Pergamon

0041-0101(95)00122-0

Toxicon, Vol. 34, No. 1, pp. 67–79, 1996

0041-0101/96 $15.00 + 0.00

ELECTROPHORETIC PROFILES AND BIOLOGICAL ACTIVITIES: INTRASPECIFIC VARIATION IN THE VENOM OF THE MALAYAN PIT VIPER (*CALLOSELASMA RHODOSTOMA*)

JENNIFER C. DALTRY,[1] GNANAJOTHY PONNUDURAI,[2] CHAI KOH SHIN,[3] NGET-HONG TAN,[2] ROGER S. THORPE[1] and WOLFGANG WÜSTER[1]

[1]School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, U.K.; [2]Department of Biochemistry, University of Malaya, Kuala Lumpur, West Malaysia; and [3]Zoo Negara, Hulu Kelang, Ampang, Selangor, West Malaysia

(*Received* 12 *June* 1995; *accepted* 28 *August* 1995)

J. C. Daltry, G. Ponnudurai, Chai Koh Shin, N.-H. Tan, R. S. Thorpe and W. Wüster. Electrophoretic profiles and biological activities: intraspecific variation in the venom of the Malayan pit viper (*Calloselasma rhodostoma*). *Toxicon* **34,** 67–80, 1996.—The Malayan pit viper (*Calloselasma rhodostoma*) is of major clinical significance both as a leading cause of snakebite and as the source of ancrod (Arvin™). Although its venom has been extensively studied, the degree to which venom composition varies between individuals is poorly known. We individually analysed the venoms of over 100 *C. rhodostoma* using isoelectric focusing. In all populations, females produced an intense band that was absent from all males, and significant ontogenetic variation was detected. Principal components analysis of the banding profiles also revealed strong geographic variation, which was significantly congruent with variation in the biological activities of the venom (phosphodiesterase, alkalinephosphoesterase, L-amino acid oxidase, arginine ester hydrolase, 5′-nucleotidase, thrombin-like enzyme, haemorrhagic activity). Studies of captive-bred snakes indicate that the intraspecific variation in venom is genetically inherited rather than environmentally induced. The intraspecific variation in venom composition and biological activity could be of applied importance to snakebite therapy, both in correct diagnosis of the source of envenomation and in the development of a more effective antivenom. Greater attention should be given to the source of *C. rhodostoma* venom used in research to ensure reproducibility of results.

INTRODUCTION

The Malayan pit viper, *Calloselasma rhodostoma* (formerly known as *Agkistrodon rhodostoma*), is a terrestrial snake occurring in southern Vietnam, Laos, Cambodia, Thailand, northern West Malaysia, Java and various offshore islands (Gloyd and Conant, 1990). Its venom has long been valued as the source of the thrombin-like enzyme ancrod (Arvin™), which has been employed as a research tool for understanding the processes

of blood coagulation and used clinically as an anticoagulant. More importantly, *C. rhodostoma* is also the leading cause of venomous snakebite throughout much of its large distribution range (Warrell, 1986). Only a small percentage of bites are fatal, but many victims suffer permanently debilitating injuries (Warrell *et al.*, 1986).

Owing to the exceptional medical significance of this snake, its venom has been the focus of numerous studies (Plagnol and Martin, 1957; Reid *et al.*, 1963; Chan *et al.*, 1965; Regoeczi *et al.*, 1966; Esnouf and Tunnah, 1967; Ashford *et al.*, 1968; Marshall and Esnouf, 1968; Reid and Chan, 1968; Denson, 1969; Regoeczi and Bell, 1969; Collins and Jones, 1972; Hatton, 1973; Ouyang *et al.*, 1983; Tan *et al.*, 1986; Chinonavanig *et al.*, 1988; Ho *et al.*, 1986*a*; Dambisya *et al.*, 1994; Ponnudurai *et al.*, 1993, 1994).

In almost every case, the venom samples were pooled from an unspecified number of individual *C. rhodostoma* of unrecorded size, gender and/or geographic origin, thus ignoring the potential existence of within-species variation in venom composition. Yet considerable intraspecific variation has been detected in the venom of many other crotaline vipers (Vellard, 1937, 1939; Barrio and Brazil, 1951; Schenberg, 1959; Jiménez-Porras, 1964; Fiéro *et al.*, 1972; Bonilla *et al.*, 1973; Jones, 1976; Glenn and Straight, 1978; Sadahiro and Omori-Satoh, 1980; Aragón-Ortiz and Gubenšek, 1981; Glenn *et al.*, 1983; Lomonte *et al.*, 1983; Rael *et al.*, 1984, 1992; Kamiguti and Hanada, 1985; Minton and Weinstein, 1986; Mackessy, 1988; Moreno *et al.*, 1988; Gutiérrez *et al.*, 1990, 1991), and can lead to difficulties when attempting to interpret the results or replicate experiments (Chippaux *et al.*, 1991). Inconsistencies in venom composition also have serious implications for snakebite therapy, since diagnosis may be confused by within-species variation in symptomatology, and antivenoms prepared against one venom variant may prove ineffective against another (Jiménez-Porras, 1964; Gutiérrez *et al.*, 1991; Anderson *et al.*, 1993). It is therefore crucial to determine accurately the patterns of venom variation within each species of snake.

The aim of this investigation is to rigorously analyse the pattern of geographic variation in the venom of *C. rhodostoma* and to determine whether snake body size and gender are also influential. This study will largely be based upon analytical isoelectric focusing since this technique provides a detailed and consistent means of qualitatively comparing the composition of small (unpooled) venom samples (Gregory-Dwyer *et al.*, 1986). A few samples were also subjected to assays of biological activity in order to determine whether variation in electrophoretic profiles corresponds to differences in the venoms' effects.

MATERIALS AND METHODS

Venom and reagents

We collected 96 wild *C. rhodostoma* in West Java, West Malaysia (Kedah state), Vietnam (province Xuyen Moc) and Thailand (provinces Trang, Krabi, Nakhon Si Thammarat, Chumphon, Prachuap Khiri Khan, Kanchanaburi and Rayong). All snakes were collected at the start of the rainy season of each locality. The gender and snout–vent length (SVL) of each specimen were recorded. Venom was extracted from each specimen within 12 hr of capture and desiccated to minimize deterioration in storage (Tan and Tan, 1987).

A further ten venom samples were collected from captive *C. rhodostoma* at Zoo Negara in Kuala Lumpur in 1993, with the permission of the zoo director. These included two adults from Trang (Thailand) and their offspring hatched in 1990, 1991, 1992 and 1993 (one male and one female per age group). The largest specimen (>6 years old) measured 950 mm SVL, and the smallest (1 month old) measured 180 mm SVL.

