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THE ACTIONS OF SNAKE VENOMS ON NERVE AND MUSCLE. THE PHARMACOLOGY OF PHOSPHOLIPASE A AND OF POLYPEPTIDE TOXINS

B. S. MELDRUM

Medical Research Council Neuropsychiatric Research Unit, Woodmansterne Road, Carshalton, Surrey

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I. INTRODUCTION

Many of the features that distinguish snakes from other reptiles have arisen as adaptations to the limbless state. The secretion of a highly toxic venom and the adaptation of the jaw and teeth to facilitate its injection are clearly an example of this. The venom has two beneficial effects. The prey is immobilised and therefore easier to swallow, and digestion, which is slow with an intact prey, is initiated. Some non-venomous snakes such as pythons and boa constrictors achieve the same results by crushing their prey. Other non-venomous snakes eat diets of insects, eggs, or vegetable foodstuffs instead of small vertebrates. Venoms immobilise the prey rapidly and by very diverse means. These can be classified into actions on the neuromuscular system, with which this review is primarily concerned, and actions on the cardiovascular system, such as massive coagulation, multiple haemorrhages, acute hypotension through blood pooling or capillary dilatation, *etc.*, which it is not intended to cover in this review.

Recent reviews by Zeller (466, 469), Slotta (412) and Boquet (33) discuss broadly the great diversity of toxic and enzymatic actions displayed by snake venoms, and the book by Kaiser and Michl (221) gives a comparative account of the entire range of animal venoms.

There is general agreement that enzymes contribute to a) the local capillary damage and tissue necrosis produced by snake venoms (proteases, phospholipase A and hyaluronidase by direct action, and possibly L-amino acid oxidase by activating tissue proteases) (212, 220, 280, 282, 339, 469), b) the diverse coagulant and anticoagulant actions of venoms (various proteases: 33, 110), and c) the release of kinins from their precursors (arginine ester hydrolase) (182, 191, 370, 401). Among other enzymes of high activity which have been proposed as toxic elements in snake venoms are 1) monophosphatases (5-nucleotidase and a non-specific alkaline monophosphatase), 2) phosphodiesterases and related enzymes (nicotinamide adenine dinucleotidase, ATPase, ribonuclease), 3) cholinesterase, 4) L-amino acid oxidase (466). However, in a large number of studies in which the venoms of many species have been fractionated by chromatography or electrophoresis and enzyme activities of the separate fractions assessed it has been demstrated that none of these enzymes is responsible for the acute toxicity of snake venoms (see Sections III and VI and reference 388).

The only venom elements known to have pathological effects on whole nerve or muscle are the enzyme, phospholipase A, and the toxins which are heat-stable polypeptides or low molecular weight proteins without known enzymatic actions. This review, therefore, begins with a general account of the effects of venoms on nerve and muscle. A discussion of the problem of venom fractionation precedes a detailed account of the biochemistry and pharmacology of phospholipase A. Our very incomplete knowledge of "toxins" is summarised. Phosphodiesterases, cholinesterase and nerve growth promoting factors are briefly discussed because of the theoretical possibility of their involvement in changes in nervous function following envenomation.

II. ACTIONS ON NERVE AND MUSCLE

A. Acute animal experiments

Peripheral paralysis as a cause of death. Flaccid paralysis is the outstanding feature of poisoning with low doses of venom from snakes belonging to the families Elapidae (*Bungarus*, kraits; *Naja*, cobras; *Notechis*, tiger snakes; *Pseudechis*, Australian black snakes; *Dendraspis*, mamba; *Micrurus*, coral snakes, *etc.*) and Hydrophiidae (*Enhydrina*, sea snakes). Convulsive movements are a common terminal event.

In the first major study of paralysis by snake venoms, Fontana (139) showed, in many mammalian and avian species, that nerve conduction is not affected by venom but that the direct excitability of skeletal muscle is abolished. A hundred years later Brunton and Fayrer (40, 41, 42) observed that cessation of respiration precedes convulsive movements in many different species injected with Indian cobra (*Naja naja*) venom, and that the heart continues to beat in totally insensi-

ble animals. The convulsions can be stopped and started by the intermittent application of artificial respiration and were presumed to be anoxic (40, 41). The venom may abolish the direct excitability of frog or mammalian muscle though indirect excitability is often abolished when direct excitability remains. Large doses of cobra venom produce death by action on the cardiovascular system, but small doses kill by peripheral paralysis (40, 41). This view that peripheral paralysis of the diaphragm is the principal cause of death was soon supported (351) but subsequently contested (65, 112, 141, 142, 371, 372, 438). The criticisms of the theory include observations that stimulation of the phrenic nerve in an animal dead from venom produces a perceptible contraction of the diaphragm—but the same is true after poisoning with curare (229)—and that direct application of venom to the floor of the fourth ventricle produces respiratory arrest (141, 142). However, subsequent evidence has clearly established that the lethal effect of paralytic venoms stems from a peripheral action (229, 259, 440). Thus rhythmic respiratory movements of the facial muscles can be observed after respiration has ceased (12, 351). In the paralysed, artificially respired animal the Hering-Breuer reflex is still demonstrable in records of the rhythmic bursts of impulses in the phrenic nerve (79, 229). Their intensity increases during asphyxia produced by interrupting artificial respiration or during the administration of 5 % CO_2 (229, 259, 440), thus demonstrating the persistence of central respiratory control. With krait (*Bungarus*) venom the intercostal muscles are affected before the diaphragm, whereas the tibialis muscle is affected after the diaphragm (259).

Direct action on skeletal muscle. A curarized frog sartorius muscle placed in 2×10^{-5} g/ml cobra venom displays twitching and contraction and then relaxes (79). Cobra venom (5×10^{-6} g/ml) produces a block of neuromuscular transmission in the same time as 10^{-5} curare but this action is always accompanied by some diminution in the direct excitability of the frog muscle (204, 230). Cobra venom which has been heated at 100°C for 10 minutes at pH 5.5 depolarizes frog sartorius muscle (431). Houssay and his collaborators noticed a correlation between the haemolytic activity of various venoms and their direct actions on muscle as judged by the production of contractures and loss of excitability (203). Sodium citrate enhances and calcium chloride diminishes the muscle fibrillation produced by venom (202). Potassium, inorganic phosphate and soluble enzymes such as aspartate aminotransferase are released from muscle treated with crude or heated venom or lysolecithin (202, 272, 301). In these experiments demonstrating changes in muscle cell permeability *in vitro*, concentrations 10 to 100 times greater than those necessary to produce changes in muscle excitability were required. Myolysis restricted to the site of the bite is seen with many venoms (282). Only one venom (*Enhydrina schistosa*) produces a generalised increase in muscle cell wall permeability *in vivo* as manifested by raised serum potassium, raised serum transaminases and myoglobinuria (360, 361, 362).

Crotalus terrificus terrificus venom from certain regions of South America has a highly specific action on skeletal muscle (19). In the conscious rat or dog extensor spasms of the limbs are observed before the flaccid paralysis develops. Study of the responses of the rat sciatic nerve soleus-gastrocnemius preparation shows that

the spasticity arises from a direct action on the muscle that closely resembles that of veratrine alkaloids (242, 410). A single stimulus produces a normal twitch followed by a sustained contraction which is associated with an exceptionally large negative afterpotential. Repetitive stimuli at 30/minute produce a sustained tetanus which slowly fatigues and is abolished by various general anaesthetics.

Curare-like action. With mammalian muscle preparations a true neuromuscular block without significant changes in the excitability of the muscle to direct stimulation can be demonstrated with some Crotalid venoms and with most Elapid venoms, but Australian black snake *Pseudechis australis* is an exception (12, 19, 38, 40, 41, 51, 57, 58, 59, 113, 151, 227, 229, 259, 298, 300, 318, 343, 389, 398, 424). The neuromuscular block is slower in onset than that produced by curare. When it is only partially established a diminution in the block can be produced by anticholinesterases or specific antivenins (38, 57, 227). Once it is fully established *in vitro*, anticholinesterases or antivenins have little effect and, after washing out the venom, the block remains complete for as long as the muscle remains excitable. Several authors have presented evidence that neuromuscular blocking venoms have a postsynaptic site of action. Formosan cobra (*Naja naja atra*) venom and banded krait (*Bungarus multicinctus*) venom block the depolarizing action of acetylcholine on the frog sartorius (343) and block the contracture of frog rectus abdominis produced by acetylcholine (58, 424). The twitch response of a rat phrenic nerve-diaphragm to acetylcholine declines in parallel with the response to nerve stimulation in the presence of *Enhydrina schistosa* venom (51). *Naja naja* venom blocks the depolarizing action of carbachol in the rat diaphragm (298, 300). This is a "non-depolarizing" block of neuromuscular transmission, like that of curare, and is apparently independent of the depolarizing action of some venoms for skeletal muscle membrane. Thus *B. multicinctus* venom lacks this depolarizing action (58, 343) and from *Naja naja atra* venom a fraction can be separated ("najatoxin") which blocks neuromuscular transmission in the rat diaphragm but has no direct action on the muscle fibres (257). Saturation with curare can protect frog rectus muscle from exposure to doses of krait or cobra venom that would otherwise irreversibly abolish its sensitivity to acetylcholine (58, 424); this protection is understandable if the same receptor sites in the subsynaptic membrane are involved in the action of curare and venom toxins.

The release of acetylcholine by the rat phrenic nerve diaphragm preparation is not altered when complete neuromuscular block with *B. multicinctus* venom is first established, but some time later it declines to a very low level (57). This venom can be separated by electrophoresis on starch at pH 5.0 into four fractions. One lacks neuromuscular blocking properties but contains cholinesterase. One called "α-bungarotoxin" produces a neuromuscular block of rapid onset *in vitro* and *in vivo*. It does not alter the acetylcholine output of a rat diaphragm but it abolishes the response of the chick biventer cervicis muscle to acetylcholine. The two most electropositive fractions both produce a neuromuscular block and a severe reduction in acetylcholine output in the rat diaphragm only after a latent period of about one hour and this period is not shortened by increasing the dose. Neuromuscular block produced by these fractions in the chick biventer cervicis

muscle is not associated with any diminution of sensitivity to acetylcholine. Mice given large doses of these fractions show hyperirritability at first and, after a latent period of one hour, die suddenly with dyspnoea and convulsions (59). Crude *Naja naja atra* venom diminishes the acetylcholine output of rat diaphragm whereas "najatoxin" does not (257), a difference that suggests that cobra venom also has two means of producing a neuromuscular block.

An electronmicroscopic study of motor endplates in mice poisoned with crude cobra venom failed to reveal any structural changes (464).

Action on smooth muscle. Australian Elapid venoms produce a sharp contraction of the isolated guinea pig uterus which shows marked tachyphylaxis, leaving unimpaired the response to histamine (226). An increase in the rhythmic contractions of the stomach and intestines is produced by cobra venom and strong solutions (10^{-3}) constrict mammalian blood vessels (79). Among the known properties of venoms relevant to an action on smooth muscles are the release of kinins, acetylcholine, 5-hydroxytryptamine, histamine, slow-reacting substance and lysolecithin. Possibly none of these events is of primary importance; histamine release, for example, cannot explain the venom-induced contraction of the virgin rat uterus since this organ relaxes with histamine (226). The heat stability of the factor contracting the rat uterus is similar to that of the polypeptide toxins and that of phospholipase A.

Action on cardiac muscle. Naja naja venom produces slowing, and an increase in systolic tone, in the frog and rabbit heart at 10^{-6} g/ml (79, 112). Similar effects have been demonstrated with many other venoms (113, 117). High concentrations of venom rapidly produce systolic arrest of the heart which is not prevented by atropine (41, 163, 174). The striking effects of Crotalid venoms and the less decisive effects of Elapid venoms on the blood pressure are due not to effects on the heart itself but to changes in the pulmonary capillary resistance, to changes in systemic capillary permeability and to peripheral blood pooling (21, 26, 27, 386, 387). These peripheral vascular effects are not produced by the venom constituents which produce paralysis or central nervous system pathology. A fraction prepared from cobra venom possessing the direct cardiac actions of the venom ("cardiotoxin") also abolished the direct excitability of frog skeletal muscle (394, 396, 398).

Actions on peripheral nerve. Venoms do not block conduction in mammalian nerve trunks *in vivo* even with supra-lethal doses. Diminution of the excitability of amphibian nerve trunks produced by cobra venom has been reported frequently (67, 150, 203). In isolated single fibres of the frog sciatic nerve, excitability can be abolished by high concentrations of crude or acid-heated cobra venom (329). Lobster giant axons can be made inexcitable and depolarized by 3×10^{-5} g/ml of crude or preheated cobra venom (431, 433) and a similar effect has been demonstrated with squid giant axons using Crotalid and other venoms, the most effective being *Ancistrodon piscivorus* (376, 377). Venoms abolish the electrical activity of nerves without producing any alterations demonstrable with the electronmicroscope (329, 432). The membrane properties of the lobster giant axon during exposure to cobra venom have been described by Narahashi and

Tobias (327). The resting potential declines irregularly, conduction being blocked when it falls to two-thirds normal. The action potential, threshold potential and membrane resistance decrease while the membrane capacity increases. *Crotalus terrificus* venom (10^{-4} g/ml) increases the threshold for electrical stimulation of an isolated frog nerve trunk without altering the magnitude of the maximal response (118) and 0.5 mg of venom can produce this effect in a 200-gram rat (19).

Actions on sensory receptors. Fontana in 1787 recorded that venom applied to the tongue produces a loss of sensation (139). More recently (228) cobra venom venom (10^{-5} g/ml) has been shown to block tactile receptors in frog skin and at higher concentrations to block the responses of the frog muscle spindle. In Macht's opinion central analgesic effects of cobra venom greatly predominate over peripheral effects in the whole animal (277).

