TOXINS.*

Their Sources, Preparation and Neutralisation

By

Dr. Chaloem Purananda.**

Introduction

Mr. Chairman, ladies and gentlemen, the subject to be dealt with to-day may sound Greek to some of you, but in reality everybody has more or less been in contact with it. You were vaccinated against small-pox when a few months old; caught cold during the wet season; some one may have an accident with poisonous animals, be stung by beetles or be bitten by snakes; all these you have to suffer and the cause of your suffering is the Toxin. I will tell you what toxins are, how they make you suffer, and show you the way to fight and overpower them. Toxins may be derived from different sources, still they possess their properties in common. The present knowledge of toxin is the result of work done for centuries by research workers and scientists; yet we need more understanding of the subject.

In descriptive natural sciences, the morphological characteristics are the criteria for their classification. Recently it has been recognized that there are present in the living organisms peculiar biochemical substances which vary with the variation of structure. This discovery was made indirectly, not as the result of studies made with that aim in view.

When one has recovered from an infectious disease there remains in that body an immunity for that particular illness, which may last a certain time depending on the types of the infection. Small-pox, when once it attacks a human being, will leave in the body a lifetime immunity, while in such maladies, as typhoid fever and diphtheria, the body will be only temporarily protected. The search for this remarkable explanation led to the discovery of a peculiar sort of substances in the serum, the socalled Antibodies which protect against infectious agents (bacteria and virus). These substances,

---

* Lecture delivered July 26th, 1940, before the Natural History Section.

** Chief of Research Section, Science Section of the Thai Red Cross Society, Bangkok.

apparently proteins in nature, are formed not only as the result of infections, but also in consequence of the administration of certain complex poisons or dead bacilli. Substances inciting the formation of and reacting with antibodies are termed Antigens; poisons inciting the formation of neutralizing antibodies are called TOXINS.

Definition:—Toxin is a complex and soluble poison produced by the action of plastids in microbes, higher plants and animals, which are termed bacterial toxin, phytotoxin and zootoxin respectively.

It is due to the toxins of pathogenic microorganisms to which we attribute nowadays the general accidents and the lesions of infectious diseases.

Toxins do not act indifferently on every anatomical element of the body of the infected hosts; most of them have selective action, for example tetanus toxin on the nervous system.

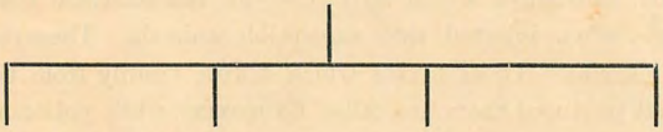
Substances of the same nature are elaborated by vegetables: the phytotoxins, and by animals: the zootoxins. Among the phytotoxins those can be cited as examples are *ricin*, in the grains of Ricinus communis, *abrin* in the grains of Abrus precatorius, and *crotin* in the grains of Croton tiglium. In the animal kingdom one finds venoms of snakes, scorpions, bees and fish. All these poisons are those little understood substances known as TOXINS; they are characterized by provoking in the animal body, when injected, a combating substance: the Anti-body, by which the system seeks to resist them so that the body can be accustomed to increasing quantities of the poison. The immune antibodies all have in common the property of specificity, that is they react as a rule only with the antigens or toxins that were used for immunizing or with similar ones. (See table page 191).

Of this subject of toxins from the bacterial, plant and animal kingdoms, I have to confine my speech to those which I am working with, that is bacterial and animal (snake) toxins.

BACTERIAL TOXINS.

There is no apparent reason why the presence in the body of any reasonable number of cells of an order of the size expressed in low multiples of a micron should produce any harmful effect on the host, that harbours them. The basis of all harmful effects of bac-

SOURCES OF TOXIN



BACTERIA	VIRUSES	PLANTS	ANIMALS
e.g.	e.g.	e.g.	e.g.
DIPHTHERIA	SMALLPOX	CROTON TIGLIUM	SNAKES
toxin, toxone		crotin	neurotoxin, hemorrhagin
TETANUS		RICINUS COMMUNIS	FISHES
tetanospasmin, tetanolysin		ricin	weevers
ANTHRAX		ABRUS PRECATORIUS	INSECTS
endotoxin		abrin	scorpions, bees

terial infection is quite certainly chemical; and only when the chemist has replaced the immunologist shall we be able to give an intellectually satisfying account of what happens when a particular germ invades a particular host. In the meantime we must be contented with the incomplete data at our disposal.

