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Snake venom action: are enzymes involved in it?

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Summary. Enzymes were the first clearly recognized components of snake venoms. When several more were discovered, attempts were made to correlate venom action with enzymic functions. The last few years have seen most successful efforts in the identification, isolation and structural elucidation of highly toxic polypeptides present in snake venoms, in particular of 'neurotoxins' and membrane-active toxins. Following this development the polypeptides were called the true toxic components and the enzymes lost their previous central position in venom pharmacology. The time, therefore, has come to re-evaluate the role of enzymes in the complex interaction between snake and prey. While highly active polypeptides indeed dominate the action of hydrophiid venoms, they appear to play a lesser role in crotalid venom action as compared with enzyme components. Enzymes are involved in many levels of venom action, e. g. by serving as spreading factors, of by producing very active agents, such as bradykinin and lysolecithins in tissues of preys or predators. Some toxins, e. g. the membrane-active polypeptides appear to participate in the interaction between membrane phospholipids and venom phospholipases. The classical neurotoxin, β-bungarotoxin, has been recognized as a powerful phospholipase. Several instances are known which indicate that some enzymes potentiate the toxic action of others; the analysis of a single enzyme may, therefore, not fully reveal its biofunction. For 3 enzymes, ophidian L-amino acid oxidase, ATPpyrophosphatase, and acetylcholinesterase, some of the problems pertaining to venom toxicity are discussed.

Heralded by MELDRUM'S review on the pharmacology of phospholipase and polypeptide toxins of snake venoms in 1965[1], the last 10 years have seen most successful efforts in the identification, isolation, and structural elucidation of highly toxic polypeptides present in snake venoms. Many of the spectacular achievements are described in the papers of BOQUET et al.[2], CONDREA[3], VON HAHN and HONEGGER[4], KARLSSON[5], MEBS[6] and ZLOTKIN[7]. It is not surprising, therefore, that during this period the other major class of venom components, the enzymes, lost their significance as an explanation for the tremendous power of venoms. A typical statement is found in TU's informative review on neurotoxins[8]: 'Many enzymes are present in snake venoms. Most are hydrolytic enzymes, with the notable exception of L-amino acid oxidase. Because of their presence, many earlier investigators were tempted to correlate enzymes to toxic action. The best-known example was an article by ZELLER, "Enzymes as Essential Components of Bacterial and Animal Toxins"[9]. With progress in techniques of protein chemistry, snake venoms eventually were fractionated, and their lethal action and enzyme activities were differentiated. For example, on fractionation of *N. naia atra* venom, phosphodiesterase, phosphomonoesterase, 5'-nucleotidase, ATPase, proteinase, and phospholipase A were separated from toxicity[10].'

Why then are enzymes present in snake venoms if they do not correlate with toxic action? Why are enzymes in many instances the dominant constituents?

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Although we do not have any exact data regarding the quantities of *all* components in a given venom, we can make a rough estimate as to the content of neurotoxins and related polypeptides in some venoms. In sea snakes the concentration of these substances is very high and can comprise as much as 85% of the protein content of the venom[5,11]. On the other hand, for many crotalid venoms, no convincing evidence for the occurrence of 'neurotoxins' has been brought forth as yet. Even if we assume that the lethal power of rattle snake venom were entirely due to neurotoxins (and related compounds) the latter would contribute only 1–2% to the total mass of the venom. The rest of the solid material seems, with few exceptions, to be taken up by enzymes. In fact, snake venoms are among the richest enzyme sources found anywhere in nature[6,13]. Even in the venom of Hydrophiidae (sea snakes), with its high level of polypeptide toxins, the usual set of enzymes does occur, viz. hyaluronidase, alkaline phosphatase, 5'-nucleotidase, phosphodiesterase, phospholipase A, acetylcholinesterase, NADase, leucine aminopeptidase, and fibrinogen clotting activity (*Enhydrina schistosa*)[14]. The statement that 'Hydrophiidae venoms contain very few or no enzymes' has to be documented before it can be accepted (p. 1332[6]). In the following sections some points regarding the biofunction of a few snake venom enzymes are discussed.

