PATHOLOGIC EFFECTS OF THE VENOMS OF CROTALUS ATROX AND NAJA NAJA IN PIGS

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ABSTRACT

PATHOLOGIC EFFECTS OF THE VENOMS OF
CROTALUS ATROX AND *NAJA NAJA* IN PIGS

by Gilberto Stephenson Trevino

The pathologic changes produced in swine tissues by the venoms of a crotalid snake (*C. atrox*) and an elapid snake (*N. naja*) were studied by assay of serum enzyme levels, histochemistry, light microscopy, electron microscopy, and electrophoresis of serum proteins.

Significant neuronal and demyelinating changes were found in the cortex and brain stem of animals venenated with the venom of *C. atrox*. These lesions consisted of focal areas of neuronal necrosis, perivascular cuffing, microhemorrhages, and foci of demyelination. Vascular lesions, characterized by hemorrhages between detached endothelial lining and the enveloping medial tunica, were observed in several tissues remote from the site of venom deposition.

In induced crotalid ophidism, serum enzyme levels of total lactate dehydrogenase, isocitrate dehydrogenase, glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, and creatine phosphokinase were increased 26 hours postinjection. Serum alkaline phosphatase remained relatively unchanged. Enzyme levels were not significantly altered by cobra venom, which produced death within 4 hours.

Changes produced in hepatocytes, muscle fibers, and erythrocytes at subcellular levels were recorded by means of electron micrographs. Complete disruption of myofibrils, swelling of mitochondria, distortion

of erythrocytes, disruption of endoplasmic reticulum and other organelles, and presence of myelin figures in liver cord cells were some of the changes observed.

Histochemically, virtual absence of cytochrome oxidase was observed in locally envenomated small intestine and striated muscle. Alkaline phosphatase in epithelial cells of renal tubules, well localized in normal sections, appeared disorderly distributed in clumps in sections of envenomated kidney.

Alteration of albumin:globulin ratios, the result of lowered albumin and increased alpha globulin levels, was detected only 3 hours postinjection.

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By

Gilberto Stephenson Trevino

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INTRODUCTION

Ophiology is the science concerned with snakes. Of approximately 2500 species of snakes in the world, about 10% are poisonous. This minority kills, through venomous inoculation, between 30,000 and 40,000 human beings annually throughout the world. In the United States, of 6500 to 7000 yearly human victims of ophidiasis, only about 15 cases terminate fatally. Many of the survivors, however, are left with serious crippling defects as a result of the extensive tissue destruction of necrotizing venoms.

For centuries man has sought to improve the efficacy of the techniques employed in the treatment of snakebite. The multitudinous arrays of chemicals and procedures used in chemotherapy and reported in numerous publications present evidence that no one method has been singularly successful. Particularly in the case of those techniques that resulted in iatrogenic aggravation of a bite from a nonpoisonous snake, it is indeed fortunate that most of the early recommended treatments have been relegated to well-deserved oblivion. In the last decade attention has been focused on the biochemical aspects of snake venom toxinology, with particular emphasis on the separation of venom components. More and more complex fractionation procedures have evolved in an effort to isolate and characterize the various constituents of venoms. Successful "vaccination" of a few human volunteers with carefully prepared gradient doses of whole elapid venoms has given impetus to investigations aimed at the development of an active immunizing agent against

many species of poisonous snakes. Despite the sophistication of modern automation and cybernetics, much remains to be learned of the exact mechanism of cellular death in ophidiasis.

OBJECTIVES

The primary objective of research in the broad area of snake venom toxicology, regardless of the specific discipline in which it is conducted, has been and is being directed toward the preservation of life of the victim of snakebite. Corollary objectives are to prevent the loss of an extremity, to prevent any loss of function in the affected part, to reduce the period of hospitalization of the patient, and to simplify, without sacrificing efficacy, the implementation of therapeutic measures. A high degree of success in the fulfillment of these objectives would go far toward the attainment of a less apparent goal, namely, the abolition of the almost universal fear of snakes.

To claim that this study proposes to attain the aforementioned objectives would be presumptuous. Its broad aims are to record certain pathologic effects of two snake venoms, one a crotalid venom, the other an elapid, in the tissues of pigs. The venoms of Crotalus atrox, the western diamondback rattlesnake, and Naja naja, the common cobra, have been chosen as being representative of crotalid and elapid snakes, respectively.

The specific aims may be briefly summarized as follows:

1. To assess the extent of the pathologic changes to swine tissues by means of the light microscope.

2. To record the effects of the venoms on the tissue activity of cholinesterase, leucine aminopeptidase, alkaline phosphatase, cytochrome oxidase and lactate dehydrogenase.

3. To examine by means of the electron microscope some of the morphologic alterations caused by the venoms at the cellular and subcellular levels following direct envenomation of liver and muscle.

4. To record the effects of the venoms on the serum levels of alkaline phosphatase, creatine phosphokinase, glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, isocitrate dehydrogenase, and lactate dehydrogenase.

5. To determine by electrophoresis the effects of the venoms on the serum proteins of pigs.

It is hoped that this study may contribute to the current body of knowledge regarding cytonecrosis following ophidiasis. By studying the biologic effects of venoms on tissue enzymes, it is further hoped that a valuable prognostic procedure for use in envenomated patients may be found. Finally, it is anticipated that this study will contribute, albeit minimally, toward the eventual synthesis of a biochemical agent that will partially or completely neutralize the lethal properties of some snake venoms.

REVIEW OF LITERATURE

Historical

Even from remotest antiquity the influence which snakes have exercised upon mankind has been apparent. In the Garden of Eden it was the despicable serpent which presented the temptation to the original sin. The dreaded asp which Cleopatra utilized as a means of suicide in 30 B.C. is now thought to have been Naja haje, the Egyptian cobra. Snakes have been held as symbols of wisdom and cunning and have been used in the religious rites of many cults, some of which exist in the United States even today. The caduceus, symbol of the medical sciences, is represented by a staff around which serpents are intertwined. According to Pollard (1956) Ovid wrote between 43 B.C. and 17 A.D. that the arrows of Hercules were charged with the venom of the Lerneian serpent. The hood of the cobra symbolized the supreme power of Egyptian rulers. Studies of fossilized remains recovered from Hitchcock County in the State of Nebraska suggest that rattlesnakes inhabited Earth as early as 4 to 12 million years ago (Klauber, 1956). Romer (1945) believed that poisonous snakes evolved from nonvenomous ancestors. An index of the intense interest generated by these reptiles is reflected by the number of references in the literature on the subject of snakes. Russell and Scharffenberg (1964) cited 5829 articles pertaining to snakes. Their bibliography included 163 references published prior to 1850.

Early References to Venoms

The earliest references to treatment of poisonous bites and stings by a variety of animals and arthropods are found in the ancient Egyptian papyri, *circa* 1600 B.C. (Leake, 1956). One of the oldest specific sources of information about snake venoms is Matthioli's edition of Discorides' *De Materia Medica*, which appeared in Venice in 1554 (Leake, 1956). In Johnson's English translation of Pare's works, entitled *The Workes of that Famous Chirurgion Ambrose Parey* [sic.], published in London in 1634, there are illustrations of vipers and discussions of bites by stingrays, snakes and other poisonous animals (Leake, 1956). Grevin in 1568 published an authoritative and systematic account of poisonous animals and plants.

The first methodical work on snake poisons was written by Francesco Redi in 1664. He demonstrated that snake venoms produce their effects only when injected and are harmless when taken orally. In 1767 Felice Fontana greatly augmented Redi's work by contributing his *Richerche Fichiche Sopra il Deleno della Vipera*, one of the first systematic works on toxicology. The first American contributions to the subject were made by Mitchell in 1860 and by Mitchell and Reichert in 1886. These workers determined that snake venoms are proteins and that some contain toxic albumins. They also noted some of the pathologic effects of snake venoms upon nervous tissue and blood.

Calmette, the great French bacteriologist and immunologist, was the first to obtain hyperimmune serum from animals injected with snake venoms. In 1895 Sir Thomas R. Fraser reported that animals could be rendered immune to cobra venom and obtained a specific antivenomous serum.

The work of these 2 distinguished scientists gave impetus to the mass production of antivenoms by the various Pasteur Institutes and other laboratories throughout the world.

Studies With Snake Venoms in the Twentieth Century

Biological effects of venoms were studied early in this century by Flexner and Noguchi in 1902, who noted hemolysis and other toxic activities of venoms on the blood. Noguchi in 1909 published a book entitled Snake Venoms.

According to Leake (1956), Preston Kyes in 1903 found lecithin to be a complement of cobra hemolysin.

Vital Brazil, the founder of the famed ophiologic study center, Instituto Butantan, of Sao Paulo, contributed much to the study of venoms, and in 1911 wrote a book entitled A Defensa comtra o Ophidismo.

A most important contribution was made by Marie Phisalix in the form of 2 volumes entitled Animaux Venimeux et Venins (1922). These volumes, with a total of 1520 pages, are considered by toxinologists to comprise all the knowledge about venoms, as well as the most nearly complete bibliography on the subject up to 1922.

Interesting clinical data were reported by Jackson, a veterinarian and physician, who experimented with the venom of C. atrox, the western diamondback rattlesnake, in dogs (1929). He also wrote regarding the management of snakebite (1926, 1927, 1944).

Interest in the biochemical fractionation of snake venoms was intensified following the successful purification by Slotta and Fraenkel-Conrat (1938) of a toxin from the venom of Crotalus terrificus terrificus, the South American rattlesnake. This toxic component, called crotoxin,

was reported by Slotta (1956) to possess the formula C_{1230} H_{1776} O_{432} N_{328} S_{36}; its molecular weight was stated to be 30,000.

A different protein, called crotamine, was separated from C. t. terrificus venom by J. Moura Goncalves (1950). Its molecular weight is between 10,000 and 15,000.

One of the most notable contributions to our over-all knowledge of snakes and their venoms was made by Klauber (1956). His work, consisting of 2 volumes, comprises the most comprehensive and exhaustive study of rattlesnakes and must be considered a classical contribution to the field of snake venom toxinology.

Other books of salient merit are Venoms, a book edited by Buckley and Porges, which contains a valuable array of papers presented at the First International Congress on Venoms in 1956; Venomous and Poisonous Animals and Noxious Plants of the Pacific Region (Keegan and Macfarlane, eds., 1963); and Bibliography of Snake Venoms and Venomous Snakes (Russell and Scharffenberg, 1964).

Taxonomic Classification of Snakes

All snakes, whether poisonous or nonpoisonous, are classified taxonomically as follows:

Animal Kingdom

Phylum Chordata

Subphylum Vertebrata

Class Reptilia

Subclass Diapsida

Order Squamata

Suborder Serpentes

Under the Suborder Serpentes, the snake fauna of the world is further divided into 13 families. Of these, 4 families (Crotalidae, Viperidae, Elapidae, and Hydrophiidae) are composed entirely of poisonous forms, while the Family Colubridae, which contains the great majority of the known types of snakes, includes 2 subfamilies, Boiginae and Homalopsinae, of rear-fanged forms. The 2 colubrid subfamilies include only 2 or 3 species that could be considered potentially dangerous to man.

