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THE FORMATION OF CL. BOTULINUM TOXIN TYPE C IN SYMBIOTIC CULTURE

by

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THE FORMATION OF CL. BOTULINUM TOXIN TYPE C IN SYMBIOTIC CULTURE

In 1956, at the time of an outbreak of botulism in mink, the type D strain of *Cl. botulinum* was isolated for the first time in the USSR (Matvyeyev et al, 1957). Subsequent research established that the toxin produced by these microbes is related to the sub-type C_β (Buletova and Matyyeyev, 1965).

Study of the first strain of the type C botulism agent, isolated in the USSR, was of interest from the point of view of further use of it for producing sera and anatoxins. At the present time, anatoxins and sera are produced from the C_α strain No. 91, obtained in 1931 from the USA, which in pure culture was weakly toxigenic. Only under conditions of symbiosis with a non-toxigenic microbe of another type, did the toxigenic properties of this agent of botulism strongly increase.

We have observed an analogous phenomenon of high toxigenicity only in symbiotic culture, in the type C strains of the botulism agent, which we have isolated from mink. However, many attempts to obtain a pure culture of these strains were unsuccessful. Microscopically, in the reseeding of these cultures, two forms of Gram-positive bacteria were always present: thin ones with rounded ends, sometimes containing spores in the shape of rockets, and larger, thick ones with a large oval spore on the end.

In 2% sucrose-blood agar and in a high column of 1% agar (Hottinger broth was taken as the base for these media), colonies of various forms grew after 2 days. In the high column there were three types of colonies: flocculant particles (usually on the very bottom of the test tube), dense particles and lens-shaped colonies with protuberances of various sizes. In the blood agar (in a vacuum) the rounded, branching colonies grew in great quantity with a hardly noticeable clearing around them, and single soft colonies with uneven borders, surrounded

by a sharp zone of hemolysis Upon seeding from the lens-shaped colonies and the dense particles from the high column and from the rounded colonies from the blood agar in Hottinger's broth, rounded gram-positive, anaerobic, spore-forming non-toxic bacilli grew, which liquified gellatin and did not decompose sucrose.

The microbes, sifted out from the high column out of the particles and out of the fine colonies with hemolysis from the blood agar, were reminescent in morphology to the botulism agent: gram-positive bacilli of average dimersions whose spores are in the form of rockets. During the first reseeding in Hottinger broth with small pieces of meat under liquid petrolatum, they formed weak (up to 20 Dlm in 1 ml) botulistic toxin, which was neutralized by a specific type C serum (serum to the strain C No. 91). Upon repeated reseeding in the same medium were casein-fungoid, weak growth was observed without toxin-formation or growth was absent. Pepsin-peptone,broth, Marten or Gluzman broth proved to be unsuitable for toxin-formations by the type C strains.

One must point out, that successful isolation of pure strains from fine colonies is extremely rare; mixed cultures were more frequently obtained, which produced as strong a toxin as the original mixed strain.

Pure strains of type C botulism agents are best grown in media with 0.05% thioglycolate and 0.01% cysteine; however, upon subsequent reseeding the toxigenic properties were lost.

Thus, division of the natural symbiosis of these microbes led to the result that the type C strain lost the ability to grow and form toxin. Mixed cultures upon repeated reseedings in the same medium continue good growth over a period of more than 9 years and produce of a strength of 20,000 to 50,000 Dlm in 1 ml (strains No. 10, 12 37, 47, 49).

Many investigators (Quortrup and Gorham, 1949; Appleton and White, 1959; McKee et al., 1958, etc.) noticed that the type C botulism agents lose their toxigenic property shortly after isolation. In the work of Quortrup and Sudheimer (1943) it was shown that only in symbiosis with *Pseudomonas aeruginosa* did they form strong specific toxin. We also noticed this in work with the type C strain No. 91.

There was great interest in clarifying whether the highly toxic Mink strain, obtained from GDP, was also symbiotic. This strain in the media which we used (Hottinger broth, casein-fungoid medium) constantly produced toxin of a strength of 50,000 Dlm in 1 ml for a period of more than 6 years.

