Toxicon Vol. 29, No. 11, pp. 1279–1303, 1991.
Printed in Great Britain.

0041–0101/91 $3.00+.00
Pergamon Press plc

REVIEW ARTICLE

SNAKE VENOM VARIABILITY: METHODS OF STUDY, RESULTS AND INTERPRETATION

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(*Received* 4 *December* 1990; *accepted* 19 *March* 1991)

J.-P. CHIPPAUX, V. WILLIAMS and J. WHITE—Snake venom variability: methods of study, results and interpretation. *Toxicon* **29,** 1279–1303, 1991.—The causes and implications of venom variability are discussed with a review of the literature. Venom variability may have an impact on both primary venom research and management of snakebite, including selection of antivenoms and selection of specimens for antivenom production. Choice of venom is reviewed, including venom collection, maintenance, and pooled venom versus venom milked from individual specimens, the latter being more reliable in many applications. Intraspecific variability resulting in clinical variability of envenomation occurs and is reviewed. Venom variability is considered at several levels; interfamily, intergenus, interspecies, intersubspecies and intraspecies, geographical variation, between individual specimens, and in individual specimens, due to seasonal variation, diet, habitat, age-dependent change, and sexual dimorphism. It is concluded that venom researchers must be aware of venom variability both in selecting their sources of venom and in interpretation of results. Producers of antivenom must utilize an understanding of such variability in selecting sources of venom for antivenom production to ensure representation of all venom types required within each antivenom. Furthermore, clinicians treating snakebite should understand the influence of venom variability on both the presentation of envenomation and the treatment implications.

INTRODUCTION

THIS review paper will discuss the various aspects of our current knowledge of venom variability, an understanding of which assumes increasing importance as attention is focused on individual venom components and not just on whole venom.

Snake venom variability was first observed in clinical cases in antiquity and the variability attributed to natural exogen factors. ARISTOTLE, in "*Historia Animalium*", believed that heat was the predisposing factor in the increase in viper venom toxicity when

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comparing viper envenomation in Greece and North Africa, but no description of species was given. In modern times, initially venom variability in yield and toxicity (AMARAL, 1929; GITHENS and GEORGE, 1930; GITHENS, 1935) and cross-reactivity (KELLAWAY, 1930) was studied in an effort to elucidate phylogenetic relationships. To some extent this is still a consideration, however, venom variability has been shown to have far wider implications.

The venoms are a complex mixture of components which have a diverse array of actions both on prey and human victims. These components are biologically highly active proteins, whose primary function is to kill or immobilize prey and also to assist in the digestion of that prey. Each population of snakes will have its own particular requirements for the action of venom and the proportionate mix of actions will at least in part be determined by the type of prey it seeks. GLOYD (1940) suggested that this requirement of specialization in venom composition may occur independently of morphological variation. To achieve the end result of prey acquistion and/or digestion, the venoms may contain a mixture of the following activities: neurotoxic (pre/post synaptic), cardiotoxic, myolytic, coagulant (anticoagulant), haemostatic (activating/inhibiting), haemorrhagic and possibly directly nephro- or hepatotoxic actions.

PARE, in "*Traite sur les Venins*", noted a seasonal variation in the European viper (*Vipera aspis*) venom, with spring venom being more effective than autumn venom and bites inflicted in spring or summer being more severe. Soon it became apparent that interspecies variation also existed and clinicians pointed out differences observed after snakebite. A reason for this variation was sought, firstly based on clinical observations and then through rudimentary experiments, with conflicting results.

The combinations of activities within the venoms and the experimental methods developed to identify these activities, e.g. chemical and immunological techniques and statistical analysis of such observations, has allowed for the determination of the level of venom variations and their origins. The importance of such studies is obvious. Therapeutically the knowledge of intraspecific variability would allow for more efficacious treatment of bite victims, and symptoms in bite victims from specific localities may aid in the choice of an appropriate antivenom. Thus the production of antivenom is reliant on the knowledge of the variability of venoms within and between specific localities (BARRIO and BRAZIL, 1951; WARRELL, 1985; WARRELL *et al.*, 1989; THEAKSTON *et al.*, 1989; BOBER *et al.*, 1988). When preparing pharmacologically active fractions from crude venoms as a research (therapeutic) tool it is essential that venoms are chosen which are known to be rich in the component of interest, and hence knowledge of the component's distribution within species and within geographic localities is essential to success (SCHENBERG, 1959; MEBS and KORNALIK, 1984; JAYANTHI and VEERABASAPPA GOWDA, 1988; SADAHIRO and OMORI-SATOH, 1980; MORENO *et al.*, 1988).

The combination of various activities in venoms is also important for zoologists, aiding in the phylogenetic characterization of snakes at species and subspecies levels (GLENN and STRAIGHT, 1978) and last but not least investigation of variability has extended our fundamental knowledge of venom chemistry.

METHODOLOGY

The question of variation in venoms has been approached from many angles and the methods employed in investigations have shown great diversity. The venom composition of individual snakes has been studied through age-dependent and seasonal changes and even in individual glands, while on the other hand far more expansive

work has been done on differences at species or even genus level. In some respects the methods employed to study variation are governed by the aim and achievable ends of the investigation.

Early reports were perhaps hampered by technical expertise, and while criticism of each method employed in venom variability studies is outside the scope of this review, care should be taken to critically evaluate the methods employed in any particular study, especially if results are compared between authors employing essentially the same experimental procedures. Methodology employed in these studies may be biased towards particular components of the venom and while advancing our knowledge in a variety of areas of venom composition it may also have produced a disjointed overall picture. The species of snake chosen in comparative studies understandably often reflects a local interest and availability of specimens. To this end North and South American species feature heavily in the literature but studies have also been performed on specimens from most parts of the world.

VENOM—CHOICE AND CONSIDERATIONS

It is obvious that the choice of venom is of considerable importance. In early studies pooled venom was employed and the poor maintenance of the collected venom was matched with limited experimental methods of analysis.

Pooled venom

The use of pooled venoms stems from a requirement for an average venom quality or a statistically representative venom. Commercially prepared venoms are a probable source of pooled venom and identification of the snakes to (sub)species is removed from the researcher's control. Collection of the specimens to be milked for a pooled venom would appear most satisfactory (BONILLA *et al.*, 1973). The snakes should be caught within a limited territory and the milking performed the same day. The season the collection was performed should be considered as a possible source of variation while the following information should be logged: morphology, sex, geographical location (perhaps topography) and the age of the specimen if possible. These factors may have a bearing when interpreting data and can provide explanations or allow exclusions of possible sources of variation.

Individual variation cannot be studied with pooled venom and it could be argued that the numerous venom components present, each able to vary independently, results in a number of different venoms (SCHENBERG, 1959; JOHNSON, 1968). The constitution of representative venom requires a great number of samples to be included and supposes a knowledge of the level and grade of variation within it.

Individual venoms

A much surer way to study variation is through individual specimens. Of course it is still necessary to consider the potential sources of variation, e.g. the origin of the snake, procedure of milking and storage of the venom. The snakes may be taken in the wild (WILLIAMS and WHITE, 1987; GREGORY-DWYER *et al.*, 1986), may be captive specimens (BOCHE *et al.*, 1981; MEIER, 1986; MINTON, 1975) or the result of breeding programmes (CHIPPAUX *et al.*, 1982; FURTADO and KAMIGUTI, 1985; MEBS and KORNALIK, 1984). The season of capture of wild specimens can thus be arranged, or in captivity the conditions can be manipulated to suit the researcher. Some studies have employed controlled year-round temperatures (GUBENSEK *et al.*, 1974; WILLEMSE *et al.*, 1979) while others have attempted to recreate seasonal variations with adjustable lighting and temperature (GREGORY-DWYER *et al.*, 1986). An alternative to this is to maintain the specimens in a 'snake-pit' where a more natural environment is available with seasonal flux in photo-

period and temperature. The effects, if any, of unnatural aggregations of snakes in captivity, and altered energy expenditure in foraging are not known. Again the means of maintaining the snakes may also be reflected in the aim of the study.

