THE EXOCELLUAR BACTERIOLYTIC SYSTEM OF SOIL ACTINOMYCES

IV. THE ACTION OF THE LYTIC SYSTEM ON GRAM-POSITIVE ORGANISMS

by

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In the previous papers of this series (JONES, SWALLOW, AND WEBB[1]; MUGGLETON AND WEBB[2,3]) the exocellular bacteriolytic system of a soil Actinomyces (Strain A) was shown to lyse both killed Gram-negative and Gram-positive bacterial cells.

The lysis of dead Gram-negative cells was brought about by a proteolytic enzyme system which consisted of a proteinase and a peptidase. The initial lysis of the cells resulted from the action of the proteinase, whilst the peptidase caused some further hydrolysis of the liberated peptides. This proteolytic enzyme system alone was without effect upon killed Gram-positive cells. Lysis of the latter was associated with the presence of an enzyme of the ribonuclease type, which rendered the cells Gram-negative. In this state they were susceptible to the lytic action of the proteolytic enzyme system. The ribonuclease was demonstrated initially in filtrates from cultures in complex media (JONES, SWALLOW, AND WEBB[1]) and was subsequently shown to be produced by cultures grown in a synthetic medium (MUGGLETON AND WEBB[2]). The present study of the Actinomyces system was undertaken to investigate further the action of the complex enzyme system on killed Gram-positive cells.

EXPERIMENTAL

Preparation of the Actinomyces culture filtrates

Cultures of the Actinomyces (Strain A) were grown in a chemically defined medium (mannitol—synthetic soil) in mould culture flasks at 25°.

Where appropriate, the culture filtrates were purified, concentrated and freeze-dried as previously described (MUGGLETON AND WEBB[2]).

The lysis of heat-killed Gram-positive cells

Measurement of cell lysis

The techniques used for the preparation of the heat-killed suspensions of bacterial cells and the estimation of cell lysis were described in a previous paper (MUGGLETON AND WEBB[2]). Particular care was observed in the preparation of the heat-killed Gram-

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positive cells, and suspensions which contained any Gram-negative forms were rejected.

The lysis of five different species of heat-killed Gram-positive bacteria (*e.g. Clostridium welchii, Staphylococcus aureus, Lactobacillus delbruckii, Corynebacterium diphtheriae* and *Streptococcus haemolyticus*) by the Actinomyces culture filtrate was studied in Part 2. The results obtained with these organisms and with various other Gram-positive species which have been examined subsequently, show that the Actinomyces enzyme system is not species-specific in its lytic action against killed Gram-positive cells. In this connection it is of interest that heat-killed cells of various strains of a given species were lysed to similar extents. Thus four strains of *Streptococcus haemolyticus*, grown under identical conditions in nutrient broth containing 10% horse serum, were lysed by the Actinomyces culture filtrate (1 ml/5 ml) to the extent of 53, 54, 59 and 56% respectively in 24 hours.

Ribonuclease activity of the culture filtrates

The activities of the various culture filtrates and enzyme solutions were compared by the following modification of the method of DAVIDSON AND WAYMOUTH[4].

Into each of a series of 5 or more 15 ml conical centrifuge tubes were placed 0.1% sodium ribonucleate (1.5 ml), 0.1 *M* veronal buffer pH 7.0 (1.0 ml), enzyme solution (1.0 ml) and 0.85% (w/v) sodium chloride (0.5 ml). The solutions were mixed and the tubes incubated at 37° in a water-bath. Immediately after mixing, and at suitable intervals thereafter, one tube was taken from the bath, cooled and 4 ml of MACFADYEN'S uranyl acetate-trichloracetic acid reagent added. The unhydrolysed ribonucleic acid which was precipitated, was collected in the centrifuge, washed in half-strength uranyl acetate reagent and analysed for total phosphorus by ALLEN'S[5] method.

