INACTIVATION OF CROTOXIN BY GROUP-SPECIFIC REAGENTS

by

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The neurotoxin of the Brazilian rattlesnake, *Crotalus terrificus terrificus*, was isolated in crystalline form by SLOTTA AND FRAENKEL-CONRAT[1]. This protein, named crotoxin, was later found to appear homogeneous in ultracentrifugal and electrophoretic studies[2, 3]. It proved to resemble insulin in its high sulfur content which was almost completely accounted for as cystine and methionine[4]. Like insulin, crotoxin was readily inactivated by reducing agents[5] which indicates that the integrity of most of its disulfide bonds is essential for its neurotoxic and hemolytic activities.

Crotoxin has now been treated with a number of protein reagents, some of which have in recent years been demonstrated as selectively affecting only one or a few types of protein groups. These studies have shown that the neurotoxic activity is lost when an appreciable fraction of the amino, phenolic, carboxyl, aliphatic hydroxyl, imidazole, indole, amide, or guanidyl groups are chemically modified. These findings, in conjunction with the previously demonstrated essentiality of the disulfide groups, characterize crotoxin as the protein with the widest range of essential groups yet recognized.

METHODS AND MATERIALS

Pure amorphous and crystalline crotoxin was prepared from dried venom as previously described[1]. The first experiments were performed with the amorphous product to avoid loss of material during crystallization. When a greater supply of the venom became available, crucial experiments were repeated with crystalline crotoxin. No differences were noted in the toxicity, nor in the reactivity of the two preparations.

1. *Acetylation*

Specific acetylation of the amino groups was achieved with acetic anhydride[6]. To 50 mg crotoxin in 1 ml half saturated sodium acetate was added 0.06 ml acetic anhydride at 0° C., with shaking, in the course of one hour. The product isolated by lyophilization after dialysis (45 mg) showed an equivalent loss in amino nitrogen and acetyl content, corresponding to five groups per 10^4 g protein. Acetylation with less acetic anhydride (5 mg for 12.5 mg protein) yielded a product retaining 72% of its amino nitrogen.

The fact that the phenolic groups did not participate in the reaction with acetic anhydride was indicated by colorimetric analyses. The FOLIN chromogenic value corresponded to 5.5 and 5.8% tyrosine for the (unhydrolysed) acetylated and unreacted protein, and the ratio of the values obtained without or with exposure to an alkaline p_H (p_H 8/p_H 11 ratio[7]) was 0.95 and 0.88 for the two preparations, respectively. While the limitations of these methods are well recognized[8], particularly in a protein which in the intact state gives only 41% of its total FOLIN colour (as ascertained by enzymatic or alkaline hydrolysis), these results do not suggest that any alkali-labile phenol-acetate groups are formed in the reaction.

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2. Esterification

46 mg crotoxin was methylated by exposing it to 5 ml 0.1 N methylalcoholic hydrochloric acid at room temperature for several days. This technique has been shown to lead to extensive and probably exclusive methylation of the carboxyl groups[8]. The product, after precipitation with ether and humidification contained 3.5% methoxyl, *i.e.*, 11.3 equivalents per 10^4 g protein. Reversal of the esterification was attempted by the same method that has been advocated for insulin[9], *i.e.*, exposure in 0.8% solution to 0.1 N sodium hydroxide at 0° C. for 24 hours, followed by neutralization.

Two partially methylated crotoxin preparations were obtained by a) treating 50 mg as above but at —5° C. instead of at room temperature (3.5 equivalents of methoxyl were introduced), and b) treating 5 mg protein with 2 ml 0.025 N methanolic hydrochloric acid for four hours at room temperature. The protein dissolved in the reaction mixture and was isolated by precipitation with ether. Control samples to the latter two preparations were exposed first to the alcohol and ether, as above, and then in aqueous solution to hydrochloric acid of the same strength as used for the experimental samples.

3. Sulfation

50 mg protein was treated with 2 ml concentrated sulfuric acid at —18° C. for five minutes, then poured over ice, neutralized and dialysed. This technique has been shown to transform into acid sulfate esters almost all the aliphatic hydroxyl groups of proteins, as well as a small fraction of the phenolic groups, and the -SH groups if such are present, which is not the case for crotoxin[10]. The sulfated product was largely soluble after dialysis and was isolated by lyophilization. It contained 2.74% sulfate S and 11.9% N (uncorrected for moisture), equivalent to the presence of 10.5 aliphatic hydroxyl groups per 10^4 g protein.

