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SUMMARY

The main purpose of this work is to demonstrate the possibility of a radioactive element biosinthetic "in vivo" incorporation to snake venoms.

South American rattlesnake (crotalus durissus terrificus) was used as pattern. S-35 was the tracer choosen for the high content of sulfurated amino-acids in this species venom.

A carrier-free, pH 8.0, $Na_2{}^{35}SO_4$ solution was administrated to have the final total activity about 0.15 mCi in the last venom sample.

One intraperitoneal injection was made in sin of the eight snakes studied.

Semanal doses, during two months, were administrated in the other two snakes.

The venom samples, extracted in intervals of 15 days, were tested for protein bound total radioactivity (determined in a liquid scintillator system) and its fractions separated by electrophoresis (revealed by autoradiography).

It was observed that labelled sulfur was incorporated to the molecular structure of venom proteins, specially crotamine and crotoxin.

The specific activity was about 0.65 nCi/mg. So, it could not be used in dinamic studies of the fractions.

This work demonstrates the biological "in vivo" radiosulfur incorporation in rattlesnake venom proteins.

Snake venoms are complex mixtures of proteins with distinct molecular weights, isoelectric points and composition. Some of these components are highly active pharmacologically while others exhibit only low toxicity or are even atoxic. Very little is known about the distribution of these proteins in the living organism. The same can be said in relation to the rate and route they are absorbed following subcutaneous or intramuscular administration, and to the process they are excreted. The use of the radioisotope tracer technique provides an efficient method for the

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investigation of these points, some of which are of fundamental importance in envenomation. The use of labelled venom components would also afford an excellent opportunity of studying their localization at cellular and subcellular levels, and, perhaps, of separating the receptors upon which they fix themselves. This is particularly true in relation to those components such as crotoxin, α-bungarotoxin and cobra neurotoxin, which produce neuromuscular blockage seemingly through combination with the specific colinergic receptors at the motor end-plate[1,3]. The advantage offered by these toxins upon the competitive or depolarizing neuromuscular blocking drugs is that they form irreversible or very slowly reversible complexes with receptors.

Labelling of crude venoms with radioiodine or of toxins separated from them has already been done[4,7]. These preparations, particularly the labelled toxins[5,6], can give some useful information concerning distribution, absorption and excretion of venom components. However, they offer the disadvantage of undergoing decomposition in the animal body. Moreover, ^{131}I-labelled toxins are not very satisfactory for autoradiography studies on account of spreading spots given by the γ-radiation of iodine. Therefore, labelling of venom components with a β-emitter isotope incorporated in their molecular structure would be highly desirable.

This paper is a preliminary report of a research designed to label ophidic venoms by injecting ^{35}S-containing compounds into snakes. A $Na_2{}^{35}SO_4$ solution was used in the present experiments. A definite incorporation of ^{35}S into venom components seems to have occurred although the specific activities obtained were still very low. Venom toxins are usually very rich in sulphur[8,9,10]. This suggested experimentation along the above referred lines.

MATERIAL AND METHODS

South American rattlesnakes (Crotalus durissus terrificus) from Southern Brazil (São Paulo State) were used in the experiments. They belonged to both varieties of C.d. terrificus, i.e. to the variety whose venom contains crotamine[11], and to that whose venom is devoid of this basic toxin. The snakes were kept in individual cages and weighed at the beginning of the experiment and at several occasions during its course. They usually were not fed; water was always left in their cages.

A $Na_2{}^{35}SO_4$ solution, pH 8.0, carrier free, was intraperitoneally injected in seven snakes. Only one injection was made in six of them. In one snake the $Na_2{}^{35}SO_4$ solution was administered several times. The activity administered was such that it was 0.16 mCi at the time the venoms were counted. Mice were also intraperitoneally injected with the $Na_2{}^{35}SO_4$ solution. Two of the snakes were fed with such mice. The $Na_2{}^{35}SO_4$ was supplied as a carrier-free product by the Radiochemical Division of the Atomic Energy Institute, São Paulo, Brazil.

As initial venom (unlabelled venom) extraction was carried out immediately before the first injection of the $Na_2{}^{35}SO_4$ solutions in order to empty the venom glands. Twenty days later the venom was again extracted (first extraction of labelled venom). Other extractions were also done at intervals of 15 or more days. The venom was always vacuum dried immediately after being extracted.

The labelled venoms dissolved in saline were submitted to electrophoresis. Whatman Nº 1 or 3 MM paper, barbiturate buffer, pH. 8.6, ionic strenght 0.05 and running time of 20 hours were employed. Fifty microlitres of a 0.1% solution of Na_2SO_4 were added to the electrophoresis strips as carrier.