It has long been known that certain bacteria produce highly poisonous substances which give rise to characteristic lesions or symptoms when injected into susceptible animals. These are the bacterial toxins. Those toxins which diffuse readily from the bacteria that produced them, are called EXOTOXINS, while poisonous substances which remain attached to the bacteria are know as ENDOTOXINS.

NATURE OF TOXINS.

Soluble toxins and endotoxins form the complex mixtures which bacteria produce in the organisms, in the common nutritive media, and in the specially prepared media.

They are recognized by certain of their characters, being in colloidal condition: filtrable resistance to dialysis and heat, absorbability action of the infinitesimal or extremely weak dose, formation of specific antibody in the animals. In general we admit that they are produced in the protoplasm of bacteria where they diffuse more or less easily.

As to the formation toxins are likened to proteins and lipoproteins. We cannot obtain toxins in a pure sample and the exact constituent rest is unknown. Some toxins possess many toxic substances which by themselves have different actions on the organisms; for example in Tetanus Toxin one finds tetanolysin which has hemolytic properties, and tetanospasmine which has the action on nerve cells, resulting in spasmodic contraction of muscles.

TOXINOGENESIS.

The formation of toxins in artificial media varies with the species of microbe and the chemical composition of the media, their reaction and the surrounding condition of the culture.

Of the same bacteria, we may come across the strain of less toxicity, strains of hypertoxicity and those between the two. This fact will be very well demonstrated by the diphtheria bacillus; certain strains produce almost no toxic substance, on the other hand such a

strain as that of Park-William possesses very high toxicity. There is an oscillation of toxicity in each strain during their passage in the culture media.

The nutritive media, which are most favourable for the production of toxins, are different for each microbe. A laboratory for the preparation of media used for bacterial culture can be compared to a restaurant or a pastryshop. Mineral salts are very important, a comparatively high dose conceded to favour the growth of the germ. Bacillus diphtheria always requires some phosphate, while bacillus tetani requires chloride.

Ordinarily the reaction of media should remain alkaline, but in case the alkalinity becomes too strong the toxicity of the culture diminishes. Martin broth, which is used for the culture of bacillus diphtheria, adjusted between p.H. 7. 5 and 8. 2. gives a very regular result. Beyond p.H. 8. 6 it was found that toxin does not form, and with a p.H. of 5. 8. to 6. 1 the filtered culture kills a guinea-pig at a dilution of 1 : 10 cc. instead of 1 : 700. Contrary to this idea, the p.H. of 6. 7, or slightly acid, is more favourable to the production of tetanustoxin than an alkaline medium.

In general the temperature for the function of toxinogenesis is 37°C and about.

Aeration plays an important part, with regard to the microbes which develop abundantly on the surface of the media (bacillus diphtheria, vibrion cholera, etc.); on the other hand anaerobic bacteria, (bacillus tetani, vibrion septique, etc.), do not produce their poison in the presence of oxygen.

To put this in short and easy terms : each species of bacteria grows and produces toxin in a specially prepared medium suited for its own species and each species has its own likings.

In our study of toxins, we have to be content with crude products which are filtrates, autolysates or extracts, which certainly contain a multitude of substances besides those which we desire to investigate ; as we are yet unable to obtain toxin in a pure state we try to get it in the highest toxic condition and this depends on the strain of the germs, the media that keep them growing and the surrounding conditions : temperature, oxygen, etc.

Toxins are soluble, noncrystallizable, do not dialyze except

through very thin membranes. Many are very labile, being sensitive to light, free oxygen or oxidizing agents and to heat, being destroyed by heating to 60°C for 30 minutes. It is believed that the toxin molecule is composed of two essential parts, one that causes toxic effects and the other has an affinity for the cell receptor. The deterioration of toxin under ordinary conditions of storage was primarily due to the deterioration of the toxic part. Apart from the deterioration by nature toxins can also be rendered atoxic by adding chemicals, e. g. iodine, formaline, etc.