4. Iodination

a. 25 mg crotoxin, dissolved in a mixture of 0.5 ml water, 0.15 ml 3 M acetate buffer (p_H 5.75), and 0.35 ml M sodium chloride, was treated at 0° C. with 0.05 ml 0.1 N iodine solution. The solution which remained yellow was dialysed after half an hour. 23 mg of the reaction product was isolated by lyophilization. It contained only 0.30% iodine. b. 31 mg crotoxin, dissolved in 10 ml water by addition of 3 drops 0.1 N sodium hydroxide was treated with 1 ml 3 M acetate buffer (p_H 5.75) and 0.7 ml 0.1 N iodine solution (in potassium iodide) for two hours at room temperature. The reaction mixture remained yellow; a precipitate formed. It was dialysed as usual. The product contained 12.3% iodine (9.7 equivalents per 10^4 g).

Indications from the literature[11, 12] suggest that in the absence of -SH groups the phenolic groups alone would react under such conditions, incompletely in case a) where the amount of iodine used corresponds to only about one sixth of that needed for complete diiodination of the tyrosine residues, and more extensively under the conditions of experiment b. The participation of histidine and tryptophane residues, however, cannot be regarded as having been strictly excluded[6]*.

5. Formaldehyde

a. At p_H 11.5 : 40 mg crotoxin was dissolved in 2 ml water and 0.2 ml N sodium hydroxide; 0.3 ml 38% formaldehyde was added, and after fifteen minutes 0.2 ml N hydrochloric acid to neutralize the reaction mixture which was then dialysed. 30 mg of an insoluble product was isolated. In the alkaline medium the indole ring is known to react most rapidly, the amide groups only slightly slower, and the guanidyl groups yet slower[15–17]; the addition of the formaldehyde to amino groups yields methylol groups which are so labile as to be hydrolysed during dialysis, as indicated by almost unchanged amino nitrogen values for the isolated proteins. The tryptophane residues had lost their chromogenic activity, thus appeared to have been transformed to methylol tryptophane. The latter may not interfere greatly with the recovery of acid-labile formaldehyde: thus the observed value of three equivalents of formaldehyde reversibly bound may be an approximation of the true amount.

b. At p_H 7.6: 25 mg crotoxin in 2.5 ml water and 0.125 ml 3.4 M phosphate buffer (p_H 7.6) was treated with 0.125 ml 38% formaldehyde for one h. at room temperature. The reaction mixture became turbid. 24.6 mg of reaction product was isolated after dialysis and lyophilization. The product showed only a slight decrease in amino nitrogen (Table I). Its tryptophane residues appeared unaffected; thus formaldehyde analysis have no quantitative significance[18].

c. In presence of alanine: In one experiment 50 mg crystalline crotoxin and 180 mg alanine were dissolved in 2 ml water, 0.5 ml M sodium chloride and 0.5 ml M phosphate buffer (p_H 7.0), and treated with 0.25 ml 38% formaldehyde. Half of the reaction mixture was dialysed after ninety

* A detailed study of the iodination of lysozyme[13] indicates that its histidine residues are more reactive than its phenolic groups at p_H 7.6 and bind some iodine even at p_H 6. A fraction of the tryptophane appears to become oxidized at p_H 5–7.6. In contrast, in insulin the phenolic groups alone react under mild conditions[13, 14]. A similarly thorough study of the effect of iodine on crotoxin under a variety of conditions would have required too much material.

