THE EXOCELLULAR BACTERIOLYTIC SYSTEM OF SOIL ACTINOMYCES

I. THE NATURE AND PROPERTIES OF THE LYTIC SYSTEM

by

A. S. JONES, A. J. SWALLOW AND M. WEBB

Chemistry Department, University of Birmingham (England)

Since the early observations of LIESKA (1921) that specific *Actinomycetes* were able to lyse many living and dead bacterial cells, numerous biological investigations of the phenomenon have been made. These have been reviewed in considerable detail by WAKSMAN (1945). Extensive studies by WELSCH (1941, 1942) showed that killed gram negative cells were readily dissolved by sterile filtrates termed "Actinomycetin", of broth cultures of his *Actinomyces* strain G obtained after sporulation. Killed gram positive cells, though susceptible to some extent, were more resistant. A few strains of streptococci and pneumococci were found to be susceptible to sterile actinomyces culture filtrates even when alive. Other living gram positives, though resistant to sterile actinomycetin, were dissolved after 2–3 days when the aqueous cell suspension was inoculated with suitable actinomyces and some actinomycetin. Living gram negative bacteria were found to be very resistant to both actinomycetin and *Actinomyces* sp. Concentration of the lytic agent was achieved by precipitation at 0.75 saturation of ammonium sulphate. Such preparations though highly active when tested on killed cells, did not clarify suspensions of living cells.

Ether extracts of actinomycetin exhibited a marked bactericidal action on gram positive bacteria suspended in an inorganic medium. This action was greatly reduced in ordinary complex culture media. The active agent appeared to be a lipoid, probably a fatty acid, and it was considered that part of it originated from the actinomyces and part from the culture medium since similar extracts were obtained from the sterile media. It was concluded that bacteriolysis of living bacteria by *Actinomyces* occurred in two stages. First, the susceptible cells were killed by the selective bactericidal lipoid and, second, these dead cells were then dissolved by the lytic agent, which alone was responsible for the lysis of heat-killed bacteria.

The lytic agent was considered to be an enzyme which required some crystalline co-enzyme (GORYUNOVA 1944), but the nature of the enzyme was not established.

In the present study, the bacteriolytic system of a soil *Actinomyces* sp. which lyses living gram positive and killed gram negative bacteria, has been shown to consist of a bactericidal substance together with a proteolytic system, which alone is responsible for the lysis of killed gram negative cells. The enzyme system is active over a wide p_H range with optimum activity at p_H 7.0–7.5 and is inhibited by reducing agents such as hydrogen sulphide and thioglycollic acid. Preliminary results indicate that this enzyme system is composed of at least two proteolytic enzymes.

References p. 182.

EXPERIMENTAL

Source of Organisms. The soil actinomycetes were obtained from Dr DAGNY OXFORD of Rothampsted Experimental Station, on whose suggestion this investigation was instigated. Of these, (A) was an *Actinomyces* sp. known to lyse suspensions of killed, gram negative and living gram positive eubacteria incorporated in agar plates, but which had no apparent action on living gram negative, or killed gram positive cells. Filtrates of broth cultures of the organism were known to lyse killed gram negative cells. Culture (h) was a true *Actinomyces*, maintained for over one year in the vegetative phase and which, if frequently subcultured in a medium containing protein, only rarely developed spores. Culture (5 f) was originally similar to (h), but was kept actively sporulating, whilst (All 17) was a *Micromonospora* sp. Cultures (19) and (T 2) were *Proactinomyces* of the "soft" and "dry" types respectively.

Choice of Media for the Production of the Actinomyces Lytic Enzyme System

50 ml of each medium recorded in Table I were distributed in 150 ml Erlenmeyer flasks, sterilised for 15 min at 18 lbs/sq. inch and inoculated with the stock *Actinomyces* (*A*), maintained on nutrient agar slopes.

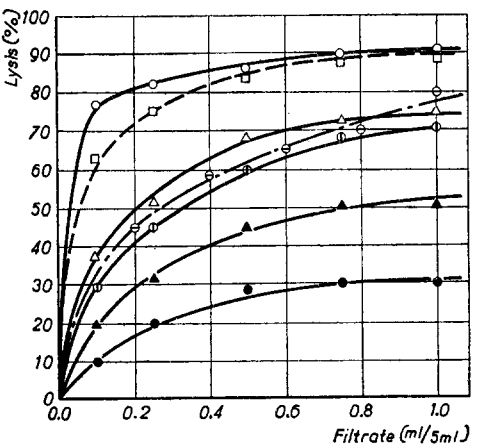

Fig. 1. Lytic activity of *Actinomyces* culture filtrates against heat-killed *Bact. lactis aerogenes* at pH 7.0

○——○ Lemco-peptone-glucose broth
□-----□ Lemco-peptone broth
△——△ EVANS peptone glucose broth
⌀——⌀ CZAPEK medium
o-----o Mannitol synthetic soil
▲——▲ Glycerol-asparagine medium
●——● STANIER's medium

TABLE I

MEDIA FOR THE PRODUCTION OF THE *Actinomyces* LYTIC ENZYME SYSTEM

Synthetic media

1. STANIER's (1942) inorganic medium (pH 7.0–7.3)
2. Mannitol synthetic soil
3. Glycerol-asparagine (CONN and CONN 1941)
4. CZAPEK-sucrose-nitrate

Complex media

5. EVANS' peptone (2 %)-glucose broth
6. LEMCO (1 %)-peptone (1 %) glucose broth
7. LEMCO (1 %)-peptone (1 %)

After 15 and 35 days at 18°, cultures in each medium were filtered and the filtrates examined for lytic activity against a substrate of heat-killed, gram negative cells (*Bact. lactis aerogenes*) at p_H 7.0 according to the method previously described (JONES, STACEY, AND WEBB 1948). The suspensions of the cells and enzyme were incubated at 37° in corked tubes with toluene (0.1 ml). After 48 hours, the corks were removed, the toluene allowed to evaporate, and the degree of lysis determined after a further 18 hours by comparison with a standard opacity scale. The lytic activities of the 35 day culture filtrates against the gram negative cells are shown in Fig. 1. The 15 day culture filtrates possessed similar properties, though the enzyme concentration, particularly in each of the synthetic media, was considerably less. The results (Fig. 1) revealed that the media most favourable for the production of the lytic enzyme system

References p. 182.

were complex and contained proteins and peptones. Such media also supported a more vigorous growth of the *Actinomyces* sp. Of the synthetic media, mannitol synthetic-soil was most favourable for the production of the enzyme system and for the growth of the organism. This medium was, in general, employed for the production of the enzyme system for chemical studies.