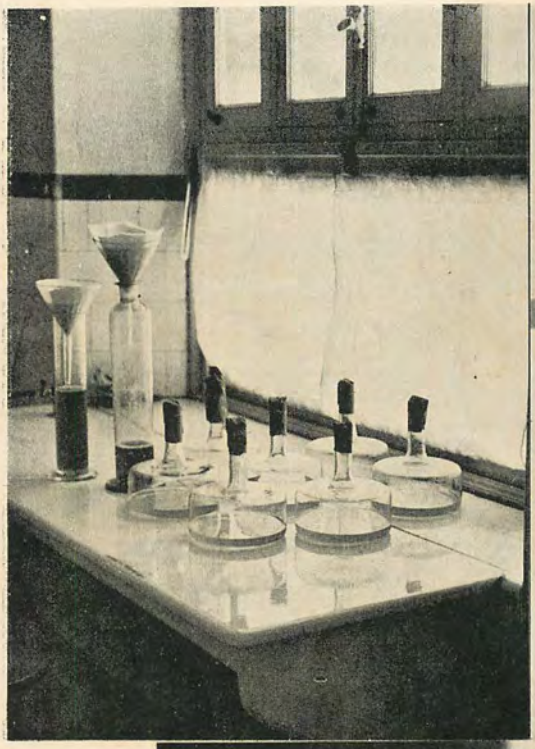
A typical exotoxin is readily separated from bacterial culture by filtration through a porcelain candle. It produces easily recognizable symptoms during life, or damages which can be detected after death, and usually is fatal in a very small dose. Exotoxins can be distiguished from one another by their pharmacological actions.

A typical endotoxin is not liberated into the fluid medium in which the microbe, producing it, is growing, and thus cannot be separated from the bacterial cells by filtration. The usual method of preparing a solution or suspension of an endotoxin is to break up the bacterial cells by prolonged grinding, by alternate freezing and thawing, or by merely allowing them to autolyse in fluid medium. To test its toxicity one usually injects the bacterial cells into the animal; in this case it is assumed that the liberation of toxin is brought about by the lysis of the cells inside the body of the experimental animal. But it does not produce any marked damage; thus the endotoxin does not possess the pharmacological actions. It needs a large dose of endotoxin to kill an animal.

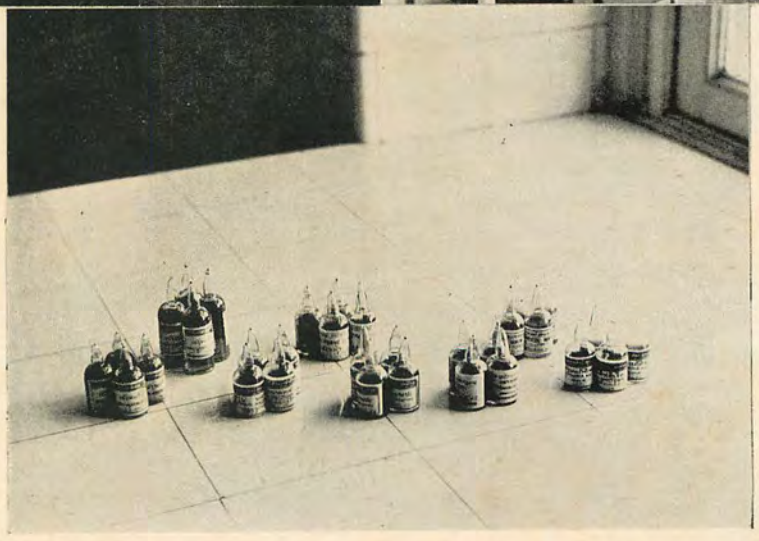
As the result of our experiments on the production of diphtheria toxin, Martin broth is used as culture media with 0.2% glucose and 1% sodium acetate; the p.H. is set at 8. 2; the culture, kept in the incubator, after twelve days, yields a toxin; the minimal lethal dose reaches 1/800 of a cc. (M. L. D. = 1/800).

