CHANGES IN THE ELECTROPHORETIC PROPERTIES OF THE TOXIN OF *Clostridium oedematiens* UNDER THE INFLUENCE OF FORMALIN

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The production of toxoids from bacterial exotoxins by means of the action of formalin and heat presupposes the removal of the toxocity of the preparations while fully preserving their antigenic properties. In several cases, however, the antigenicity of the resulting toxoids is inadequate. It has been shown [1] that the loss of antigenic properties is to a large extent dependent on the conditions of detoxication. Meanwhile the mechanism of action of formalin, which is a fundamental factor in the conversion of toxin into toxoid, has received insufficient study. The investigation of this problem is of great importance from the point of view of the production of high-quality preparations for active immunization of human subjects.

In the present research we studied the changes in the electrophoretic mobility of the toxin of *Clostridium oedematiens* in the process of its detoxication with formalin. These changes were compared with the changes in the electrophoretic properties of the toxin of *Cl. perfringens* in the process of toxoid formation, which we have described previously [3].

EXPERIMENTAL METHOD

We studied the toxins of *Cl. oedematiens* strain No. 794 grown on media based on acid casein hydrolyzate. Optimal accululation of toxin in the medium was observed on the 3rd-4th day. The crude toxins thus obtained contained 6000-10,000 MLD/ml and 30-50 units/ml and were suitable for immediate electrophoretic investigation without preliminary concentration. A single dose of formalin was added to give a concentration of 0.4%. The process of detoxication was conducted at 37° and lasted 2-3 days. As detoxication proceeded samples of toxin were withdrawn at known intervals of time, and subjected to electrophoretic fractionation in a starch block [2]. In each sample we determined the protein content by Lowry's method [6], and the biological activity of the toxin and toxoid by experiments on white mice. The intervals between taking the samples and the beginning of detoxication were 1, 2, 3 and 4 days. The electrophoretic fractionation of the toxins of *Cl. oedematiens* in the course of detoxication was carried out in conditions similar to those previously described [3]. The results of electrophoretic fractionation were shown as electrophoregram graphs. The graphs were provided with a scale of electrophoretic mobility previously drawn up from the results of experiments carried out in a Tiselius apparatus [3].

Control determinations of the amino nitrogen in the initial toxin and also in the preparation during detoxication with formalin were made by Tsuverkalov's method.

EXPERIMENTAL RESULTS

In Fig. 1 we show the result of one typical experiment of electrophoretic fractionation of the toxin of *Cl. oedematiens* in a starch block.

* Deceased.

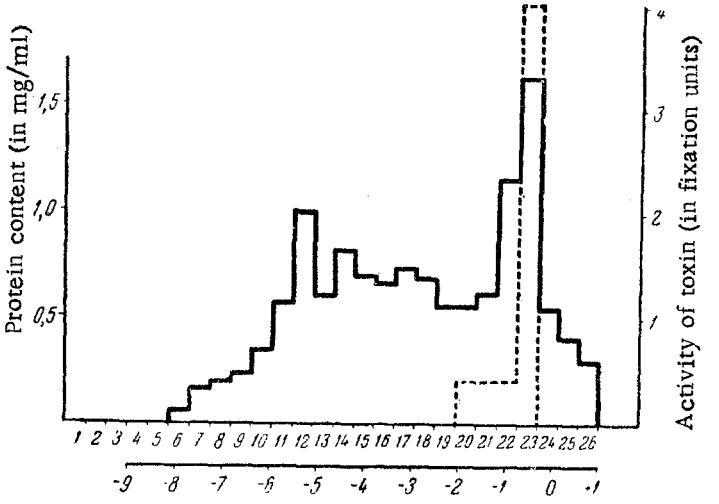

Fig. 1. Electrophoretic fractionation of the toxin of Cl. oedematiens. ——— protein content (in mg/ml); – – – – activity of toxin in fixation units; 1-9 corresponds to the point of application of the toxin. Along the axis of abscissas are plotted the positions on the starch block in cm, and the scale of electrophoretic mobility in a boric acid-borate buffer (pH 8.6; electrical conductivity 0.0035 $ohm^{-1}cm^{-1}$).