In studying microscopic preparations, stained according to Gram, we did not succeed in revealing a difference in the dimensions of the bacilli of this strain, and assumed that it represents a mixture of two forms of microbes. Later, in research in the phase-contrast microscope, it was established that the strains of *Cl. botulinum* No. 91, Mink, No. 37 and 47 represent a mixture of 2 bacilli of different dimensions (cf. figures in inset).

Upon seeding in a high cylinder of agar, we succeeded in isolating microbe-symbionts, which upon repeated reseeding grew well in nutritive media, but their filtrates always remained non-toxic even when grown in a vacuum. These were the round Gram-positive spore-forming anaerobic bacilli One of them dissolved gelatin and clotted serum (symbionts of the strains No. 37 and 47); others did not dissolve (symbionts No. 91, Mink); not one of them decomposed sugars.

With the help of the reaction of indirect hemagglutination, it was shown that the microbe-symbiont antigens related to *Cl. botulinum*, and also *Cl. sporogenes* and *Cl. putrificum*.

The question arises, which form these bacilli-symbionts which differ in antigenic relations and in other properties, found in the mixture with strains *Cl. botulinum* No. 91, Mink, No. 37 and 47, aided growth in toxin-formation of these microbes.

From the work of many investigators it is known that the type C botulism agents are strict anaerobes. Upon seeding in the high column of agar, toxigenic bacilli of this type of botulism agent usually grow in the form of soft particles at the bottom of the test tube, where, as was demonstrated by the research of a series of authors (Aubel and Aubertin, 1927; Prevot, 1938), most favorable anaerobic conditions are created. We assume that the bacilli-symbionts most probably, in liquid media, sharply lower the oxidation-reduction potential (rH_2), and this facilitates rapid growth, propagation, and toxin-formation.

The principle according to which anaerobes do not transfer the high oxidation-reduction potential of the surrounding medium was explained by Engel'gardt (1944). He showed that several vitally important enzymes are inactivated at high rH_2. Moreover, the anaerobic organism loses the ability for normal feeding, for the most important constructive processes and dies from hunger.

For the purpose of studying the mechanism the influence of associated mocrobes on the growth of the type C botulism agents, we carried out experiments in the measurement of pH, Eh and calculation of rH_2 during the growth of microbe-symbionts in casein-fungoid medium. Of the 14 experiments carried out, 10 were with microbe-symbionts, isolated from the No. 91 strains (symbionts No. 6, 7, 8, 9), 37 (symbionts No. 19, 20, 23), 47 (symbionts No. 38, 39), Mink (symbiont No. 30); in 4 control experiments we grew *Cl. sporogenes* and *Cl. putrificum* in the same media.

Pure strains of type C botulism agents No. 91, 37, 47 could not be used as controls, since they did not grow in the media which we used. The experiments were set up as follows: after sterilization in the medium (in a 2-liter bottle) 0.5% glucose was added and the cotton plug was replaced by a sterile rubber stopper, into which were fastened two platinum electrodes for measuring the oxidation-reduction potential, a tube, filled with agar and KCl solution for combination with a calmel electrode, and a tube for seeding and sampling culture fluid. Thirty minutes after immersing the electrodes in the medium (to a depth of 10 cm) the potential was determined (emf in millivolts), the pH of the nutritive medium and seeding of the culture was carried out; after seeding the bottle was mixed in a thermostat of 35° and after a specific time (2, 4, 6 and 24 hours of growth) the potential is measured by the electrometric method; for this, platinum electrodes, immersed in the culture, with an electrolytic bridge combined with the standard saturated calmel electrode and switched into the circuit of a lamp potentiometer LP-5. By the compensation method, the pH was determined analagously, and the value of the potential calculated (in millivolts). Simultaneously with the measurements of the potential, a sample of the culture was selected for determination of pH (on the same potentiometer). According to the amount of the potential and the pH, the quantity rH_2 was calculated according to the nomogram proposed by Usov and Pogodãyev (1956).