Toxins are liable to deteriorate easily; we therefore have to keep them in a condition which is free from heat, light and chemicals. We are now able to concentrate and dry them and keep them as a powder; by this method the toxins retain their toxicity indefinitely. For those toxins in daily use we usually keep them in an amber bottle at 4°C; since we use it every other day it does not change

First Stage of Filtrations of Toxin with Filter Paper.



Second or Last Stage of Filtration of Toxin with Porcelain Candle.



Samples of Sera produced by the Science Section of Thai Red Cross Society.

much. It is from toxin that we prepare antitoxin, for neutralizing toxin; and toxoid, for the preventive treatment and the preparation of antitoxin. The process of their preparation will be mentioned later together with the animal antitoxin.

Animal Toxins.

With regard to the zootoxins the most important in Thailand is the snake venom. The venoms of bee, spider, scorpion, miriapede and fish are of less significance. We have treated few cases of poisoning a year and besides they are not very dangerous.

In nonpoisonous as well as poisonous snakes there are present glands, capable of secreting venom; in the former category the secretion is just for the purpose of enabling them to digest their preys, while the venom is able to kill their victims and serves as a means of defence.

The gland of a poisonous snake is situated behind the eye on each side of the skull; it may attain a size of a large almond. Each gland is surrounded by a thick capsule of fibrous tissue with two prolongations, inserted beneath the masseter muscle. When the snake closes its jaws to bite the gland is forcibly compressed and the contained fluid is squeezed through the excretory duct which has an opening in the fang. The quantity of venom secreted each time varies greatly according to the length of time which has elapsed since its last meal and its previous bite.

Freshly collected venom is a syrup-like fluid, pale yellow in colour and clear in consistency; if it has opalescence, white pus-like in character, it means that there is an infection in the mouth of the snake. It can be dried in the desiccator with calcium chloride and appears in cracked translucent lamellae of the same colour. When a venom is dried it leaves about 30 to 45% of its original weight.

Venom is collected from living or freshly killed snakes. At our Institute we apply the former process, the technician will catch the snake by its head in one hand and hold a sterile watch glass in another. By pushing the edge of the watch glass into the snake's mouth, the snake is stimulated to bite the watch glass; at this very moment the thumb and the fore-finger which catch hold of the head of the snake, are pressed on the gland on both sides, helping the flow

of the venom into the recipient. This collected venom is then dried as mentioned previously. The dried venom can be kept indefinitely, if protected from light, air and moisture. It dissolves readily in water ; when we want to use it in an experiment we have to grind the dry venom in large quantity so that we obtain a homogenous powder and enough for the whole experiment. This is then dissolved in normal saline solution; the quantity dissolved should not exceed much the quantity required as it deteriorates easier in form of solution.

All these stock toxins, bacterial and animal, are to be tested for their toxicity before use. In case of diphtheria toxin we use guinea-pigs, weighing 250 grammes, to find out the smallest dose or toxin that will, by subcutaneous injection, kill the guinea-pig after 96 hours and we call this the minimal lethal dose (M. L. D.). If the M. L. D. is low then the toxicity is high and vice versa. The M. L. D. figure thus represents the measurement of the toxicity of the toxin. The same process is practised for tetanus toxin with the difference that we employ a bigger guinea-pig 300 to 350 grammes and the injection is intramuscular. There is another method of determining the toxicity of a toxin by injecting intracutaneously on the shaved skin of a rabbit or guinea-pig, and reading the reaction on the site of the inoculation.

As for the snake venoms, various animals are used, white mouse, guinea-pig, pigeon and rabbit, depending upon the facility to obtain the animals. In South America they use pigeon; in France rabbit, in Denmark and Yugoslavia white mouse ; here at our Institute we use the white mouse and rabbit. The results which I worked out on various kinds of venom, using different animals and with the variation of mode injection, are tabulated on the next page.

Minimum Lethal Dose (M. L. D.).