Central actions when systemically applied. Technical difficulties produced by concurrent peripheral muscular paralysis or circulatory disturbances have led most authors to deal cautiously with this subject. The evidence supposedly demonstrating an action on the respiratory centre has been criticised above (II A). We are left with many observations of questionable significance. Brunton and Fayrer found that dogs given cobra venom lost some spinal reflexes when sensation as judged by cranial reflexes was unimpaired (40, 41). Houssay (201) reported that *Crotalus terrificus* venom in the "isolated" dog head produced transient vagal inhibition of the heart and stimulation of respiration with a latency of less than a minute. Impairment of cranial reflexes has been demonstrated in similar cross-perfusion experiments (144, 229) but simultaneous vascular changes led these authors to question the significance of the effect. Macht (277, 279) stated that a "neurotoxic" preparation from cobra venom antagonised the convulsant action of picrotoxin or camphor in mice, raised the pain threshold in guinea pigs, and in low dosage in cats produced signs of pleasure and in higher dosage sedation, ataxia and miosis of central origin. Other authors (81) were unable to detect any analgesic action in rats.

A recent report that 0.5 mg/kg of *Naja naja* venom or *Crotalus adamanteus* venom given intravenously to dogs or monkeys under pentobarbital anaesthesia produces complete electroencephalographic silence in less than one minute (although the average survival time is 1 to 2 hours) (439) is surprising as unanaesthetised animals receiving doses of this order exhibit organized behaviour for 10 to 100 minutes after the injection. Possibly the venom potentiates the anaesthetic action of the barbiturate.

Crude krait or cobra venom labelled with I^{131} passes into the cerebrospinal fluid of rabbits very slowly. Two hours after intravenous injection the ratio of blood counts to cerebrospinal fluid counts is 30/1 or 50/1. The kidneys become most heavily labelled, followed by the lungs, spleen, liver, stomach and intestines. The brain contains only 0.3 % of the injected dose (257). If these venoms act directly on the brain either the active component does not label well with I^{131} or the brain is exceedingly sensitive to the venoms and they should act when given intracerebrally in a dose less than one-hundredth of that needed systemically.

Actions when applied directly to the central nervous system. Fraser and Elliot (141, 142) produced respiratory arrest by applying *Enhydrina* venom to the floor of the fourth ventricle. Subsequently Pacella (341) reported that injection of 10 mg of the venom of *Naja naja*, *Crotalus terrificus* or *Lachesis alternatus* into the subarachnoid space in dogs produces a rapid rise in arterial blood pressure, slowing of the pulse, and later diminution in and arrest of respiration. Kellaway, Cherry, and Williams (229) found that powerfully paralytic Australian venoms (death adder *Acanthophis antarcticus*, copperhead, *Denisonia superba*) applied to the floor of the fourth ventricle in cats, produce only a transient increase in respiratory rate and depth. They felt that venoms locally applied to the brain might alter vascular tone and permeability rather than act directly on brain cells. This criticism likewise applies to a recent study in which intraventricular injections of up to 5 mg of venom from various Crotalidae (*C. adamanteus*, *C. atrox*, *C. horridus*, *Ancistrodon piscivorus*, *A. contortix*) were shown to produce parasympathetic and locomotor effects in cats (385). A recent study employing the same venom as Fraser and Elliot (*Enhydrina schistosa*) showed that the crude venom given directly into the medulla oblongata of rabbits is 10 to 100 times more toxic than when given subcutaneously (51). A fraction obtained by dialysis and containing the lethal and paralytic properties of whole venom when given systemically is not effective when injected into the medulla, but a fraction containing phospholipase A activity which has very low toxicity when given systemically is as effective in arresting respiration as whole venom when given into the medulla (51, 52). The lethal dose of *Naja nigricollis* venom injected into rabbits in the region of Ammon's horn is one-hundredth of the lethal dose injected intravenously, 0.004 mg producing recurrent bouts of severe motor excitement and death within a few hours (175). *Naja naja* venom, previously heated to 100°C for 15 minutes, injected into the lumbar intrathecal space in rats does not have a lower lethal dose than intraperitoneally administered venom, but sublethal doses (0.5 mg/kg) produce a paralysis of the hind limbs in a few minutes which improves in the following four weeks (205).

Actions on the blood-brain barrier. Cobra venom given into the carotid artery of guinea pigs or rabbits increases the permeability of the cerebral vessels, enabling trypan blue to enter the brain tissue (39). Various enzymes enhance the speed of action of barbiturates on mouse brain including trypsin, hyaluronidase and phospholipase C (from *Clostridium welchii*) (22). This action possibly arises through the modification of permeability barriers. Many Crotalid venoms have their most striking actions on capillary permeability and it is clearly established that basic toxins contribute to this effect (178, 180). Thus venoms potentially have several different ways of increasing the permeability of the blood-brain barrier.

B. Neurological signs in man

Envenomation. The clinical features of poisoning by elapid snakes from all parts of the world are similar. The variation in response between victims bitten by one species of snake is more impressive than the variation due to species differences (1, 2, 5, 23, 47, 48, 274, 363). Symptoms of systemic intoxication develop within 1

to 12 hours of envenomation and include drowsiness, apathy, a feeling of alcoholic intoxication or of receding consciousness, diplopia, blurred vision, headaches and heaviness of the eyelids. Among objective signs of elapid envenomation ptosis is the commonest, occurring for example in 39 of 54 cases described by Campbell in Papua (47). Paralysis of the extrinsic ocular muscles is common and the palate, jaw, tongue and neck muscles are also frequently affected. The clinical picture may resemble bulbar palsy. In cases showing complete paralysis the intercostal muscles are usually affected before the diaphragm and limbs. Sensation as tested clinically is not usually impaired even in severely paralysed subjects. Coma and twitching movements or convulsions may be seen as terminal events. After cobra bites, patients with partial paralysis, or with complete paralysis if artificially respired, recover full motor function in 3 to 7 days. The diaphragm recovers sufficiently to sustain life in 1 to 4 days, the ocular muscles recover in 2 to 4 days. Deep tendon reflexes may be absent when limb power has returned to normal. Following krait bites an acute ascending paralysis has been reported and this may be followed by chronic paralysis of the lower limbs.

Sea snake poisoning (360, 361) is characterised by early generalised muscle pains with myoglobinuria and, later, paresis and trismus. Victims surviving regain muscle power very slowly and may exhibit dystrophic signs for up to six months.

The systemic features of Crotalid poisoning are predominantly those of cardiovascular shock and include nausea, vomiting, pallor, weakness, apathy, circumoral paraesthesiae, muscle fasciculation, and incontinence (7, 273, 342, 364, 384, 446, 456). Cranial nerve palsies or generalised paralysis are sometimes seen following rattlesnake (*Crotalus*) or moccasin (*Ancistrodon*) bites. Some species, *e.g.*, *Ancistrodon rhodostoma*, never produce paralytic signs.

Use in therapy. The bibliography of Russell and Scharffenberg (390) cited more than 400 papers describing therapeutic uses of snake venoms. Some of these refer to effects on the coagulation of the blood (see 239) but a large proportion are concerned with actions on the nervous system.

A preparation from rattlesnake venom ("crotalin") was extensively used in the management of epilepsy at the beginning of this century (417) but its clinical effectiveness was never established.

Between 1933 and 1943, preparations of cobra venom were widely employed for analgesia, principally in the treatment of tabetic crises, trigeminal neuralgia, arthritis, angina and terminal carcinoma (45, 66, 78, 245, 246, 278, 319, 380, 421). It was commonly given daily by intramuscular injection in amounts representing 5 to 20 lethal doses for a 24-gram mouse (equivalent to 25 to 100 μg of crude cobra venom) and required several days to achieve a full effect. About 20 clinical studies testify to its effectiveness as an analgesic, permitting the discontinuance of morphine in many cases. It was markedly inferior to aspirin in the treatment of rheumatoid arthritis (429). Improvement was reported in locomotor ataxia (380) and in Parkinsonism (152). Very few toxic reactions were reported; nausea, vomiting and local pain were sometimes seen.

In the last ten years cobra venom therapy has been neglected but not abandoned. Its successful use in 6 cases of trigeminal neuralgia has been described

(455), and 100 to 200 mouse units given daily for several weeks appeared to produce an analgesic effect in terminal carcinoma (196). Reports that cobra venom benefited 80 to 90 % of two large series of arthritic patients (43, 271) were followed by a double-blind trial which failed to show any effect of venom treatment on arthritic pain (296).

C. Neuropathological findings in man and animals

Neuropathological changes in rabbits and monkeys have been demonstrated after the systemic injection of various Elapid venoms (208, 233, 247, 248, 250, 252, 253, 392, 393). The most constantly reported change is in the anterior horn cells and the cranial nerve nuclei depending on the type and dose of venom and the time to death; these cells show every stage of acute chromatolysis with ultimately cell vacuolation and nuclear degeneration. Similar changes are found in pyramidal and Purkinje cells in the monkey. Chromatolysis begins earliest (in 2 to 3 hours) and is most acute with the venoms of cobra (*Naja naja*) and sea snake (*Enhydrina valakadien*) but is also seen in the more chronic poisoning produced by venom from the krait (*Bungarus fasciatus* and *B. coeruleus*). It was not produced by viperid venom (*Daboia russelli*) (208, 251). Fragmentation of the myelin sheaths in peripheral nerves was sometimes seen, especially with sea snake venom. A Crotalid venom (*Trimeresurus flavoviridis*) produces severe degenerative changes of an unspecific nature throughout the guinea pig nervous system (340). Monkeys dying 36 hours after receiving *Crotalus* venom showed some chromatolysis in the brain and spinal cord (128). The chromatolysis induced in monkeys by cobra venom is apparently reversible. Monkeys treated with antivenin when paralysis appears (2 to 3 hours after the venom injection) show little or no chromatolysis if killed when the paralysis has improved on the same or the following day (248).

Some authors (229) have felt that the morphological changes described by Lamb and Hunter (248–253) can be attributed to anoxia or ischaemia rather than to a direct action of the venom on the nerve cells. Anoxic or ischaemic cell damage usually does not involve the anterior horn cells (198, 303), however, and there is a delay of several hours between the anoxic episode and the appearance of ischaemic cell changes; yet Lamb and Hunter saw changes 1 and ⅓ to 4 hours after giving the venom and within one hour of the onset of paralysis. Furthermore, Sanders, Akin, and Soret (392) have reported neuronal damage in monkey anterior horn cells after the injection of quantities of heated *Naja flava* venom insufficient to produce clinical evidence of paralysis. Although further study is needed, a direct action of some venom component on the motor cells of the brain and spinal cord seems probable. There is no evidence to link these morphological changes with the paralysis, hyperexcitability and convulsions produced by venom. It is possible that components of the venom responsible for the morphological changes are entirely different from those bringing about the functional changes.

The lack of data on the neuropathology of snakebite in man is surprising in view of the annual death rate of 30,000 for India alone (5). Lamb and Hunter

were unable to identify changes in the brain of a Krait-bite victim comparable to those seen in monkeys. No histological changes were detectable in the brains of eight victims of sea snake bite (288).

Positive reports concern venoms producing vascular lesions. Three victims of *Bothrops atrox* showed angionecrosis and focal perivascular lesions in the brain (379).

III. VENOM FRACTIONATION

A. Non-proteins

Invertebrate venoms contain many pharmacologically active substances either known to occur in the mammalian nervous system or closely related to such compounds. These include acetylcholine, urocanylcholine, histamine, dopamine, 5-hydroxytryptamine, homarine, octopamine, tetramine, various kinins and eledoisin (3, 6, 294, 404, 449). None of these substances has been identified in snake venoms. However, Fischer and Dorfel (129) found adenosine in certain venoms (12% by weight in *Bitis arietans* venom, 2.6% in *Dendraspis viridis*). They also reported a positive test for sugars with *Bothrops* venom and with several *Viperid* venoms. Cobra venom contains some cholesterol, free lecithin and protein-bound lecithin (147). Fractions with absorption maxima at 252 and 257 mμ can be separated from Formosan cobra venom and are probably nucleic acid derivatives (461).

Zinc occurs at a concentration of 5 mg/g in cobra venom and rather less in krait, viper and other venoms (92, 223, 224, 357). It is not significantly associated with the phospholipase A or the neurotoxin. Possibly, by inhibiting the phosphatases and other enzymes, it protects the venom gland from damage by its own secretion (134, 135). Protein-bound copper at a concentration of 1 to 4 mg/g is present in *Naja naja, Bitis arietans, Vipera palestinae* and other venoms (156). Magnesium (5 mg/g) has been reported in cobra venom (224).

B. Fractional precipitation of proteins

Around the end of the nineteenth century many authors reported that snake venoms could be separated into toxic and non-toxic protein fractions by precipitation with salt, ammonium sulphate, alcohol or heat (222, 289, 290, 316, 317, 332). Mitchell found that some venoms contained a dialysable toxic factor which he described as a peptone. However, Faust (119, 120) claimed that the toxicity of cobra and rattlesnake venoms belonged to nitrogen-free components. Some uncertainty remained (238, 240, 448) until 1936, when many reports of fractional precipitation of venoms into toxic and non-toxic fractions began to appear from Germany and India (88, 146, 147, 154, 154a, 155, 304, 305, 381, 397, 453). This work was reviewed by Slotta (412) and much of it has now been superseded. The two aspects which will be discussed are the isolation of crotoxin from rattlesnake venom and the preparation of "neurotoxin" from cobra venom.

Slotta and Fraenkel-Conrat (411, 414, 415) isolated from *Crotalus t. terrificus* venom by heat treatment, ammonium sulphate precipitation and crystallisation

with acetic acid-pyridine, crotoxin, a protein crystallising in quadratic plates. Gralen and Svedberg (164) undertook sedimentation and diffusion measurements and reported that crotoxin was a single protein with a molecular weight of 30,000. Li and Fraenkel-Conrat (266) showed that crotoxin was electrophoretically homogeneous from pH 3.91 to 7.20 and had an isoelectric point at pH 4.71. This protein possessed the paralytic and toxic actions of the original venom and also the phospholipase A and hyaluronidase activity (412). However, Slotta later (412, 413) separated crotoxin chromatographically into two fractions, only one of which was haemolytic. Neumann and Habermann first (330) isolated chromatographically from whole *Crotalus t. terrificus* venom a non-toxic preparation of phospholipase A. Later they (178, 187) prepared from crotoxin by ion-exchange chromatography both a toxic substance, "crotactin," and a non-toxic preparation of phospholipase A. Fraenkel-Conrat and Singer (139a) obtained two dinitrophenol derivatives from crotoxin, the larger one (M.W. 18,000) having a higher content of dibasic amino acids than the smaller one (M.W. 12,000). The history of crotoxin illustrates a recurrent difficulty in the fractionation of venoms, namely the tendency for two venom components to occur bound together either in a highly specific fashion or in simple electrostatic attraction.