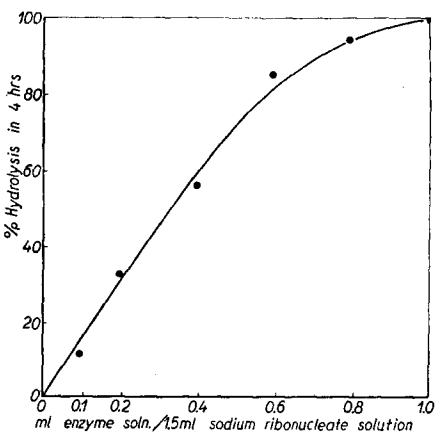

Fig. 1. The relationship between hydrolysis of sodium ribonucleate and Actinomyces ribonuclease concentration. Hydrolysis of the substrate expressed as the percentage of the total phosphorus rendered acid-soluble after 4 hours at 37°

The activities of the various enzyme solutions were compared by means of the activity curves, constructed by plotting percentage hydrolysis against time of incubation. Under suitable conditions of enzyme and substrate concentration it was possible to compare enzyme activities by measuring the degree of hydrolysis of the ribonucleic acid at an appropriately chosen time, since, for values up to 80% hydrolysis, the hydrolysis of the substrate in a given time was proportional to the enzyme concentration (Fig. 1).

Deoxyribonuclease activity of the culture filtrates

It was established that the Actinomyces strain grown in the synthetic medium produced, in addition to the ribonuclease, an enzyme of the deoxyribonuclease type. The deoxyribonuclease activity of the culture filtrates was determined against 0.1% sodium deoxyribonucleate as described by GILBERT, OVEREND, AND WEBB[6].

Typical ribo- and deoxyribo-nuclease activities of an Actinomyces culture filtrate are shown in Fig. 2.

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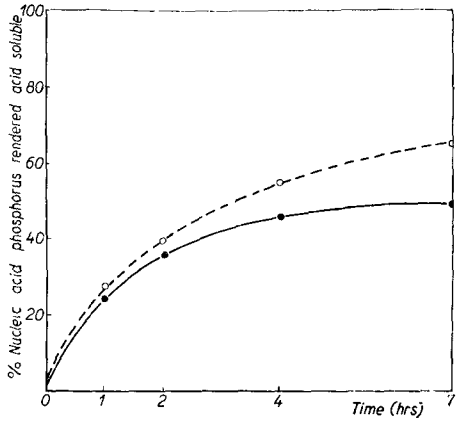

Fig. 2. Ribonuclease (O---O) and deoxyribonuclease (●——●) activities of the filtrate from a 4 weeks old Actinomyces culture

Proteinase activity of the culture filtrates

Proteinase activity, measured viscosimetrically against a casein substrate, was expressed in the units previously defined (MUGGLETON AND WEBB[3]).

The production of the nucleases by the Actinomyces strain in a synthetic medium

Three cultures of the Actinomyces in mould culture flasks containing the mannitol—synthetic soil medium (600 ml) were incubated at 25°. After 1, 2, 3, 4, 6 and 8 weeks, aliquots (20 ml) were removed aseptically from each flask without disturbing the growth. The three samples removed on each occasion were combined, filtered through paper and the filtrate assayed for ribonuclease and deoxyribonuclease activity. The results obtained (Fig. 3) show that the nucleases were present in greatest concentration at the second week of incubation and thereafter a rapid decrease in activity occurred. In this connection it may be recalled that the proteolytic enzymes of the Actinomyces cultures do not reach their greatest concentration until the 4th to 5th week of growth (MUGGLETON AND WEBB[2]). Thus it appears possible that the rapid decrease in the activities of the nucleases after 2–3 weeks growth may be due to the destruction of enzymes by the proteolytic system.

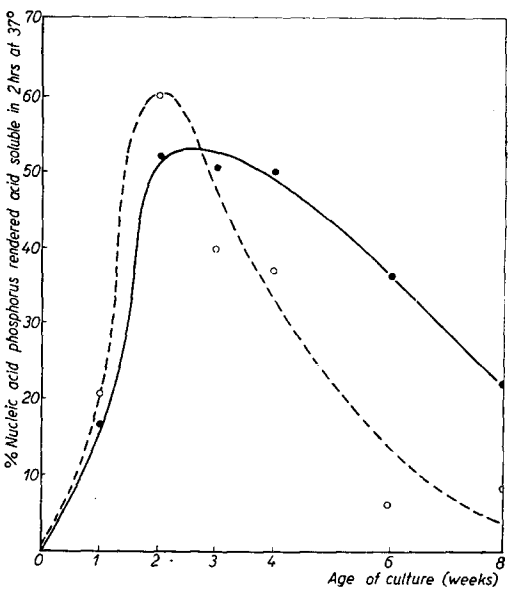

Fig. 3. The production of ribonuclease (●——●) and deoxyribonuclease (O---O) during the growth of the Actinomyces (Strain "A") in a chemically-defined medium

Concentration of the Actinomyces ribonuclease

1. *Evaporation of the culture filtrates*

The combined filtrates from 21 day Actinomyces cultures were dialysed for 48 hours in cellophane tubes against running tap water. The dialysed solution was divided into two equal parts which were concentrated to 1/5 volume by "pervaporation" and by distillation *in vacuo* at 30° respectively. The ribonuclease and proteinase activities of the culture filtrate, before and after dialysis, and of the two concentrated solutions are shown in Table I. The lytic activities of the enzyme solutions against heat-killed Gram-positive *Cl. welchii* are shown in Fig. 4.