Venom of		White mouse 18-25 grammes. Intravenously	Guinea-pig 400-500 grammes.	Rabbit Intravenously mgm./1000 gms.
		mgm.	mgm.	mgm.
Paris.	V. Aspis	—	—	0.2
	V. Russelli	0.02	0.06	0.4
Copenhagen				
	V. Aspis	0.015	—	—
	Ammodytes	0.02	—	—
	King Cobra	0.011	—	0.55
	Cobra	0.0025	—	0.06
Bangkok	Banded Krait	0.018	—	0.9
	Common Krait	0.014	—	—
	V. Russelli	0.007	—	0.35
	Ngu Kapa	0.0035	—	0.08

It was mentioned in the beginning of this paper that, when one has recovered from a contagious disease, one is generally protected from that illness; the subject is said to be immune to the disease, This protective state of the body is acquired at its own expense, by the reaction created in the body after the fight with the infective agent during the period of illness; whence comes the term acquired immunity after the previous infection. From this fact, Jenner realized his vaccination against small-pox in 1798; Pasteur in 1885 discovered the preventive injection against rabies. In 1877 Reynaud injected a new born heifer with the blood of a cow, infected with cow-pox, and found that the heifer had become immune to cow-pox. In 1888 Richet and Héricourt reported that a rabbit was protected against staphylococcus infection after being inoculated with the blood of the dog having pustules. The same authors then experimented with the injection of blood from a tuberculous dog into a rabbit, but this experiment did not give any practical result.

The discovery of Roux and Yersin of diphtheria toxin enabled Behring in 1889 to discover the serum used for the neutralization of diphtheria toxin. He demonstrated that the serum of an animal, injected with the products from a culture of B. diphtheria, possesses the specific antidiphtheretic properties. In 1890 Behring and Kitasato

were able to demonstrate the same phenomenon with the serum of animals vaccinated with tetanus toxin. At Saigon Calmette in 1892 succeeded in preparing serum against the bite of snakes. Marchoux obtained a serum against streptococcal infection in the year 1894.

