All the morphologically pneumococcal cultures from the same plate were mixed, about 5 c.cm. were removed to a sterile test tube and the rest was killed in the water-bath at 56°C. for 30 minutes. The 5 c.cm. quantity was then tested for bile solubility. In the beginning the reagent employed was 10 per cent sodium taurocholate adding 5 drops of it to 5 c.cm. of culture, but it was unsatisfactory often producing only a slight turbidity, even in some of those cases where the organism was subsequently proved to be pneumococcus by the serological methods. Later, a 10 per cent sodium desoxycholate was substituted with entirely satisfactory results. Use of Hartley's broth is preferable to serum broth in the execution of this test.

Next, the agglutination test following Dreyer's method was done with the killed culture, incubating the stands for two hours at 56°C. and reading the results on the following morning, although in most of the cases rapid agglutination took place as in the case of flagellar agglutination. The results were entered parallel with those of capsular reaction, inulin fermentation, and bile solubility (*see* table).

In the above series only those cases where clumping took place in a dilution of 1 in 80 or more have been included. The blank in the 'remarks' column represents cases of lobar pneumonia.

It was noted that the method of dissolving the organism with bile salt does not interfere with its antigenic property; for when the homologous serum was added to the clear solution a thick precipitate was formed while the control remained unaffected. In the case of empyema fluid, or cerebro-spinal fluid, the supernatant fluid from a centrifugalized specimen also produced in low dilutions the precipitation phenomenon on the addition of the specific serum.

The table brings out the fact that there was agreement between the capsular reaction and the agglutination reaction in 58 out of 60 cases. In specimen 61 the smear did not show the presence of pneumococcus while the culture was positive, and in specimen 70 there was disagreement between these two reactions. About 75 per cent of cases are due to the first and the second types. The table also reveals a close correlation between the capsular and the agglutination reactions. Therefore, it would appear that the method of direct typing is adequate, at any rate, in those positive cases of capsular reaction and to proceed further to mouse inoculation or culture for confirming the type for therapeutic purposes would seem to be unnecessary. Only when the organism is very scanty in the specimen does enrichment by mouse inoculation become necessary.

Conclusion

Direct typing of pneumococcus from the sputum or other material is, with rare exceptions, as reliable as the agglutination reaction. The addition of Löffler's methylene blue to the reagents in the slide method does not interfere with the capsular reaction and is advantageous.

(*Concluded at foot of next column*)

(*Continued from previous column*)

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IN VIVO ACTION OF SOME SUBSTANCES ON THE PROTEOLYTIC SYSTEM IN BLOOD

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THE possibility of the changes in the proteolytic system that may be brought about by certain therapeutic substances has been studied in the present paper. The changes in the trypsin content of blood in certain pathological conditions have been reported by Iyengar *et al.* (1942). From these experiments it was considered likely that changes in the trypsin content may be reliable index of changes in nitrogen metabolism. The accumulation of the proteolytic enzymes in blood in certain diseases like nephritis, and its significant reduction in other diseases like cancer and anæmia, etc., were particularly observed. If such abnormal changes in an important constituent of blood take place as a result of the specific pathological condition, it should be the endeavour of the biochemist to investigate the action of drugs that may be able to counteract this tendency. It is, however, not suggested that the rectification of this particular abnormality will cure the specific disease. A study of this kind may help the clinician to give a fair therapeutic trial to those drugs which may be found to have an effect on the proteolytic system opposite to the one brought about by the specific pathological condition. The present study has, therefore, been undertaken with this object, as well as to throw light on the complex proteolytic system reported to be present in blood.

The use of cobra venom in the therapy of cancer has been recommended by Calmette *et al.* (1933) and largely extended by Chopra and Chowhan (1935) and his collaborators. They

report that in most cases the patient is considerably relieved of the pain and this they ascribe to the neurotoxin that is reported to be present in cobra venom. The other explanation is that the venom contains a proteolytic enzyme which acts on the tumour tissue, thus bringing about an improvement in the condition of the patient. This is rather untenable on account of the fact that the amount of trypsin present in the cobra venom is too small to exert any lytic effect, when injected in the doses recommended by the above authors.