Venom collection

A number of extraction techniques have been employed in these studies. Voluntary injection of the venom into a receptacle through a rubber or parafilm membrane (WILLEMSE *et al.*, 1979; THEAKSTON and REID, 1978; WILLEMSE, 1978), manual extraction by massaging the venom glands (GLENN and STRAIGHT, 1989; JIMENEZ-PORRAS, 1961; BOBER *et al.*, 1988; GITHENS, 1935; MINTON, 1953) and electrical stimulation to improve venom extraction (GLENN and STRAIGHT, 1977; MARSH and GLATSON, 1974; JOHNSON *et al.*, 1987). The majority of reports, however, do not stipulate the collection technique. MINTON (1967) reported that venom extraction from young snakes in his study had been traumatic and that infection and injuries incurred during the milking were responsible for the deaths of several animals and therefore care should be taken when handling and milking specimens. The effects of frequent or repeated milking have been compared for both yield and composition (KOCHVA, 1960; SCHENBERG *et al.*, 1970; ISHII *et al.*, 1970; WILLEMSE *et al.*, 1979; MARSH and GLATSTON, 1974) (see individual variation).

Sample maintenance

Venom samples can be studied fresh, and while in the fresh state variables such as colour and turbidity can be determined (MACKESSY, 1988). Alternatively, filtrates may be employed or the venom may be centrifuged, frosted, dried or lyophilized. WILLEMSE and HATTINGH (1980) warned of the problems associated with venom preservation prior to use. Poor preservation techniques can lead to protein degeneration and the appearance of split fractions, with loss of original activity. Both toxicity and electrophoretic patterns could be modified. Reconstitution of dried venoms may also introduce artefacts, by altering the concentration of components with synergistic or antagonistic activity.

CLINICAL OBSERVATIONS AND REPORTS

Numerous authors have described differences in symptomatology after envenomation by snakes belonging to the same species and this is more apparent from widely distributed species. VELLARD (1937, 1939) described geographical variations in *Bothrops atrox* and *Crotalus terrificus* venom. Epidemiological studies have permitted further delineation of these observations and specific clinical manifestations appear to result from intraspecies venom variability.

The thrombin-like enzyme of *Echis carinatus,* a species widely distributed from West Africa through Central Asia and into Sri Lanka, is considered to be the principal cause of morbidity and death in its victims. In some areas of its distribution, however, necrosis would appear to be the major clinical problem (CHIPPAUX *et al.*, 1961). Zoologists have divided this species into several species and subspecies which concurs with these observations of clinical variability.

Crotalus scutulatus envenomation in the south-western United States has also produced variations in clinical pictures, (GLENN and STRAIGHT, 1978; GLENN *et al.*, 1983; GLENN and STRAIGHT, 1989) and initially were thought due to misidentification. Envenomation

by this species was characterized by predominantly neurotoxic effects (including paralysis) with little or no local effects. Further clinical studies in Arizona indicated some envenomations did in fact produce local tissue effects, sometimes severe (HARDY, 1983). The appearance of two venom types within this species provided an explanation for the anomalous clinical presentations and is discussed in more detail later. Clinically, this variability has had repercussions in the choice of antisera. JIMENEZ-PORRAS (1964) reported that antisera prepared with rattlesnake venom from northern Brazil provided no protection in victims bitten in the southern regions of Brazil and thus confirmed a requirement for local antiseras to be produced (GONCALVES and VEIRA, 1950; GONCALVES, 1956). BOBER *et al.* (1988) agreed that the differences in venom properties within species or subspecies of rattlesnakes may be significant enough to affect the clinical signs as well as the ability of commercial antivenom to neutralize venom toxicity.

Studies of *Vipera russelli* showed similar variations in clinical findings. JAYANTHI and VEERABASAPPA GOWDA (1988) indicated the lack of protection afforded southern Indian victims of *V. russelli* when given antivenom produced at the Haffkine Institute, Bombay. WARRELL (1985) detailed the clinical signs of *V. russelli* across central and south-east Asia, with neurotoxicity and intravascular haemolysis in Sri Lanka, and pituitary haemorrhage in Burma and southern India while increased capillary permeability was also found in Burma. The requirement for locally produced antivenom was supported with clinical responses to Indian poly-specific antivenom in Sri Lankan victims (WARRELL *et al.*, 1989). No reversal of neurotoxic signs was evident and up to 200 ml of antivenom was necessary to reverse the coagulation defect (THEAKSTON *et al.*, 1989). Clinical studies of *Pseudonaja* spp. envenomation in Australia have also raised the possibility of clinically significant variability in symptomatology (WHITE, 1987; ACOTT, 1988).

Clinical assessment of variation in venom activity must, however, allow for individual sensitivity to particular venoms and should not exclude therapeutic manipulations as a possible cause of apparent venom variation. Clinical observations have an important role to play in the identification of venom variation and may be the first clue to composition variability.

TOXICITY/LETHALITY STUDIES

Venom lethality has been extensively used in venom variation investigations and a number of targets for these venoms have been employed. MACHT (1937) used the minimal lethal dose in white mice and the inhibition of the growth of seedlings of *Lupinus albinus* watered with solutions of *Crotalus* venoms as an indicator of toxicity in comparing 16 samples of venom from *Crotalus* species. GITHENS (1935), studying North American pit vipers, employed pigeons as the target, giving venom intravenously. In studying age variation in toxicity of *Bothrops atrox, Echis carinatus* and *Naja nigricollis,* MEIER and FREYVOGEL (1980) used both mice and female crickets (*Grillus bimaculatus*) as the target organisms. MACKESSY (1988) determined the toxicity of venoms from adult and juvenile *Crotalus viridis helleri* and *C. v. oreganus* using the sagebrush lizard, *Sceloporus graciosus.* JOHNSON (1968) used the lethal dose 50% (LD_{50}) of *Paramecium multimicronucleatum* with crotalid venoms in determining possible taxonomic criteria for the species. In a study of the neuromuscular action of *Crotalus terrificus terrificus* venom from various South American countries, BARRIO and BRAZIL (1951) used a number of animals for their study including rats, mice, dogs, guinea-pigs, rabbits and the batrachians *Bufo arenarum* and *Leptodactylus ocellatus.*

KORNALIK and MASTER (1964) used the toxic unit of lethality in mice; however, the more common model for venom toxicity studies is the LD_{50} determination in mice. JOHNSON *et al.* (1987) compared LD_{50} determinations in venom from each gland of a southern Pacific rattlesnake (*C. viridis helleri*) and they have been used in comparing venom effectiveness with the age of the snake (MINTON, 1967, 1975; FIERO *et al.*, 1972; THEAKSTON and REID, 1978; REID and THEAKSTON, 1978; LOMONTE *et al.*, 1983). DETRAIT and DUGUY (1966) used LD_{50} measurements in comparing seasonal and individual variations of *Vipera aspis* venom, while GLENN and STRAIGHT (1977) used lethal toxicity for individual variability in *Crotalus viridis concolor* specimens. MINTON (1953) also used LD_{50} determinations in a study of individual variation in toxicity of *Agkistrodon contortrix mokason* and *Crotalus horridus horridus* venoms. The geographical variation studies of *Crotalus* (GLENN and STRAIGHT, 1978, 1985; GLENN *et al.*, 1983) and Elapid venoms (IRWIN *et al.*, 1970; SUTHERLAND, 1983) used this method as a basis of comparison and TABORSKA (1971) used the LD_{50} when determining intraspecies variability in *Echis carinatus* venoms.

The route of introduction of venom in LD_{50} studies may be intraperitoneal (i.p.), intravenous (i.v.), intramuscular (i.m.) or subcutaneous (s.c.) and in the majority of cases employs whole venom. In using whole venom, however, variations in pharmacologically less active components may be concealed by a highly potent protein. The validity of LD_{50} studies on mice when comparisons are made above species or possibly subspecies level is debatable as natural prey of the snakes in question may be more susceptible to their own particular predator's venom.