Lytic Activity of other Actinomycetes

The non-sporulating (h) and sporulating (5 f) *Actinomyces*, *Micromonospora* (All 17) and *Proactinomyces* (19 and T 2) were inoculated into flasks of mannitol synthetic soil medium (50 ml). In these cultures (h) grew mainly throughout the medium with the production of a yellow pigment. However, possibly owing to the absence of protein from the culture medium, some surface growth, with the production of spores, occurred. The sporulating form (5 f) grew entirely at the surface of the medium with the formation of a light brown pigment. The remaining strains gave rise to submerged growth and produced little or no pigment.

The cultures were filtered after 50 days at 18° and the filtrates examined for lytic activity against heat-killed *Bact. lactis aerogenes* at p_H 7.0 according to the standard procedure. Filtrates of cultures of the *Proactinomyces* (19 and T 2) were without lytic activity against the killed gram negative cells while that of the *Micromonospora* sp. (All 17) was without activity in low concentrations. The sporulating *Actinomyces* (5 f) culture filtrate had considerably greater activity than the filtrate of the non-sporulating form (h) (Fig. 2).

As it has been reported (GORYUNOVA 1944) that the lytic enzyme does not appear in broth cultures until the actinomyces have reached the sporulating stage, the following experiments, in which spore formation was respectively stimulated and reduced, were devised.

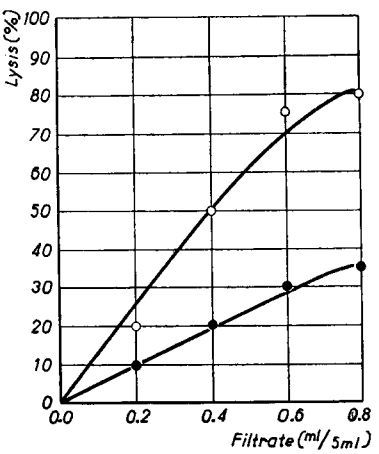

Fig. 2. Lytic activity of non-sporulating (●——●) and sporulating (○——○) *Actinomyces* culture filtrates against heat-killed *Bact. lactis aerogenes* at p_H 7.0

Lytic Enzyme Production in Actinomyces cultures with Restricted Air Supply

700 ml of mannitol synthetic soil medium were placed in each of two 750 ml Erlenmeyer flasks, sterilised and inoculated immediately after cooling with *Actinomyces* (*A*). One culture flask was covered with a rubber cap which was then sealed with paraffin wax. The culture with the restricted air supply (A 22) was free from pigment, whereas the second culture (A 23) produced a light brown pigment which diffused into the medium. After 30 days at 18°, the cultures were filtered and increasing concentrations of the filtrates examined for lytic activity against killed *Bact. lactis aerogenes* at p_H 7.0. Solution (A 22) exhibited no action against the gram negative cells, whereas solution A 23 (0.5 ml) lysed a suspension of the killed cells (5 ml), of opacity corresponding to No. 10 on MACFARLAND'S standard barium sulphate opacity scale, to the extent of 50 %.

Lytic Enzyme Production in Aerated Actinomyces Cultures

Actinomyces (*A*) was inoculated into each of two 3 l bolt-head flasks containing man-

References p. 182.

nitol synthetic-soil medium (1.5 l). One of the cultures was continuously aerated with sterile air to prevent a stationary surface and spore formation. The second culture was allowed to stand undisturbed, when surface growth with the production of spores took place. After 28 days at room temperature the cultures were centrifuged and the supernatants decanted. Determination of the lytic activity of these solutions against killed *Bact. lactis aerogenes* showed that the filtrate from the aerated culture contained the greater enzyme concentration (Fig. 3).

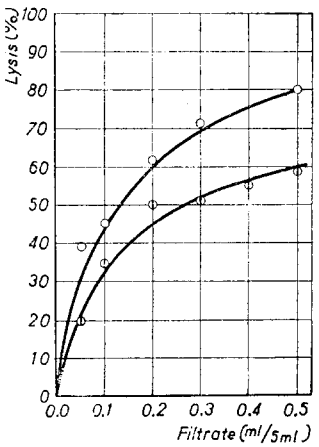

Fig. 3. Lytic activity of aerated (O——O) and stationary (⊙——⊙) *Actinomyces* culture filtrates against heat-killed *Bact. lactis aerogenes* at pH 7.0

From these results it was concluded that the production of the lytic enzyme was dependent upon the strain of actinomyces and upon the free access of air, but not upon spore formation. Accordingly, all subsequent cultures were grown in shallow layers of the medium in penicillin pans. It is to be noted that WELSCH (1941) in his studies on the actinomyces lytic system, found that the best results were obtained when the organism was cultivated in very shallow layers of ordinary broth.

Enzyme Production with time of Growth of the Actinomyces

Aliquot fractions of a culture of *Actinomyces* (*A*) in mannitol synthetic soil medium at 18° were withdrawn under sterile conditions at weekly intervals, filtered and the filtrates (0.05, 0.1–0.5 ml) examined for lytic activity against killed *Bact. lactis aerogenes* at p_H 7.0 under identical conditions. From the corresponding lysis-concentration curves thus obtained, the mean lytic activity of 0.15 ml culture filtrate was determined for each interval of time. The curve expressing these results (Fig. 4) shows that the production of the enzyme at first increases with the time of growth, becomes stationary and then after 7–8 weaks again increases. It is considered that this increase in lytic activity may be in part due to autolysis of the *Actinomyces*, with the liberation of proteolytic enzymes, occurring when the culture reaches a certain age. Such proteinases, as for example those of the staphylococcus autolytic system (JONES, STACEY, AND WEBB 1948) are not species specific in their action and readily lyse killed *Bact. lactis aerogenes*.

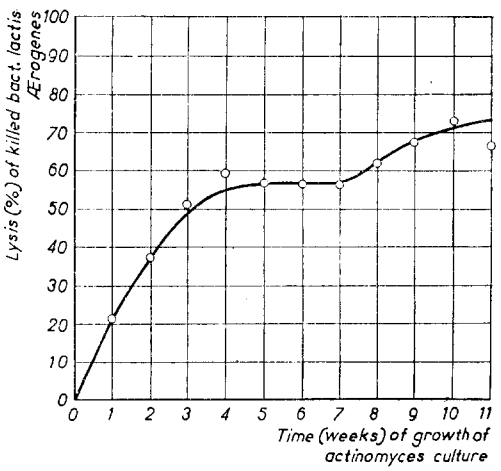

Fig. 4. Lytic enzyme production with time of growth of *Actinomyces* culture

In order to avoid the possible contamination of the exocellular bacteriolytic system with the endocellular autolytic system, preparations of the former were made from 30–35 day cultures.

The remarkable stability of the lytic enzymes of *Actinomyces* sp. is evidenced by the fact that cultures in both synthetic and complex media possessed considerable lytic activity against killed gram negative cells after 6 months or 1 year at room temperature.

References p. 182.

