SNAKE VENOM INSTABILITY

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Accepted: March 1978

ABSTRACT

Comparative electrophoretic studies were conducted on the venom of the rinkals (*Hemachatus haemachatus*), Egyptian cobra (*Naja haje haje*) and puffadder (*Bitis arietans*). Considerable differences in electrophoretic characteristics were found between fresh venom and commercial venom samples from the same species of snake. These differences could be attributed partly to the instability of snake venom under conditions of drying and storage.

INTRODUCTION

It is generally accepted that the biological activities of snake venom dried in vacuum at room temperature remain unaltered (Christensen 1955). The possibility of an alteration in biochemical properties due to the method of drying has been demonstrated by Björk & Boman (1959), but this would not necessarily influence the lethality of the venom (Irwin *et al.* 1970; Russel *et al.* 1960). Although it is usually emphasized that freeze-dried samples are preferable to desiccated samples for use in snake venom research (Björk & Boman 1959; Christensen 1968), the majority of reports make no mention of the length of time the venom samples were stored before they were used for experimental purposes. The instability of liquid venom (Brunton & Fayrer 1873; Marsh 1975) as well as desiccated venom (Schöttler 1951) has been shown under conditions of storage. Contrary to this, the stability of freeze-dried and desiccated venom samples when stored for up to 26 years has also been reported by various authors (Christensen 1955; Marsh 1975; Russel *et al.* 1960; Schöttler 1951). It has also been reported that toxic activity of snake venoms decreases markedly upon storage of the dried product for long periods of time (Klobusitzky 1971). Some inconsistencies in experimental results suggest that dried venom samples may be influenced by different methods of drying and storage, whereas they are ascribed by some workers to differences in experimental technique or geographic variation (Christensen 1955; Irwin *et al.* 1970). In order to explore this possibility, we made a systematic investigation of the effects of drying and storage on the electrophoretic pattern of venom of three South African snakes.

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MATERIALS AND METHODS

Venom

Freeze-dried and desiccated venom samples of the rinkals (*Hemachatus haemachatus*) and puffadder (*Bitis arietans*) were obtained from various commercial sources in South Africa. Electrophoresis was carried out on these samples within 24 hours of receiving them.

Healthy adult snakes of the species *H. haemachatus, B. arietans* and *Naja haje haje* (Egyptian cobra) were kindly donated by the Hartebeespoortdam Snake and Animal Park. Venom samples were collected and pooled according to species in conical glass vessels placed in a bath of crushed ice. Snakes were "milked" by inducing them to bite through a plastic membrane stretched over the glass vessel. These samples were then immediatey employed as fresh venom for electrophoresis, or rapidly freeze-dried at —70°C in a Virtis 10–147 MRBA freeze-drying apparatus. In order to investigate the effect of storage on snake venom, electrophoresis was carried out immediately after the freeze-drying process on each sample, whereafter the samples were divided into two portions for storage for 14 days. One portion of each sample was stored at 4°C in the dark in a stoppered glass vessel, while the second portion was stored in an open vessel in a desiccator over anhydrone *in vacuo* at 4°C in the dark before being employed for electrophoretic purposes. This procedure was carried out on the venoms of the rinkals and Egyptian cobra.

Electrophoresis

Electrophoresis was done on 7,5% (m/v) polyacrylamide gels in 0,05M Tris-glycine buffer at pH 8,5 for 45 minutes at 160V, according to the method described by Davis (1964). This technique has the advantage of considerable resolving power while requiring minimal amounts of venom. The gels were stained with saturated Amido Black in 7,5% glacial acetic acid and after destaining in 7,5% glacial acetic acid the gels were scanned in a Beckman R110 Microzone Densitometer equipped with an integrator. The protein fractions were numbered according to increasing relative mobility, with the prefixes A and C indicating respectively anodal and cathodal protein fractions and the letters S (puffadder), R (rinkals) and E (Egyptian cobra) characterizing the fractions of the venom types. Similar proteins in comparable venom samples thus received the same denomination. Statistical results are expressed as means ± standard error.