MEASURES OF BIOLOGICAL ACTIVITY

This method relies on the intrinsic enzymatic activities of the venoms, possibly revealed *in vitro* on selected substrates, or producing a measurable effect *in vivo*, on removed skin samples or cells in culture. The haemorrhagic zone produced in mice, rats, guinea-pigs and rabbits *in vivo* by intradermal injection of venom samples has commonly been used in venom variation studies. White and yellow venoms from an individual snake (JOHNSON *et al.*, 1987), specimens of *Vipera russelli* (DIMITROV and KANKONKAR, 1968) and *Vipera ammodytes* (MASTER and KORNALIK 1965) were compared through haemorrhagic activity. The haemorrhagic action of venoms has also been used for geographic variation studies (GLENN *et al.*, 1983; GLENN and STRAIGHT, 1985; MINTON and WEINSTEIN, 1986) while MINTON (1956, 1967) used areas of necrosis and haemorrhage and paramecium lysis in correlating the phylogeny of North American pit vipers and in examining the activity of juvenile snake venoms. The defibrinating capacity of venoms *in vivo* is another biological activity used as a comparative technique. This method was used in studies on *Crotalus atrox* venom changes with age (REID and THEAKSTON, 1978; THEAKSTON and REID, 1978) and intraspecies venom variability of *Echis carinatus* (TABORSKA 1971; KORNALIK and TABORSKA, 1988). JOHNSON *et al.* (1987) looked for myonecrotic effects of venom through histological examination of mouse thigh tissue injected with venom, while in a comparative study of venom from adult and young *Crotalus durissus durissus* specimens, LOMONTE *et al.* (1983) measured haemorrhagic activity and also the oedema producing ability of the venom.

BIOCHEMICAL ANALYSIS

Biochemical analysis of venoms can be considered under two headings, biochemical activity and separation techniques.

Biochemical activities

TABORSKA and KORNALIK (1985) categorized these activities into three groups. 1. Pathophysiologically active substances: *in vitro* procoagulants, fibrinogenolytics and inhibitors. 2. Enzymatic: including phospholipase, proteolytics, phosphodiesterase, 5′ nucleotidase, and L-amino oxidase. 3. Amidolytic: which has more recently employed chromogenic substrates as convenient monitors of the activity. These activities have been used to some degree in most studies both qualitatively and quantitatively and the method employed is often related to the purpose of the study. However, biochemical activities may not relate species to any greater degree than protein composition (see below) since LEVITON *et al.* (1964) suggested that distantly related (rattle) snakes whose venom activity seems pharmacologically close may in fact possess groups of non-homologous but functionally analogous proteins.

Separation techniques

Separation (and identification) of venom components may be achieved by a variety of techniques, most notably by electrophoretic techniques and liquid chromatography. These techniques have become more sophisticated with time and those working in the field have not hesitated in using the latest technology in the elucidation of levels of venom variation (GUBENSEK *et al.*, 1974; SADAHIRO and OMORI-SATOH, 1980; RAEL *et al.*, 1984; WEINSTEIN *et al.*, 1985). Liquid chromatography has been employed to isolate fractions of venoms which have then been used in variation studies (AIRD and KAISER, 1985) but in the main, elution profiles are used to compare venom composition with the appearance or absence of known fractions in the elution profiles. Gel filtration would appear to be the most frequently employed method, with venom composition compared by overlaying elution profiles (AIRD, 1985; WILLIAMS and WHITE, 1987) or by placing the elution profiles one above the other (BDOLAH, 1986; MEBS and KORNALIK, 1984; SCHAEFFER, 1987; WOODHAMS *et al.*, 1990; DIMITROV and KANKONKAR, 1968; BERNADSKY *et al.*, 1986; WILLIAMS *et al.*, 1988). GLENN and STRAIGHT (1989) used reverse phase chromatography to isolate the acidic and basic subunits of Mojave toxin and determine the presence of absence of this toxin in the venom of *Crotalus scutulatus scutulatus* from Arizona. Comparison of elution profiles, however, is limited by the possibility of like-elution of components whereby all components may not be visualized. The difficulty of equating shouldered regions of peaks must also be considered, particularly when an increased quantity of a component may produce a broader peak with the resultant masking of other components (MARSH and GLATSTON, 1974). Fractionation of the venom with liquid chromatography does have the advantage of allowing collected fractions to be tested for activities and thus compared to other venoms.

Electrophoretic methods and modifications have been extensively employed in comparative studies. In the 1960s starch-gel electrophoresis appeared to be the method of choice (JIMENEZ-PORRAS, 1961, 1964, 1967; MASTER and KORNALIK, 1965) and BERTKE *et al.* (1966) suggested that the electrophoretic patterns were distinct from a given species. JONES (1976) and LEVITON *et al.* (1964) used cellulose acetate strips to compare venom

proteins in *Agkistrodon* snakes from North America and rattlesnakes, respectively. CHIPPAUX *et al.* (1982) used Cellogel strips to compare the electrophoretic patterns of the venoms from a litter of *Bitis gabonica*. Isoelectric focusing of whole venoms has been performed in polyacrylamide gels (GREGORY *et al.*, 1984; GREGORY-DWYER *et al.*, 1986; LEVY and BDOLAH, 1976; ARAGON-ORTIZ and GUBENSEK, 1981) and using commercial gels (MEIER, 1986) with comparison of the electrophoretic patterns to determine variability of venom composition due to age, seasonal and geographic distribution. MORENO *et al.* (1988) used agarose isoelectric focusing to separate phospholipase A_2 isozymes and confirmed differences found in adult and juvenile specimens of Atlantic and Pacific venoms of *Bothrops asper*. TU *et al.* (1980) used isotacophoretic patterns to compare venom from different species of *Crotalus* and *Trimeresurus flavoviridis* venoms from different islands. By far the most popular electrophoretic technique for comparing whole venoms or individual components is polyacrylamide gel electrophoresis, with or without SDS, and in a native or mercaptoethanol reduced state. The methods of DAVIS (1964) and LAEMMLI (1970) (in some cases with modifications) have allowed venom components to be separated, stained and in gel slabs visually compared for component variation.

IMMUNOLOGICAL ANALYSIS

KELLAWAY (1930) used active immunity in guinea-pigs produced against various Australian venoms to determine protective cross-reactivity when challenged by venoms of other species. Studies by CLAUS and MEBS (1989) and GENE *et al.* (1989) used polyvalent antivenoms to determine cross-reactivity in thrombin-like enzymes of various snake genera and the coagulant and fibrinolytic activities of Costa Rican crotaline venoms, respectively, by neutralization of these activities. RUSSO *et al.* (1983) raised monoclonal antibodies to specific coelenterate lethal toxins and used these to determine common antigenic sites in lethal proteins of non-related animal venoms. KORNALIK and TABORSKA (1989) examined cross-reactivity in venoms of vipers and crotalids with mono- and commercial polyvalent antivenoms and determined neutralizing ability of these antivenoms against various venom activities.

The development of the immunodiffusion technique by OUCHTERLONY (1949) allowed for simple and convenient examination of venom components and comparisons between antigenic components of species and genus. TU and ADAMS (1968) used this technique in determining phylogenetic relationships in snakes of the genus *Agkistrodon* from Asia and North America. Immunodiffusion has also been used in comparing specific venom principles (phospholipases, haemorrhagic activity and toxins) between genus and within species (NAIR *et al.*, 1980; CHEN *et al.*, 1984; WEINSTEIN *et al.*, 1985; MANDELBAUM *et al.*, 1989). MINTON (1957*a*) and KAWAMURA (1974) used this technique to investigate the immunological relations between rattlesnake venoms and Asiatic *Agkistrodon* venoms, respectively. SCHENBERG (1963) used gel double-diffusion to identify intra subspecies qualitative differences in *Bothrops neuwiedi* and it was also used in comparing differences in the venoms of the subspecies *Vipera russelli pulchella* and *Vipera russelli siamensis* (WOODHAMS *et al.*, 1990). Comparisons of the yellow and white venoms of *Vipera ammodytes* (MASTER and KORNALIK, 1965) and *Crotalus viridis helleri* (JOHNSON *et al.*, 1987) used immunodiffusion to show qualitative differences between these venoms. The changing antigenic make-up of venoms with age has also used these simple immunodiffusion techniques (MINTON, 1967, 1975; THEAKSTON and REID, 1978).

