ESTIMATION OF SOME OF THE AMINO-ACIDS IN COBRA (*NAJA NAJA*) NEUROTOXIN

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A highly toxic substance which paralyses the respiratory movements but has no action on heart, has been isolated form cobra venom. The diamino-acids contained in this active principle has been estimated. It has been found that the composition of this active principle is similar to that of thymus histone.

It has been reported in a previous communication by Ghosh and De (*Indian J. Med. Res*, 1938, 25, 784) that the iso-electric point of the cobra-neurotoxin lies above *p*H 8·0. It may be inferred from this observation that the neurotoxin has got basic properties and probably belongs to the class of proteins known as histones or protamines. Since the basic properties of the histones and protamines have been traced to the relatively high proportion of the diamino-acids contained in their molecules, an estimation of these amino-acids in the cobra-neurotoxin may lead to valuable information regarding its chemical nature. With this object in view, the neurotoxin was separated from the various constituents such as trypsin, haemolysin, choline esterase, etc. which are present in the crude cobra venom. The preparation which we finally obtained was weight for weight, about 15 times more toxic than the crude venom with which we started. The toxicity of this neurotoxin could not be further increased either by fractional precipitation with electrolytes or by cataphoretic experiments in a multi-chambered cell. This neurotoxin produces stoppage of respiratory movements, but it has no action on heart. It will be referred to as neurotoxin (R). The diamino-acids of the crude cobra venom were also estimated for the purpose of comparison, as it would indicate how the composition changed with the increase in purity of the neurotoxin. The results are recorded in this paper. The sample of crude venom used in this experiment was highly toxic and its m.l.d. for pigeons (300. g.) was 0·095 mg.

Experimental

Isolation of the Neurotoxin (R).—The crude cobra venom contains, besides the neurotoxin, other active principles like the haemolysin and also a considerable amount of inactive proteins. It was found by experiment that the major portion of these latter substances could be removed by fractional precipitation with sodium sulphate. A stock solution of sodium sulphate was prepared for this purpose using 44g. of anhydrous sodium sulphate per 100 c.c. of water at 37° and the precipitation was carried out in two steps using different concentrations of sodium sulphate. In the first step (*a*), 300 c.c. of a 0·5% solution of the cobra venom were added with constant stirring to an equal volume of the sodium sulphate and the mixtute was incubated at 37° for 30 minutes. It was then filtered, the precipitate dissolved in 150 c.c. of water and reprecipitated by the addition of 150 c.c. of the sodium sulphate solution. This process was repeated once more and the different filtrates were combined. The combined filtrate was found to contain more than 80%

of the neurotoxin. The next step (*b*) consisted of further fractionation of this combined filtrate. The volume of the filtrate was measured and for every 200 c.c., 100 c.c. of the sodium sulphate solution were added with constant stirring. The mixture was left in a thermostat at 37° for 30 minutes and then filtered. The precipitate was disolved in 200 c.c. of water and then treated with 420 c.c. of sodium sulphate solution. After incubarion at 37° for 30 minutes, it was filtered. The filtrates were combined and this mixture was found to contain about 55% neurotoxin.

The volume of the combined filtrate was measured and to every 100 c.c. of the filtrate 3·3 c.c. of 2/3 *N*-sulphuric acid and 3·3 c.c. of 10% solution of sodium tungstate were added with constant stirring. The mixture was left at room temperature for about 10 to 15 minutes and then centrifuged. The supernatant liquid was decanted off and the precipitate washed twice by centrifugalisation using each time 30 c.c. of an aqueous solution of sodium tungstate and sulphuric acid, in the same proportion in which they were used previously. After the washing was completed, the precipitate was suspended in 30 c.c. of water and *N*/5-sodium hydroxide solution was added to it drop by drop with repeated stirring until the solution became alkaline (p_H 8·6 to 8·8). To this solution was then added barium chloride in slight excess to remove the tungstic acid as insoluble barium tungstate. The mixture was centrifuged and the supernatant liquid containing the neurotoxin was withdrawn. It was neutralised with dilute sulphuric acid solution and any barium ions contained in the solution were removed by treatment with just sufficient quantity of dilute sodium sulphate solution. It was filtered, the filtrate cooled in an ice-chest and then treated with three times its volume of ice-cold methyl alcohol when a precipitate was formed containing the neurotoxin. The mixture was centrifuged, the supernatant liquid was decanted off and the precipitate dried in a vacuum desiccator.

The dried substance was dissolved in water, cooled to about 2° and subjected to fractional precipitation between p_H 1·8 and p_H 2·0, with ice-cold methyl alcohol. The experiments were carried out at a low temperature in order to avoid destruction of the neurotoxin owing to denaturation. It was noticed that when a volume of alcohol equal to 1·8 times that of the aqueous solution was used, a precipitate was formed which had the highest toxicity per unit weight. The precipitate obtained under the above condition was washed several times by centrifugalisation, using a mixture of methyl alcohol and water in the proportion of two volumes of alcohol to one volume of water. Finally it was washed with absolute methyl alcohol and dried in a vacuum desiccator. This dried substance will be referred to as neurotoxin (R). The minimum lethal dose for pigeons of this neurotoxin (R) was found to be 0·0064 mg. only.

Hydrolysis of the Neurotoxin.

The separation and estimation of the basic amino-acids were carried out by the method of Kossell and Kutscher (Kossell, "The Protamines and Histones", 1927, p. 4, ; *Physiol. Chem.*, 1900, 31, 165) A weighed quantity of the material not exceeding 0·5g. was digested with 10 c.c. of 30% (by weight) sulphuric acid in a conical

flask fitted with a reflux condenser and the flask was placed in an oil-bath maintained at a temperature of about 130° to 140°. The digestion was continued for 18 hours, as preliminary experiments showed that this period was required for completing the hydrolysis. After cooling, the volume was made up to 50 c.c. and 40 c.c. of it were used for the estimation of the diamino-acids and 5 c.c. for the estimation of total amino-acid nitrogen after the removal of the humin matter by centrifuging.

Estimation of Histidine, Arginine and Lysine.

40 C.c. of the hydrolysate were neutralised with concentrated baryta and again filtered. The combined filtrate was evaporated to 20 c.c. and divided into two equal parts each of which was separately analysed for its histidine, arginine and lysine content by the method of Kossel and Kutscher (*loc. cit.*). The results are recorded in Table I.

TABLE I.

Substance.	Nitrogen as per cent of total nitrogen.		
	Arginine.	Histidine.	Lysine.
Dry crude cobra venom	7·7	4·1	14·2
Purified cobra neurotoxin	28·5	7·3	5·2
Thymus histone	27·1	5·8	8·04
Histopeptone from thymus	28·4	2·9	12·1

DISCUSSION.

It will be noticed from the data recorded in Table I that the neurotoxin (R) which we have isolated from cobra venom (*Naja Naja*) is similar to thymus histone, in so far as its composition with respect to arginine, histidine and lysine is concerned. It will also be noticed that while the neurotoxin is rich in arginine the crude cobra venom is rich in lysine. It is, therefore, worth investigating which of the active principles of cobra venom is rich in lysine. Further work on this line is in progress.

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