RESULTS

For the purpose of comparison, commercially supplied puffadder venom from a source in the south-eastern Cape Province (freeze-dried) was assessed against fresh venom from puffadders originating in the same area. It was attempted in this way to eliminate any possible geographic variation in venom characteristics such as has been reported for other snakes (Barrio & Brazil 1951; Irwin *et al.* 1970; Schenberg 1959).

Some differences in electrophoretic properties could be demonstrated between the fresh

and commercial (freeze-dried) samples (Figure 1). A total of 21 protein fractions could be clearly distinguished in the fresh venom sample (SP). Nineteen of these fractions migrated towards the anode while two migrated towards the cathode at pH 8,5. The anodal and cathodal protein fractions contained respectively 90% (± 1%) and 10% (± 1%) of the total protein content of the venom. Fraction SA_{16}, found in fresh venom samples, did not appear in the commercial sample. Apart from this difference, the electrophoretic pattern of both samples was similar. As a whole, the anodal fractions in the commercial sample contained

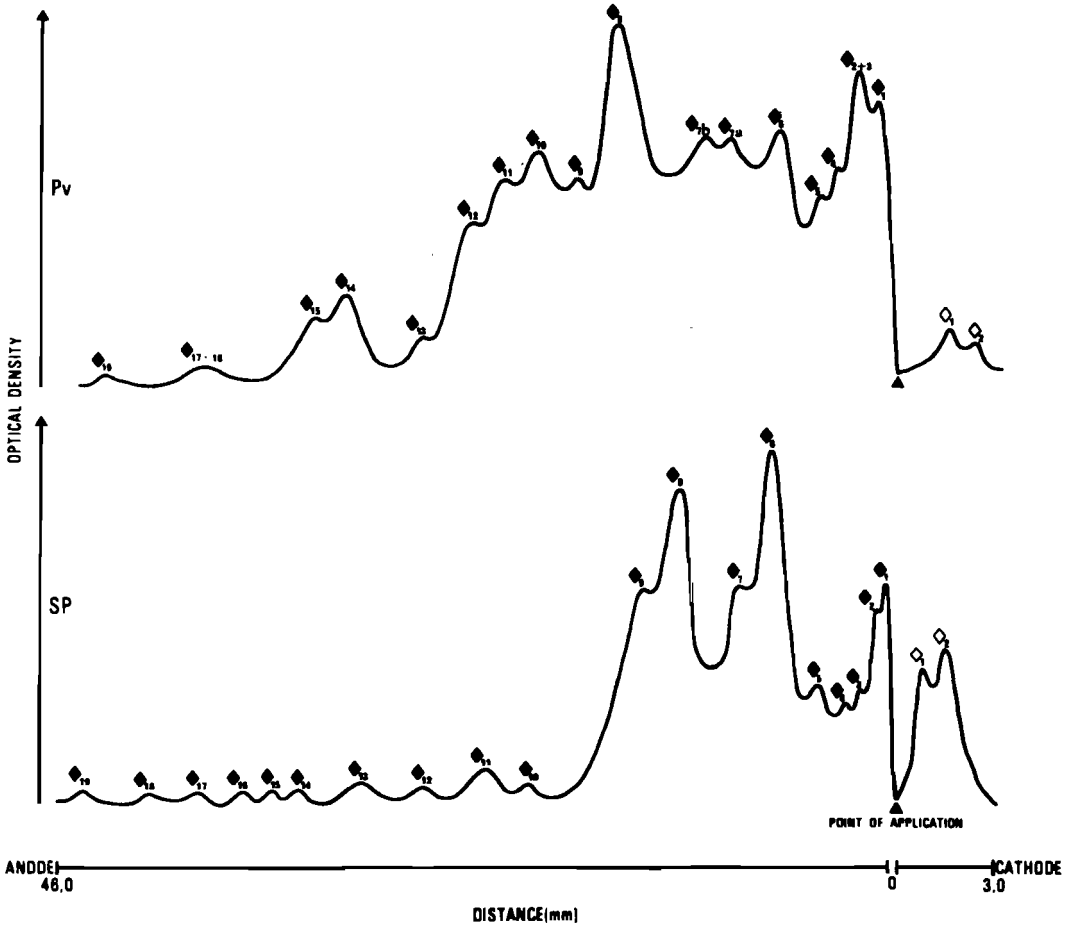

FIGURE 1.

Polyacrylamide gel electrophoretograms of puffadder venom from the south-eastern Cape Province. Comparison of a freeze-dried commercial venom sample (Pv) and fresh venom (SP).

Key ◆ —denotes anodal puffadder venom protein fractions SA_1–SA_{19}
◇ —denotes cathodal puffadder venom protein fractions SC_1–SC_2

99% (± 1%) and the cathodal fractions only 1% (± 1%) of the total protein content. This shift in relative prominence was caused by a lower relative concentration of the cathodal protein fractions as well as an increased relative concentration of some anodal fractions (SA_{10}–SA_{11}) in comparison with the fresh venom sample. Differences between fresh venom and commercially dried samples from the same species of snake could also be shown for the rinkals (Figure 2). Preliminary comparative electrophoresis of fresh venom failed to show