On the basis of their dental morphology, snakes may be grouped into the following categories (Ditmars, 1946):

Aglypha: Without venom-conducting teeth
Representative: Family Colubridae (except Boiginae and Homalopsinae)

Glypha: With venom-conducting teeth
Opisthoglypha: Enlarged grooved teeth at rear of upper jaw
Proglypha: Groove on front of enlarged tooth
Representative: Genus Dispholidus
Pleuroglypha: Groove on side of enlarged tooth
Representative: Genus Tantilla
Proteroglypha: Fixed hollow fangs
Representatives: Family Elapidae (includes 29 genera of such dangerously poisonous forms as the cobras, kraits, coral snakes, mambas, the Taipan and tiger snake of Australia)
Family Hydrophiidae (15 genera with about 50 species; the sea snakes)
Solenoglypha: Long, hollow, movable front fangs that fold against upper jaw when mouth is closed and that represent the most highly developed venom-injecting apparatus.
Representatives: Family Viperidae (Old World true vipers; about 50 species, including the gaboon viper, puff adder, and Russell's viper)
Family Crotalidae (New World pit vipers, so-called because of the characteristic facial opening located below and behind the nostril; 6 genera and about 150 species, including the rattlesnakes, copperheads, cottonmouths, bushmaster, and fer-de-lance)

Some taxonomists prefer to treat the true vipers and pit vipers as subfamilies (Viperinae and Crotalinae, respectively) under a single family, the Viperidae.

The scientific designation of snakes consists of generic and specific names, and, in many instances, a subspecific name to indicate a subspecies, such as Crotalus viridis helleri.

Approximately 10% of a total of 2500 species of snakes are poisonous. Less than 200 are considered dangerous to man (Pollard, 1956).

In the United States, 3 families include venomous snakes; namely, Colubridae, Crotalidae, and Elapidae. The family Colubridae is not considered a menace to man because of the inefficiency of this group's venom-injecting apparatus, combined with a low toxicity of the venom. However, the Elapidae, represented by the coral snakes (2 genera and 4 species), and the 3 genera and 36 species and subspecies of Crotalidae comprise a total of 40 forms which are considered dangerously venomous.

Fatalities from Snakebites

Although precise world-wide accounts of yearly deaths attributable to snakebite are difficult to obtain, Swaroop and Grab (1956) of the Statistical Studies Section of the World Health Organization, compiled a country-by-country numerical estimate of mortalities due to ophidiasis. Their report of 30,000 to 40,000 deaths a year is probably the most nearly accurate estimate yet made, despite the fact that data from many countries were incomplete. Most of the fatalities occur in India. Ajuja and Singh (1956) reported that in India about 300,000 persons become victims of ophidism annually, with an over-all mortality of 9.5%. Brazil, with 2000 annual deaths, leads all countries of the Western Hemisphere. No precise figures were available for Mexico; but Venezuela, with 150 annual deaths, and Costa Rica, with about 30, probably rank next in total numbers of fatalities from bites of venomous snakes. The

figures given for Brazil, Venezuela, and Costa Rica seem to indicate that yearly deaths from ophidism in these countries correspond to about 3 or 4 per 100,000 inhabitants.

As stated before, in the United States from 6500 to 7000 persons are bitten by snakes every year. Whereas the number of deaths in this country amounts to less than 15 per year (Parrish, 1959), many victims of snakebite are left with crippling defects, such as the loss of a limb from amputation, a permanent ankylosis, or some other cicatricial deformity. Losses among domestic animals would be difficult to estimate, but Klauber (1956) quotes Amaral as estimating the annual livestock losses in Texas at "over a million dollars". No substantiative figures exist to support this estimate.

There have not been any poisonous terrestrial snakes found in Cuba, Jamaica, Haiti, the Dominican Republic, Puerto Rico, Iceland, Ireland, Chile, New Zealand, Madagascar, or the Polynesian, Canary and Azores Islands. Alaska, Hawaii, and Maine are the only states in America free of native venomous land-dwelling snakes.

Physical Characteristics of Venom

Freshly extracted rattlesnake venom is a viscous, yellow fluid with a specific gravity varying from 1.07 to 1.10. Slight species differences exist. Moreover, the venoms of juvenile rattlesnakes have lower specific gravities than those of normal adults. In general the difference in depth of the yellow color is directly proportional to the solid content (Klauber, 1956). Bacteria are often present in the mouths and venom glands of snakes (Parrish *et al.*, 1956), but controversy exists about the incidence and significance of septicemic sequelae following snakebite (Jackson, 1944; Reid, 1958). The mean solid content of pure, fresh

rattlesnake venom by weight is about 24.34 $\pm$ .30%, and its pH ranges from 5.6 to 6.2 (Klauber, 1956). According to Gennaro *et al.* (1961) snakes have the capacity to control the amount of venom injected into the prey. There is a direct correlation between the size of a snake and the amount of venom yielded, and a fully grown eastern diamondback rattlesnake may yield up to 5 ml. of venom, amounting to about 1250 mg. of dried venom. This quantity of venom would be sufficient to kill about 8 adult men.

Chemical Composition of Venoms

In contrast to the ease with which the physical properties of venom can be studied, it has thus far been impossible to define with precision the qualitative and quantitative chemical composition of snake venom. Stanic (1956) stated:

> "The chemical composition of snake venoms is still an unsolved problem. The hemorrhagic, hemolytic and neurotoxic action is the result of numerous and complicated biologically active enzymes."

Klauber (1956) described venoms as "organic chemical mixtures of great complexity, of which gross chemical analyses have little value." Gennaro (1963) wrote:

> "Venoms are heterogeneous mixtures of enzymes and toxins probably protein in nature...they are composed of a mosaic of factors which both kill the prey and digest it."

It has been convenient to describe some components of venoms in terms of the pathophysiologic effects elicited in experimental animals. Therefore, in the search for information pertaining to the chemical composition of venom, one commonly encounters cryptic and nebulous terms, such as "cytolysins" and "hemotoxins" applied to certain principles of venoms; to the critical reader these terms stand out as

monuments to man's frustrations in his attempts to characterize and describe precisely the active principles in venoms.

Amaral (1928) summarized the constituents of venom as follows: (1) proteins (albumin and globulin); (2) proteases and peptones; (3) mucin and mucinoid substances; (4) ferments; (5) fat; (6) cellular detritus (not present in purified venom); and (7) salts, such as calcium chloride, and calcium, magnesium, and ammonium phosphates.

It has been customary and convenient in the past to divide snake venoms into 2 broad categories, the hemolytic and the neurotoxic, depending on whether the destructive properties are exerted primarily on the vascular or nervous system. Venoms of crotalid snakes were said to be hemolytic and proteolytic; elapid venoms were described as neurotoxic; and viperid venoms were characterized as mixtures of these two. In modern toxinology this classification is untenable, for it is well known that venoms of one group of snakes possess activities that formerly were attributed only to venoms of another family. For instance, at least 5 crotalid snakes produce potent neurotoxins; Githens and Wolff (1939) reported these to be Crotalus s. scutalatus, C. t. terrificus, C. tigris, C. m. mitchelli, and S. c. catenatus. Furthermore, Naja naja, the common cobra, was shown by Reid in Malaya (1963) to cause extensive local necrotizing effects in 29 out of 33 patients and neurotoxic symptoms only in the other 4.

In the last decade there has been a large volume of research performed with venoms by biochemists and toxinologists. Jimenez Porras (1963) presented his doctoral dissertation on the electrophoretic and biochemical characteristics of venoms of Costa Rican snakes. C. C. Yang of Formosa has been a prolific author in enzymic biochemistry of venoms

of Asiatic snakes (1954-1960). The best review of venom phospholipase A was given by Condrea and de Vries (1965). A comprehensive survey of available information on the subject of enzymes in snake venom is given by the 3 aforementioned sources.

Methods Employed in the Study of Venoms

A diversity of methods has been utilized in the biochemical and toxinologic study of various components of snake venom. Although a detailed discussion of intricate methodological procedures is beyond the scope of this presentation, it seems appropriate to list the more common techniques employed for the assay of venoms.

1. Salt precipitation
2. Ultracentrifugation
3. Paper electrophoresis
4. Free boundary electrophoresis
5. Zone electrophoresis on starch columns
6. Starch-gel electrophoresis
7. Dialysis
8. Paper chromatography
9. Chromatography on amerlite column, on DEAE-cellulose and carboxymethyl cellulose
10. Gel filtration
11. Electrophoresis in a density gradient
12. Enzymic procedures, using natural and artificial substrates, activators, and inhibitors
13. Separation of phospholipase A by boiling crude venom at an acid pH
14. *In vitro* and *in vivo* determinations of effects of venom on hemoglobin, fibrinogen, fibrin, erythrocytes, leukocytes, platelets, tissue cell preparations, homogenates, mitochondria, and specimens of muscle and nerve

15. Agar gel diffusion tests

16. Serologic neutralization of venom and determination of protective capacity of gamma globulin of animals immunized against snake venom

17. Toxicologic studies to determine LD_{50} and LD_{100} in experimental animals

18. Evaluation of drugs and operative procedures, such as EDTA, dihydrolipoic acid, corticosteroids, heparin, lactic acid, tetracyclines, carbolic acid soaps, incision-ligation-suction, cryotherapy, and resection, with or without antivenins or other adjunctive therapy

19. Appraisal of clinical signs and lesions in animals experimentally injected with venom and the use of special procedures to study the micropathologic changes in tissues

20. Immunization of animals and human volunteers with crude and altered venom antigens

Numerous enzymes have been isolated from venom by the utilization of some of the biochemical procedures listed above, some of which require complex laboratory apparatus and a high degree of skill. The most important of these are listed in Table 1.

Some Physiologic and Pharmacologic Activities of Venoms

Most snake venoms possess proteolytic activity; however, even closely related species may exert different degrees of proteolysis on the same substrates. For example, the venom of Crotalus adamanteus, the eastern diamondback rattlesnake, was reported by Deutsch and Diniz (1956) to coagulate purified fibrinogen, while that of C. atrox and of Agkistrodon piscivorus, the cottonmouth water moccasin, did not. This variance in behavior of the venoms occurred despite the fact that all 3 of these snakes are crotalid.

Haas (1946) reported the discovery of "proinvasin I", a factor in the venom of A. piscivorus which destroys the nonspecific hyaluronidase inhibitor present in the serums of many different vertebrates.

Table 1. Specific enzyme activities demonstrated in snake venoms

Enzyme Activity	Type of venom from which derived	Reference(s)
Phosphodiesterase	Crotalid	Uzawa (1932)
Phosphomonoesterase	Viperid	Gulland and Jackson (1938)
Phospholipase A	Crotalid	Chargaff and Cohen (1939)
	Crotalid, elapid, viperid	Condrea and de Vries (1965)
L-amino acid oxidase	Crotalid	Singer and Kearney (1950)
Diphenylphosphatase	Crotalid	Gulland and Jackson (1938)
Adenosine-5'-phosphatase	Crotalid	Gulland and Jackson (1938)
Adenosine triphosphatase	Crotalid	Zeller (1950)
	Elapid and viperid	Ghosh and Bhattacharjee (1951)
Hyaluronidase	Crotalid	Duran-Reynals (1939)
Dehydrogenase inhibitors	Elapid	Chain (1939)
Acetylcholinesterase	All elapids	Ghosh *et al.* (1956)
Noncholinesterases	Elapid (cobra)	Ghosh *et al.* (1956)
Dipeptidase, tripeptidase, polypeptidase	Various	Ghosh *et al.* (1939)
Nucleotidase (endonuclease)	Crotalid (*B. atrox*)	Georgatsos and Laskowski (1962)

Hadidian (1956) studied the action of the venom of A. piscivorus on hemoglobin, casein, and gelatin, as well as on certain circulatory, muscular, and nervous functions of the dog and the frog. He found that this venom broke down casein and hemoglobin and also liquefied gelatin. It also destroyed both prothrombin and fibrinogen. When given intravenously to anesthetized dogs at a dose of 1 mg./Kg. body weight, there was a precipitous fall in the arterial blood pressure to 30 or 40 mm. of mercury. If the dog were initially given one or more small doses of 0.02 to 0.05 mg./Kg. body weight, it was then able to tolerate subsequent larger doses of venom, up to 0.5 mg./Kg., with only a moderate fall in blood pressure. Animals dying an hour or more after administration of a large quantity of venom had widespread hemorrhages; those dying more rapidly had no such evidence. Dogs given venom intravenously or intra-arterially manifested initially a decreased muscular response to direct stimulation of the peroneal nerve, then secondarily a decreased response to muscular stimulation of the tibialis anterior muscle. Eventually the muscle became refractory to both nervous and muscular stimuli. Hadidian was unable to explain precisely the bases for the actions of the venom on the muscle and the nerve, but he did conclude that the effect on the myoneural junctions was different from that observed with curare. The response to curare was immediate, while that observed with venom was indicative of a gradual deterioration in the conductive process.

