutralized per ml of serum *	MLD (mg) for 2-kg rabbit
Bitis arietans	24.0	1.5
Bitis gabonica	20.0	2.0
Bitis nasicornis	6.0	1.8
Naja flava	1.9	0.35
Naja haja	2.4	0.9
Naja nigricollis	2.4	1.2
Sepedon haemachates	1.7	1.0
Dendrapsis angusticeps	0.8	0.35
Naja melanoleuca	0.5	0.35

* Modified globulin.

The *Institut Pasteur, Paris*, also prepares in the affiliated Institute at Garches (department of Dr P. Boquet) two polyvalent antiviper sera against the venoms of African vipers. These are:

(1) serum AO anti-*B. arietans* and anti-*B. gabonica*;

(2) serum AO-EC anti-*B. arietans*, anti-*B. gabonica* and anti-*Echis carinatus*.

The neutralizing properties of these sera are determined against homologous venoms according to the multiple-level Ipsen method and expressed in terms of the CLD for a mouse of 18-20 g. The mixtures are injected

intravenously and extemporaneously, two animals being used per venom-antivenin mixture.

One ml of AO serum must neutralize 30 LD of *B. arietans* venom, as well as a similar amount of *B. gabonica* venom. In addition, the AO-EC serum must also neutralize 30 LD of *E. carinatus* venom.

The recommended therapeutic dose of these two sera is 10 ml injected subcutaneously, if possible in association with hyaluronidase; the period of validity is 3 years.

As already stated, the Behringwerke, Marburg, also prepare, in addition to European antiviper sera, monovalent sera against the venoms of various African vipers, namely those of *B. arietans*, *B. gabonica* and *E. carinatus* as well as *V. russellii* of Asia.

The sera of horses immunized by means of non-modified and formolized venoms are titrated at a single level. After 30 minutes' contact at 37°C, the mixtures are injected intravenously into mice weighing 14-16 g, two animals being used per mixture.

The neutralizing power of the respective monovalent sera is defined in terms of CLD of homologous venom per ml of the sera. One ml of the serum must, in the case of the mouse, neutralize at least 100 CLD of the homologous venoms. The potency of the serum is expressed in *neutralizing units*. A serum neutralizing 100 CLD per ml is considered to have a strength of 100 neutralizing units. In addition, a reference standard serum is kept for each of the monovalent antivenins. For therapeutic use, polyvalent sera of varying composition are prepared according to requirements, by mixing monovalent antiviper or anticobra sera in a form concentrated and purified by an enzymatic process. The therapeutic dose of these sera is at least 10 ml, administered subcutaneously; their period of validity is 3 years.

In the Union of South Africa, a polyvalent African antiviper-anticobra serum has, since 1929, been prepared by the *South African Institute for Medical Research, Johannesburg*, under the direction of Dr E. H. Cluver.

The horses producing this serum are immunized by a mixture of formolized anavenin of *B. arietans* and of *N. flava* absorbed by bentonite.

These two venoms are characterized by having the widest antigenic spectrum in relation to the different members of the viper and elapine species of southern Africa, respectively.

The satisfactory results of the assay of the neutralizing activity of the *B. arietans* and *N. flava* antibodies in accordance with the multiple-level method of Ipsen, as well as by the selected single-level assay technique, show a limited spread not exceeding 5% (Grasset [39]), a fact which has already been mentioned by the author. The single-level method is, at present, used for the assay of polyvalent serum concentrated by means of Pope's method, as manufactured in the South African Institute for Medical Research. After allowing to stand for 30 minutes at room temperature, the mixtures are injected intravenously into mice weighing 16-18 g, at least

3 animals being used per mixture. The neutralizing power of the serum is expressed in terms of mg of venom per ml of serum.[a]

In addition to in vivo neutralization titration, the determination of the *B. arietans* antibodies is supplemented by the following tests:

(1) Neutralization of the antihaemorrhagic properties by intracutaneous injection of guinea-pig with mixtures of *Bitis* venom and antivenomous serum, in accordance with the Pratt-Johnson method [72] described below.

(2) " Anti-rennin-like test " in vitro on milk.

The recommended dose of concentrated polyvalent antivenin is at least 20 ml; one injection of up to 30-40 ml must be administered in cases of elapine bites, the serum being injected intravenously; the period of validity is 2 years.

The neutralizing potency, expressed in terms of mg of the homologous venoms of *B. arietans* and *N. flava* is shown below as well as the group neutralizing potency of the serum (in the concentrated form of globulins) against the main viper and elapine species of southern Africa (Grasset & Christensen [40]).

Species	*Weight of venom (mg) neutralized per ml of serum*
Causus rhombeatus	35.0
Bitis arietans	25.0
Naja flava	3.0
Naja haja	3.3
Naja nigricollis	3.6
Sepedon haemachates	3.0
Dendrapsis angusticeps	1.2
Naja melanolenca	1.1

It can be seen from these figures that the serum titres in respect of the venoms of *N. naja*, *N. nigricollis* and *S. haemachates* closely resemble the titre of *N. flava*. As far as the *N. melanoleuca* and *D. augusticeps* venoms are concerned, the group neutralizing potency, although considerably less, reaches therapeutic levels in this concentrated form.

In a recent general study on the venoms and antivenins of snakes from South Africa, Christensen [26] gives new precisions concerning the mode of action, in vivo and in vitro, of various Elapidae and Viperidae. He discusses the relationship between the various constituents of the venom-serum mixtures which intervene in neutralization when increasing doses are used.

As regards the venom of Viperidae, e.g., *B. arietans*, Christensen notes that in numerous cases the neutralization line is incurved and not straight as is the case for *N. flava* venom-antivenin mixtures.

The South African Institute for Medical Research also prepares a monovalent antiscorpion serum against the bite of *parabuthus* as well as

[a] The neutralizing potency of the elapine antibodies (*N. flava*) is expressed in terms of provisional units, introduced by the Department of Public Health of the Union of South Africa.

a monovalent antispider serum against poisoning caused by *Latrodectus indistinctus*.

A polyvalent antivenin is also prepared by the *Clinsearch Laboratories, Johannesburg*. According to information received from Dr Pratt-Johnson, director of this institute, the horses producing therapeutic serum are immunized by means of anavenins formolized according to Grasset's method, using venoms supplied by the Fitzsimmonz Snake Park Laboratories, Durban.

The composition of the antigens is as follows:

(1) *Viperidae*. A 1% solution of *B. arietans* venom in normal saline, detoxicated by the addition of 1% of formol over a period of 4 weeks at 37°C. The neutralizing properties in respect of *B. arietans* venom are determined according to the method described by Pratt-Johnson [72] which is based on the lesion produced by the haemorrhagin contained in this venom when it is injected intracutaneously into the guinea-pig and the neutralization of this haemorrhagin by mixing with the antivenin.

(2) *Elapidae*. A 1% solution of *N. flava* venom in Difco peptone, also detoxicated by contact with 1% of formol for a period of 6 weeks at 37°C.

For the assay of the *N. flava* antibody, mice weighing 17-22 g are injected intravenously with the serum-venom mixture after standing for 45 minutes at 37°C. Four animals are utilized per mixture. The anticobra neutralizing potency is expressed in terms of the antitoxic units introduced, as already stated by the public health authorities of the Union of South Africa. The polyvalent serum concentrated by ammonium sulfate precipitation has a period of validity of 3 years. The recommended therapeutic dose is 10-30 ml.

According to the opinion expressed by Pratt-Johnson on the accuracy of tests carried out by means of the above-mentioned methods and assays:

" The general impression is that standardization of antivenenes is not as satisfactory as in the case of diphtheria and tetanus antitoxins and is probably not very accurate." (Personal communication)

African Anticobra Sera (Elapidae)

The determination of the neutralizing potency of anticobra serum is generally based on and expressed in relation to measurement of the neutralization of the neurotoxin which is the most important element of this venom. The assay is carried out by means of technical methods which vary considerably according to the institute producing the sera. In his early work in the Institut Pasteur, Saigon, on the assay of anticobra serum *Naja naja*, Calmette used the rabbit as test animal. This animal continues to be used at present in certain institutions, but in others preference is shown for pigeons or mice.