Various authors by fractional precipitation, adsorption and electrophoresis have prepared fractions of cobra venom with high toxicity and low enzymic activity. These preparations have been called "neurotoxin" without any specific proof of their purity (147, 154, 154a, 155, 305, 453). Measurement of dialysis coefficients suggested a molecular weight between 1,500 and 4,000 (304, 453). The toxic substance appears to be a basic polypeptide containing 5% sulphur (in cystine) and arginine, histidine and lysine (153). More recent studies on substances of this type are considered in Section VII.

A fraction of cobra venom with a marked toxicity for the perfused toad heart was prepared by precipitation procedures and called "cardiotoxin" (394, 396). By diffusion its molecular weight was found to be about 46,200 (395) but this value is possibly far too high (see Section VII).

Several enzymes including cholinesterase and phospholipase A were prepared by fractional precipitation. The phospholipase A was called "haemolysin" and assayed by the production of haemolysis in the presence of lecithin. Although a crystalline preparation was obtained, it was probably contaminated with basic substances of low molecular weight because its apparent isoelectric point was too basic (84, 85, 86, 87, 154a) compared with the recent data of Dawson (82).

C. Electrophoresis

Electrophoretic and chromatographic techniques for protein separation have been applied to a wide range of snake venoms. Most crude venoms display 6 to 12 different enzymic activities and about 8 to 30 separate measurable actions on biological systems. To measure the percentage recovery of all these in each separable fraction is impracticable. Most studies have been designed to show that particular enzymes and biological actions cannot be attributed to a single substance as the activities can be separated.

Polson, Joubert, and Haig (348), using free electrophoresis of various cobra venoms at pH 6.2, found some non-toxic fractions and some which possessed both haemolytic and toxic activity. Goncalves and Polson (161) identified protease and toxicity in fractions obtained by free electrophoresis of *Crotalus terrificus* and *Bothrops jaracacussu* venoms at different pHs. They recognised that complex formation between proteins with opposite charges is a critical factor in venom fractionation.

Paper electrophoresis (131, 160, 167, 177, 186, 306) revealed as many as eight components in cobra venoms (*Naja naja*, *Naja nigricollis* and *Naja haje*), and, although most fractions possessed toxic activity, it was possible to show that the phospholipase had been separated from a factor acting on skeletal muscle and from a factor producing direct haemolysis. The presence in some samples of *Crotalus t. terrificus* venom of a basic toxin, called crotamine, can also be demonstrated by this means.

The use of supporting media for electrophoresis permitted further advances. Bussard and Côté (46) running *Naja naja* venom on a starch column at pH 9.0 separated the phospholipase from a toxin which will pass through a cellophane dialysis membrane. Starch column electrophoresis fractionates the venoms of Habu (*Trimeresurus*), Formosan cobra (*Naja naja atra*) and *Ancistrodon actus* (337, 339, 459, 462). With the latter venom, for example, the toxicity is recovered separate from the phospholipase A, phosphodiesterase, phosphomonoesterase, 5-nucleotidase, ATPase and proteinase activities.

Agar gel electrophoresis of *Naja naja* venom at pH 6.8 reveals five fractions all moving to the cathode (102, 103). From the most mobile band 60% of the initial toxicity is recoverable. Other bands contain cholinesterase, phospholipase A and L-amino acid oxidase.

Starch gel electrophoresis (349, 416) was first applied to snake venoms by Michl and Kiss (307) and subsequently by Master and Rao (291, 292) and Jimenez-Porras (215, 216, 217). Cobra venom was shown to yield nine separate fractions identifiable as cholinesterase, phospholipase A (plus anticoagulant), L-amino acid oxidase, protease, phosphodiesterase, 5-nucleotidase, protease, neurotoxin and phosphomonoesterase. Subsequent authors (297, 300, 328) have found additional stainable components—13 or 14—but have not identified the fractions so fully. Russell's viper venom separates into 8 fractions, none of which is toxic when given separately; recombination of the anode fractions, which include coagulase, protease and L-amino acid oxidase, restores the toxicity. Krait (*Bungarus caeruleus*) venom displays 10 stainable components of which five are toxic. *Crotalus atrox atrox* venom shows 10 stainable bands on starch gel electrophoresis at pH 8.9 (215). The enzymatic activities show some overlap between bands. Phosphodiesterase, ribonuclease, ATPase, NADase and 5-nucleotidase move to the cathode; trypsin, phospholipase A and L-amino acid oxidase move toward the anode. Similar separations were achieved with *Bothrops atrox* and *Bothrops numifera* venoms (216, 217).

Immunoelectrophoresis or the simpler gel diffusion procedures have not been applied to the study of snake venoms as extensively as might be expected in view

of the availability of antisera. These techniques not only provide information about the number of components in crude preparations and the purity of specific fractions but can also be used as a guide to the relationships between venoms of different species (52, 102, 104, 166, 241).

Chromatography. Ion-exchange chromatography has been extensively applied to the fractionation of snake venoms. Combining ion-exchange chromatography with gel filtration, Bjork (28, 31) has separated from the venom of Ringhals cobra (*Hemachatus haemachates*) two phosphodiesterases, lecithinase A, 5-nucleotidase, cholinesterase and two toxins. Formosan cobra venom on carboxymethylcellulose column chromatography yields seven fractions, two of which are basic (461). Indian cobra venom on dextran polymer (Sephadex) columns yields five fractions, two of which are toxic (but each contains at least three components on cellulose acetate strip electrophoresis) (368). Further fractionation on CM cellulose indicates that there are two toxic factors, one of which is stable to heat, the other labile (367).

Vipera palestinae venom on DEAE cellulose separates into five fractions (241). One fraction which has 10 antigens demonstrable by gel diffusion also contains a neurotoxin producing respiratory paralysis. Another fraction with 4 antigens contains a toxin which produces tremor. A neurotoxin fraction free of proteolytic and anticoagulant activity can be prepared by ammonium sulphate precipitation of the DEAE cellulose fraction (323). The toxicity of this fraction is trebled by recombination with a non-toxic protease fraction of the venom. Preincubation experiments suggest that this enhancement of the toxicity is truly synergistic and not the result of degradation of the neurotoxic molecule (323). *Walterinnesia aegyptia* venom similarly yields on chromatography two complex fractions which produce haemorrhages, paralysis and convulsions, a non-lethal fraction, and a fraction containing phospholipase (157).

Japanese Habu (*Trimeresurus flavoviridis*) venom on CM cellulose yields two RNAases, one DNAase, two phosphodiesterases, phosphomonoesterase, and three 5-nucleotidases (280, 281, 338). The toxicity is associated with two of the 5-nucleotidases.

Two neurotoxins have been separated from *Notechis scutatus scutatus* venom using Amberlite IRC 50 (106). One toxin produces paralysis after 1 to 7 days; the other produces convulsions within a few hours.

In summary, no venom has yet been comprehensively fractionated and there is no one procedure that gives a complete separation of the toxic fractions and enzymes. Starch gel electrophoresis gives the most complete one-stage fractionation, but several of the pharmacological activities of venoms, including nerve growth factor, have yet to be assayed in these fractionations. Ion-exchange chromatography, either in multiple stages or combined with fractional precipitations, tends to be the best preparative procedure. Selecting satisfactory criteria of purity has proved to be surprisingly difficult. Gel diffusion analysis against crude antisera appears very valuable for this purpose and could be more widely applied.

The separation of the components of snake venoms has proved more difficult than that of other body fluids containing several proteins primarily because of the

way toxic fragments are bound to, or combine with, other proteins. The nature of this combination is obscure. In some venoms, such as *Naja naja*, simple electrostatic forces seem to be involved, while in other venoms, such as *Crotalus terrificus*, more highly specific mechanisms seem probable. We cannot exclude the possibility that disruption of chemical bonds by enzymic or physical means occurs in the preparation of some of the low molecular weight toxins described in Section VII.

IV. PHOSPHOLIPASE A

A. Biochemical aspects

Substrate specificity. Phospholipase A (phosphatide-acyl hydrolase, E.C. 3.1.1.4) characteristically hydrolyses only one of the fatty acyl ester linkages in diacyl phosphatides. The phospholipase A found in mammalian pancreas closely resembles snake venom phospholipase A in substrate specificity, "activation" requirements and heat stability, and most of the following account applies equally to enzyme preparations from both sources. The naturally occurring phosphatides that serve as substrates (see Figure 1) are phosphatidyl-choline, -serine and -ethanolamine, phosphatidal-choline (and probably phosphatidal-serine and -ethanolamine), phosphatidic acid and cardiolipin. Hydrolysis of phosphatidyl inositol has recently been reported but diphosphoinositide and sphingomyelin are apparently not attacked (90, 225, 269, 285, 286, 352, 353, 437).

The view that only α-phosphatides are hydrolysed by phospholipase A (269) has been revised since it has been shown that (91a) *Crotalus* venom releases one equivalent of fatty acid from α-γ-distearoyl β-glyceryl phosphoryl choline. However, the naturally occurring phospholipids are predominantly if not entirely α forms. The enzyme has steric specificity, attacking L-α forms and not D-α forms (89, 269). It is now clear that in α-phosphatides the β-ester linkage is specifically attacked (91, 193, 287) by pancreatic phospholipase A and by all snake venoms specifically tested but not by splenic phospholipase A (267). Both saturated and unsaturated fatty acids are released (286). An apparent preference for unsaturated fatty acids arises because of a preponderance of unsaturated fatty acids in the β position in naturally occurring phosphatides (391). Synthetic phosphatides with short-chain fatty acids are attacked (373) but at a slower rate than long-chain fatty acids (437). Van Deenen and De Haas (437), by testing a wide range of synthetic substrates, have been able to define the chemical structure essential for a compound to serve as a substrate for *Crotalus* phospholipase A. From the enzyme's known positional specificity and the fact that it can hydrolyse β-acyl lysocompounds (β-acyl glycerylphosphoryl ethanolamine) and the glycol homologue of lecithin, it appears that the essential requirement is for a fatty acyl ester bond adjacent to the phosphoryl alcohol linkage with a favourable steric configuration. A study of the products released from egg yolk lecithin after short incubations with *Crotalus* venom has shown that the rate of hydrolysis depends not only on the saturation of the fatty acid in the β position but also on that of the α-fatty acid (320, 321).

Assay systems, "activators," and pH optimum. Phospholipase A presents a

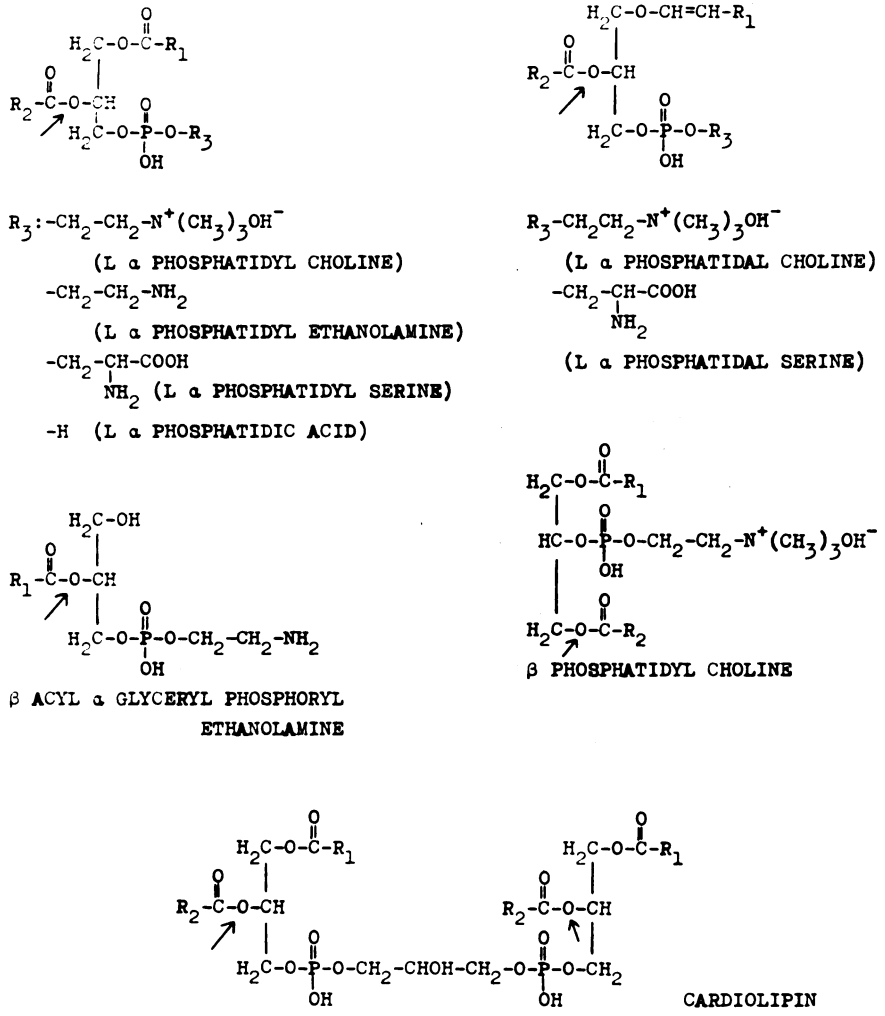

FIG. 1. Structural formulae of the main classes of substrates for phospholipase A.