For isoelectric focusing, Coomassie brilliant blue R-250, orthophosphoric acid, sodium hydroxide, sulphosalicyclic acid, trichloroacetic acid, ethanol, acetic acid and glycerol were purchased from Sigma Chemical Company (Dorset, U.K.) and BDH Chemicals (Leics, U.K.). A broad p*I* calibration kit (pH 3–10), Ampholine PAGplates™ (pH 3.5–9.5), sample application pieces and anode strips were supplied by Pharmacia (Herts,

U.K.). For the enzyme assays, calcium-bis-*p*-nitrophenylphosphate, barbituric acid, *p*-nitrophenyl-phosphate, 5′-adenosinemonophosphate, trichloroacetic acid, Tris–(hydromethyl)-methylamine, α-benzoyl L-arginine ethylester, triethanolamine, horseradish peroxidase, L-leucine, *O*-dianisidine and bovine fibrinogen were purchased from Sigma Chemical Company (St Louis, U.S.A.). Magnesium sulphate, sodium hydroxide, sulphuric acid and ascorbic acid were obtained from Merck (Darmstadt, Germany). Ammonium molybdate was purchased from Ajax chemicals (Australia). Mice were supplied by the Central Animal House, Faculty of Medicine, University of Malaya.

Isoelectric focusing

Each venom sample ($n = 106$) was subjected to analytical isoelectric focusing using an LKB flat-bed electrofocusing apparatus Multiphor II 2117 (Pharmacia) in pH range 3.5–9.5. The venoms were individually rehydrated with reverse osmosis water to a concentration of 10 mg/ml soluble protein and briefly centrifuged at 6000 ***g***. Fifteen microlitres of each supernatant was applied to the gel using sample application pieces alongside pH markers. The anode strip was soaked in 1 M orthophosphoric acid and the cathode strip was soaked in 1 M sodium hydroxide. The entire system was maintained at 10°C for 20 min prior to and during electrophoresis. Focusing was carried out for 80 min at 1500 V. The gel was fixed and stained with Coomassie blue R-250 according to the instructions in the Multiphor II system handbook (Pharmacia). Each venom was focused at least three times on different gels to ensure that the electrophoretic profiles were not affected by any inconsistencies in gel structure or running conditions. We found no perceptible differences in p*I* values of individual between different runs of the same sample.

Determination of biological activities

Venoms from 17 wild adult *C. rhodostoma*, representing a wide range of capture localities, were subjected to the seven assays of biological activity. All assays were carried out in triplicate, with the exception of that for thrombin-like activity, which was carried out in duplicate. The activities tested were as follows.

Phosphodiesterase assay. Phosphodiesterase activity was determined by a method modified from Lo *et al.* (1966): we added 0.1 ml venom solution to an assay mixture containing 0.5 ml of 2.5 mM calcium-bis-*p*-nitrophenylphosphate, 0.3 ml of 0.01 M $MgSO_4$ and 0.5 ml of 0.17 M veronal buffer, pH 9.0. The hydrolysis of the substrate was followed by measuring the rate of increase of absorbance at 400 nm. Enzymatic activity was expressed in nmole product released/min/mg. The extinction coefficient at 400 nm was 8100 cm^{-1} M^{-1}.

Alkaline phosphomonoesterase assay. Alkaline phosphomonoesterase activity was assayed by a method modified from Lo *et al.* (1966): 0.1 ml venom solution was added to an assay mixture containing 0.5 ml of 0.5 M glycine buffer, pH 8.5, 0.5 ml of 0.01 M *p*-nitrophenylphosphate and 0.3 ml of 0.01 M $MgSO_4$. After the mixture was incubated at 37°C for 30 min, 2 ml of 0.2 M sodium hydroxide was added and allowed to stand for 20 min at room temperature. The absorbance at 400 nm was then measured. Enzyme activity was expressed in nmole product released/min/mg. The extinction coefficient at 400 nm was 18,500 cm^{-1} M^{-1}.

L-*amino acid oxidase assay.* L-amino acid oxidase activity was determined as described in the Worthington Enzyme Manual (Worthington Biochemical Corporation, U.S.A., 1977), with some modifications: 50 μl of 0.0075% horseradish peroxidase (100 purpurogalin units/mg) was added to 0.9 ml of 0.2 M triethanolamine buffer, pH 7.6, containing 0.1% L-leucine and 0.0075% *o*-dianisidine and incubated for 3 min at room temperature. Venom solution (50 μl) was then added and the increase in absorbance at 436 nm was measured. Enzyme activity was expressed in μmoles of L-leucine oxidized/min. The difference in absorbance coefficient was 8.31×10^{-3} cm^{-1} M^{-1}.

Arginine ester hydrolase assay. Arginine ester hydrolase activity was determined as described by Collins and Jones (1972): the assay mixture contained 0.95 ml of 0.8 mM α-benzoyl L-arginine ethyl ester (BAEE) in 0.05 Tris–HCl buffer, pH 7.8, and 0.05 ml venom solution. The reaction was followed by measuring the increase in absorbance at 255 nm. Enzyme activity was expressed in terms of mole substrate transformed/min/mg. The extinction coefficient at 255 nm was 815 cm^{-1} M^{-1}.

5′-*nucleotidase assay.* This was determined from a method modified from Heppel and Hilmore (1955): 0.1 ml venom solution was added to an assay mixture containing 0.5 ml of 0.02 M 5′-adenosinemonophosphate (preadjusted to pH 8.5), 0.5 ml of 0.2 M glycine buffer, pH 8.5, and 0.1 ml of 0.1 M $MgSO_4$. The mixture was incubated for 10 min at 37°C before terminating the reaction by adding 1.5 ml of 10% trichloroacetic acid. The

ascorbic acid method (Chen *et al.*, 1956) was used to determine the inorganic phosphate. One millilitre of ascorbic acid reagent, containing equal parts of 3 M sulphuric acid, 2.5% ammonium molybdate, 10% ascorbic acid and water, was added to the assay mixture. This was left at room temperature for 30 min and the absorbance at 820 nm was determined. Enzymatic activity was expressed in nmoles phosphate released/min/mg. (A standard curve was constructed using known concentrations of inorganic phosphate.)

Thrombin-like activity. This was determined using the method of Denson (1969): 0.4 ml of 0.5% bovine fibrinogen was incubated at 37°C for 3 min. The clotting time was recorded upon the addition of 0.1 ml venom solution.

Haemorrhagic activity. Haemorrhagic activity in mice was determined according to the method of Kondo *et al.* (1960): 50 μl venom solution was injected intradermally into the dorsum of unanaesthetized mice (25 ± 2 g). After 1 hr, the mice were killed by cervical dislocation and the skin was carefully removed. Both the longest diameter of the haemorrhage spot and the diameter perpendicular to the longest diameter were measured. Haemorrhagic activity was defined as the product of these two measures. Minimum haemorrhagic dose (MHD_{10}) was defined as the quantity of venom inducing a haemorrhagic spot of 10 mm diameter, 1 hr after subcutaneous injection. Tests with each venom dose were carried out in triplicate, and the minimum haemorrhagic dose was determined graphically by plotting the logarithm of venom dose versus the size of the haemorrhagic spot. This was determined by plotting log dose versus cross-diameters of the haemorrhagic spots (Kondo *et al.*, 1960).