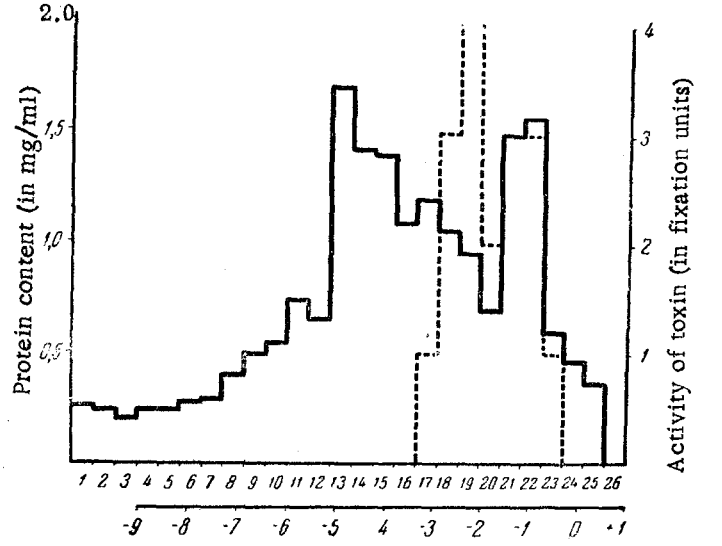

Fig. 2. Electrophoretic properties of the toxin of Cl. oedematiens 24 hours after the addition of formalin. ——— protein content (in mg/ml); – – – – activity of toxin in fixation units.

It will be seen from Fig. 1 that the greater part of the activity of the Cl. oedematiens toxin was included in 23 fractions from the block counting from the anode, with an electrophoretic mobility of 0.3 to $-0.731 \cdot 10^{-5} cm^2 sec^{-1} v^{-1}$. The mobility and negative charge of the Cl. oedematiens toxin were a little lower than those of phospholipase C, the main component of the toxin of Cl. perfringens, which had the value 1.2 to $-1.6 \cdot 10^{-5} cm^2 sec^{-1} v^{-1}$ [3]. Fractions 22, 21 and 20 also possessed slight toxicity and had a mobility similar to that of the phospholipase C of Cl. perfringens toxin. The attempt to find this phospholipase in the 22nd, 21st and 20th fractions by means of its enzymic activity was unsuccessful, for this activity was extremely small.

Figure 2 shows the activity of the toxin of Cl. oedematiens 24 hours after the addition of formalin. It is clear that under the influence of formalin an appreciable change takes place in the electrophoretic properties

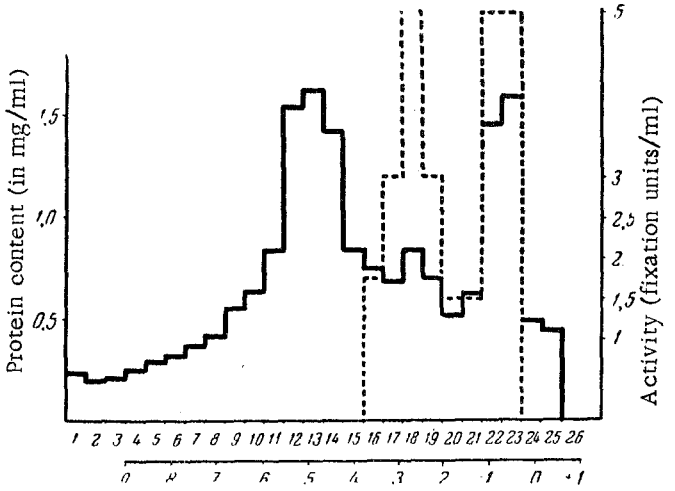

Fig. 3. Change in the electrophoretic mobility of the toxin of Cl. oedematiens 48 hours after the addition of formalin. ——— protein content (in mg/ml); – – – – activity of toxin in fixation units.

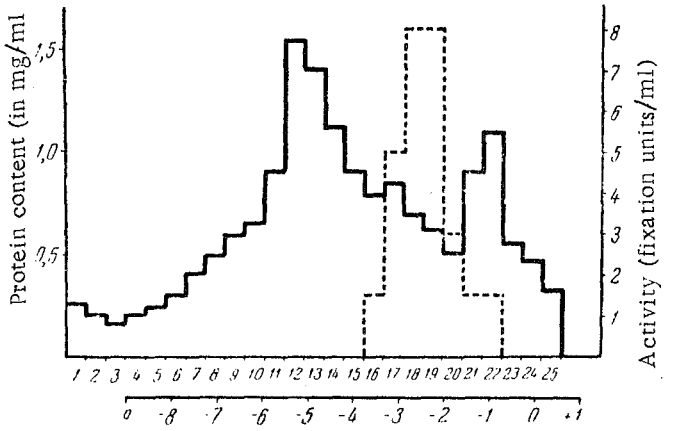

Fig. 4. Electrophoretic fractionation of Cl. oedematiens toxoid. ——— protein content (in mg/ml); – – – – activity of toxin in fixation units.