LOMONTE *et al.* (1983) and WEINSTEIN and MINTON (1984) used immunoelectrophoresis in comparative studies of species and age-dependent changes in venom composition. Recently MOURA DA SILVA *et al.* (1990) used transblotted antigens, after SDS-PAGE fractionation, revealed by homologous and heterologous antivenoms in determining antigenic cross-reactivity in venoms from snakes of the genus *Bothrops*.

VENOM VARIATION

RUSSO *et al.* (1988) looked at venom variability at its most extreme when looking for homology in toxin composition of unrelated animals. Monoclonal antibodies raised against sea-nettle and Portuguese-man-of-war lethal factors cross-reacted with the venom of *Crotalus durissus terrificus* suggesting antigenic recognition of an active moiety with toxic action being present in these animals.

INTERFAMILY VARIABILITY

The comparison of venom composition at this higher phylogenetic classification centres both on variability and commonality of components. In the late 1800s observations on the effects of snake venoms on prey, pharmacological effects, tissue changes and chemical differences were reported (FAYRER, 1872; MITCHELL and REICHERT, 1886; WOLFENDEN, 1886). With the advent of antisera to snake venoms, LAMB (1902, 1904) used precipitin reactions in an antigenic comparison of the venoms from *Vipera russelli, Echis carinatus, Bungarus fasciatus, Notechis scutatus, Ophiophagus hannah, Bungarus caeruleus, Enhydrina schistosa, Trimerusurus gramineus* and *Crotalus adamanteus* against cobra (*Naja naja*) antivenom with little effect. Cross-reactivity was observed only with Russell's viper and a weak reaction with *T. gramineus* and the sea snake, *Enhydrina schistosa*. It was concluded from this that there was little correlation between the precipitin reaction and the phylogenetic relationship of these snakes.

Neutralization abilities of antivenoms showed in some cases cross-reactive protection between families and subfamilies with some unexpected results indicative of shared antigens between distantly related snakes (SCHOTTLER, 1951; KEEGAN *et al.*, 1962; MINTON, 1979, TU *et al.*, 1980). MINTON (1979) used the Ouchterlony immunodiffusion method to determine common antigenic sites in a number of venoms against six commercial monovalent antisera and considerable cross-reactivity was observed. The use of immunological parameters has shown large numbers of common antigens in venoms of phylogenetically well separated snakes.

BERTKE *et al.* (1966) examined electrophoretic patterns of both crotalids and elapids. The crotalids predominantly showed movement of bands towards the anode in the starch gels while those of the two elapids tested showed a cathodic trend. Of 119 distinct bands from all species only 22 were common to two or more species and only three were present in both the elapids and crotalids, one from *Naja naja atra* being found in four of the crotalids and two in *Bungarus multicinctus* being present in only one of the crotalids, *Trimerusurus mucrosquamatus*. These results suggest the physical properties of elapid and crotalid venom components show considerable variability and common antigens do not necessarily imply the same proteins are responsible for pathophysiological effects.

WEINSTEIN *et al.* (1985) looked at the distribution of a specific toxin isolated from *Crotalus scutulatus scutalus* in venoms of species representing crotalids, viperids, elapids, hydrophids and colubrids. In this specific study the toxin was found in a number of

TABLE 1. CLASSIFICATION OF VENOMOUS SNAKES AND LEVELS OF VENOM VARIABILITY EXAMINED

Classification level			Level of venom variability
Phylum	Chordata		Inter (between) phylum
Class	Reptilia		
Order	Squamata		
Suborder	Serpentes		
Family	Colubridae		Inter family/subfamily
	Viperidae		
	Subfamily	Viperinae	
		Crotalinae	
	Elapidae		
	Hydrophiidae		
Genus (multiple)			Intergenus
Species (multiple)			Interspecies/subspecies
Subspecies (multiple)			Intra (within) species including geographical variability
			Individuals seasonal
			diet/habitat
			age-dependent
			sex

species of the genus *Crotalus* from high to trace concentrations while only one venom from *Trimerusurus flavoviridis* outside of the rattlesnakes was found to contain this toxin. The hyaluronidase activities of elapids and viperids of south-east Asian snakes were determined by PUKRITTAYAKAMEE *et al.* (1988). They found little or no activity in elapid venoms, with the exception of the Malayan Krait (*Bungarus candidus*). The presence of the hyaluronidase did not appear to be related to the apperance of local toxicity, a consistent feature of viper bites but a variable effect with elapids. The elapid found to contain hyaluronidase elicits little or no local toxicity. WARRELL *et al.* (1989) reported the appearance of neurotoxic symptoms from envenomations by two elapids (*Bungarus caerulus, Naja naja naja*) and *Vipera russelli pulchella.* Local symptoms were found (swelling, blistering and necrosis) in victims of the cobra and viper, while respiratory paralysis occurred in two elapid victims. Generalized muscle tenderness was a feature of approximately one-third of the viper victims.

At subfamily level KORNALIK and TABORSKA (1989) found cross-reactivity between commercial polyvalent antiviperid and anticrotalid antivenoms in neutralizing the lethal effects of two African Viperidae and four Crotalidae, while monovalent antivenom was ineffective. Neutralization of skin haemorrhagic ability of the venoms showed almost complete cross-reactivity with polyvalent antivenom, but less with monovalent antivenoms. MANDELBAUM *et al.* (1989) using antibodies raised against the specific haemorrhagic factors of *Bothrops jararaca* and *Bothrops neuwiedi* found similarity in these components within the *Bothrops* genus while some resemblance was found in the haemorrhagic principles from other crotalid venoms (*Crotalus, Trimeresurus* and *Agkistrodon*). The venoms from the Viperinae subfamily (*Bitis* and *Vipera*), however, showed a much reduced similarity with only a partial neutralization by these sera.

The coagulant activity of four Crotalinae and one Viperinae were shown to be neutralized by a number of commercial antivenoms for North and Central Africa, Orient (Near and Middle East), from the South African Institute of Medical Research and Wyeth antivenom, U.S.A. (CLAUS and MEBS, 1989). The anti-thrombin-like antibodies removed from Orient antivenom by affinity chromatography using purified enzyme from *Bitis*

gabonica had the ability to neutralize the coagulant activity of the venoms from the crotalids, *Agkistrodon acutus, Bothrops asper* and *Bothrops atrox* (CLAUS and MEBS, 1989). KORNALIK and TABORSKA (1989) found a cross-neutralizing effect of anti-crotalid and anti-viperid polyvalent antivenoms on the defibrinating ability of the venoms of *Echis carinatus* and *Bothrops asper* despite the different modes of actions of these two coagulant enzymes and suggested the probability of common antigenic determinants existing between the thrombic proteases and prothrombin-converting enzymes of these venoms.

The presence of myotoxin *a*, a basic protein responsible for muscle necrosis, was determined for the venoms of seven viperids and one elapid (BOBER *et al.*, 1988). The toxin was present in a number of *Crotalus* and one *Sistrurus* species (see interspecies variation). There was no evidence of a myotoxin *a*-like substance in the venom of the elapid (*Naja naja kaouthia*) nor in the venom of *Vipera russelli russelli, Bitis arietans* or species of *Agkistrodon* also tested.