Phosphodiesterase vs. ATPpyrophosphatase

RUSSELL et al.[15] isolated phosphodiesterase (oligonucleate 5'-nucleotide hydrolase, 3.1.4.1) from several snake venoms. When they injected it i.v. into mice an immediate and profound hypotensive crisis ensued. 2 mechanisms accounting for this reaction are suggested here.

1. The phosphodiesterase successively releases 5'-nucleotides from the 3' terminus of nucleic acids (RNA and DNA)[16]. If messenger RNA with about 200 successive adenylate residues at the 3' terminus were the substrate, a considerable amount of adenylic acid would be set free. The latter in turn is dephosphorylated to adenosine by the 5'-nucleotidase, usually the most active of all phosphatases present in snake venoms. Adenosine is known for its ability to produce a transient reduction of blood pressure by arterial dilatation (vide infra). From the venom of *Crotalus adamanteus*, the purification leads to a 70fold increase of activity[17]. Similarly, for various *Crotalus* species and for *Vipera Russelli*, an increase in activity in the course of purification from 60- to 340fold was obtained[15]. This means that the original diesterase content must have been at most between 0.3 and 1.6%. Whether the quantities of the phosphodiesterase introduced into the body of the prey by the snake bite produces sufficient adenosine to lower the blood pressure, remains to be tested.

2. A second answer may come from our work on the occurrence of a phosphatase in snake venoms which catalyzes the degradation of ATP. This enzyme was discovered in 1948[9,18,19] and was shown to occur in venoms of more than 30 species[20]. So far, only one species, the sea snake *Laticauda semifasciata*[21] has been reported not to produce this enzyme. We elucidated the overall mechanism of the reaction by isolating inorganic pyrophosphate from the reaction mixture[22]. It catalyzes, therefore, the hydrolysis of ATP to AMP and pyrophosphate. Accordingly, it is an ATPpyrophosphatase (ATPpyrophosphohydrolase, 3.6.1.8)[23]. In 1953, KREBS et al.[24] confirmed our results. They labelled the ultimate and penultimate phosphate (^{32}P) and found the label in inorganic pyrophosphate in the chromatographically separated products of the action of cobra venom on ATP[24]. By still another method, the formation of inorganic pyrophosphate from ATP in the presence of snake venom (*C. adamanteus*) was demonstrated by FELLIG[25].

In 1959 BOMAN observed that the phosphodiesterase was always accompanied by ATPpyrophosphatase activity[26]. Similarly, PFLEIDERER and ORTANDERL, while isolating the phosphodiesterase, found the ratio between the 2 enzyme activities to remain the same[27]. The 2 enzymes, classified as 3.4.1.4 and 3.6.1.8, therefore, were considered to be identical. This conclusion is supported by the recent report that in the *L. semifasciata* venom mentioned above not only the phosphodiesterase but also the ATPpyrophosphatase is absent[21]. On the other hand, PEREIRA LIMA et al. investigated the 2 enzyme activities in several venoms and recorded marked differences between them with respect to activation by bivalent cations, inactivation by basic and acidic media, and 2 other criteria[28]. It is, however, conceivable that these observations are based on the marked structural differences between the re-

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active groups of the 2 classes of substrates: one responding with the hydrolysis of a pyrophosphate linkage, the other with the hydrolysis of a 3'-phosphate ester bond.

Whichever way this problem is going to be solved, we can assume that ATPase activity is very widespread among snake venoms. Although it seems to be present in a relatively small quantity (see section of phosphodiesterase), antisera against the whole venom markedly reduce its activity[29]. Again, we want to know the role, if any, of this enzyme in venom action. JOHNSON et al.[24] considered 2 products of the ATPase reaction, inorganic pyrophosphate and adenosine (the latter formed in the presence of 5'-nucleotidase) as contributing to the toxicity by referring to the blood pressure reducing power of both compounds. No quantitative data regarding the concentration of these agents in critical loci after venom application are available, however. There is one bit of evidence which supports the assumption that adenosine could be involved: as in anaphylactic shock, the so-called Kurloff bodies[29] present in guinea-pig blood disappear completely after the administration of *Bitis gabonica* venom[19], one of the richest sources of ATPase as well as 5'-nucleotidase[9]. This vanishing act is prevented by adding Zn^{2+} to the venom; Zn^{2+} is an activator of ATPase[28] but a powerful inhibitor of the 5'-nucleotidase[9].