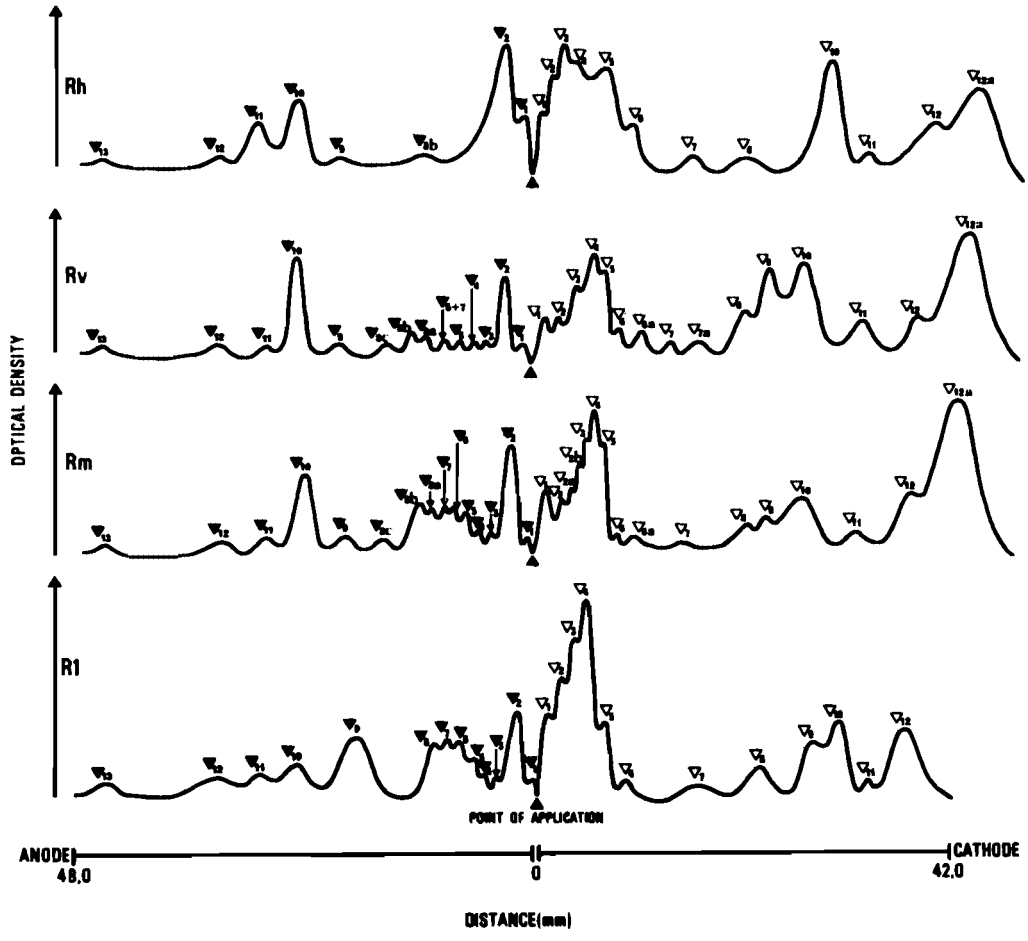

FIGURE 2.

Comparative polyacrylamide gel electrophoretogram of rinkals venom. R_1: fresh venom; Rm and Rv: freeze-dried commercial venom samples; Rh: desiccated commercial venom sample.

Key ▼—denotes anodal rinkals venom protein fractions RA_1–RA_{13}
▽—denotes cathodal rinkals venom protein fractions RC_1–$RC_{12}a$

any differences attributable specifically to geographic variation, and therefore freeze-dried and desiccated commercial venom samples obtained from various sources in the country are here compared with a single pooled fresh venom sample.

Thirteen anodal and 12 cathodal protein fractions could be clearly identified in fresh rinkals venom. The anodal protein fractions in fresh venom were found to constitute 33% (± 1%) of the total protein content of the venom and the cathodal fractions 67% (± 1%). The corresponding figures for the commercial venom samples are: Rm: 26% (± 1%) anodal and 74% (± 1%) cathodal; Rv: 18% (± 1%) anodal and 82% (± 1%) cathodal; Rh: 13% (± 1%) anodal and 87% (± 1%) cathodal. From Figure 2 it can be seen that fraction RA_9, which is one of the more prominent anodal fractions in fresh venom, could hardly be detected in the commercial venom samples, whereas the relative prominence of fraction RA_{10}, and in the case of commercial sample Rh, fraction RA_{11} as well, was markedly higher in relation to the same fraction(s) in fresh venom. The trend towards lower prominence of corresponding fractions in the commercial venom samples compared with fresh venom could also be seen in the protein fraction group RA_3–RA_8. In addition to the lower prominence of this group of fractions, recognition of the individual protein components in the group became more difficult because the individual protein bands tended to overlap. Two protein fractions, RA_8a & RA_8b, could be seen in the freeze-dried samples, which corresponded in mobility to fraction RA_8 in the fresh venom. This was also the case with freeze-dried puffadder venom (Figure 1) where fractions SA_7a and SA_7b corresponded to fraction SA_7 in the fresh venom. In contrast with the freeze-dried rinkals venom, only fraction RA_8b could be identified in desiccated rinkals venom (Figure 2). The differences in electrophoretic properties between the fresh venom samples and the commercially dried preparations were further illustrated by the presence in the commercial samples of some fractions which could not be detected in the fresh venom (RA_8c, RC_2a, RC_2b, RC_6a, RC_7a, $RC_{12}a$).

In order to determine whether the differences in electrophoretic properties between fresh and commercial venom samples could possibly be attributed to drying and storage, the effect of these factors on freeze-dried rinkals and Egyptian cobra venom was investigated. From the results it can be seen that drying and storage had a profound effect (Figures 3, 4) but that the latter was much more marked than the former for both venoms.