The method of expressing the potency of the serum also varies with the institutes manufacturing them. The potency is calculated either in terms of the MLD for the test animal used, in weight (mg) of venom, or in neutralizing units.

The *Institut Pasteur, Paris,* prepares an antivenin against the venom of the north African cobra *N. haja* in the preparation of which enters, in addition to the homologous *N. haja* venom, that of the *N. naja* of India in view of the difficulties encountered in obtaining adequate supplies of north African cobra venom.

The possibility of using the venom of Asiatic cobras in this way is based on the group neutralizing power exercised by anti-*N. naja* serum on the venom of the various African Elapidae, including *N. haja, N. flava, N. nigricollis,* and *S. haemachates* (Ahuja ;[1] Grasset & Zoutendyk [43]).

The monovalent anticobra serum C produced by the Institut Pasteur is obtained, according to information supplied by Dr P. Boquet, from horses immunized with the venom of the Asiatic *N. naja.* The titration is carried out on mice weighing 18-20 g in accordance with the multiple-level method of Ipsen. Two mice are utilized per mixture, the latter being injected immediately after preparation. According to the norms in force in the Institut Pasteur, 1 ml of anti-*N. naja* serum neutralize at least 40 LD of this venom in the mouse. The recommended therapeutic dose is 10-20 ml of serum in association with hyaluronidase; the period of validity of this serum is 3 years.

A polyvalent African anticobra serum prepared also in the *Behringwerke, Marburg,* is obtained from horses which are hyperimmunized with the venom of the following 4 African elapines: *N. flava*; *N. haja*; *N. nigricollis* and *S. haemachates.* Its neutralizing power is established with regard to these 4 venoms as well as that of *Dendrapses angusticeps* and of *D. viridans* of southern Africa, and the venom of the *N. naja* of India.

The assay of the serum is effected at a single level. After allowing to stand for 30 minutes at 37°C, the mixtures are injected intravenously into mice weighing 14-16 g, two animals being used per dose. The strength of the serum is calculated in terms of LD of the corresponding venom neutralized by 1 ml.

According to the minimum requirements, 1 ml of this anticobra serum must neutralize at least 50 LD of the corresponding elapine venom. The potency of the serum is finally expressed in terms of neutralizing units in accordance with the principle used in the Behringwerke for antiviper sera. An anticobra serum neutralizing 50 CLD of venom will thus have a titre of 50 units. Serum intended for therapeutic use is purified by an enzymatic process. The recommended dose is 10 ml as above; the period of validity is 3 years.

ANTIVENINS PRODUCED IN SOUTH-EAST ASIA

At the *Central Research Institute, Kasauli,* India, the name of the present director, Dr M. L. Ahuja, has been associated with much of the research work carried out during the last 30 years in connexion with venoms and antivenomous serotherapy.

According to detailed information supplied by Dr Ahuja, the polyvalent antivenin produced by the institute is obtained from horses immunized by means of the venoms of *N. naja* and *V. russellii.* The specific neutralizing potency is determined in respect of these two venoms by using pigeons weighing 300-320 g and mice weighing 18-20 g.

Assays are carried out by the multiple-level method, for both viperine and elapine antibodies. After keeping 30 minutes at 37°C, the antivenomous mixtures are injected intravenously, for the assay of *V. russellii* antibody, into the pigeon or mouse. For the assay of *N. naja* antibody, the mixtures are injected intramuscularly into the pigeon and intravenously into the mouse. The neutralizing power is expressed as the weight in mg of venom neutralized per ml of antivenin. Allowance is made in this test for the amount of venom tolerated by the animal in the absence of a specific antivenin.

When rabbits are used, 4 animals are injected per mixture, the corresponding figures being 6 for the pigeon and 20 for the mouse. According to studies carried out by Ahuja [1] and by Grasset & Zoutendyk,[42, 43, 44] the polyvalent antivenin prepared by the Central Research Institute has a high neutralizing power against the venoms of the following snakes:

Viperidae	Elapidae
V. aspis	*N. bungarus*
B. jararaca	*N. flava*
B. atrox	*N. nigricollis*
C. terrificus	*S. haemachates*
C. cornutus	*D. angusticeps*

In view of its group neutralizing properties in respect of the above-mentioned vipers and cobras, this serum is considered by Indian authors to be one of the most active against the bite of the various venomous snakes in the different countries. Serum intended for therapeutic use is concentrated by fractional precipitation with ammonium sulfate.

According to standards fixed to meet the minimum requirements established by the control authorities, 1 ml of the polyvalent antivenin prepared by the Kasauli Institute must neutralize, in the pigeon, at least 4 mg of *V. russellii* venom, and 2 mg of *N. naja* venom. The recommended therapeutic doses are from 20-40 ml or more, according to the gravity of the symptoms and the response to treatment; the period of validity of the serum is 2 years.

Since 1945, the *Haffkine Institute, Bombay*, has, under the direction of Dr S. S. Sokhey and, later, of Dr P. M. Wagle, been making a polyvalent antivenin against the venom of the 4 principal snakes of India, namely, *N. naja*, *B. coeruleus*, *V. russellii* and *E. carinatus*.

Hazra, Lahiri & Sokhey [47] described the reasons which convinced them of the necessity of including in the preparation of the polyvalent antivenins (*N. naja* and *V. russellii*) the venoms of two other species (*B. coeruleus* and *E. carinatus*) since the anticobra-antiviper serum formerly produced in the institute had no neutralizing effect on them.

For reasons of stability, the venoms of these snakes, after being harvested at the institute, are freeze-dried and redissolved at the time of use. The horses producing polyvalent serum are immunized with a non-modified mixture of the 4 venoms.

In connexion with the assay of this serum, these authors point out that after obtaining unsatisfactory results with the Anderson & Caius [3] assay method which was formerly in common use and according to which 300 g pigeons are injected with mixtures consisting of a fixed amount of 1 ml of serum added to lethal doses of venom, they took advantage of the opportunity offered by the preparation of the new type of serum to use the Ipsen assay method, in accordance with the suggestions made by the Permanent Commission on Biological Standardization of the League of Nations.

In view of the satisfactory results obtained with this method by Grasset [37, 39] in the assay of the monovalent African anticobra-antiviper serum and of the polyvalent serum prepared at the South African Institute for Medical Research, Johannesburg, Anderson & Caius applied the said technique with a few modifications (30 minutes contact at 37°C) for the respective assays against the above-mentioned venoms, 20 mice being used per venom and per assay. As shown by the reports and diagrams relating to these assays, the results of the determination of the neutralizing power of this serum against the venoms of *N. naja*, *V. russelli* and *B. coeruleus* are characterized by a final neutralization line which deviates but little from the preliminary neutralization line.

On the other hand, the tests made in connexion with *E. carinatus* venom show more marked divergencies. The scattering observed in this case may be imputed, according to the authors, to the fact that a considerable part of the venom is neutralized or inhibited by the natural defensive reactions of the mouse.

The neutralizing titre of the serum concentrated after precipitation with ammonium sulfate is expressed in mg of the respective venom per ml of serum. So far, no attempt has been made to establish a local standard venom. Research into in vitro assay by the flocculation method has so far not permitted the adoption of this method. Indeed, in certain cases, several zones of optimum flocculation, which may overlap, have been noted.

The serum is distributed for clinical use in a freeze-dried form which guarantees the retention of its activity, however unfavourable the climatic conditions, for an estimated period of 10 years. The initial therapeutic dose of this serum is 20 ml, which may be increased to 40 ml or more in serious cases.

In Thailand, polyvalent antivenomous sera are prepared by the *Queen Saovabha Institute, Bangkok*, against the bite of the principal snakes, i.e., vipers and cobras, of Malaya. According to information supplied by Dr C. Purananda, director of this institute, an antiviper serum is prepared against the venoms of *V. russellii* and *Ancistrodon rhodostoma* as is a monovalent anticobra serum against *N. tripudians* venom. A polyvalent antiviper-anticobra serum is prepared by making a mixture of these two sera. The assay of the neutralizing power of these sera is determined in respect of the three homologous venoms by using 2-kg rabbits as test animals, into which the mixtures are injected intravenously, after standing for 30 minutes at laboratory temperature (30°C).