Hemodynamic alterations in dogs envenomated intravenously with the venoms of C. adamanteus and N. naja were reported by Morales et al. (1956). There was an abrupt fall in peripheral arterial pressure immediately after administration of either venom. With the rattlesnake

venom, a rise in portal pressure coincided with the fall in arterial pressure; then both arterial and portal pressures gradually decreased until the death of the animal. A different response was obtained with the cobra venom. In this instance there was an abrupt fall in both arterial and portal blood pressures. The use of a respirator partially restored both arterial and venous pressures, the portal pressure remaining higher than normal, and the arterial at nearly normal levels, until just before death. With both venoms there was a drop in the plasma volume and a rise in the hematocrit level.

Results conflicting with those of Morales et al. were obtained by Malette et al. (1963). These investigators, working with C. atrox venom in splenectomized dogs, reported that both the plasma volume and red blood cell volume were decreased following subcutaneous envenomation. Arterial blood pressure was determined by an indwelling catheter in the femoral artery and decreased from a normal of 122 mm. of mercury to 90 mm. about 2 to 4 hours after a lethal injection of venom.

At the U. S. Army Medical Research Laboratory in Fort Knox, Kentucky (Maurer et al., 1964), hematocrit values of nonsplenectomized dogs given lethal doses of C. atrox venom subcutaneously were in agreement with those recorded by Morales et al. Increased hematocrit values were observed as early as 4 hours after envenomation. Thirty-six hours after envenomation hematocrit values dropped below normal and persisted at subnormal levels thereafter until the termination of the experiments 120 hours postvenomization. Blood sedimentation rates fell from a normal of 1 to 4 mm. per hour to 0 mm. per hour between Postinjection Hours 8 and 96. After 96 hours the sedimentation rates climbed to a peak level of 46 mm. per hour. The rise in the sedimentation rate was synchronous

with a fall in the hemoglobin value, which decreased to 11.2 Gm. per 100 ml. of blood from a preinjection index of 15.8 Gm.

The anticoagulating action of cobra venom was demonstrated by Devi et al. (1956) by the use of citrated plasma of humans, monkeys, rabbits, sheep, guinea pigs, and cattle. There was no effect on the plasma of humans or oxen, even when 4 mg. of cobra venom were added to the specimens; but the coagulation of plasmas of sheep, guinea pigs, and rabbits was partially or completely prevented by relatively low amounts of venom.

Symptoms of Ophidiasis

A number of factors affecting the pathogenesis of ophidism must be reviewed before one can consider intelligently the sequence of events that usually follows envenomation. Some of these variable factors influence profoundly the outcome of the snake bite, and Klauber (1956) has enumerated these so capably that they are listed here only with minor modification:

1. The age, size, vigor and health of the victim
2. The patient's sensitivity to the venom
3. The overlay of psychogenic influences
4. The site of the bite and the tissues involved
5. The dose of venom actually injected
6. The protection afforded by clothing and boots or shoes
7. The number of bites and the depth to which the venom is injected
8. The size, species, and age of the snake and the condition of its fangs
9. The presence of pathogenic bacteria in the mouth of the snake
10. The efficacy of first-aid measures undertaken

Symptoms of Crotalid Envenomation

In bites by crotalid snakes other than those which produce potent neurotoxins, such as C. t. terrificus and C. s. scutulatus, the most common symptoms, listed in the order of frequency, are the following: (1) swelling, (2) pain, (3) weakness, (4) giddiness, (5) respiratory difficulty, (6) nausea and/or vomiting, (7) hemorrhages, (8) weak pulse or heart failure.

Symptoms of Elapid Ophidism

Bites by elapid snakes, such as cobras and coral snakes, usually produce the following symptoms: (1) pain, (2) swelling, (3) lassitude, drowsiness and giddiness, (4) respiratory difficulty, (5) receding consciousness, (6) weak pulse, (7) tachycardia, (8) blurring and dimming of vision, (9) ptosis, (10) labored speech, (11) dribbling of saliva and mucus with lingual and laryngeal paralysis, (12) coma, (13) paralysis, (14) loss of sphincter control, and (15) cessation of respiration.

The interval between the bite and the onset of neurotoxic symptoms is seldom longer than 2 to 3 hours.

Symptoms of Hydrophiid Ophidism

Very few cases of bites by sea snakes have been reported. Reid (1956) listed the following main clinical features: (1) complete absence of pain after the bite, (2) delayed onset of symptoms, (3) generalized paresis, slightly spastic initially, then rapidly becoming flaccid with loss of reflexes, (4) ptosis, (5) trismus, (6) myoglobinuria and hemoglobinuria, (7) terminal hypertension, sweating, and respiratory failure.

I have personally observed several cases of ophidiasis in human beings. Two of them occurred at the herpetarium of the U. S. Army

Medical Research Laboratory in Fort Knox, Kentucky (1963-1964). In one instance an enlisted man engaged in extracting venom from the snakes in the colony was bitten on the ring finger of the left hand by a large eastern diamondback rattlesnake. Despite the fact that only one fang penetrated the distal phalanx of his finger, and despite prompt ligation, incision, and suction of the wound, there developed after 18 hours an alarming edematous swelling and discoloration of the left arm up to the level of the shoulder. Within 24 hours the ecchymosis advanced to the dorsal anterior area of the left thoracic region. No antivenin was administered to this patient because he had a marked sensitivity to horse serum, and after a protracted stay in the hospital, he was released following the amputation of the third phalanx of the affected digit.

The other accident occurred to an Army veterinarian who was bitten by a large common cobra, N. naja. Over a period of months preceding the bite, this officer had received weekly injections of gradually increasing doses of crude cobra venom in the deltoid muscles. That his serum possessed a marked capacity to neutralize cobra venom was known by periodic assays in mice. At the time of the bite, which occurred at the distal end of the first metacarpal on the dorsum of the left hand, he felt instant burning pain in the area and, after an estimated 15 or 20 seconds, he managed to pry the snake loose from his hand. At no time after the bite were any signs of neurotoxicity detected. However, there was severe local reaction, with the swelling progressing to the left shoulder by the following day. An ulcer about 4 cm. long and 2 cm. wide formed at the site of the bite. This lesion failed to heal, and after 2 months in the hospital, the patient received a skin graft, following

which he regained complete use of the hand. The course of this subject's illness corroborated Reid's observation that the bite of the common cobra in Malaya resulted in pronounced necrotizing effects.

Pathogenetic Sequelae and Gross Lesions of Ophidiasis

As can be deduced from the foregoing paragraphs, the lesions produced by the bite of a poisonous snake vary greatly with the species of snake involved, the amount of venom injected, the site of venom deposition, and with the many other factors already listed. Envenomation by crotalid snakes other than by those which produce markedly neurotoxic venoms is usually characterized by intense burning pain at the site of the bite, spontaneous bleeding from the fang wounds, rapidly spreading swelling with discoloration of the skin due to ecchymoses, rapid formation of vesicles, involuntary muscular twitching, hematemesis, bloody stools, shock, dyspnea, giddiness, polydipsia, and central nervous disturbances, such as syncope, blurring of vision, and slurring of speech. Some victims have stated that everything they saw appeared yellow to them; this unusual sequela has been reported by a number of persons bitten by C. adamanteus (Klauber, 1956). The writer has induced crotalid ophidism in about 175 experimental animals, including dogs, rabbits, and pigs. The constancy of the effects produced by intramuscular and subcutaneous injections in these species was striking. The first symtom of intramuscular envenomation noted in the unanesthetized animals was immediate lameness. The animals bore no weight on the injected limb, even when the amount of venom was considerably less than a lethal dose, and walked with the affected member in a flexed position. When C. atrox venom was injected in dogs subcutaneously in the distal phalanges, vesicles became apparent between 25 and 30 minutes after the

injection. Bullae and vesicles were not observed following subcutaneous envenomation at sites other than the digits. When the venom was deposited under the skin of the thorax, for example, the animals developed a marked edema which was most pronounced at the ventral portions of the body; i.e., along the sternum and ventral abdomen. There was thus a clear gravitation of the venom and of the swelling to the most dependent body areas and, in many instances, a black, necrotic lesion in the skin was followed by an ulcer which discharged a serosanguineous, brownish fluid for days afterward. The results of intravenous ophidism were especially dramatic. When 2 dogs weighing 12.5 Kg. were each given a total of 5 mg. of venom of A. piscivorus, they vomited within 2 minutes; 5 minutes afterward they were cyanotic; and within 6 minutes both had lost consciousness. Dyspnea was apparent. The femoral pulse was barely detectable, but after about 9 or 10 minutes it became stronger, the animals recovered consciousness, and had bloody stools and hematemesis for approximately 12 hours. One animal died 20 hours after receiving the venom, but the other survived.

In Gennaro's experiments (1966) with the venom of the coral snake, Micrurus fulvius, in dogs, symptoms of central nervous intoxication were characterized by salivation, defecation, emesis, dyspnea, impairment of the blink reflex, central fixation of the pupils, and paralysis. It is interesting to note that the venom of the coral snake, generally regarded as being solely neurotoxic, also produced hematuria and bleeding from most of the body orifices. Pain and tenderness was always noted at the site of venom deposition.

A detailed description of 3 fatal human cases of sea snake ophidism was given by Reid (1956). He observed that no sensory abnormalities

were detectable in any of his patients and that their symptoms resembled bulbar poliomyelitis. According to Reid, the hemoglobinuria observed in patients bitten by sea snakes was confirmed by spectroscopy, but later (1963) he stated that the urine pigment was myoglobin, not hemoglobin. It was his impression that the venom of the sea snakes produced neurotoxicity in experimental animals but caused myotoxicity in human patients.

Many writers have described the gross and microscopic lesions of ophidism in various species. Much of the information is repetitive and, for the sake of brevity, only the work of Beamer et al. (1960), who induced crotalid ophidism in rabbits, will be cited here. These workers made a comparative study of the effects of subcutaneous (S.C.) and intramuscular (I.M.) envenomation of rabbits with the venom of C. atrox. They noted that edema and necrosis of skin and muscle were more pronounced in the rabbits injected I.M. but hemorrhage was more extensive in those injected S.C. They also reported that tissues receiving identical amounts of venom presented identical appearances regardless of the route of administration used. In the animals injected I.M. the skin over the injection site became necrotic and usually sloughed by the end of 48 hours, exposing the underlying necrotic muscles. Those rabbits injected S.C. had more edema and hemorrhage, but no extensive skin sloughs.

Microscopic Lesions of Ophidiasis

Beamer et al. (1960) described the histologic changes in envenomated muscles as being characterized by swelling of fibers, pyknosis, karyolysis, eosinophilic infiltration, and later by liquefaction. Often the sarcoplasm became liquefied, leaving the sarcolemma with a fine eosinophilic granular

material within it; at times there was a complete absence of stainable material within the sarcolemma. When the animals survived longer than 36 hours, heterophils and mononuclear cells invaded the site of venom deposition. Microscopic lesions were found consistently in the liver, kidney, lung, heart, and brain in all envenomated animals regardless of whether they were injected I.M. or S.C. Changes in the kidney varied from cloudy swelling to necrosis of the tubular epithelium. Granular and hyaline tubular casts were observed, and a proteinaceous material was seen within Bowman's space. The lesions in the liver had a variable pattern of necrosis; the swelling of the hepatic cells was classed by the writers as hydropic infiltration. "Microhemorrhages" and albuminous degeneration in the myocardium were described. Extensive alveolar hemorrhages, edema, and the presence of large cells resembling megakaryocytes were observed in the lungs. Hyperemia, edema, and scattered hemorrhages were noted in the cerebrum, cerebellum, and brain stem, but no distinct degenerative lesions were found in these tissues.