The radioactivity was determined in a liquid scintillator

system (Model 725 of Nuclear Chicago). The venom, dissolved in saline, was incorporated into 15 ml of a liquid scintillation medium, as described by Bray[12]. Three to 5% of finely divided silica (Ca-O-Sil) was added to Bray's solution to form a gel in order to avoid protein precipitation. Quenching was weak because the samples contained small amounts of venom (1 mg/0.1 ml). Correction for quenching effects was provided by the channel ratio method. As counting rates were low, long counting times were required to measure the ratio with precision. The standard solutions were prepared with and without carrier for determination of self-absorption.

The incorporation of sulphur-35 into the molecules of venom components was ascertained by the trichloroacetic acid test and by autoradiography of paper electrophoresis strips. The trichloroacetic acid test was performed by precipitating a solution containing 30 mg of venom with a 10% solution of trichloroacetic acid, the precipitate obtained being washed several times. The radioactivity of this precipitate as well as that from the supernatant liquor was determined by the procedure described.

RESULTS

Table 1 summarizes the results of the experiments. As can be seen, the specific activities of the venoms from the first extractions were very low. The radioactivity obtained in the successive extractions was usually even lower. However, the venom from the second extraction of snake No. 6 showed a much increased specific activity. This snake received four injections of the $Na_2{}^{35}SO_4$ solution after the first extraction of labelled venom and was also fed at two different times with a mouse injected with the ^{35}S-containing salt. The venom from the third extraction of snake No. 3 also showed an increase in specific activity. This snake was fed with a mouse injected with $Na_2{}^{35}SO_4$ after the second extraction of labelled venom.

TABLE 1. Incorporation of ^{35}S into the rattlesnake (C.d. terrificus) venom

Snake No.	Weight (g)	Venom extraction No.	Specific activity (nCi/mg)
1	165	1	0.48
2	450	1	0.73
	450	2	0.25
3[a]	1500	1	0.15
	1500	2	0.02
	1500	3	0.30
	1500	4	0.14
4	380	1	0.78
5	300	1	0.15
6[b]	155	1	0.65
	160	2	3.10
7	850	1	0.30

The snakes were injected only once with the $Na_2{}^{35}SO_4$ solution. An exception was snake No. 6.

a This snake was fed with a mouse injected with $Na_2{}^{35}SO_4$ after the second venom extraction.

b Beside receiving four injections of $Na_2{}^{35}SO_4$ solution after the first venom extraction, this snake was also fed twice (at 30 days' interval) with a mouse injected with the ^{35}S-containing salt.

The result given by the trichloroacetic acid test showed that almost all radioactivity accompanied the precipitate (Table 2).

TABLE 2. Trichloroacetic acid test. Snake No. 6, 155 g, first extraction of venom

	Specific activity (nCi/mg)
Whole venom	0.65
Precipitate	0.63
Supernatant	0.012

Autoradiograms of the electrophoretic strips of venoms devoid of crotamine showed only one spot (Fig. 1 and Fig. 2) while two spots were obtained in the autoradiograms of crotamine-containing venoms (Fig. 1D).

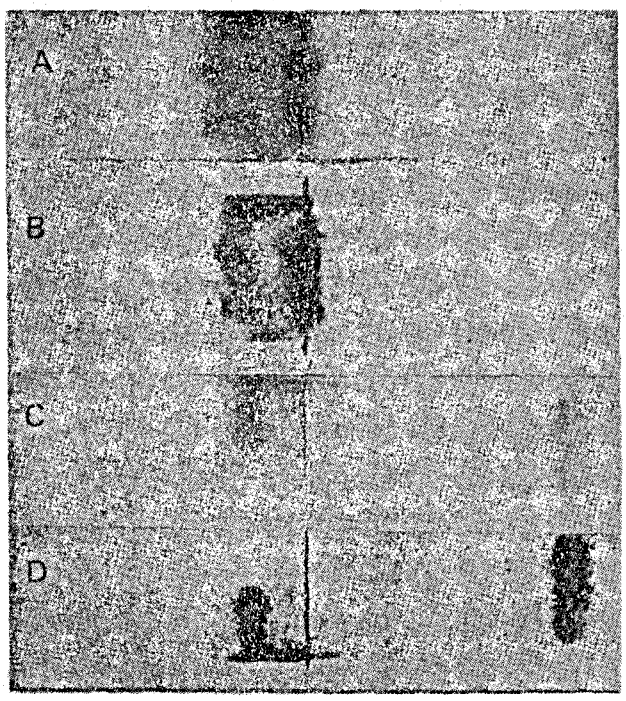

FIGURE 1. A and C: Venom electrophoresis. Rattlesnakes (*C. d. terrificus*) injected with $Na_2{}^{35}SO_4$. Whatman paper No. 1. Barbiturate buffer, pH 8·6, μ 0·05, running time 20 hours. A—crotamine negative venom. B—crotamine containing venom. B and D: Autoradiograms of the strips. No screen x-ray film. Exposure time 20 days.

