STANDARDISATION OF CALMETTE'S ANTI-
VENOMOUS SERUM WITH PURE
COBRA VENOM:THE DETERIORATION OF THIS SERUM THROUGH KEEPING
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THE standardisation of an anti-venomous serum is of great practical as well as theoretical importance. It is the practical side of the question to which we propose to confine ourselves in this paper.

The snake venom which M. Calmette¹ employs both for the purpose of immunisation of his horses and for the purpose of standardisation of the serum recovered from these immune horses is a mixture of colubrine and viperine poisons, the former making up about 80 per cent. of the mixture. A solution of this mixture is heated at about 73° C. for half an hour and then filtered. It is this mixed and heated poison which is employed at Lille for both the purposes above mentioned.

Myers² has correctly pointed out that as we have in all cases clinically to deal with intoxication by a pure venom, either colubrine or viperine, and also by an unheated venom, it is preferable, if the true value of this serum as a curative agent against snake-bite is to be determined, to use a pure unheated poison for its standardisation. The object, then, of our investigation was to ascertain as exactly as possible the neutralising power of Calmette's anti-venomous serum when tested with pure, fresh, and carefully dried cobra poison. Further, in consequence of the observations made by us in a recent case of cobra intoxication,³ we have extended our investigations so as to ascertain if any deterioration of the serum takes place through keeping in this country. In another communication we hope to be able to record the results of similar experiments made with the venom of Russell's viper (*Daboia Russellii*).

The method of standardisation of the anti-venomous serum which we adopted in our experiments was the method now recognised as giving the most accurate results. It is based on the now almost generally-acknowledged fact that snake-poison toxin and its antitoxin interact directly and that this neutralisation is, so far as our knowledge goes, independent of vital activity. The evolution of this method is briefly stated as follows. The method which M. Calmette⁴ recommends and still practises is the following:—The dose of venom (mixed and heated) which will kill a rabbit of two kilos in weight in from 15 to 20 minutes when injected into the marginal vein of the ear is determined; then the minimum amount of serum which will prevent death when injected intravenously five minutes before this dose is ascertained, which is done by experimenting with varying quantities of serum. If one cubic centimetre is able to accomplish this M. Calmette says that the serum contains 2000 immunity units per cubic centimetre or 20,000 per 10 cubic centimetres.

Martin of Melbourne⁵ was the first to use the method of mixing the venom and serum *in vitro* before the injection. In some early experiments with the poison of the Australian tiger snake (*Hoplocephalus curtus*) he used only a single lethal dose in estimating the curative value of Calmette's serum. However, in some later researches he recognised that this method was fallacious inasmuch as if the amount of serum was able to neutralise even a mere fraction of the single lethal dose the animal would recover even though the larger part of the poison remained unneutralised. He therefore recommended the employment of at least 10 lethal doses.

Along with one of us Major D. Semple, R.A.M.C.,⁶ has pointed out that the following objections could be taken to Calmette's method: (1) that there is no direct estimation of the amount of venom which a given quantity of serum will neutralise;⁷ and (2) that there is no estimation made of the amount of venom the injection of which an untreated animal is capable of surviving. Further, we showed that Calmette's test dose for the rabbit amounted to about three lethal doses for that animal. We thus gave a value for the serum in terms of its neutralising power and not as in the method used at Lille in terms of its preventive power. With such a small test dose as three lethal doses, unless careful attention be paid to the second objection mentioned above, it is evident that a much enhanced neutralising power would be given to the serum.

The next step in advance was made by Myers.⁸ As previously mentioned this observer pointed out that in order to ascertain the strength of an anti-venomous serum for practical purposes it was *a priori* preferable to test it against a pure venom, such as pure cobra poison or pure Daboia poison. He further showed that it was preferable also to use unheated venom, inasmuch as heating at the temperature employed by M. Calmette causes important changes in cobra venom, altering it considerably from the natural poison as injected by a snake. Further, Myers recommended that a cheap, small, and susceptible animal should be employed. He showed that mice of about 15 grammes in weight answer all requirements, and recommended that these animals should be used. He further designated Calmette's method as unscientific, inasmuch as by injecting the serum before the venom the completion of the reaction between these two bodies does not take place before they enter the blood-stream. He showed that much more accurate results are attained when the poison and serum are mixed *in vitro* than by Calmette's method. Finally, he recognised the necessity of using a multiple, and if possible a large multiple, of the minimum lethal dose.

In the series of experiments which we have now to present these various points were kept carefully in view. We may preface the protocols by giving a short account of the method employed and of the general results obtained.

The animals which we used were rats from 115 to 120 grammes in weight. These fulfil all the conditions indicated by Myers. They are cheap and the supply is abundant, while with care no difficulty is experienced in handling them. At the same time they are extremely susceptible to cobra poison, as susceptible as, if not more so than, any of the other laboratory animals. Thus for a mouse of 15 grammes Myers⁹ states the minimum lethal dose of cobra venom to be 0.012 milligramme, or in other words 0.8 milligramme per kilo. For rabbits Elliot¹⁰ has found that a certain lethal dose of pure cobra poison injected subcutaneously is 0.7 milligramme per kilo. In our experiments we found that the minimum lethal dose of this poison for a rat of about 120 grammes was 0.04, or at the rate of 0.33 milligramme per kilo. Another reason for selecting rats was that horses' serum, even in large doses, has no lethal action on them as it has on mice. As in the course of our experiments large doses of serum had to be given this was an important point. Great care was taken in choosing rats of one size for all experiments—viz., of about the weight mentioned above. The venom used was pure, fresh, unheated cobra poison. This was collected from a number of living, full-sized, healthy cobras by "milking" the glands after the method practised by M. Calmette. The poison was then thoroughly dried over lime and kept till used in an air-tight tube away from light. Before weighing it was pounded in a mortar and was then again dried over sulphuric acid. A solution of this powdered venom in normal salt solution was made. The strength of this solution was 0.1 per cent. When necessary a tenfold dilution of this material was employed. All the solutions were freshly made, as required, so that no deterioration of the poison was possible through keeping in solution. All injections were made in the inner side of the thigh.