The protease in cobra venom has been investigated by Iyengar *et al.* (1938) and found to be a trypsin which is not highly active at a pH near neutrality. The *in vivo* action of the venom on the proteolytic system of blood has not been reported. In view of the significant decrease of the trypsin content of blood in cancer, the rationale of the treatment of this malignant disease by cobra venom may be explained in the light of the results of such a study. Further, Chopra and his collaborators (1937) have reported that the clotting time of blood from a monkey is accelerated by the injection of small doses of cobra venom and is inhibited when the dose of the venom exceeds a particular limit. No satisfactory explanation based on any experimental evidence is offered by them for this rather interesting observation. They merely visualize the possibility of digestion of fibrinogen by the large dose of cobra venom. In view of the rôle played by trypsin in the process of coagulation, it occurred to us that *in vivo* changes in plasma trypsin might be brought about by the injection of the venom.

Current evidence indicates that the oxidation-reduction state of the medium probably plays a determining rôle with respect to the direction in which proteolytic enzymes (particularly cathepsin) act. Bersin and Logemann (1933) investigated enzymic proteolysis as influenced by oxidizing and reducing agents. They observed that mild reducing agents activate proteolysis, whereas oxidants, such as hydrogen peroxide and benzo-quinone, inhibit the reaction. Excessive oxidation or reduction destroys the enzyme itself. Reiss (1938, 1939), who studied this problem in its physico-chemical relation, finds that at a pH near 7.4 the proteolytic activity decreases sharply in a medium where the potential is more positive than + 50 millivolts or more negative than — 100 millivolts. With lower pH value (4.9) there is a shift to a more positive range between + 500 millivolts and + 50 millivolts.

In the light of this interesting phenomena, it was considered exceedingly interesting to study the *in vivo* action of substances like vitamin K. The study of the action of vitamin K in this respect is of particular interest, since it plays an important rôle in the synthesis of prothrombin which is a globulin protein. One of the suggested mechanisms of prothrombin formation is that vitamin K activates protein synthesis, by tending to inhibit proteolysis. There is, however, no suggestion that vitamin K promotes the synthesis of any other protein except prothrombin. Its action in synthesizing prothrombin should be regarded as remarkably specific.

The *in vivo* action of various doses of 2–methyl–1 : 4 naphthaquinone (synthetic vitamin K) is reported in the present communication.

Material and methods

Cobra venom.—This was obtained from the School of Tropical Medicine through Capt. J. S. Chowhan.

2–methyl–1 : 4 naphthaquinone.—This was prepared by us in the laboratory by the oxidation of 2–methyl naphthalene. The crystals were dissolved in olive oil under sterile conditions before using for injections.

Determinations of free plasma trypsin and total plasma trypsin were made according to the method described in our previous communications (Iyengar *et al.*, 1942).

Determination of trypsin content in red blood corpuscles.—The blood corpuscles are first washed with normal saline and centrifuged twice. Two c.cm. of the washed corpuscles are laked with distilled water and the solution is then precipitated with 4 volumes of acetone. The precipitate obtained after centrifuging is washed well with acetone. The tryptic activity of this precipitate is determined as above.

Determination of catheptic activity of serum.—Two c.cm. serum are precipitated with acetone, centrifuged, and the residue washed twice with acetone. After ordinary drying this precipitate is made into a fine suspension with 10 c.cm. of 1 per cent casein in phosphate buffer of pH 7.0 and the increase in NPN determined after incubation as usual.

Experimental

The experiments were carried out on two dogs kept under controlled conditions in the laboratory animal house. Before we study the action of drugs on the trypsin content of plasma, it is necessary to ascertain the limits within which the trypsin content of the same dog may vary from day to day.

TABLE I

Days	Dog no. 1		Dog no. 2	
	Free	Total	Plasma trysin expressed as increase in NPN for 100 c.cm. plasma	
			Free	Total
1	18.5 mg.	61.8	22.8	69.4
2	23.2 mg.	58.8	28.4	76.5
3	20.9 mg.	65.6	20.5	62.6
4	24.8 mg.	69.8	26.6	65.8

The free trypsin and the total trypsin in plasma appear to remain fairly constant in each dog.