R_1 and R_2 are saturated or unsaturated hydrocarbon chains. The arrows indicate the site of enzymatic hydrolysis. (An alternative structural formula to that shown for cardiolipin has recently been proposed (374) but the diphosphatidyl glycerol structure has now been proved by synthesis (91b)).

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particular difficulty for the enzymologist in that its principal substrates ar. insoluble in water. Early authors used an emulsion or suspension of egg yolk Hughes, having shown that the surface potential of a unimolecular film of lysolecithin was only ⅗ that of a lecithin film, used the reduction of this potential of a lecithin film as a measure of phospholipase A activity (206, 207). Although it is possible to use a water soluble substance such as dicaproyl lecithin (373) or serum lipoproteins (73) most authors have used synthetic or chromatographically purified phospholipids either in wet diethyl ether (192, 269) or in water with an

emulsifying agent such as sodium deoxycholate or an "activator" such as ether (82, 283, 284). The reaction can be followed by measuring the rate of release of fatty acid (manometric or titrimetric) or of lysolecithin formation (chromatographic) or ester-linkage loss (hydroxylamine reaction). The requirements for "activators" and the pH optimum vary according to the source of the enzyme, the buffer system and the substrate used. Magee and Thompson (284), using *Ancistrodon piscivorus* venom to hydrolyse ovolecithin in 2–4–6 collidine buffer, found that the addition of 10% ether greatly increases the rate of hydrolysis; calcium ions are necessary, and variations in pH between 6.5 and 8.5 have little effect on the initial reaction velocity. Dawson (82), using an enzyme prepared from cobra venom by density gradient electrophoresis, found that it was necessary to add ether to the buffer in order to activate the hydrolysis of lecithin but not of phosphatidylethanolamine; the addition of calcium ions was obligatory. The rate of hydrolysis of lecithin did not vary much between pH 6 and 9, but phosphatidylethanolamine was hydrolysed more at higher pH. Ether apparently owes its "activating" power to its ability to disperse lecithin particles into smaller units and to prevent the orientation of fatty acids at the lipid water interface. Ultrasonic dispersion of a lecithin sol also permits more rapid hydrolysis by phospholipase A (83).

Physical properties: isoenzymes. Hughes (207) showed that phospholipase A in cobra venom is not affected by boiling for 15 minutes at pHs below 5.9 but that heating at pH 7 or above destroys it. Subsequently it was shown that pancreatic phospholipase A has a similar heat stability (170, 171, 283).

Assessment of the enzyme's isoelectric point is made difficult by the possibility that basic toxins are adsorbed to, or complexed with, the enzyme. The isoelectric points of enzyme from different venoms vary widely; many venoms contain 2 or 3 enzymes with phospholipase A activity separable by chromatography or electrophoresis. Saito and Hanahan (391) prepared two phospholipases from *Crotalus adamanteus* venom which were 80 to 85% pure and were found by sedimentation analysis to have molecular weights around 30,000 to 35,000. Their enzymic activities were similar but their isoelectric points were at pH 4.40 and 4.55. Only one phospholipase A has been found in *Crotalus t. terrificus* and this has an isoelectric point near pH 6.5 (178). Electrophoresis of *Vipera russellii* venom reveals 3 separate phospholipases, two of which are capable of hydrolysing the phospholipids in osmotically prepared red cell ghosts directly, while the third one can do so only if an activator (such as DLF—see below) is added (74). *Pseudechis porphyriacus* venom contains a family of phospholipases separable by chromatography (108). Two lecithinases separated from Formosan cobra venom were found to have different pH optima and different enzymic properties (comparing rates of hydrolysis of egg and yeast lecithins in an unconventional assay system) (445). Two enzymes able to pass through a dialysis membrane are distinguishable by electrophoresis in the venom of Ringhals cobra (*Hemachatus haemachetes*) (28). *Naja flava* venom has two phospholipases (328) but *Naja naja* has only one. Dawson (82) found the phospholipase from *Naja naja* venom had an isoelectric point at pH 5.2 compared with values of 8.55 and 8.62 found by De (87) for crystalline "haemolysin" prepared from the same venom.

B. Actions in vitro

Cell lysis. Haemolysis. Haemolysis by snake venoms was described in the nineteenth century (315, 422) and the ability of lecithin to act as a "complement" in haemolysis was soon appreciated (243, 244). In a thesis in 1905, Ludecke suggested that venom produced a lytic compound by enzymatic action on lecithin, and Delezenne and Ledebt (94, 95, 96, 97) clearly demonstrated that an enzyme in cobra venom formed a haemolytic substance when it acted on horse serum or egg yolk. They showed that lecithin was the substrate and fatty acid a reaction product. Subsequently Delezenne and Fourneau (93) reported that "lysocithine" was palmito-glycero-phosphorylcholine. From this time onwards there has been widespread acceptance of the view that haemolysis by snake venoms is due to the action of phospholipase A, either "indirectly" by producing lysophosphatides from serum or added lecithin, or "directly" by hydrolysing phospholipids in the red cell membrane. Hughes (207) and others subsequently (74, 301) have shown that if snake venoms from different species are compared there is no correlation between their phospholipase A content and their ability to haemolyse washed red cells. Phospholipases from different species of snake undoubtedly differ, but this is not the principal explanation. Grassmann and Hannig (167), using paper electrophoresis at pH 8.6, separated the direct haemolytic action of *Naja naja* from its phospholipase A activity. Habermann and Neumann (186) separated *Naja nigricollis* by paper electrophoresis at pH 7.0 into 5 fractions moving to the cathode, of which the second and third possessed the direct lytic action and the fourth and fifth (the least mobile) contained the phospholipase A. Doery and Pearson (108) prepared chromatographically from *Pseudechis porphyriacus* venom a phospholipase A free from direct haemolytic action. The powerful direct haemolytic activity of the crude venom was accounted for by the most electropositive component of the venom. Condrea, De Vries, and Mager (74) fractionated *Hemachatus haemachates* and *Naja naja* by paper electrophoresis at pH 7.0 and found a direct lytic factor (DLF) among the most electropositive fractions of both venoms and the phospholipase A (free from direct lytic activity) among the least electropositive fractions. The DLF closely resembles phospholipase A in its heat stability. Increasing concentrations of whole venom produce rates of haemolysis and of fatty acid release from the washed red cells which increase in a closely parallel fashion. Combining a non-haemolytic concentration of DLF from cobra venom with a phospholipase A preparation from a venom incapable of causing direct haemolysis (*Vipera palestinae*) produced rapid haemolysis and phospholipid hydrolysis with washed red cells. Thus a basic component of cobra venom can make phospholipids in the red cell membrane accessible to phospholipase A, as also can surface active agents such as digitonin and saponin. Protamine and polylysine, which can produce haemolysis (20), are not "activators" for phospholipase A at the red cell membrane. A difference between enzymes was shown, in that *Naja naja* phospholipase A can attack osmotic haemolysates of red cells without any activator whereas *V. palestinae* phospholipase A can do so only in the presence of DLF or digitonin.

Red cells of different mammalian species show striking variations in their

susceptibility to haemolysis by cobra and other venoms (232). Some resistant cells such as those from ox, sheep and goats have a low lecithin content (434, 435) and the resistant cells of the camel liberate their lecithin less readily into ether-ethanol than do cells susceptible to haemolysis. However, it is clear that washed cells which resist lysis by *Naja naja* venom do so because the direct lytic factor does not affect them. *V. palestinae* venom hydrolyses the phospholipids in sheep and camel red cell ghosts in the presence of saponin but not in the presence of DLF from cobra venom even though this permits it to hydrolyse human or rabbit ghost phospholipids (75).

Other cell types. Snake venoms produce microscopically observable lysis in a wide range of cell types including spermatozoa, ova, nerve cells and liver cells (137, 138). This is not prevented by heating the venom in solution but is prevented by heating the tissue to 56°C for 30 minutes. Preheated tissues can be lysed by venom if unheated serum is added.

Houssay (202, 203) found that snake venom or lysolecithin applied to frog skeletal muscle produced contractures, loss of excitability, muscle swelling and release of potassium and inorganic phosphate. A compound resembling lysolecithin could be prepared from muscle treated with venom. He concluded that phospholipase A was responsible for the direct action of venom on muscle. Subsequent authors (272, 301) have shown that lysolecithin and heated snake venom promote the efflux of a soluble enzyme, aspartate aminotransferase (E.C.2.6.1.1.), from brain slices and rat diaphragm.

Pancreatic phospholipase A does not release fatty acids from rat diaphragm or increase the outflow of transaminase from it in the absence of any activator (211). Although whole cobra venom releases fatty acids and promotes enzyme outflow from the rat diaphragm, the ability of phospholipase A to attack the undamaged muscle cell membrane in the absence of toxins or activators remains unproved. Reports of the action of phospholipase A on brain slices differ. The release of fatty acids and of transaminase from rat brain slices in glycylglycine buffer are linearly related to each other using either pancreatic phospholipase A or cobra venom. Apparently no activator is required for phospholipase A to be able to hydrolyse brain phospholipids (210, 211). However, chromatographic examination of phospholipids from rat brain slices incubated in saline with whole cobra venom or purified phospholipase shows that, even in the presence of DLF, no significant hydrolysis of phospholipids occurs. Yet brain homogenates incubated with whole venom or purified phospholipase show a rapid conversion of phospholipids to lysophosphatides (237).

Demyelination of spinal cord slices *in vitro* by snake venom and by *Cl. welchii* α-toxin (324, 447) is apparently attributable to the action of phospholipases.

Human blood platelets are lysed by cobra venom phospholipase (separated by paper electrophoresis) and their lecithins, phosphatidyl-ethanolamines, and phosphatidyl-serines are converted to lyso compounds. *Vipera palestinae* phospholipase can attack platelet phospholipids only in the presence of cobra venom DLF or other surface-active agent (234).

Release of histamine and similar substances. Feldberg and Kellaway (126)

showed that cobra venom releases histamine from the perfused lung of a cat or dog. A substance which produces a slow contraction in the guinea-pig jejunum also appears in the effluent. There is no doubt that phospholipase A from snake or bee venom can release histamine from rat diaphragm or mast cells (179). The degranulation of rat mesentery mast cells produced by phospholipase A or by compound 48/80 is prevented by anoxia, substrate deficiency, metabolic inhibitors, or warming the mast cells to 45°C, (436). Apparently phospholipase A or 48/80 merely triggers a process requiring metabolic energy that leads to the release of histamine and 5-hydroxytryptamine.

The slow-reacting substance released by phospholipase A from perfused tissues or from lung lipid extracts is a mixture of polyunsaturated fatty acids which are capable of producing haemolysis (308, 443). These fatty acids probably owe their smooth muscle stimulating action to the formation of hydroxy fatty acids or hydroperoxides (15, 80, 444). Both groups of reaction products of phospholipase A (lysophosphatides and fatty acids) are potentially significant pharmacological agents. The contraction of the guinea pig ileum produced by a bee venom fraction containing phospholipase A (179) which exhibits marked tachyphylaxis might involve a direct action of the enzyme or a release of histamine, lysolecithin, fatty acids, or some other transmitter.

Venoms can release other neurohormones from tissue preparations, and these effects may be due to phospholipase A. The cat adrenal gland perfused with cobra venom or lysolecithin releases adrenaline (122). If this happens *in vivo* it could explain the rise in blood sugar in cobra venom poisoning (24, 25, 113, 165). There is no decisive evidence that phospholipase A either directly or indirectly causes a release of adrenaline *in vivo*. Another component of crude venom which could release adrenaline is the enzyme which forms bradykinin, since in low doses in the rabbit bradykinin causes the release of adrenaline (265). Cobra venom and lysolecithin release "bound" acetylcholine from guinea pig brain homogenates (148, 149). The conversion of "bound" to "free" acetylcholine has been proposed as an explanation for the increased synthesis of acetylcholine by brain tissue produced by venom *in vitro* (36). This distinction between "bound" and "free" acetylcholine is probably without physiological significance, but in brain homogenates there are real differences in the solubility of acetylcholine. Whittaker (452) found that all the acetylcholine in a subcellular brain fraction was released by chloroform or ether but only part of it by hypotonic solutions or cobra venom.

Effects on oxidative metabolism. Inhibition of glycolysis and of oxygen uptake by cobra venom was first demonstrated in cancer cells by Mellanby (302). Chain (53, 54) showed that many venoms prevented glycolysis by muscle extracts and that various dehydrogenases were inhibited by black tiger snake venom (55). He suggested that inactivation of nicotinamide-adenine dinucleotide by a nucleotidase was responsible. (Actions of venoms on nucleotides are discussed in Section VI.) Subsequent authors have paid more attention to effects on the later stages of the respiratory chain. Braganca and Quastel (37) found that cobra venom heated to 100°C for 15 minutes inhibits the oxygen uptake of brain slices. Succinic dehydrogenase and cytochrome oxidase are inhibited by venom but "soluble"

enzymes are unaffected. Crystalline crotoxin (0.005 mg in 5 ml) or a pancreatic phospholipase A preparation (100 mg in 5 ml) was found by Nygaard and Sumner (336) to abolish the succinoxidase activity of rat liver mitochondria. The effect is complete when less than 10 % of the mitochondrial lecithin has been hydrolysed. Inhibition of succinic dehydrogenase or cytochrome oxidase requires a greater concentration of crotoxin. The factor most sensitive to phospholipase A is located between cytochromes b and c (334). Crotoxin or lysophosphatides produce swelling of isolated mitochondria and disruption of mitochondrial membranes (335, 457). These effects of venoms on mitochondrial oxidations have been confirmed and extended (17, 111, 345, 346, 430). Formosan workers (458, 460, 463) concluded that the inhibition by cobra venom fractions and by crotoxin of the succinate cytochrome-c reductase activity of heart muscle homogenates was not produced by enzymic destruction of lecithin because heat treatment and reduced glutathione do not affect the lecithinase activity and the inhibitory potency to the same extent. However, the effects on oxidative phosphorylation of fractions from *Naja flava* venom and from bee venom vary much more closely with their phospholipase A activity than with their toxicity (176, 350). Phospholipids play an essential role in most of the stages of electron transfer; indeed extracting phospholipids from mitochondrial preparations with acetone-water destroys their capacity to carry out electron transfer. This capacity can be restored by the addition of the appropriate phospholipids (168). These critical phospholipids are probably accessible to the enzyme of cobra or rattlesnake venom.