Changes of Eh, pH and rH_2 During Growth of Microbe-Symbionts on Casein-Fungoid Medium

Time of Growth in Hours	Dynamics of Changes During Culturing															
	Microbe-Symbionts								Control Strains							
	№ 19 (From Strain No. 35)				№ 7 (From Strain No. 5ff)				Cl. sporogenes № 276				Cl. putrificum № 1051			
	pH	EMF (in mv)	Eh	rH_2	pH	EMF (in mv)	Eh	rH_2	pH	EMF (in mv)	Eh	rH_2	pH	EMF (in mv)	Eh	rH_2
Original Medium	7,4	—180	+70	18,0	7,5	—160	+90	18,8	7,4	—230	20	16,0	7,4	—230	+20	16,0
2	7,3	—500	—280	7,0	7,2	—250	0	15,0	7,3	—230	20	15,8	7,2	—230	+20	15,6
4	7,0	—530	—280	4,0	7,0	—360	—110	11,0	7,2	—230	15	15,6	7,2	—210	+10	15,5
6	6,9	—530	—280	3,8	6,8	—530	—280	4,0	7,0	—235	15	15,0	7,1	—210	+10	15,0
24	6,8	—530	—280	3,6	6,2	—530	—280	3,0	6,9	—530	—280	7,0	7,0	—520	—270	7,0

The research indicated (cf. table) that the majority of strains of symbionts possessed the ability to reduce the rH_2 of the medium rapidly. In the period of the first six hours, the quantity rH_2 fell from 18 to 21.5 to 5.5 to 9, at the same time as [illegible] produced such a reduction only after 24 hours. In symbiotic culture of toxigenic strains of the C type, changes in pH, Eh and rH_2 were also detected, as well as in media in which only non-toxic strains of symbionts were grown.

We are not inclined to consider that the mechanism of the favorable effect of microbe-symbionts on growth and toxin-formation of the type C botulism agent leads only to a sharp reduction of oxidation-reduction potential of the medium. This question must be studied in greater detail.

We did not succeed during joint growth of strain-symbionts and other weakly toxigenic strains of type C botulism agents (No. 36, 91--the original variants, obtained from the State control institute) and having lost the toxigenic properties (No. 185, 1861) two strains in their toxin-formation. This suggests that each type C strain may form a toxin only in a mixture with its symbiont, with which it was isolated from the organism or from the external medium.

Scientists have long been interested in the question of the influence of various microbes on the growth and toxin-formation of botulism agents. In a series of reports, antagonism, symbiosis, destruction of toxin under the influence of accompanying microbes, were noted. At the present time the mechanism of the influence of accompanying flora on toxin-formation of type E botulism agents has been relatively well studied (Sakaguchi and Tohyama, 1955). The work was connected with the study of the influence of accompanying microbes on the type D microbe (Prevot et al., 1962).

In our opinion, the study of symbiotic strains is of great interest from the point of view of obtaining highly toxigenic strains for the objective produced. At the present time one may assume with high probability, that the strains of type C botulism agents produced, which are used not only in the Soviet Union (No. 91) but also abroad (No. 468, 573), are symbiotic, since over a period of many years (as indicated by data of the literature) they remain highly toxigenic. The C strain No. 468, isolated by Prevot (1950), constantly produces toxin of a strength of 1 million to 500 thousand DLm for mice (Prevot et al., 1953; Appleton and White, 1959).

The question of maintaining toxigenic properties and the reasons for their rapid loss (with type C strains of botulism agents, and also type D), the study of the mechanism of enhancement of their toxigenic properties by accompanying microbe-symbionts is of great theoretical and practical interest and therefore requires further comprehensive study.

Conclusions

1. The type C botulism agent, isolated from mink in the USSR, is a symbiotic strain (mixture of bacilli of two types) and maintains its toxigenic properties only in a mixture with the strain-symbiont.

2. The microbe symbionts, isolated from various strains of type C botulism agents, possess the ability to reduce sharply the oxidation-reduction potential of the medium in the first 6 hours of cultivation, which facilitates growth and toxin-formation of these microbes.

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The type C botulism agent, isolated from mink in the USSR, is a symbiotic strain (a mixture of bacilli of two types) and retains its toxigenic properties only in a mixture with the strain-symbiont. Microbe-symbionts, isolated from various strains of the type C botulism agent, possess the ability to reduce sharply the oxidationreduction potential of the medium in the first six hours of cultivation. This facilitates the growth and toxin-formation of these microbes.

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