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TABLE I

ANALYSES OF CROTOXIN AND DERIVATIVES[e]

Reaction	NH_2-N	Groups per 10^4 g[22]		Tyrosine		Tryptophane[24]	Tyrosine plus Tryptophane[a] (as tyrosine)
				Folin[a]	Gern-Gross[23]		
	percent	Acid	Basic	percent	percent	percent	percent
None	0.78, 0.89[b]	14.2	11.1	12.0	10.5	2.7	14.3
Acetylation (partial)	0.56						
Acetylation (intense)[c]	0.15, 0.24[b]						
Formaldehyde, pH 11.5	0.77					0	
Formaldehyde, pH 7.6	0.68					2.4	
Formaldehyde plus alanine, pH 7.6		17.4				3.0	
Formaldehyde plus alanine, pH 4.2	0.90	19.8				2.1[f]	
Sulfation[d]					7.6	2.0	14.5
Iodination (partial)					7.1		13.1
Iodination (intense)					3.9		10.3
Coupling (4 equiv.)		15.8			5.9		
Coupling (12 equiv.)		18.6					

[a] The *tyrosine* of crotoxin was estimated by means of the Folin reagent[7] after acid hydrolysis, which causes the destruction of all tryptophane present. Without hydrolysis, only 6.6% tyrosine is available to the Folin reagent, *i.e.*, 47% of the total chromogenic activity. After 10 minutes exposure to pH 11[7], 1.14 times as much colour is obtained with untreated crotoxin, 1.05 times as much with the intensively acetylated derivative which is evidence for the non-participation of phenolic groups in the acetylation reaction.

Tyrosine plus tryptophane was determined in alkaline or enzymatic hydrolysates[15] with the Folin reagent.

[b] Van Slyke analyses[20]. The others are colorimetric[19].

[c] 5.3 acetyl equivalents bound per 10^4 g protein.

[d] 5.0 sulfate equivalents bound per 10^4 g protein.

[e] None of the analyses are corrected for the moisture content (about 8%). Crotoxin contains 0.83% amide-N.

[f] A typical purple colour.

minutes, the rest after twenty four h. Another similar experiment was performed in acid solution. 50 mg crystalline crotoxin, dissolved in 0.5 ml 0.1 N hydrochloric acid was treated for twenty four hours with 250 mg alanine, 0.5 ml water, 0.25 ml 3 M acetate buffer (pH 4.2) and 0.25 ml 38% formaldehyde. Both products were largely soluble after dialysis, in contrast to a sample treated in acid solution as above, but without the addition of alanine.

It has been shown that the conditions of experiments b. and c. would favour condensation reactions of formaldehyde between pairs composed of amino and amide, or amino and guanidyl groups[16]. More slowly (*i.e.*, in several days) the aminomethylol also condense with phenolic groups[17]. When a great excess of a small-molecular amine, *e.g.*, alanine, is present, its amino groups will react, particularly in acid solution, with the amide, guanidyl (and possibly phenolic) groups of the protein while most of the amino groups of the latter will be prevented from participating in the reaction. Thus formaldehyde, in conjunction with alanine, can be used as a semi-specific reagent to study the essentiality of amide and guanidyl groups in biologically active proteins. In the above experiments, both the fixation of alanine, through methylene groups, and the non-involvement of most of the amino groups of the protein was demonstrated analytically.

6. Coupling.

Two samples of 40 mg of crystalline crotoxin, dissolved in 1 ml water containing 2 drops of 3.4 M phosphate buffer (pH 7.6), were treated with 0.9 ml or 0.3 ml of a fresh 1% solution of diazobenzene sulfonic acid in 0.34 M phosphate (pH 7.6) for thirty to forty minutes at room temperature. Both samples were dialysed and lyophilized. The samples that had been treated with 12 equivalents (per 10^4 g) were dark red, the one treated with 4 equivalents bright yellow. They contained 4.4 and 1.6 newly introduced acid groups, respectively.

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Treatment of proteins with excessive amounts of diazo compounds causes coupling with imidazole, phenolic, and possibly other groups. When insulin was treated with limited amounts (up to 4 equivalents per 10^4 g) of such compounds, active derivatives were obtained[18]. Surprisingly, both phenolic and imidazole groups were found affected even when only small amounts of the reagent were added to insulin or lysozyme[13, 14].

Methods of analysis and assay

Amino nitrogen analyses were generally performed by the colorimetric ninhydrin method of HARDING AND MACLEAN[19], which necessitates only a few mg of material. Occasionally VAN SLYKE's manometric method was used[20]. Methoxyl groups were determined by the ZEISEL method as modified by CLARK[21]. Formaldehyde was determined with chromotropic acid after distillation from acid solution[15]. Acid and basic groups were determined by a dye method[22]. The FOLIN[7] and THOMAS[23] methods were used for tyrosine plus tryptophane and tyrosine analyses, respectively. Tryptophane was determined by the method of HORN AND JONES[24]; sulfate-S according to MEASE[25]; amide-nitrogen as previously described[16]. Acetyl groups were determined by vacuum distillation after acid hydrolysis in sealed tubes[26].

