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Recent reports of poisoning following ingestion of fish products has enhanced interest in the problem of Clostridium botulinum in foodstuffs.

Hitherto the search for inhibitors of the growth of Clostridium botulinum and thereby of its production of toxin has been concerned mainly with type A of the bacteria. It was therefore thought worth while studying a variety of chemicals and preservatives for their inhibitory effect on the production of toxin by Clostridium botulinum, type E.

Materials and Methods

Organism. Clostridium botulinum, type E, strain 1537, obtained from Dr. A. Johannsen, Lund. The organism was isolated from blood obtained from a patient before death from botulism.

Culturing methods. The organism was cultured under anaerobic conditions in Beef Heart Infusion Medium prepared according to Recommended Methods for the Microbiological Examination of Foods, 1958. The pH was adjusted to 7.2. The bacteria were cultured for 10 days at 30°C, after which the spores were centrifuged and washed three times with 0.85% NaCl. The spores were then dried over night at 60° in test tubes which were afterwards sealed by melting.

A suspension of spores in a concentration of $10^6 - 10^7$/ml. was prepared by addition of 0.85% NaCl. In the investigation of the effect of various chemicals on growth and the production of toxin, the bacteria were cultured in 50 ml bottles with screw caps.

The bottles contained 25 ml of Beef Heart Infusion Medium in addition to the chemicals to be tested. Every bottle was inoculated with 1 ml of spore suspension, and then incubated under anaerobic conditions at 30°C for 4 days. The production of toxin was compared with that in the controls, i.e. broth cultures without addition of inhibitors, and any difference found was taken as a measure of the effect of the chemical added to the suspension.

Inhibitors. The following substances were studied for any inhibitory effect on the production of toxins: NaCl, $NaNO_2$, hexamethylene-tetramine, tylosin, oxytetracycline and the amino acids D-serine and DL-serine.

Toxin Assay. The strength of the toxin was estimated from the effect of intraperitoneal injection of 0.5 ml of the cell-free supernatant into white mice weighing 16 - 18 g. Most of the mice that received more than a tenfold LD_{50} showed the first symptoms of botulism within 2 - 4 hours. Rarely did an animal die without overt symptoms within 6 hours of the injection. Each test was usually carried out on 6 mice, which were observed for 24 hours, i.e. the period within which mice will usually succumb to LD_{50} of toxin, type E. The LD_{50} had been estimated beforehand. The interval between inoculation and death of the animals could therefore be used as a measure of the strength of toxin in the cultures.

Results

I. Standard curve for correlation between the strength of the toxin and survival period

Doses of 0.5 ml of 1:10, 1:100, 1:1000, 1:10,000 dilutions of toxin, type E, were injected intraperitoneally into mice and the survival times were noted. The values were then used as a basis for the standard curve given in Fig. 1.

II. Effect of chemical inhibitors on amount of toxin

NaCl. The effect of incorporation of NaCl in the culture medium on the production of toxin in Cl. botulinum, type E, is apparent from Fig. 2. The production of toxin was reduced by 2.8% NaCl and completely inhibited by 3.4%.

$NaNO_2$. Fig. 3 shows that 0.017% $NaNO_2$ was sufficient markedly to reduce the production of toxin which was completely inhibited by 0.034%.

Tylosin. Addition to 10 µg tylosin per ml considerably supposed the production of toxin, which was completely inhibited by 50 µg/ml (Fig. 4).

Oxytetracycline. In a concentration of 0.5 µg/ml this agent completely inhibited the production of toxin.

Peroxide of hydrogen. This agent reduced the production of toxin but did not inhibit it completely.

Hexamethylene tetramine. In concentrations between 0.025 - 0.1% this

preservative produced no demonstrable inhibitory effect, but in a concentration of 0.2% it completely inhibited the production of toxin.

DL-serine and D-serine. In concentrations up to 50 µg/ml these substances had only a very weak inhibitory effect.

Discussion

When assaying chemical substances for their effect on the growth of Clostridium botulinum and its production of toxin, nutrient medium should be such as to give rapid germination within the period the test agent is active. The absence of demonstrable growth does not necessarily mean that the spores have been killed by the chemical used.