Numerical analysis

To test whether the venom composition of *C. rhodostoma* changes with body size, the banding profiles of the 10 captive specimens were recorded as a series of binary digits denoting the occurrence of each variable band (1 = band present; 0 = absent). A 10×10 taxonomic distance (similarity) matrix was constructed from these banding scores and compared to a distance matrix based upon the SVL of each snake by means of a Mantel matrix association test (Thorpe and Báez, 1993). To determine whether the venom composition of wild populations shows a similar relationship with body size, a Mantel test was used to compare the venom electrophoretic profiles and SVL of wild *C. rhodostoma* from the largest regional group (West Java: $n = 20$). Each Mantel test was executed using 10,000 randomizations (Jackson and Somers, 1989).

To elucidate the pattern of geographic variation in the venom electrophoretograms, each adult specimen ($n = 67$) was given a score of digits denoting the occurrence of each variable band of known isoelectric point (1 = band present, 0 = absent). A principal components analysis (PCA) was executed on the banding scores [program written by Davies (Imperial College London) and modified by Thorpe (University of Wales, Bangor)]. This technique summarizes variation in a large number of characters along a reduced number of axes, and is therefore a useful method for visualizing complex patterns of variation.

To clarify the overall pattern of variation in venom biological activities, both a PCA and a canonical variate analysis (CVA) were executed on the seven biological activities exhibited by each sample. The CVA was executed using program 7M, BMDP package (Dixon, 1990) with the venom samples grouped by geographic origin. To test whether there is a significant correlation between the electrophoretic profiles and biological activities of the venom, a 17×17 distance matrix was constructed from the seven activity scores of each of the 17 venom samples tested and compared to a distance matrix derived from the electrophoretic profiles of these samples using a Mantel test.

RESULTS

Sexual variation in isoelectric banding profiles

Across all 96 venom samples from wild snakes, two bands were gender specific. Females from Java, Malaysia and Thailand ($n = 33$) produced an intense band of p*I* 6.90 that was absent from all males in these regions ($n = 48$). In Vietnam, *all* venom samples lacked the band of p*I* 6.90, but every female ($n = 9$) produced an intense band of p*I* 5.90 which was absent from all six Vietnam males.

Ontogenetic variation in isoelectric banding profiles

Twenty distinct bands were visible in all venoms from the zoo group, while seven bands were present in only a few. Band p*I* 6.90 was produced only by the females (see above). A Mantel correlation test on the distribution of the other six variable bands revealed a

highly significant association between venom composition and snake size ($P = 0.0004$). Band p*I* 7.00, for instance, was only produced by hatchlings, whereas band p*I* 8.25 was only produced by specimens exceeding 1 year of age (>400 mm SVL).

Strong ontogenetic variation was also revealed in the wild populations. For example, 11 distinct bands varied in occurrence among the pit vipers from West Java ($n = 20$). After excluding the female marker band of p*I* 6.90 (present in the nine females only), body size and venom composition were again found to be significantly associated ($P = 0.0190$). However, although ontogenetic variation appears to be a feature of all populations, the affected bands are also subjected to geographic variation; the venoms of juvenile *C. rhodostoma* from Java, for instance, are not identical to those of the juveniles in Thailand. The venom of adult snakes also varies geographically (see below).

Geographic variation in isoelectric banding profiles

Owing to the strong influence of ontogeny, only venoms from adult specimens exceeding 400 mm SVL ($n = 67$) were compared in order to elucidate the pattern of geographic variation. Across these samples, bands of 37 different isoelectric points could easily and consistently be distinguished, of which 18 were present in all samples. The 19 variable bands correspond to p*I* 9.40, 9.15, 8.55, 8.45, 8.25, 7.85, 7.45, 7.00, 6.90, 6.55, 6.20, 6.10, 6.00, 5.90, 5.80, 5.70, 5.60, 5.50, 5.45. Two of these are gender dependent (p*I* 5.90 and p*I* 6.90; see above) and were therefore excluded from the principal components analysis.

Fifty-three variants were identified (specimens with identical venom profiles invariably originated from the same geographic region). The venom composition of *C. rhodostoma* showed strong geographic variation (Fig. 1). The first two principal components (PC1,

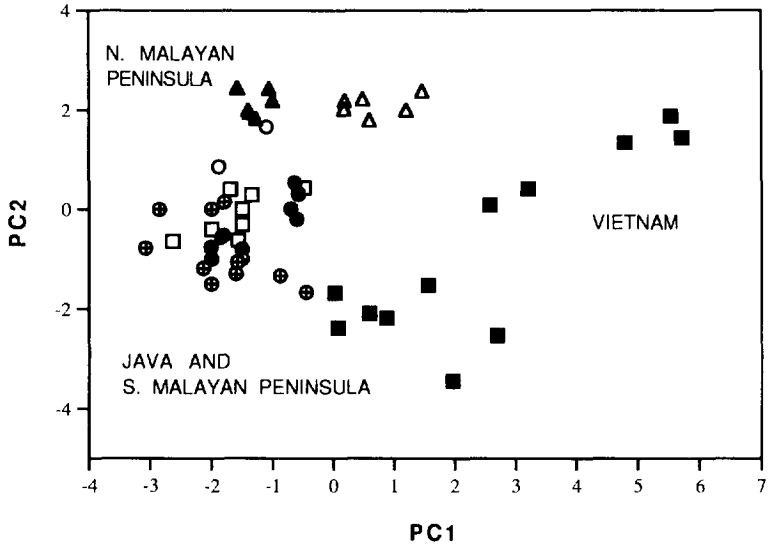

Fig. 1. Principal components analysis of isoelectrically focused electrophoretograms of venom samples from adult *C. rhodostoma* collected in Vietnam (■), Malaysia (●), Java (⊕) and the Thai provinces of Kanchanaburi (△), Prachuap Khiri Khan and Chumphon (▲), Trang, Nakhon Si Thammarat and Krabi (□), and Rayong (○).
The axes are graded in units of standard deviation of the total sample.

PC2) account for 27.97% and 14.67% of the total variation, respectively. The variants from Vietnam form a large yet discrete group characterized by high PC1 scores. Venoms from Java, Malaysia and South Thailand (provinces Trang, Nakhon Si Thammarat and Krabi) share low PC1 scores, but note that the venoms from South Thailand are clearly distinguished from the Java variants when the third component scores are compared. Further up the Malayan Peninsula, venoms from the provinces Prachuap Khiri Khan and Chumphon form another group. The six samples from West Thailand were similar in composition and form a discrete group with relatively high PC1 and PC2 scores.

Variation in venom biological activities

The results of the assays of biological activity are presented in Table 1. Figure 2 shows the results of the canonical variate analysis of the seven biological activities exhibited by each sample (the principal components analysis of the same data gave essentially the same pattern). The first two canonical variate scores (CV1, CV2) account for 81.19% and 13.32% of the total variation, respectively (Fig. 2). The biological activity of *C. rhodostoma* venom shows strong geographic variation.

The electrophoretic profiles of these venom samples are presented in Table 1 (excluding bands common to all samples). These profiles were compared to the seven enzymatic activities of each venom sample using a Mantel test, and were found to be highly significantly correlated ($P = 0.0009$); that is, the more dissimilar the venom electrophoretic profile, the more dissimilar the effects of the venom.