The effect of injecting various doses of cobra venom intramuscularly on the free trypsin content of plasma was next studied, and the results are given below:—

Weight of the dog = 7 kilograms. Blood was taken for each determination 2 hours after injection of the venom. The injections were given once in 72 hours.

TABLE II

Dose of venom	Trypsin in 100 c.cm. plasma	Dose of venom	Trypsin in 100 c.cm. plasma
nil	25.5 mg.	0.10 mg./kilo	90.9 mg.
0.03 mg./kilo	65.3 „	0.12 „	107.2 „
0.05 „	72.2 „	0.15 „	46.6 „
0.07 „	79.2 „	0.19 „	17.3 „

There is a significant rise in the free trypsin content of plasma after the injection of cobra venom. This increase is, however, not maintained. At the end of 24 hours the trypsin content tends to decrease and within 48 hours it comes to its original level. The cause of this increase in trypsin can be explained in different ways. The venom itself has tryptic activity (Ghosh, 1936; Ghosh and De, 1936; Iyengar, Sehra and Mukherji, 1938) and the increase brought about may simply be an additive effect. This is extremely unlikely, since the actual amount of trypsin that is present in the quantities of the venom injected is extremely small and is further diluted by the circulating blood. The dilution of the venom in the blood after injecting the dose of 0.12 mg./kilo, which brings about a maximum increase in trypsin, is found by calculation to be 1 in 700,000. In such a microscopic quantity of the venom, the trypsin present is practically negligible. Since the blood plasma contains, in addition to free trypsin, a compound of trypsin-inhibitor which is normally inactive, but which can be activated by a kinase, the possibility that the venom might contain the trypsin-kinase has to be considered. In order to test this possibility the following experiments were undertaken :—

Plasma was precipitated directly with acetone and the precipitate was dried after washing with acetone twice. This contains the trypsin-inhibitor compound as well as the small quantity of free trypsin. If this compound could be split up by the addition of cobra venom in a concentration of 1 in 20,000 (which is very much higher than the maximum concentration of the venom in blood after the injection), and consequently increased trypsin activity demonstrated, it could then be regarded as sufficient evidence of the presence of trypsin-kinase.

The above results clearly disprove the possibility of the presence of trypsin-kinase in cobra venom, since there is practically no increase in the tryptic activity of plasma trypsin on the *in vitro* addition of the venom.

TABLE III

In vitro *action of cobra venom on plasma proteins*

	Increase in NPN after 48 hours
1. (*a*) 100 mg. acetone precipitated plasma proteins incubated in 10 c.cm. buffer of pH 8.4.	0.25
2. (*a*) + 0.5 mg. cobra venom in 10 c.cm. buffer of pH 8.4.	0.27
3. 100 mg. casein + 0.5 mg. cobra venom in 10 c.cm. buffer of pH 8.4.	*nil*

The next possibility of the mechanism of this *in vivo* increase of plasma trypsin is the increased capacity of the red blood corpuscles, leucocytes or platelets which are the chief sources of trypsin in blood, to synthesize the enzyme. Determination of the trypsin content of red corpuscles and the mixture of leucocytes and platelets made before and after the injection do not show any significant difference.

In the light of the above results, the only reasonable explanation that can be offered for the observed increase is that the proteolytic enzyme is released into the blood from the tissues under the influence of the venom. This tentative hypothesis could not be put to test.

By an examination of table II, it can be seen that the plasma trypsin content which has a progressive tendency to rise until a dose of 0.12 mg./kilo of cobra venom is reached, falls down suddenly when the amount of venom is increased to 0.15 mg./kilo. If the dose is further increased, the fall in the plasma trypsin content is much more significant. The presence of trypsin-inhibitor in the cobra venom (Ghosh, 1936, and Iyengar *et al.*, 1938) might be responsible for this strange finding. This inhibitor is present in such small quantity that it cannot effectively exert its action until sufficient concentration of the venom is obtained. When a dose of 0.15 mg./kilo is reached, the inhibitor probably begins to exert its effect and inactivates the trypsin that is released from the tissues. The inactivation of the normal plasma trypsin is also noticed when the amount of the venom injected is further increased to 0.19 mg./kilo. In this case the plasma trypsin content (17.3 mg.) is lower than the normal value (25.3 mg.).