Concentration of the Actinomyces Lytic System

In preliminary experiments to ascertain the best procedure, 2 l of a 35 day culture of *Actinomyces* (A) in Lemco-peptone medium were filtered through paper and the filtrate (A 2) fractionated as follows:

(I). The filtrate (650 ml) was saturated with solid ammonium sulphate and the resulting precipitate collected (filtration) after 15 minutes. A solution of the precipitate in distilled water (50 ml) was dialysed against tap-water until free from ammonium sulphate, centrifuged and the clear supernatant (A 3) diluted to 100 ml.

(II). Saturated ammonium sulphate solution (3 l) adjusted to p_H 8 with ammonium hydroxide was added to the culture filtrate (600 ml) (Final concentration, 0.8 saturation of ammonium sulphate). The precipitate was collected (filtration), dissolved in distilled water and dialysed. After centrifuging free from the insoluble material the clear solution (A 4) was diluted to 92 ml.

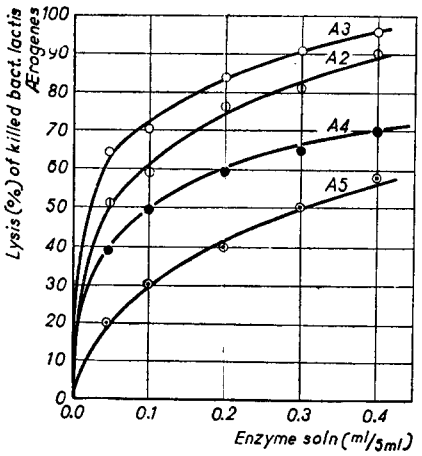

Fig. 5. Lytic activities of *Actinomyces* culture filtrate (A 2) and fractions separated therefrom by saturation with $(NH_4)_2SO_4$ (A 3), 0.8 saturation with $(NH_4)_2SO_4$ (A 4) and by ethanol precipitation (A 5)

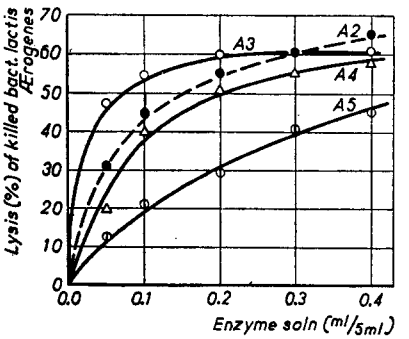

Fig. 6. Lytic activities of *Actinomyces* enzymes preparations shown in Fig. 5 after standing for 50 days at room temperature

(III). Absolute ethanol (250 ml) cooled to 0°, was added to the culture filtrate (250 ml). No precipitation occurred, and after 15 minutes at 0° a further 250 ml ethanol were added. The resulting precipitate was then separated, dialysed and the final volume of the crude enzyme solution (A 5) adjusted to 32 ml.

Thus the dilutions of A 3, A 4, and A 5 corresponded to 6.5 fold concentration of the initial filtrate (A 2).

The preparations were examined for lytic activity against heat-killed *Bact. lactis aerogenes* immediately after isolation and again after standing for 50 days at room temperature. The results (Fig. 5) show that the enzyme was more completely precipitated by saturation with solid ammonium sulphate (A 3) than at 0.8 saturation of the salt (A 4) or with ethanol (A 5) and indicate that the enzyme(s) is of relatively small molecular size. The activity of A 3 was, however, relatively less than the activity of the initial filtrate (A 2) indicating that some inactivation of the enzyme occurred on fractionation. Activities of the preparations decreased on standing at room temperature and the form

References p. 182.

of the concentration-activity curves (Fig. 6) would suggest that the lytic system consists of more than one enzyme, one of which is more stable than the other.

Although active against killed gram negative cells these preparations were without activity against heat-killed gram positive cells.

Large Scale Fractionation of Culture Filtrates

The *Actinomyces* (A) culture in complex Lemco-peptone medium or mannitol synthetic soil was filtered and the filtrate saturated with solid ammonium sulphate. The resulting precipitate collected at the liquid surface and was concentrated by means of a large tap-funnel. The suspension was then transferred to hardened paper on a Buchner funnel and filtered with suction. The solid was dissolved in distilled water and the solution dialysed against running tap-water for 18 hours. After centrifuging from the small inactive precipitate the clear solution was diluted to 50 ml for each litre of initial culture filtrate.

Refractionation of this solution by the addition of ammonium sulphate to 0.5 and 1.0 saturation yielded precipitates which possessed approximately the same lytic activity against heat-killed *Bact. lactis aerogenes*, and accordingly, in this preliminary investigation, concentration of the enzyme was not extended further than the initial precipitation by saturation with ammonium sulphate.

Optimum p_H *for the Activity of Actinomyces Bacteriolytic System*

A series of uniform test-tubes was prepared, each tube of the series containing 0.5 ml of the *Actinomyces* enzyme (Prepn A 6) 2.0 of the appropriate buffer and physiological saline (2.5 ml). 10 Drops of a suitable uniform suspension of heat killed *Bact. lactis aerogenes* were added to each tube such that the resulting opacity corresponded approximately to No. 10 on MACFARLAND'S barium sulphate opacity scale. Toluene (0.1 ml) was added to each tube to prevent contamination. The tubes were corked and incubated at 37°. After 3 days the corks were removed and the toluene allowed to evaporate during the course of a further 16 hours at 37°. The lysis in each tube was then determined by comparison with a standard opacity scale. The results (Fig. 7) show that the bacteriolytic enzyme is active over a wide p_H range with optimal activity at p_H 7.0–7.5.

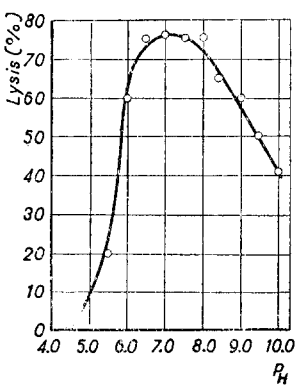

Fig. 7. Optimum p_H for the lysis of heat-killed *Bact. lactis aerogenes* by the *Actinomyces* lytic enzyme system

Inhibition of the Bacteriolytic Enzyme

The enzyme was completely inhibited by formaldehyde at concentrations of 0.05–0.1% (v/v). It was inhibited by hydrogen sulphide as shown by the following experiment.

To a series of tubes containing 0.00, 0.25, 0.5, 0.75 and 1.0 ml respectively of a saturated aqueous solution of hydrogen sulphide were added 0.2 M phosphate buffer p_H 7.5 (1.0 ml), the *Actinomyces* enzyme (0.5 ml) and physiological saline to 5.0 ml. A suspension of heat-killed *Bact. lactis aerogenes* was added to each tube to give an opacity corresponding to No. 10 on MACFARLAND'S scale. The tubes were corked and incubated at 37°. After 48 hours the experimental series was compared with a standard opacity scale. The results (Fig. 8) show that the enzyme activity was decreased by hydrogen

References p. 182.

sulphide and that the degree of inactivation was dependent upon the concentration of the reducing agent. It was observed that the addition of hydrogen sulphide to relatively large volumes of the enzyme solution resulted in the formation of a black precipitate, an observation which may suggest the precipitation of an activating metal.