Series No. 1 was undertaken to determine the minimum lethal dose for a rat of from 115 to 120 grammes in weight.

⁶ Brit. Med. Jour., April 1st, 1899, p. 781.

⁷ This is evident from the description of Calmette's method already stated. From this it will be seen that the standard which he adopts is the weight in grammes of a rabbit which one cubic centimetre of serum can protect against a quantity of poison which would kill a control animal in 20 minutes.

⁸ Loc. cit.

⁹ Loc. cit.

¹⁰ Brit. Med. Jour., Feb. 10th, 1900, p. 309.

¹ Annales de l'Institut Pasteur, 1897, p. 225.

² THE LANCET, May 19th, 1900, p. 1433.

³ THE LANCET, Jan. 5th, 1901, p. 25.

⁴ Loc. cit.

⁵ Intercolonial Medical Journal of Australasia, August 20th, 1897. Ibid., April 20th, 1898.

Brit. Med. Jour., 1898, p. 1120.

A certain lethal dose was found to be 0.04 milligramme and the maximum non-lethal dose was found to be 0.035 milligramme.

The remaining series of experiments were undertaken with a view of determining the neutralising power of various samples of anti-venomous serum. These samples were of different ages and had been in India for varying periods of time. They had been stored in different places and had therefore been exposed to different degrees of heat. The test dose of venom which was used in all cases consisted of ten times the minimum lethal dose—i.e., 0.4 milligramme. This quantity was mixed *in vitro* with varying amounts of the serum to be tested. The mixture was allowed to stand at laboratory temperature (about 25° C.) for at least half an hour. All injections were made, as in the experiments detailed in Series No. 1, subcutaneously in the inner side of the thigh. In calculating the amount of venom which the serum had been able to neutralise or had failed to neutralise the maximum non-lethal dose—i.e., 0.035 milligramme—was deducted from the test dose in all cases.

In Series No. 2 a serum which had recently been received from Lille was used. We take this opportunity of thanking M. Calmette for his courtesy in placing this serum at our disposal. This sample was dated Sept. 17th, 1900. It arrived in Bombay on Oct. 14th of the same year. Our experiments with it were made between Nov. 10th and Dec. 5th. We are justified, therefore, in considering this as perfectly fresh serum, as fresh as it would be possible to get in this country. A reference to the protocols will show that 0.5 cubic centimetre was able to prevent death while 0.45 cubic centimetre did not accomplish this. In other words, 0.5 cubic centimetre of this serum was able to neutralise 0.4—0.035 milligramme = 0.365 milligramme—namely, 1 cubic centimetre could neutralise 0.73 milligramme. Working with a mixed and heated venom Semple and one of us¹¹ found that one cubic centimetre of fresh serum was able to neutralise 1.5 milligrammes of this mixed poison. This is the same value which M. Calmette attributes to his serum when this is expressed in terms of the neutralising power of the serum. Comparing these three results we see at once, therefore, the importance of testing the serum against a pure unheated venom and not with a mixed and heated poison.¹²

The serum employed to perform the experiments detailed in Series No. 3 was obtained by purchase from Messrs. Phillips and Co., chemists, Bombay. It bore the date May 8th, 1900. The vendors informed us that they had received it from Europe about the end of August, 1900 and that it had not been out of Bombay. Our experiments were made between Dec. 5th and 12th, 1900. It will be seen therefore that this serum had been in Bombay for about three months. One of these months—viz., October—is one of the hottest months of the year.

A reference to the protocols will show that 0.6 cubic centimetre was able to prevent death, while 0.5 cubic centimetre would not accomplish this. It is evident therefore that one cubic centimetre of this serum was able to neutralise 0.61 milligramme. This result would appear to point to a slight deterioration of the serum having taken place through keeping in Bombay, even for the short time mentioned above.

The serum used in Series No. 4 of our experiments was obtained from the Natural History Society of Bombay. We take this opportunity of thanking Mr. Phipson, the honorary secretary of that society, for his kindness in placing it at our disposal. This sample of serum was not dated. Mr. Phipson, however, thought that it had been in his possession for about four years. On reference to Lille, M. Calmette informed us that he did not know the exact age of the serum, but it was at least more than three years old. From the protocols it will be seen that although one rat which received 0.75 cubic centimetre of serum recovered after being very ill the quantity of serum necessary to prevent a fatal result with certainty was 1.25 cubic centimetres. It is evident therefore that one cubic centimetre of this serum was able to neutralise only 0.29 milligramme of pure cobra venom.