With rinkals venom (Figure 3) it was found that freeze-dried venom compared closely with fresh venom. Exceptions were fractions RA_8a and RA_8b and $RC_{12}a$ which indicated some degree of correspondence with the commercial venom samples (Figure 2). The correspondence between freeze-dried rinkals venom and the commercial rinkals venom samples became more evident upon storage of the freeze-dried samples for 14 days. In the latter case the protein fraction group RA_3–RA_8 took on a much more diffuse appearance and, in fact, only fractions RA_7, RA_8a and RA_8b could be clearly distinguished, whereas fractions RA_3–RA_6 were fused in a broad diffuse band bordering on fraction RA_2 in a fashion resembling a tailing of this prominent anodal protein fraction. Storage of the freeze-dried sample also produced an increase in the relative prominence of fraction $RC_{12}a$ while fraction RA_9 all but disappeared upon storage. Furthermore, the anodal fractions of the air-stored venom sample constituted 24% (± 1%) of the total protein content of the venom and the

cathodal fractions 76% (± 1%), while the corresponding figures for vacuum-stored venom were 27% (± 1%) and 73% (± 1%) respectively. The anodal protein fractions of the fresh freeze-dried venom constituted 35% (± 1%) and the cathodal protein fractions 64% (± 1%) of the total protein content of the venom.

The same trend, although in reverse, could be observed for the venom of the Egyptian

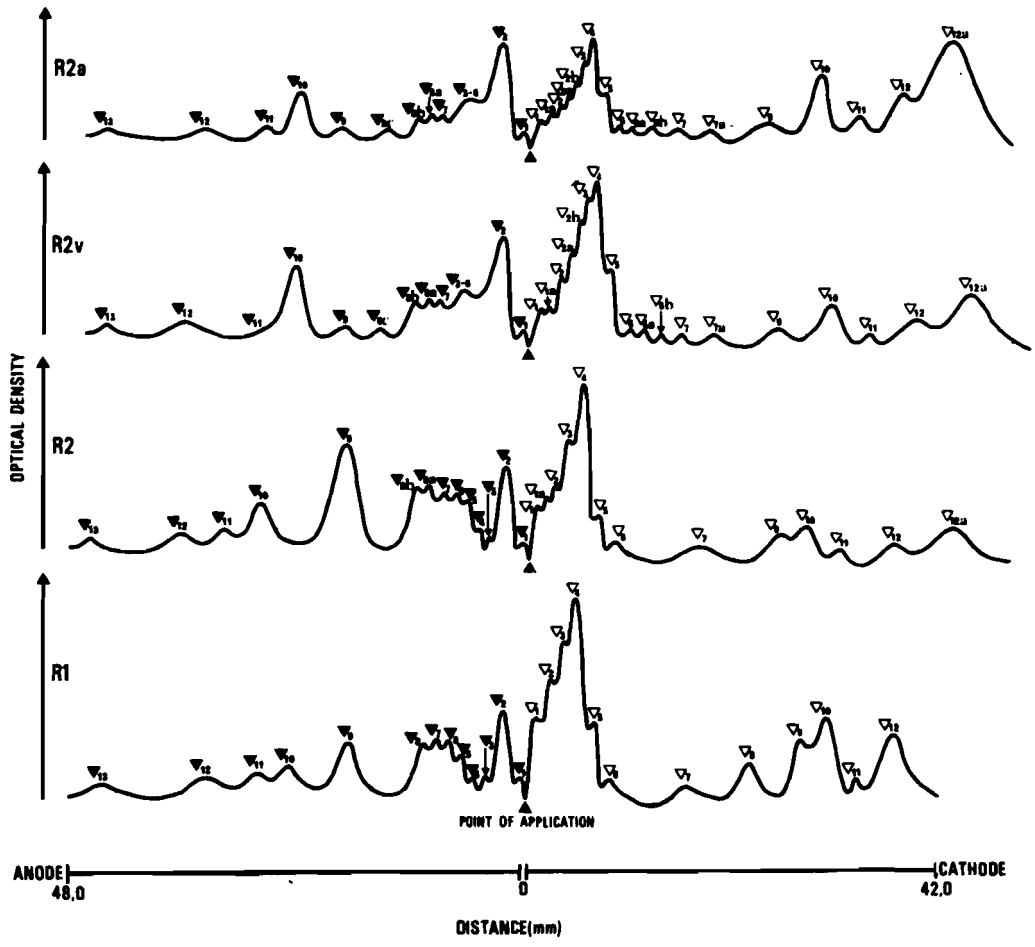

FIGURE 3.