Serum Proteins

Separation of serum proteins by electrophoresis has been accomplished and studied by numerous investigators (Jacobson et al., 1950; Rutqvist, 1958; Miller et al., 1961). A surprisingly high number of variable factors, both extrinsic and intrinsic, influence the quantitative results of serum protein resolution. Among these are the type of procedure employed in fractionation, diet, and age. Commonly employed methods, such as salt precipitation, free electrophoresis, paper electrophoresis, dye-binding, and refractive index, may yield quantitatively discordant data even in analyses of the same sample. In order to minimize these quantitative differences it is imperative, as stressed by

Jordan and White (1965), that the numerous variable factors influencing the assay of serum proteins be controlled.

According to Albritton (1952), the total serum protein level of swine is approximately 6.3 Gm./100 ml. Albumin comprises about 2.5 Gm./100 ml., while globulin levels approximate 3.8 Gm. Unfed newborn pigs have no gamma globulin detectable by electrophoresis (Brooks, 1966), but with advancing maturity and immunologic competency, the alteration in the proportion of electrophoretically separated components due to increase in gamma globulin synthesis becomes apparent (Lecce et al., 1962).

Although changes in distribution of serum proteins are not specific for a particular disease (Dimopoullos, 1963), higher or lower values for a certain fraction during the course of an illness may be helpful in understanding the pathogenetic sequence of events. This is exemplified by the extreme hypergammaglobulinemia which occurs in Aleutian disease of mink and the hypoglobulinemia of cattle with eperythrozoonosis (Cornelius and Kaneko, 1963).

Serum and Tissue Enzymes

Valuable data are derived from serum and tissue enzymes not only for diagnosis but also for observation of the course of a disease. In human medicine, the serum enzymes which have proved to be most valuable for diagnosis are creatine phosphokinase, glutamate-oxalate transaminase, and lactic dehydrogenase isozymes in myocardial infarction; glutamate-oxalate transaminase and glutamate-pyruvate transminase in liver diseases and heart failure; acid phosphatase in prostatic carcinoma; creatine phosphokinase and aldolase in muscular dystrophies; alkaline phosphatase

in tumors of bone and certain liver diseases; and complete assays with computation for quantitative ratios of several enzymes for such diseases as widespread neoplasia, toxemia of pregnancy, anemias, and leukemias.

The significance of quantitative serum enzyme assays in animals does not always parallel in importance their analogues in human medicine. For example, assays of acid phosphatase, which are of great value in the diagnosis of prostatic carcinoma of man, would probably be beneficial only in the dog, since prostatic disease is common only in this species. The relative significance of serum and tissue enzymes is also influenced by inherent species differences. This is corroborated by the "liver specificity" of glutamate-pyruvate transaminase for the dog, cat, and man.

Cornelius et al. (1959), Cornelius and Kaneko (1963), and Loeb (1965, 1967) have published comprehensive reviews of serum enzyme assays in animal diseases.

MATERIALS AND METHODS

Animals

Twenty Yorkshire pigs comprising 2 groups of 10 each were used in this study. Fortuitously, all the pigs within each group were litter mates. At the time of the experiments the animals were between 6 and 7 weeks of age and weighed between 35 and 40 pounds each. Both groups were weaned at about 4 weeks of age. During the experiments the animals were given free access to feed and water.

Source and Potency of Venoms

Two snake venoms, one a crotalid, the other an elapid, were used to envenomate the animals. The crotalid venom was obtained from pooled batches extracted from western diamondback rattlesnakes (*Crotalus atrox*) and the elapid venom was derived from the common cobra of Pakistan (*Naja naja*). The purified venoms used were furnished by the Biochemistry Division, U. S. Army Medical Research Laboratory, Fort Knox, Kentucky. After extraction the venoms were frozen, lyophilized, and stored in a dessicator at -40 C. Those portions used in these experiments were maintained in a refrigerator at 4 C. for approximately 18 months after being collected. Prior to their use the potency of the venoms was assayed in mice. The LD_{50} for the venom of *C. atrox* in 16- to 18-Gm. mice was 88 μg. per mouse; that of *N. naja* was 9 μg. per mouse. The LD_{50} determinations were made by intraperitoneal injections. Specimens of these venoms in solution were streaked on blood agar and failed to show bacterial growth after incubation at 37 C. for 48 hours.

Blood Counts

Total white blood cell and differential counts and hematocrit and hemoglobin indices were made on each animal before and after the administration of venom.

Electrophoresis of Serum Proteins

Fractionation of serum proteins was accomplished by using a Spinco-Durrum-type cell electrophoresis chamber.* Four milliliters of a 1% agar solution were used to coat pieces of film leaders approximately 16 cm. long. Beckman B-2 buffer, with a pH of 8.6 and an ionic strength of 0.075, was used to fill the chamber. Approximately 0.004 ml. of serum was used for each determination. Separation of serum proteins proceeded for 60 minutes with a current of 75 milliamps. Thereafter the strips were immersed in methanol for 15 to 30 minutes. The strips were then stained for 15 minutes in 0.2% thiazine red and, after 3 decolorizing baths in 5% acetic acid, they were dried. Quantitation of serum protein fractions was then accomplished with a Spinco Analytrol.* This procedure was followed for the serum collected before and after envenomation of the animals.

Serum Enzymes

Assay of serum enzymes was accomplished before and after envenomation of those pigs given the venom S.C. or I.M. The effects of the venoms on the serum levels of alkaline phosphatase, creatine phosphokinase,

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* Beckman Instruments, Inc., Fullerton, Calif.

glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, isocitrate dehydrogenase, and lactate dehydrogenase were recorded. Individual kits for the determination of each of the aforementioned enzymes were obtained from the Sigma Chemical Company,* and the exact procedures described in the technical bulletins accompanying each kit were followed. Since these determinations are all made colorimetrically at a wave length specific for each enzyme, the Coleman Spectrophotometer** used in these assays had to be calibrated for each enzyme. Because of the lability of lactic dehydrogenases the levels of these enzymes were determined first, using only fresh serum. In all other cases the serum specimens were frozen and maintained at -40 C. until used.

Histochemical and Electron Microscopic Studies

Selected tissues of 4 pigs were harvested for histochemical and electron microscopic studies. These 4 animals were anesthetized with sodium pentobarbital given via the marginal ear vein. When a plane of surgical anesthesia had been attained, a laparotomy was performed. Skin, muscle, liver, kidney, and small intestine, to be used as normal controls, were first injected with 1 ml. of physiological saline solution. Two of the animals received injections of 1 ml. of C. atrox venom solution at a concentration of 10 mg./ml. directly into each organ and the other 2 received similar volumes of a N. naja venom solution at a

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* Sigma Chemical Company, St. Louis, Mo.

** Coleman Instruments, Inc., Maywood, Ill.

concentration of 2 mg./ml. The skin and small intestine were envenomated, respectively, by intradermal and subserosal injections of venom; the muscle, by intramuscular injection; and the liver and kidney, by direct intraparenchymal injection. In all instances the venom was weighed and dissolved in physiological saline solution immediately prior to its use. All injections were made with a hypodermic syringe fitted with a 27-gauge needle 1/2 inch long. After 15 minutes, samples of envenomated and control tissues were collected, frozen, and labeled.

Tissues for histochemical studies were frozen immediately in individual cryostat chucks by immersing the chucks in acetone and dry ice. Sections were cut 10 microns thick with an International-Harris Cryostat,* Model CT, and stained with standard procedures outlined by Thompson (1966) and Pearce (1961). Effects of the venoms on tissue cholinesterase, leucine aminopeptidase, alkaline phosphatase, cytochrome oxidase, and lactic dehydrogenase were recorded. All histochemical procedures were performed within a few hours after collection of the tissues.

Specimens for electron microscopy were fixed in 6.25% glutaraldehyde at a pH of 7.2. They were embedded in epoxy resin in 400 mesh grids and then cut with a Porter-Blum Ultramicrotome,** Model MT-2.

Envenomation of Animals

Solutions of purified, lyophilized venoms of *C. atrox* and *N. naja* were prepared by dissolving previously weighed quantities of these in physiological saline solution. *Crotalus atrox* venom solution was made

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* International Equipment Co., Boston, Mass.

** Ivan Sorrall, Inc., Norwalk, Conn.

in a concentration of 100 mg./ml., and N. naja at a concentration of 10 mg./ml. Two uninoculated pigs served as controls for the assays of serum enzymes and serum proteins. The routes of envenomation employed and the amount given in each of the others are indicated in Table 2.

Gross and Microscopic Pathologic Studies

Autopsies were performed on all animals within minutes after they died or were killed. Electrocution was the method of euthanasia used. Gross photographs were taken of significant pathologic findings. Tissues for microscopic appraisal of changes were fixed in 10% buffered formalin and stained routinely with hematoxylin and eosin. Special stains were used on tissues when indicated.

Table 2. Sites of envenomation and quantities of venom administered

Pig No.	Type and amount of venom given	Route and Site
1*		
2*		
3	10 mg. C. atrox, each site	Skin, muscle, liver, kidney, intestine
4	2 mg. N. naja	Skin, muscle, liver, kidney, intestine
5	25 mg. C. atrox	Intravenous, marginal ear vein
6	50 mg. C. atrox	Subcutaneous, hock
7	50 mg. C. atrox	Subcutaneous, thoracic area
8	50 mg. C. atrox	Subcutaneous, hock
9	50 mg. C. atrox	Intramuscular, thigh
10	50 mg. C. atrox	Intramuscular, thigh
11	50 mg. C. atrox	Subcutaneous, thoracic area
12	50 mg. C. atrox	Subcutaneous, hock
13	10 mg. C. atrox, each site	Skin, muscle, liver, kidney, intestine
14	50 mg. C. atrox	Intramuscular, thigh
15	50 mg. C. atrox	Intramuscular, thigh
16	10 mg. N. naja	Subcutaneous, hock
17	10 mg. N. naja	Subcutaneous, hock
18	2 mg. N. naja, each site	Skin, muscle, liver, kidney, intestine
19	10 mg. N. naja	Intramuscular, thigh
20	10 mg. N. naja	Intramuscular, thigh

* Controls; not envenomated.

RESULTS

ENVENOMATION WITH *CROTALUS ATROX* VENOM

Clinical Observations and Gross Lesions

Intramuscular route (I.M.). Pigs given 0.5 ml. (50 mg.) of venom solution into the dorsolateral femoral muscles squealed loudly, carried the limb in a flexed position, and bore no weight on the appendage thereafter. Although the affected members started to swell visibly within 45 minutes, the animals continued to eat and drink intermittently for 6 to 8 hours. After this time they ate less frequently and appeared to be more depressed than the animals injected S.C. However, they still resisted the restraint necessary for the drawing of blood samples. At the end of 26 hours, all the pigs injected I.M. were still alive but noticeably depressed. By this time there was a marked swelling and discoloration of the entire lateral aspect of the affected limb from the pastern to the hip. On the ventromedial aspect the swelling extended to the inguinal region and flank. One pig had a large subcapsular hemorrhage measuring about 3 cm. in diameter at the anterior aspect of the right kidney.

Subcutaneous route (S.C.). Animals which received the venom S.C. in the dorsal thoracic area had much more severe symptoms than those envenomated via the same route midway between the pastern and hock. They also fared worse than the pigs given the venom I.M. Edema and hemorrhage

were much more evident in the subcutis overlying the sternum and ventral abdomen, a clear indication of gravitational flux of extravascular fluids. One animal out of 2 injected S.C. in the left dorsal thoracic area died about 20 hours postinjection. Pig 6, injected S.C. midway between the pastern and hock, was moribund when killed 26 hours after injection. In general the edema and discoloration in the pigs receiving the venom S.C. (Figures 1 and 2) seemed to be more extensive than in those injected I.M., but the "S.C." group remained more alert than the "I.M." group.