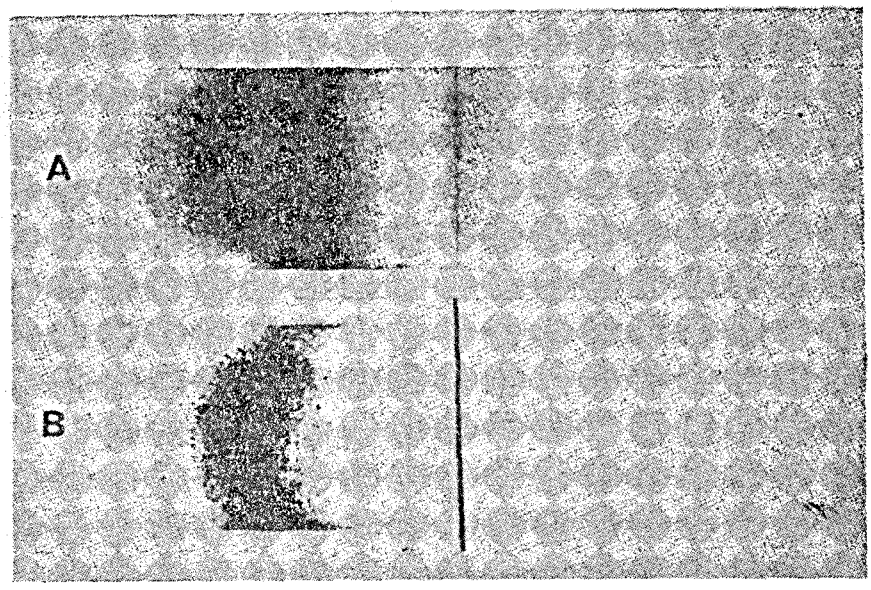

FIGURE 2. A: Venom electrophoresis. Rattlesnake (*C. d. terrificus*) injected with $Na_2{}^{35}SO_4$. 3 MM paper. Barbiturate buffer, pH 8·6, 0·05 μ, running time 20 hours. Crotamine negative venom. B: Autoradiograms of the strip. No screen x-ray film. Exposure time 20 days.

DISCUSSION

Radiosulphur could be found in the venom (a) as inorganic sulphates, (b) as acid, sulphate-containing polysaccharides and (c) incorporated in the molecular structure of some venom proteins. The first and the second hypotheses are not supported by the results of the trichloroacetic test or by the autoradiograms of the electrophoresis strips. Therefore, it seems very probable that the ^{35}S was incorporated in the molecular structure of venom proteins. However, it is not yet possible to decide if all or only some proteins of the venom were labelled. The autoradiograms seem to demonstrate that at least crotamine and crotoxin had radiosulphur incorporated in their molecules.

Slotta and Fraenkel-Conrat[9] demonstrated that most of the sulphur present in snake neurotoxins is in the form of -S-S-bridges i.e. in the form of cystine. Radiosulphur would therefore be found in this form. Another possibility is that it would be incorporated as methionine in the venom proteins. Experiments designed to clear the point or points of capture of radioactive sulphur are in progress.

The specific activities obtained in the present experiments were too low to permit the utilization of the labelled venom components in pharmacological investigations. However, an improvement in labelling can very probably be obtained by a more prolonged administration of $Na_2{}^{35}SO_4$ solution as is suggested by the result given by snake No. 6. Injection of ^{35}S-cystine or methionine can also give better results and will be tried.

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RESUMO

Objetivo fundamental dêste trabalho foi demonstrar a possibilidade de se incorporar um elemento radioativo à peçonha de cobras através de sua biossíntese "in vivo". Escolheu-se como modêlo a cascavel sul-americana (crotalus durissus terrificus) e como indicador o ^{35}S em virtude do alto teor de amino-ácidos sulfurados do veneno dessa espécie. Administrou-se.. $Na_2{}^{35}SO_4$ em solução a pH 8,0, livre de carregador e em dose tal que por ocasião da última colheita de veneno a atividade total fôsse da ordem de 0,15 mCi. O radiosulfato foi administrado por via intraperitoneal, uma única vez em seis, das oitos cobras estudadas. Nas duas restantes, doses semanais foram repetidas durante mais de dois meses. Cada partida de veneno,extraída cada 15 dias, foi testada separadamente quanto ao seu teor de radioatividade total ligada às proteínas do veneno precipitadas pelo ácido tricloroacético (medida em cintilador líquido) e nas suas diversas frações separadas eletroforèticamente (reveladas por autoradiografia). Verificou-se que o radioenxôfre se incorporou às moléculas protéicas do veneno, em particular às de crotamina e crotoxina. A atividade específica foi da ordem de 0,65 nCi/mg, não sendo, pois, compatível com o emprêgo em estudos de dinâmica das próprias frações. Ficou demonstrada, no entanto, a incorporação de radioenxôfre, por via biológica "in vivo" nas proteínas da peçonha da cascavel.

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