INTERGENUS VARIABILITY

KELLAWAY (1930) produced active immunity in guinea-pigs against a number of elapid venoms and determined the protection afforded the animals against envenomation by other genera. Immunity against tiger snake (*Notechis scutatus*) protected the guinea-pigs against challenges by the venom from both copperheads (*Austrelaps*) and the giant brown snake (*Pseudechis australis*). No protection was noted against brown snake (*Pseudonaja*) venom, however. KELLAWAY found protection against the 'thrombase' activity of tiger snake venom when immunization was by a closely related venom, even though this did not contain coagulant activity. The conclusion drawn was that active immunity produced greater ability to cross-neutralize than passive immunity. The similarity of the venoms of these genera did not appear to relate to their similarity of neurotoxic effect or coagulant activity.

MINTON (1957*a*) used precipitin lines developed in agar to determine comparative similarity in the venom composition of various Crotalinae species. Antisera against *Sistrurus catenatus* produced precipitin lines with each of the *Crotalus* species and the two *Sistrurus* species, while cross-reactivity was also noted with an antisera against *Agkistrodon contortrix* producing at least one precipitin line against each of the *Crotalus* species, except *Crotalus durissus terrificus,* and lines with the two *Sistrurus* species. DETRAIT and SAINT GIRONS (1979) compared antigens of venoms from 21 species of Viperidae representing 10 genera. Results were concordant with the classification of the Viperidae family. SAINT GIRONS and DETRAIT (1980), studying venoms from 29 species belonging to 15 genera of Elapidae, showed the heterogeneity of the Australian Elapinae group, and the difference between venoms from the African *Naja* group and those from Asiatic *Naja.* In both papers it was concluded that there was a good correlation between immunological results and morphological observations, but poor correlation with venom functions (e.g. choice of prey and ability to kill it), ecological studies, and geographical similarities. However, the authors assumed that shared antigens indicates affinity between two taxa, but differences in antigens may not necessarily prove a lack of affinity.

Comparison of protein content, *Paramecium multimicronucleatum* LD_{50} and disc electrophoretograms of *Crotalus atrox, C. scutulatus* and *Agkistrodon contortrix mokason* (JOHNSON, 1968) found little difference in protein content between these animals but considerably increased toxicity in the *A. c. mokason.* The electrophoretograms from pooled specimens of the three species were distinctly different; however, they also differed

from the patterns of the individual snakes of those species and thus were not regarded as a suitable taxonomic criterion.

ROSENFELD *et al.* (1959) examined venoms for their coagulant and fibrinolytic capacities. They found that the venoms can be markedly coagulant with little fibrinolytic activity (*Bothrops cotiara*, and young *B. jararaca*), have both activities to high degree (*B. jararaca, B. insularis, B. atrox*), have low coagulant with high fibrinolytic activity (*Agkistrodon piscivorus, Crotalus durissus durissus, B. neuwiedi*) or be weak in both (*Crotalus durissus terrificus* -white venom). No distinctive genus-specific pattern of these emerged, however. GENE *et al.* (1989) looked at the coagulant, defibrinating, fibrin and fibrinogenolytic action of Costa Rican crotaline snake venoms. Again a genus-specific activity was not found with a number of *Bothrops* spp., *Lachesis muta* and *Crotalus durissus* inducing *in vitro* coagulation. Four of the *Bothrops* species, however, did not produce coagulation. All of these venoms are fibrinolytic *in vitro* and neutralization of the coagulant and fibrinolytic action of all venoms was accomplished with a polyvalent antivenom indicating cross-reactivity between the components affecting coagulation in Costa Rican Crotalids.

GITHENS (1935) and MINTON (1956) examined the venoms of the North American pit vipers with particular reference to their toxicity. The results from both of these studies showed that variation in the lethal toxicity, necrotizing actions, haemagglutinin and haemolytic activities of these venoms was no greater between the three genera of *Agkistrodon, Crotalus* and *Sistrurus* than between the species representing them. GITHENS suggested a possible phylogenetic relationship between neurotoxic dominance in a venom through to local reactions being indicative of advancement from a primitive state. MINTON, however, cited the potent neurotoxic venom of *C. durissus terrificus* as an end form while indicating that the loss of haemagglutinin and haemolysin from the venoms with an increased lethal toxicity was evidence of phylogenetic advancement. The venoms of *Sistrurus catenatus* and *S. miliarius* showed considerable divergence in their activities in accord with taxonomic evidence of their long separation from a common ancestor.

INTERSPECIES AND SUBSPECIES VARIATION

Venom variability at this level is also obvious, and as mentioned above, variation between species is often as marked as that found at intergenus level. Observed venom variations correspond to morphological differences in accord with zoological classification. The description of various properties of *Vipera aspis* venoms by BOQUET (1948) was followed by the zoological classification of a new subspecies *V. aspis zinnikeri* based upon scalation differences.

The genus *Agkistrodon* presents an interesting case, being the only genus with species representation in both Asia and the Americas. However, the genus *Agkistodon* has recently been split into several genera based on geographic range, and morphologic variation. The morphological similarity of these snakes did not appear to relate to the immunological status of the venoms of these snakes (TU and ADAMS, 1968). From the immunological studies performed a genetic kinship existed between those snakes of Asian and American origin which became less distinct with geographical separation and indicated probable migration sometime earlier than 22,000 years ago. The variability within the Asian populations of *Agkistrodon* was examined by KAWAMURA (1974) and suggested from cross-protection tests that a number of species were genetically closely linked with abilities to neutralize heterologous venoms, while some venoms appeared to have differentiated beyond cross-reactivity. CHEN *et al.* (1984) attempted to classify the

species of *Agkistrodon* in China on the variability of venoms in polyacrylamide gels and in Ouchterlony diffusion tests. JONES (1976) compared electrophoretograms of *Agkistrodon* species of North America and suggested that variability of these patterns was sufficiently distinct to separate venoms from various species; however, at levels below species the taxonomic usefulness of these patterns was lost. However, TU *et al.* (1980) compared venoms of Asian *Trimeresurus* and Central American *Bothrops* using immunodiffusion and isotachophoresis techniques, and found that venoms from geographically separated species are immunologically related.

Variation in venom composition for species identification was also employed by JIMENEZ-PORRAS (1967) for distinction between *Bothrops nummifera* and *B. picadoi,* morphologically closely related in shape and colour patterns. Analysis of the venoms by electrophoresis and biochemical characteristics showed dramatic differences in the venom compositions. The most interesting find was the absence of a coagulant effect in the venom of *B. picadoi,* while both exhibited the presence of an anticoagulant. It was concluded that the number of differences noted in these venoms was taxonomically significant. BERNADSKY *et al.* (1986) examined the gel filtration patterns of nine species of *Vipera.* The results of this queried the inclusion of *V. russelli* within the genus while dividing the remaining species into two groups according to their patterns. The general pattern obtained from the Sephadex filtration, however, was characteristic for the whole genus excluding *V. russelli.* Despite similarities in the gel filtration patterns of members of the genus *Vipera,* examination of immunological reactions (WEINSTEIN and MINTON, 1984) and venom proteins (NILSON and SUNDBERG, 1981) certainly produced evidence for the separation of these snakes into valid species. NAIR *et al.* (1980) compared the venoms of a number of *Crotalus* species for antigenically similar forms of phospholipase A_2. Considerable difference was noted in the immunological activities of phospholipase in venoms of closely related species and indeed at subspecies level between *Crotalus scutulatus salvini* and *C. s. scutulatus.* Thus considerable contention would appear to exist as to the value of these studies for taxonomic purposes. AIRD (1985) stated that biochemical variations of venoms were linked to taxonomic findings based upon morphology and geographical data. However, quantitative analysis of linkage or degree of variation between taxa are restricted by the presence of other causes of venom variation. SCHAEFFER (1987) working with *Echis* venom lots suggested variation in venom activities from snakes within the same species could lead to contradictory biochemical or physiological test results. Physiologically, this was found by MINTON (1953) in comparing toxicity in *Agkistrodon* and *Crotalus* venoms. It was found that variation in toxicity is a random occurrence, with 'weak' and 'strong' venoms in snakes captured within a locality on the same day.