The analytical results obtained in the course of the present study with crotoxin and its derivatives are summarized on Table I.

Toxicity assays were performed by subcutaneous injection in mice weighing 18–25 g. Groups of 3–8 mice were used at different dose levels. Death usually occurred withing eighteen hours at a clearly defined threshold dosage.

DISCUSSION OF RESULTS

The results of the toxicity assays of crotoxin and its derivatives have been summarized on Table II. It is evident that modification of the molecule by any of the reagents used causes extensive detoxication.

The inactivity of the acetyl derivative in which an appreciable proportion of the amino groups has been acetylated is good evidence that these groups are essential for the toxic activity. The fact that no reactivation resulted from treatment with alkali under conditions which would cause hydrolysis of any phenol-acetate linkages supports this conclusion.

The methylation experiments, particularly the inactivation under the mildest reaction conditions and the stability of the toxin in the control solutions, indicate the essentiality of most of the carboxyl groups. Attempts to regenerate the activity by a reversal of the esterifying reaction by mild alkaline hydrolysis, however, were unsuccessful. The same had been found with the pituitary lactogenic hormone[27], in contrast to insulin which can be at least partially reactivated after esterification by controlled alkaline hydrolysis[9].

The inactivity of the sulfated derivative points to the essentiality of the aliphatic hydroxyl groups, although either nonspecific denaturation or the great change in the isoelectric point and in the net charge of the protein may well be responsible for its detoxification. The sulfation of a small fraction of the phenolic groups probably is not responsible for the detoxication, since the iodination experiments under gentle conditions suggest that the most reactive phenolic group is not essential. On the other hand, intensive iodination caused marked inactivation, which, for lack of evidence to the contrary, is regarded as indicating the essentiality of the bulk of the phenolic groups*.

Formaldehyde in alkaline solution combines rapidly with all the indole, most of the amide and many guanidyl groups, and the complete detoxication of crotoxin under such conditions might be attributed to any or all of these reactions. By analogy with gramicidin which is detoxified under similar conditions by combination of its numerous

* See footnote on p. 99.

TABLE II

TOXICITY OF CROTOXIN DERIVATIVES

No.	Nature of Derivative	Approximate LD^{50}	Death Rate at Various Dosages		Activity Recovered
		γ/mouse	γ	Percent	Percent
	Untreated	8	10	100	100
			8	43	
			6.5	0	
1	Alkali (control to #6, 15)	18	20	77	45
			15	33	
2	Alkali (control to #7, 11)	< 10	10	100	>80
3	Methanol, then acid (control to #8)	8	10	100	100
			6.7	0	
4	Acetylated, partly	10	10	50	80
			5	0	
5	Acetylated, intensively	>200	200	0	< 4
6	As #5, then 10 min. 25° C., p_H 12	> 56	56	0	<14
7	As #5, then 24 hrs. 0° C., p_H 10	>120	120	0	< 7
8	Esterified, partly (0.1 N HCl, —5° C.)	35	50	75	24
			25	0	
9	Esterified, partly (0.025 N HCl, 4 hrs.)	90	100	67	9
			50	0	
10	Esterified, intensively	>160	160	0	< 5
11	As #10, then 24 hrs. 0° C., p_H 10	> 80	80	0	<10
12	Sulfated	>200	200	0	< 4
13	Iodinated, partly	13	15	100	62
			10	0	
14	Iodinated, intensively	> 54	54	0	<15
15	Formaldehyde, p_H 12 (10 min.)	>500	500	0	< 2
16	Formaldehyde, p_H 7.3 (60 min.)	35	54	100	23
			20	0	
17	Formaldehyde plus alanine, p_H 7 (90 min.)	>100	100	0	< 8
18	Formaldehyde plus alanine, p_H 4.2 (24 hrs.)*	>400	400	0	< 2
19	Coupled, partly (4 equiv.)	>100	100	0	< 8
20	Precipitated with Orange G	15	19	100	53
			12.5	20	

* Without alanine, crotoxin was rendered insoluble by formaldehyde at p_H 4.2.

indole groups with formaldehyde[15], one may favour the same reaction as primarily responsible for the observed detoxication. The stable fixation of formaldehyde by the indole groups may also play a rôle in the commercial preparation of the anatoxin (toxoid) if the prolonged incubation with formaldehyde is performed in slightly alkaline solution.

