THE PROPERTIES OF SULFITE-TREATED TETANUS TOXIN

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1. Introduction

In the earlier work which examined the action of sulfite on tetanus toxin in buffers without denaturants [1–3] the results have been inconclusive. In the presence of urea and sodium dodecyl sulfate (SDS), sulfite and reductants have been shown to cleave rapidly the toxin prepared from culture filtrates into chains of approx. mol. wt of 95 000 and 55 000 daltons, but the toxin prepated from cell extracts resisted this change [4].

This work studies the prolonged action of sulfite on tetanus toxin in sodium phosphate buffer at pH 7.5 in the presence of 50 μM $CuSO_4$ and similarly in the absence of Cu^{2+} and protected from catalyst metals with EDTA (sodium salt) and glycine. It was found that in the presence of Cu^{2+}, sulfite brought about considerable scission of both cell extract and culture filtrate toxins and it is estimated that at least 3 sites other than disulfide bridges are involved. The treatment also reduced the toxicity approx. 1000-fold. Rather unexpectedly the humoral responses to the cleaved toxins were comparable to those of the complete toxins. The use of EDTA and glycine prevented cleavage of both cell extract and culture filtrate toxins by sulfite and preserved their biological properties.

The work indicates that disulfide cleavage of proteins by sulfite requires at least traces of heavy metal ions and that in these conditions the action is not necessarily restricted to sulphenyl-sulfite formation.

2. Materials and methods

The cell extract and culture filtrate toxins were prepared and checked for homogeneity as described earlier [4]. The toxins (5 mg/ml) were dialysed for 10 days at 6°C with fourfold changes against solutions in excess containing either (a) 30 mM Na_2SO_3, 50 μM $CuSO_4$, 70 mM sodium phosphate, pH 7.5 or (b) 30 mM Na_2SO_3, 10 mM EDTA, 50 mM glycine, 70 mM sodium phosphate, pH 7.5 or (c) 10 mM EDTA, 50 mM glycine, 70 mM sodium phosphate, pH 7.5.

For preparation of toxoids, the treated toxins were prepared at a concentration of 0.06 mg protein/ml in 100 mM sodium phosphate buffer, pH 7.0 and detoxified by making 35 mM with respect to formaldehyde and holding for 2 days at 20°C and then 3 days at 37°C. The toxoids were evaluated first in mice, by determining the effective dose [6] against a challenge of 50 MLD of culture filtrate toxin and using a dose range of 4, 2, 1, 0.5 Lf units of toxoid in groups of 10 mice; secondly in guinea pigs, by determining the neutralization value (I.U./ml) in the pooled sera of 9 animals. These animals received doses of 5 and 10 Lf units at a 3 week interval and the sera was collected 2 weeks after the second dose. Electrophoresis was carried out in SDS buffers according to the method of Weber and Osborn [5] treating the samples with urea, mercaptoethanol as appropriate. Flocculation values, (Lf) by optimal proportions; minimum lethal dose, (MLD); neutralization values (I.U.) were obtained by standard procedures.

3. Results

Table 1 gives the results of the biological tests. The original MLD values of the toxins were maintained after treatment for 10 days in phosphate buffer containing EDTA and glycine. However there was at least a 100-fold drop in toxicity after treatment with sulfite

Table 1

	Cell extract toxin			Culture filtrate toxin		
	Treatment					
	Sulfite Cu^{2+}	Sulfite EDTA glycine	EDTA glycine	Sulfite Cu^{2+}	Sulfite EDTA glycine	EDTA glycine
Flocculation value Lf units/mg protein	400	340	330	350	340	340
Flocculation time minutes	90	30	26	150	32	28
Minimum lethal doses/mg protein	2.5×10^3	1.0×10^7	1.7×10^7	1.7×10^3	1.0×10^7	1.0×10^7
Effective dose of corresponding toxoid (Lf units)	5.7	2.8	2.4	5.0	4.0	2.1
Sera neutralization values produced by corresponding toxoid (International units) per ml.	24	24	12	28	14	12

in the presence of copper. When toxins were protected from trace metals by EDTA, sulfite did not substantially alter their toxicities. The Lf values of all treated toxins were similar but the flocculation times of the toxins treated with sulfite and Cu^{2+} were greatly lengthened. The effective dose values, (which are approximately the Lf dose of toxoid required to protect 50% of the animals) and the neutralization values indicate that no sharp change in the immunizing properties of the toxoids was introduced by the sulfite treatments. Differences in responses were shown but these would be complicated by the secondary effects of the formaldehyde binding and animal species response.

The results obtained in the SDS acrylamide gels are shown in fig.1. Both the cell extract and the culture filtrate toxins showed considerable cleavage when treated with sulfite and Cu^{2+} (disc 1 and 2.) Further treatment of the cleavage products with urea, SDS and mercaptoethanol did not alter the acrylamide gel patterns. Although the proportions of the cleavage products of the cell extract and culture-filtrate toxins are obviously different, electrophoresis of their mixture indicated similar products were present. Increase in the time of sulfite and Cu^{2+} treatment from 10 to 21 days did not alter the result. The results shown in disc 1 and 2 are compared with those obtained when the toxins are treated with urea, SDS and mercaptoethanol [4]; in which case only the culture filtrate toxin is substantially cleaved into 2 products (disc 3 and 4). When the toxins were held in the presence of EDTA and glycine or with sulfite in addition to these practically no cleavage occurred and only traces of smaller molecular weight products are shown (discs 5–8). In discs (5–8) traces of the slower-moving dimer of tetanus toxin remain.