INTERSUBSPECIES VARIABILITY

Several studies have found quite different compositions for venoms at the level of subspecies. Ion exchange chromatography of the subspecies *pulchella* and *siamensis* of *Vipera russelli* revealed different elution positions for haemolytic, and procoagulant activities, while one possessed platelet aggregating activity (WOODHAMS *et al.*, 1990). AIRD (1985) compared the venoms from three subspecies of *Crotalus viridis* (*concolor, viridis* and *lutosus*) through their gel elution profiles and concluded that they were distinguishable on the basis of quantitative and qualitative differences in their profiles. The venom composition of the *Pseudocerastes persicus* subspecies *P. p. fieldi* and *P. p. persicus* revealed a simple pattern of few bands for *P. p. fieldi* and a more complex banding pattern for

P. p. persicus with gel isoelectric focusing (BDOLAH, 1986). *Pseudocerostes persicus persicus* showed haemorrhagic and L-amino acid oxidase activity which was absent in *P. p. fieldi*. SUTHERLAND (1983) found unique banding patterns on polyacrylamide gels for the venoms from each of the subspecies *serventyi, niger, occidentalis* and *humphreysi* of *Notechis ater*. Although the specimens were from isolated populations, similarity was noted between the banding in that a distinct separation was present between the high and low molecular weight components of each venom, suggesting a possible common ancestor. Variability was also noted in the LD_{50} values for these venoms.

INTRASPECIFIC VARIATION

At the level of intraspecific variation a number of factors must necessarily complicate the picture. At levels of comparison above this, gross comparisons are drawn between the contents of venoms with minor regard for the individuals of populations examined. However, at this level the variation noted considers the individual input into venom composition but also considers the effects contributed by the geographical conditions, age-dependent effects, feeding habits and seasonal changes. Each of these latter effects will be considered separately later. The existence of considerable variability at intraspecific level may be debatable and perhaps to some extent is reliant on the interpretation of similarity.

SCHENBERG (1963), in an extensive study of *Bothrops neuwiedi*, found consistency in the immunological patterns of venoms from specific areas and suggested that the place of origin of the venom could be determined. Variability, however, was found between localities. Variations noted, however, did not necessarily correlate with morphological differences. JIMENEZ-PORRAS (1964) found similar consistency within geographic areas and that the venom pattern could identify the zone of capture. LEVITON *et al.* (1964) compared the electrophoretic patterns of three individuals of *Crotalus viridis helleri* and found homogeneity of the protein composition between these specimens. Homogeneity was also found in the venoms of a population of *Notechis ater niger* although this could be considered a special case as the population was small and isolated on an island (WILLIAMS and WHITE, 1987). RAEL *et al.* (1984) found two distinct variants of *Crotalus scutulatus scutulatus* corresponding to two geographical areas. However, within these populations again the venoms were extremely similar suggesting that in fact two genetically divergent populations existed.

TABORSKA (1971) examined the venoms from 21 individuals of *Echis carinatus* from a climatically, geographically and nutritionally homogeneous habitat. Electrophoretic results showed both qualitative and quantitative variability in the fractions of the venom samples in the majority of cases while several had identical or very similar patterns. Enzymic activity showed homogeneity in phosphodiesterase, 5′-nucleotidase and caseinolytic activity while the individual variation in phospholipase A and L-amino oxidase was pronounced. JONES (1976) found that the variation between individual specimens of *Agkistrodon* could not be related to diet, hibernation, recency of last venom extraction, temperature or age and indicated that there was great variability within a population but a more common pattern from one population to another. Electrophoretic variation was found within all of the species examined by WILLEMSE (1978) with none of the individual venoms containing all fractions identified for that species, although major bands did appear to be in common. GLENN and STRAIGHT (1977) observed great variability in the electrophoretic patterns from eight individual *Crotalus viridis concolor* venom samples and that these patterns were a poor marker for the species, and a similar result was found with

Bitis species venoms (BOCHE *et al.*, 1981). GLENN and STRAIGHT (1985) examined protease, esterase and phosphodiesterase activity in populations of *Crotalus* species and found as much variation in activity within populations as between them. BOCHE *et al.* (1981) found that the neurotoxin content was different from individual to individual in both *Naja melanoleuca* and *Naja nigricollis*. The assumption that the quality of *Echis carinatus* venoms was determined by the geographical origin of the snakes proved incorrect, with individual animals from the same area showing as much variability in their venoms as that observed between pooled venoms from different geographical habitats (KORNALIK and TABORSKA, 1988).

Intraspecific variability of venoms is also noted in the phenomenom of yellow and white venom production. Venoms collected from *Vipera ammodytes* and *Vipera russelli* specimens exhibit this variability. The yellow venoms of *V. ammodytes* contained a considerably greater quantity of L-amino acid oxidase while other activities (coagulase, tryptic, gelatinase, fibrinolytic, phospholipase A, toxicty and phosphodiesterase) were equivalent between the white and yellow venoms (KORNALIK and MASTER, 1964; MASTER and KORNALIK, 1965). KORNALIK and MASTER (1964) found that the yellow and white venoms of *V. russelli* contained similar quantities of L-amino acid oxidase, gelatinase and protease activity, however, the necrotizing action of the yellow venom was much stronger. DIMITROV and KANKONKAR (1968) also found a lack of necrotizing activity in white venom but in this study there was an absence of proteolytic enzymes, and L-amino acid oxidase was present in only small amounts in these venoms.

The appearance of detectable variability at this level has been ascribed to the genetic make-up of the population and several litter studies have given credence to this theory. CHIPPAUX *et al.* (1982) showed the genetic origin of individual variation within a litter of *Bitis gabonica*, with four distinct groups being found from their venom electrophoretic patterns. MEBS and KORNALIK (1984) examined the venom from a litter of four *Crotalus adamanteus* snakes and found that two of the four were lacking a basic toxin, which was a consistent finding from these snakes. FAURE and BON (1987) discovered different proportions and different sequences in isoforms of crotoxin in venoms from various single specimens of *Crotalus durissus terrificus*. These modifications did not appear to change the toxicity or enzymatic activity of the venom but are the result of the expression of several isogenes. TABORSKA and KORNALIK (1985) found considerable individual variability in both pathophysiological and enzymatic activity between parents and siblings of a family of *Bothrops asper* snakes. Coagulant activities between members of three generations of *Bothrops asper* snakes and seven adult siblings of *Vipera russelli* showed as much variability as that found between unrelated snakes (KORNALIK and TABORSKA, 1988). They concluded that the biological activity of a pooled venom was dependent on the proportional composition of venoms of the individuals making up that pool. WILLEMSE (1978) suggested that there were no other causes of variability other than that between individuals and that this was genetically predetermined. Variability between individuals would appear to be a general feature of venoms, as it also has been demonstrated in scorpion venoms (MARTIN *et al.*, 1987).

GEOGRAPHICAL VARIATION

The variation in venom composition with geography is necessarily an integral part of instraspecific variability; however, as many studies have focused on this level of variation, we have considered it under a separate section.

BARRIO and BRAZIL (1951) examined the neuromuscular action of *Crotalus terrificus terrificus* venom in rats and found two distinct responses. These activities were geographically distributed, with Type I (characterized by seizures, hypotonia and paralysis) being present in venoms from Argentina, Paraguay and Bolivia, while Type II (characterized by initial hypotonia and muscle flaccidity) was found in areas of Brazil along with areas of Type I. Snakes from the Sao Paulo area, however, had venoms of either type. SCHENBERG (1959) established a distribution map of crotamine containing venoms from *Crotalus durrissus* specimens in the Sao Paulo region. Two areas were described, one where crotamine secretors were present and a hybrid area where crotamine was either present or absent in venom. SCHENBERG (1963) also found distinct geographical delineations in the south of Brazil of five variants of *Bothrops neuwiedi* venoms. The venoms of *Bothrops nummifera* specimens collected in Costa Rica clearly showed biochemical variation associated with their Pacific or Atlantic zone origins (JIMENEZ-PORRAS, 1964). The reproductive isolation of these populations by the mountain range running the length of the country being responsible for the evolution of these variations. Hybrid variants may be found where mountain passes allow for interbreeding of these populations. A similar situation of Atlantic and Pacific zone variability was found in the venom composition of *Bothrops asper* specimens (ARAGON-ORTIZ and GUBENSEK, 1981; MORENO *et al.*, 1988). Geographical variation in the content and lethal toxicity of *Vipera russelli* was reported by JAYANTHI and VEERABASAPPA GOWDA (1988) with high proteolytic activity and low lethality in specimens from northern and western India with the reverse being the case from southern India. Clinical studies of *V. russelli* bites have confirmed the geographical variation of venom composition with symptomatology varying with locality (WARRELL, 1985; WARRELL *et al.*, 1989; THEAKSTON *et al.*, 1989).