The assay details reported by Dr Purananda are reproduced below:

The assays are begun by using a minimum quantity of venom and a fixed quantity of antivenin. If the animal survives, the dose of venom is increased up to the maximum quantity of venom tolerated without harm by the animal, one rabbit being used for each level of venom. The neutralizing power is expressed as the weight (mg) of venom neutralized by 5 ml of antivenin. No account is taken of the quantity of venom tolerated by the animal's organism in the absence of antivenin. The minimum requirements laid down for the therapeutic use of the serum stipulate that 5 ml of natural antivenin must neutralize at least 3 mg of *V. russellii* venom, 2 mg of *A. rhodostoma* venom and 2 mg of *N. tripudians* venom.

This polyvalent serum possesses neutralizing properties against a number of other snakes encountered locally, including *B. fasciatus*, *N. hannah* (King cobra) and other varieties of land and water snakes.

The recommended therapeutic dose is 10 ml for monovalent sera and 20 ml for polyvalent sera. Injections are given subcutaneously in benign cases and intravenously in severe cases; the period of validity of the serum is 2 years.

In Indonesia, the *Institut Pasteur, Bandung*, Java, produces under the direction of Dr Soemiatno, an antivenin against the bite of the principal vipers and cobras of the Indonesian Archipelago.

Horses imported from Australia (local horses are too small) are immunized by means of formolized anavenins from *Ancistrodon rhodostoma*, *B. fasciatus* and *N. sputatrix*, respectively.

The neutralizing potency of these three monovalent sera is determined using mice weighing 20-25 g. These animals are injected intravenously with mixtures containing a fixed volume of 0.2 ml of serum and increasing doses of venom corresponding to 1.5-2 MLD. A lethal dose corresponds to the

quantity of venom which kills the mouse in 5-10 minutes. After one hour's contact at 35°C, 0.2 ml of each of the three respective sera must be sufficient to neutralize a minimum of 2 LD of the corresponding venoms.

Serum intended for clinical use is a mixture of equal volumes of the three monovalent sera *A. rhodostoma*, *B. fasciatus* and *N. sputatrix*. A 10-ml ampoule represents the minimum dose recommended for therapeutic use.

The personal opinion of Dr Soemiatno concerning the importance of snake-bite in Indonesia is that: " generally speaking, snake-bite has never been a problem at all in Indonesia. Cases do occur, of course, but never to such an extent that they alarm us ".

In Japan, an antiviper serum is prepared at the *National Institute of Health, Tokyo*, under the direction of Dr Kozima. According to the information kindly forwarded by Dr K. Nakamura, head of the serum department, horses are immunized with unmodified venom of *Agkistrodon blomhoffi* and *Trimeresurus flavoviridis*.

In vivo neutralization method

For the titration of the serum, aliquots of a solution of viper venoms of *A. blomhoffi* and *T. flavoviridis* containing 25 LD for mice per ml, are mixed with fixed volumes of decreasing dilutions of the antivenin. After one hour of contact at room temperature, the mixtures are injected intravenously into mice of 14-16 g. Four animals are injected with the same mixture. The activity of the serum is expressed in terms of units, in relation to the number of LD_{50} of venom neutralized per ml of serum. No local standard serum has yet been established. However, standardization of antivenin is under consideration.

Investigations have been carried out on the possibility of applying in vitro assay methods of titration, based on the titration of the antihaemolytic properties of the serum, as well as by means of floculation. However, the titres thus obtained vary to such a degree from those obtained by the in vivo method, that the in vitro techniques cannot be used for the appreciation of the activity of this type of antivenin.

Natural, as well as concentrated and refined antivenins obtained by ammonium sulfate fractionation have been used for therapeutic purposes. A purified product has been prepared by means of Pope's method, but so far has not been produced commercially.

The period of validity of the serum is one year. Doses of 40-60 ml are recommended for therapeutic use. The number of bites notified per year in Japan reaches about 1000. Death-rate due to snake-bite in the absence of antivenin therapy corresponds to 0.1%; no deaths have been reported among snake-bite cases treated with antivenin.

ANTI-ELAPINE SERA PRODUCED IN AUSTRALIA

In Australia, the *Commonwealth Serum Laboratories, Melbourne*, under the direction of Dr F. G. Morgan, prepare an antivenin against the bite of Australian elapines.

The work done by this institute brings out the following facts:

The dominant element in the venom of most of these Australian reptiles is a neurotoxin. A second characteristic factor is the cytolytic properties of the venom. Feldberg & Kellaway [31a] showed that this factor leads to the liberation of histamine from the non-striated muscle cells, resulting in a collapse of the peripheral circulation. A third factor is haemolytic, while a fourth is characterized by a coagulating action and is present in concentrated form in the venoms of *Notechis* and other Elapidae, but is absent from the venoms of *Acanthopis antarcticus* and *Denisonia superba.*

According to information received from Dr Morgan, the serum produced at Melbourne is a monovalent one prepared from the venom of *Notechis scutatus scutatus* (mainland tiger snake). The immunization of horses is carried out by means of non-modified venom injected in a form precipitated by means of alum with a view to stimulating the antibody response of the horses producing the serum.

The Commonwealth Institute also prepares, on an experimental basis only, monovalent antivenins against the venoms of the following elapines:

Denisonia superba
Notechis scutatus (var. *Niger*)
Acanthopis antarcticus
Pseudechis porphyriacus
Oxyuranus scutellatus

The assay of the neutralizing power of anti-*Notechis* serum, determined against a single toxin dose, is carried out on guinea-pigs weighing 400 g or on mice weighing 25-30 g. To a fixed dose of venom are added volumes of anti-*Notechis* serum, decreasing in concentration; the test-dose of venom used is 0.1 mg, corresponding to 12.5 CLD for a 400-g guinea-pig. The mixtures, made up to constant volume by the addition of normal saline, are injected subcutaneously into the guinea-pig or mouse after leaving for 30 minutes at 37°C. One animal is used per dose. The entire assay is carried out in four series. The neutralizing potency of the serum is expressed in units. One unit represents the quantity of serum capable of neutralizing 0.01 mg of standard *N. scutatus* venom, obtained by mixing venom from a large number of serpents of this species.

In expressing the neutralizing potency, the quantity of *N. scutatus* venom tolerated by the test animal, in the absence of antivenin, is taken into account.

Antivenin intended for therapeutic use is supplied in a form concentrated and purified in accordance with Pope's method. Its neutralizing potency is generally 500-580 units per ml, i.e., 1 ml of this serum neutralizes from 5-5.8 mg of *N. scutatus* venom. A standard anti-*Notechis* serum has not yet been established as such. Nevertheless, a batch of anti-*Notechis* serum has been freeze-dried in the laboratories of the Commonwealth Institute. This product serves as a reference standard for the standardization of the sera manufactured. Moreover, a large quantity of *Notechis* venom from different batches has been dried, pulverized and preserved for use in connexion with standardization.

There are no legislative regulations laying down minimum requirements in connexion with the strength of antivenins produced in Australia.

Group neutralizing power against other species of Australian elapines

The anti-*Notechis* serum has a group neutralizing power against other species of Australian elapines. Relevant information, based on investigations made by the Commonwealth Institute (Kellaway & Morgan [50]), and showing the weight in mg of the venom of various Australian snakes which is neutralized by 1 ml of anti-*Notechis* serum follows:

Type of venom	*Weight (mg) of venom neutralized by anti-*Notechis *serum*
Mainland tiger snake (*Notechis scutatus scutatus*)	2.016
Reevesby Island, black tiger (*Notechis scutatus*)	1.73
Chappel Island, black tiger (*Notechis scutatus*)	0.93
Copperhead (*Denisonia superba*)	0.89
Black snake (*Pseudechis porphyriacus*)	0.413
Death adder (*Acanthophis antarcticus*)	0.276
Brown snake (*Demansia textilis*)	0.006

Certain batches of antivenin intended for therapeutic use are checked periodically or occasionally for this group-neutralizing action. The recommended minimum dose of antivenin is 3000 units, administered intravenously, this dose being increased according to the gravity of the case; the period of validity of this serum is 2 years.