In view of the central position of ATP (and related triphosphates) in cellular energy transformations, and considering its role in the synthesis of cyclic monophosphates, in NAD, NADP, coenzyme A, etc., one may suspect that the loss of ATP caused by the ATPpyrophosphatase activity may contribute to the snake venom action. I have previously suggested[9] that Page's hypothesis could serve as a useful paradigm for the analysis of this facet of venom action: Page proposed that shock, accompanied by a sharp reduction of blood pressure may be caused by a reduction of ATP below a critical level[30,31]. No measurements have come forth of ATP levels in pertinent tissues after snake bite.

Ophidian L-*amino acid oxidase (L-AAO)*

Starting from the observation that large amounts of riboflavin occur in snake venoms[32], we discovered a new amino acid oxidase in this material[32–34]. Out of more than 30 species only 2 did not display the enzyme. They were also the ones devoid of flavin[13]. The enzyme does not only occur in the venoms, but also in several organs of poisonous and non-poisonous snakes[35]. It was, therefore, called ophidian L-amino acid oxidase[36] (L-amino-acid oxygen: oxidoreductase, deaminating, 1.4.3.2). The enzyme differs in many ways from the enzyme found in mammalian kidney, not only by the nature of the flavin component (FAD vs. FMN), but also by its specificity pattern. The mammalian L-AAO attacks not only L-amino acids but also the corresponding α-hydroxy acids; in fact, the latter enzyme may be functionally more of an α-hydroxy acid than a L-amino acid oxidase[37,38]. In contrast, the ophidian oxidase is quite unable to catalyze the oxidation of α-hydroxy acids[39], contrary to some statements found in the literature, e.g.[15]. Since the ophidian L-AAO can be readily purified within a short time and obtained in crystalline form[40], it has been used in countless studies of the mechanism of flavin enzymes in general and L-amino acid oxidases in particular[41–43]. Before the biofunction of this enzyme can be discussed, some phantom enzymes have to be eliminated first.

a) MOHAMED et al. reported the presence of alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, 2.6.1.2) in several snake venoms[44], while the aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, 2.6.1.1) was absent. The transaminase measurement was carried out in the presence of approximately 0.2 *M* L-alanine and 0.34 mg of venom for 30 min. Under these circumstances, L-alanine is extensively oxidized[33,34]. A large fraction, if not all, of the pyruvate formed and measured photometrically must have been produced by the L-AAO reaction. Since this enzyme is incapable of attacking glutamate and aspartate[45], no aspartate aminotransferase action can be simulated by the L-AAO action. Accordingly, in snake venom this transaminase could not be demonstrated.

b) When a slow degradation of serotonin by snake venom was observed it was concluded that monoamine oxidase (MAO, amine: oxygen oxidoreductase (deaminating) (flavin-containing), 1.4.3.4) was responsible for this reaction[46]. In all animals, including reptiles, MAO so far has been found to be tightly bound within mitochondria and never seems to occur in solu-

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ble form inside and outside cells with more than minute activities. Although we have tried many times with several substrates, we have been unable to demonstrate unequivocally MAO to be part of snake venoms. Even our more sensitive methods, e.g. spectrophotofluorometric detection of peroxide formation, the large amounts of venom and long incubation periods did not help[47].