The results of Table I show that a considerable loss of ribonuclease activity occurred during both dialysis and concentration. Experiments, in which the culture filtrate was

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TABLE I

THE EFFECT OF DIALYSIS AND CONCENTRATION OF THE CULTURE FILTRATE ON THE ACTIVITIES OF ACTINOMYCES RIBONUCLEASE AND PROTEINASE

Solution	*Proteinase (U/ml)*	*Ribonuclease (% hydrolysis of ribonucleic acid at pH 7 after 2 hrs at 37°)*
Crude culture filtrate	175.5	84.7
Dialysed filtrate	140.6	49.2
Dialysed filtrate after concentration by "pervaporation"	262.0	79.4
Dialysed filtrate after concentration under reduced pressure	270.0	3.0

dialysed for 48 hours against a small volume of distilled water, showed that the loss of ribonuclease activity which occurred on dialysis was due to factors such as those described by GILBERT AND SWALLOW[7] and not to the diffusion of the enzyme through the membrane.

It is seen from the results of Table I and Fig. 4 that although the pervaporated solution (Table I), had a ribonuclease activity almost equal to that of the crude filtrate and possessed considerably greater proteinase activity, its lytic activity against heat-killed *Cl. welchii* was less than that of the initial filtrate. This was found to be due to the loss of calcium ions during dialysis, since, as previously shown (MUGGLETON AND WEBB[3]), the proteinase of the bacteriolytic system requires ionic calcium for activation.

The effect of a deficiency of calcium ions was not apparent in the estimation of the proteinase activity, as the casein substrate contained sufficient calcium for activation of the enzyme. Also it was not apparent in the determination of lytic activity against killed *Bact. lactis aerogenes* cells which contained more calcium than *Cl. welchii* cells*.

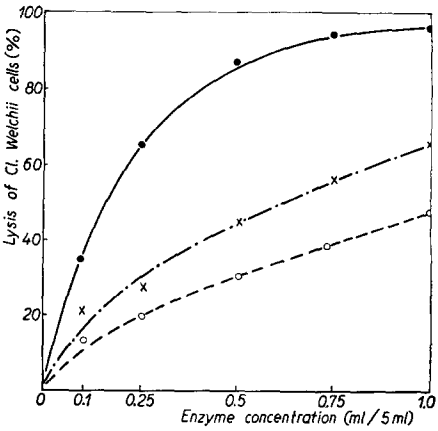

Fig. 4. The lysis of heat-killed Gram-positive cells (*Cl. welchii*) by a dialysed and concentrated Actinomyces culture filtrate.

●——● Crude culture filtrate
O---O Dialysed filtrate
×-·-·-× Concentrated, dialysed filtrate

2. Precipitation of the ribonuclease with ammonium sulphate

The culture filtrate (100 ml) was saturated with ammonium sulphate and the resulting precipitate, which floated to the surface, collected in a tap funnel. The precipitate was redissolved in water (50 ml) and the solution dialysed against running tap

* Washed cells of *Cl. welchii* and of *Bact. lactis aerogenes* were dried to constant weight at 105° and incinerated in a platinum boat. The calcium content of the ash was estimated in the usual way by titration with permanganate after precipitation of the calcium as the oxalate. The calcium content of the *Cl. welchii* cells was found to be 0.08% of the dry weight, whereas that of the *Bact. lactis aerogenes* cells was 0.42%.

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water for 48 hours. After dilution to 100 ml, the proteinase and ribonuclease activities of the solution were determined. The results (Table II) show that approximately 50% of the total activity of both the ribonuclease and the proteinase were recovered under these conditions.

The lytic activities of these solutions against a suspension of heat-killed *Cl. welchii* cells ran approximately parallel to their ribonuclease and proteinase activities.