DISCUSSION

Intraspecific variation in the venom of Calloselasma rhodostoma

This study has revealed hitherto unrecorded variability in the venom composition of *C. rhodostoma*. Strong geographic variation was evident among different populations and, within these populations, significant sexual, ontogenetic and individual variation was also detected.

Geographic variation. The pattern of geographic variation is remarkably well defined: pit vipers from the same locality tend to produce more similar venoms than snakes from different localities (Fig. 1). Interestingly, this pattern is not strongly congruent with the spatial distribution of the collection sites; for example, the venom electrophoretograms of Vietnamese *C. rhodostoma* were more similar to those from Kanchanaburi province (West Thailand) than the geographically closer Rayong province (East Thailand).

When venom profiles of adult *C. rhodostoma* from Zoo Negara (captive-bred from specimens caught in the Thai province of Trang) were compared to the venoms of wild snakes, they proved to be almost identical to those from Trang. Since the captive specimens are maintained under somewhat unnatural conditions, this indicates that the geographic venom composition is strictly genetically inherited rather than environmentally induced (see also Gregory-Dwyer *et al.*, 1986; Chippaux *et al.*, 1991).

Of course, since structurally (and functionally) dissimilar proteins can share equal net charge, the presence of bands of identical isoelectric point in two venom samples does not necessarily mean that venoms will exhibit the same biological effects. Conversely, the absence of a band in one sample does not necessarily relate to differences in venom activity because enzymes which perform the same function may differ in isoelectric point.

Table 1. Biological activities and isoelectric banding profiles of venom from 17 individual *Calloselasma rhodostoma*

Source	PDE (nmoles/min/mg)	PME (nmoles/min/mg)	LAAO (nmoles/min/mg)	AEH (μmoles/min/mg)	NUC (μmoles/min/mg)	TLE (sec)	HEM (μg)	Isoelectric banding profile
West Java (M)	13.1 ± 0.4	2.6 ± 0.0	476 ± 11	2.1 ± 0.1	4.2 ± 0.0	45–45	15	1 0 0 1 0 0 0 0 0 1 0 0 0 1 1 1 0 1
West Java (M)	9.3 ± 0.4	2.5 ± 0.1	417 ± 2	2.6 ± 0.0	5.0 ± 0.0	45–50	10	1 0 0 1 0 0 0 0 0 1 0 0 0 1 1 1 0 1
West Java (F)	11.4 ± 0.6	0.8 ± 0.1	278 ± 8	1.9 ± 0.0	1.4 ± 0.2	65–75	11	1 0 0 1 1 0 0 1 0 0 0 0 0 1 1 1 0 1
West Java (F)	26.5 ± 0.6	6.9 ± 0.1	462 ± 3	2.4 ± 0.0	2.6 ± 0.1	45–50	9	1 0 0 1 0 1 0 1 0 1 1 1 0 1 1 1 1 1
Malaysia (M)	18.2 ± 0.6	6.8 ± 0.0	397 ± 1	1.9 ± 0.2	4.7 ± 0.1	60–70	13	1 1 0 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1
Malaysia (M)	19.9 ± 0.4	5.1 ± 0.2	442 ± 6	2.3 ± 0.2	2.9 ± 0.1	45 50	9	1 0 0 0 0 0 0 1 0 1 0 1 0 1 1 1 1 1
Malaysia (F)	17.2 ± 0.4	1.6 ± 0.1	331 ± 1	2.1 ± 0.0	2.1 ± 0.0	45–50	7	1 1 0 0 0 0 0 0 0 0 1 1 0 0 1 1 1 1 1
Nakhon Si Thammarat (F)	16.4 ± 0.9	16.4 ± 0.5	298 ± 9	2.2 ± 0.1	5.0 ± 0.1	50 60	9	1 0 0 1 0 1 0 1 0 1 0 1 0 1 1 1 1 1
Krabi (F)	18.2 ± 0.1	15.6 ± 0.1	367 ± 1	1.5 ± 0.0	2.7 ± 0.1	35–40	10	1 0 1 0 0 1 0 1 1 1 1 1 0 1 1 1 1 0
Chumphon (M)	12.6 ± 0.7	2.8 ± 0.2	369 ± 2	1.4 ± 0.1	8.4 ± 0.1	90–90	15	0 1 0 0 0 0 0 0 0 0 1 0 0 0 1 1 0 1 1
Kanchanaburi (M)	17.5 ± 0.2	1.1 ± 0.0	416 ± 3	2.3 ± 0.1	3.2 ± 0.0	50 50	24	0 1 0 0 0 0 0 0 0 0 1 0 1 0 0 1 1 0 0
Rayong (M)	29.0 ± 0.7	2.7 ± 0.1	404 ± 4	1.5 ± 0.1	5.8 ± 0.2	75 80	10	1 0 0 1 0 1 1 0 0 1 0 1 0 0 0 1 1 1
Vietnam (F)	17.4 ± 0.6	1.8 ± 0.1	484 ± 2	2.2 ± 0.1	6.3 ± 0.1	45–45	19	1 1 0 1 0 0 0 0 0 1 1 1 1 1 1 1 0 0
Vietnam (F)	17.2 ± 0.9	1.2 ± 0.2	479 ± 9	2.2 ± 0.0	3.2 ± 0.0	60 60	18	1 0 0 1 0 0 0 0 1 1 1 1 1 0 1 1 0 1
Vietnam (F)	21.2 ± 1.1	1.5 ± 0.1	476 ± 3	2.1 ± 0.0	5.7 ± 0.1	70–70	17	0 1 0 1 0 0 0 0 1 1 1 1 1 0 1 1 0 1
Vietnam (M)	26.7 ± 0.7	4.6 ± 0.2	410 ± 5	2.1 ± 0.0	2.8 ± 0.0	40–40	21	1 0 0 1 0 0 0 0 0 0 1 1 1 0 0 1 1 1 0
Vietnam (M)	19.9 ± 0.4	16.2 ± 0.1	431 ± 3	1.8 ± 0.0	2.1 ± 0.1	50–55	24	1 1 0 1 0 0 0 0 0 0 1 1 1 0 0 1 1 0 1

Abbreviations and units of biological activities: PDE, phosphodiesterase (nmole/min/mg); PME, alkaline phosphomonoesterase (nmoles/min/mg); LAAO, L-amino acid oxidase (nmoles/min/mg); AEH, arginine ester hydrolase (μmoles/min/mg); NUC, 5′-nucleotidase (μmoles/min/mg); TLE, thrombin-like enzyme (sec); HEM, haemorrhagic activity (minimum hamorrhagic dose, μg). Figures are given ± 1 S.D., except in the case of TLE, where the range of values from two tests is given. Isoelectric banding profile of the venoms shows the occurrence of bands p*I* 9.40, 9.15, 8.55, 8.45, 8.25, 7.85, 7.45, 6.90, 6.55, 6.20, 6.10, 6.00, 5.90, 5.80, 5.70, 5.60, 5.50, 5.45. 1, Band present; 0, band absent. Bands present in all samples are not shown. M, Male; F, Female.

Capture localities of snakes: 1–4, West Java; 5–7, Malaysia; 8, Nakhon Si Thammarat; 9, Krabi; 10, Chumphon; 11, Kanchanaburi; 12, Rayong, 13–17, Vietnam.