c) As described by EICHBAUM and STAMMREICH, several authors have reported that blood or erythrocytes turn brown in the presence of snake venoms[48]. The darkening, as first shown by GHOSH, was due to the formation of methemoglobin[49]. The effect is enhanced by the addition of blood serum, and was considered to be related to catalase action. No indication, however, was given where the hydrogen peroxide would come from. Since L-AAO is the only peroxide-producing enzyme in the snake venoms, apparently it must be the source of the agent which converts hemoglobin to methemoglobin without need of another oxidase. Before more efficient methods became available, often the generation of H_2O_2 in certain enzyme reactions was proved by the addition of hemoglobin to the reaction system and by the measurement of the increase in absorption at 614 nm due to methemoglobin production[50]. Addition of serum to the system venom/hemoglobin increased methemoglobin, obviously by supplying L-amino acids as substrates of L-AAO[47]. In complete accordance with this conclusion is the observation that the venom obtained from *Bothrops itapetiningae* does not lead to methemoglobin production[48]. This venom is one of the very few which is white and does not contain even traces of L-amino acid oxidase[13]. Thus, methemoglobin formation cannot be ascribed to an enzyme sui generis. Recently we have shown that the system L-AAO (from *Bothrops jararaca*), L-leucine, washed human erythrocytes, and oxygen are capable of converting hemoglobin to methemoglobin within the intact red cells[47]. In short, from the list of snake venom enzymes we have to delete transaminase, MAO, 'hemoglobin oxidase' and α-hydroxyacid oxidase.

With few exceptions (vide supra), we find the L-AAO in the venoms collected from elapids, crotalids, and viperids. Recently, we had a chance to test its presence in a member of the opistoglyph Colubridae, viz. *Dispholidus typus* and of the hydrophiids, viz. *Laticauda semifasciata*. With L-leucine and L-phenylalanine as substrates, no degradation could be registered with the spectrophotofluorometric method[47]. Thus, this enzyme seems to occur in 3 of the 5 major groups of venom producing snakes.

In dry venoms, we find 3% and less of L-AAO. The smallness of these quantities may be responsible for the fact that antisera produced against whole venoms showed little influence on L-AAO activity[13]. More recently, however, the antigenicity of purified L-AAO has been positively demonstrated[51]. The isolated purified enzyme is fairly toxic (LD_{50}: 9 mg/kg mouse i.v.[51]) and approximately to the same degree as the crude venom of copperheads (*Agkistrodon contortrix*)[12]. SATO et al. separated the venom of the dreaded habu (*Trimeresurus flavoviridis*) into 11 fractions and attributed lethal activity to the combined action of L-AAO, protease, and phospholipase A[52]. The cause for this toxicity is not known. Some authors considered the appearance of methemoglobin as a factor contributing to the toxicity of venom[48]. However, if we make a rough estimate of the actual rate of oxidation of amino acids in blood, we arrive at a value of 10^{-6} moles $\times$ (min)$^{-1}$ $\times$ (mg venom)$^{-1}$. This calculation is based on V_{max} for leucine[36] and gives us an upper limit rather than the actual rate, because leucine is one of the best substrates of L-AAO and because the actual amino acid concentration in blood plasma is on the order of $< K_m/10$. Even so, only 10^{-3}% of the hemoglobin could have been oxidized in 1 min. This calculation is in accordance with our recent experiments: when rats were killed by i.v. injection of *Jararaca* venom, no methemoglobin could be detected spectroscopically in their blood[47].

Acetylcholinesterase

IYENGAR et al.[53] and GHOSH et al.[54] discovered the presence of cholinesterase (ChE) in certain snake venoms. In our laboratory, their observations were confirmed and extended to the study of venoms of over 50 species[9,13,55,56]. The enzyme was present in *all* elapid venoms and completely absent in the venoms of *all* members of the Viperidae family (viperids and crotalids)[55]. Later, the enzyme activity was also found in hydrophiid venoms, e.g. of *Enhydrina schistosa*[14]. KUMAR et al.[57] could not detect the enzyme in the venoms of *Dendroaspis angusticeps* (Mamba) and of *Naia nigricollis*. In our investigation, however, the venom of both species while displaying low ChE activity were still clearly separated from the entirely negative *Viperidae* venoms. Thus, a variable, but consistent ChE activity appears to be present in all venoms collected from elapids and hydrophiids.