The fifth series of experiments detailed in the protocols was made with serum which was sent to us by Captain Mitchell of Nasik. In view of the results obtained the history of this sample of serum is interesting. It was originally the property of the Bombay Natural History Society and was got from Europe at the same time as the serum used in Series No. 4 of our experiments. It was, therefore, more than three years old. Soon after its arrival in Bombay it was sent to Nasik. It was kept there till Captain Mitchell sent it to us in October, 1900. Our experiments with this serum were made between Oct. 16th and Nov. 1st, 1900. A reference to the protocols will show that 0.8 cubic centimetre was able to prevent death when our test dose was employed. In other words one cubic centimetre of this serum was able to neutralise with certainty 0.46 milligramme of pure cobra poison.

For Series No. 6 of our experiments the sample of serum was obtained from Lieutenant-Colonel Gimlette, I.M.S., residency surgeon, Indore. This sample was dated Oct. 17th, 1896. Its history was as follows. It was imported to India by Messrs. Smith, Stanistreet, and Co., chemists, Calcutta, arriving in this country about the end of August, 1899. It was sent to Indore on Sept. 6th of the same year. Colonel Gimlette despatched it to Bombay at the end of November, 1900, and our experiments were completed between Nov. 29th and Dec. 3rd. It will be seen, therefore, that for over a year it lay at Indore and was thus subjected to the great heat of this place during one hot season. A reference to the protocols will show that 1.7 cubic centimetres of this serum failed to neutralise the test dose, while two cubic centimetres were able to accomplish this. The rat which received this latter quantity was ill for a short time. We may take it therefore that two cubic centimetres were the amount which could neutralise just over nine lethal doses, or in other words that one cubic centimetre was able to neutralise only 0.18 milligramme of venom.

The experiments detailed in Series No. 7 of the protocols were made with a serum received from Mr. Hankin of Agra. This sample bore the date Sept. 17th, 1895. It had been stored in Agra for over four years. It was received in Bombay on Oct. 17th, 1900, and our experiments with it were completed between Nov. 27th and Dec. 10th. A glance at the protocols will show that 2.5 cubic centimetres of this serum—i.e., the largest amount used—were not able to prevent death when our test dose of 10 lethal doses was employed. From this it is evident that one cubic centimetre of this Agra serum could not neutralise even 0.14 milligramme of poison. The serum can therefore be said to have lost all neutralising power for practical purposes.

The serum employed in the eighth and last series of experiments detailed in the protocols was received from Lieutenant W. G. Liston, I.M.S., Civil Surgeon, Ellichpur, Berar. It was dated Nov. 7th, 1896. Lieutenant Liston had purchased it from Lille in September, 1898, and had brought it with him to India in the following month. During the hot seasons of 1899 and 1900 the serum had been subjected to the heat of Berar at two stations—viz., Hingoli and Ellichpur. It was sent to Bombay in December, 1900. The experiments made by us were completed between Dec. 10th and 14th. From the protocols it will be seen that two cubic centimetres of this serum failed to neutralise the test dose. We can therefore conclude that one cubic centimetre was not able to neutralise 0.18 milligramme of venom. It is unfortunate that no more serum was available to complete this series. It is, however, evident that like the Agra serum it had lost for all therapeutical purposes its power to neutralise pure cobra poison.

We may now pass on to consider some of the practical bearings which the data collated above and detailed in the protocols have on the standardisation of anti-venomous serum and on the therapeutics of cobra poisoning. First, our experiments conclusively prove Myers's statement that it is necessary in standardising this serum to use a pure unheated poison. Both in Calmette's method of standardisation and in the method followed by Semple and one of us a mixed venom containing about 80 per cent. of colubrine poison was employed. The solution of this mixture was heated at 73° C. for half an hour and was then filtered. The conclusion arrived at in both cases was the same—viz., that one cubic centimetre of serum was able to neutralise 1.5 milligramme of this venom. As this mixture contained only a fifth part of viperine poison it appeared justifiable to conclude that one cubic centimetre of serum would be able to neutralise at least about 1.2 milligrammes of pure cobra

¹¹ Loc. cit.

¹² This comparison, as well as other similar comparisons to be detailed later, is made on the assumption that all specimens of anti-venomous sera sent out from Lille have when fresh the same neutralising power. There can be no doubt, however, that such a desirable result cannot practically be obtained. It is probable, therefore, that a small proportion of the differences in the neutralising power of the sera with which we experimented may be due to slight initial differences in this neutralising power.

poison. Our experiments, however, have shown that such a hypothesis is not borne out by fact and that one cubic centimetre of serum can only neutralise about 0·7 milligramme of pure cobra venom.¹³ Secondly, it is evident that any attempt to calculate the therapeutic dose of the serum can only be made when the exact measure of its power of neutralising a natural poison has been ascertained. Let us consider this point in some detail.

Calmette recommends that from 10 to 20 cubic centimetres¹⁴ be given as the dose in all cases of bites from poisonous snakes. We shall see later that such a dose would probably be insufficient in some cases of cobra bite.