The absence of toxin may be ascribable to one or more of the following causes. The chemical substance added may have killed the spores. Since usually up to 90% of the clostridium spores germinate within 1 - 24 hours, it is likely that the spores that have germinated in this period are killed. Another possibility is that the production of toxin in the vegetative cells is inhibited, for example, by inactivation of enzymes in the cell metabolism, or the chemical added may directly inactivate the toxin already produced by the cells.

The inhibitors studied for their effect on the production of toxin by Cl. botulinum, type E, were selected because of their present or possible use in the food industry and secondly, because of the literature about their effect on the species of Clostridium. All strains were cultured and assayed at pH 7.2. This pH is slightly higher than that of food products. Both NaCl (Ingram, 1958) and $NaNO_2$ (Tarr, 1942) are more bactericidal at lower pH. The effect of preservatives in such tests on the inhibition of Cl. botulinum would therefore probably be stronger at lower pH despite an apparent, but not true, increase of the production of toxin owing to increased autolysis of the cells at lower pH.

NaCl is a common preservative. Opinions differ on the effect of NaCl on the growth and the production of toxin by Cl. botulinum. Moreever different species of Cl. botulinum differ in their tolerance to salt. Most investigations have been carried out with type A. Thom el al. (1919) reported formation of toxin in cooked meat medium containing 5% NaCl and Dozier (1924) demonstrated production of toxin in medium containing 6 and 7%. Type E is less tolerant to NaCl and, according

to Pedersen (1957), 4% NaCl inhibits the germination of spores of type E. Salt tolerance varies somewhat with the nutrient medium. Tanner & Evans (1933) found that several strains of type A did not produce toxin in nutrient broth containing 8% NaCl. In pork infusion medium, 10.5% NaCl was required to prevent the production of toxin. According to Halvorson (1955), NaCl, KCl and $MgCl_2$ partly inhibited the germination of the spores of type A at a salt concentration of 4 - 9%. Neither Na_2SO_4 nor $MgSO_4$ in concentrations up to 15% had any effect on germination. The mechanism by which NaCl inhibits spore germination, growth and toxin production is obscure. Fig. 2 shows that 2.8% NaCl was necessary to produce any demonstrable reduction in the toxin formation and that 3.4% NaCl completely inhibited it. At the beginning of the present investigation nothing was known of the effect of $NaNO_2$ on the toxin formation by type E. Fig. 3 shows that 0.017% $NaNO_2$ considerably reduced toxin formation, which was completely inhibited by 0.034%. Tarr (1942) found that 0.02% $NaNO_2$ inhibited germination of Cl. botulinum, type A, at pH 6.0. No attempt was made to explain the mechanism of inhibition. Quastrel & Wooldridge (1927) suggested that the nitrite inactivates some enzymes, owing to the nitrite being bound to the amino groups. Other data available in the literature about the effectiveness of $NaNO_2$ in Cl. botulinum are inconsistent. Tanner & Evans (1934) reported that concentrations between 0.05 - 0.4% did not prevent the reproduction, while Yesair & Cameron (1942) maintained that 0.005% is sufficient to reduce the number of germinated spores in meat infusion agar by 70%. The effect of H_2O_2 on toxin production was much less than that expected from its general bactericidal effect described in the literature (Fig. 5). Wheater (1953) showed that 0.03% H_2O_2 is sufficient to stop gas production by Cl. sporogenes but for complete inhibition of growth 0.05 - 0.06% is required. The bactericidal effect of penicillium B, according to van Bruggen, is due to the presence of H_2O_2 formed by enzymatic reactions. According to McCulloch (1936), H_2O_2 prevents growth in anaerobes, but does not kill the spores.

Benzoic acid and hexamethylene tetramine were excluded in the present investigation because they do not affect toxin formation by type E (Molin, 1964).

Tylosin and oxytetracycline were found to inhibit toxin production by Cl. botulinum. The tests show that oxytetracycline is active in

a concentration 0.5 µg/ml. This is in agreement with the finding of Sakaguchi et al. (1960), who reported inhibition of the toxin production at 0.2 ppm OTC. OTC is believed to inhibit germination. According to Sakaguchi, the antibacterial effect of OTC can be entranced considerably by addition of NaCl (1% or more). Tylosin differs from most of the antibiotics by its relatively high degree of heat stability. Fig. 4 shows that toxin formation was considerably reduced by this substance in a concentration of 10 µg/ml and completely inhibited by 50 µg/ml. Here we may be dealing with a direct inhibition of toxin synthesis, since Greenberg et al. (1962) showed that germination of spores of Cl. botulinum was not inhibited by tylosin in the concentrations 10 and 100 µg/ml. Tylosin has no effect on existing toxin.