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To find out if there is any contribution of ChE to the envenimation process we would like to know to what type of ChE the snake venom belongs because the substrate (and inhibitor) pattern for the 2 major types of ChE is quite different. Once the assignment is made we can search for the *physiological* substrate within a narrower set of compounds. A few notes about the origin of differentiation of ChE into 2 types are given here because snake venoms played a decisive role in this investigation. In 1942–3, the first experimental observations clearly suggesting that 2 fundamentally different cholinesterases are capable of catalyzing acetylcholine came from English[58], Canadian[59] and Swiss[60,61] laboratories. Our studies included the analysis of inhibitor pattern and physicochemical properties of ChEs, e.g. inhibition by high substrate concentration and culminated in the proposal of a model of enzyme-substrate interaction. For the acetylcholinesterase, the importance of the trimethyl ammonium and ester moieties for the binding were stressed. A similar, but more detailed model developed by Nachmansohn and Wilson became later a textbook classic.

In a series of publications we described some of the properties of snake venom ChE[9,13,62–64]. We found the enzyme to be closely related to the one present in red cells and in brain. In addition to acetylcholine, it accelerated the hydrolysis of several alcohols and phenols, including deoxycorticosterone, cortisone, phenol, and *p*-nitrophenol. Later, MOUNTER and WHITTAKER[65], successfully tested several ring-substituted phenylacetates as substrates of cobra venom ChE. More recently, KUMAR and ELLIOTT demonstrated the degradation of phenylacetate by a highly purified ChE obtained from *Bungarus fasciatus*[66]. However, CoA acetate, although closely related to the excellent substrate thiocholine acetate – both compounds contain the sequence $N–CH_2–CH_2S–CO–CH_3$ – was not attacked by the venom of *Naia melanoleuca* as yet, one of the richest sources of ChE in nature[56]. In short, the snake venom ChE is a typical acetylcholineesterase and its substrate pattern is not limited by any means to acetylcholine. Henceforth, the abbreviation ChE refers to this particular type of enzyme (acetylcholine hydrolase, 3.1.1.7).

We have to tackle now the thorny problem regarding the participation, if any, of this enzyme in the overall toxic effect of the crude venom. A priori, the amounts found in some elapid venoms would point toward a substantial role of ChE. Unfortunately, no LD_{50}-data have been reported for a really pure ChE. Since it can be purified 11- and 26fold from the venoms of *Naia naia atra* (Formosan cobra) and *Bungarus multicinctus* (banded krait) the ChE content of the 2 venoms apparently is $\geq 9\%$ and $\geq 4\%$ respectively[67]. As the earlier studies on elapid venoms showed the marked effects of these venoms on neuromuscular activity it was thought that the enzyme was responsible for the curare-like effect of the whole venom and the death of the bitten animal[53]. However, a number of experimental data are known which do not support this conclusion: a) ChE can be separated from the major toxic constituents of cobra venom which cause paralysis in mammals[68,69]. Recent experiments confirmed these early observations: by zone electrophoresis the neurotoxin was removed from ChE (and other enzymes)[70]. b) The role of neurotoxins such as the α- and β-bungarotoxins in the neuromuscular transmission has been elucidated to a considerable degree[4] so that apparently in this process no niche appears to be left for ChE.

A testable concept concerning the biofunction of venom ChE is proposed here. It is based on the observation that in Colubridae venoms we find powerful polypeptides, e.g. neurotoxins and membrane-active polypeptides as well as ChE, and on the action of the 2 bungarotoxins (as clearly described by VON HAHN and HONEGGER, whose review has to be consulted for details and references[4]).

β-Bungarotoxin (β-BuTX) acts exclusively on the presynaptic side of the neuromuscular junction. Its effect is associated with an almost total loss of synaptic vesicles. The free acetylcholine is then destroyed by the postjunctional membrane ChE. The system β-BuTX bears some resemblance to that of the reserpine-MAO relationship. When the monoamines are prevented by reserpine from being stored in specific vesicles, they are destroyed by MAO. If the capacity of the ChE were not sufficient for the precipitate influx of acetylcholine caused by the BuTX action some acetylcholine molecules may escape immediate hydrolysis. By reacting with some postjunctional receptors, they could protect them competitively against the

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α-bungarotoxin. Thus, the additional ChE supplied by the venom could prevent this reaction and potentiate the immobilizing power of the bungarus venom.