Thioglycollic acid also inhibited the enzyme. At concentrations of 0.2% thioglycollic acid, the enzyme activity was reduced by 50 %.

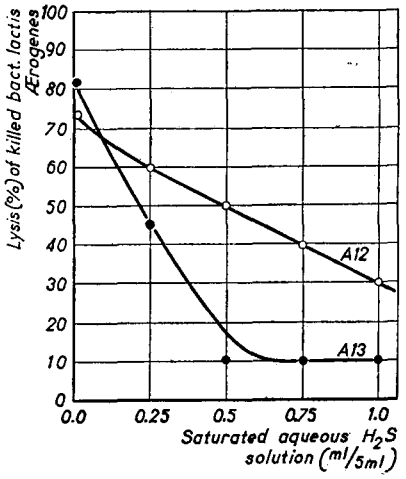

Fig. 8. Inhibition of the activity of the *Actinomyces* lytic enzyme system (Preparations A 12 and A 13) by hydrogen sulphide

Variation of Activity with Enzyme Concentration and Heat Inactivation of Actinomyces Bacteriolytic System

The inactivation of the enzyme at 60° was studied by immersing tubes containing the enzyme (Prepn A 15) buffered to different p_H values in a water-bath at 60°. At intervals of 30 minutes the tubes were withdrawn from the water-bath, cooled and the solution adjusted to p_H 7.0. The bacteriolytic activities of equivalent concentrations of these solutions were then examined for lytic activity against heat-killed *Bact. lactis aerogenes* according to the method previously described. The results (Figs. 9–12) show that the enzyme system is stable over a wide range and is most stable at alkaline p_H values.

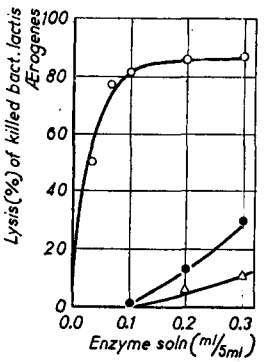

Fig. 9 (p_H = 4.0)

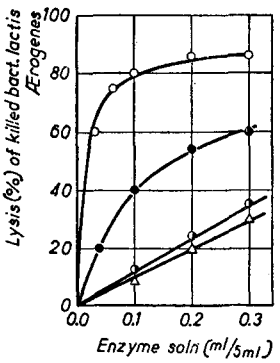

Fig. 10 (p_H = 7.0)

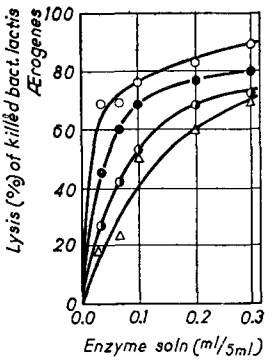

Fig. 11 (p_H = 7.9)

Figs. 9–12. Heat inactivation of the *Actinomyces* lytic enzyme system
Enzyme heated at 60° for 0 min O——O Enzyme heated at 60° for 60 min ◑——◑
Enzyme heated at 60° for 30 min ●——● Enzyme heated at 60° for 90 min △——△

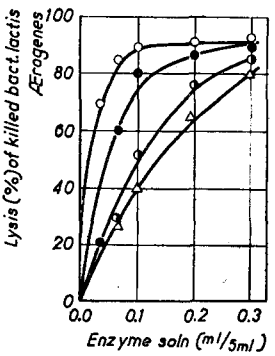

Fig. 12 (p_H = 9.26)

Nature of the Actinomyces Bacteriolytic Enzyme System

Studies on the autolysis of gram positive bacteria have shown that the action of the autolytic enzyme system may be divided into two separate and distinct stages. The first stage, the conversion of the gram positive cell to the gram negative form, is brought about by a species specific enzyme which also

References p. 182.

has the property of hydrolysing ribonucleic acid. The second stage, the lysis of the residual gram negative cell body, is caused by an enzyme system composed of at least two proteolytic enzymes (JONES, STACEY, AND WEBB 1948). This proteolytic system is not species specific for it readily dissolves the gram negative forms of all gram positive organisms and also heat-killed gram negative bacteria. It has no action on living gram negative, or on heat-killed gram positive micro-organisms. The close similarity in the properties of this enzyme system andthose of the *Actinomyces* bacteriolytic system suggested that the latter was composed of proteolytic enzymes. This was confirmed when preparations of the *Actinomyces* enzyme were examined for hydrolytic activity against protein substrates. Determination of the increase in amino nitrogen as measured by NORTHROP's (1926) modification of the formol titration showed that these preparations possessed considerable proteolytic activity (Tables II and III).

TABLE II

PROTEOLYTIC ACTIVITY OF PREPARATIONS OF THE *Actinomyces* BACTERIOLYTIC ENZYME SYSTEM AT pH 7.0

Enzyme Preparation	Time (h at 37°)	Substrate	Increase in formol titration (ml 0.01 N NaOH)
A 1	18	WITTE peptone (1 %)*	1.32
	41		2.01
	18	Casein (2 %)*	1.10
	41		1.70
A 8	18	WITTE peptone (1 %)	2.40
	18	Casein (2 %)	3.90
A 12	22	WITTE peptone (1 %)	4.00
	22	Casein (2 %)	7.22
A 13	22.5	WITTE peptone (1 %)	2.20
	22.5	Casein (2 %)	4.20

* Final concentration of substrate in 5 ml solution containing enzyme preparation (2 ml), substrate and distilled water.

TABLE III

HYDROLYTIC ACTIVITY OF *Actinomyces* BACTERIOLYTIC ENZYME AGAINST PROTEIN SUBSTRATES AT pH 7.0

Substrate	Increase in formol titration (ml 0.01 N NaOH) after 72 hours at 37°			
	Enzyme preparation: –			
	A 2	A 3	A 4	A 5
	1 ml enzyme/5 ml solution			
2 % Casein	2.27	3.92	2.70	0.87
1 % WITTE peptone . . .	1.75	2.67	2.07	0.75
1 % Edestin	0.65	1.30	0.75	0.30
1 % Gelatin	1.65	1.95	1.65	0.63
1 % Albumin	0.00	0.35	0.45	0.15

Comparison of the results of Table III with those of Fig. 5, in which are recorded the bacteriolytic activities of the same enzyme preparations against heat-killed *Bact. lactis aerogenes*, shows that proteolytic activity is proportional to bacteriolytic activity. Furthermore, decreased proteolytic activity resulted when the enzyme preparations

References p. 182.