Recently krait (*Bungarus fasciatus*) venom has been shown to have an effect on mitochondrial preparations besides the uncoupling of oxidative phosphorylation produced by phospholipase A. After exposure to venom the mitochondria display inhibition by adenosine 5′-diphosphate (reverse acceptor control) and it is suggested that this effect is produced by an esterase present in fresh venom (471).

Some of the inhibitory action of cobra venom on the oxidation of succinate by mitochondrial preparations is probably attributable to free or protein-bound metals. Ashed cobra venom retains 60 % of this inhibitory action on mitochondria, and EDTA can abolish this inhibition (266a). Copper is known to inhibit mitochondrial oxidations (344a) and zinc, copper and magnesium are present in venoms, as described in Section IIIA above.

Miscellaneous effects. There are other effects of venoms *in vitro* that are apparently caused by the hydrolysis of phospholipids. These include the inactivation of brain or lung thromboplastin by venom from *Naja naja* or *Naja nigricollis* (177); this probably contributes to the anticoagulant action of cobra venoms. Heated cobra venom inhibits the synthesis of corticoids from cholesterol or other intermediates by rat adrenal homogenates (344). *Crotalus* venom prevents the esterification of cholesterol by acetone powders of rat plasma, apparently by destroying phospholipids (409).

C. Actions in vivo

There are few reports of experiments in which satisfactory preparations of phospholipase A were employed *in vivo*. Phospholipase A from any source will

rapidly produce lysophosphatides and free fatty acid from serum. Activators are not necessary and the phospholipids in lipoproteins are among the most accessible forms of substrate (73). Phospholipase A separated chromatographically from *Vipera palestinae* venom when given intravenously to rabbits produces within 5 or 10 minutes a three- to six-fold reduction in plasma lecithin and a smaller increase in plasma lysolecithin. The apparent loss of phospholipid is partially accounted for by adsorption of lysolecithin onto red blood cells. These changes progressively diminish over the following two hours. The same phenomena are seen after the injection of bee venom phospholipase A (185). Red cell sphering occurs concurrently with the change in plasma phospholipid and a decrease in red cell survival can be demonstrated in the following days (105). No pigments are detectable in the urine. Apparently the rate of red cell destruction in the spleen increases. This failure of lysophosphatides to produce any striking intravascular haemolysis is probably due to the protective action of plasma cholesterol, phospholipids and proteins (14, 93, 232). A transient increase in respiratory and heart rates with pupillary constriction is produced in rabbits by intravenous *V. palestinae* phospholipase A (235). An impure phospholipase A fraction from bee venom produces a prompt, transient fall in cat blood pressure which shows marked tachyphylaxis, without any change in the response to acetylcholine or histamine (180).

Phospholipase A is probably responsible for the action of venoms in protecting animals against some bacterial toxins. The protective effect of fractions from *Pseudechis porphyriacus* venom against staphylococcal toxin in mice varies with the phospholipase A content, and a similar protective action is displayed by both lysophosphatides and unsaturated fatty acids (107, 333, 418, 419).

It has been claimed that effects of phospholipase A on brain lipids and oxidative phosphorylation can be demonstrated *in vivo* (9, 10, 11). The fact that brain lipid preparations from mice injected with lethal doses of heated cobra venom are more haemolytic than control preparations (9) is not (as was claimed) proof of lysophosphatide formation, as basic polypeptides resembling the haemolytic toxin in heated cobra venom are known to bind readily to phospholipids (168). The effects of lethal doses of heated cobra venom and "crystalline phospholipase A" on oxidative phosphorylation in mitochondrial preparations from the brains of mice injected with the agent thirty minutes prior to sacrifice, are of considerable interest (10, 11). The crystalline phospholipase A used here was prepared by a precipitation method (426) and showed marked toxicity (75 μg of heated venom or 9 μg of crystalline phospholipase A produced death in 2½ hours in a mouse). As phospholipase A prepared from cobra venom by gel electrophoresis is non-toxic (103, 291, 297, 298), it seems probable that this precipitated preparation is contaminated with toxins. Restating the observation of Aravindakshan and Braganca (10, 11), mitochondrial preparations from brains of mice injected with enzyme plus toxins show a preferential inactivation of the phosphorylation located in the cytochrome oxidase system.

A synthetic inhibitor of phospholipase A [2, 3 distearoyl oxypropyl (dimethyl) β-hydroxyethylammonium acetate] protects mice against the lethal action of

moccasin (*Ancistrodon piscivorus*) venom if premixed with the venom (378). This is not good evidence that phospholipase A contributes to the venom's toxicity as cobra venom is effectively inactivated by mixing it with an oil-in-water emulsion (143).

V. PHOSPHOLIPASE B AND C

Phospholipase B activity (lysolecithin acyl-hydrolase E.C.3.1.1.5, effecting the hydrolysis of lysolecithin to fatty acid and glycerophosphocholine—see Figure 2) has recently been demonstrated in a wide range of snake venoms (109). *Ancistrodon piscivorus* and *Naja naja* venoms display activity only at pH ranges above 8.0, whereas *Pseudechis porphyriacus* venom is active over the pH range 6.5 to 11.0. The latter enzyme was not inactivated by heating at 100°C at pH 7.0. Calcium ions have an activating effect. Phospholipase A can hydrolyse β-acyl glycerophosphorylcholine but not the α-acyl form, whereas in these experiments the lysolecithin formed by the action of phospholipase A (*i.e.*, α-acyl forms) was hydrolysed. However, this activity has not been demonstrated in the absence of phospholipase A (in fact the three chromatographic fractions from *P. porphyriacus* venom containing phospholipase A all contained phospholipase B activity) and so the possibility of spontaneous or enzymatically induced transacylation must be considered, although it seems improbable. The possible presence of phospholipase B should be remembered in future studies. Other authors (236) were unable to detect phospholipase B in Ringhals cobra (*Hemachatus haemachetes*). This enzyme is found in wasp and hornet venoms (76, 77, 140) and in toxin from *Vibrio cholerae* (60). Nothing is known of its pharmacology.

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, E.C.3.1.4.3—see Figure 2), which hydrolyses phosphatidylcholine to a diglyceride and choline phosphate, has been stated to occur in the venom of *Bothrops alternatus* (441). Incubation of a 1 % emulsion of egg yolk lecithin at pH 7.1 and 37°C for 1 to 3 hours with low concentrations of whole venom (1 μg in 10 ml) leads (after treat-

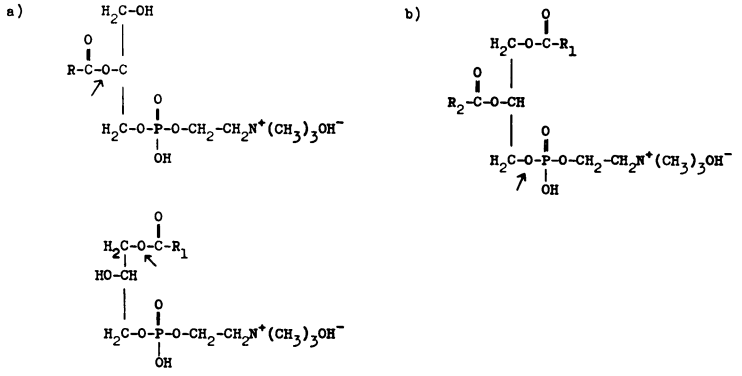

FIG. 2. Structural formulae of the substrates for a) lysolecithin acyl hydrolase, phospholipase B (E.C.3.1.1.5.), b) phosphatidylcholine cholinephosphohydrolase, phospholipase C (E.C.3.1.4.3.).

ment with 10% trichloroacetic acid) to the release of acid-soluble phosphorus (measured by the method of Fiske and Subbarrow). Choline was measured after treatment of the incubated emulsion-venom mixture with 2% HCl and found to be in equimolar proportion to the acid-soluble phosphate. Higher concentrations of venom did not lead to a greater release of acid soluble phosphate. No conclusions on the presence of phospholipase C in snake venom can be drawn from these experiments

It is well established that phospholipase C occurs in the α-toxin of *Clostridium welchii* and in toxins from related organisms (194, 268, 275, 276). This enzyme is highly toxic and has striking pharmacological effects including haemolysis, increase of serum potassium and effects on capillary permeability *in vivo*, and effects *in vitro* on smooth and skeletal muscle, histamine release from mast cells and demyelination of the spinal cord (181, 185, 324).

VI. ENZYMES WITH DOUBTFUL CLAIMS TO "NEUROTOXICITY"

A. Monophosphatases, phosphodiesterase and endonuclease

The recent statement by Chain (56) "that the powerful neurotoxic effect of certain snake venoms [is] due to their action on the respiratory chains. The link affected [is] the nicotinamide adenine dinucleotide coenzyme, which [is] broken down into two mononucleotides by an enzyme of nucleotidase nature contained in the venom" lacks experimental proof. However, the various enzymes in snake venoms that hydrolyse phosphate ester or acid anhydride bonds in mono- or poly-nucleotides can impair energy metabolism in tissue homogenates and must be considered as possible causes for some of the effects of crude venom on brain, muscle and the circulatory system.

In snake venoms there are four (perhaps six) distinct enzymes hydrolysing phosphate bonds in nucleotides. They are 5-nucleotidase, nonspecific alkaline monophosphatase, phosphodiesterase (which may also be responsible for the ATPase and NADase activity of venoms) and endonuclease. The 5-nucleotidase and phosphodiesterase can be further fractionated into isoenzymes on the basis of slight differences in physical properties. Very little is known about their pharmacological effects *in vivo*. Phosphodiesterase can probably be excluded as a major contributor to the lethality of neurotoxic venoms as it is not stable to heat.

5-Nucleotidase. 5-Nucleotidase (or AMPase) is known to occur in mammalian brain (365, 369) and was identified in Russell's viper venom by Gulland and Jackson (173). It is a 5′-ribonucleotide phosphohydrolase (E.C.3.1.3.5.) hydrolysing 5′-mononucleotides to the ribonucleoside and orthophosphate. It is present in all snake venoms so far tested, including species from *Bothrops, Naja, Bungarus, Denisonia, Notechis, Pseudechis, Hemachatus, Ancistrodon, Bitis, Crotalus, Sistrurus, Trimesurus* and *Vipera* (173, 425, 468). The 5′-nucleotidase in crude *Naja naja* venom shows optimum activity at pH 6.8 to 7.0, is activated by Mg^{++} or Mn^{++} and inhibited by Zn^{++}. It is inactivated by heating at 75° for 30 minutes at pH 7.0 or 8.4. Like the enzymes from the venoms of *Crotalus atrox, Bungarus caeruleus, Vipera russelli* and *Bothrops atrox*, it has an isoelectric point

about 8.6 (the saw-scaled viper *Echis carinatus* enzyme has a lower isoelectric point) (215, 216, 223, 224, 291, 292). 5′-Nucleotidase prepared from *Hemachatus haemachates* or from *Crotalus adamanteus* apparently had a pH optimum at 7.5 (28, 72) but further purification led to values between pH 8.0 and 9.0 (30, 173, 425). The rattlesnake enzyme has been extensively purified and shown to be highly specific for 5′-mononucleotides, not attacking 3′-mononucleotides, ribose-5-phosphate or oligonucleotides. Purified 5′-nucleotidase from *H. haemachates* venom is inactivated at 60°C at pH 6.0 or 9.0 and is activated by 2′- and 3′-mononucleotides (30).

5-′Nucleotidase fractions have on occasion shown toxicity. Thus 5′-nucleotidase separated on starch gel from krait venom was in one of four toxic zones (292) and of three 5-nucleotidase fractions separated chromatographically from Habu venom two were toxic (281). Contamination with toxins is of course possible. 5′-Nucleotides are produced by the action of venom phosphodiesterases on naturally occurring substrates. Secondary pharmacological effects could follow their conversion to nucleosides.

Nonspecific alkaline monophosphatase. This enzyme was detected in a proportion of snake venoms by Gulland and Jackson (172) and has been isolated from *Bothrops atrox* venom (425). It has the properties of an orthophosphoric monoester phosphohydrolase (E.C.3.1.3.1.) and hydrolyses 3′-AMP nearly as fast as 5′-AMP and also releases phosphate from ATP, ribose 5-phosphate, dinucleotides, mononucleoside-3′5′-diphosphates, monophenylphosphate, *etc.* It shows optimum activity at pH 9.5 and is activated by Ca^{++} and Mg^{++}. The enzyme from *Naja naja* or from *Vipera russelli* has an isoelectric point above pH 8.6. It is present in much greater amount in *Naja nigricollis* venom than in Crotalid venoms (366). Nothing significant is known about its pharmacology.

Phosphodiesterase. An orthophosphoric diester phosphohydrolase (E.C.3.1.4.1.) was first identified in Habu and Mamushi venoms by Uzawa and later in many other venoms by Gulland and Jackson (172). Bjork and Boman (28, 29, 31, 32, 358, 359), Laskowski, and others (127, 256, 454) have shown that this enzyme also acts as an exonucleotidase, releasing 5′-nucleotides in a sequential fashion from polynucleotides. It has been suggested that this enzyme is also responsible for the ATPpyrophosphohydrolase and the dinucleotide nucleotidehydrolase (NADase) activity of venoms (31).

Venom phosphodiesterases so far tested (including those from rattlesnake, Ringhals cobra and Russell's viper) show optimum activity around pH 9.0, both for calcium di-*p*-nitrophenyl phosphate and for DNA (28, 29, 32, 209, 331). Their activity towards both substrates is enhanced by Ca^{++} or Mg^{++} and inhibited by EDTA or univalent cations (358, 454). Several authors have reported that various venoms yield two or three chromatographically or electrophoretically separate phosphodiesterases (with identical enzymic properties). It is possible that these apparent "isoenzymes" arise by proteolysis in the course of the preparations (358). The isoelectric points of the enzymes are not well established. In starch gel at pH 8.6 with some venoms the enzyme fails to enter the gel and in other cases it moves slowly either to the anode or the cathode (215, 216, 291, 292).