Effect of drying and storage on rinkals venom. R_1: fresh venom; R_2: venom rapidly freeze-dried *in vacuo* at —70°C; R_2v: freeze-dried venom stored at 4°C *in vacuo* over anhydrone; R_2a: freeze-dried venom stored at 4°C at atmospheric conditions.

Key: ▼ —denotes anodal rinkals venom protein fractions RA_1–RA_{13}
▽ —denotes cathodal rinkals venom protein fractions RC_1–$RC_{12}a$

cobra (Figure 4) where 17% (± 1%) of the total protein content of fresh venom consisted of anodal protein fractions and 83% (± 1%) cathodal, while the freeze-dried and stored samples yielded the following figures: freeze-dried: 12% (± 1%) anodal, 88% (± 1%) cathodal; vacuum-stored: 21% (± 1%) anodal, 79% (± 1%) cathodal; air-stored: 23% (± 1%) anodal, 77% (± 1%) cathodal. By inference it could probably be assumed that the same phenomenon occurs with puffadder venom where, in both cases, the relative prominence of the anodal and

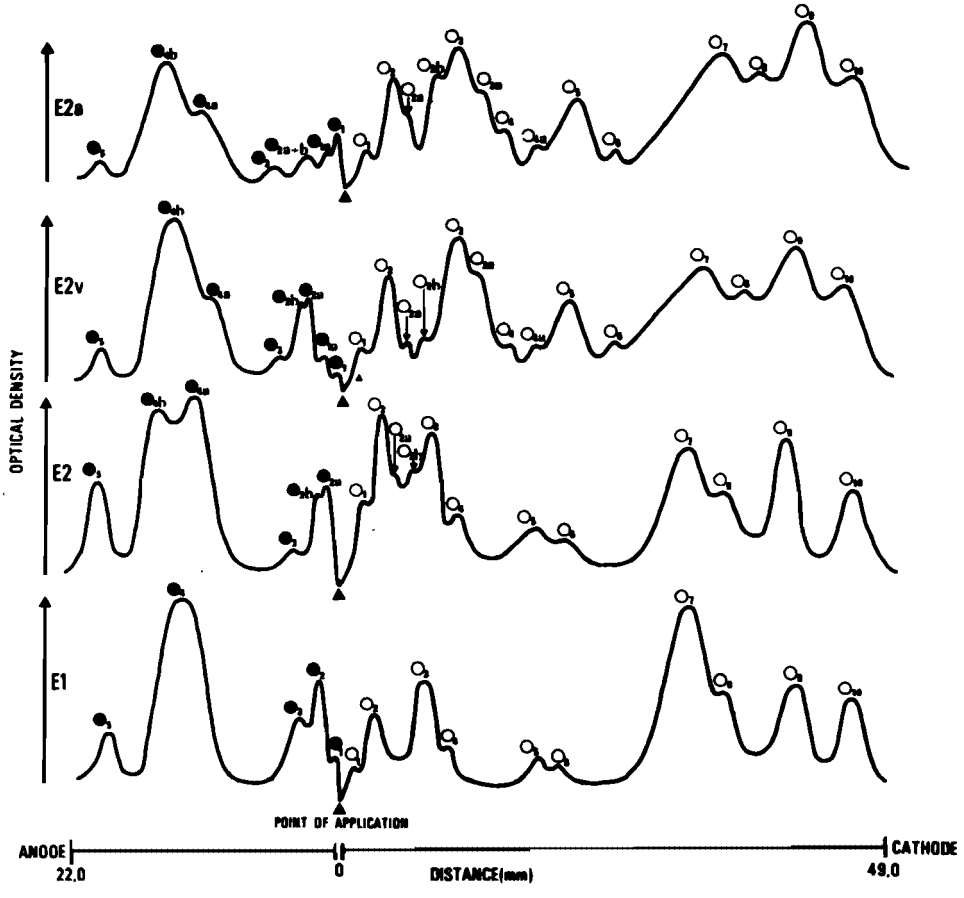

FIGURE 4.