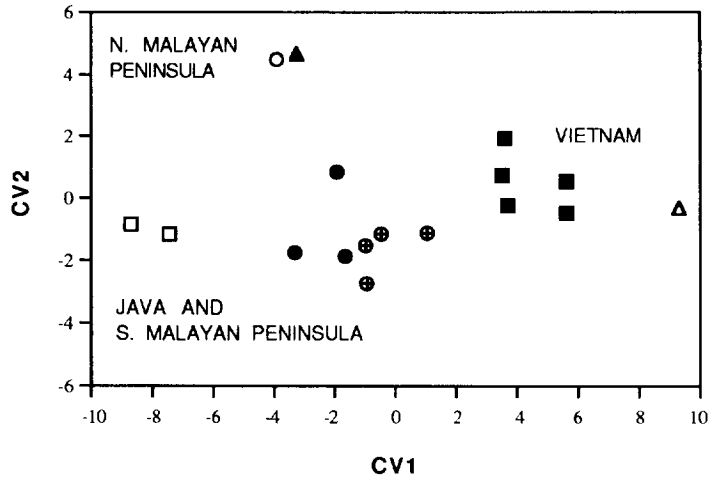

Fig. 2. Canonical variate analysis of various biological activities (phosphodiesterase, alkaline phosphomonoesterase, L-amino acid oxidase, arginine ester hydrolase, 5′-nucleotidase, thrombin-like enzyme, haemorrhagic activity) of 17 *C. rhodostoma* venom samples from Vietnam (■), Malaysia (●), Java (⊕), and the Thai provinces of Kanchanaburi (△), Chumphon (▲), Nakhon Si Thammarat and Krabi (□), and Rayong (○) (see Table 1).
Axes are graded in units of pooled within-group standard deviation.

Moreover, some of the variable bands may have no perceptible biological action. Nevertheless, it does appear that venom samples with more disparate electrophoretic profiles display more dissimilar enzymatic activities (the pattern of variation in the profiles of 17 venom samples from across South-east Asia was highly significantly congruent with the overall variation in their biological activities). Recent studies have shown that the geographic variation probably resulted from natural selection upon different populations of *C. rhodostoma* for venoms appropriate for subduing and digesting their local prey; wild populations with similar natural diets produce electrophoretically and functionally similar venoms (Daltry, J. C., Ph.D. Thesis, University of Aberdeen, 1995; Daltry *et al.*, 1995; Thorpe *et al.*, 1995).

Sexual variation. Isoelectric focusing of 106 venom samples of *C. rhodostoma* revealed that all captive bred and wild-caught females ($n = 48$) produced a strong band which was absent from the males from the same localities ($n = 58$). The function, if any, of this dimorphism is unknown; venoms from both genders were included in the assays of biological activity, but there did not appear to be any consistent differences that could be unequivocally attributed to the banding discrepancy (see Table 1). However, the activities studied here are by no means exhaustive and, when more samples become available, other biologically important properties, such as protease activity and toxicity, should also be compared.

Prior to this study, there have been sporadic reports of gender differences in snake venoms, but these were based upon too few samples for statistical credibility. Interestingly, however, it appears that, like female *C. rhodostoma*, female *Bitis nasicornis* (Marsh and Glatston, 1974) and *Crotalus adamanteus* (Mebs and Kornalik, 1984) produce an extra venom component which is absent from conspecific males.

Ontogenetic variation. Distinct banding differences were also evident between the venom electrophoretograms of juvenile and adult *C. rhodostoma* from the same geographic areas. Ideally, the venom of a group of pit vipers should be monitored throughout their lives to discount the possibility that the disparities between the age groups are attributable to individual variation (see below). That seems unlikely, however, given the strong correlation between banding pattern and body size even within the relatively inbred zoo group. Moreover, many other crotaline snakes are known to undergo similar ontogenetic changes in venom electrophoretograms (e.g. Lomonte *et al.*, 1983; Meier, 1986; Gutiérrez *et al.*, 1990). The effects of the ontogenetically variable components of *C. rhodostoma* venom have not yet been tested, but as a general rule, juvenile viperid snakes exhibit higher toxicity, higher coagulant activity and lower proteolytic activity than adults (Fiéro *et al.*, 1972; Bonilla *et al.*, 1973; Minton and Weinstein, 1986; Mackessy, 1988; Gutiérrez *et al.*, 1991; Chippaux *et al.*, 1991; Andrade and Abe, 1993). There is mounting evidence that such changes represent an inherent adaptation to ontogenetic changes in the snake's diet (Mackessy, 1988).

Individual variation. Although the bulk of the variation in venom can be classified as geographic, sexual or ontogenetic, some differences in banding profiles and biological activities were detected in the venoms of *C. rhodostoma* collected from the same geographic area on a similar date, of the same gender and similar body size. These probably reflect innate individual variation in venom synthesis, since genetic variation is expected in the gene pool of most sexually reproducing species. Williams and White (1987) describe immense venom variability within large populations of *Notechis ater*, while in small, isolated populations the gene pool is restricted and is associated with more homogeneous venoms. Judging by the high incidence of bites, *C. rhodostoma* is abundant in many regions and most populations can be expected to exhibit appreciable heterozygosity in venom-coding genes.

An alternative explanation for discrepancies among individual *C. rhodostoma* is that they reflect differing lengths of time between each snake's previous release of venom (before capture) and the date of extraction for this study (Willemse *et al.*, 1979). However, Paine *et al.* (1992) found that although electrophoretic profiles vary quantitatively over the first few days after venom extraction, new proteins are transcribed synchronously, so there is no *qualitative* change in venom composition.

Implications of intraspecific variation in venom composition

Identification of the species responsible for envenomation is often essential in order to deploy the most effective treatment. Since relatively few victims of snakebite can reliably describe the snake which bit them, physicians frequently must infer the species from the presentation of the bite alone. However, species diagnosis can be seriously confounded by intraspecific variability in venom composition; for example, the symptomatology of bites by *Daboia* (= *Vipera*) *russelli* shows immense geographic variation (Warrell, 1986; Wüster *et al.*, 1992) and, in some species, the venoms of juvenile and adults within the same population differ markedly in clinical effects (see Russell, 1983).

In view of the considerable variability in venom composition and biological activity revealed in this study, it is hardly surprising that symptomatological variation has also been detected in *C. rhodostoma* bites. In Thailand, for example, the most common site of spontaneous systemic haemorrhage is the gingival sulci whereas in Malaysia it is the lungs (Warrell, 1986). Unfortunately, many of the typical symptoms of *C. rhodostoma* bite are

also associated with the bites of *Daboia russelli* and green pit vipers (*Trimeresurus* spp.), which can lead to erroneous diagnosis and inappropriate treatment (Ho *et al.*, 1986*b*). Therefore, it is essential that medical practitioners are familiar with the specific clinical features which characterize envenomation by their local population of *C. rhodostoma*.