of the Cl. oedematiens toxin, as shown by a widening of the zone of activity to give a range of electrophoretic mobilities from 0.6 to $-3.34 \cdot 10^{-5}$ $cm^2 sec^{-1} v^{-1}$. In contrast to the concentrated Cl. perfringens toxin, detoxication of which continued for 60 days, the conversion of Cl. oedematiens toxin into toxoid took place much more quickly. Only 24 hours after the addition of formalin the greater part of the toxin was converted into toxoid with a maximum activity in fractions 22, 21, 20, 19 and 18 and with slight activity in fractions 17 and 23. After detoxication for 48 hours a certain redistribution of activity took place along the length of the starch block. The maximum of toxoid activity fell in fractions 22, 23 and 18, and also in fractions 20, 21 and 16, forming two peaks of activity on the curve. The active fraction extended over a range of values of electrophoretic mobility from 0.6 to $-3.77 \cdot 10^{-5}$ $cm^2 sec^{-1} v^{-1}$. At the end of the second day of detoxication practically the whole of the toxin was converted into toxoid. After nine days of contact with formalin the toxin which was obtained possessed electrophoretic properties which differed qualitatively from those of the original toxin. Instead of a mobility of $-0.6 \cdot 10^{-5}$ $cm^2 sec^{-1} v^{-1}$, characterizing the lethality factor of the toxin, the main activity of the toxin was concentrated in the fractions with a mobility of $-2 \cdot 10^{-5}$ $cm^2 sec^{-1} v^{-1}$ and $-2.9 \cdot 10^{-5}$ $cm^2 sec^{-1} v^{-1}$, i.e., the negative charge of the system of proteins forming the toxin complex was increased fourfold.

The action of formalin on the protein molecule is known to proceed in several directions and not to be confined to blocking amino groups [4]. Nevertheless, in connection with the considerable increase in the negative charge and, consequently, in the acid properties of the toxin in the process of detoxication, it was of great interest to study the changes in the amino nitrogen content of the toxin in the process of detoxication. In the table we give figures showing changes in the content of amino nitrogen during the gradual change of Cl. oedematiens toxin into toxoid. Detoxication of the toxin was complete at the end of the second day.

Change in the Amino Nitrogen Content in the Process of Detoxication of Cl. oedematiens Toxin

Name of preparation	Amino nitrogen content (in mg%)		
	before addition of formalin	after addition of formalin	
		24 hours	48 hours
Crude toxin I	309	275	261.0
" " II	280	204	207.0
Active fraction I	19	12	16.6
" " II	12	12	10.0
" " III	20	20	15.0

The figures in the table show that the amino nitrogen content in the original toxin and in its active fraction, separated by electrophoretic fractionation on starch at different intervals after the addition of formalin, was roughly equal, and could not be used as a criterion to account for the change in the charge of the protein and its acquisition of acid properties in the course of toxoid formation.

We concluded from the experimental findings described above that during detoxication with formalin the toxin of Cl. oedematiens, like that of Cl. perfringens which we studied previously, when undergoing conversion into toxoid gradually changed its electrophoretic properties. The toxoid possessed a much greater negative charge and was electrophoretically heterogeneous. The increase in the negative charge was not associated with any decrease in the content of amino nitrogen in either the original toxin or in its active fraction. The amino nitrogen content of the latter was very low throughout the entire period of detoxication. The development of electrophoretic heterogeneity may be due to a change in the spatial configuration of the protein molecule of the toxin under the influence of formalin. Pillemer [7], for instance, cites evidence in support of the view that tetanus toxoid is the dimer of toxin molecules, condensed through their toxic groups. In Van Heyningen's view [5], the idea that the conversion of toxin into toxoid may involve condensation, which may be catalyzed by various reagents, is most likely. This question may be answered after the further study of the mechanism of detoxication by means of modern chemical and physico-chemical methods.

SUMMARY

In detoxication of Cl. oedematiens toxin with formalin there occur distinct changes in the electrophoretic mobility with a considerable rise of its acid properties, i.e., of the negative charge of the preparation. The mobility of the toxoid, a qualitatively new protein, is about 4 times greater than that of the original toxin. During the process of toxoid formation no interrelationship was noted between the change of the protein charge and the amino nitrogen content.

LITERATURE CITED

1. P. M. Dreitser, Zhur. Mikrobiol., Epidemiol. i Immunobiol. 14, 6, 845 (1935).
2. M. V. Isopolatovskaya, et al., Zhur. Mikrobiol., Epidemiol. i Immunobiol. 1, 54 (1959).
3. M. V. Ispolatovskaya and I. A. Larina, Biokhimiya 4, 738 (1959).
4. D. French, J. T. Edsall, in: Advances in Protein Chemistry (New York, 1945) 2, 277.
5. W. E. Heyningen, Bacterial Toxins (Oxford, 1950).
6. O. H. Lowry, et al., J. biol. Chem. 193, 265 (1951).
7. L. Pillemer, et al., J. Immunol. 58, 223 (1948).