Effect of drying and storage on the venom of the Egyptian cobra. E_1: fresh venom; E_2: venom rapidly freeze-dried *in vacuo* at —70°C; E_2v: freeze-dried venom stored at 4°C *in vacuo* over anhydrone; E_2a: freeze-dried venom stored at 4°C at atmospheric conditions.

Key: ○—denotes anodal Egyptian cobra venom protein fractions EA_1–EA_5
●—denotes cathodal Egyptian cobra venom protein fractions EC_1–EC_{10}

cathodal protein fractions respectively differed markedly between the commercial samples and the corresponding fresh venom samples. The effect of drying and storage on the electrophoretic profiles of snake venom could also be demonstrated for the venom of the Egyptian cobra (Figure 4). Once again the electrophoretogram of the freeze-dried sample corresponds fairly well with that of fresh venom except for fractions EA_2 and EA_4 which seem to have split up into fractions EA_2a and EA_2b, and fractions EA_4a and EA_4b respectively in the freeze-dried sample. Two extra cathodal fractions EC_2a and EC_2b also appeared between fractions EC_2 and EC_3 in the freeze-dried sample. Upon storage these differences became more pronounced and more additional fractions appeared. Cathodal fractions EC_7–EC_{10} also became more fused in the stored venom samples. With the exception of fraction EA_1 in freeze-dried venom, however, all the fractions present in fresh venom could be observed in the other samples, indicating that the change in cobra venom when stored probably progressed more slowly than was the case with rinkals venom.

DISCUSSION

In the context of the results presented here, the method of venom collection could be of paramount importance. It is quite possible that the precise method chosen for venom collection could influence venom composition. Consequently the nature and proportion of the fractions—many of them enzymes—initially present may influence the nature of subsequent changes in the material under storage. In this regard we have noticed that chloroform anaesthesia caused, in some instances, spontaneous venom loss from the venom glands in the puffadder. It is therefore debatable whether venom collected by electrical stimulation from anaesthetised snakes could be regarded as representative of natural ejection conditions such as occur in the case of snakebite. It was thought that the method of venom collection chosen for this study, the "conventional" method of milking, represented as much as was practically possible the natural conditions of venom ejection.

The possible importance of centrifugation of venom after collection should also be considered. Solid debris may constitute a large proportion of the volume of the expressed venom. This characteristic is for instance especially noticeable in the venom of the puffadder and rhombic night adder (*Causus rhombeatus*). It seems possible that unstable equilibria of some proteins between the solids and the venom solution might influence the composition of the material as finally assayed and, as a result, the reaction of individual venoms to storage. Centrifugation of venom samples before drying could thus possibly contribute to a more stable dried product.

From the results obtained with commercially prepared venom samples of the rinkals and puffadder in comparison with fresh venom from the corresponding species of snakes, it can clearly be seen that appreciable differences exist in their electrophoretic properties. In some cases these differences entail the absence from commercial samples of fractions which can clearly be identified in the fresh venom, and in other cases the presence of protein fractions in the commercial samples which could not be detected in the fresh venom. These findings

support the view of some authors that the quality of desiccated snake venom is decidedly inferior to that of freeze-dried samples and that commercially freeze-dried venom samples lack some of the characteristics of fresh venom (Björk & Boman 1959; Marsh 1975). Not only were there considerable differences in electrophoretic properties between commercially dried preparations and fresh venom from the same species of snake, but the different commercial samples also exhibited a marked degree of difference between themselves. This possibly explains differences in experimental results previously ascribed to other factors (Christensen 1955) and could probably be attributed to differences in collection and drying methods as well as storage time of the venom samples.