DL-serine and D-serine were tested because these amino acids reduce the formation of tetanus toxin (Mueller, 1949). They did not have any noteworthy effect on the toxin formation by Cl. botulinum.

Oxytetracycline proved to be the strongest inhibitor of the toxin formation by Cl. botulinum, type E, and was active in the concentration 0.5 µg/ml, followed by tylosin 50 µg/ml, sodium nitrite 0.034% and NaCl 3.4% in the orden given. These inhibition effects are at pH 7.2.

Additional studies on the effect of chemicals, especially disinfecting media, on Cl. botulinum, type E are being planned.

Summary

A number of chemicals in different concentrations were studied for their inhibitory effect on the toxinproduction by Cl. botulinum type E.

Beef Heart Infusion medium containing the chemical to be tested was inoculated with the spore suspension and incubated anaerobically at 30°C for 4 days after which the toxin production was estimated.

The following chemicals completly inhibited toxin production: Sodium chloride (3.4%), Sodium nitrite (0.034%), tylosin (50 µg/ml) oxytetracycline (0.5 µg/ml) and hexamethylentetramine (0.2%). The aminoacids DL-serine and D-serine (concentrations up to 100 µg/ml) had no greater effect on the toxin production. Hydrogen peroxide reduced the toxin production, but at a concentration of 0,04% the toxin titre was still at a level of 100 LD_{50}.

Göteborg den 3 januari 1964

2127 E Abn/Bkg

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Fig. 1 Survivaltime for mice after injection of dilutions of toxin by Cl. botulinum, type E.