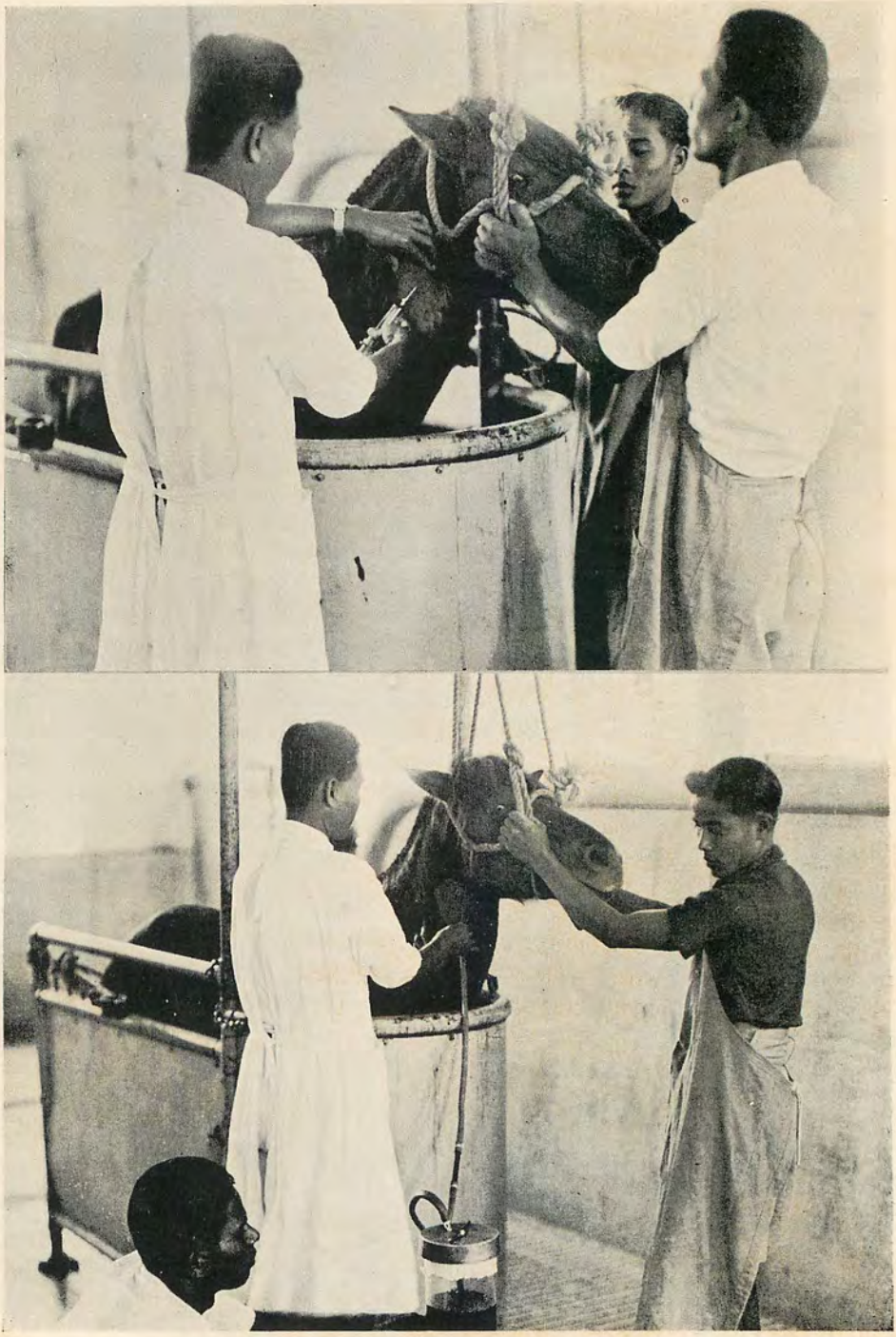
The practical work of producing therapeutic sera is based on researches of the process of immunity, that are being pursued in laboratories in many countries, and the method of production is constantly undergoing improvement. In the early days of the manufacture of diphtheria antitoxin, difficulties were encountered in obtaining a regular supply of uniformly strong toxin from successive lots of broth cultures; this difficulty is still a problem at present if we are not careful enough during the process of culturing the bacteria.

We start the preparation of antitoxin by injection of strong and uniform toxins (tetanus, diphtheria or snake venom) into animals. preferably horses, (the donkey is also used by some laboratories): with increasing doses at reasonable intervals. (Plate II). After a certain time, when the animal has received a good quantity of toxin, we bleed the animal about 50 cc. (Plate II). This is the test bleeding from which serum is separated and tested for its neutralizing property against the toxin injected. If it possesses the neutralizing property up to standard, we bleed the animal in large quantity, say from 4 to 8 litres, the clot is then separated, the serum preserved, filtered and put in ampoules ready for use. (See plate 1).

The estimation of the strength of serum known as titration is the most important step in the preparation of antitoxin. Each kind of therapeutic sera or antitoxin is titrated differently and each laboratory has its own method. Recently under the auspices, of the Permanent Commission of Health Organization of the League of Nations a union of serologists from different countries was formed, with a view to co-ordinating and improving the method of standardization of therapeutic sera. Since then the antitoxin is issued with its strength expressed in International Units (I. U.), and the method of titration of each serum is the same in every laboratory. In this process of standardization one must get the standard toxin and antitoxin from laboratories named by the Health Organization of the League of Nations.

The therapeutic sera prepared at our Institute are standardized

Immunizing Horse with Toxin.



Bleeding of Immunized Horse.

with standard toxins and antitoxins received from the State Serum Institute, Copenhagen, which is the laboratory that prepares the standard samples used all over the world. In most countries a Government Control Department exists, which regulates the standard of purity and potency to which therapeutic sera must conform before they are released for use. Especially in those countries where a large amount of therapeutic sera is needed, there are many commercial laboratories preparing them for exportation and local use. In the United States of America the supervising authorities have their Headquarters in the National Institute of Health, Washington. In the United Kingdom, the co-operative body of the Medical Research Council and the Ministry of Health exercise this control. In Denmark, the State Serum Institute occupies itself with this work. I hear that the Government of Thailand is going to put this control in action very soon.