Even when the species responsible for envenomation has been accurately identified, however, effective therapy is not always assured. Antivenom is the single most effective treatment of snakebite, but its efficacy can be seriously diminished if the venoms used to prepare the antibodies differ from the venom of the snake that bit the victim. Antivenom prepared against *D. russelli* from Western India, for example, only weakly neutralizes the venom of populations in Southern India or Sri Lanka (Phillips *et al.*, 1988), while antivenom prepared from adult *Crotalus durissus* in Costa Rica is a poor neutralizer of the venom of local juveniles (Gutiérrez *et al.*, 1991). In the case of *C. rhodostoma*, antivenom manufacturers typically obtain venom from large specimens from a limited part of the distribution range. The Queen Saovabha Memorial Institute (Thai Red Cross Society) and Government Pharmaceutical Organization, for instance, prepare antivenoms chiefly from the venom of large (mostly female) specimens from southern Thailand, but it is not yet known whether these are equally effective against bites by snakes of both genders, all age groups and from all parts of the distribution range. However, physicians in Malaysia suspect that antivenoms prepared against Thai *C. rhodostoma* are less effective for treating bites in Malaysia than antivenoms prepared from Malaysian *C. rhodostoma* (Dr S. Ambu, Institute of Medical Research, personal communication), and immunological differences have been demonstrated between pooled venoms from Thailand and Java (Tu and Ganthavorn, 1978).

Perhaps a more widely effective antivenom could be developed by taking venom from *C. rhodostoma* of a range of age groups, of both genders and from all parts of the geographic area in which the antivenom is likely to be used (Chippaux *et al.*, 1991). This strategy would inevitably increase production costs, however, so it is important to ascertain the extent to which variation detected using isoelectric focusing is of real significance to antivenom efficacy. After all, some of the variable components might be of no pharmacological significance, while others may have potent clinical effects yet fail to stimulate an antigenic response (Chinonavanig *et al.*, 1988).

In addition to antivenom manufacture, *C. rhodostoma* is used to produce ancrod, the predominant thrombin-like enzyme in its venom (Tan, 1991). This study has shown considerable variation between individuals in the level of thrombin-like activity (see Table 1); venom from the Krabi province female, for instance, exhibited more than twice the potency of venom from the Chumphon male. Perhaps ancrod production could be significantly improved by selecting the groups which naturally synthesize higher amounts of this important drug.

The venom of *C. rhodostoma* is also widely used in academic research. Numerous workers have studied the venom in order to understand its biochemistry and pharmacological action (see Introduction for references). Snake venoms are also commonly studied by taxonomists in attempt to elucidate the phylogenetic relationships among species (Githens and George, 1931; Foote and MacMahon, 1977; Tu *et al.*, 1980); recently, venom electrophoretograms from *C. rhodostoma* and other pit vipers were compared for this purpose (Knight *et al.*, 1992). In almost every investigation, however, only the name and address of the supplier of the venom (typically a pooled sample) was recorded. Since this study has shown that the biological activities and electrophoretic properties of *C. rhodostoma* venom are greatly influenced by the geographic origin, gender and size

of the specimens whose venom constitutes these pools, the interpretation and reproducibility of many studies must be called into question. The i.v. LD_{50} of *C. rhodostoma* venom in mice, for instance, has often been analysed, but findings vary widely among authors (see Mebs, 1978). Owing to the paucity of information regarding the source of the venom samples, however, it is impossible to determine whether the inconsistent findings reflect intraspecific variation in venom toxicity (see Fiéro *et al.*, 1972; Theakston and Reid, 1978; Minton and Weinstein, 1986; Mackessy, 1988; Gutiérrez *et al.*, 1990, 1991; Rael *et al.*, 1992) or methodological differences, such as the use of different strains of mice (Russell, 1983).

Therefore, whenever possible, venom suppliers should inform researchers of the capture locality, size and gender of the specimens that provided the samples, and these data should be clearly indicated in ensuing publications. Other factors, such as the date of venom extraction and the nutritional health and reproductive condition of the snakes, may also be important (see Chippaux *et al.*, 1991). Greater attention to such details would assuredly contribute towards a more rigorous understanding of the biochemistry, pharmacology, treatment and uses of the venom of *C. rhodostoma* and other snakes.

Acknowledgements—For assistance and advice, we gratefully acknowledge the staff of Choray Hospital (Ho Chi Minh City), especially Trinh Kiem Anh and Trinh Xuan Kiem, S. Ambu, B. L. Lim (Institute of Medical Research, Malaysia), M. Grisley, A. Cumming (University of Aberdeen), G. Laing, R. D. G. Theakston (Liverpool School of Tropical Medicine) and M. J. Cox (Bangkok). We are indebted to B. J. F. Manly for the Fortran source code of his Mantel test, and the University of Aberdeen's Department of Zoology for the use of facilities. This research was funded by a SERC studentship (J.C.D.), NERC (W.W.) and the Leverhulme Trust (R.S.T.).

REFERENCES

Anderson, S. G., Gutiérrez, J. M. and Ownby, C. L. (1993) Comparison of the immunogenicity and antigenic composition of ten Central American snake venoms. *Toxicon* **31,** 1051–1059.

Andrade, D. V. and Abe, A. S. (1993) Toxicidade do veneno de *Bothrops moojeni* e sua relação com a variação ontogenética dieta. In: *III Congresso Latino Americano de Herpetologia*, p. 76 (Abstract). Campinas: UNICAMP.

Aragón-Ortiz, F. and Gubenšek, F. (1981) *Bothrops asper* venom from the Atlantic and Pacific zones of Costa Rica. *Toxicon* **19,** 797–805.

Ashford, A., Ross, J. W. and Southgate, P. (1968) Pharmacology and toxicology of a defibrinating substance from Malayan pit viper venom. *Lancet* **i,** 486–489.

Barrio, A. and Brazil, O. V. (1951) Neuromuscular action of *Crotalus terrificus terrificus* poisons. *Acta physiol. Latinoam.* **1,** 291–308.

Bonilla, C. A., Faith, M. R. and Minton, S. A. (1973) L-amino acid oxidase, phosphodiesterase, total protein and other properties of juvenile timber rattlesnakes (*Crotalus h. horridus*) venom at different stages of growth. *Toxicon* **11,** 301–303.

Chan, K-E., Rizza, C. R. and Henderson, M. P. (1965) A study of the coagulant properties of Malayan pit viper venom. *Br. J. Haematol.* **11,** 646–653.

Chen, P. S., Toribara, T. Y. and Warner, H. (1956) Microdetermination of phosphorus. *Analyt. Chem.* **28,** 1756–1759.

Chinonavanig, L., Billing, P. B., Matangkasombut, P. and Ratanabanangkoon, K. (1988) Antigenic relationships and relative immunogenicities of venom proteins from six poisonous snakes. *Toxicon* **26,** 883–890.

Chippaux, J-P., Williams, V. and White, J. (1991) Snake venom variability: methods of study, results and interpretation. *Toxicon* **29,** 1279–1303.

Collins, J. P. and Jones, J. G. (1972) Studies on the active site of IRC-50 arvin, the purified coagulant enzyme from *Agkistrodon rhodostoma*. *Eur. J. Biochem.* **26,** 510–519.