Semple and one of us¹⁵ calculated that 15 cubic centimetres were sufficient to save a patient if this quantity were injected into the blood before the lapse of such period of time as may be required for the absorption of the lethal dose of venom. This calculation, however, was made on the following assumptions:—1. That the minimum lethal dose of cobra venom for a man of 30 kilos in weight was, as estimated by Fraser¹⁶ about 31 milligrammes and that consequently we may take 30 milligrammes as the maximum non-lethal dose for an average man. 2. That a full-sized cobra can inject at each bite a quantity of venom which in the dried state amounts to from 30 to 45 milligrammes. These amounts have been demonstrated experimentally by Calmette.¹⁷ To save a man bitten by a cobra, injecting the maximum quantity possible, we should therefore have to give an amount of serum capable of neutralising 45—30 milligrammes = 15 milligrammes of cobra poison. 3. That one cubic centimetre of serum was capable of neutralising one milligramme of pure cobra venom. This figure was arrived at by assuming that pure unheated cobra poison was not more than a third more deadly than the mixed venom which we employed. This mixture it will be remembered, contained 80 per cent. of colubrine poison, and one cubic centimetre of serum was able to neutralise 1·5 milligrammes of it. It is evident, therefore, that were these three assumptions correct 15 cubic centimetres of serum would be a safe dose to employ in all cases of cobra-bite. Our experiments, however, have demonstrated that one cubic centimetre of serum is only capable of neutralising about 0·7 milligramme of pure cobra venom, while Myers¹⁸ from his experiments on mice makes the quantity neutralised by one cubic centimetre even less than this—viz., 0·6 milligramme. Now, basing the calculation on this fact—viz., that one cubic centimetre of serum can neutralise from 0·6 to 0·7 milligramme of cobra venom—and on the first two assumptions stated above, we arrive at the conclusion that instead of 15 cubic centimetres of serum, a dose of from 22 to 25 cubic centimetres would have to be given to prevent death from the bite of a full-sized cobra.

As we have already stated, one of the assumptions on which the above calculation is founded is that the minimum lethal dose of cobra venom for a man is as much as 31 milligrammes. This assumption is based partly on the fact, experimentally shown by Calmette¹⁹ and other observers, that for such animals as the guinea-pig, the rabbit, the dog, the monkey and the ass the larger the animal the greater is the amount of cobra venom, weight for weight, required to cause death. This rule, however, does not hold good for all species of animals. Thus Myers²⁰ has shown that the minimum lethal dose of cobra poison for a mouse of 15 grammes in weight is 0·012 milligramme. If the rat were as susceptible to this poison as is the mouse the lethal dose for a rat of 120 grammes weight would be 0·096 milligramme. We have, however, seen above that the minimal lethal dose for a rat of this size is only 0·04 milligramme—namely, that the rat is at least twice as susceptible to cobra venom as the mouse.

In view of this fact it would seem expedient in estimating the dose of anti-venomous serum to be given in a case of cobra-bite to base our calculations on the assumption that man is as susceptible as the most susceptible animal with which experiments have been made—viz., the rat. Assuming, then, that man is as susceptible as the rat, it is evident that

the minimum lethal dose for a man of 60 kilogrammes in weight would be about 20 milligrammes. We can therefore calculate that if a cobra had injected the maximum amount of venom possible—viz., 45 milligrammes (an assumption which it would be necessary to make in every case of snake-bite)—the quantity of serum required to merely save the life of anyone receiving this injection would be the amount which was capable of neutralising at least 45—19 milligrammes = 26 milligrammes. This amount of fresh serum—namely, serum of which one cubic centimetre was able to neutralise about 0·7 milligramme of cobra venom, would be about 37 cubic centimetres. It is interesting in this connexion to point out that Calmette²¹ himself has arrived at the conclusion that the minimum lethal dose of cobra venom for a man is only about 10 milligrammes. Working on this hypothesis it is evident that it would require about 50 cubic centimetres of serum to save a patient who had received the maximum quantity of venom which a cobra can inject.

Our contention that 15 cubic centimetres of serum is quite an inadequate dose for the treatment of a case of cobra bite receives corroboration from consideration of the accidental case which occurred in this laboratory a short time ago.²² The only treatment employed in this case was the subcutaneous injection of anti-venomous serum. Immediately after the accident 18 cubic centimetres of old serum were injected. This was the serum obtained from the Bombay Natural History Society the neutralising power of which was tested in the experiments detailed in Series No. 4 of the protocols. From a reference to the protocols it will be seen that one cubic centimetre of this serum was able to neutralise 0·29 milligramme of pure cobra venom. It follows, then, that 18 cubic centimetres of such a serum was equivalent to 7·5 cubic centimetres of fresh serum—that is, of serum one cubic centimetre of which was capable of neutralising 0·7 milligramme of venom. Our patient therefore received as a preliminary dose a quantity of serum equivalent to 7·5 cubic centimetres of a perfectly fresh serum. In spite of this injection well-marked general symptoms referable to the central nervous system set in from about two and a half to three hours after the accident. The case in all probability would have had a fatal ending had not a second injection of fresh serum been given before the symptoms had progressed too far.