ANTIVENINS AGAINST THE VENOMS OF AMERICAN VIPERS

North America

In north America, a polyvalent antivenin is prepared in the USA in the *Wyeth Laboratories, Marietta*, Pennsylvania, under the name of " Polyvalent *Crotalidae*-Antivenin (North and South American Antisnake Serum) " and under the direction of Dr B. Scott Fritz. According to information provided by the latter, this serum which, as its name shows, is prepared against the bite of north and south American *Crotalidae*, is obtained from horses immunized by means of the venoms of *Crotalus adamanteus*, *C. atrox*, *C. durissus*, and *C. terrificus*, as well as of *Bothrops atrox*, with an admixture of " Amphojel Wyeth " (aluminate and carbonate gel), and of penicillin and streptomycin.

The neutralizing potency of the polyvalent serum thus obtained is determined with regard to the above-mentioned venoms with the exception of that of *C. durissus*. Group neutralization tests are also effected in respect of the venoms of the following reptiles:

Crotalus horridus	*Agkistrodon bilineatus*
Crotalus viridis	*Lachesis muta*
Crotalus oreganus	*Bothrops jararaca*
Crotalus basiliscus	*Bothrops neuweidii*
Agkistrodon mokasen	*Bothrops jaracucu*
Agkistrodon piscivorus	*Bothrops alternatus*

The assay of the neutralizing activity of this polyvalent serum is carried out in vivo, using mice weighing 18 g. The neutralizing power of the natural serum is determined in relation to the number of LD_{50} of venom neutralized by 10 ml of the serum, and expressed in mg of dried venom. At least 6 mice are injected subcutaneously with each mixture, after one hour's standing at laboratory temperature.

This antivenin is concentrated by fractional precipitation with ammonium sulfate. A standard, working serum has been established in the Wyeth Laboratories. To satisfy the minimum requirements of this manufacturer, 10 ml of serum must neutralize at least 180 LD_{50} of *C. atrox* venom, 540 LD_{50} of *C. adamanteus* venom, 1320 LD_{50} of *C. terrificus* venom and 780 LD_{50} of *B. atrox* venom. After filling into ampoules holding 10 ml plus 10% excess antibody, the serum is freeze-dried. When used, it is dissolved by adding 10 ml of sterile, normal saline contained in a sealed ampoule which accompanies each dose of antivenin intended for clinical

use. The recommended therapeutic dose is 10-20 ml of the serum obtained on re-dissolving in normal saline.

According to Dr Scott Fritz, an average of 1000-2000 rattlesnake bites are reported every year; mortality among persons not receiving serotherapy is estimated at 10.8%; it is reduced to 2% for persons receiving inadequate treatment, while it is considered that no deaths occur among persons receiving full initial serotherapeutic treatment.

Sharp and Dohme's Biological Laboratory, Glenolden, Pennsylvania, no longer prepares antivenins against snake-bites, but it does produce, under the direction of Dr E. S. Barclay, a monovalent antiarachnid serum against the bite of *Latrodectus mactans.*

In South America, where snake-bite is a serious risk, antivenins are prepared in a number of institutes against the venom of various types of vipers which are responsible for the majority of snake-bites in South America.

The *Instituto Vital Brazil, Niteroi,* Brazil, according to information provided by Dr Roched A. Seba, director, prepares 3 types of antivenins against south American vipers:

(1) Anticrotalic serum (monovalent)

(2) Anti-*Bothrops* serum (polyvalent)

(3) Antisnake-bite serum (polyvalent)

The anticrotalic serum is derived from horses immunized by means of the venom of *Crotalus terrificus terrificus* (Cascavel), captured in various areas of Brazil.

The anti-*Bothrops* serum is obtained from horses injected with the venoms of the following reptiles: *B. jararaca, B. atrox, B. jararacussu, B. alternatus, B. neuweidii, B. cotiara,* and *Lachesis muta.*

Lastly, the polyvalent antisnake-bite serum is really a mixture of sera from horses immunized against the venom of various Brazilian *Bothrops* vipers and *Crotalus* and *Lachesis* of south and central America. The venoms used for the immunization of horses are treated previously with a lipoidic substance of hepatic origin. When the sera are assayed, the neutralizing power of the anti-*Bothrops* serum is determined in respect of *B. jararaca* venom as well as that of *Crotalus terrificus terrificus.*

In vivo assay, using the pigeon, is carried out by the method of Vital Brazil,[11, 12, 13] in which a constant dose of undiluted serum is mixed with lethal doses of the respective venoms; after 30 minutes standing at room temperature (20°-37°C), the mixtures are injected intravenously, two animals being used per dose. In addition to in vivo tests of the neutralizing power, an assay is also made by flocculation in vitro.

For therapeutic use, the antivenins are purified and concentrated according to Pope's method and by fractional precipitation with ammonium sulfate. The recommended therapeutic doses are 20-30 ml injected subcutaneously or intramuscularly, intravenous injections being given under medical supervision.

According to the minimum requirements established, the strength of the antivenins is expressed in terms of mg of dried venom neutralized per ml of the respective serum.

1 ml of anticrotalic serum must neutralize 0.8 mg of *C. terrificus* venom.

1 ml of anti-*Bothrops* serum must neutralize 1.5 mg of *B. jararaca* venom.

1 ml of antisnake serum must neutralize 0.6 mg of *C. terrificus* venom.

1 ml of antisnake serum must neutralize 1.0 mg of *B. jararaca* venom.

No use is made of a standard serum in assaying the three above-mentioned sera.

According to the requirements prescribed in the first and second supplements to the Brazilian pharmacopoeia,[31] the neutralizing potency as determined by the Vital Brazil method, of the anti-*Bothrops* serum must be such that 1 ml of the latter neutralizes in vitro a minimum of 0.0015 g of dried *B. jararaca* venom. The MLD of the venom must be equal to, or less than, 0.04 mg. This method requires the use of the pigeon, into which the antivenins are injected intravenously.

As for the anticrotalic serum, its strength must be such that 1 ml neutralizes in vitro at least 0.0005 g of dried crotalic venom, the MLD of which must be equal to, or less than, 0.0015 mg.

Lastly, the neutralizing power of the polyvalent antisnake serum must be such that it neutralizes 0.0004 g of *C. terrificus* venom, and 0.001 g of *B. jararaca* venom. The period of validity of the three sera prepared by the Instituto Vital Brazil is fixed at 4 years.

Clinical results

Antivenin therapy is considered by Dr R. A. Seba to be effective in most cases where an adequate dose of the specific antivenin has been given; the therapeutic effect of the latter appears to be increased by association with hyaluronidase.

The *Instituto Butantan, São Paulo*, prepares, under the direction of Dr Afranio do Amaral, various types of antivenins against the bites of the Crotalidae and Elapidae species of south America as well as against the sting of south American scorpions and spiders.

As regards the preparation of sera against poisoning due to snake-bite (to which we are obliged to confine this report) the detailed information supplied by Dr Amaral are reproduced below in their original form.

Preparation of antivenins

(A) Type of antivenins for

			Antivenin number
(*a*) Serpents			
1. Crotalidae:	*Crotalus terrificus terrificus*	=	1
	Bothrops atrox *Bothrops alternata* *Bothrops jararaca* *Bothrops jararacussu* *Bothrops neuwiedii* * *Bothrops cotiara* *	=	2
	Bothrops jararaca	=	2-A
	Bothrops neuwiedii	=	2-B
	Lachesis muta	=	4
	Crotalus terrificus terrificus *Bothrops atrox* *Bothrops alternata* *Bothrops jararaca* *Bothrops jararacussu* *Bothrops neuwiedii* * *Bothrops cotiara* *	=	3
	Crotalus terrificus durissus *Bothrops atrox*	=	3-A
2. Elapidae:	*Micrurus corallinus* *Micrurus frontalis* *Micrurus lemniscatus*	=	5
(*b*) Scorpions:	*Tityus bahiensis* *Tityus serrulatus*	=	9
(*c*) Spiders:	*Ctenus nigriventer*	=	7
	Lycosa raptoria	=	6
	Ctenus nigriventer *Lycosa raptoria*	=	8

(B) Venoms entering into the preparation of antigens injected into horses for the production of antivenins:

Every venom cited, respectively, under (A) (*a*) 1, (A) (*a*) 2, (A) (*b*) and (A) (*c*).