(2 ml) in physiological saline (1 ml) and 0.2 M phosphate buffer (1 ml) were allowed to stand for 18 hours at room temperature with 1.0 ml of a saturated aqueous solution of hydrogen sulphide (Table IV). It has previously been shown (Fig. 8) that the bacteriolytic activity of the enzyme preparations is decreased by hydrogen sulphide.

TABLE IV

INHIBITION OF PROTEOLYTIC ACTIVITY OF *Actinomyces* LYTIC ENZYME SYSTEM WITH HYDROGEN SULPHIDE

Enzyme Preparation	Time (H at 37°)	Increase in Formol Titration (ml 0.01 N NaOH)			
		Enzyme alone		Enzyme with hydrogen sulphide	
		Substrate			
		2 % Casein	1 % WITTE peptone	2 % Casein	1 % WITTE peptone
A 7	66	2.85	1.45	0.00	0.55
A 8	41	6.15	3.15	3.30	2.30
A 12	20	6.37	2.30	2.98	1.50
A 13	20	3.75	1.60	0.57	1.42

Further correlation of the bacteriolytic and proteolytic activities of the *Actinomyces* lytic system was provided by the inactivation studies. 2 ml of the enzyme preparations (A 15) buffered to p_H 7 and heated at 60° for the given time intervals, were examined by the Formol titration for hydrolytic activity against protein substrates. The results (Table V) show that heat inactivation caused a decrease of proteolytic activity parallel to the decrease in bacteriolytic activity.

TABLE V

HEAT INACTIVATION OF THE PROTEOLYTIC ENZYMES OF THE *Actinomyces* LYTIC SYSTEM (PREPARATION A 15) AT p_H 7.0

Substrate	Time (h at 37°)	Activity (increase in Formol titration ml 0.01 N NaOH) of enzyme solution heated at 60° for			
		0 min	30 min	60 min	90 min
2 % Casein	23	2.85	1.05	0.78	0.58
1 % WITTE peptone . . .	23	1.10	0.60	0.40	0.36
	51	1.75	1.00	0.90	0.76
1 % Edestin	23	0.78	0.33	0.25	0.20
	51	1.13	0.53	0.40	0.35
1 % Gelatin	23	1.65	0.65	0.50	0.40
	51	2.00	0.90	0.70	0.58

The results of Table IV show that the ratio: hydrolysis of casein: hydrolysis of peptone for each enzyme preparation was considerably altered by the presence of hydrogen sulphide. An alteration in this ratio from 1.81 immediately after the preparation of the enzyme (A 12) to 2.77 after 15 days also occurred when the solution was allowed to stand at room temperature.

Since the ratio of the hydrolysis of one substrate to the rate of hydrolysis of a second substrate is constant for a particular enzyme under standard conditions (IRVING, FRUTON, AND BERGMANN 1941) these results indicate that the *Actinomyces* bacteriolytic system is composed of at least two proteolytic enzymes. It was found possible to alter

References p. 182.

considerably the ratio of the enzymes by fractionation on foam according to the method of SCHÜTZ (1937), but complete separation was not achieved.

As a preliminary experiment to determine the stability of the enzyme on foam, a current of nitrogen was passed through the enzyme solution (preparation A 19) for 18 hours. The rate of the nitrogen flow was adjusted such that foam was produced but did not pass into the receiver. Comparison of the proteolytic activities of the initial solution (A 19, 0.7 ml in 5 ml solution) and of the solution remaining after foaming (A 19 a, 0.7 ml in 5 ml solution) showed that no inactivation of the enzyme occurred on the foam (Table VI). The rate of the nitrogen flow was then increased until foam was collected in the receiver. After 2 hours the foam fraction (A 19 b, 0.7 ml in 5 ml solution) and the residual solution (A 19 c, 0.7 ml in 5 ml solution) were examined for hydrolytic activity against casein and WITTE peptone. The alteration in the ratio of the hydrolysis of the two substrates achieved by this procedure is shown in Table VI. Extension of this method of separation is being investigated further.

TABLE VI

PARTIAL SEPARATION OF THE PROTEOLYTIC ENZYMES OF THE *Actinomyces* BACTERIOLYTIC SYSTEM ON FOAM

Enzyme Solution	Increase in Formol titration (ml 0.01 N NaOH) after 24 hours at 37°		Ratio: $\frac{\text{Casein activity}}{\text{Peptone activity}}$
	2 % Casein Substrate	1 % WITTE Peptone Substrate	
A 19	1.58	1.00	1.58
A 19a	1.65	0.95	1.74
A 19b	2.30	0.83	2.77
A 19c	1.45	0.60	2.42

Proteolytic Activity of Culture filtrates of other Actinomycetes

Filtrates of cultures of the sporulating (5 f) and non-sporulating (h) *Actinomyces*, *Micromonospora* sp. (All 17) and *Proactinomyces* (19 and T 2) in the mannitol-synthetic soil medium which were examined for lytic activity against killed *Bact. lactis aerogenes* (p. 169), were also examined for hydrolytic activity against casein and WITTE peptone. The results (Table VII) are of considerable interest as the *Proactinomyces* culture filtrates, which exhibited no lytic activity against killed gram negative cells, were able to hydrolyse peptone but not casein. By analogy with the proteolytic enzymes of the stap-

TABLE VII

PROTEOLYTIC ACTIVITIES OF CULTURE FILTRATES OF OTHER *Actinomycetes*

Culture filtrate of	Increase in formol titration (ml 0.01 N NaOH) due to culture filtrate (2.0 ml in 5 ml solution) after 48 hours at 37°	
	2 % Casein Substrate	1 % WITTE peptone Substrate
Actinomyces (h)	0.90	0.81
Actinomyces (5 f)	0.70	0.40
Micromonospora (All 17)	0.20	0.31
Proactinomyces (19)	0.00	0.15
Proactinomyces (T 2)	0.00	0.52

References p. 182.

hylococcus autolytic system it would therefore appear that the hydrolysis of casein on the one hand, and of peptone on the other, is due to two separate and distinct enzymes. The enzyme which hydrolyses casein is responsible for the initial disintegration of the killed, gram negative cells and the enzyme which hydrolyses peptone, and which itself has no action on the killed cells, is responsible for the further hydrolysis of the split products resulting from the action of the casein hydrolysing enzyme. The complete separation of these activities, a problem also of considerable importance in studies of the autolytic enzyme systems, is at present under investigation.

Action of the Actinomyces Lytic Enzyme System on the Culture Medium

As the media most favourable for the production of the *Actinomyces* lytic system contained proteins and peptides, it was of interest to determine whether the isolated system was able to hydrolyse these complex molecules into smaller units capable of being utilised by the cell. It was established that the enzyme system, concentrated by precipitation with ammonium sulphate, hydrolysed the proteins of the growth medium for when the enzyme preparation (A 27, 2.0 ml) was incubated at 37° with the Lemco peptone medium (1.0 ml) diluted with distilled water (2.0 ml), an increase in the Formol titration value of 1.05 ml 0.01 N NaOH was obtained.