The therapeutic use of diphtheria antitoxin gives a very good result. It has saved a great number of lives and improved a lot of sufferings. For tetanus antitoxin we obtained a positive result in the prophylactic treatment, but as a specific therapeutic application it rests on a much less solid basis. However, it was agreed that soon after the manifestations of tetanus are observed, a large dose of specific serum should be given to flood the various systems of the body with antitoxin, with the aim to neutralize any toxin circulating in the body. In the treatment of snake bite cases with specific antivenin sera, early administration and a sufficient dose are to be considered. As the result of our experience the mortality is zero, if the patient receives the sera in time. As a conclusion, the specific therapeutic use of antivenin sera gives a hundred per cent. cure. (see Plate III).

I cannot terminate my speech without a word on Toxoid, which is modified toxin or anatoxin. The fact that toxin deteriorates by nature after storage under ordinary conditions led to the investigation by immunologists of many countries and at last they found out that only the toxic part of the toxin loses its action, but the power of producing antibodies in the host is still intact. Toxoid is prepared by adding certain chemicals, iodine or formalin to strong toxin and keeping it in the incubator for a period of time ; it should be tested for its toxicity once a week. After a certain time this modified toxin is no more toxic to susceptible animals. The last step is to titrate the

toxoid using standard antitoxin. This toxoid is to be used for the preventive inoculation of the specific disease and is also used in the immunization of animals in order to obtain therapeutic serum.

Special properties of Zootoxin.

Though animal toxin or venoms are usually fatal to susceptible animals at infinitesimal doses, yet we are able to make use of them for therapeutic purposes. Bee venom was used in old days as a relief in case of rheumatic arthritis. Modern chemists found out that there is histamine in the bee venom, and now they collect bee venom and sell it in ampoules ready for injection against rheumatism.

Viper venom is prescribed by surgeons as a haemostatic agent and cobra venom is also used to ameliorate pain in cancer.

Summary and Conclusion.

(I) Toxins, little known substance of protein in nature, formed from the result of catabolism in bacteria, some plants and animals, are capable of causing diseases and sufferings.

(II) The discovery of toxin was made indirectly by the fact that it leaves a specific resisting substance (antibody), and renders the infected body unyielding to that toxin (immunity).

(III) Toxin plays a part in the classification in natural science.

(IV) Toxin can be obtained, not in a pure state, but sufficient for study, from which we are able to produce antitoxin, for therapeutic use against that toxin, and toxoid for prophylaxis and immunization.

(V) Toxin, in infinitesimal doses, can be used for nonspecific therapeutic purposes.

(VI) Toxins are very noxious substances; yet we are able to make use of them directly and indirectly. From this I am inclined to think that there is nothing in the world which is not useful to human beings if one knows how to work it out. This knowledge requires not only regular and tedious work but also a right tract to follow.

References.

1. L. Nattan—Larrier—Traité de Microbiologie.
2. Cours de l'Institut Pasteur, Paris, 1935-1936.
3. F. Bezançon—Precis de Microbiologie clinique.
4. A. Calmette—Les Venins.
5. Annales de l'Institut Pasteur, Tome LVII, 1936.
6. Annales de l'Institut Pasteur, Tome LXII, 1939.

Amount of Antivenine Serum produced in each year,
B. E. 2468-2482 (1925-26 to 1939-40).

Number of patients treated at the Institute in each year,
B. E. 2460-2482 (1917-18 to 1939-40).

7. Topley & Wilson—The Principles of Bacteriology and Immunity.
8. Park & Williams—Pathogenic Microorganisms.
9. A. Besredka—Études sur l'Immunité.
10. Topley—Outline of Immunity.
11. Fleming & Petrie—Recent advances in Vaccine & Serum Therapy.
12. Landsteiner—Specificity of Serological Reactions.
13. I. H. Burkill—A Dictionary of The Economic Products of the Malay Peninsula.