Cooperativity between various venom components

To gain some understanding of the role of various parts of snake venoms it seems necessary to find out whether some measure of cooperativity between the 10–15 components exists. a) The case of phosphodiesterase-5'nucleotidase (or ATPpyrophosphatase-5'nucleotidase) or that of ophidian L-AAO, phospholipase, and protease are to be mentioned here again. b) Of great interest is the cooperation between phospholipases with membrane-active polypeptides. Phospholipase A plus direct lytic factor (DLF) from cobra venom were found to inhibit the brain cell membrane sodium- and potassium-activated ATPase to a much greater extent than either compound alone[72]. In one instance, purified phospholipase from a sea-snake, *L. semifasciata*, was observed to be nontoxic[73]. c) In 1938, SLOTTA and FRAENKEL-CONRAT isolated crystalline crotoxin from the venom of *Crotalus terrificus*[74], which, according to numerous tests appeared to be homogenous. Only after 19 years of investigations carried in several laboratories, HABERMANN et al. were able to separate crotoxin into several fractions[75,76]. The toxicity of 'crotoxin' is attributable to more than one component. 2 of its phospholipases possess some genuine toxicity which is greatly enhanced by nontoxic, strongly acidic constituents. The difficulties experienced in the separation of the various components from each other could indicate a genuine aggregation which even may be classified as a quarternary structure with 'synergistic toxicity of its subunits'[57]. d) CONDREA[3] and ZLOTKIN[7] give a very clear outline of the most intensively investigated case, that of the interaction of the direct lytic factors (DLF) with phospholipases. The polypeptide appears to modify membrane conformation so that the proper bonds are exposed to the active site of the phospholipase. This interaction between polypeptide and enzyme is directed not only toward the classical object, the red cell membrane, but also toward the membranes of lysosomes[77], platelets[78], mast cells[77] and toward the membranes which form the structural basis for axonal nerve conduction. It has been repeatedly suggested that all biomembranes are damaged by the combined action of polypeptides and phospholipases. Thus, the membrane-active polypeptides which as cardiotoxins, cytotoxins, etc., contribute a substantial part to the toxicity of elapid venoms, seem to act by being a part of a complex enzyme system. f) Recently, a lucid example of a cooperative effect involving phospholipase A (*Naia naia*) and a proteolytic enzyme has been described. It 'suggests that phospholipases may make protein more exposed to proteolytic enzyme for its digestion'[79]. g) A final example pertaining to bee venoms may be presented here: venoms collected from some bees contain phospholipase, others do not; the former turned out to be more toxic than the latter[80].

Snake-prey interactions

We can recognize not only interactions between various venom components, but also between enzymes of the venom with certain biochemical systems of the prey or predator. Only a few examples can be mentioned here. a) The venom phospholipases will act on the prey's free phosphoglycerides and liberate the corresponding lysophosphoglycerides. The latter are capable of destroying membrane-structure and -function. b) The combined action of phospholipases + DLF on lysosomes will release digestive enzymes and thus initiate autodigestive processes. A similar lysis of mast cell membranes will release histamine. c) The combined action of phosphodiesterase + 5'-nucleotidase or ATP pyrophosphatase on the prey's nucleic acids and ATP could set free substantial amounts of adenosine. d) Finally, the degradation of the plasma α_2-globulins of the prey by a venom peptidase (kininase) leads to the formation of the nonapeptide bradykinin. Kininase appears to occur in the venom of various species of the genera *Crotalus, Agkistrodon, Bothrops* – all crotalids – and of the viperid Echis[81]. Bradykinin is known for its power of strongly enhancing the permeability of the capillary wall.

Several of these snake-prey interactions could contribute to the onset of fatal shock by a variety of mechanisms, including loss of plasma fluid through the capillary wall. Marked fall in blood pressure is often observed as a consequence of bites from crotalid snakes[12,15]. Again, we have to stress that only few quantitative data have been brought forth as a basis for these tentative conclusions.