The possibility exists that the level of variation may correspond to an unknown or undescribed subspecies as was the case previously for *V. aspis* subspecies. In such cases this cannot be considered as true geographical variation. This is certainly the case for *Echis carinatus* which is a species complex (e.g. morphologically indistinguishable different species). SCHAEFFER (1987) showed the existence of geographical variation in *Echis carinatus* venoms; however, the venom lots used were from such different sources that the possibility of variation due to subspecies could not be discounted and in fact the development of new taxa for the African *Echis carinatus* is in progress. The situation with the *Naja nigricollis* species complex was clarified by BROADLEY (1968). The toxin-alpha from *N. nigricollis* showed geographical variations, both qualitative and quantitative which followed species distribution within the former species complex. It is clear that the original toxin-alpha of *N. nigricollis* (BOQUET *et al.*, 1966) in reality belonged to one of the subspecies of *Naja mossambica* (probably *N. mossambica pallida*). ZHAO (1980) confirmed the existence of a new species of *Agkistrodon,* previously identified as *A. halys* (Pallas) from Shedao by venom analysis.

There are possibly two situations where true geographical variation can be observed. The first concerns close or sympatric populations. In 1978 GLENN and STRAIGHT described a venom type in *Crotalus scutulatus scutulatus* from the north-eastern extreme of their range in Arizona with consistently higher LD_{50} values. GLENN *et al.* (1983) and RAEL *et al.* (1984) further elucidated the situation describing the two divergent populations with no significant external morphological differences, but differing on the basis of presence or absence of Mojave toxin. These findings also helped to clarify previously conflicting clinical reports after envenomation by *C. s. scutulatus*. There is evidence that the venom A and B populations were historically isolated; however, there is no present barrier to

interbreeding between these populations and indeed an intergrade population has been described (GLENN and STRAIGHT, 1989). A cline of variation corresponds to a hybridization of characters. This has also been described by MINTON and WEINSTEIN (1986) with *Crotalus atrox* from Texas to Arizona. The Mojave toxin was found in both *C. atrox* and *C. scutulatus* from the same locality (although in trace amounts only in *C. atrox*) while being absent in venoms of both species from other areas. This suggests the possibility of a common ancestor with parallel evolution related to geographical distribution.

The second form of geographical venom variation is seen in investigations of isolated populations. SADAHIRO and OMORI-SATOH (1980) found that specimens of *Trimeresurus flavoviridis* from the Okinawa Islands lacked a haemorrhagic fraction present in the morphologically indistinguishable snakes from Amami Oshima Islands. This is in accordance with the results reported by TU *et al.* (1980) although these authors described quite similar isotachophoretic profiles for venoms from four different populations of *T. flavoviridis* from four different Japanese islands. However, several venoms, including that from Okinawa Island, lacked two non-identified proteins. Thus isolation alone would appear to be the contributory factor in this divergence. Two of us have described this phenomenon in homogeneous island populations of *Notechis ater niger* (WILLIAMS and WHITE, 1987; WILLIAMS *et al.*, 1988). Isolated small populations tend to homogeneous venom production while isolation between populations showed conservation of several components but variation in the total venom spectrum in relation to time of isolation one from another. MEBS and KORNALIK (1984) suggested that genetic variation should affect those components with minor biological roles while changes in the major toxic fractions may be characteristic of species. Thus genetic drift may produce variability in minor components with no loss in toxicity. Few authors have found changes in the major toxic principles, for example Mojave toxin (GLENN and STRAIGHT, 1977), or in the haemorrhagic properties of *T. flavoviridis* (SADAHIRO and OMORI-SATOH, 1980). As demonstrated by WILLIAMS *et al.* (1988), the venom variations are not directly explained by ecological pressure. There was no relation between snake venom composition and potential prey. Possibly with time environmental factors select genetic characters responsible for changes in venom composition.

INDIVIDUAL VARIABILITY

Age-dependent variation in specimens is well documented and is considered as a separate section while variability in venom composition within individuals has produced divergent results.

MARSH and GLATSTON (1974) found no major changes in the number or quantity of components on gel electrophoresis in the venom from *Bitis nasicornis* irrespective of the milking interval. ISHII *et al.* (1970) reported a similar situation in *Trimeresurus flavoviridis* with no differences noted in the four major bands from disc electrophoresis with consecutive milkings. Despite variation noted within populations of *Agkistrodon,* JONES (1976) could find no such variability in electrophoretic patterns in venom of an individual *A. c. mokason* collected at intervals over a seven-month period.

The frequency of milking does appear to affect venom production with decreased mean dry weights with increased frequency or short interval consecutive milkings (ISHII *et al.*, 1970; MARSH and GLATSTON, 1974; KOCHVA, 1960; WILLEMSE *et al.*, 1979). MINTON (1957*b*) observed an increase in venom yield in a specimen of *Crotalus atrox* with monthly

milkings over a period of 19 months, while an inverse trend was noted in the toxicity. During this period, however, the snake doubled its size.

SCHENBERG *et al.* (1970) found considerable variability in the enzymatic constitution of venoms from *Bothrops jararaca* in 5'nucleotidase, ADP-ase, ATP-ase, phosphodiesterase and caseinolytic activity with reduction due to milking frequency. The coagulant activity was unaffected, however, and showed an increase in specific activity. Regeneration of the activities showed individual variation. Disparity was also noted in the activity levels of 5'nucleotidase and ATP-ase prior to captivity, which were never attained at any time during captivity. WILLEMSE *et al.* (1979) found that daily venom extractions from *Bitis arietans* produced a different electrophoretic pattern over the first three days but this seemed to disappear on days 4 and 5. The changes noted included both the disappearance of certain bands and the appearance of new ones, while concentration changes occurred in those that remained. SCHENBERG (1963) also observed this type of variation in a single specimen of *Bothrops neuwiedi*. The variability with milking frequency may relate to autolysis and/or other breakdown processes in stored venom, with freshly secreted venom being homogeneous (WILLEMSE *et al.*, 1979).

JOHNSON *et al.* (1987) found a case of individual variation unrelated to milking frequency in a specimen of *Crotalus viridis helleri* which secreted white and yellow venoms from individual glands. Biochemically these venoms were quite different and the white venom had a significant reduction in corresponding bands to yellow venoms when electrophoresed. CHIPPAUX *et al.* (1982) did not observe any variation between the venoms from the right and left glands of 30 *Bitis gabonica* from the same litter.