The partially purified enzyme is rapidly inactivated by heating about 70°C at neutral or alkaline conditions, but is slightly more stable in acid conditions, losing only 22% of its activity in 30 minutes at 60°C (29). It seems unlikely that any activity would survive 10 minutes at 100°C at pH 5.0.

Possibly venom phosphodiesterase produces the immediate fall in blood pressure that follows the intravenous injection of many venoms. Epstein (113) showed that a lethal dose of Cape cobra (*Naja flava* or *Naja nivea*) given intravenously to cats or rabbits produced within 30 seconds a transient bradycardia and arterial hypotension that was mediated by the vagus (atropine or vagal section diminished or prevented it). This was confirmed in chloralosed cats by Westermann and Klapper (451), who also noticed a transient apnoea simultaneous with the hypotension and showed that a non-dialysable component of venom was responsible. Russell, Buess, and Woo (387) found that phosphodiesterase (prepared from various rattlesnake venoms by the method of Williams, Sung, and Laskowski, 454) when given intravenously to cats produced an immediate transient fall in arterial blood pressure and slight alterations in respiratory rate. Since disc electrophoresis showed that the phosphodiesterase preparations had two or three components, the attribution of the immediate hypotension to the action of phosphodiesterase is only provisional. Kellaway and Trethewie (231) demonstrated the presence of a cardiac depressant thought to be an adenosine derivative in the perfusate from organs treated with cobra venom. Adenosine 5′-phosphate (formed by the action of venom phosphodiesterase) and adenosine (formed by the further action of 5′-nucleotidase) both produce in anaesthetised cats and dogs a transient fall in blood pressure with a time course closely resembling that produced by venoms or phosphodiesterase preparations (8, 169). Adenosine and the adenine nucleotides lower blood pressure by producing peripheral vasodilatation, by direct action on the heart, and by vagal reflex action. Probably vagal slowing of the heart and the transient apnoea produced by adenine nucleotides result from a direct action on the brain stem (169).

Habermann (182) gave evidence that the immediate hypotension produced by venom is, like the release of bradykinin, due to an esterase which can be inhibited with DFP. Phosphodiesterase preparations do not produce paralysis in the cat and do not have a deleterious effect on the isolated phrenic nerve diaphragm preparation (387).

ATP pyrophosphohydrolase. The hydrolysis of adenosine and inosine triphosphates by snake venoms has been studied by Zeller and others (71, 195, 218, 224, 467, 468). Many venoms rapidly convert ATP to AMP and pyrophosphate and this activity has been assigned to an α-β"ATPase" (E.C.3.6.1.8.). Probably because the action is on an acid anhydride bond, the Enzyme Commission and others have classified this enzyme as separate from phosphodiesterase. It displays many features in common with phosphodiesterase, including optimum activity near pH 9.0, marked activation by Mg^{++} and less by Ca^{++}, and complete inactivation on heating to 60°C for 15 minutes at pH 8.3. All the phosphodiesterase fractions prepared chromatographically from Ringhals cobra

venom by Bjork and Boman (31) displayed pyrophosphatase activity towards ATP and NAD.

Nucleotide pyrophosphatase. The ability of snake venoms to hydrolyse dinucleotides including NAD and CoA to mononucleotides (E.C.3.6.1.9.) was first shown by Chain (55) and subsequently studied by Bhattacharya (27a) and Kaye (224). This too displays optimum activity at pH 9.0 and is activated by Mg^{++}. It is destroyed by heating to 70°C. Chain showed that dehydrogenases which require NAD as coenzyme, namely lactic, malic, β-hydroxybutyric and amino-acid dehydrogenases, are completely inactivated by *Notechis scutatus* venom and suggested that the nucleotidase in the venom was responsible. Fleckenstein (133, 136) compared the dehydrogenase-inactivating properties of many venoms, and Braganca and Quastel (37) showed that venoms heated at 100°C for 15 minutes did not inactivate lactic and malic dehydrogenases. A nicotinamide antagonist, which *in vivo* inhibits enzymes requiring NAD as cofactor, produces chromatolysis in anterior horn cells in rats (407, 423) and thus closely mimics the effects of some snake venoms. It is possible that destruction of NAD is the critical step causing the chromatolysis but heated venom is also alleged to produce the effect (392).

Endonucleases. Delezenne and Morel in 1919 (98) demonstrated that venoms hydrolyse nucleic acids, and Taborda *et al.* (427, 428) made a manometric study of the hydrolysis of DNA and RNA by various venoms, but it was only in 1957 that a clear differentiation was made between the phosphodiesterase action on DNA (splitting off 5′-nucleotides) and the DNA depolymerase type of activity displayed by venoms in viscosimetric assay systems (189, 256). From these studies using *Crotalus adamanteus* venom and the later work on a purified preparation from *Bothrops atrox* venom by Georgatsos and Laskowski (152a), the latter enzyme appears to be of the DNAase II kind (E.C.3.1.4.6.). It shows optimum activity at around pH 5.0, and it does not require activation by Mg^{++}. It splits deoxyribonuclease and ribonuclease at similar rates and produces tri- or higher oligonucleotides terminating in 3′-monoesterified phosphate. Its activity is increased by heating at 60°C for 30 minutes and it is stable at pH 6.0 to heating at 100°C.

Nothing definite is known of the pharmacology of these endonucleases. In view of their heat stability they should be considered as possible contributors to the "neurotoxic" actions of venoms. The inhibitory actions of venoms on the growth of cancer and other cells in tissue culture might owe something to their action.

Ribonuclease. Babkina and Vasilenko (16) have reported that Asian cobra (*Naja oxiana*) and two viper venoms contain an enzyme that is not phosphodiesterase, that hydrolyses RNA but not DNA, that shows optimum activity at pH 7.8 and that is activated by Mg^{++}. This enzyme has not yet been defined with sufficient precision to differentiate it from the enzymes described above.

B. Cholinesterase

Hydrolysis of acetylcholine by *Naja naja* venom was demonstrated by Iyengar *et al.* in 1938 (213). Unlike most enzymes in venoms, cholinesterase's distri-

bution can be precisely specified in taxonomic terms. It is found in all Elapid venoms (*Bungarus, Naja, Notechis, etc.*) but not in the Viperidae, Crotalidae or Hydrophiidae (465, 466). Studies of the substrate specificity of the enzyme (13, 34, 61, 325, 465) have shown it to be an acetylcholinesterase (E.C.3.1.1.7.). Acetyl β-methylcholine is rapidly hydrolysed and benzoyl- and butyryl-choline are not. Crude cobra venom hydrolyses various non-choline esters but purification of the enzyme by fractional precipitation leads to loss of the ability to hydrolyse tributyrin and methyl butyrate (61). However, various acetic acid esters are hydrolysed by the venom cholinesterase. The enzyme is inhibited by physostigmine and by DFP. Hydrolysis of acetyl- or propionyl-choline is diminished in the presence of excess substrate. Heating crude venom or the purified enzyme to 60°C for 60 minutes at pH 7.4 completely inactivates it. It is not more stable at other pHs (61).

As the neuromuscular blocking action of snake venoms is not abolished by heating them or by adding physostigmine it is clear that cholinesterase does not contribute significantly to this action. Although it seems prudent to remember the presence of cholinesterase in crude venoms when conducting experiments on nerve-muscle preparations, no pharmacological effects of venom cholinesterase have yet been demonstrated. Presumably where the transmitter role of acetylcholine is important there is already an excess of acetylcholinesterase.

Apparently Formosan cobra (*Naja naja atra*) venom contains a heat-labile acetylcholinesterase-inactivating factor (258). This is not effective against the cholinesterase activity of mammalian tissue preparations.

Some venoms, free from cholinesterase (*Ancistrodon piscivorus, Crotalus adamanteus*), increase the cholinesterase activity of intact squid and lobster nerves, probably by attacking permeability barriers that prevent the access of substrate (375).

VII. POLYPEPTIDE TOXINS

A. Biochemical properties

This is a difficult field to discuss because findings in one species, or sub-species, cannot be assumed to apply to other species even when the pharmacological actions of the toxins are very similar. No one species has been studied sufficiently to permit the establishment of correlations between the chemical composition of the toxins and their pharmacological actions.

Crotalus terrificus terrificus. Crotamine: This toxin has an isoelectric point at pH 10.3 and can be separated by electrophoresis from the venom of the Brazilian rattlesnake (158, 159, 162, 178, 180). It passes through cellophane dialysis membranes, and sedimentation studies suggest a molecular weight between 10,000 and 15,000. Its amino acid composition is not known.

On injection in mice it produces a spastic paralysis of the hind limbs and subsequently death from respiratory paralysis. It is undoubtedly responsible for the veratrine-like actions of crude rattlesnake venom described by Barrio and Brazil (19) (Section II). A sustained contraction of the rat gastrocnemius in response to a single stimulus to the sciatic nerve is seen after intravenous in-

jection of 200 μg/kg of crotamine (62, 326). A marked enhancement of the negative afterpotential is the basis of this effect, implying that crotamine produces a specific alteration in the ionic permeability properties of the muscle cell membrane. It also releases histamine from the rat diaphragm and increases capillary permeability (178, 180).

Crotactin: This is the name given by Habermann and Neumann (178, 180, 187, 330) to a toxic fraction, with no enzymic activity, prepared from crotoxin by ion-exchange chromatography. Its isoelectric point is at pH 4.3. Its molecular weight and amino acid composition are not known.

Mice injected intraperitoneally with crotactin die with a flaccid paralysis. It also releases histamine from the rat diaphragm and increases capillary permeability. Crotactin is curiously unlike the other venom toxins that release histamine and produce paralysis, in that it is acidic.

Toxin III: A much less well defined component of rattlesnake venom has been called "toxin III" by Habermann (178, 180). Paper electrophoresis at pH 6.0 separates it from crotamine because it remains at the origin. Its biochemical and pharmacological properties are not precisely known. Death in mice is usually preceded by flaccid paralysis but some spasticity may be seen. The possibility that it is a combined form of the other two toxins cannot be excluded.

Naja naja. "Neurotoxin": The isolation of a "neurotoxin" from Indian cobra venom, and its characterization as a basic polypeptide with a molecular weight between 1,500 and 4,000 by German and Indian scientists in 1936 has already been described (Section III B). An isoelectric point at pH 9.4 was found by Ghosh, De and Chaudhuri (155). Unfortunately the physical chemistry and amino acid composition have not been re-examined using current procedures.

Fractions producing paralysis have been prepared from *Naja naja* venom by electrophoresis on starch columns, agar gel and starch gel (see Section III C). Detrait and Boquet (102) found that when prepared by agar gel electrophoresis the fraction producing paralysis shows only one antigen with gel diffusion analysis against antisera. In no case, however, has the paralysing toxin been proved to be free from any direct activity against skeletal or cardiac muscle. Starch gel fractions producing a neuromuscular block also depolarize skeletal muscle (297, 298, 300). Either a toxin capable of depolarizing skeletal muscle contaminates the neurotoxin or the neurotoxin can itself depolarize skeletal muscle. The neuromuscular blocking action of cobra neurotoxin is curare-like in that the depolarizing action of carbachol or acetylcholine at the motor endplate is abolished. Unlike curare the block cannot be removed by washing but is sustained for several hours after exposure to toxin.

"Cardiotoxin," DLF, skeletal muscle depolarizing factor: A fraction producing cardiac slowing and ultimately systolic arrest of the frog or mammalian heart was isolated from *Naja naja* venom by fractional precipitation (394). This fraction inhibits skeletal muscle contraction not by a neuromuscular block but by a direct action on the muscle (396, 398). Sarkar (394, 395) determined the isoelectric point of cardiotoxin as pH 8.2 and the molecular weight at 46,200 (the true values are probably higher for the isoelectric point and much lower for the molecular weight).

It is possible that the basic polypeptide in cobra venom which lyses washed red blood cells and is referred to by Condrea, De Vries and Mager (74) as DLF is identical with cardiotoxin. It is found in the two bands moving fastest to the cathode on paper electrophoresis. Depolarizing activity against skeletal muscle is also found in the two bands moving fastest to the cathode on starch gel electrophoresis (296, 297).

Naja naja atra. From the venom of the Formosan cobra, Sasaki (399, 400) has prepared, by acetone and ammonium sulphate fractionation, a toxic component which gives a single spot on paper electrophoresis. Determination of its amino acid composition (see Table 1) indicates a molecular weight of 6,000. Thus, if Sasaki's toxic fraction contains only one polypeptide, the Formosan and Indian cobras have different neurotoxins.

Experiments demonstrating the irreversible curare-like action of this venom have been referred to in Section II. The neurotoxin separated by electrophoresis on starch at pH 5.0 (257), unlike the whole venom, does not have any direct action on muscle, cardiotoxic action, or histamine-releasing action and does not reduce the acetylcholine output of the rat diaphragm.

Naja nivea. By dialysis of the liquid venom of the Cape cobra (*Naja nivea*, *Naja flava*) Raudonat (355, 356) has separated the toxic polypeptides from most of the enzymes (a protease is able to pass through the membrane). Chromatographic procedures yielded three fractions all possessing toxicity. A fraction stopping the frog heart in systole contained zinc. Qualitative amino acid analysis of the most toxic fraction showed that, unlike the neurotoxins of *Naja naja* or *Naja naja atra*, this polypeptide contains methionine.