Now, in this case the poison from one gland only was injected. We can therefore conclude that 15 cubic centimetres of fresh serum would have been quite inadequate to have prevented the onset of symptoms of intoxication and probably would not have prevented death had the patient received the quantity of poison contained in both glands. Shortly after the onset of symptoms 10 cubic centimetres of comparatively fresh serum were injected. This serum was got from Messrs. Phillips and Co. (*vide* Series No. 3 in the protocols). We have seen that one cubic centimetre of this serum was capable of neutralising 0·61 milligramme of cobra poison. The dose which was injected was therefore equivalent to 8·5 cubic centimetres of perfectly fresh serum such as was used in the experiments detailed in Series No. 2 of the protocols. Our patient therefore received in all a quantity of serum equivalent to 16 cubic centimetres of perfectly fresh material. All symptoms in this case, in which the bite was inflicted by only one fang, soon passed off after the second injection of serum. Since, however, the second dose was given after some of the poison had already joined on to the central nervous system it is for *a priori* reasons necessary to conclude that a smaller dose would have sufficed if the whole quantity had been injected before the onset of any symptoms. On the other hand, it must be remembered that the cobra which inflicted this bite had been in captivity for some months and would not consequently yield as much poison as an ordinary snake in nature would do. For is it an observation of common occurrence in this laboratory that a cobra newly caught will yield from 20 to 30 large drops of poison, while after he has been a captive for some time this quantity will have diminished to from six to 10 drops and in time to *nil*.

From what we have stated above it is evident that the question of the dose of serum to be injected in cases of cobra bite turns upon factors which cannot be directly experimentally determined—namely, (1) upon the amount of poison which the snake has injected and (2) upon the amount of cobra venom which an average man can survive. In view of the fact, however, that in the treatment of any

¹³ The serum which we used in these experiments although newly arrived in India had necessarily been subjected for a short time to the heat of the voyage out—viz., in the Red Sea and the Indian Ocean. A slight deterioration may thus have taken place. This, however, could only account for a small proportion of this great difference.

¹⁴ This is the dose prescribed in the printed instructions sent out with every bottle of serum.

¹⁵ *Loc. cit.*

¹⁶ *Brit. Med. Jour.*, 1896, p. 957.

¹⁷ *Le Venin des Serpents*, 1896.

¹⁸ *Loc. cit.*

¹⁹ *Loc. cit.*

²⁰ *Loc. cit.*

²¹ *Loc. cit.*

²² *THE LANCET*, Jan. 5th, 1901, p. 25.

case of cobra-bite we must always estimate the dose of anti-venine to meet the possibility of the snake having injected the maximum quantity possible—i e., about 45 milligrammes—and in view of the fact that man may be as susceptible, weight for weight, as the rat, or perhaps more susceptible, it seems to us, from the considerations put forward above, that a dose of from 30 to 35 cubic centimetres of fresh serum or the equivalent of this should be given in every case of cobra-bite. A larger dose would of course be necessary if symptoms had set in before the treatment was begun. At any rate the history of this case shows that a dose of about 15 cubic centimetres would be quite inadequate in most cases of cobra-bite.

We may now pass on, in conclusion, to consider briefly the question of the deterioration which anti-venomous serum undergoes by keeping in India. The data bearing on this subject are contained in detail in the protocols, Series No. 2 to No. 8 inclusive. These data may be briefly summarised in tabular form as follows:—

TABLE I.—The Deterioration of Anti-venomous Serum by Keeping in India.

Series in protocols.	Age of the serum.	Length of time in India.	Where kept in India.	Neutralising power.	Remarks.
No. 2	Two months.	One month.	Bombay.	1 cubic centimetre neutralised 0·73 milligramme.	This serum may be regarded as perfectly fresh serum.
No. 3	Seven months.	Three months.	Bombay.	1 cubic centimetre neutralised 0·61 milligramme.	—
No. 4	Over three years.	About three years.	Bombay.	1 cubic centimetre neutralised 0·29 milligramme.	—
No. 5	Over three years.	About three years.	Nasik and Bombay.	1 cubic centimetre neutralised 0·46 milligramme.	It is uncertain how long this sample had been in Bombay and how long in Nasik.
No. 6	Four years.	One and a quarter years.	Indore.	1 cubic centimetre neutralised 0·13 milligramme.	—
No. 7	Five years.	Over four years.	Agra.	1 cubic centimetre failed to neutralise 0·14 milligramme.	—
No. 8	Four years.	Two and a quarter years.	Bombay and Berar.	1 cubic centimetre failed to neutralise 0·18 milligramme.	In Bombay six months and in Berar one and three-quarter years.

The protective power ascribed by M. Calmette to all samples of sera sent out from Lille is 20,000 units per 10 cubic centimetres. We may, therefore, assume that all brews when fresh possess practically the same neutralising power (*vide* Footnote 12). A study of the above table shows that a progressive deterioration takes place from keeping the serum in India. Thus it will be noticed on the one hand that while the serum which was seven months old and had been in India for only three months (Series No. 3) already showed a slight diminution in its neutralising power, on the other hand, the sera which had been kept for some years in the Berar (Hingoli and Ellichpur), i.e., Series No. 8 and in Agra, i.e., Series No. 7, had practically lost all neutralising power, and the sera which had been stored at Indore, Bombay, and Nasik had deteriorated to a considerable extent but had not completely lost all neutralising power.

Let us consider for a moment the climatic conditions as regards temperature which prevail at these different places. In the cold season Agra and the Berars have a comparatively low temperature; but for three or four months of the year the heat in these districts is excessive, the maximum temperature in the shade being seldom below 105° F. and often reaching 110° F. and over. Indore also has a severe hot season and a comparatively cool "cold" season, a similar climate in fact to that of Berar, although, perhaps, the maximum temperature does not reach so high as in the other two cases. The great neutralising power of the serum which had been kept at Indore, compared with the samples

kept at Berar and Agra, can be accounted for by the fact that the former sample had been subjected to this great heat for only one hot season, while the sera kept at Berar and Agra had been stored in these districts during two and four hot seasons respectively.