Daltry, J. C., Wüster, W. and Thorpe, R. S. (1995) The role of ecology in determining venom variation in the Malayan pit viper. In: *Venomous Snakes: Ecology, Evolution and Snakebite; A Zoological Society of London Symposium* (Thorpe, R. S., Wüster, W. and Malhotra, A., Eds). London: Academic Press. In press.

Dambisya, Y. M., Lee, T-L. and Gopalakrishnakone, P. (1994) Action of *Calloselasma rhodostoma* (Malayan pit viper) venom on human blood coagulation and fibrinolysis using computerized thromboelastography (CTEG). *Toxicon* **32,** 1619–1626.

Denson, K. W. E. (1969) Coagulant and anticoagulant action of snake venoms. *Toxicon* **7,** 5–11.

Dixon, W. J. (1990) *BMDP Statistical Software Version* 1990. Los Angeles: BMDP Statistical Software.
Esnouf, M. P. and Tunnah, G. W. (1967) The isolation and properties of the thrombin-like activity from *Ancistrodon rhodostoma* venom. *Br. J. Haematol.* **13,** 581–590.
Fiéro, M. K., Seifert, M. W., Weaver, T. J. and Bonilla, C. A. (1972) Comparative study of juvenile and adult prairie rattlesnake (*Crotalus viridis viridis*) venoms. *Toxicon* **10,** 81–82.
Foote, R. and MacMahon, J. A. (1977) Electrophoretic studies on rattlesnake (*Crotalus* and *Sistrurus*) venom: taxonomic implications. *J. Biochem. Physiol.* **57B,** 235–241.
Githens, T. S. and George, I. D. (1931) Comparative studies of the venoms of certain rattlesnakes. *Bull. Antivenin Inst. Amer.* **5,** 31–35.
Glenn, J. L. and Straight, R. C. (1978) Mojave rattlesnake *Crotalus scutulatus scutulatus* venom: variation in toxicity with geographic location. *Toxicon* **16,** 81–84.
Glenn, J. L., Straight, R. C., Wolfe, M. C. and Hardy, D. L. (1983) Geographic variation in *Crotalus scutulatus scutulatus* (Mojave rattlesnake) venom properties. *Toxicon* **21,** 119–130.
Gloyd, H. K. and Conant, R. (1990) *Snakes of the Agkistrodon Complex: A Monographic Review*. Oxford, OH: Society for the Study of Reptiles and Amphibians.
Gregory-Dwyer, V. M., Egen, N. B., Bosisio, A. B., Righetti, P. G. and Russell, F. E. (1986) An isoelectric focusing study of seasonal variation in rattlesnake venom proteins. *Toxicon* **24,** 995–1000.
Gutiérrez, J. M., Avila, C., Camacho, Z. and Lomonte, B. (1990) Ontogenetic changes in the venom of the snake *Lachesis muta stenophrys* (bushmaster) from Costa Rica. *Toxicon* **28,** 419–426.
Gutiérrez, J. M., dos Santos, M. C., Furtado, M. F. and Rojas, G. (1991) Biochemical and pharmacological similarities between the venoms of newborn *Crotalus durissus durissus* and adult *Crotalus durissus terrificus* rattlesnakes. *Toxicon* **29,** 1273–1277.
Hatton, M. W. C. (1973) Studies on the coagulation enzyme from *Agkistrodon rhodostoma* venom: isolation and some properties of the enzyme. *Biochem. J.* **131,** 799–807.
Heppel, L. A. and Hilmore, R. J. (1955) 5′ nucleotidase. In: *Methods in Enzymology*, Vol. II, p. 547 (Colowick, S. P. and Kaplan, N. O., Eds). New York: Academic Press.
Ho, M., Warrell, D. A., Looareesuwan, S., Phillips, R. E., Chanthavanich, P., Karbwang, J., Supanaranond, W., Viravan, C., Hutton, R. A. and Vejcho, S. (1986*a*) Clinical significance of venom antigen levels in patients envenomed by the Malayan pit viper (*Calloselasma rhodostoma*). *Am. J. trop. Med. Hyg.* **19,** 880–884.
Ho, M., Warrell, M. J., Warrell, D. A., Bidwell, D. and Voller, A. (1986*b*) A critical reappraisal of the use of enzyme-linked immunosorbant assays in the study of snake bite. *Toxicon* **24,** 211–221.
Jackson, D. A. and Somers, K. M. (1989) Are probability estimates from the permutation model of Mantel's test stable? *Can. J. Zool.* **67,** 766–769.
Jiménez-Porras, J. M. (1964) Intraspecific variations in composition of venom of the jumping viper, *Bothrops nummifera*. *Toxicon* **2,** 187–195.
Jones, J. M. (1976) Variations of venom proteins in *Agkistrodon* snakes from North America. *Copeia* **1976,** 558–562.
Kamiguti, A. S. and Hanada, S. (1985) Study of the coagulant and proteolytic activities of new born *Bothrops jararaca* venom. *Toxicon* **23,** 580.
Knight, A., Densmore, L. D. and Rael, E. D. (1992) Molecular systematics of the *Agkistrodon* complex. In: *Biology of the Pitvipers*, pp. 49–69 (Campbell, J. A. and Brodie, E. D., Eds). Tyler, TX: Selva.
Kondo, H., Kondo, S., Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of habu snake venom. *Jpn. J. med. Sci. Biol.* **13,** 43–51.
Lo, T. B., Chen, C. H. and Lee, C. Y. (1966) Chemical studies of Formosan cobra (*Naja naja atra*) venom (I). Chromatographic separation of crude venom on CM-Sephadex and preliminary characterization of its components. *J. Chin. chem. Soc. Ser. II,* **13,** 25–37.
Lomonte, B., Gene, J. A., Gutiérrez, J. M. and Cerdas, L. (1983) Estudio comparativo de los venenos de serpiente Cascabel (*Crotalus durissus durissus*) de ejemplares adultos y recién nacidos. *Toxicon* **21,** 379–384.
Mackessy, S. P. (1988) Venom ontogeny in the Pacific rattlesnakes, *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia* **1988,** 92–101.
Marsh, N. and Glatston, A. (1974) Venom of the rhinoceros horned viper, *Bitis nasicornis*. *Toxicon* **12,** 621–628.
Marshall, R. and Esnouf, M. P. (1968) The effect of *Ancistrodon rhodostoma* venom in the dog. *Clin. Sci.* **35,** 251–259.
Mebs, D. (1978) Pharmacology of reptilian venoms. In: *Biology of the Reptilia*, Vol. 8, *Physiology B*, pp. 437–560 (Gans, C. and Gans, K. A., Eds). London: Academic Press.
Mebs, D. and Kornalik, F. (1984) Intraspecific variation in content of a basic toxin in eastern diamondback rattlesnake (*Crotalus adamanteus*) venom. *Toxicon* **22,** 831–833.
Meier, J. (1986) Individual and age-dependent variations in the venom of the fer-de-lance (*Bothrops atrox*). *Toxicon* **24,** 41–46.
Minton, S. A. and Weinstein, S. A. (1986) Geographic and ontogenetic variation in the venom of the western diamondback rattlesnake (*Crotalus atrox*). *Toxicon* **24,** 71–80.
Moreno, E., Alape, A., Sanchel, M. and Gutiérrez, J. M. (1988) A new method for the detection of phospholipase A_2 variants: identification of isozymes in the venoms of newborn and adult *Bothrops asper* (terciopelo) snakes. *Toxicon* **26,** 363–371.

