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by

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13. ABSTRACT

Three strains of Cl. histolyticum were grown on various nutritive media, and a-toxin and collagenase were extracted, purified, and concentrated from culture filtrates. The a-toxin was present at levels of 500-1,000 Dlm/ml, along with 0.5-2 units of collagenase (as indicated by viscometry). Data are presented on the dynamics of a a-toxin and collagenase formation on the suggested nutritive media.

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PRODUCTION OF A LETHAL TOXIN AND Cl. HISTOLYTICUM COLLAGENASE ON CASEIN NUTRITIVE MEDIA

Cl. histolyticum is one of the inducers of gaseous gangrene and, although the disease in encountered in a small percent of cases, one must nevertheless consider its influence during construction of imnunogenic associated preparations for the prophylaxis of gaseous gangrene.

Interest in this microbe has not been exhausted. It is one of the few microorganisms which produces the enzyme collagenase. Moreover, it is one of the most active producers of collagenase, which is the only enzyme which cleaves native collagen.

In recent years there have been a great number of reports, connected with extraction of collagenase, its purification and concentration, and also its application to the study of the structure of collagen. There have also bee reports on the application of collagenase with a medical purpose--for the resolution of keloid projections, treatment of burns, etc. The literature on these questions is already so broad, that we have allowed ourselves to cite only the available reviews of Kunina and Shpikiter (1960) and Mandl (1961).

In agreement with the data of Oakley and Warrak (1950), a number of antigenic components are observed in the filtrate of *Cl. histolyticum* cultures, which they designated α, β, and γ; of these, α is a lethal toxin, which appears to also have the necrotic effect of β-collagenase and γ-cystein-activating protease. Antibodies towards all three components were detected in the serum of immunized animals. The roles of each of these components in infection and immunity has not at this time been determined, since they have not been obtained in pure form.

Alcmeier (1952) has shown that immunization with anacollagenase (of which the method of extraction has not been specified) insures better protection from infection than immunization with anatoxin.

From all that has been stated above, it is clear that it is of theoretical and practical interest to obtain filtrates of *Cl. histolyticum*, containing maximal quantities of the α, β, and γ components. The aim of the present work was also the development of a recipe for nutritive medium and conditions of cultivation of *Cl. histolyticum*, in order to obtain maximally active filtrates, containing α-toxin and collagenase, which would later serve as the starting material for extracting purified and concentrated toxins and anatoxins and collagenase.

Four strains of *Cl. histolyticum*--No. 5, 247, H_4 and 230-2--were taken for the work. The strains were kept in nutritive medium from Poup broth with small pieces of fresh meat, 0.1% agar, and 0.4% gelatine under liquid petrolatum.

The toxin was titrated by the generally accepted method in mice by intravenous injection.

The activity of the collagenase was determined by a viscometric method.[1] The activity was marked under conditions of unit-cotangent angles of a straight slope, obtained by means of division of the difference in quantities of the logarithms of the specific viscosity by the difference in the corresponding time intervals in minutes (Kazakova et al., 1958).

Acid, pancreatic hydrolysates of casein and a hydrolysate, obtained with the help of *Aspergillus terricola* protease (Vinogradova, et al., 1958) served as a basis for obtaining various nutritive media. To this hydrolysate were added gross factors--various concentrations of corn extract, yeast water, vitamin B complex (Vinogradova, et al., 1963).

Preliminary data indicated that the best results in relation to production both of α-toxins, and of collagenase, were obtained in nutritive media based on the acid hydrolysate of casein. Precisely these media were used for the preparation of large quantities of filtrates for the purpose indicated above. Inoculations were carried out in a bottle with cotton in a 2.5 *l* of nutritive medium and remained in a thermostat at 37°.