The results obtained with Series No. 4 and Series No. 5 are especially interesting. Both these sera were of the same age and were imported to Bombay at the same time. One sample—viz., No. 5—had been stored for a considerable time at Nasik after Bombay. Now, the temperature of Nasik during the cool season of the year is much lower than is that of Bombay. On the other hand, during the hot months, March to May inclusive, the maximum shade temperature of Nasik is only slightly lower than is that of Bombay, while the minimum temperature is much lower. On the whole, therefore, we may take it that the serum which had been kept at Nasik had not been subjected to nearly such a high mean temperature during the year as had that serum which had been stored in Bombay. In consequence, the neutralising power of the former was more than a half greater than the neutralising power of the latter. We may conclude then from these data that anti-venomous serum undergoes a progressive and fairly rapid deterioration when stored in hot climates, and that this deterioration is greater and more rapid the higher the mean temperature to which it is subjected. The practical bearings of the results of these observations are so patent as to require no further discussion.

PROTOCOLS.

Series No. 1.—The poison used was carefully dried, pure, fresh cobra venom. It was unheated. A 0·1 per cent. solution in sterile normal saline solution was made. Ten-fold and hundred-fold dilutions of the original solution were used when necessary. The weights refer to the original dried venom. All rats were carefully selected of about 118 grammes weight. The injections were made subcutaneously in the inner side of the thigh.

TABLE II.—Experiments to determine the Minimum Lethal Dose of Pure Cobra Venom for a Rat of about 118 grammes.

Animal.	Amount of dried venom in milligrammes.	Result.
Rat 1	0·1	Died within 2 hours.
" 2	0·09	" " "
" 3	0·08	" " "
" 4	0·07	" " 3 "
" 5	0·06	" " "
" 6	0·05	" " "
" 7	0·05	" " "
" 8	0·045	Very ill for 24 hours; died in four days.
" 9	0·04	Died in 3½ hours.
" 10	0·04	" 2½ "
" 11	0·035	Ill for 16 hours; recovered.
" 12	0·035	" 6 " "
" 13	0·03	Slightly ill for 3 hours; recovered.
" 14	0·03	No symptoms.
" 15	0·025	" "
" 16	0·02	Slight symptoms; recovered.
" 17	0·01	No symptoms.
" 18	0·005	" "

From the above table it appears, then, that 0·04 milligramme is a certain lethal dose and that 0·035 milligramme is a dose which a rat of this size can survive—i.e., a maximum non-lethal dose. In the series of experiments No. 2 to No. 8, detailed hereafter, 10 lethal doses—namely, 0·4 milligramme—were mixed *in vitro* with varying amounts of sera of different ages and which had been kept under different conditions of temperature. The mixtures were allowed to stand at laboratory temperature (about 25° C.) for at least half an hour and were then injected subcutaneously in the inner side of the thigh. Carefully selected rats of about 118 grammes were used in all cases. In calculating the amount of venom which the serum had been able to neutralise or had failed to neutralise the maximum non-lethal dose—

namely, 0.035 milligramme—was deducted in all cases. These series are as follows.

Series No. 2.—The serum used in this series of experiments was received from Lille in October, 1900. It was dated Sept. 17th, 1900. The experiments were completed between Nov. 10th and Dec. 5th.

TABLE III.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of Fresh Serum newly imported was capable of neutralising.

Animal.	Amount of dried venom in milligrammes.	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.15	Died in 5 hours,
" 2 ...	0.4	0.2	" 10½ "
" 3 ...	0.4	0.2	" 7½ "
" 4 ...	0.4	0.2	" 2¾ "
" 5 ...	0.4	0.2	Died within 48 hours.
" 6 ...	0.4	0.25	Died in 2¾ hours.
" 7 ...	0.4	0.25	Died within 17 hours.
" 8 ...	0.4	0.25	" " 5 "
" 9 ...	0.4	0.3	Very ill for 48 hours; recovered.
" 10 ...	0.4	0.3	Died in 5 hours.
" 11 ...	0.4	0.3	" 2¾ "
" 12 ...	0.4	0.3	Died within 5 days.
" 13 ...	0.4	0.35	" " 48 hours.
" 14 ...	0.4	0.35	" " " "
" 15 ...	0.4	0.35	Died in 17 hours.
" 16 ...	0.4	0.4	Died within 40 hours.
" 17 ...	0.4	0.4	Ill for 5 days; recovered.
" 18 ...	0.4	0.4	Died within 48 hours.
" 19 ...	0.4	0.45	" " 3 days.
" 20 ...	0.4	0.45	Died after 8 days.
" 21 ...	0.4	0.5	No symptoms.
" 22 ...	0.4	0.5	Ill for 2 days; recovered.
" 23 ...	0.4	0.6	" " "
" 24 ...	0.4	0.7	No symptoms.

From the above series we can conclude that 0.45 cubic centimetre of this serum failed to neutralise with certainty 0.4 — 0.035 milligramme = 0.365 milligramme, while 0.5 cubic centimetre could neutralise this amount. In other words, one cubic centimetre was able to neutralise 0.73 milligramme.