The rapid inactivation by formaldehyde at p_H 7.6 may be ascribed to its tendency to produce crosslinks between amino and amide or guanidyl groups. The inactivation by formaldehyde and alanine in neutral or acid solution is probably due to the fixation of $-CH_2-NH-CH(CH_3)-COOH$ groups on amide or guanidyl groups, but recent experiments with gramicidin[17] suggest that the imino groups of the indole rings may also participate in methylene condensation with amines under such conditions.

The marked inactivation of crotoxin treated with only 4 equivalents of diazotized sulfanilic acid suggests the essentiality of the most reactive phenolic and imidazole groups.

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Slight loss in activity was observed with crotoxin which had been precipitated by salt-formation of all its basic groups with the divalent dye, Orange G. This was probably due to incipient denaturation during the twenty four hour period of shaking at room temperature.

The marked sensitivity of crotoxin to all reagents used might be regarded as evidence that either the protein must be particularly unstable, or the reaction conditions too severe for a biologically active protein. The control experiments listed on Table II show that crotoxin is quite stable in acid solution, and only moderately unstable in alkali. The preparation of crotoxin involves a step during which inert proteins are heat-coagulated at 70° C. and p_H 4. Thus the protein appears in no way exceptionally susceptible to denaturation. Concerning the reaction conditions employed, it can only be stated that each reaction that has been used has yielded fully active derivatives with one or several bioactive proteins (*e.g.*, insulin[13], trypsin inhibitors and trypsin[26]). Thus it appears that the observed marked detoxications of crotoxin are actually indicative of the essentiality of a great variety of protein groups.

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SUMMARY

Acetylation of most of the amino groups, or esterification of the carboxyl groups of crotoxin causes extensive detoxication.

Sulfation of the aliphatic hydroxyl groups, or iodination of most of the phenolic groups, or coupling of these and imidazole groups of the rattlesnake neurotoxin also causes inactivation.

Reaction with formaldehyde, particularly in alkaline solution, or in the presence of alanine in neutral or acid solution, causes complete inactivation. This is regarded as evidence for the essentiality of any or all of the following groups: indole, guanidyl and amide.

RÉSUMÉ

La crotoxine perd sa toxicité par acétylation de la plupart de ses groupes amino ou par estérification de ses groupes carboxyl.

Cette neurotoxine du serpent à sonnettes (crotale) est également désactivée par sulfonation de ses groupements hydroxyl aliphatiques, par ioduration de la plupart de ses groupes phénoliques et par copulation avec les diazoïques de ses groupes phénoliques et imidazoliques.

De plus la toxine est complètement désactivée par réaction avec la formaldéhyde, surtout en solution alcaline ou, en présence d'alanine, en milieu neutre ou acide. Ceci semble prouver que l'un ou tous les groupes suivants: indol, guanidyl et amido sont essentiels pour d'action toxique de la crotoxine.

ZUSAMMENFASSUNG

Das Crotoxin wird durch Acetylierung seiner Aminogruppen oder durch Veresterung seiner Carboxylgruppen weitgehend entgiftet.

Dieses Neurotoxin der Klapperschlange wird durch Verestern seiner aliphatischen Hydroxylgruppen mit Schwefelsäure, durch Jodieren der meisten seiner Phenolgruppen oder durch Kuppeln der Phenol- und Imidazolgruppen mit Diazoverbindungen inaktiviert.

Durch Einwirkung von Formaldehyd, besonders in alkalischer Lösung,oder in Gegenwart von Alanin in neutraler oder saurer Lösung tritt vollständige Entgiftung ein.

Dies wird als Beweis dafür angesehen, dass die Indol-, Guanidyl- und Amidogruppe entweder einzeln oder zusammen für die Giftigkeit des Crotoxins notwendig sind.

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