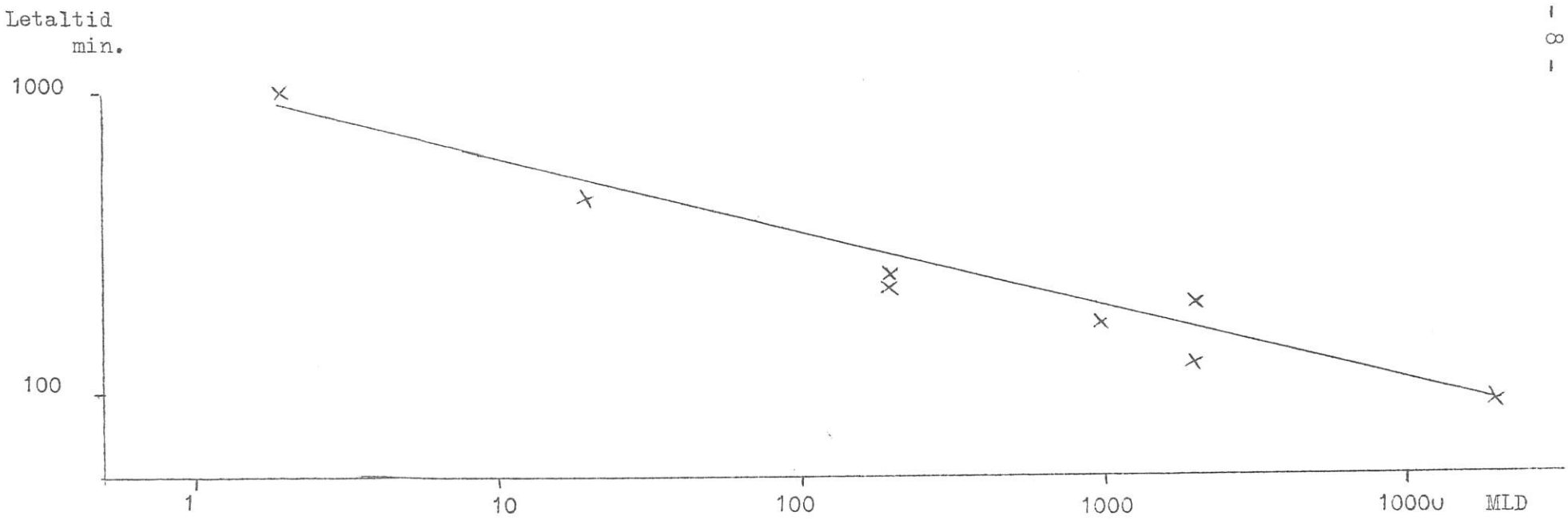
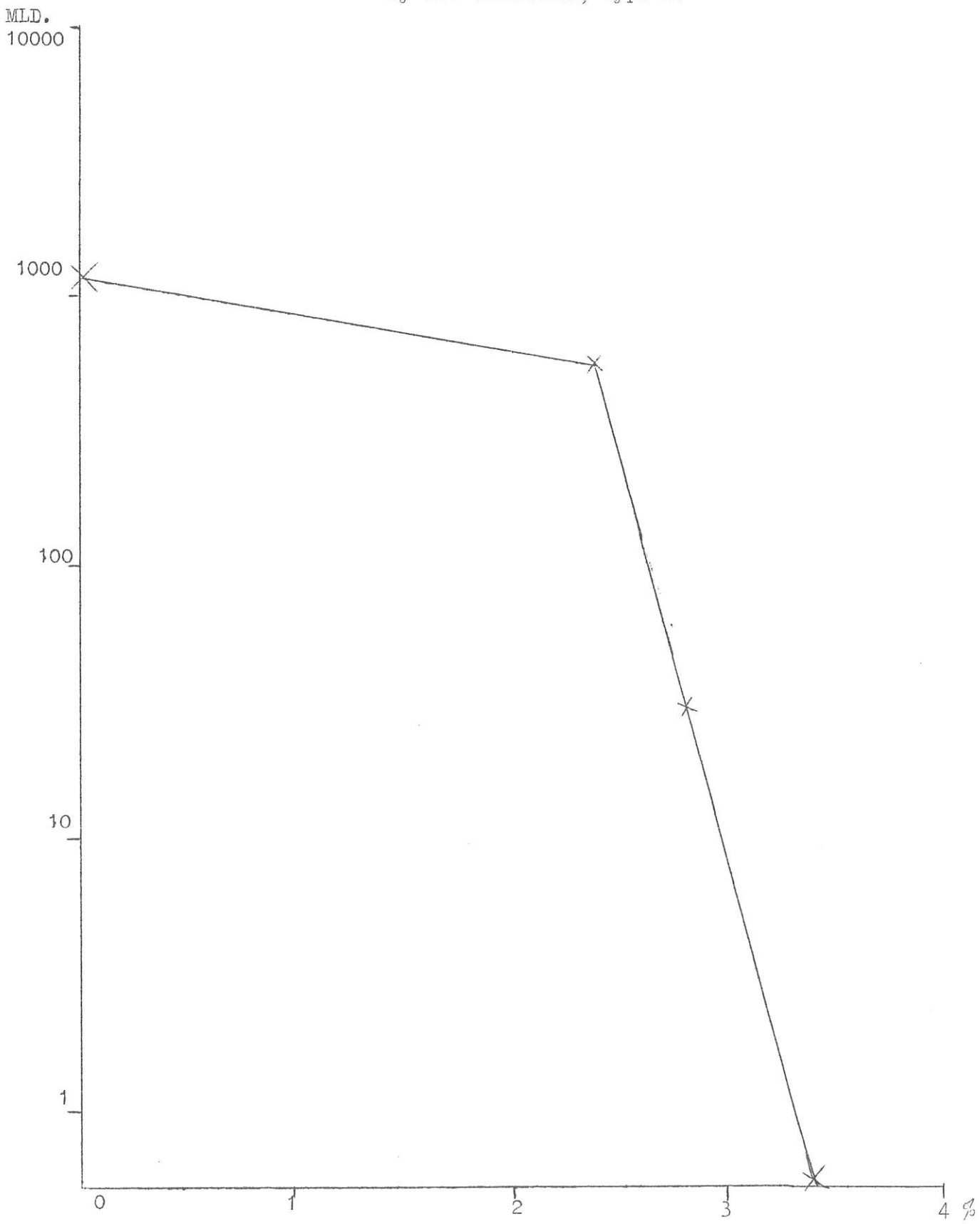


Fig. 2 Effect of NaCl on the toxinformation by Cl. botulinum, type E.