TABLE II

PRECIPITATION OF THE ACTINOMYCES RIBONUCLEASE AND PROTEINASE FROM A CULTURE FILTRATE BY SATURATION WITH AMMONIUM SULPHATE

Enzyme solution	*Ribonuclease activity (% hydrolysis of sodium ribonucleate) after*			*Proteinase U/ml*
	2 hrs	*4 hrs*	*6 hrs*	
Culture filtrate	30.6	63.5	80.6	185.0
Ammonium sulphate precipitated enzymes	19.7	27.2	43.3	95.8

Deactivation of the Actinomyces nuclease enzymes by heat

Both the ribonuclease and deoxyribonuclease were extremely heat labile. Thus they were completely deactivated when maintained at 60° for 10 minutes (pH 7.3); after 5 minutes at 60°, *c.* 10% of each activity remained.

Inactivation of the enzymes at 60° occurred equally rapidly at an acid pH (pH 4.9).

The effect of heat inactivation of the ribonuclease on the lytic activity of the enzyme solution against killed Gram-positive cells

An aliquot of an Actinomyces enzyme solution was heated at 60° for 15 minutes to inactivate the ribonuclease. The lytic activities of the initial enzyme solution and of the heated (*i.e.* ribonuclease-free) solution (proteinase content = 76 U/ml), were determined against heat-killed *Bact. lactis aerogenes* and *Cl. welchii* cells. The Gram-negative cells (*Bact. lactis aerogenes*) were lysed by both solutions. The Gram-positive cells (*Cl. welchii*) however, were lysed by the unheated solution, but were unaffected by the heated, ribonuclease free, solution. Furthermore, in the latter case, stained films of the cells showed that they remained Gram-positive.

These results show conclusively that the lysis of killed Gram-positive cells by culture filtrates of Actinomyces "A" is dependent upon the presence of an enzyme of the ribonuclease type which renders the cells Gram-negative and therefore susceptible to the action of the proteolytic enzyme system.

Adsorption of the Actinomyces bacteriolytic enzymes on heat-killed Gram-positive cells

In Part 2 of this series it was shown that when heat-killed Gram-negative cells are lysed by the Actinomyces lytic system, first the proteinase and later the peptidase are adsorbed from the solution onto the cells. This work was extended to the study of the lysis of heat-killed Gram-positive cells (*Cl. welchii*) by the lytic enzyme system, as follows:

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To the Actinomyces culture filtrate (50 ml) was added 0.1 *M* veronal buffer pH 7.0 (10 ml) and a suitable suspension of washed, heat-killed *Cl. welchii* cells in 0.85% sodium chloride (40 ml) such that the opacity of final cell suspension was equivalent to 5 times that of tube 10 of the MacFarland standard scale. After the addition of toluene (2 ml), the mixture was incubated in a waterbath at 37°. Controls, in which (a) the cell suspension was replaced by 0.85% sodium chloride (the enzyme control) and (b) the enzyme solution was replaced by 0.85% sodium chloride (the cell control), were also incubated at 37°. At intervals up to 43 hours, aliquots (10 ml) of the cell-enzyme mixture and of the cell control were centrifuged (Angle head centrifuge) and the clear supernatants removed. The cell deposits were resuspended in 0.85% sodium chloride (50 ml) and the cell lysis determined turbidimetrically with the Spekker photoelectric absorptiometer. Films, stained by GRAM's method using a standardised technique, were also prepared from these suspensions and the percentage of Gram-positive cells determined by a method similar to that used for carrying out a differential leucocyte count.

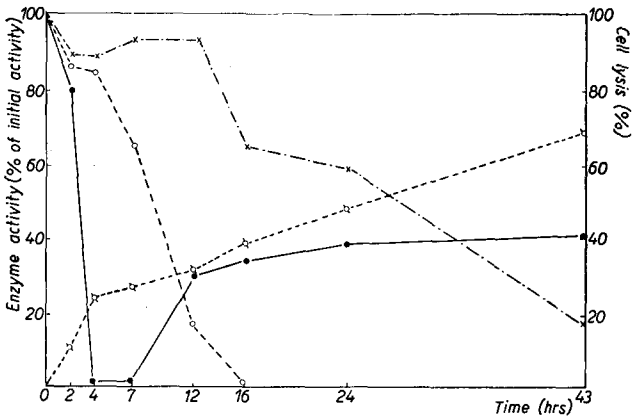

Fig. 5. Adsorption of the enzymes of the Actinomyces lytic system during the lysis of heat-killed Gram-positive *Cl. welchii* cells. ●——● Ribonuclease activity; O---O Proteinase activity; ×–·–·× Peptidase activity; O----O Cell lysis.