Ouyang, C., Hwang, L. J. and Huang, T. F. (1983) A-fibrinogenase from *Agkistrodon rhodostoma* (Malayan pit viper) snake venom. *Toxicon* **21,** 25–33.

Paine, M. J. I., Desmond, H. P., Theakston, R. D. G. and Crampton, J. M. (1992) Gene expression in *Echis carinatus* (carpet viper) venom glands following milking. *Toxicon* **30,** 379–386.

Phillips, R. E., Theakston, R. D. G., Warrell, D. A., Galigedara, Y., Abeyesekera, D. T. D. J., Dissanayaka, P., Hutton, R. A. and Aloysius, D. J. (1988) Paralysis, rhabdomyolysis and haemolysis caused by bites of Russell's viper (*Vipera russelli pulchella*) in Sri Lanka—failure of Indian (Haffkine) antivenom. *Q. J. Med.* **68,** 691–717.

Plagnol, H. and Martin, P. (1957) Electrophorèse du venin d'*Ancistrodon rhodostoma* Boie. *Ann. Inst. Pasteur* **92,** 525.

Ponnudurai, G., Chung, M. C. M. and Tan, N-H. (1993) Isolation and characterization of a hemorrhagin from the venom of *Calloselasma rhodostoma* (Malayan pit viper). *Toxicon* **31,** 997–1005.

Ponnudurai, G., Chung, M. C. M. and Tan, N-H. (1994) Purification and properties of the L-amino acid oxidase from Malayan pit viper (*Calloselasma rhodostoma*) venom. *Archs biochem. Biophys.* **313,** 373–378.

Rael, E. D., Knight, R. A. and Zepada, H. (1984) Electrophoretic variants of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) venoms and migration differences of Mojave toxin. *Toxicon* **22,** 980–985.

Rael, E. D., Johnson, J. D., Molina, O. and McCrystal, H. K. (1992) Distribution of a Mojave toxin-like protein in rock rattlesnake (*Crotalus lepidus*) venom. In: *Biology of the Pitvipers*, pp. 163–168 (Campbell, J. A. and Brodie, E. D., Eds). Tyler, TX: Selva.

Regoeczi, E. and Bell, W. R. (1969) *In vivo* behaviour of the coagulant enzyme from *Agkistrodon rhodostoma* venom: studies using I-Arvin. *Br. J. Haematol.* **16,** 573–587.

Regoeczi, E., Gergley, J. and McFarlane, A. S. (1966) *In vivo* effects of *Agkistrodon rhodostoma* venom: studies with fibrinogen. *Int. J. clin. Invest.* **45,** 1202–1212.

Reid, H. A. and Chan, K. E. (1968) The paradox in therapeutic defibrination. *Lancet* **i,** 485–486.

Reid, H. A., Thean, P. C., Chan, K. E. and Baharou, A. R. (1963) Clinical effects of bites by Malayan pit viper (*Ancistrodon rhodostoma*). *Lancet* **i,** 617–621.

Russell, F. E. (1983) *Snake Venom Poisoning*. New York: Scholium International.

Sadahiro, S. and Omori-Satoh, T. (1980) Lack of a hemorrhagic principle in habu snake venom, *Trimeresurus flavoviridis*, from the Okinawa Islands. *Toxicon* **18,** 366–368.

Schenberg, S. (1959) Geographical pattern of crotamine distribution in the same rattlesnake subspecies. *Science* **129,** 1361–1363.

Tan, N.-H. (1991) The biochemistry of venoms of some venomous snakes of Malaysia—a review. *Trop. Biomed.* **8,** 91–103.

Tan, N-H. and Tan, C-S. (1987) Thermal stability of venom enzymatic activities. In: *Progress in Venom and Toxin Research*, pp. 188–198 (Gopalakrishnakone, P. and Tan, C. K., Eds). Singapore: National University of Singapore.

Tan, N.-H., Kanthimathi, M. S. and Tan, C.-S. (1986) Enzymatic activities of *Calloselasma rhodostoma* (Malayan pit viper) venom. *Toxicon* **24,** 626–630.

Theakston, R. D. G. and Reid, H. A. (1978) Changes in the biological properties of venom from *Crotalus atrox* with ageing. *Period. Biol.* **80,** 123–133.

Thorpe, R. S. and Báez, M. (1993) Geographic variation in scalation of the lizard *Gallotia stehlini* within the island of Gran Canaria. *Biol. J. Linn. Soc.* **48,** 75–87.

Thorpe, R. S., Malhotra, A., Black, H., Daltry, J. C. and Wüster, W. (1995) Relating geographic pattern to phylogenetic process. *Phil. Trans. R. Soc. B.*, **349,** 61–68.

Tu, A. T. and Ganthavorn, S. (1978) Comparison of snake venoms (Reptilia, Serpentes) from Java, Indonesia and Thailand and its significance in evolution and zoogeography. *J. Herp.* **12,** 105–107.

Tu, A.-T., Stermitz, J. and Ishizaki, H. (1980) Comparative study of pit viper venoms of genera *Trimeresurus* from Asia and *Bothrops* from America: an immunological and isotachophoretic study. *Comp. Biochem. Physiol.* **66B,** 249–254.

Vellard, J. (1937) Variation géographique du venin de *Bothrops atrox*. *C. R. Acad. Sci.* **204,** 1369–1371.

Vellard, J. (1939) Variation géographique du venin de *Crotalus terrificus*. *C. R. Soc. Biol.* **130,** 463–464.

Warrell, D. A. (1986) Tropical snake bite: clinical studies in South-east Asia. In: *Natural Toxins—Animal, Plant and Microbial*, pp. 25–45 (Harris, J. B., Ed.). Oxford: Clarendon Press.

Warrell, D. A., Looareesuwan, S., Theakston, R. D. G., Phillips, R. E., Chanthavanich, P., Viravan, C., Supanoranond, W., Karbwang, J., Ho, M., Hutton, R. A. and Vejcho, S. (1986) Randomised comparative trial of three monospecific antivenoms for bites by the Malayan pit viper (*Calloselasma rhodostoma*) in southern Thailand: clinical and laboratory correlations. *Am. J. trop. Med. Hyg.* **35,** 1235–1247.

Willemse, G. T., Hattingh, J., Karlsson, R. M., Levy, S. and Parker, C. (1979) Changes in composition and protein concentration of puff adder (*Bitis arietans*) venom due to frequent milking. *Toxicon* **17,** 37–42.

Williams, V. and White, J. (1987) Variation in venom constituents within a single isolated population of peninsula tiger snake (*Notechis ater niger*). *Toxicon* **25,** 1240–1243.

Wüster, W., Otsuka, S., Malhotra, A. and Thorpe, R. S. (1992) Population systematics of Russell's viper: a multivariate study. *Biol. J. Linn. Soc.* **47,** 97–113.