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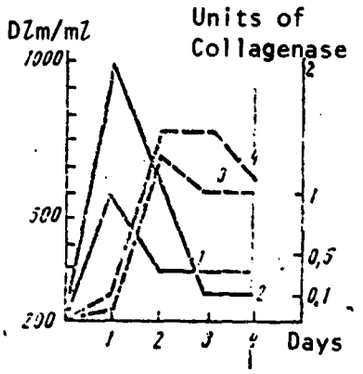
[1]The determination of collagenase activity was carried out by colleagues at the Institute of Biological and Medical Chemistry by G. A. Levdikov and N. I. Solov'ev, and also a colleague of the Department of Wound Infections of the Gamalei Institute of Epidemiology and Microbiology, G. F. Shemanov.

In the first experiments, samples were taken from the bottles 24, 48, 72, and 96 hours after innoculation, with the purpose of determining the time of maximal accumulation of α-toxins and collagenase.



Accumulation of α-Toxin (1, 2) and Collagenase (3, 4) in Two Experiments.

The maximal accumulation of α-toxins was observed 24 hours, and of collagenase--48 hours after innoculation, while the accumulation of these components occurred independently of each other (cf. Figure). In order to obtain anatoxins, 0.4% formalin was added to the filtrate of the 24-hour culture, and placed in a thermostat at 37° to render it harmless.

In order to obtain purified collagenase, the filtrate of the 48-hour culture was taken. In 1962 we had taken the additional strains of *Cl. histolyticum*, H_4 and 230-2. According to the data of MacLennan et al. (1953) and Mandl and Zaffuto (1960), these strains are the best producers of collagenase. Moreover, addition of 5% peptone to the casein nutritive medium seems to be foolproof for the production of collagenase. These data are confirmed in our preliminary experiments.

In our application of these media without addition of peptone, the growth of the microbes was weak, as was the production of α-toxin and collagenase. Upon addition of 3 to 5% peptone to the medium, good results were observed in relation both to production of α-toxin, and collagenase, while the strain *Cl. histolyticum* H_4 gave the worst results in comparison with the strain 230-2, and therefore the latter was taken for further work.

As can be seen from the preliminary data in the table, in our use of casein nutritive media toxin was obtained with a strength of 500 to 1,000 Dlm/ml, i.e. according to activity, they did not yield the toxins, which were obtained by domestic and foreign authors in meat nutritive media (Zyelyevinskaya, 1943; Stewart, 1936; Walbum and Raymann, 1938; Bowen, 1952; Raynand et al., 1954; Wildfuhr, 1950, etc.).

Unfortunately, we cannot compare our data on the activity of collagenase with the data of other authors, because the determination of collagenase activity was carried by different methods. However, the possibility of obtaining sufficient quantities of partially purified collagenase from these filtrates (Vlasova and Solov'eva, 1962) already speaks about collagenase activity obtained from filtrates.

Extraction of Toxin and Collagenase During Cultivation of *Cl. Histolyticum* in Casein Nutritive Media

Nutritive Medium	Strain	Number of Inoculations With Various Strains of Toxin (in DZm)			Number of Innoculations with Various Collagenase Activities		
		200—500	500—1000	1000—2000	0.25—0.5	0.5—1	1—2
Acid Hydrolysate of Casein and 3% Corn Extract	S	3	9	1	Not Determined		
Acid Hydrolysate of Casein and 5% Corn Extract		10	14	8	1	5	14
Acid Hydrolysate of Casein and 0.3-0.5% Vitamin B (Complex)	247	—	3	—	—	—	3
Acid Hydrolysate of Casein, 5% Yeast Water and 3-5% Peptone		—	3	7	—	3	4
Acid Hydrolysate of Casein, 5% Yeast Water and 3-5% Peptone	230-2		6	4		3	4
Acid Hydrolysate of Casein, 0.5% Vitamin B (Complex) and 3% Peptone			5				5

Conclusions

1. The nutritive media and conditions of culturing of *Cl. histolyticum* which we used, permit one to obtain filtrates of the culture, which are active in relation both to α-toxin, and collagenase.

2. The filtrates which we obtain served as the starting raw material for obtaining purified and concentrated anatoxin of *Cl. histolyticum* for active immunization, and also as the starting raw material for obtaining purified collagenase.

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