Intravenous route (I.V.). Only one animal (Pig 5) was injected I.V., and it received .25 ml. (25 mg.) of venom solution via the marginal ear vein. Approximately 3 minutes after the injection, the animal squealed, fell to its side, and made violent efforts to vomit. A frothy vomitus, ultimately streaked with blood, was expelled. Cyanosis, observed easily because of the pig's white skin, rapidly ensued. At about 5 minutes postinjection the animal gasped as if in terminal agony and then lay still. No movement was detected for 2 minutes, during which the animal appeared to have died. Abruptly it gasped again, the gasps became more frequent, and then its respiration, at first extremely labored, slowly returned to normal. Incredibly, within 15 minutes the pig regained consciousness, arose unsteadily, and walked off. The animal not only survived, but failed to show any sign of illness thereafter. It remained under observation for a period of several months, during which it appeared to grow and gain normally; it was ultimately killed. This one incident contrasts sharply with Pearce's experiments with rabbits (1909), in which some of the animals succumbed 5 or 6 days following the I.V. injection of small doses of C. adamanteus venom.

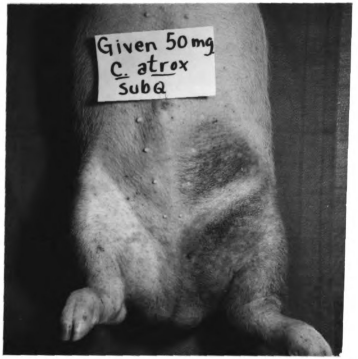

Figure 1. Hemorrhage and edema in a pig injected subcutaneously between the hock and the pastern with 50 mg. of venom of C. atrox, 24 hours postinjection.

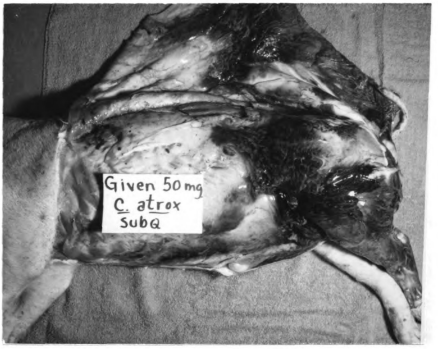

Figure 2. Skin reflected over site of subcutaneous deposition of 50 mg. of venom of C. atrox to show lesions in subcutis and muscle, 24 hours after envenomation.

Microscopic Lesions

Microscopically the lesions found in pigs given C. atrox venom were qualitatively similar whether the venom was given S.C. or I.M. The main differences observed in the tissues of S.C.- and I.M.-envenomated animals were quantitative. This conclusion is in harmony with that of Beamer et al. (1960), who studied the histopathologic lesions in rabbits given the same type of venom. The salient pathologic features noted in the various tissues will be discussed separately below.

Skin. At the site of venom deposition there was severe hemorrhage. Dermal collagen was fragmented and in complete disarray. Bundles of collagen were widely separated by a proteinaceous fluid interpreted as edema. The lumens of small blood vessels were filled with blood, and vascular endothelial cells were swollen, their cytoplasm at times appearing granular and markedly eosinophilic. In some places actual rupture of vessel walls with escape of blood into surrounding tissues was observed. In some intances necrosis of vascular walls was accompanied by accumulations of neutrophils and lymphocytes. The foregoing effects were more pronounced in animals given the venom S.C. Figures 3 and 4 illustrate the appearance of normal and envenomated skin, respectively.

Muscle and blood vessels. The hemorrhages seen in muscle can best be explained by noting first the damage to vessels nourishing larger muscular fasciculi and then following the wake of the injury to smaller arteriolar branches supplying progressively smaller bundles. From the site of deposition of venom the hemorrhage spreads along epimysial, perimysial, and finally endomysial planes. There are swelling and necrosis

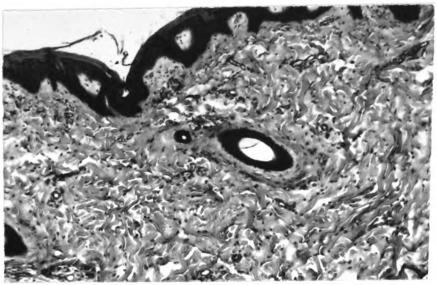

Figure 3. Normal skin of pig. Hematoxylin and eosin. x75.

Figure 4. Skin of pig envenomated subcutaneously with 50 mg. of C. atrox venom. Hematoxylin and eosin. x75.

of endothelial cells and, in some instances, a frank discontinuity of vascular walls with consequent extravasation of blood. A few instances were observed in which the endothelial lining was actually detached from the tunica media. Hemorrhage could be observed between the detached endothelium and the enveloping medial tunic (Figure 5). This change was also observed in vessels supplying tissues remote from the actual site of venom injection. Edema was marked in the interfascicular connective tissues and in the adventitia of blood vessels. No damage could be detected in large muscular or elastic arteries beyond a variable degree of swelling in the endothelial lining cells.

In contrast to the hemorrhagic effects, the necrosis of skeletal muscle can be understood best by first describing the damage to individual fibers, then tracing the pathogenetic progression to bundles of increasingly larger mass. Early in this sequence of events the myofibrils became fused, the fibers lost their striations, and the individual fibers appeared homogeneously eosinophilic. At this stage the effect on the fiber is compatible with that seen in Zenker's degeneration of muscle due to other causes. Later there were vacuoles within the fiber, either because of loss of sarcoplasm through a damaged sarcolemma or because of lysis and precipitation of the proteins within the fiber. At times a change resembling "hyaline droplet" degeneration could be seen. A patchy basophilia, probably from imbibition of lysed nuclear material, could sometimes be demonstrated. Ultimately liquefaction necrosis and invasion of the sarcolemma by neutrophils, which at times replaced necrotic fibers, initiated the stage of resolution of the entire process. Figure 6 depicts the appearance of normal porcine muscle. Figures 7, 8 and 9 illustrate various degrees of injury to envenomated

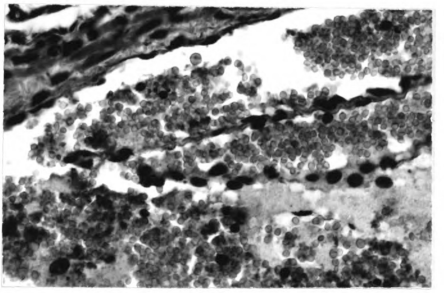

Figure 5. Blood vessel with hemorrhage between detached endothelium and medial tunic. Hematoxylin and eosin. x312.

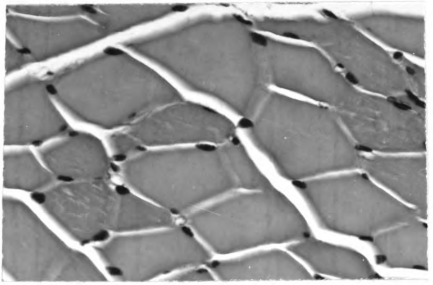

Figure 6. Normal muscle of pig. Hematoxylin and eosin. x312.

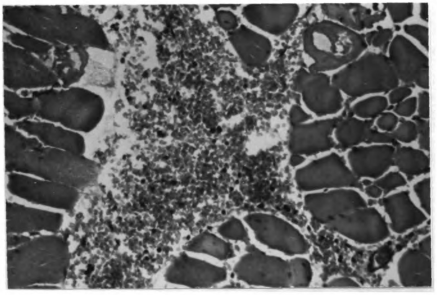

Figure 7. Muscle of pig envenomated intramuscularly with 50 mg. of C. atrox venom. Perimysial hemorrhage. Hematoxylin and eosin. x187.

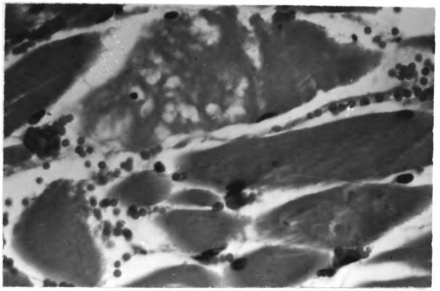

Figure 8. Envenomated porcine muscle. Beginning necrosis of fiber. Hematoxylin and eosin. x750.

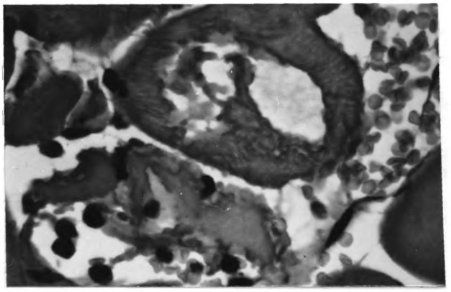

Figure 9. Envenomated pig muscle with more advanced necrosis of fiber than in Figure 8. Hematoxylin and eosin. x750.

muscle taken at variable distances from the site of venom deposition.

Regional lymph nodes. The medullary sinuses were markedly hyperemic. Numerous instances of erythrophagocytosis were in evidence, some large mononuclear phagocytes bearing as many as 15 red blood cells within their cytoplasm (Figure 10). At times a complete corona of erythrocytes was seen around an already replete macrophage. Although blood vessels were greatly dilated, only an occasional hemorrhage could be seen.

Kidney. Although the renal vessels of most of the animals were engorged, hemorrhages were exceptional, occurring in only 3 pigs. In one instance, a large subcapsular hemorrhage measuring about 3 cm. in diameter was seen, but this suffusion did not extend into the renal parenchyma. Many glomeruli had proteinaceous globules in Bowman's space (Figure 11) but these lesions did not approach in number or extent the renal lesions reported in rabbits by Pearce (1909, 1913). At times the tubules in entire medullary pyramids appeared swollen, with no visible lumens, while those of adjoining pyramids were apparently normal. In some of the swollen tubules hyaline droplet degeneration was seen. Whether renal changes of greater magnitude might have developed had the animals lived longer is a moot point.

Liver. There was engorgement of all vascular channels in this organ. In most instances necrosis and degeneration became apparent first in the centrilobular areas and then in the midzone of the lobule. Several instances were observed in which entire lobules were replaced by blood (Figure 12), a process which closely resembled gossypol or clay-pigeon poisoning but differing from the latter in that only an occasional

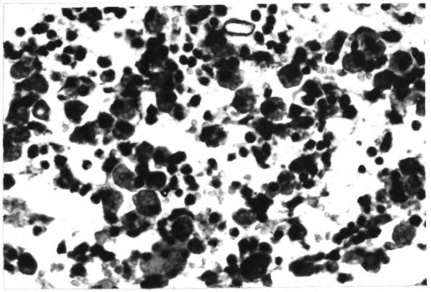

Figure 10. Erythrophagocytosis in lymph node of pig given 50 mg. of C. atrox venom subcutaneously. Hematoxylin and eosin. x312.

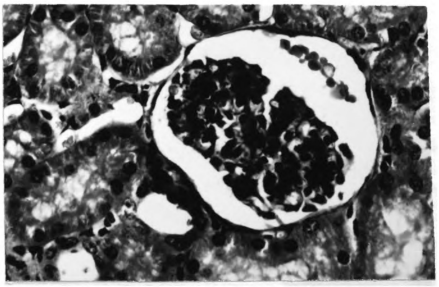

Figure 11. Glomerulus with globules of proteinaceous material in Bowman's space. Hematoxylin and eosin. x750.