The above, cell-free supernatant solutions from the experimental series and aliquots of the enzyme control mixture were assayed for ribonuclease, proteinase and peptidase activities. In addition the total phosphorus, the inorganic phosphorus and the phosphorus content of the material precipitable with uranyl acetate-trichloracetic acid were estimated by ALLEN's[5] method in aliquots of these solutions. The results of these determinations are shown in Figs. 5 and 6.

The control suspension of cells without enzyme showed no lysis. Only about 5% of the cells became Gram-negative and negligible amounts of phosphorus were liberated during the course of the experiment. The enzyme control (without cells) showed that no inactivation of the proteinase or peptidase occurred during the 43 hours, but that 18% of the ribonuclease activity was lost between the 24 h and 43 h measurements.

The results show that the ribonuclease was adsorbed by the killed *Cl. welchii* cells more rapidly than the proteolytic enzymes and was completely removed from solution after 4 hours. Following the adsorption of the enzyme there was a rapid change in the Gram staining reaction of the cells. After 12 hours, when the cells were more or less

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completely Gram-negative, the ribonuclease was again liberated into solution. With the formation of the substrate of Gram-negative cells after 4 hours, adsorption of the proteinase commenced, and after 16 hours, the supernatant solution was free from proteinase activity. Unlike the ribonuclease, this enzyme was not re-liberated. The removal of 14% of the proteinase activity during the first 2 hours of the experiment was of interest, since a more rapid lysis of *c.* 24% of the cells also occurred during the first 4 hours (Fig. 5). It is possible that this was due to an initial rapid formation of the proteinase substrate by the action of the ribonuclease on a fraction of the cells in which the Gram complex (*cf.* HENRY AND STACEY[8]) was less firmly bound.

No marked adsorption of the peptidase occurred before 12 hours, when over 80% of the proteinase had been adsorbed. The initial small decrease in peptidase activity in the first few hours was probably due to the introduction of some free peptidase (*e.g.* peptone) with the cell substrate and is not considered to be of any significance.

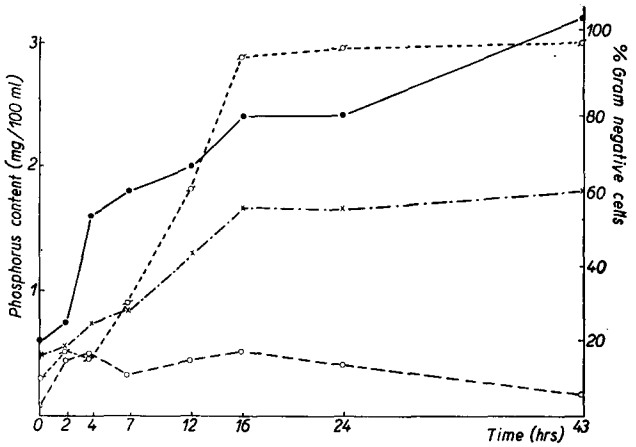

Fig. 6. Liberation of phosphorus compounds during the lysis of heat-killed Gram-positive *Cl. welchii* cells by the Actinomyces lytic system. ●——● Total phosphorus; O---O Acid-precipitable phosphorus; ×-·-·-× Inorganic phosphate; ○------○ Percent. Gram negative cells

Since only a small percentage of the phosphorus liberated during the lysis of the cells (Fig. 6) was precipitable at any time with uranyl acetate-trichloracetic acid, it follows that the ribonucleic acid removed from the cells was hydrolysed rapidly. Whether the nucleic acid was degraded before or after it was removed from the cells remains to be determined. The inorganic phosphorus liberated into solution increased markedly during the first 16 hours of lysis and then remained constant.

Similar results were obtained when the above experiments were repeated with the purified culture filtrate.

Liberation of inorganic phosphorus from sodium ribonucleate by the Actinomyces culture filtrates

From the results of the experiments described above it was apparent that little acid-soluble organic phosphorus (*i.e.* Total P — (acid precipitable P + inorganic P)) was present in the supernatant solution at any stage in the lysis of the cells. Thus it appeared that the hydrolysis of the ribonucleic acid of the Gram complex was accompa-

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nied by the liberation of inorganic phosphorus. Accordingly the phosphatase (nucleotidase) activity of the Actinomyces solution was studied as follows:

A series of tubes containing sodium ribonucleate and the culture filtrate, was prepared as described for the estimation of ribonuclease activity, and incubated at 37°. At intervals, the uranyl acetate-trichloracetic acid reagent was added to the contents of one tube and the resulting precipitate centrifuged off. The precipitate and the supernatant solution were then analysed for total phosphorus and inorganic phosphate respectively.