Role of enzymes in spreading of venom in the animal organism

From the locus of the deposit, the subcutaneous or muscular tissue, the venom has to spread out and reach its target, be it a neuromuscular endplate, the circulating blood, or the central nervous system. However, even the smaller components, viz. the membrane-

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active polypeptides and the so-called neurotoxins of 7000 daltons, are likely to diffuse rather slowly through the ground substance of the connective tissue. It is generally assumed that the process is sped up by breaking down hyaluronic acid, the major component of the ground substance and by transforming it from a highly viscous material into one with not much more viscosity than water. Actually, hyaluronidase was discovered in snake venom as a 'spreading factor'. It is found in the products of many poisonous animals as seen in Table 1 of ZLOTKIN's paper[7] (p. 1454). The role of hyaluronidase is briefly discussed in the same paper (p. 1457) and more extensively in the reviews of DEVI[82], KAISER and MICHEL[83], MELDRUM[1], RUSSELL[12], SLOTTA[84] and ZELLER[9,13]. Possibly, other enzymes are involved in the spreading process, viz. proteases, phospholipases + DLF. Without the spreading power of some enzymes, the venoms may produce some local effects, but probably would not accomplish the rapid immobilization of the prey.

Snake venoms as digestive secretion

For the understanding of the consequences of snake bite it is of great importance to be aware that the venoms are the products of digestive glands. Recently, the venom glands were called analogous rather than homologous to the mammalian salivary parotids (for discussion of the problem, see[85]). Homology or analogy notwithstanding, biochemical and physiological facts are strongly suggesting an originally digestive function of the venom producing glands. The more primitive genera, Colubridae opisthoglyphae, have developed a simple fang structure in the rear of the upper jaw and a comparatively mild venom. These reptiles have never been included among the dangerous serpents, apparently because they cannot use their grooved fangs as weapons. While they swallow their prey they inject the 'venom' into it and thus initiate the digestive process. Unlike many other animals the snakes swallow their catch as a whole and, consequently, the digestive enzymes rather slowly penetrate into the more or less intact body of the prey. Only one member of the *C. opisthoglyphae*, *Dispholidus typus*, can inflict serious injury to man. It is also the only species with a mobile maxilla which permits the grooved fangs to move forward during the attack[86]. In the course of phylogenetic development, the length of the maxilla decreased and the fangs gradually shifted toward the frontal part of the oral cavity. Thus, these teeth developed into weapons of attack and defense (*Colubridae proteroglyphae*). But even in snakes with the most advanced venom apparatus, the digestive power of the venoms is still in force. When human beings are bitten by large pit vipers and do survive long enough, all soft tissues of the legs and arms may be destroyed with concomitant mummification of hand or foot. Another demonstration of the digestive function of venom was given by C. STEMMLER-MORATH[13]: A mouse killed by the action of viper venom (*Vipera aspis*) was digested by the snake in about 3 days; but when the snake was prevented from using its fangs, the process lasted for 5–8 days.

The set of enzymes found in most snake venoms closely resembles that established for lysosomes. Endo- and exopeptidases, nucleases, hyaluronidase, phospholipases and several phosphatases are found in both sources. It may be worthwhile to mention that, in contrast to intestinal proteases, the venom proteases appear to be secreted in fully active form and stored chiefly in the central and tubular lumina of the glands. There must exist, therefore, efficient ways to protect the gland and its tubules against the attack by the powerful proteases. This interesting problem has not as yet been given much consideration. The observation that *Naia naia atra* venom contains a heat labile acetylcholinesterase-inactivating factor which is not effective against cholinesterase activity of *mammalian* tissue[87] may give us a hint about self-protecting devices.

Functional classification of enzymes

When we conceive that snake venoms essentially are products of digestive glands we gain a new vantage point for a functional classification of their enzymes: a) enzymes with exclusive digestive function; b) enzymes which, in addition to their original role, became part of the venom action; c) enzymes which are not known to be ever involved in digestive activity.

a) At the present we do not have enough facts for assigning exclusive digestive action to some enzymes. The extensive destruction of soft tissues mentioned above, however, is obviously an expression of a predominant digestive power. Similarly, the resolution of thrombi which often is induced by venom proteases could be classified here, because the destruction of the thrombus may take place many hours after the venom administration and thus does not contribute to the immobilization of the prey.