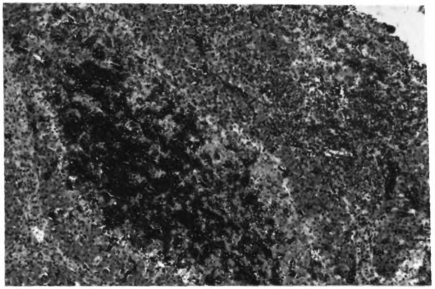

Figure 12. Hepatic lobule with centrilobular necrosis resembling clay-pigeon poisoning. Hematoxylin and eosin. x75.

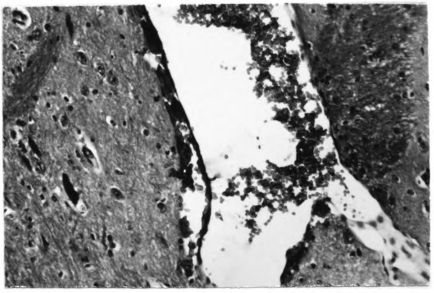

Figure 13. Hemorrhage around a cerebral vessel with detachment of endothelial lining from tunica media. Hematoxylin and eosin. x187.

lobule was so involved. A lesion found more frequently was hydropic degeneration of the peripheral portions of the lobule. Cloudy swelling of liver cord cells around central veins was also a frequent observation.

Myocardium. Areas in the myocardium which grossly appeared as pale, poorly circumscribed foci of variable size proved to be zones of albuminous degeneration under microscopic examination. An occasional microhemorrhage was present. A finding noted inconstantly was an abnormally wide spacing of myocardial fibers.

Brain. Impressive changes were noted in the brain stem and cortex of most of the animals. A narrow cuff, 1 or 2 cell layers thick, appeared around many small vessels. Microhemorrhages were also observed (Figures 13 and 14). Gliosis and neuronophagia were noted with unexpected regularity. Hyperemia and microhemorrhages of meningeal and cerebral vessels were seen, but grossly visible extravasations of blood were not observed. Foci of neuronal necrosis were in the cerebral cortex, but these areas were not sufficiently identified with specific anatomic regions to warrant their being considered characteristically constant lesions. Irregular foci of demyelination such as that illustrated in Figure 15 were found in the cerebellum.

Spleen. The hyperemia noted in the spleen resembled that seen in barbiturate intoxication. There was an abundance of reticuloendothelial elements in the red pulp.

Lung. Marked hyperemia was present, but no significant extravasations were found.

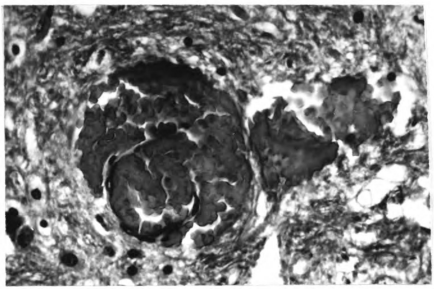

Figure 14. Hemorrhage around a cerebral vessel with filling of the Virchow-Robin space. Hematoxylin and eosin. x312.

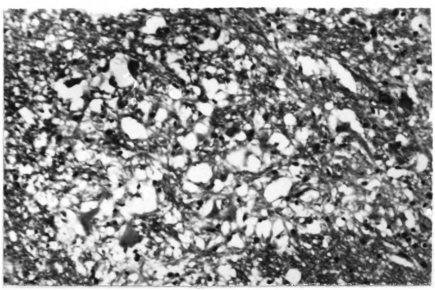

Figure 15. A focus of demyelination in the cerebellum of a pig given 50 mg. of venom of C. atrox intramuscularly. Hematoxylin and eosin. x187.

Other organs. Microscopic lesions in other tissues, such as gastrointestinal tract, pancreas, thymus, and adrenals, were inconstant and minor.

ENVENOMATION WITH NAJA NAJA VENOM

Clinical Observations and Gross Lesions

Four pigs were injected with 1 ml. each of a solution of venom of Naja naja containing 10 mg. of lyophilized venom per milliliter. Pigs 16 and 17 received the venom S.C. at a point about midway between the pastern and hock, and Pigs 19 and 20 received it I.M. in the lateral femoral muscles. Because the symptoms were similar regardless of the route of envenomation used, the clinical observations recorded here apply to the entire group.

Immediately after injection the pigs returned to their troughs, ate, and remained free of signs until 2-1/2 hours afterward. The first sign observed was a posterior weakness manifested initially by a slight weaving of the hindquarters and a sudden spasmodic flexion of the joints which would cause them to assume abruptly a sitting posture. The weakness progressed rapidly to a posterior paresis and was followed shortly by a peculiar dyspnea during which the animals, in a sitting attitude and with neck stretched forward, would open their mouths and gasp at each inspiration (Figure 16). Then, in rapid sequence, the inspirations became more labored; cyanosis became more marked; the animals assumed a lateral recumbency; and all died approximately 4 hours postinjection.

The gross lesions were more easily demonstrable in the pigs receiving the venom S.C. Figures 17 and 18 demonstrate the contrast between pigs given venom of N. naja and those injected with C. atrox. In these

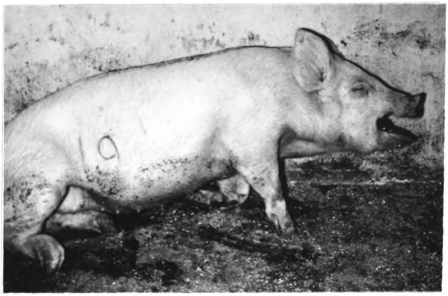

Figure 16. Characteristic dyspnea in pig given 10 mg. of venom of N. naja intramuscularly.

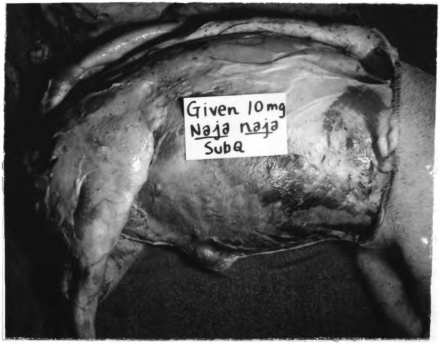

Figure 17. Site of subcutaneous deposition of 10 mg. of venom of N. naja.

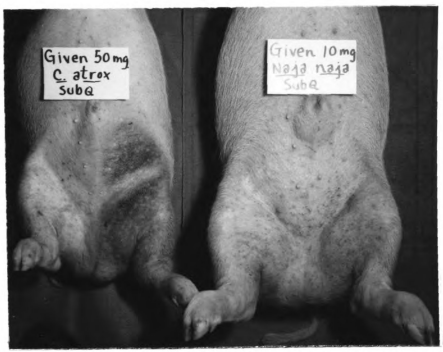

Figure 18. Comparison of gross effects of rattlesnake and cobra venom. Left, pig injected with 50 mg. of venom of C. atrox into subcutis of left hock, 26 hours postinjection. Right, pig injected with 10 mg. of venom of N. naja into subcutis of right hock, 4 hours postinjection.

there was a very slight gelatinous edema and a mild discoloration in the subcutis, both limited to a radius of 3.0 cm. around the site of the injection. In the animals injected I.M. there was an area about 2.5 cm. in diameter in which the muscles appeared dark. A mild edema was seen in the intermuscular septa adjoining the presumed site of venom deposition.

No significant gross lesions were detected in any of the other organs except the lungs, which were hyperemic.

Microscopic Lesions

Skin, subcutis, and muscle. The lesions in tissues collected from the immediate vicinity of the site of venom deposition resembled in character those previously described for C. atrox envenomation. However, an important point of difference in the action of the 2 venoms was the extent of the damage produced. In pigs which received the cobra venom the resultant edema and hemorrhage were confined to a zone no more than 2 to 3 cm. beyond the actual site of injection. Necrosis of muscle fibers was present, but this effect was also limited to the venomized area. Extravasation of blood was minimal in contrast to the massive hemorrhages observed with the rattlesnake venom.

Regional lymph nodes. The popliteal lymph nodes had a large amount of inflammatory reaction. Surrounding the lymph follicles and adjacent to the trabeculae were large numbers of neutrophils. This inflammatory component was only rarely seen in the medullary sinuses.

Lungs. The microscopic appearance of the lungs corroborated the gross observations. In addition to marked hyperemia, there were foci of

hemorrhage under the epithelium of numerous bronchioles. Many alveoli were filled with blood.

In the ventral portions of all the lobes numerous alveoli had a faintly eosinophilic acellular fluid interpreted as edema.

Other tissues. No significant lesions were recognized in any of the other tissues examined.

Histochemistry

Good localization of some enzymes, notably alkaline phosphatase (AP), leucine aminopeptidase (LAP), and cytochrome oxidase (CO) was obtained in the normal pig tissues. In sections of normal small intestine and kidney, AP activity was most intense in the cytoplasm of cells lining the villi, in the crypts, and in the brush border of the convoluted tubules. The CO was best visualized in the cytoplasm of the cells lining the villi and in the skin and hair follicles, but in none of these locations was the reaction strong. Difficulty was encountered with the demonstration of CO, and it became necessary to use a negative control to assure that the staining observed was the result of enzymatic activity and not artifactitious. The negative control slides were prepared by the addition of potassium cyanide to the substrate solution. Leucine aminopeptidase was well localized in the cytoplasm of intestinal villous cells and in the epithelial cells of the kidney tubules. It was not possible to demonstrate unequivocally lactate dehydrogenase (LDH) activity in the sections examined. Although Nachlas et al. (1958) reported LDH activity to be high in smooth muscle, the sections of both normal and envenomated pig intestine had such inconstant and erratic results that no interpretations were made.

Sections of intestine, skin, and kidney, whether envenomated with cobra or rattlesnake venom, had no AP or CO activity at the site of venom deposition. However, enzymatic activity was present 2.0 cm. from the actual site of injection, where epithelial cells were still relatively intact. The intensity of the staining reactions varied proportionally as the distance from the site of envenomation. At the periphery of the venomized areas AP activity could be seen extracellularly in clumps of granules that stained blue with Barka's modification of Burstone's technic. Activity of CO was not as clearly demarcated, for relatively intact intestinal villi at the periphery of the venomized zones had no enzymatic activity. An unusual result was observed in sections stained for leucine aminopeptidase. In these, even in areas next to venomous deposition, activity of the enzyme could be seen diffusely distributed in agglutinated aggregations which stained red with the Burstone and Folk diazo coupling method.

Cholinesterase (ChE) activity was strong in sections of normal muscle and nerve. Envenomated tissues had so much demonstrable enzyme activity that it was widely dispersed and unsuitable for interpretation. Some of the enzyme activity in tissue envenomated with venom of N. naja may have been contributed by the venom itself, since ChE is reported to be present in the venoms of all elapid snakes (Ghosh et al., 1956).

Electron Microscopy

Because of the backlog and the large volume of work in progress at the only available electron microscopic processing laboratory, it was necessary to limit the study of tissues to liver and muscle specimens envenomated with venom of C. atrox.

Sections from several blocks were examined at different magnifications. Electron micrographs at a magnification of x12,000 were made of envenomated liver and muscle; micrographs of similar normal tissues were also taken for comparison.

Specimens taken from the periphery of the venomized areas proved to be most desirable for study. Liver cord cells had swollen mitochondria and a loss of sharp detail in other structures, such as the Golgi complex and endoplasmic reticulum. Myelin figures, which appeared as concentrically laminated structures, were very common in damaged hepatocytes. An increased number of lysosomes and dense bodies were seen. Erythrocytes within sinusoids appeared unduly distorted and almost invariably had a film of dark granular material adherent to the plasma membranes. The nearer the samples were to the actual site of venomous injection, the more difficult it was to discern with precision the organelles within the hepatocytes.