Only in the case of cobra and rattlesnake venoms is there anything approaching precise chemical information about the composition of the polypeptide toxins. This is given in Table 1. However, the data of Fischer and Dorfel (130) and Fraenkel-Conrat and Singer (139a) refer to the amino acid composition of crotoxin and its two DNP derivatives, and we have no information concerning the relationship between these two fragments and the phospholipase A activity, the hyaluronidase activity and crotactin. As the isoelectric point of crotactin is at pH 4.3 it cannot be identical with the insoluble DNP fragment. Comparison of the amino acid composition of *Naja naja atra* "neurotoxin" with that of the polypeptide toxins in bee and scorpion venoms on the one hand and two low molecular weight basic enzymes (ribonuclease and lysozyme or muramidase) on the other (Table 1), permits certain speculations but establishes no conclusions. The absence of methionine from the polypeptides appears consistent but it is found in *Naja nivea* neurotoxin (356). The excess of aspartic over glutamic acid seen in the scorpion toxins is not found in the others, and the high proportion of cysteine is seen in all the toxins except melittin.

Other venoms. The three neuromuscular blocking fractions isolated from Formosan krait (*Bungarus multicinctus*) venom (59) (discussed in Section II and below) have not been shown to be free from enzymic activity but it is a reasonable working hypothesis that there are two or more polypeptide toxins, one appearing in the α-bungarotoxin fraction and the other in the β- and γ-bungarotoxin fractions.

TABLE 1

Comparison of the amino acid composition of toxins from snake, bee and scorpion venoms with that of two small, basic enzymes

Protein or Polypeptide	Crotoxin	Insol. DNP Derivative	Sol. DNP Derivative	Neurotoxin	Apamin	Melittin	Androctonus I	Androctonus II	Ribonuclease	Muramidase
Biological source	*Crotalus terrificus*			*Naja naja atra*	*Apis mellifica*		*Androctonus australis*		Ox pancreas	Rabbit spleen
Molecular weight	30,000	17,000	13,000	6,000	2,100	5,700	15,970	11,000	13,895	14,600
c-terminal	aspartic	0	aspartic	glycine						leucine
n-terminal	serine	0	serine	leucine		glycine				lycine
Amino acid residues g/100 g										
ala.	3.69	4.2	5.4	—	10.2	3.8	2.00	2.93	6.14	6.62
gly.	4.96	6.1	7.5	5.82	—	6.1	4.99	6.02	1.21	4.56
val.	1.46	2.4	1.9	1.64	—	7.0	6.21	4.55	6.34	4.06
leuc.	3.12	9.6	6.6	4.26	5.4	16.3	5.67	3.11	1.72	8.10
i. leuc.	2.56			—	—	12.0	3.75	1.04	2.32	5.77
pro.	4.48	3.8	5.0	3.98	4.7	3.5	6.99	4.45	3.28	3.54
phe.	8.60	6.6	5.2	—	—	—	1.84	2.70	3.11	3.20
tyr.	8.58	8.5	4.3	3.54	—	—	6.64	11.97	6.84	3.69
try.	3.81	2.6	2.2	3.42	—	6.6	4.66	4.27	—	2.46
ser.	4.16	4.2	4.9	3.28	—	3.1	7.09	3.19	9.44	5.46
thre.	4.21	5.2	4.8	11.99	4.8	7.1	2.53	4.64	7.55	5.00
cys.	11.33	10.4	10.7	10.64	23.0	—	11.18	8.89	6.47	3.79
met.	1.83	1.1	0.9	—	—	—	—	—	3.51	1.58
arg.	7.60	10.5	4.6	13.51	14.9	11.0	4.40	7.16	4.39	6.63
his.	1.98	2.3	1.4	3.96	6.6	—	3.43	3.77	3.71	1.06
lys.	5.52	8.5	3.3	6.06	6.1	13.6	12.05	9.40	9.20	5.08
asp.	10.56	9.8	16.8	13.48	5.5	—	14.77	13.72	12.96	16.51
glu.	12.16	10.9	20.2	15.26	18.5	9.1	2.30	8.09	10.88	10.09
Total	100.61	98.7	105.7	100.93	99.7	99.2	100.50	99.90	99.07	97.2
References	(130, 139a,)			(399, 400)	(183)		(314)		(197)	(219)

The toxic fractions prepared from Australian tiger snake (*Notechis scutatus scutatus*) venom by Doery (106) (whose pharmacological properties are discussed below) behave differently on chromatography and electrophoresis. Fraction A was stationary on paper electrophoresis at pH 8.6, fraction B moved to the cathode at this pH, and the components producing direct lysis of red cells moved to the anode.

Dialysis and subsequent chromatography of the venom of sea snake, *Enhydrina schistosa*, which is remarkable for its ability to produce myolysis *in vivo*, permits the preparation of a compound of low molecular weight producing paralysis and a postsynaptic neuromuscular block. This gives only one line on immunoelectrophoresis (52).

From *Bothrops jararacussu* venom a basic component whose effects in the mouse resemble those of crotamine has been separated chromatographically. The name "botramine" has been proposed (159).

B. Pharmacology

Some of the pharmacological properties of the toxins mentioned above are shown in Table 2, which includes, for comparison, data relating to the two toxins found in the venom of the honey bee and to the basic toxins ("Scorpamines") found in scorpion venoms. The LD_{50} values are not sufficiently certain to permit detailed comparisons but there is little doubt that the toxins whose major action is the production of peripheral paralysis are the most toxic and about 1 μg of cobra toxin or rather less sea snake toxin can kill a 20-gram mouse.

Increases in capillary permeability, assessed by the local spread of intravenous dyes following intradermal injection of the venom or toxin, can be demonstrated with very small quantities of venom (less than 1/100 the LD_{50}). By the use of specific antagonists, it has been shown that the local release of histamine and serotonin plays a major part in mediating this response (121). In the whole venom, phospholipase A as well as the polypeptide toxins will contribute to this effect. *Naja naja atra* venom is a very powerful histamine releaser but its paralytic toxin has no such activity (63). In general Crotalid and Viperid venoms are more notable for capillary damage than are Elapid venoms, but the toxins from the Australian tiger snake (which belongs to the Elapidae) have a powerful effect on capillary permeability.

Lysis of washed red blood cells by a basic component of several cobra venoms has been described by Grassmann and Hannig (167), Habermann (177, 186), and De Vries and his colleagues (74, 75). These venoms also contain a factor which arrests the frog heart in systole and abolishes the response of skeletal muscle to direct stimulation. This factor is dialysable (in *Naja nivea*) and moves with the neurotoxin to the cathode on starch gel at pH 8.6 (297, 298). Thus the "DLF," "cardiotoxin" and the skeletal muscle depolarizing factor might all be one basic polypeptide. As described above, the action of crotamine on the skeletal muscle membrane is completely different from that of the cobra toxins in that crotamine prolongs the negative afterpotential without altering the resting potential. In the case of sea snake venom it was clearly established by Carey and

TABLE 2

Pharmacological effects of toxins from snake, bee and scorpion venoms

	Crotalus T. Terrificus			*Naja n. atra*	*Naja naja*	*Bungarus multicinctus*		*Notechis scutatus*		*Enhydrina schistosa*	*Apis mellifica*		*Androctonus australis*
	Crotamine	Crotactin	Toxin III	Najatoxin	Cardiotoxin	α Bungarotoxin	β and γ Bungarotoxins	Fraction A	Fraction B	Dialysable toxin	Melittin	Apamin	Scorpamines
LD_{50} mouse μg/g i.p.	1.0												
	0.07	0.06	0.15	0.05		0.3	0.1	0.2	0.4	<0.05	3.5	4.0	0.05
Increases capillary permeability	+	+	++					++	+		++	+	
In vitro histamine release	+	+		0							+		
5HT release from platelets											+		
In vitro red blood cell lysis				0	+ ?			0	0	0 (+)	+		
Action on skeletal muscle membrane	+				+ ?	0	0			?			
Toxic action on heart				0	+								
Smooth muscle contraction	+	++						+	+		+		+
Flaccid paralysis		+	+	++		+	+	+	?	+	+		
Spastic paralysis	+		+	0		0							+
Neuromuscular block a) postsynaptic		?		+		+				+	?		
Neuromuscular block b) presynaptic		?		0	?		+				?		
CNS excitant action	+ ?						+ ?		?			+	+
Impairment of oxidative phosphorylation											+		
References	(178, 180, 187)			(257, 259)	(300, 396, 398)	(57, 58, 59)		(106)		(51, 52)	(183)		(313, 314)

Wright (49, 50, 51, 52) that the neuromuscular block and the acute paralysis are produced by a single dialysable toxin but it is not known whether the myolysis, biochemically and histologically demonstrable in man and the rabbit, is produced by a dialysable toxin, by the phospholipase A or other enzymes, or by enzymes and toxins working synergistically.

Some of the complex effects of dialysable toxins on heart rate and blood pressure are secondary to the release of histamine and some are probably the result of alterations in vascular tone in the lungs and capillaries (124, 125, 451). Thus it is not only the directly cardiotoxic polypeptides that lower blood pressure.

Several of the toxins produce a slow contraction of the isolated guinea pig gut and other smooth muscle preparations. This is not due to histamine release and is not antagonised by antihistamines. It shows marked tachyphylaxis.

The ability to produce a flaccid paralysis is for most of these toxins the lethal property and is their most distinctive feature. Apparently depolarization of skeletal muscle plays little part in this. In three cases (cobra neurotoxin, α-bungarotoxin and *Enhydrina* toxin) there is fairly good evidence that neuromuscular transmission is blocked in a "curare-like" way, that is to say that the endplate region is not depolarized but the depolarizing action of acetylcholine is abolished. However, unlike curare's the toxins' action is not a competitive one; increasing the amount of acetylcholine applied, or adding anticholinesterases, does not alleviate the block, nor does washing the preparation with venom-free solutions. In the case of β- and γ-bungarotoxins there is evidence that a presynaptic block occurs. The action of acetylcholine on the frog rectus abdominis is not prevented but the release of acetylcholine from the stimulated rat phrenic nerve diaphragm is diminished with a time course matching that of the neuromuscular block. Whole Formosan cobra venom also has this presynaptic blocking action. It is known that the curarizing "najatoxin" is not responsible for this. Whichever element is responsible is likely to be capable of producing a flaccid paralysis.

Statements that snake venoms are central nervous system excitants are widespread in the venom literature. These are based on the apparent hyperexcitability of some experimental animals after envenomation (which might be the product of pain and malaise) and the frequent terminal occurrence of convulsions. Depending on the venom the convulsions may be accompanied by intravascular thrombosis, multiple petechial haemorrhages, acute profound hypotension, severe electrolytic imbalance or anoxia with respiratory paralysis. Not all convulsions following envenomation are attributable to a direct action of venom toxins on the central nervous system. In fact, although it has been shown that a polypeptide in bee venom (apamin—see below) can produce convulsions by a central action, the attribution of central convulsive activity to any snake venom toxin at the time of writing requires further study.

A chromatographic fraction from *Vipera palestinae* venom produces generalised tremors in mice (241) and another, basic, fraction produces paralysis (323).

The small molecular size of the toxins in cobra and some other venoms has two important consequences when they are inoculated into the whole animal. Firstly,

as shown by Barnes and Trueta (18) the toxic factors in Indian cobra venom pass straight from the tissues into the blood stream instead of travelling *via* the lymphatics. Black tiger snake venom on the other hand was shown to be absorbed only through the lymphatic system. Of course changes in capillary permeability may contribute to the absorption of cobra toxins. The other consequence of their small molecular size is that they may be only feebly antigenic. This has been shown for the neurotoxin prepared from *Vipera palestinae* venom (322) and for the toxic polypeptides in bee venom (184). In practice inoculation with whole snake venoms usually produces antisera which neutralise the polypeptide toxins. The complexing of toxin to protein may be important in this respect.

C. Comparison with some mammalian polypeptides

There are several polypeptides found in mammalian tissues whose pharmacological properties resemble in some respects those described above (reviewed by Schachter, 403, 404, 405). The kinins which lower blood pressure, contract isolated smooth muscle preparations and increase capillary permeability, are nona- or decapeptides which can be released from a precursor in plasma (an α_2 globulin) by the action of kallikrein, trypsin or various snake venoms. They have no neuromuscular blocking or central nervous actions. Compounds with similar effects are found in wasp and hornet venoms. The structural sequence of bradykinin (H Arg Pro Pro Gly Phe Ser Pro Phe Arg OH) is notable in that arginine occurs in both the C- and the N-terminal positions. Kallidin II has the same structure as bradykinin but with lysine added in the N-terminal position (347, 450). As shown in Table 1, arginine and lysine are conspicuous in the composition of venom toxins.

Other mammalian polypeptides of similar size have highly specific actions on smooth muscle. Angiotensin is found in two active forms, a decapeptide and an octapeptide, both of which have marked hypertensive potency. Oxytocin and vasopressin are octapeptide amides which resemble some of the venom toxins in possessing a cysteine pair with a disulphide linkage. As well as acting on smooth muscle, vasopressin alters the permeability of the distal tubules of the kidney apparently by an indirect action on the intercellular substance, suggesting an analogy with the actions of toxins on capillary permeability. Substance P is a polypeptide found in the gut and the brain which stimulates smooth muscle preparations and dilates blood vessels. Crude preparations have a sedative action in many species. The convulsant action of strychnine and of picrotoxin is antagonised and barbiturate hypnosis is potentiated. It is curious that the analgesia and respiratory depression produced by morphine are antagonised (470). Pure substance P lacks these actions (442).

D. Comparison with polypeptides in other venoms

The only reptile outside the order Ophidia that secretes a lethal venom is the Gila monster (Heloderma). This contains nerve growth factors but the biochemical nature of its toxins is not known.

Toxins with diverse actions on the heart, gut and nervous system are found

in the skin and the associated venom glands of amphibia (reviewed by Kaiser and Michl, 221). A wide range has been isolated and chemically identified from frog and toad skins. They fall broadly into three classes, 1) organic bases with an indole nucleus related to 5-hydroxytryptamine, *e.g.*, bufotenin (5-hydroxy-N, N-dimethyltryptamine), 2) more complex compounds containing a steroid nucleus such as bufotoxin (which is the aglucone bufotalin linked at the 14 C position to suberylarginine), 3) polypeptides in the kinin size range (7–12 residues) which lower blood pressure and contract non-vascular smooth muscles. In the last group is physalaemin, which has recently been isolated from the skin of South American amphibian *Physalaemus fuscumaculatus* and shown to be an endacapeptide with the structure Pyroglu-Ala-Asp(OH)-Pro-Asp(NH_2)-Lys-Phe-Tyr-Gly-Leu-Met-NH_2 (114). On a weight basis it is more than 100 times more potent in lowering the dog blood pressure than bradykinin. It also increases capillary permeability and contracts gut preparations. Its pharmacological similarity and chemical dissimilarity compared with mammalian kinins is astonishing when it is remembered that omitting one amino acid from bradykinin can deprive it of all activity.