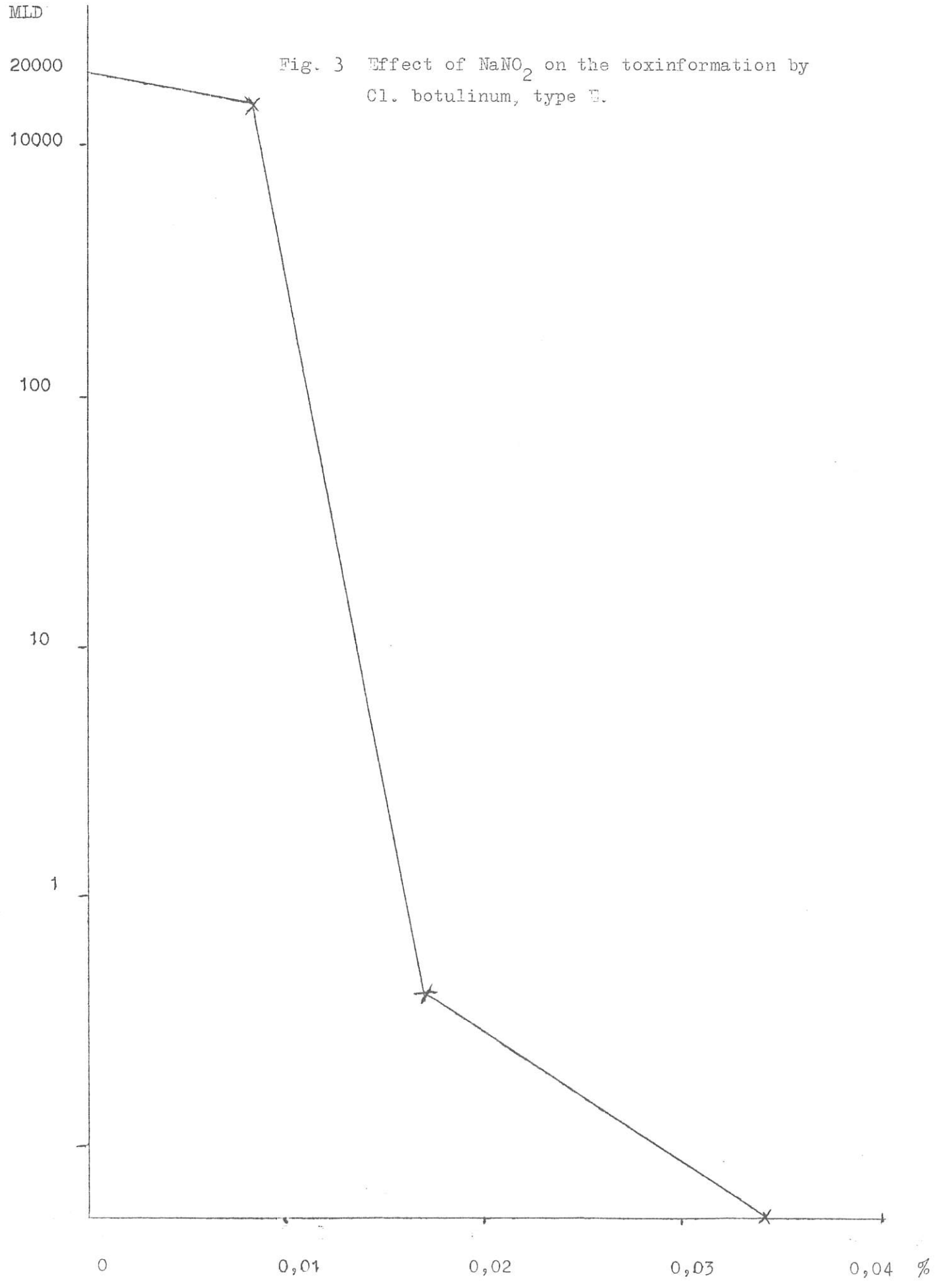


Fig. 3 Effect of $NaNO_2$ on the toxinformation by Cl. botulinum, type E.