In a further experiment, the enzyme (A 27) and the Lemco peptone medium were dialysed for 72 hours against running tap water. Each solution was analysed for total nitrogen by the micro KJELDAHL method and the enzyme (50 ml) added to the dialysed medium (100 ml). The solution was incubated at 37° for 48 hours and then dialysed through cellophane in the concentrating dialyser. The results (Table VIII) show that during this period 82.9 % of the total nitrogen of the substrate had been rendered dialysable.

TABLE VIII

ACTION OF THE *Actinomyces* BACTERIOLYTIC SYSTEM (PREPARATION A 27) ON LEMCO-PEPTONE CULTURE MEDIA

Total nitrogen (Micro Kjeldahl) of:	100 ml dialysed culture medium	86.34 mg
	50 ml dialysed enzyme solution	3.71 mg
	System (enzyme + substrate)	90.05 ml
After 48 hours at 37°, total nitrogen of:	dialysed material	71.60 mg
	non-dialysable residue	18.68 mg
	System (dialysate + residue)	90.28 mg

During the routine examination of *actinomyces* culture filtrates and the concentrated enzyme preparations for lytic activity against killed cells, it was found that a culture filtrate and the corresponding enzyme preparation of a 35 day culture of the organism in Lemco-peptone medium were capable of lysing heat-killed gram positive *Staph. albus* (9238) as well as the killed gram negative organisms. The concentrated enzyme solutions (1 ml/5 ml) completely lysed suspensions of heat-killed gram positive *Sarcinae, Micrococci, Streptococcus faecalis* and *Staphylococcus aureus* (3661). At lower concentrations partial lysis was obtained (Fig. 13).

The experimental procedure was as follows: Increasing concentrations (see Fig. 13) of the enzyme preparation and 0.2 M phosphate buffer p_H 7.0 (1.0 ml) were added to a

References p. 182.

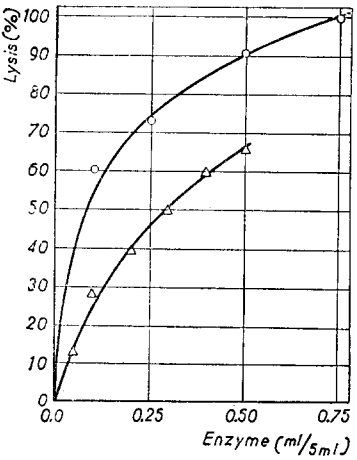

Fig. 13. Lysis of heat-killed, gram positive micro-organisms by the *Actinomyces* lytic system from 35 day cultures in Lemco peptone medium
○——○ *Sarcina sp.*
△——△ *Staph. aureus* (3661)

series of uniform test tubes. The total volume in each tube was adjusted to 5.0 ml with physiological saline. 10 Drops of a uniform suspension of the heat killed, gram positive organism were added to each tube together with toluene (0.1 ml). The tubes were corked and incubated at 37°. After 3 days the corks were removed, the toluene allowed to evaporate during the course of a further 16 hours and the degree of lysis in each tube determined by comparison with a standard opacity scale.

Examination of further filtrates of Lemco-peptone cultures of *Actinomyces* (A) showed that as the age of the culture increased the activity of the filtrate against gram positives decreased. Thus, a 43 day culture filt rate produced only slight lysis of suspensions of heat-killed *Staph. albus* at p_H 7.0 and a 50 day culture filtrate failed to produce any lysis in suspensions of the killed gram positive organisms.

In view of the studies on the bacterial autolytic enzyme systems (JONES, STACEY, AND WEBB, 1948) and of the present demonstration that the proteolytic enzymes of *Actinomyces* culture filtrate were capable of lysing gram negative organisms, it appeared that this activity against killed gram positives was due to the presence in the 35 day culture filtrate of a nucleinase capable of converting the killed gram positive organisms to the gram negative state. In this condition the cells would be readily lysed by the proteolytic enzyme system.

The existence of a nucleinase in such enzyme preparations was confirmed by following the hydrolysis of ribonucleic acid produced by the enzyme by the method of DAVIDSON AND WAYMOUTH (1944). The bacteriolytic system from 35 day cultures of *Actinomyces* (A) in Lemco peptone medium exhibited a relatively strong ribonucleinase activity (Fig. 14). This enzyme activity was considerably reduced in 43 day cultures and was not present in 50 day cultures. The decrease in ribonucleinase activity, with consequent decrease in lytic activity against heat killed gram positive cells, may possibly be due to the digestion of the nucleinase by the enzymes of the proteolytic system. In support of this is the experimental finding that gram positive cells rendered gram negative by the action of ribonucleinase were completely lysed by the subsequent addition of the *Actinomyces* proteolytic enzyme system, but only slight lysis was obtained when these enzymes were added together to the suspension of heat-killed, gram positive cells.

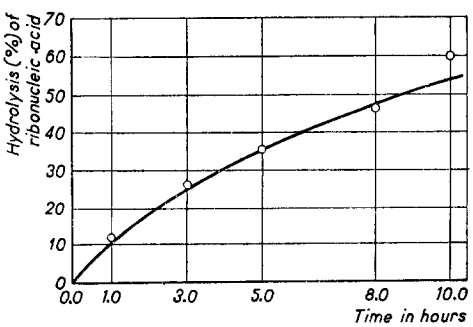

Fig. 14. Hydrolysis of ribonucleic acid by the *Actinomyces* lytic enzyme system from 35 day cultures in Lemco-peptone medium

Nucleinase activity could only be demonstrated in *Actinomyces* (A) cultures in Lemco peptone medium, Filtrates from 35 day cultures in synthetic media were without

References p. 182.

action upon heat killed, gram positive cells. It was at first considered that the production of the nucleinase in the complex was due to the presence of ribonucleic acid in the constituents of the medium. The presence of ribonucleic acid in either Lab-Lemco or commercial peptones could not, however, be established. Furthermore, the production of ribonucleinase activity by *Actinomyces* (A) could not be induced by continued subculture of the organism in STANIER's (1942) inorganic salt medium containing, in addition, 0.7 % sodium ribonucleate. Filtrates from 35 day cultures of *Actinomyces* (A) in this medium exhibited considerable lytic activity against killed gram negative cells, but had no action on heat killed gram positive organisms.

ACTION OF *Actinomyces* CULTURE FILTRATES ON LIVING GRAM POSITIVE CELLS

Actinomyces (A) plated onto agar containing living gram positive organisms was known to bring about the lysis of the these cells. Sterile filtrates of cultures of *Actinomyces* (A) in either complex or synthetic media were also found to lyse living gram positive cells, as shown by the following representative experiments, but had no action on living gram negative cells.