A polypeptide closely resembling physalaemin is found in the salivary gland of the octopus *Eledone moschata*. It is called eledoisin and has the structure Pyroglu-Pro-Ser-Lys-Asp(OH)-Ala-Phe-Ileu-Gly-Leu-Met-NH_2 and its effects on blood pressure and smooth muscle are similar to those of physalaemin (6, 115, 116). A study of synthetic analogues has shown that the C-terminal part is necessary for hypotensive activity and that a chain length of 7 (starting from methionine) is adequate (270, 408). It is clear that pharmacologically similar polypeptides are not necessarily chemically similar.

The most striking parallels with snake venoms are found among the arthropod venoms. Many insects and spiders live by preying on other insects and have developed venoms with paralytic and digestive functions. Species which use other insects as food for their larvae often have very effective paralytic toxins. Present knowledge finds the closest resemblance to snake venoms in the venom of the honey bee (*Apis mellifica*). Fractionation of the toxic components was reported by Hahn and Leditschke in 1937 (190). More recently two toxic polypeptides have been isolated and their amino acid composition determined (Table 1) (132, 183, 188).

Melittin, from the venom of the honey bee, probably has a molecular weight of 5,700 and has a wide range of pharmacological activity (as indicated in Table 2). Like DLF it is basic and its direct haemolytic action is inhibited by acidic compounds such as heparin and lecithin. Habermann considered that its haemolytic activity results from its high surface activity. Apamin is a smaller compound with an isoelectric point at pH 12. As well as an action on vascular permeability it produces hypermotility, hypersensitivity and death with convulsions in mice and rats. The neuromuscular blocking action of the venom on the rat diaphragm resembles that of cobra venom in its time course and failure to reverse with washing or eserine (199, 200). Bee venom also contains phospholipase A and hyaluronidase. Bee venom phospholipase A as prepared chromatograph-

ically by Habermann, is relatively toxic (the mouse LD_{50} is 7 μg/g), raises capillary permeability, lowers blood pressure and contracts smooth muscle.

Wasp (*Vespa vulgaris*) and hornet (*V. crabro*) venom contain "kinins" which lower blood pressure and contract smooth muscle (182, 214). Their structures are not known, but Schachter suggested on pharmacological grounds that they are closely related to mammalian kinins, and Bradbury (35) found a high arginine content, indicating a chemical resemblance to bradykinin rather than to eledoisin. The *Vespa* venoms do not contain paralytic polypeptides, but the venom of the digger wasp (*Philanthus triangulum*) may do so. It has a very powerful peripheral paralytic action on the honey bee (which it stores alive in brood cells) (354).

Scorpion venoms also have some features in common with snake venoms. The biochemical studies of Miranda, Rochat, and Lissitzky (309, 310, 311, 312, 313, 314) on the venoms of the North African scorpions *Androctonus australis* and *Buthus occitanus* have shown that each venom contains two similar "scorpamines" which can be purified chromatographically. These have mouse LD_{50}s in the range 0.05 to 0.15 μg/g. Their isoelectric pH is between 8.0 and 9.0, and they can form stable complexes with proteins. The amino acid composition of the two scorpamines from *Androctonus* is given in Table 1. These values and sedimentation studies suggest a molecular weight of 11,000 for *Androctonus* II and one between 12,000 and 16,000 for *Androctonus* I.

Various enzymes also occur in scorpion venoms. Protease, 5′-nucleotidase and phosphodiesterase but no phospholipase A are found in the venom of Indian scorpions (293).

Pharmacological studies of the venom of the Mexican scorpion (Centruroides) by Del Pozo (99, 100, 101) have indicated that the spastic paralysis of the hind limbs results partly from an action at the neuromuscular junction and partly from an action on the spinal cord. Veratrine-like effects on isolated frog and rat muscle have been described (4). Probably central depression also contributes to the respiratory paralysis (100). Morphine and some other central depressant drugs enhance the toxicity of scorpion venom (420).

VIII. NERVE GROWTH FACTOR

Toxicity of crude venoms to tissue cultures. Inhibition of cell growth *in vitro* by venoms has been familiar from the time tissue culture techniques were first used. Levaditi and Mutermilch in 1913 (260) showed that less than 10 μg/ml crude or heated cobra venom inhibited the multiplication of chicken embryo heart cells. A similar effect was later shown with 50 μg/ml *Vipera aspis* venom (255) and a concomitant increase in the cell membrane permeability to dyestuffs was demonstrated. After the introduction of 10 μg/ml of *Naja naja* or *Trimeresurus flavoviridis* venoms the onset of cytotoxic effects is much more rapid in stable tumour cell cultures (Hela, LI, KB) than it is in cultures of mouse monocytes (402). In these experiments cytotoxicity could be completely abolished by heating the venom solution to 100°C at pH 5.6. The cytotoxic effect against tumour cell lines can be used as a means of titrating antisera (254). Trypsinized

preparations of mouse or chick embryo cells are even more sensitive than stable tumour cell lines to the cytopathic effects of venoms. The use of crude fractions (from DEAE cellulose chromatography) of *Vipera palestinae* and *Echis colorata* venoms showed that haemorrhagic, protease and cytopathic activities tended to occur together. Heating these venoms destroyed the cytopathic activity (145).

It is probable that the cytopathic action of crude venoms in tissue culture is not attributable primarily to either the polypeptides that produce paralysis or to the phospholipase A. Proteases may be important and so may polypeptides altering membrane permeability.

Nerve growth factor. Chopra, Das, and Mukherjee in 1936 (64) reported that crude cobra venom (10–20 μg/ml) enhances the growth of choroid tissue from 10-day chick embryo eyes. The effect was well established within 12 hours and was sustained for 60 hours.

Through the observations of Bueker (44) and of Levi-Montalcini (263, 264) it was discovered that a protein which enhances the outgrowth of nervous filaments from explants of chick embryo sensory or sympathetic ganglia and which augments the growth of the sympathetic chain in newborn mice can be isolated from a transplantable mouse sarcoma (S 180). Levi-Montalcini and Cohen (70) subsequently discovered a similar factor in a wide variety of snake venoms and Cohen later isolated it (68). The amount of the factor in different venoms was assayed by incubation with cultures of chick embryo sensory cells for 18 hours. Venoms from the Viperidae are the most active—1 to 5 μg of *Vipera russelli* or *V. aspis* venom per ml of culture fluid give a marked response. *Naja naja* venom is nearly as active and the Crotalid venoms are the least active—for an equivalent response, 6 μg/ml of *Ancistrodon piscivorus* or *Crotalus atrox* venoms is necessary. The possible interfering effect of cytolytic toxins was not discussed. The active factor was purified about 40-fold by fractional precipitation and ion-exchange chromatography from *Ancistrodon piscivorus* venom. It is non-dialyzable, destroyed by heat at pH 7.4, and unstable to acid but stable to alkali. Sedimentation studies indicate a molecular weight around 20,000. Acid hydrolysis gives a typical protein amino acid pattern, and proteolytic enzymes destroy the biological activity, as does antiserum to snake venom. The only enzyme activities detectable in the preparation are protease and ribonuclease, but these are contaminants, not the effective agent. The data clearly establish that the snake venom nerve growth factor is unlike the enzymes and toxins previously identified in venoms.

May and Guimard (295), using chick spinal cord explants, found that *Vipera aspis* venom had only a feeble stimulant action on nerve filament growth, that *Naja naja* and *Bitis gabonica* venoms were effective at concentrations below 5 μg/ml (5 μg/ml produced degenerative changes in the nerve filaments in 24 hours), and that *Echis carinatus* venom was the most active and least toxic.

A factor with the same biological activity can be isolated from the salivary gland of the mouse (69, 261) and an antiserum prepared by injecting a purified preparation of this into rabbits neutralises the *in vitro* activity of the snake venom factor (262). In newborn mice the antiserum inhibits the development of

the sympathetic nervous system. The salivary gland factor appears to have a molecular weight of 44,000. Schenkein and Bueker (406) have further separated the salivary gland factor into two components both of which are required for nerve growth promotion. One has a molecular weight of 20,000, the other possibly 3,500. The snake venom factor and the mouse salivary gland factor have many similarities but seem unlikely to be identical.

The nerve growth factor apparently plays a significant role in the morphogenesis of the nervous system. Why it occurs in snake venom and in salivary glands remains obscure. It plays no part in venom toxicity. Possibly salivary and venom glands are here performing an excretory function, removing excess nerve growth factor from the circulation.

Venom teratogenicity. The introduction of 2 μg of *Naja naja* venom into the developing hen's egg leads to a great increase in the incidence of malformations of several organ systems, especially the heart, and there is a parallel increase in overall mortality (382, 383). The factors responsible have not been identified.

IX. SUMMARY OF THE PROBLEM OF VENOM "NEUROTOXICITY"

The term "neurotoxic" is widely used in the venom literature. It usually describes a venom or venom fraction which when injected into experimental animals produces paralysis or convulsions before death. Unfortunately biochemists have commonly used "neurotoxic" to describe the deleterious effects of venoms or enzymes on the metabolism of brain or muscle homogenates, with the inescapable implication that the *in vitro* effects of phospholipase A or nicotinamide adenine dinucleotidase are related in some way to the paralysis and convulsions produced by whole venoms. We can avoid this source of confusion by using only the descriptive terms "paralytic" and "convulsive," provided we remember that any convulsions observed may be secondary to a primary action on a system other than the brain and spinal cord.

The peripheral paralytic actions of "neurotoxic" snake venoms contribute most to the lethality of such venoms, and we know more of the mechanism of these effects than we do of any central effects. They include actions on the muscle cell membrane of two types. A basic polypeptide (crotamine) has a veratrine-like action and is responsible for the spasticity produced in rats by rattlesnake venom. A dialysable basic polypeptide in cobra venom depolarizes skeletal muscle and is presumably responsible for the fibrillations produced by the whole venom. It is not known if phospholipase A can contribute to damage to the muscle cell membrane.

At the neuromuscular junction two effects are observable. A "curare-like" block is produced by polypeptide toxins. This is not relieved by eserine or reversed by washing-out. The other type of effect is impairment of acetylcholine release from the presynaptic terminals. This can be produced by whole cobra venom or by "β and γ bungarotoxins." At present the possibility that enzymes contribute to this effect is still open. The cholinesterase present in many venoms is not thought to contribute to their pharmacological actions.

Many venoms produce chromatolysis in anterior horn cells, motor cells of the

cranial nerve nuclei, and pyramidal cells. The functional significance of this change, and which venom components are responsible is not known.

Convulsions are a prominent feature in experimental poisoning with many different snake venoms. They may be feeble, occurring late in the course of peripheral paralysis, or they may be violent extensor spasms with no signs of paralysis. There are no studies to show that these result from a direct action on the central nervous system, or what parts thereof may be involved. Now that a polypeptide from bee venom has been shown to possess convulsive activity, a closer search for convulsive agents in venoms and an attempt to identify their site and mode of action seems indicated.

There are also widespread but imprecise indications that many venoms have other central actions mainly depressant in nature. These range from reports of euphoria, sedation and coma in people and cats, to potentiation of barbiturate anaesthesia in dogs and monkeys. Here also the venom components and pharmacological mechanisms concerned remain obscure.

The actions of venoms and venom fractions when applied intracerebrally or intraventricularly are enormously varied and quite unlike their actions when applied systemically. However, as Feldberg (123) has shown, the same is true of familiar compounds such as curare whose pharmacological mode of action we imagine we understand.

The role of the snake venom enzymes in these actions on the nervous system remains to be elucidated. Some confusion has arisen in the recent literature through the statement that venom solutions which have been heated to 100°C for 10 minutes can be regarded as preparations of phospholipase A (37). If such preparations are heated between pH 6.0 and 8.0, however, they may contain an endonuclease and the esterolytic enzyme that releases bradykinin, or, if heated between pH 4.5 and 6.5 they may contain, as well as phospholipase A, various polypeptide toxins. The use of "acid-heated" venom solutions in the study of nerve and muscle membrane properties is particularly likely to mislead, as both the polypeptide toxins and the enzyme may be involved in such effects.

On the other hand, because there are good reasons for thinking that phospholipase A gained its place in the venom enzyme team through its reputation as a top-ranking digestive enzyme, we must be careful not to underestimate it as a pharmacological agent. It and the polypeptide toxins have in common actions on capillaries and the ability to release histamine, contract smooth muscles and lyse red cell membranes. In some situations they have synergistic actions.

Some of the enzymes in snake venoms appear to be superfluous. This is also the case with the nerve growth factor and suggests that the venom gland may be performing an excretory role. If this is so, the polypeptide toxins might not be highly specialised substances which evolved only in certain species that needed venoms. They might instead belong to a class of compounds occurring in a wide range of animal species and performing an important physiological role. It is clear that polypeptides with 7 to 12 amino acid residues occur throughout the animal kingdom and have neurohumoral functions related to smooth muscles.

The toxins in snake venoms are the first example of polypeptides having an action on neuromuscular synapses. If this class of compounds has a physiological role in higher vertebrates it is more likely to be at central synapses than at the neuromuscular junction (see 299).

Further research on snake venoms, besides helping snakebite victims, is likely to provide techniques and information of value in the study of enzymes, and important advances in the understanding of synapses and excitable membranes.

Note Added in Proof

Since this review was prepared a paper has been published by Condrea, E. and De Vries, A.: Venom phospholipase A, a review. Toxicon **2**: 261–273, 1965.

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