4. Discussion

In earlier studies the action of sulfite on culture filtrate toxin in phosphate buffers gave only a partial cleavage [1] and when Cu^{2+} was introduced the products aggregated and could not be satisfactorily resolved on chromatographic gels [2]. In this study electrophoresis in SDS gels has been used to resolve the cleavage products. It shows that prolonged exposure of cell extract and culture filtrate toxin to sulfite and Cu^{2+} produces chain cleavage by a mechanism other than that of disulfide scission. In addition the culture fil-

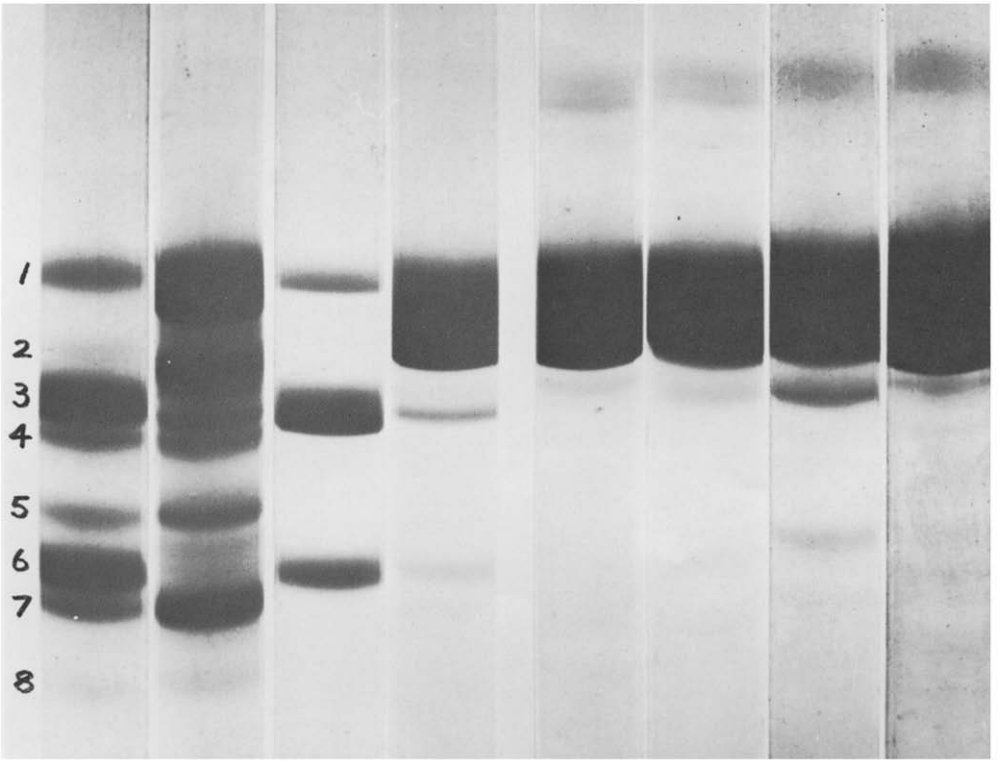

Fig. 1. Disc polyacrylamide gel electrophoresis (5% gels) in 0.1% SDS. Discs 1 and 2: culture filtrate and cell extract toxin after treatment with sulfite and Cu^{2+}. Discs 3 and 4: culture filtrate and cell extract toxin after treatment in SDS, 8 M urea and 1% mercaptoethanol. Discs 5 and 6: culture filtrate and cell extract toxin after treatment with EDTA and glycine. Discs 7 and 8: culture filtrate and cell extract toxin after treatment with EDTA, glycine and sulfite.

trate toxin gave products corresponding to disulfide cleavage of its two chains. Although Swan [7] showed that Cu^{2+} accelerated sulfitolysis, the absolute need of a catalytic ion does not appear to have been reported. In the case of culture filtrate toxin sulfitolysis does not take place when protected by EDTA or glycine.

An explanation based on the relative mobility and density of acrylamide bands indicates that cleavage has occurred at at least three sites other than those of the disulfide bands. It is considered that band 1 in the gels is the single chain protein, typified by the cell extract toxin. Bands 3 and 6 represent the large and small chains given by disulfide cleavage and which are typical of the culture filtrate toxin. Bands 2 and 7 characterize the cell extract toxin and represent cleavage by sulfite and Cu^{2+} in that part of the toxin molecule corresponding to the small chain of the culture filtrate toxin. Band 7 occurs in the gels of the culture filtrate toxin by reason of a similar scission in the small chain. Bands 4 and 5 are considered to be cleavage products of the toxins in that part of the toxin molecule corresponding to the large chain and are therefore found in the gels from both cell extract and culture filtrate toxins. Band 8 may be a fragment residual to other cleavage. Diffusible fragments would be lost during the prolonged dialysis.

It is of interest that even though a 1000-fold loss in toxicity in the culture filtrate and cell extract toxins is evident and considerable cleavage occurs in sulfitolysis with Cu^{2+}, no sharp change in antigenicity is found. The differences that do occur are considered to be due at least in part to the degree of formaldehyde binding; in which the minimum consistent with detoxification yields the better antigen [1]. The degree of formal-

dehyde treatment used in preparation of the toxoids was the minimum required for them to be non toxic in guinea pigs but in excess of that required for detoxification when used in the mouse. It is generally considered [8] that the three dimensional structure is necessary to maintain the antigenicity of native globular proteins and it can be shown that SDS destroys the protective antigens of tetanus toxin. A possible explanation in this case is that the aggregation that occurs after sulfite and Cu^{2+} treatment preserves its antigenic structure and this is stabilised by formaldehyde cross-linking.

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