Figures 19 and 20 illustrate normal and envenomated liver tissue, respectively. The sample of envenomated muscle shown in Figure 22 was taken from an area bordered by perimysium. It illustrates the type of damage observed in muscle fibers and surrounding connective tissue. There are numerous erythrocytes, most with a film of dark granular material around their plasma membranes. Fragmented collagenous fibers and particles of myofibrils are seen. Clumps of hazily outlined material, probably precipitated protein globules, are seen diffusely scattered throughout the field. An irregularly shaped cell with a bilobed nucleus, probably a neutrophil, can be observed. Some lysosomes and mitochondria may also be seen. Figure 21, a portion of normal muscle fiber, is presented for comparison.

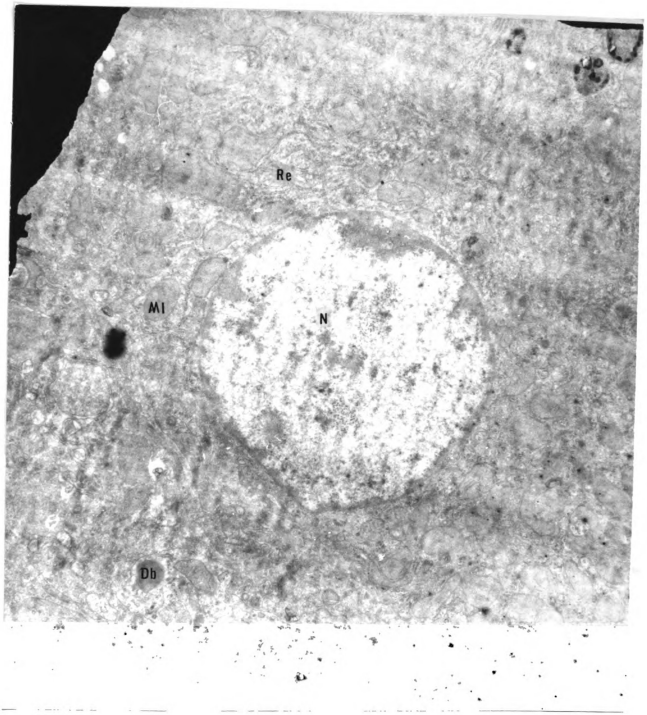

Figure 19. Specimen of normal hepatocyte, x12,000. N, nucleus; MI, mitochondrion; Db, dense body; Re, endoplasmic reticulum.

Figure 20. Specimen of envenomated hepatocyte, x12,000. Nu, nucleolus; MI, mitochondrion; Ly, lysosome; MF, myelin figure; G, glycogen; Db, dense body; Rbc, erythrocytes.

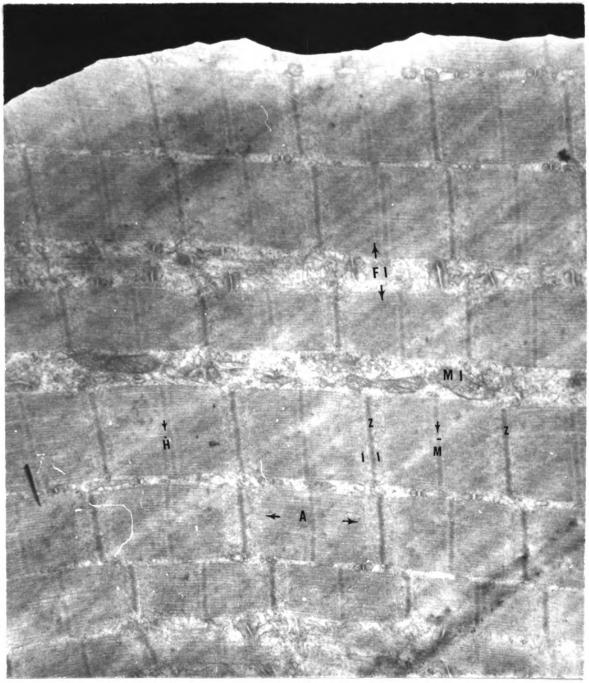

Figure 21. Portion of a normal muscle fiber, x12,000. FI, myofibril; MI, mitochondrion; A, "A" band; Z, "Z" band; M, dark "M" band traversing sarcomere; I, "I" band; H, "H" band.

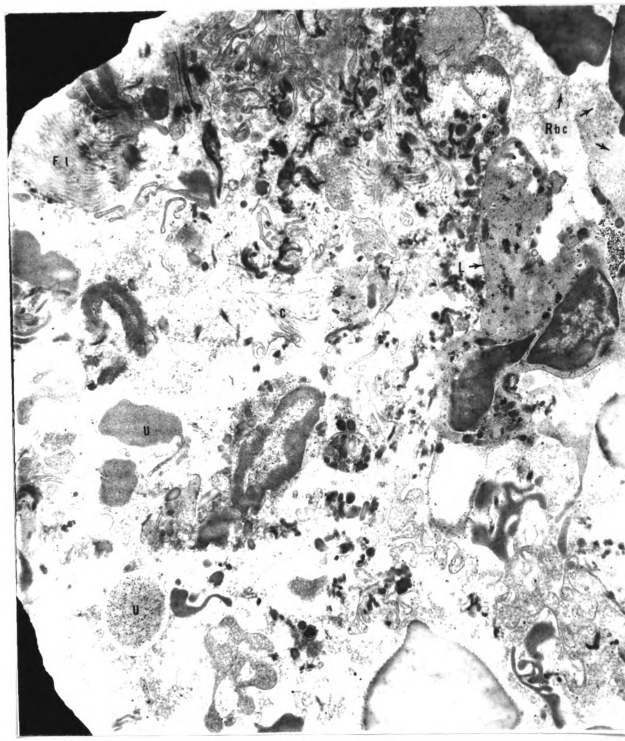

Figure 22. Portion of envenomated muscle fiber at perimysial border, x12,000. Rbc, erythrocytes; FI, fragmented myofibril; L, leukocyte; C, fragmented collagenous fiber; U, unidentified material, probably precipitated protein.

Serum Enzyme Assays

Ranges in the preinjection levels of the 6 enzymes studied in these experiments are summarized in Table 3.

Alterations in the serum enzyme levels produced by the subcutaneous or intramuscular injection of 50 mg. of venom of C. atrox are summarized in Table 4. Since no significant differences were found between pigs given the venom S.C. and those injected I.M., the results given in Table 4 represent the data obtained in envenomated animals regardless of the route of envenomation used.

Levels of serum enzymes indicated wide ranges in both normal and envenomated animals. Cornelius and Kaneko (1963) reported the normal levels of SGPT and SGOT for swine 1 to 3 years of age. The normal value of SGPT ranged from 19 to 35 Sigma-Frankel units/ml., with a mean of 27. For SGOT the normal range was 17 to 45 Sigma-Frankel units/ml., with a mean of 31. Long et al. (1965) reported the normal alkaline phosphatase levels in pigs. Their assays gave extremely variable ranges of AP; in newborn animals the serum content of this enzyme was several times higher than in mature swine.

Statistical analysis of the data was conducted with the Fisher "t" test of significance between 2 sample means. The most significant values occurred with SGOT and LDH; alterations in the other enzymes were not statistically significant, although a twofold increase in CPK was observed in the envenomated animals.

A great deal of difficulty was encountered with hemolysis of the samples drawn from the envenomated animals. Despite careful handling of the specimens, numerous instances were experienced in which hemolysis occurred. Venipunctures were repeated in Pigs 11, 14, and 15. Samples

Table 3. Ranges in levels of serum enzymes in normal serums of 7-week-old pigs

Enzyme	Range	Mean*
Alkaline phosphatase (AP), Sigma-Frankel units/ml.	4.16- 6.17	4.88
Glutamate-oxaloacetate transaminase (SGOT), Sigma-Frankel units/ml.	42-72	54.13
Glutamate-pyruvate transaminase (SGPT), Sigma-Frankel units/ml.	34.60-46.00	42.30
Creatine phosphokinase (CPK), CPK units/ml.	74-125	101.00
Lactate dehydrogenase (LDH), Berger-Broida units/ml.	870-1580	1342.00
Isocitrate dehydrogenase (ICD), ICD units/ml.	160-333	253.00

* Values represent average of 15 pigs.

Table 4. Assays of serum enzymes in pigs given 50 mg. of C. atrox venom

Enzyme	Pre-injection Mean*	Post-injection Mean*	Mean Difference	Standard Dev.	t value
Alkaline phosphatase (AP), Sigma-Frankel units/ml.	4.91	4.58	-.33	.8693	.48
Glutamate-oxaloacetate transaminase (SGOT), Sigma-Frankel units/ml.	55.44	197.76	+142.32	28.40	5.01
Glutamate-pyruvate transaminase (SGPT), Sigma-Frankel units/ml.	43.16	55.85	+12.69	6.75	1.88
Creatine phosphokinase (CPK), CPK units/ml.	110.22	345.44	+235.22	137.20	1.71
Lactate dehydrogenase (LDH), Berger-Broida units/ml.	1286.00	1810.00	+524.0	226.00	2.32
Isocitrate dehydrogenase (ICD), ICD units/ml.	253.88	426.60	+172.8	117.00	1.47

* Values represent average of 9 pigs except for AP, in which only 8 were used.

in which visually detectable hemolysis occurred were not used for analysis because of the normally high content of transaminases in erythrocytes.

One enzyme, cholinesterase, was found significantly elevated in the 4 pigs given 10 mg. of cobra venom. Cholinesterase increased from a pre-injection mean of 0.58 Rappaport unit/ml. to a postinjection level of 2.02 units/ml. The small number of animals given venom of N. naja precludes precise statistical analysis in this group; however, the mean post-injection value for cholinesterase represents a gross increase of 250%.

Serum Protein Studies

Tables 5 and 6 present the changes observed in the serum proteins of pigs given the venom of C. atrox and N. naja, respectively. The most significant alteration was seen in the decreased total protein content of the serum of the animals injected with the venom of the rattlesnake. The blood samples from the pigs given the crotalid venom were collected approximately 26 hours after envenomation, while those specimens from the animals injected with the cobra venom were drawn about 3 hours post-injection. It may be seen from Table 5 that a net loss of 1.05 Gm./100 ml. occurred in the total serum protein level; of this loss, 0.75 Gm./100 ml. was albumin and 0.30 Gm./100 ml. consisted of globulins. Statistical analysis of the data contained in Tables 5 and 6, performed with the Fisher "t" test of significance between 2 sample means, revealed that, in the group given the rattlesnake venom, the loss of total proteins and the decrease in the serum content of albumin were statistically significant; but the decrease in globulins was not. The slight changes observed in the serum proteins of the animals envenomated with cobra venom were not statistically significant.

Table 5. Changes in serum proteins of pigs given 50 mg. venom of C. atrox

	Pre-injection mean (Gm./ 100 ml.)	Post-injection mean (Gm./ 100 ml.)	Mean Difference	Standard Deviation	t value
Total protein (Gm./ 100 ml.)	5.47	4.42	-1.05	.31	3.38
Albumin	3.06	2.31	-0.75	.33	2.30
Globulins	2.41	2.11	-0.30	.88	.25

Table 6. Changes in serum proteins of pigs given 10 mg. venom of N. naja

	Pre-injection mean (Gm./ 100 ml.)	Post-injection mean (Gm./ 100 ml.)	Mean Difference	Standard Deviation	t value
Total protein (Gm./ 100 ml.)	4.75	4.41	-0.34	1.24	.27
Albumin	2.41	2.00	-0.41	0.92	.44
Globulins	2.34	2.41	+0.07	0.15	1.73

Table 7. Changes in albumin:globulin ratios in envenomated pigs

	A:G ratio, preinjection	A:G ratio, postinjection
Pigs given *C. atrox*	1.26	1.09
Pigs given *N. naja*	1.02	0.82

Table 8. Effects of venom on total white blood cell count, hemoglobin, and hematocrit

	Preinjection Means*			Postinjection Means*		
	Total WBC	Hb	Hct	Total WBC	Hb	Hct
Pigs given *C. atrox* venom	17,375(9)	11.1	35.64	22,025(9)	10.14	31.21
Pigs given *N. naja* venom	19,775(4)	10.7	33.12	17,500(4)	10.62	33.25

* Numbers in parentheses indicate number of animals.