The *in vivo* action of an analogue of vitamin K (2–methyl–1 : 4 naphthaquinone) was next investigated and the results are given below. Since the substance was injected in an oil medium, the time allowed for complete absorption was six hours. In some cases, the blood was taken even after 24 hours for trypsin determination. It is reported that prothrombin increase can be noticed 6 hours after the injection of vitamin K, although a significant rise can be observed only at the end of 12 to 24 hours.

There does not seem to be any change in the plasma trypsin activity, either free or combined, on the administration of 2-methyl-1 : 4 naphthaquinone, a synthetic analogue of vitamin K.

TABLE IV

Dog weighing 7 kilos.

Amount injected	ACTION OF 2-METHYL-1 : 4 NAPHTHAQUINONE ON PLASMA TRYPSIN	
	Free plasma trypsin 6 hours after injection	Total trypsin 6 hours after injection
nil	23.5 mg.	62.6
1 mg.	22.8 „	68.5
2 „	18.5 „	64.2
3 „	24.6 „	61.9
4 „	17.9 „	67.2
5 „	14.8 „	68.4
	18 hours after injection	18 hours after injection
3 mg.	14.5 mg.	65.6
4 „	18.2 „	62.8
5 „	16.8 „	67.9

Trypsin, however, is not known to be influenced by mild oxidizing or reducing agents while cathepsin is known to be definitely affected by the oxidation-reduction state of the medium. It is, therefore, reasonable to expect changes in the catheptic activity of serum under the influence of this drug.

TABLE V

Action of 2-methyl-1 : 4 naphthaquinone on serum cathepsin

Amount injected	Serum cathepsin (6 hours after injection)	Amount injected	Serum cathepsin (6 hours after injection)
nil	42.6 mg.	3 mg.	28.2 mg.
1 mg.	41.2 „	4 „	29.5 „
2 „	36.5 „	5 „	30.8 „

There is a significant reduction in the catheptic activity of the serum after the injection of the drug. This inhibitory effect must be due to change in the oxidation-reduction state of the medium brought about by the anti-hæmorrhagic substance. This substance is a reversible oxidation-reduction catalyst, the hydroquinone form of which is readily oxidized by molecular oxygen. This action of inhibiting the catheptic proteolysis by 2-methyl-1 : 4 napthaquinone may be regarded as favouring protein synthesis since normal anabolism and catabolism of proteins tend towards equilibrium. The net effect of inhibiting proteolysis must be to favour protein synthesis in the system. This action of vitamin K will therefore serve as a link to explain the mechanism of its action of increasing prothrombin protein as visualized by McCawley and Gurchot (1940).

Summary

The action of cobra venom on the tryptic activity of plasma has been studied. It has been found that up to a dose of 0.12 mg./kilo, there is a remarkable increase in the trypsin content of plasma and it begins to go down if the dose is increased beyond this limit. The various possibilities regarding this increase have been investigated and it is suggested that the venom may be releasing into the blood stream trypsin from the tissues. It is interesting to recall that Iyengar *et al.* (1942) have reported that plasma trypsin is reduced considerably in cases of malignant growth and that Chopra and Chowhan (1935) have recommended the administration of cobra venom solutions in the therapy of cancer. The finding in the present investigation that the plasma trypsin is increased by the administration of the cobra venom may therefore be useful partially to explain the rationale of the treatment of cancer by cobra venom. The decrease in plasma trypsin brought about by larger doses of the venom is explained as due to the trypsin inhibitor reported to be present in the venom. This inhibitor effectively comes into action only when the venom is administered in larger doses not encountered in clinical practice. 2-methyl-1 : 4 naphthaquinone does not exert any action on the plasma trypsin, but appears to inhibit the catheptic activity of serum. It is suggested that this action of the drug may serve to explain the mechanism of the action of vitamin K in synthesizing prothrombin.

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