Series No. 3.—The serum used in this series was dated May 8th, 1900. It was purchased from Messrs. Phillips and Co., chemists, in Bombay, who had received it from England about the end of August. The experiments were completed between Dec 5th and 12th. The serum had, therefore, been in Bombay during one of the hottest months of the year—viz., October.

TABLE IV.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of Comparatively Fresh Serum was capable of neutralising.

Animal.	Amount of dried venom in milligrammes.	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.2	Died in 2 hours.
" 2 ...	0.4	0.3	" " "
" 3 ...	0.4	0.4	Died within 42 hours.
" 4 ...	0.4	0.5	" " " "
" 5 ...	0.4	0.5	" " 46 "
" 6 ...	0.4	0.6	Ill for one day; recovered.
" 7 ...	0.4	0.6	No symptoms.
" 8 ...	0.4	0.7	" "
" 9 ...	0.4	0.7	" "
" 10 ...	0.4	0.8	" "

From the above series we can conclude that 0.5 cubic centimetre of this serum failed to neutralise 0.4 — 0.035 milligramme = 0.365 milligramme, while 0.6 cubic centimetre could neutralise this amount at least—namely, that

one cubic centimetre was able to neutralise 0.61 milligramme.

Series No. 4.—This serum had been in the possession of the secretary of the Bombay Natural History Society for at least three years. M. Calmette informed us that it was at least three years old, probably more.

TABLE V.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of a Serum which had been in Bombay for over Three Years was capable of neutralising.

Animal.	Amount of dried venom in Milli-grammes.	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.25	Died in 1½ hours.
" 2 ...	0.4	0.5	" " "
" 3 ...	0.4	0.75	" 3 "
" 4 ...	0.4	0.75	Very ill for 48 hours; recovered.
" 5 ...	0.4	0.85	Died within 36 hours.
" 6 ...	0.4	1.0	Died in 6½ hours.
" 7 ...	0.4	1.0	" 9 "
" 8 ...	0.4	1.0	Ill; recovered.
" 9 ...	0.4	1.15	Died after 3 days.
" 10 ...	0.4	1.25	No symptoms.
" 11 ...	0.4	1.5	" "

From the above series of experiments we can conclude that 1.15 cubic-centimetres of this serum failed to completely neutralise 0.4 — 0.035 milligramme = 0.365 milligramme, while 1.25 cubic-centimetres did neutralise this amount at least. Therefore, one cubic centimetre of this serum was able to neutralise 0.29 milligramme.

Series No. 5.—The serum used in this series of experiments was got from Captain Mitchell of Nasik. It was imported to India at the same time as the serum used in the experiments detailed in Series No. 4. After being stored in Bombay it was sent to Nasik. The exact date when this happened cannot be ascertained as no record was kept of the event. It was received at the laboratory from Nasik in October, 1900. The experiments were completed between Oct. 16th and Nov. 13th.

TABLE VI.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of a Serum of the same age as that used in Series No. 4, but which had been kept for most of the time in a comparatively cool place, was capable of neutralising.

Animal.	Amount of dried venom in milli-grammes.	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.25	Died within 3½ hours.
" 2 ...	0.4	0.25	" " 3 "
" 3 ...	0.4	0.25	" " 7 "
" 4 ...	0.4	0.3	" " 4 "
" 5 ...	0.4	0.35	" " 4½ "
" 6 ...	0.4	0.4	" " 4 "
" 7 ...	0.4	0.45	" " 5 "
" 8 ...	0.4	0.5	" " 14 "
" 9 ...	0.4	0.5	" " 4½ "
" 10 ...	0.4	0.5	Very ill for 24 hours; recovered.
" 11 ...	0.4	0.55	Died within 40 hours.
" 12 ...	0.4	0.6	" " 50 "
" 13 ...	0.4	0.65	" " 54 "
" 14 ...	0.4	0.7	" " 30 "
" 15 ...	0.4	0.7	Ill; recovered.
" 16 ...	0.4	0.75	No symptoms.
" 17 ...	0.4	0.75	Died within 8 days.
" 18 ...	0.4	0.8	No symptoms.
" 19 ...	0.4	1.0	" "
" 20 ...	0.4	1.25	" "
" 21 ...	0.4	1.5	" "

From the above series of experiments we can conclude that 0.75 cubic centimetre of this serum did not completely

neutralise 0.4-0.035 milligramme = 0.365 milligramme, but that 0.8 cubic centimetre was able to do this. It is evident, therefore, that one cubic centimetre of this sample could neutralise 0.46 milligramme.

Series No. 6.—This serum was dated Oct. 17th, 1896. At the time of our experiments it was therefore five years old. It was imported to India in September, 1899. It arrived in Indore the same month and was kept there till November, 1900, when it was sent to Bombay. The experiments were completed between Nov. 27th and Dec. 3rd.

TABLE VII.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of Serum received from Lieutenant-Colonel Gimlette of Indore was able to neutralise.

Animal.	Amount of dried venom in milligrammes	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.5	Died in 2 hours.
" 2 ...	0.4	0.6	" " "
" 3 ...	0.4	0.7	" 1½ "
" 4 ...	0.4	0.8	" " "
" 5 ...	0.4	0.9	" 2 "
" 6 ...	0.4	1.0	" 1½ "
" 7 ...	0.4	1.1	" 20 "
" 8 ...	0.4	1.2	" " "
" 9 ...	0.4	1.3	" 2½ "
" 10 ...	0.4	1.4	" 7½ "
" 11 ...	0.4	1.5	" 19 "
" 12 ...	0.4	1.6	" 90 "
" 13 ...	0.4	1.7	Died within 67 hours.
" 14 ...	0.4	2.0	Ill for 2 days; recovered.