(C) Nature of antigens injected early in the immunization:

(*a*) Pure venom, preserved in glycerin + saline and diluted at the moment of injection.

(*b*) Exceptionally, venom modified by formalin (venomoid).

(*c*) Exceptionally, venomoid at first, followed by pure venom.

* Eventually

Methods of titration of the neutralizing properties of antivenins

The following venoms an used in titrating and standardizing the respective antivenins:

1. Crotalidae

Antivenin No. 1:	venom of *Crotalus terrificus terrificus*
Antivenin No. 2 and 2-A:	venom of *Bothrops jararaca*
Antivenin No. 2-B:	venom of *Bothrops neuwiedii*
Antivenin No. 3:	venom of *Crotalus terrificus durissus* and *Bothrops jararaca*
Antivenin No. 3-A:	venom of *Crotalus terrificus terrificus* and *Bothrops atrox*
Antivenin No. 4:	venom of *Lachesis muta*

2. Elapidae

Antivenin No. 5:	venom of *Micrurus frontalis*

The specific neutralizing properties in respect of the homologous venoms are determined for the Crotalidae and Elapidae using the species mentioned above under (A) (*a*) 1, (A) (*a*) 2, (A) (*b*) and (A) (*c*).

The group neutralizing action against venoms not included in the antigens injected into the horses is determined for the various antivenins as follows:

Antivenin No. 2-A (for *Bothrops jararaca*) has, in respect of the venoms of *B. alternata*, *B. atrox*, *B. cotiara*, *B. jararacussu* and *B. neuwiedii*, a neutralizing activity practically identical with that exercised on the homologous venom.

Antivenin No. 2-B (for *Bothrops neuwiedii*) neutralizes the venoms of *B. alternata*, *B. atrox*, *B. cotiara*, *B. jararaca* and *B. jararacussu*, in practically the same way as it does towards the homologous venoms.

Assay techniques

The in vivo assay method is used in assaying the following antivenins, the test animal used being the pigeon, the guinea-pig or the rabbit:

pigeon (250-320 g): Nos. 1, 2, 2-A, 2-B, 3, 3-A, 4, 5
rabbit (+ 2 kg): Nos. 6, 8 (partly)
guinea-pig (350-400 g): No. 7
guinea-pig (400-450 g): No. 9

The number of animals injected per mixture is as follows:

4 animals: Nos. 1, 2, 2-A, 2-B, 3, 3-A, 4, 5
2 animals: Nos. 6, 7, 8, 9

The route of injection is as follows:

intravenous: Nos. 1, 2, 2-A, 2-B, 3, 3-A, 4, 5
subcutanous: Nos. 7, 8 (partly), 9
intradermic: Nos. 6, 8 (partly)

Methods of expressing assay results

(*a*) In LD of venom: Nos. 7, 8 (partly), 9

(*b*) In weight (mg) of dried venom: Nos. 1, 2, 2-A, 2-B, 3, 3-A, 4, 5.

(*c*) The principle of necrotic units is used for sera Nos. 6 and 8 (partly). This method employs a principle similar to that used by Pratt-Johnson for the determination of the antihaemorrhagic action of the venoms of African vipers, which is assayed in terms of the inhibiting effect of the serum when mixed with such venoms.

The assay of all the antivenins considered is carried out in accordance with the single level method, in which no account is taken of the correction factor proposed by Ipsen for the quantity of venom tolerated by the animal in the absence of antivenins. The serum-venom mixtures are always injected after a contact period of 30 minutes in the oven at a temperature of 37°C.

The neutralizing potency of the sera thus assayed is expressed in accordance with the requirements of the Brazilian legislation and the Brazilian pharmacopoeia [31] (second supplement). At present, the legislation is officially applied only to the main antivenins Nos. 1, 2 and 3 for the species of *Crotalus* and *Bothrops* used in the preparation of the sera.

According to the requirements of the Brazilian pharmacopoeia, 1 ml of the undermentioned sera must neutralize the indicated doses (in mg) of dried venom of the following species:

(*a*) No. 1 = 0.5 mg of *C. terrificus terrificus*

(*b*) No. 2 = 1.5 mg of *B. jararaca*

(*c*) No. 3 = 0.4 mg of *C. terrificus terrificus*, and 1.0 mg of *B. jararaca*.

In connexion with the possible utilization of a local standard serum, Dr do Amaral considers that it is " unnecessary, as long as the principle of intra-specific and intra-generic specificity in treatment is adhered to." The antivenins are no longer employed in their original form for therapeutic purposes. Indeed, " although the avidity of the natural antivenin (serum) seems to be greater than that of the purified one, the increased potency of the latter seems to be more advantageous." (Personal communication.)

In the past, sera of all types were concentrated by the Banzaf method. At present they are all purified and concentrated by Pope's enzymic method. The utilization of antivenins in freeze-dried form is now spreading and it is felt that this should become the general method in the near future. The validity of concentrated and purified serum produced at the present time is 5 years, although that period can be considerably exceeded if the sera are not exposed to heat and light. The recommended therapeutic dose for an adult is, in principle, in inverse proportion to the weight of the person bitten and to the distance of the bite from the centre of the body:

Nos. 1, 2, 3: 30 ml, repeated when necessary after 4-6 hours
No. 3-A: 20-60 ml

Nos. 4, 5: 40-120 ml
Nos. 6, 7, 8: 5-10 ml
No. 9: 10-20 ml.

Method of injection of antivenins

(*a*) Intramuscularly or intravenously, according to the gravity of the poisoning, for sera Nos. 1, 2, 2-A, 2-B, 3, 3-A, 4, 5, 7, and 9.

(*b*) Subcutaneously (preferably near the bitten part), for sera Nos. 6, 8 (a half-dose combined with another half-dose administered intravenously) and hyaluronidase. However, this substance, which prevents the occurrence of severe local necrosis, must also be injected in all cases of poisoning resulting from a *Bothrops* bite.

As regards the relative importance and severity of snake-bites and poisoning by scorpions, the following figures were communicated by Dr do Amaral:

	Species	*Incidence among ophidic accidents*	*Mortality*
Crotalidae:	*B. jararaca*	51%	22%
	C. terrificus	17%	62%
	Other species	32%	16%
Elapidae:		0.3%	

Snake venoms utilized for assays of sera are, after harvesting by the Instituto Butantan, centrifuged, freeze-dried and stored in tubes under vacuum, in a cool, dark place. The various immunological aspects of the properties of the venoms used in the preparation and assay of antivenins prepared in the Instituto Butantan are dealt with in two recent detailed publications by Schöttler.[77]

From the large series of assays made by Schöttler, it appears that the union between venom and serum does not occur in multiple proportions, so that the amount of venom neutralized by equal amounts of different antivenins is not proportional to the potencies of these sera.

Nevertheless, the method is capable of answering the question of whether one antivenin is equal, inferior or superior to another, provided that the doses of venom involved in the assay are below the maximum dose which can be neutralized. For these reasons, Schöttler considers that the method of choice should therefore be the assay of a serum against a standard antivenin.

He also advocates the preparation of a homologous standard venom, composed of venom obtained from a large number of specimens of reptiles of the same species. In connexion with the specific group neutralizing power of monovalent and polyvalent *Bothrops* sera, the tests made by Schöttler show a neutralization spectrum for the sera of horses immunized with a mixture of venoms of the 6 species of *Bothrops* which is superior to that of the respective homologous sera.