10 Drops of a uniform suspension of the cells from a 24 hour culture of *Staph. citreus* (B 9) on peptone-agar were added to each of a series of uniform tubes containing increasing concentrations (0.0–1.0 ml) of the *Actinomyces* culture filtrate (A 26), 0.2 M phosphate buffer p_H 7.0 (1.0 ml) and physiological saline to 5.0 ml. The tubes were corked and incubated at 37°. After 3 days the degree of lysis in each tube was estimated by comparison with a standard opacity scale. The results (Fig. 15) show that the clearing of the bacterial suspension in the presence of the *Actinomyces* culture filtrate was considerably in excess of that due to the spontaneous autolysis of the cells occurring at zero concentration of the culture filtrate.

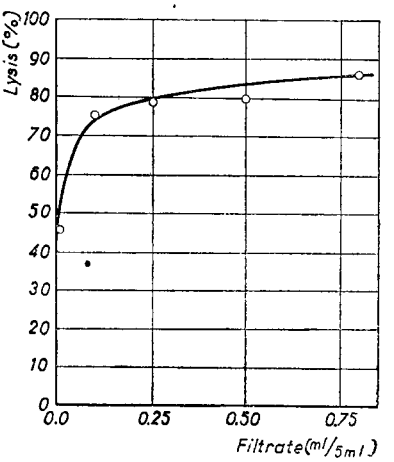

Fig. 15. Lysis of living gram positive cells (*Staph. citreus B 9*) by *Actinomyces* culture filtrate (A 26) at p_H 7.0

Since lysis of the cell suspensions in excess of that due to autolysis was not observed in suspensions of living *Staph. citreus* containing the *Actinomyces* system concentrated by ammonium sulphate precipitation, it was concluded that some additional component

TABLE IX

BACTERIOSTATIC ACTIVITIES OF ETHER EXTRACTS OF *Actinomyces* CELLS AND CULTURE FILTRATES AGAINST *Staph. aureus* IN GLUCOSE PEPTONE BROTH (SERIAL DILUTION METHOD)

Ether Extract of	Bacteriostatic Activity after	
	24 hours	48 hours
Actinomyces cells and mycelium	1 : 4000	1 : 4000
Actinomyces culture filtrate (mannitol synthetic soil medium) saturated with $(NH_4)_2SO_4$	1 : 1000	1 : 500
Actinomyces culture filtrate (Lemco-peptone medium) saturated with $(NH_4)_2SO_4$	1 : 2600	1 : 2600

References p. 182.

of the bacteriolytic system was present in culture filtrates. Following the method of WELSCH (1941) we were able to isolate, by ether extraction of *Actinomyces* filtrates after saturation with ammonium sulphate or by ether extraction of dried *Actinomyces*, an antibacterial substance which after removal of the solvent, formed a brown syrup and appeared to be a lipoid or a fatty acid. Solutions of such extracts in 0.05 M phosphate buffer p_H 8.2 were inactive against gram negative organisms, but were bacteriostatic (Table IX) and bactericidal (Table X) for gram positive organisms.

TABLE X

BACTERICIDAL ACTIVITIES OF ETHER EXTRACTS OF *Actinomyces* CELLS AND CULTURE FILTRATES AGAINST *Staph. aureus* (RIDEAL-WALKER METHOD)

Dilution of ether extract	Period of Incubation of Subcultures at 37° (h)	Ether Extract of											
		Actinomyces cells and mycelium				*Actinomyces* culture filtrate (mannitol synthetic soil medium) saturated with $(NH_4)_2SO_4$				*Actinomyces* culture filtrate (Lemco-peptone medium) saturated with $(NH_4)_2SO_4$			
		Time (min) ether extract in contact with cells											
		5	10	15	20	5	10	15	20	5	10	15	20
1 : 100	24	—	—	—	—	+	—	—	—	—	—	—	—
1 : 500	24	+	+	+	—	+	+	+	—	+	+	—	—
1 : 2500	24	+	+	+	+	+	+	+	+	+	+	+	+
1 : 12500	24	+	+	+	+	+	+	+	+	+	+	+	+

— no growth
\+ growth in subcultures

Action of the Actinomyces Bactericidal Fractions on Living gram positive Cells

Gram positive organisms were inoculated into nutrient broth in sterile centrifuge tubes and the cultures centrifuged after 18–24 hours at 37°. The cell deposits were suspended in sterile physiological saline (1 ml) and incubated at 37° with an equal volume of a sterile 1 % solution of the *Actinomyces* bactericidal substance.

After 3 days at 37° examination of stained smears prepared from the suspension revealed that in each case the cells were more or less completely gram negative (Table XI).

It was also observed that this effect, namely the death of the organism followed by the change from gram positive to gram negative, occurred when living gram positive cells were incubated at 37° with solutions of streptothricin and streptomycin (1 ml/1 ml), the antibacterial substances produced by *Streptomyces lavendulae* and *Streptomyces griseus*.

Since the change from gram positive to gram negative was not observed when suspensions of living cells containing the *Actinomyces* antibacterial fractions were kept at 0° or 60°, or when killed gram positive cells were incubated at 37° with the active extracts, it is concluded that the change in the gram staining reaction is not directly due to the action of the bactericidal substance, but is a secondary effect, autolysis following the death of the cells. A similar conclusion has been reached to explain the lysis which follows the action of tyrocidin on certain bacterial species (HEILMAN AND HERRELL 1941; MANN, HEILMAN, AND HERRELL 1943).

References p. 182.

TABLE XI

ACTION OF ETHER EXTRACTS OF *Actinomyces* CELLS AND CULTURE FILTRATES ON LIVING GRAM POSITIVE CELLS

Organism	Approx. Percentage Gram Negative Cells			
	Ether Extract *Actinomyces* cells and mycelium	Ether Extract *Actinomyces* culture filtrate (Mannitol synthetic soil medium)*	Ether Extract *Actinomyces* culture filtrate (Lemco-peptone medium)*	
			Prepn I	Prepn II
Staph. citreus (B 9) . .	90	90	100	80–90
Micrococcus 10470 . . .	80	100	100	70–80
Micrococcus 382	100	60	100	—
Cl. welchii	100	100	100	100

* Culture filtrates saturated with ammonium sulphate

Gram positive organisms, killed and rendered gram negative by the action by the bactericidal fractions from *Actinomyces* (A) (Table XI) were readily lysed by the subsequent addition of the *Actinomyces* bacteriolytic enzyme system. Furthermore, living gram positive cells were completely lysed after 3 days when incubated at 37° with a 1 % solution of the bactericidal substance and the lytic enzyme system (1 ml) at p_H 7.0.