Although pooling of the venom could possibly affect the electrophoretic properties of the fresh samples as well as the dried samples used for subsequent storage, this certainly holds true for the commercial samples, as these are collectively pooled before drying in the same fashion as in the method used for this study. In a study of the electrophoretic properties of individual venom samples it was found that a wide range of individual variation exists (still to be published) and it would therefore be meaningless to compare individual venom samples with the commercial samples which consist of the pooled venom of many individuals. One would also expect pooling to average out the peak intensities of ubiquitous components. Comparison of the electrophoretic patterns of fresh venom and commercial venom samples is made difficult due to unknown differences in pool size, season of milking and specific differences between the individual pool members such as geographic origin, age, sex and diet. It should be emphasized that these factors could be important with regard to venom differences in the same species of snake (Barrio & Brazil 1951; Gubensek *et al.* 1974; Irwin *et al.* 1970; Marsh & Glatson 1974; Schenberg 1959). In the study of individual venom samples from various species of snakes, however, no specific differences attributable to sex could be found (to be published).

With the exception of a possible effect of pooling on the electrophoretic properties of the venom, all the other factors are eliminated in the comparison of the fresh venom samples with the dried and stored samples as all these factors are constant. The results obtained with stored freeze-dried venom samples of the rinkals indicate that some of the characteristic electrophoretic properties of the commercial venom samples used in this study strongly resemble the stored freeze-dried samples. It seems reasonable, therefore, to assume that the differences between commercially freeze-dried venom and freshly prepared freeze-dried venom could, in part, be explained by the influence of storage on the electrophoretic properties. Pooling of the venom was necessary in order to compare fresh venom with commercial samples as individual variation could have masked any differences due to other causes. Investigation of breakdown of individual venom samples is, however, desirable in order to eliminate any possible effects due to pooling of the venom.

With regard to the venom of the Egyptian cobra (Figure 4), it is interesting that fraction EA_1 should be present in the stored samples and not in the freeze-dried sample before storage, but this could be due to a close association of fraction EA_1 with fraction EA_2, causing a protein complex where the two proteins are indistinguishable as separate bands in the gel. This has been known to occur with some protein fractions in snake venom (Meldrum 1965).

Similarly, the presence of more than one fraction in freeze-dried and stored venom samples corresponding to only one protein fraction in fresh venom could be due to a disruption of the specific or electrostatic bonds between the proteins which form a complex in the fresh venom. On the other hand this may also have been caused by autolysis of some proteins.

The results of this study emphasize the need for standardization of venom-drying methods as well as the fact that dried venom preparations should be used immediately after preparation, and storage of the dried product avoided. The instability of at least a few snake venoms under these conditions is probably responsible for some characteristics being attributed to snake venom which would not ordinarily be present in the fresh venom, while other characteristics could be masked. At least some doubt should also exist as to the uniform effectiveness of anti-snakebite sera. It has recently been shown by Trethewie & Khaled (1973) that dried Australian snake venom develops a cross-reaction against its specific anti-serum after 2–3 months storage of the venom, but that no such cross-reaction can be demonstrated between anti-serum and fresh venom. This suggests that the venom used in the production of the anti-snakebite serum had been stored for a considerable time, during which its overall antigenic characteristics changed. As a consequence the anti-serum would be effective against the stored venom sample but not against fresh venom which would be injected in the case of snakebite. In the production of anti-snakebite sera, therefore, the use of fresh venom or fresh freeze-dried venom would be preferable to desiccated or stored freeze-dried venom because of the immunological effectiveness of these antisera.

ACKNOWLEDGEMENT

The authors wish to thank Mr Jack Seale, Director of Hartebeespoortdam Snake and Animal Park, for donating the snakes used in this study. Financial support was received from the CSIR.

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