From the results of these tests, subjected to probit analysis (Finney [32]), Schöttler draws [76] the following conclusions:

" Throughout the various types of experiments, the ratio $LD_{100} : LD_0$ in weight of venom was practically the same, regardless of how much venom was involved, which means that the difference between individual LD_0 and LD_{100}, expressed in number of lethal venom doses, increased along with the larger amounts of venom neutralized by increasing antivenin concentration. These observations lend support to the Bordet theory of antigen-antibody union.

" The average standard error of the figures representing the amounts of venom neutralized by antivenin was 5.8% in the subcutaneous and 5.5% in the intravenous tests with *Bothrops* venom, and 6.6% (subcutaneous) and 8.8% (intravenous) with *Crotalus* venom. The average deviation of single results obtained in repeated experiments for the evaluation of the therapeutic efficacy of the antibothropic serum was 9.2% around their arithmetic means.

" The lack of multiple proportions in venom neutralization by antiserum makes direct antivenin titration impossible and leaves the indirect assay by comparison of the unknown with a standard serum as the method of choice."

Dr A. A. Pareira, director of the *Instituto Pinheiros, São Paulo*, was kind enough to supply us with the following information:

In this institute three kinds of antivenins are prepared:

(1) Anti-*C. terrificus terrificus* serum (monovalent)

(2) Anti-*B. jararacussu, B. jararaca, B. alternata, B. atrox, B. neuwiedii* serum (polyvalent)

(3) Anti-ophidic serum (polyvalent) obtained by mixture of monovalent anti-*Crotalus* serum and of a polyvalent anti-*Bothrops* serum.

The immunization of the horses is carried out by means of unmodified venoms.

Assay methods

(1) The neutralizing activity of the monovalent anti-*Crotalus* serum is determined against the venom of *C. terrificus terrificus*, the only type of *Crotalus* found in Brazil.

(2) For the anti-*Bothrops* polyvalent serum, neutralizing properties are determined against the venom of *B. atrox, B. jararaca, B. jararacussu, B. neuwiedii* and *B. alternata*. This serum exerces also group neutralizing action against the venom of *B. itapetiningae, B. bilineata*, et *B. insularis*.

(3) The neutralizing potency of anti-ophidic serum is determined for the respective venoms used as antigens in the immunization of horses.

(*a*) In vitro method: anti-*Crotalus* serum is titrated by flocculation.

(*b*) In vivo method: assays of anti-*Crotalus* and anti-*Bothrops* sera are carried out according to the Vital Brazil method, i.e., by mixing variable doses of standard venoms of known DMM (*Crotalus*: 0.0000015 g; *Bothrops*: 0.000034 g) with fixed volumes of 1 ml of the serum tested.

After 40 minutes contact at 37°C, the mixtures are injected intravenously into pigeons weighing 260-380 g. Usually two pigeons are injected per mixture, this number being increased if necessary.

The activity of the antivenin is expressed in terms of weight (in mg) of dessicated venom per ml of antivenin. No account is taken of the amount of venom tolerated by the animal injected in the absence of antivenin.

According to minimum requirements:

(1) anti-*Bothrops* serum should neutralize 0.0018 g of *B. jararaca* per ml of serum;

(2) anti-*Crotalus* serum should neutralize at least 0.0008 g *C. terrificus terrificus* per ml of serum;

(3) for anti-ophidic serum, a minimum of 0.0006 g of *C. terrificus terrificus* and of 0.0015 g of *B. jararaca* should be neutralized by 1 ml of serum.

The LD_{50} of standard venom of *B. jararaca* and of *C. terrificus terrificus* should be, respectively, of 0.00004 g and 0.0000015 g for the pigeon.

For therapeutic use, antivenins are concentrated and refined according to Pope's method. The period of validity is of 3 years. The recommended therapeutic dose is of a minimum of 60 ml of serum, to be injected intramuscularly. An anti-*Lachesis muta* serum, as well as an anti-*Micrurus frontalis* serum is prepared, but their use is reserved for eventual bites during manipulations of snakes at the institutes. For the same purpose an anti-*Bothrops* serum is also produced in sheep for the treatment of persons possibly rendered allergic to horse serum by previous antivenin treatment.

Argentina

At the *Instituto Malbran, Buenos Aires*, several types of monovalent and polyvalent antivenins have been prepared for many years in the serum department, now under the supervision of Dr I. Pyrosky, against the venom of *Crotalus* and of the main *Bothrops* representatives found in Argentina.

The Instituto Malbran prepares three types of sera:

(*a*) A polyvalent antivenin, active against the venom of *Crotalus terrificus, Bothrops alternata* and of *B. neuwiedii.*

(*b*) A bivalent antivenin against *B. alternata* and *B. neuwiedii.*

(*c*) A polyvalent " Misiones " antivenin, possessing neutralizing properties against the venom of *C. terrificus, B. alternata, B. neuwiedii, B. tararaca* and *B. jararacusu.*

Immunization is carried out from desiccated venoms which after redissolution, are injected subcutaneously into the horse. For the production

of polyvalent antivenins, horses are treated with a mixture of venoms of *C. terrificus*, *B. alternata* and *B. neuwiedii*; for the bivalent serum, venoms of *B. alternata* and *B. neuwiedii* are similarly injected; while for the polyvalent " Misiones " serum, venoms of *C. terrificus*, *B. alternata*, *B. neuwiedii*, *B. jararaca* and *B. jararacusu* are used.

These antivenins are purified and concentrated by means of the peptic digest method and differential thermocoagulation.

The neutralizing activity of the polyvalent antivenin is determined respectively against the venoms of *C. terrificus* and *B. alternata*. For the bivalent antivenin, only the venom of *B. alternata* is used. Pigeons of 300 g are injected intravenously in decreasing doses with mixtures of serum added to a constant dose of venom. The test dose of venom used for *C. terrificus* corresponds to 0.4 mg and for the venom of *B. alternata* to 2.5 mg. The venom-antivenin mixtures are injected after contact of 2 hours at 37°C. During the last two years, mice of 20 g, injected intravenously, have also been used as test animals. Three to six mice are used per dose.

The neutralizing dose of serum corresponds to the minimum dose which will protect the pigeon during 48 hours.

The therapeutic recommended dose is of 30 ml to 50 ml of serum, injected intramuscularly. This dose should be repeated every 6 hours up to control of clinical symptoms. Specific serum therapy against snake bite in Argentina, gives satisfactory results on the whole.

The Instituto Malbran produces also a *Latrodectus mactans* antivenin. This serum is produced by immunization of the horse with venom of this spider injected subcutaneously. It is also concentrated and refined by peptic digestion and differential thermocoagulation. Titration is carried out on mice of 20 g, injected intravenously; six mice are used per dose. The test dose of venoms corresponds to 12 minimum lethal doses. The therapeutic dose is of 2 ml; excellent clinical results have been obtained.

The well known works of Houssay & Negrette [48] and their co-workers on the physiological action of various toxic and antigenic constituents of the venoms of the snakes mentioned above and of their neutralization by specific antivenins constitute an important contribution to the problem of ophidism in the American continent.

IMMUNOLOGICAL FACTORS AND CRITERIA AFFECTING THE REGIONAL AND INTERNATIONAL STANDARDIZATION OF ANTIVENINS

An analysis of the methods of assay and evaluation of the potency of antivenins prepared to counteract the venom of the Viperidae and Elapidae of the different continents brings out the great diversity in the methods of establishing the neutralizing properties and in the modes of expressing them.

These differences concern as much the criteria used to assess the activity of the sera as the choice of the test animals, the weight, the method of injection of the mixtures, and the methods of evaluating given antibodies in terms of lethal doses for the species of animal envisaged, in weight of dried venom, or in neutralizing units with respect to the homologous venom.

The basic biological principles underlying the various assays used to determine the antitoxic properties of antivenins are based on one of the following procedures:

(1) Use of increasing volumes of serum mixed with a fixed concentration of homologous venom.

(2) Use of increasing doses of venom mixed with a fixed volume of homologous antivenin.