Seasonal variation

The possibility of seasonal variation in venom composition first arose from early clinical observations. Following PARE (*Traite sur les venins*), numerous European physicians showed increased lethality of viper bites occurring in spring compared to autumn (quoted by BOQUET, 1948, DETRAIT and DUGUY, 1966). However, epidemiological findings from strict surveys do not confirm these observations. Few studies consider the possible implications of seasonal variations. Seasonal variability in the toxicity of *Vipera aspis* venom observed by DETRAIT and DUGUY (1966) was based on work with pooled venoms from different snake populations and individual variability may have been a contributing factor. GUBENSEK *et al.* (1974) noted seasonal variation in the electrophoretic patterns of *Vipera ammodytes* with the virtual loss of two major components (identified as lethal) during the winter months and with some minor component variation. LATIFI (1984) did not observe significant seasonal variation in pooled venoms milked from Iranian snakes living in a continental harsh climate. Most authors have failed to demonstrate any seasonal variations using venom from individual specimens milked at various periods throughout a year. BOCHE *et al.* (1981) used venoms from species living in tropical and equatorial regions where the change in seasons is not marked. Using individual venoms MEBS and KORNALIK (1984) found no variation in the venom composition over a 12-month period. GREGORY *et al.* (1984) and GREGORY-DWYER *et al.* (1986) found no variation in the isoelectric focusing patterns from *Crotalus viridis helleri, C. molossus molossus* and *C. atrox* individuals milked monthly over a 20-month period. The snakes were kept in controlled temperatures and photoperiods to simulate seasonal conditions during the study period.

Diet/habitat variation

A relationship of venom composition with diet or habitat has never been demonstrated. BOCHE *et al.* (1981) and GREGORY-DWYER *et al.* (1986) did not find any modification of venom due to diet. WILLIAMS *et al.* (1988) showed that potential prey did not have an influence on venom composition.

Age-dependent variation

Numerous reports exist detailing differences in the venoms of snakes with age. Several authors used venoms collected from individual snakes of different ages milked at the same time in their studies (GUTIERREZ *et al.*, 1980; MEIER and FREYVOGEL, 1980; LOMONTE *et al.*, 1983; MACKESSY, 1988) and in some cases large amounts of venom from the various age groups were pooled. Individual variation was therefore not addressed and may mask or exaggerate differences found. MACKESSY (1988), however, noted that the subspecies employed showed no venom differences. Toxicity studies demonstrate that venom toxicity decreases with age until the end of youth at which point a levelling in toxicity is most often observed (MINTON, 1967, 1975; FIERO *et al.*, 1972; THEAKSTON and REID, 1978; REID and THEAKSTON, 1978; MEIER and FREYVOGEL, 1980; LOMONTE *et al.*, 1983; MINTON and WEINSTEIN, 1986; MACKESSY, 1988). One exception has recently been reported by GUTIERREZ *et al.* (1990) for *Lachesis muta stenophrys* where lethality increased with age while newborn specimens were almost devoid of toxicity. Electrophoretic analysis of newborn and adult venoms of *Crotalus viridis viridis* showed qualitatively similar patterns (FIERO *et al.*, 1972) while LOMONTE *et al.* (1983) and GUTIERREZ *et al.* (1990) found conspicuous differences in protein banding. MEIER (1986) found a decrease in the number of protein bands and a shift to components of lower molecular weights with increasing age in *Bothrops atrox*. LOMONTE *et al.* (1983) also found differences in the immunoeletrophoretic patterns between adults and newborns and MINTON (1967) found marked differences in juvenile and adult *Crotalus horridus atricaudatus* venoms but could not relate these to changes in toxicity.

Coagulant activity in venoms also appears to be related to age with decreased coagulant activity being reported with increasing age (BONILLA *et al.*, 1973; KAMIGUTI and HANADA, 1985; GUTIERREZ *et al.*, 1990). REID and THEAKSTON (1978) found qualitative differences in *Crotalus atrox* venom dependent on the age of the specimen. Venom from specimens up to 8 months old had a thrombin-like action (direct clotting of fibrinogen) after which the activity became procoagulant, disappearing completely from 11 months on. A similar qualitative change has been reported for *Bothrops moojeni* venom (FURTADO and KAMIGUTI, 1985). Variation in enzymic activity has been extensively studied with ageing, with the general finding that proteolytic activity increased with age (THEAKSTON and REID, 1978; LOMONTE *et al.*, 1983; MINTON and WEINSTEIN, 1986; MACKESSY, 1988). Haemorrhagic and haemolytic activities appear to follow no particular trend, while phospholipase A_2 activity may decrease with age (THEAKSTON and REID, 1978; MACKESSY, 1988) and may in part reflect decreasing toxicity. L-Amino acid oxidase appears to be a feature of adult venoms (JIMENEZ-PORRAS, 1964; FIERO *et al.*, 1972; BONILLA *et al.*, 1973).

Sex variation

Little attention has been given to this possible source of variation and in most cases has been treated as an incidental observation. In most cases no variation has been reported

between the venoms of males and females and WILLEMSE (1978) suggested that variation found was explicable through other factors. GLENN and STRAIGHT (1977) observed a significant difference in the yield of venom milked from males compared to females. However, the protein composition and toxicity were equal for both sexes. From investigations of large numbers of snake venoms, SCHENBERG (1959) and LATIFI (1984) concluded that the sex of a snake was not a contributing factor to variability. No correlation to sex could be found in *Echis carinatus* or *Bitis gabonica* venom variability (TABORSKA, 1971; CHIPPAUX *et al.*, 1982). Two reports, however, have suggested the possibility of sex-linkage in the appearance of components in venom. MARSH and GLATSTON (1974) noted that a female *Bitis nasicornis* invariably contained an extra protein band, while MEBS and KORNALIK (1984) found a basic toxin to be present only in the venom of the females from a single litter, although these results may be explained by individual variability.

CONCLUSIONS

Venom variation has been considered at practically every level and was considered initially as a possible aid in phylogenetics. However, this would appear not to be the case, although similarities or dissimilarities between venom components may provide clues to the taxonomists (Tu and ADAMS, 1968). Thus comparisons of venom composition at interfamily, intergenus, or even interspecies level, in the context of taxonomic clarification, is perhaps justified. In such comparisons similarities in composition and antigenic make-up are suggestive of common ancestry. Alternatively, the finding of toxic components with similar composition may be an incidental finding with the origin of a toxic component being genetically divergent. Selective pressures on unrelated or distantly related animals may result in the appearance of antigenically similar toxic components in their venom.

The variability in venom composition found between individuals is of great importance and may dominate any other levels of variation (JOHNSON, 1968). The variability at this level gives strong supportive evidence to venom composition being under genetic control. The concentration of specific components varying between specimens which is commonly observed, may mask or enhance specific biological activities being investigated. Slight structural modifications of proteins may be found due to substitutions of amino acids within populations and will be progenerated where no deleterious effect is noted. In large populations with free interbreeding, the individual variability may be great while in small and isolated populations the gene pool is restricted and results in a homogeneous venom production (WILLIAMS and WHITE, 1987).

In studying variation in venom composition we must not lose sight of the purpose for the existence of venom in snakes, the procurement of prey. While variability may be noted either at the level of individuals within a population or between species or at levels above this, the composition of the venom and continuance of that composition is reliant upon the effectiveness of that venom in procuring prey. Minor variations are tolerated within the population while those affecting the likely survival of an individual are most likely lost. The effectiveness of venom in this regard is most notably exemplified in age-dependent changes in venom activity. The requirement for the venom to immobilize prey and initiate digestion quite often changes with the size of the snake and the subsequent prey taken with a greater requirement for digestive aid through proteolytic activity with larger mammalian prey being taken in adulthood.

The validity of venom composition comparisons at the higher levels of family and genus may lie in the information it provides taxonomists; however, the variability noted in venom composition within species has two important consequences. The first is the use of venoms as research tools where consistency of venom composition is an important consideration. Careful choice of the venom source is an important initial step with the possibility that individual venom composition may affect the outcome of investigations and may make the use of pooled venoms an unattractive proposition. Secondly, knowledge of the geographical variability in venoms has become extremely important in the production of effective antivenoms. The disparity of symptoms in victims of the same species of snakes has alerted clinicians to the requirement for more specific antivenoms (HARDY, 1983; JIMENEZ-PORRAS, 1964; JAYANTHI and VEERABASAPPA-GOWDA, 1988; WARRELL *et al.*, 1989) and has emphasized the inefficiency of antivenom produced with venom samples from limited areas. The development of the most effective treatment can only benefit from the continued expansion of knowledge of venom variation.

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