DISCUSSION

In agreement with the work of WELSCH (1941, 1942) the foregoing experimental has shown that the bacteriolytic system of a soil *Actinomyces* is composed of an antibacterial substance and a proteolytic enzyme system. The latter enzyme system lyses killed gram negative organisms and the gram negative forms of gram positives, but has no action on living gram positive or gram negative cells. The antibacterial substance has no action on living gram negative organisms, but gram positives are killed under conditions which are favourable for the action of the autolytic enzymes of the cell. The action of these enzymes results, in the first instance, in a change in the gram staining reaction of the cells which therefore become susceptible to the action of the *Actinomyces* lytic enzyme system.

WELSCH (1941) was of the opinion that part of the antibacterial activity of *Actinomyces* culture filtrates was due to the presence in the medium of bactericidal fatty acids. Such substances have been isolated from the peptones which have been used in this work (WEBB, 1948 a) and have approximately the same bactericidal activity as the active fractions herein described. However, although gram positive organisms are killed by the action of these extracts, conditions are not favourable for the action of the autolytic enzymes and the cells do not become gram negative. Thus, such fatty acids in the complex culture media, though possibly increasing the bactericidal activity of *Actinomyces* cultures, do not themselves play any part in the bacteriolysis of living gram positive organisms.

Certain cultures of *Actinomyces* (A) in complex media contain a ribonucleinase and

References p. 182.

are able to lyse heat killed gram positive organisms. This enzyme is only produced in complex media, is relatively unstable and cannot be considered as forming part of the normal bacteriolytic system of the *Actinomyces*. WELSCH (1941) has stated that killed gram-positive cells, though more resistant, are to some extent susceptible to the *Actinomyces* lytic system. It may, however, be pointed out that, unless special precautions are taken, a variable percentage of gram negative cells result when gram positive bacteria are killed by heat (WEBB 1948 b) and these are dissolved by proteolytic enzymes.

The bacteriolytic system herein described appears closely similar to that of myxococci which has been shown to consist of a relatively unstable, ethanol soluble, antibacterial agent and an exocellular proteinase (OXFORD, 1947). The proteolytic enzyme was found to lyse killed gram negative eubacteria, but had no action on living gram negative cells. The antibacterial substance was bactericidal for gram positive but had no action on gram negative bacteria. In view of the present work, it would be of interest to determine whether gram positive cells killed by this bactericidal substance subsequently became gram negative.

Thanks are due to Professor M. STACEY for his interest, and to Dr DAGNY OXFORD for many helpful discussions, suggestions and constructive criticism, throughout this work.

SUMMARY

The bacteriolytic system of a soil *Actinomyces* has been shown to consist of a bactericidal substance and a proteolytic enzyme system. The latter enzyme system lyses killed gram negative cells and killed gram positive cells rendered gram negative (with ribonucleinase), but has no action on the intact gram positive cells. Under the influence of the bactericidal substance, gram positive cells are killed and become gram negative. In this state they are susceptible to the action of the proteolytic enzymes.

RÉSUMÉ

Il a été démontré que le système bactériolytique d'un *actinomyces* du sol se compose d'une substance bactéricide et d'un complexe d'enzymes protéolytiques. Sous l'action de ce dernier complexe d'enzymes, des cellules mortes gram-négatives et des cellules mortes gram-positives rendus gram-négatives (par la ribonucléinase) sont lysées. Par contre des cellules gram-positives intactes ne subissent pas cette action. Sous l'influence de la substance bactéricide, des cellules gram-positives sont tuées et deviennent gram-négatives. Dans cet état, elles sont sensibles a l'action des enzymes protéolytiques.

ZUSAMMENFASSUNG

Das bakteriolytische System einer Erde-*Actinomyces* besteht aus einer bakterientötenden Substanz und einem proteolytischen Enzymsystem. Letzteres Enzymsystem löst getötete gramnegative Zellen und löst ebenfalls diejenigen grampositiven Zellen, die (durch Ribonucleinase) in gramnegative umgewandelt worden sind, aber es ist ohne irgendeinen Einfluss auf die unbeschädigten grampositiven Zellen. Die grampositiven Zellen werden durch Einwirkung der bakterientötenden Substanz getötet und in gramnegative übergeführt. In diesem Stadium sind sie für die Einwirkung der proteolytischen Enzyme empfindlich.

REFERENCES

H. J. CONN AND JEAN E. CONN, Value of pigmentation in classifying actinomycetes, *J. Bact.*, 42 (1941) 791.

J. N. DAVIDSON AND C. WAYMOUTH, Tissue nucleic acids. II. The isolation and properties of liver nucleic acid, *Biochem. J.*, 38 (1944) 375.

S. V. GORYUNOVA, Mechanism of lysis. I. Comparison of processes of lysis and autolysis in actinomyces of MAKHLIN strain, *Microbiology (U.S.S.R)*, 13 (1944) 226.
HEILMAN, DOROTHY AND W. E. HERRELL, Mode of action of gramicidin, *Proc. Soc. Exp. Biol. Med.*, 47 (1941) 480.
G. W. IRVING, J. S. FRUTON, AND M. BERGMANN, Kinetics of protease action. Application to specificity problems, *J. Biol. Chem.*, 138 (1941) 231
A. S. JONES, M. STACEY, AND M. WEBB, The autolytic enzyme system of gram positive micro-organisms. The proteolytic system of staphylococci, *Proc. Roy. Soc. B.*, in the press (1948).
R. LIESKA, *Morphologie und Biologie der Strahlenpilze*, Leipzig (1921), p. 138.
F. C. MANN, DOROTHY HEILMAN, AND W. E. HERRELL, Effect of serum on haemolysis by gramicidin and tyrocidin, *Proc. Soc. Exp. Biol. Med.*, 52 (1943) 31.
J. H. NORTHROP, A convenient method for the formol titration, *J. Gen. Physiol.*, 9 (1926) 767.
A. E. OXFORD, Observations concerning the growth and metabolic activities of myxococci in a simple, protein-free liquid medium, *J. Bact.*, 53 (1947) 129.
F. SCHÜTZ, Adsorption on Foam, *Nature*, 139 (1937) 629.
R. Y. STANIER, Agar decomposing strains of the actinomyces-coelicolor species group, *J. Bact.*, 44 (1942) 555.
S. A. WAKSMAN, *Microbial antagonists and antibiotic substances*, Commonwealth Fund, New York (1945) Chap. 6.
M. WEBB, The influence of magnesium on cell division. I. The growth of Cl. welchii in complex media deficient in magnesium, *J. Gen. Microbiol.*, in the press (1948, a).
M. WEBB, The action of lysozyme on gram positive micro-organisms, *J. Gen. Microbiol.*, in the press (1948, b).
M. WELSCH, Bactericidal substances from sterile culture media and bacterial cultures, *J. Bact.*, 42 (1941) 801.
M. WELSCH, Bacteriostatic and bacteriolytic properties of actinomycetes, *J. Bact.*, 44 (1942) 571.

Received January 30th, 1948