(3) Use of venom doses and volumes of homologous serum in proportions which, following the method of Ipsen, are chosen according to a preliminary established neutralization line and read from a co-ordinate system in which the abscissa corresponds to venom doses and the ordinate to volumes of serum.

In the formula expressing the neutralizing capacity of the serum under study, allowance is made for the amount of venom tolerated by the animal in the absence of the antivenin.

This diversity in the methods used in vivo and in vitro is particularly striking for antiviper sera because of the multiplicity of the antigenic elements characterizing these venoms.

The greater homogeneity in the antigenic constitution of the venoms of the proteroglyphic Colubridae, and in particular of their chief constituent, neurotoxin, is brought out by the results of cross-neutralization between the venoms and the anticobra sera of Africa and Asia.

Although it is not customary for the WHO Expert Committee on Biological Standardization to choose methods for the carrying out of proposed assays on biological standardization, it is difficult in the case of antivenins to disregard this technical element. It was in recognition of this necessity that the Permanent Commission on Biological Standardization recommended, in 1936,[56] that studies be undertaken to prepare a suitable method for expressing the activity of European antiviper serum. The technique of multiple-level assays proposed by Ipsen seemed to satisfy these deciderata.

Moreover, a method of assay at one selected level, applied in conditions satisfying biological requirements, also seems, according to comparative studies made by the present author with the Ipsen method, capable of rendering the same services and offering equal reliability.

Studies carried out during the last 10 years on the application of the Ipsen multiple-level method in various institutes all indicate the possibility of adapting this method to the assay of the various types of antivenins,

and show that the same accuracy was obtained in assays of the European anti-*aspis* serum (Ipsen) and of anti-*ammodytes* serum (Institute of Hygiene, Zagreb, and the Istituto Sieroterapico Milanese). This is the case for the antivenins prepared against African vipers, and for anti-*B. arietans* serum, according to studies carried out at the South African Institute for Medical Research and the Institut Pasteur, Paris, as well as for anti-*B. gabonica* serum. Lastly, the same is true of the Asiatic vipers *V. russellii* and *E. carinatus*, the sera of which are assayed by the same method at the Haffkine Institute, Bombay.

Concordant results have likewise been obtained in the assay of African anti-cobra sera, e.g., the anti-*N. flava* serum (South African Institute for Medical Research) and Asian anticobra serum (*N. naja*) by the Haffkine Institute and the Institut Pasteur, Paris.

Lastly, the anti-*Bungarus coeruleus* serum is assayed by the same method at the Central Research Institute, Kasauli, as is the *N. naja* antibody contained in the polyvalent serum already referred to. The neutralizing potency of the anti-*Notechis* serum, produced by the Commonwealth Institute, Melbourne, is expressed in units in relation to a local working standard.

The practical value of establishing a reference standard for the assay of antivenins has been recognized in various institutes, as has the importance of establishing standard venoms for the homologous sera, which would ensure the uniformity and repeatability of assay methods over a period of several years.

The idea of measurement by units has also been introduced in certain institutes in connexion with, or independently of, the use of a standard serum. This is the case in the Behringwerke, Marburg, for antiviper and anticobra sera, in the Commonwealth Institute, Australia, for cobra antibodies, and, as a temporary measure, it has been introduced by the public health authorities in the Union of South Africa.

The establishment of such a unit is, in general, based on the neutralizing potency of a given volume of serum expressed either in terms of one or more LD of venom, or in terms of the mg of homologous venom neutralized by such a unit.

Nature of Venoms Used as Antigens for the Titration of Antivenins

At present, generally speaking, all institutes producing antivenins use non-modified crude venoms as antigens for assessing the neutralizing power of antivenins obtained from horses which have been hyperimmunized with various types of venoms. These venom antigens should comply with the following requirements:

Homogeneity of the venom antigens and importance of test venoms

As stressed above, the test venoms should be a homogeneous mixture of venoms, collected from a large number of specimens of snakes belonging to the same species, coming from the region or continent which is the habitat of the Viperidae and Elapidae families concerned.

These standard venoms should have been collected, if possible, over several years and at different seasons, if this factor is known to be of particular importance, as in the case of *V. aspis.*

Stability of the venom antigens

Every precaution should be taken to ensure that the standard venoms are of the highest possible uniformity, from the zoological and qualitative viewpoints, as well as stability. The collection of venom from snakes in specialized institutes has the advantage that the specimens can be directly checked from the zoological viewpoint, as can the apparent health of the snakes, and that the venom can be collected under satisfactory conditions avoiding the admixture with the venom proper of cellular debris from the salivary glands resulting from unduly brutal handling.

Similarly, precautions should be taken, after harvesting, so as to dry the venom as quickly as possible, thus ensuring that they retain their toxic biochemical and antigenic properties.

In various institutes, drying by means of certain chemicals, such as calcium chloride or phosphorus pentoxide, has been replaced by the more advanced technique of freeze-drying of the venoms, thus ensuring the maximum stability in biological and antigenic properties over a number of years. The venoms thus preserved should be re-dissolved just before use. In some cases, the solution should be centrifuged so as to remove debris and insoluble matter. The venom solutions prepared are used within the next few hours and kept for this short period in the refrigerator at 2°-4°C, in case additional tests may be necessary. New solutions should be made daily if proved necessary by the assay experiments. The stability of the biological properties of the venoms should be controlled experimentally by determining the MLD per mg of standard venom for a test animal of given weight and sex.

Use of stabilizers

When working conditions call for the keeping of a solution without it having been subjected to freezing, the process of re-freeze-drying of the parent solutions may be employed. Solutions of venom antigens may be kept at 2°-4°C on the addition of thymol. In certain institutes, glycerol is added to venom solutions before storage.

Precautions aiming at safeguarding the stability of the venoms as far as possible are particularly necessary in view of the labile nature of various

components of certain venoms, in particular of the haemotoxic and cytotoxic as well as coagulant or anticoagulant constituents of the venoms of the Viperidae—constituents which can be rapidly broken down by various physical and chemical factors.

The neuro-toxic component in venoms from various Elapidae, however, is considerably more resistant and has a substantially higher degree of stability.

Fractionation of Venoms and Neutralization of the Isolated Fractions by Homologous Antivenins

Work carried out in various institutes, over the last ten years, on the constitution and fractionation of venoms has considerably enlarged our knowledge of their physiopathological properties. The experiments of Slotta [79, 80] on the *Crotalus* venom, carried out in the Instituto Butantan, have permitted the extraction of a highly purified substance: Crotoxin.

The application of electrophoretic [69] analysis to venoms, introduced for the African venoms by Polson, Joubert & Haig [69] has shown the possibility of using this method to isolate the toxic and antigenic principles entering into the constitution of the venoms. In this way, their intrinsic properties can be studied, either in vivo or in vitro, according to the nature of the venoms, as well as their specific relation to the homologous antivenins, as was done by Schöttler [76] for the venoms and homologous sera of *B. jararaca* and *C. terrificus*. The proteins of the venom of *V. ammodytes* have been separated by precipitation under iso-electric conditions by Muic & Piantanida.[62]

Moreover, the application to the venoms of the method of electrophoretic [82] analysis on paper has considerably facilitated such research (Wieland & Wirth [81]). By mixing the antigen fractions from the venoms thus isolated with the homologous antivenin, it was possible to study the mode of interaction in vivo and in vitro of various constituents of Viperidae and Elapidae venom as was done by Grasset & Schwartz [56] for the venom of *V. russellii* and, more recently, for that of *V. aspis* and *B. arietans*.

Titration of antivenins by flocculation

As early as 1902, Lamb,[53, 54] followed by Calmette & Massol in 1909,[20] reported on the precipitation observed on mixing cobra venom with the serum of immunized horses. They concluded that this precipitation reaction could serve to measure approximately, in vitro, the antitoxic value of an antivenin. Work carried out in numerous institutes on the reaction in vitro between venoms and specific sera confirmed that flocculation phenomena appeared in these mixtures. Most authors expressed, however, reservations as regards the application of this method for estimating the antigenic power

of the sera thus studied and the very relative parallelism between the titre obtained in vitro and the neutralization of the venom by the homologous sera in vivo. In this connexion, mention may be made of the work of Lamb in India, with *V. russellii* venom, that of Arantes, Koormann & Bier [4] and Bier [8] with *C. terrificus* and *B. jararaca* venoms confirmed by Schöttler.[76] The same irregularities in the results of flocculation experiments were reported for the venoms of *V. aspis*, *C. cornutus* and *N. tripudians*, by Cesari & Boquet,[24] as well as by Mallick [60] for flocculation taking place between *V. russellii* and *N. naja* venoms and antisera.