From the above series of experiments we can conclude that two cubic centimetres were the minimum amount of serum which was able to neutralise 0.4-0.035 milligramme = 0.365 milligramme. In other words, one cubic centimetre was able to neutralise 0.18 milligramme.

Series No. 7.—This sample of serum bore the date Sept 17th, 1895. Mr. Hankin informed us that he had kept no record

TABLE VIII.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of Serum received from Mr. Hankin of Agra was able to neutralise.

Animal.	Amount of dried venom in milligrammes.	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.7	Died in 5 hours.
" 2 ...	0.4	0.8	" " "
" 3 ...	0.4	0.9	" 8 "
" 4 ...	0.4	1.0	Died within 48 hours.
" 5 ...	0.4	1.1	Died in 3½ hours.
" 6 ...	0.4	1.2	" 9 "
" 7 ...	0.4	1.25	" 19 "
" 8 ...	0.4	1.3	" 9 "
" 9 ...	0.4	1.4	" " "
" 10 ...	0.4	1.4	" 30 "
" 11 ...	0.4	1.5	No symptoms.
" 12 ...	0.4	1.5	Died within 18 hours.
" 13 ...	0.4	1.5	Died in 38 hours.
" 14 ...	0.4	1.6	Died within 22½ hours.
" 15 ...	0.4	1.6	Died in 18 hours.
" 16 ...	0.4	1.7	Died within 18 hours.
" 17 ...	0.4	1.7	Died in 16½ hours.
" 18 ...	0.4	1.8	Died within 18 hours.
" 19 ...	0.4	1.9	" " "
" 20 ...	0.4	2.0	Died in 24 hours.
" 21 ...	0.4	2.0	Died within 17 hours.
" 22 ...	0.4	2.25	" " "
" 23 ...	0.4	2.5	Died in 46 hours.

of the exact date when he received this sample of serum, but that he had little or no doubt that he received it in the

year in which it was prepared. We may, therefore, take it that this serum had been in Agra for over four years.

From the above series of experiments we can conclude that 2.5 cubic centimetres of this serum could not neutralise 0.4-0.035 milligramme = 0.365 milligramme. It is evident, therefore, that one cubic centimetre could not neutralise even 0.14 milligramme.

Series No. 8.—This serum was dated Nov. 7th, 1896. It was purchased from Lille by Lieutenant Liston, I.M.S., in September, 1898. It was imported to India the following month. For six months it was kept in Bombay, but during the hot season of 1899 and 1900 it was exposed to the great heat of Berar. It was sent to Bombay in December, 1900. The experiments were completed between Dec. 10th and 14th, 1900.

TABLE IX.—Experiments to ascertain the Amount of Venom which One Cubic Centimetre of Serum received from Lieutenant Liston, I.M.S., Ellichpur, Berar, was capable of neutralising.

Animal.	Amount of venom in milligrammes.	Amount of serum in cubic centimetres.	Result.
Rat 1 ...	0.4	0.5	Died within 3½ hours.
" 2 ...	0.4	0.8	" " " "
" 3 ...	0.4	1.0	" " " "
" 4 ...	0.4	1.25	Died in 22 hours.
" 5 ...	0.4	1.5	Died within 42 hours.
" 6 ...	0.4	1.75	" " 50 "
" 7 ...	0.4	1.75	" " 48 "
" 8 ...	0.4	2.0	" " 42 "

From the above series of experiments we can say that two cubic centimetres of this serum failed to neutralise 0.4-0.35 milligramme = 0.365 milligramme—namely, that one cubic centimetre could not neutralise 0.18 milligramme.

THE FEEDING OF DIPHTHERIA PATIENTS, WITH SPECIAL REFERENCE TO CHILDREN AND SEVERE CASES.

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In the treatment of diphtheria the feeding is of great importance, while at the same time it is attended with the greatest difficulty, owing to the inability of many patients to take or to retain nourishment given by the mouth. So much depends upon the nurse that it is essential to obtain the best nursing available. Feeding may be carried out by (1) the mouth; (2) the nasal tube; (3) the rectum; and (4) subcutaneous injections. In all cases, where practicable, feeding should be performed by the mouth. Certain conditions, however, render mouth feeding impossible or inadvisable. Chief amongst these are the following: (1) inability to swallow owing to pain or faucial swelling; (2) some cases of regurgitation, which will be referred to later; (3) entrance of food into the larynx in spite of slow and careful feeding indicated by the patient coughing when fed; (4) in certain cases, owing to struggling, the time taken and the exhaustion consequent on mouth feeding; and (5) continued vomiting—(a) vomiting may be present almost from the onset of diphtheria; (b) early vomiting, commencing about the middle or end of the second week; and (c) late vomiting, which occurs after some weeks of the disease. Generally speaking, vomiting which begins early is of graver import than that which starts late in the disease. It is well to remember that cases occur in which patients can be fed with a nasal tube without vomiting, although they vomit when fed by the mouth. In some this is probably due to distaste for their nourishment.

I.—MOUTH FEEDING.

In the early stages of diphtheria milk should be the chief