Results of Total White Blood Cell Counts and Hemoglobin and Hematocrit Indices

These results are given in Table 8. The animals given the crotalid venom had a total increase in the white blood cell count of 4650 cells/mm^3 at the end of Postinjection Hour 26. At this time the mean hemoglobin (Hb) and hematocrit (Hct) indices had decreased to 10.14 Gm./100 ml. and 31.21 mm., respectively, from preinjection levels of 11.1 Gm./100 ml. and 35.64 mm., respectively.

Blood specimens from the pigs injected with the cobra venom were collected only 3 hours after envenomation, since observation of clinical signs indicated that they might not live much beyond this time. At the end of Postinjection Hour 3, this group had experienced a mean decrease in the total leukocyte count of 2275 cells/mm^3. The hemoglobin and hematocrit indices of this group had changed only slightly; the hemoglobin level decreased 0.08 Gm./100 ml. and the hematocrit increased .13 mm.

DISCUSSION

Gross and Microscopic Lesions

The effects of snake venoms in the tissues of the animals used in this study confirmed and extended previous research on this subject. The lesions observed in the brains of the pigs envenomated with C. atrox during this study warrant emphasis because no reference was found in the literature reviewed that adequately described them.

Although the venom of C. atrox is not generally regarded as being potently neurotoxic, most of the pigs given this venom had demonstrable lesions in the gray and white matter of the cerebral cortex, cerebellum, midbrain, and brain stem. These changes consisted of microhemorrhages, perivascular cuffing, neuronal necrosis, and focal areas of demyelination and gliosis.

Another lesion to which no reference could be found in the literature was the peculiar intramural hemorrhage observed in a few blood vessels. The endothelial lining of these was detached from the medial tunic, and extravasated blood was observed between the endothelial layer and the tunica media. This pathologic feature indicates that, although there may be hemorrhage by diapedesis in ophidiasis, ecchymoses may also occur by direct damage to vascular endothelium, perhaps by lysis of the cells, permitting the escape of blood through the intima. If these extravasations occur in significant numbers, they might have an effect resembling that of multiple dissecting aneurysms.

Whereas all but one of the pigs given the crotalid venom were alive at the end of 26 hours (8 of 9), those given the elapid venom died within 4 hours (4 of 4). The rapid demise of the latter group resulted in few recongizable lesions except around the site of venomous deposition. One lesion observed in the animals given the cobra venom is worthy of discussion. There was a noticeable degree of inflammatory exudate, consisting mainly of neutrophils and eosinophils, around lymphoid follicles and next to trabeculae.

Histochemical Studies

The concept of injecting venom directly into parenchymatous organs, such as liver, kidney, and intestine, with the animal under general anesthesia seems to be desirable in that it affords an opportunity to study the necrotizing effects in gradient degrees. The farther the sample is from the precise site of venom deposition, the more nearly normal the tissue and the enzymes elaborated by it will be. Furthermore, by collecting the samples from the live animal and freezing these immediately one is assured that the tissues will be fresh and that there will be little or no loss of tissue enzymes.

For the visualization of alkaline phosphatase in the tissues it is best to collect both small intestine and kidney. Leucine aminopeptidase is best studied with samples of small intestine. In order to discern with precision the exact localization of tissue enzymes following envenomation, it is advisable to make positive control slides of the same tissues. Serial sections of the envenomated area are necessary in order to follow the necrotizing effects of the venom.

For the study of cytochrome oxidase it would be preferable to envenomate small areas of myocardium, since this enzyme seems to be in greatest abundance in the heart muscle. Negative controls must be used in studying cytochrome oxidase.

It is probable that histochemical study of envenomated organs might be more fruitful if the selected tissues were observed with the electron microscope.

Serum Enzymes

In general the results of enzymic alterations in the serum of the envenomated pigs did not contribute as much to this research as had been expected. Whereas enzymic activity of venoms *in vitro* can be well controlled, *in vivo* study of serum enzymes is fraught with an infinite number of problems because of the multiplicity of enzymes produced by the living animal, the multifaceted action of the venom, and the considerable number of steps involved between collection of the specimen and final interpretation of the results. As stated by Cliffton (1960):

> "The use of enzymes in clinical medicine is so new and expanding so rapidly that a critical review and evaluation is difficult. A previous attempt by [this] speaker in 1954 is now hopelessly outmoded."

Adding to the frustrations of the student of serum enzymes is the large number of isozymes. For example, lactate dehydrogenase has at least 5 isozymes, and alkaline phosphatase has at least 2.

Statistically significant increases were observed after crotalid envenomation in the levels of serum glutamate-oxaloacetate transaminase and lactate dehydrogenase. Although SGOT is not "liver specific", Cornelius (1963) reported it to be elevated in liver necrosis and in certain diseases of muscle, both of which are damaged by crotalid venom, as proved by histopathologic assessment of the lesions of ophidiasis.

An attempt to determine which of the isozymes of lactate dehydrogenase was elevated the most was made in 2 pigs. Both of the animals had markedly elevated total LDH 24 hours after envenomation; when their serums were heated to 65 C., it was found that the heat stable isozyme remained at the preinjection level. This indicated that the increase in total LDH was due to the heat labile isozymes, which are produced mainly by the liver. Had the heat stable fractions been elevated, the analysis would have indicated myocardial damage.

An enzyme which on cursory examination of results seemed to be greatly elevated was creatine phosphokinase (CPK). The mean preinjection level was 110.22; after 24 hours the mean was 345.44, an increase of 235.22; however, statistical analysis revealed that the t value was less than 2.00.

Elevation of serum enzymes can occur for several reasons. The cell membrane may be damaged to the point where its altered permeability permits a free efflux of the enzymes it produces; altered metabolism of the cell may stimulate it to produce an enzyme at a greater than normal rate; or the rate of inactivation of a particular enzyme may be slow. Another factor to consider in the explanation of an unduly high level of an enzyme is the activity of the enzyme in question. In serum it is the activity of an enzyme which is measured, not the amount, although the two may be related.

It would appear from these studies that the elevation in the levels of SGOT and LDH resulting from crotalid envenomation was due mainly to hepatic injury. Although high alkaline phosphatase levels, another index of liver disease, was not increased in the venomized animals, it is possible that the venom partially inactivated this enzyme.

Only one serum enzyme, cholinesterase (ChE), was elevated in the pigs injected with cobra venom. Some of the ChE activity may have been that of the cobra venom itself. Since the group injected with the cobra venom lived less than 4 hours after receiving the venom, the failure of the other enzymes to reach demonstrably higher levels may have been due to the relatively short time elapsing between envenomation and death. However, a more tenable explanation is that the venom of N. naja is so strongly neurotoxic that it affects the higher brain centers, producing paralysis and death before demonstrable lesions appear in other organs.

Serum Proteins

The greatest decrease in serum proteins after envenomation occurred with albumin. This resulted in a narrowing of the A:G ratio, as indicated in Tables 5, 6, and 7. The most significant decrease in the globulins was in the beta fraction. There was a consistent increase in the concentration of the alpha globulin, although the net amount, measured in Gm./100 ml., was decreased.

The foregoing changes are not specific for ophidism, since they commonly occur in many diseases. The loss of albumin from damaged capillary endothelium, through the efflux of fluid into the interstitial tissue, is marked in ophidiasis. The increased concentration of alpha globulin is probably the result of accumulation of leukotaxine, products of necrosis, and pyrexin in the globulins of inflammatory exudates.

Electron Microscopic Studies

The response of the tissues to the action of the crotalid venom as observed with the electron microscope makes this instrument valuable in the study of ultrastructural alterations in ophidiasis. As illustrated

in Figures 20 and 22, the effect on the cells is remarkable. Further work with the electron microscope and different amounts of venom may permit more precise study of ultrastructural alterations.

SUMMARY AND CONCLUSIONS

The pathologic effects of 2 snake venoms, namely, that of Crotalus atrox, the western diamondback rattlesnake, and that of the common cobra of Pakistan, Naja naja, were studied in pigs 7 weeks of age by the following procedures: (1) assessing the gross and microscopic lesions; (2) selectively staining, by histochemical means, samples of locally envenomated organs obtained by laparotomy; (3) separating serum protein fractions by electrophoresis and computing A:G ratios; (4) determining the effects on 6 serum enzymes; and (5) examining ultrastructural changes in liver and muscle tissue by means of the electron microscope.

Several brain lesions, to which no comprehensive reference could be found in the review of the literature, were described and illustrated. These consisted of microhemorrhages, demyelination, neuronal necrosis, and perivascular cuffing, and occurred in the cerebral cortex, cerebellum, midbrain, and brain stem. Hitherto undescribed lesions in the vascular endothelium, characterized by a detachment of the endothelial lining from the medial tunic, were found and illustrated in this research.

Sections of directly envenomated intestine, kidney, and liver were particularly informative in the study of tissue enzymes. Alkaline phosphatase, leucine aminopeptidase, and cytochrome oxidase were best visualized in intestine. Some difficulty was encountered in staining cytochrome oxidase, and it was necessary to use negative control slides to interpret the results with this enzyme. It might have been more desirable to use myocardium to study cytochrome oxidase.

Although important alterations occurred in the distribution of serum proteins in the envenomated animals, the changes are not specific for ophidiasis.

Statistically significant increases in serum levels of serum glutamate-oxaloacetate transaminase and lactate dehydrogenase followed induced crotalid ophidiasis. The probability that this finding represents hepatic injury is supported by the fact that the increase in LDH, determined in 2 animals, was primarily in the heat labile isozymes; by the concomitant elevation in SGOT; and by microscopic demonstration of foci of centrilobular hemorrhagic necrosis resembling clay-pigeon poisoning.

Remarkable ultrastructural changes were observed in envenomated liver and skeletal muscle with the electron microscope. In hepatocytes, there was swelling of mitochondria and apparent injury to the Golgi complex, in addition to an increase in the number of myelin figures and lysosome particles. A specimen of skeletal muscle taken near the site of venom deposition was altered so markedly that no intact myofibrils were visible. Clumps of what appeared to be precipitated protein globules were seen. Erythrocytes were unduly distorted and had a dark, granular substance adhering to the plasma membrane. This material may be a conjugation of venom with phospholipids. The electron microscope, combined with histochemistry, could evolve as two of the most valuable tools to use in understanding the pathogenetic sequence of cellular death in ophidiasis.

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VITA

Gilberto S. Trevino was born in Laredo, Texas, on January 11, 1925. After attending the public schools of that city, he enrolled at Texas A&M University in September of 1942. In May of 1944 his education was interrupted, and for 2 years he served as a private in the infantry of the U. S. Marine Corps. He participated in the invasion of Iwo Jima in February of 1945.

In 1952 the writer graduated from the School of Veterinary Medicine at Texas A&M University. He then practiced veterinary medicine in North Hollywood, California, until November of that year. He was again called to active duty, as a second lieutenant in the U. S. Army Veterinary Corps, and served until February, 1954. After his discharge he again entered into private veterinary practice, this time in El Paso, Texas.

In September of 1954 he accepted an appointment with the teaching staff in the small animal clinic at Texas A&M University. While there he enrolled in the graduate school and, in 1959, obtained the degree of Master of Science in veterinary medicine and surgery. He resigned his position and again entered the U. S. Army in May of 1959.

In May of 1960 the writer was married to Christine Van Dam of Climax, Michigan. They have 2 children, Lisa, 7, and Steve, 6.

In September, 1965, the writer was assigned by the Army to the graduate school at Michigan State University.

During his career, the writer has had 20 articles published on various topics of veterinary pathologic and clinical interest. He is presently serving as a lieutenant colonel in the U. S. Army Veterinary Corps.