Grasset [38] expressed a similar opinion in the conclusions of his papers dealing with the flocculation phenomena observed both in *B. arietans* and in *N. flava* venom-serum mixtures. More recently, Christensen [25] in studies dealing with these venoms, showed the possibility of recovering cobra neurotoxin by exposing the venom-antivenin flocculates to a temperature of 70°C at pH 2, which made it possible to isolate 3 thermostable toxins, α, β and γ, from the venom of *N. flava*. On the other hand, as regards viperine venoms, the flocculation reaction obtained with the venom of *B. arietans* cannot be considered, according to Christensen, as giving an indication of the protective power of this serum.

On replacing the flocculation technique for the study of the interaction of antivenins and venoms by precipitation in a gel column, developed by Oudin [64] as well as the analogous agar-plate method of Outcherlony [63] it is possible, according to observations made by Grasset et al.[45, 46] to study the line spectrum of precipitation resulting in agar from the reaction between venoms and homologous antivenins, as well as group-antigen components which play a part in the precipitation of these mixtures.

Apart from the immunological interest attached to these studies, there is a possibility that such research will lead subsequently to the development of physico-chemical methods for the in vitro assay of antivenins, in relation to certain of the most important antigenic components of the venoms.[a]

Immunological Considerations and their Bearing on the Establishment of Standard Antivenins

The considerable biological differences existing in the composition and the distribution of the antibodies present in the specific antivenins in respect of the homologous Elapidae, and still more Viperidae, venoms of the various continents, imply from an immunological standpoint, the use of monovalent standard sera which are specific towards the homologous standard-venoms.

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[a] The texts of the communications presented at the First International Conference on Venoms held in Berkeley, California, in December 1954,[1] which dealt with various pharmacological properties of the venoms and the standardization of antivenins, were not received early enough to permit them to be analysed in the present paper. This remark refers, in particular, to the papers by Cindrich & Hohenabel, Keegan and Ahuja.

In view of the nature of these facts it might even be advisable to have several of such standard sera in the various areas infested by species of snakes with antigenically different venoms. The geographical distribution of such standard sera would be based on the presence in such areas of groups of venomous snakes having venoms of a common or related antigenic composition.

TABLE II. GEOGRAPHICAL DISTRIBUTION OF SNAKES WHOSE ANTIGENIC CONSTITUTION INFLUENCES THE ESTABLISHMENT OF STANDARD VENOMS AND ANTIVENINS

Geographical distribution	Type of venomous snakes		
	Viperidae	Crotalidae	Elapidae
Europe	*V. aspis* *V. ammodytes*		
Africa			
northern	*V. lebetina* *C. cornutus* *C. carinatus*		*N. naja*
equatorial	*B. gabonica*		
southern	*B. arietans*		*N. flava*
Asia			
India and Middle East	*V. russellii* *E. carinatus*		*N. naja* *B. coeruleus*
Malaya (South-east)	*Ancistrodon*		
Americas			
(north and south)		*C. terrificus terrificus* *B. araraca*	
(south)			*M. frontalis*
Australia			*N. scutatus*

Seen as a whole, measures taken in view of the adoption of standard sera for the international standardization of antivenins might be spread

over several stages, whatever the nature of the sera considered, i.e., antiviper, anticobra, antiscorpion, or antispider.

Preliminary steps for the establishment of a standard antivenin

(1) Adoption of an homologous "*standard*" *test venom* by the regional institutes engaged in the production of the respective type of serum.

(2) Selection of a suitable homologous, monovalent antivenin to be eventually adopted as *standard serum* according to the following procedure:

Determination of the neutralization potency of the serum by an adequate assay method, such as the multiple level titration method or by using an adequate single-level method. This will permit a provisional expression of the neutralization potency of the serum, in terms of mg of dried homologous venom or in multiples of the CLD per ml of the serum, and will serve as criterion for appreciating its suitability as a standard antivenin.

(3) Adoption of a convenient *antitoxic unit* chosen so that it will meet the biological and technical assay requirements. This unit might be related to a basic method of expressing activity in weight (mg) of dried venom, such that 0.1 ml or 1 ml would neutralize, for example, 5 or 10 CLD of the homologous standard-venom.

By making use of these principles it would be possible to determine the potency of the neutralizing antitoxic activity of monovalent or polyvalent commercial sera in relation to such a standard serum and in terms of the antitoxic unit adopted for expressing its neutralizing potency.

RÉSUMÉ

Les études sur le titrage des sérums antivenimeux poursuivies dans les divers instituts où ces sérums sont préparés reflètent la complexité du problème et la diversité des méthodes mises en œuvre in vivo et in vitro pour apprécier le pouvoir neutralisant des sérums des animaux soumis à l'immunisation de venins de constitution variée.

Les recherches entreprises dès 1935 dans le cadre international, sur la recommandation de la Commission permanente de Standardisation biologique de la Société des Nations, conduisirent à l'élaboration d'une méthode de titrage, à plusieurs niveaux, du sérum anti-vipères Europe qui se révéla applicable à une variété de sérums anti-vipéridés et anti-cobras.

Considérant la diversité des méthodes de titrages, l'OMS décida en 1954 d'inclure cette question au programme du Comité d'experts de la Standardisation biologique. Il apparut judicieux de procéder tout d'abord à la comparaison des méthodes de titrages utilisées. Grâce à l'obligeante collaboration des directeurs des instituts producteurs de sérums antivenimeux, l'auteur fut à même de collationner les méthodes de titrages employées dans 22 instituts; elles sont reproduites dans cet article avec l'autorisation des intéressés. L'analyse des éléments biologiques et techniques de ces méthodes fait ressortir les différences qui existent entre elles et qui portent aussi bien sur le principe d'évaluation de l'activité des sérums que sur le choix des animaux d'expérience, le mode d'injection

des mélanges que sur les méthodes d'appréciation des anticorps déterminés soit en fonction de doses minimums mortelles, soit en poids (mg) de venin desséché ou, encore, en unités neutralisantes envers le venin homologue.

Le principe biologique de ces méthodes est basé sur l'une des trois modalités suivantes: 1) mélange de doses croissantes de sérum antivenimeux avec des doses fixes de venin homologue; 2) mélange de doses croissantes de venin avec un volume fixe de sérum; 3) emploi, selon Ipsen, de doses de venin et de sérum antivenimeux en proportions variables déterminées pour une série de points sur une ligne préliminaire de neutralisation dans un système de coordonnées où les abscisses correspondent au venin et les ordonnées au sérum antivenimeux. Il est tenu compte dans la formule d'expression du pouvoir neutralisant du sérum de la résistance de l'animal envers le venin en l'absence de sérum antivenimeux.

Les résultats obtenus pour un même sérum sont susceptibles de varier considérablement selon les méthodes de titrages employées.

Le principe de l'utilité de référence à des sérums-étalons antivenimeux, de même que d'unités neutralisantes, a déjà trouvé son application dans certains instituts.

L'adoption de sérums-étalons antivenimeux sur le plan international se heurte à des difficultés de nature immunologique étant donné la composition complexe et la distribution géographique des anticorps constituant des sérums antivenimeux à l'égard des venins homologues des élapidés et plus encore des vipéridés des divers continents.

Ces différences dans la constitution des venins impliquent l'emploi de sérums-étalons monovalents envers des venins types homologues, de haute activité et stabilité, et l'établissement de sérums-étalons sur une base régionale correspondant aux groupes de serpents venimeux caractérisés par des venins de composition antigénique commune ou proche.

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