Fig. 4 Effect of some chemical inhibitors on toxinformation by Cl. botulinum, type E.

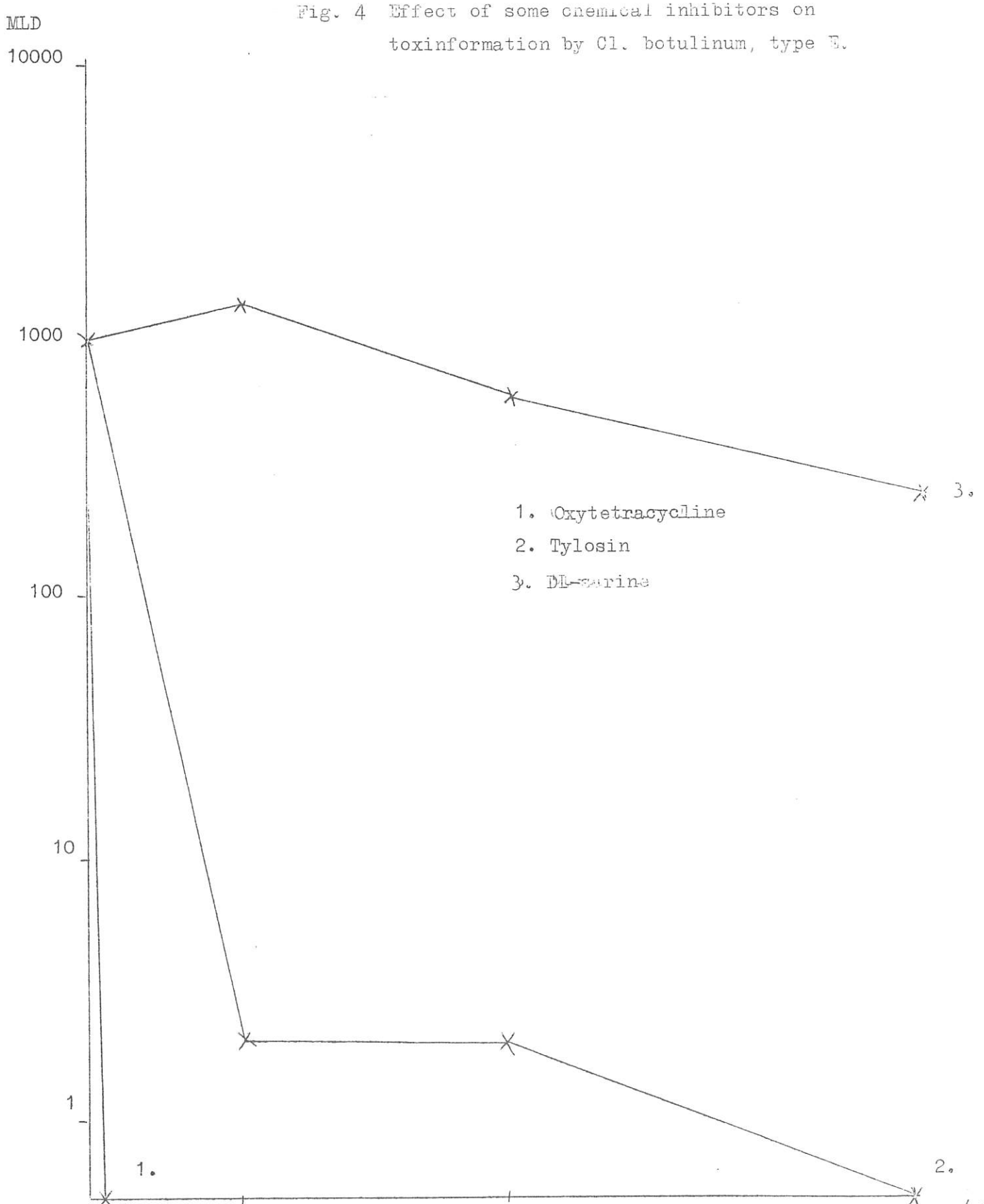


Fig. 5 Influence of hydrogen peroxide on toxinformation by Cl. botulinum, type D.