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b) When snake venom reaches its target it will doubtlessly cause considerable metabolic perturbation with its high concentration of very active enzymes. The following example may illustrate this point: nobody considers urease, a plant enzyme, to be a poison. And yet, upon injection of 0.15 mg of the crystalline enzyme i.v. into rabbit, the animal is killed[88]. The lethal dose is much smaller than for many powerful snake venoms (see Table 1, ref.[12]). Further analysis showed that the toxic effect was due to the ammonia produced by the action of this enzyme on urea. As to snake venoms, a few examples appear to belong to this group: phosphatases which catalyze the hydrolysis of various phosphate ester- and pyrophosphate-bonds – after being forced into tissues – could perform the same reaction there and destroy metabolically active nucleotides such as nicotinamide adenine dinucleotides, coenzyme A, etc. and, thus, could disturb the enzyme apparatus. If it is true that the phosphodiesterases are identical with ATP pyrophosphatases we could visualize that a transition has taken place from a digestive agent enhancing the hydrolysis of nucleic acids to an enzyme destroying ATP and related nucleotides. Similarly, kininases may have evolved from an ordinary peptidase to one specifically producing bradykinin.

c) In digestive secretions, acetylcholinesterase, hyaluronidase, and L-aminoacid oxidase appear to be absent. Since hyaluronidase does occur in lysosomes and since the enzyme sets in lysosomes and snake venoms are very similar, the appearance of hyaluronidase is not too surprising. For the 2 other enzymes, no direct relationship to digestive activities have been found as yet. From these few paragraphs it becomes obvious that our proposal is a program for classification rather than a system itself.

Complementarity between physical and chemical apparatus

When we compare the envenimation apparatus of the 2 major groups of poisonous snakes, the Colubridae (hydrophiids and elapids) and Viperidae (viperids and crotalids), we find marked differences between them. The former and phylogenetically older group displays relatively short, grooved, and non-movable fangs, while the latter group, representing a different phylogenetic line, is characterized by long, hollow, curved and erigible fangs. In considering the toxicity of the venoms, as expressed by LD_{50}-values, we arrive at the following series (Table 1, ref.[12]): hydrophiids > elapids > viperids > crotalids. While the 'toxins' appear to dominate the venoms of the hydrophids, the enzymes reach a maximum in the crotalids. Thus, the most effective chemical constituents are associated with a relatively primitive physical apparatus and vice versa.

Comparative biochemistry of small molecular protein components

If the origin of digestive enzymes or modified digestive enzymes can be understood as products of salivary glands, where do 'neurotoxins' and membrane-active polypeptides enter our overall picture? Do they constitute a contradiction to our concept? There begins to emerge, however, an answer to this question based on the investigations of BONILLA et al.[85]. These authors found that some of the small molecular weight basic proteins (SMBP) in human parotid fluid closely resemble those of some 7 crotalid venoms in their physico-chemical and biological properties. One of the SMBP isolated from human parotid secretion has identical biochemical properties of *Crotalus viridis viridis* basic protein. The biofunction of these polypeptides in mammalian saliva remains to be elucidated. This is also true of the proteinaceous nerve growth factor which seems to activate tyrosine hydroxylase in cell bodies of sympathetic ganglia[89].

Quintessentia

Again and again, the action of neurotoxins, cardiotoxins, and other membrane-active factors has been sharply differentiated from enzyme reactions. Some evidence suggests, however, that the membrane-active polypeptides, including DLF, are part of phospholipase systems (see section on cooperativity). The term 'non-enzymic', therefore, has to be taken with a grain of salt. One wonders whether even the neurotoxins may also be parts of complex catalytic systems. Recently, it has been reported that β-BuTX, a classical neurotoxin, displays a phospholipase activity as high as that of purified phospholipases A_2 from *Naia naia* and *Vipera Russelli*[90]. Among 'neurotoxins' we find, thus, enzymes, components of enzyme complexes, and polypeptides such as β-BuTX which need some special considerations before they can be related to enzyme processes[3,91]. In summary, enzymes are involved in many levels of venom action. It would make more sense to study each component as part of the intricate pattern of venom-prey interaction rather than to worry which is the most important one.

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