The results (Table III) show that in addition to the formation of acid-soluble polynucleotides, the action of the Actinomyces enzyme solution on ribonucleic acid also results in the liberation of inorganic phosphorus. It is apparent that the latter is due specially to a phosphatase and not to the ribonuclease, since a lag (Table III) occurs between the hydrolysis of the ribonucleic acid and the formation of inorganic phosphate.

TABLE III

LIBERATION OF INORGANIC PHOSPHORUS FROM SODIUM RIBONUCLEATE BY THE ACTINOMYCES CULTURE FILTRATE

	Time of incubation (hrs)				
	0	*2*	*4*	*7*	*24*
Phosphorus content of acid precipitate (mg)	0.102	0.049	0.034	0.029	0.002
Hydrolysis (%) of the nucleic acid	0	52	67	72	98
Inorganic phosphorus (% of acid soluble P)	0	0	7	10	33

The action of the phosphatase on mononucleotides

In order to confirm the presence of a phosphatase in the Actinomyces enzyme system, the activity of the latter was determined against adenylic acid. A 0.1% solution of adenylic acid in distilled water was adjusted to pH 7.0 with sodium hydroxide. A series of tubes, each containing 1.5 ml of the adenylic acid solution, 0.1 *M* veronal buffer pH 7.0 (1.0 ml), purified enzyme solution (1.0 ml), 0.85% sodium chloride (0.5 ml) and toluene (0.05 ml) was incubated at 37°. At suitable intervals, a tube was removed from the incubator and its contents analysed for inorganic phosphorus. The ribonuclease activity of the enzyme solution was also determined in the usual way. In addition, a second sample of the same enzyme solution was heated at 60° for 5 minutes, and then assayed for phosphatase and ribonuclease activity. The results of these experiments

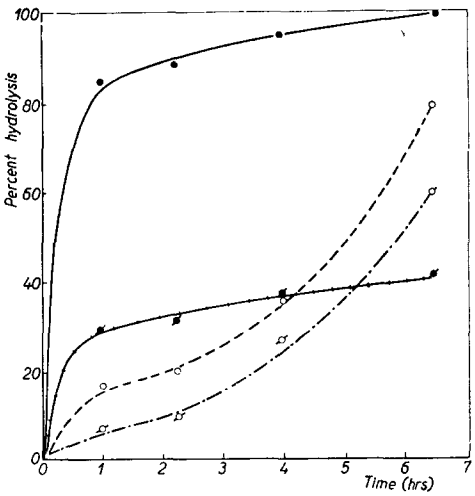

Fig. 7. The effect of heat on the activities of the Actinomyces ribonuclease and phosphatase.
● Ribonuclease
● Ribonuclease activity after 5 min at 60°
O Phosphatase
O Phosphatase activity after 5 min at 60°

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(Fig. 7) established the separate identities of the two enzymes. Thus inorganic phosphorus was liberated from the adenylic acid by the phosphatase which was slightly activated by the heat treatment, whereas the ribonuclease activity was markedly reduced after 5 minutes at 60°.

The phosphatase was not only active against nucleotides, but also hydrolysed sodium glycerophosphate at pH 7–8 and has been used successfully for the hydrolysis of phosphoric acid derivatives of D-galactose and 2 deoxy-D-galactose (FOSTER AND OVEREND[9]).

Action of the phosphatase on the hydrolysis products resulting from the action of pancreatic ribonuclease on sodium ribonucleate

A solution of the Actinomyces phosphatase was prepared free from ribonuclease activity by heating a sample of culture filtrate at 60°. After 15 minutes at 60°, the ribonuclease activity was completely destroyed, whilst as previously observed, the phosphatase was slightly activated. An aqueous solution (10 ml) of crystalline pancreatic ribonuclease (KUNITZ[10]), containing 0.25 mg protein/ml, was added to a mixture of 0.1% sodium ribonucleate (15 ml), 0.1 *M* veronal buffer pH 7.0 (10 ml) and 0.85% sodium chloride (5 ml), in a stoppered flask. 1 ml of toluene was added and the mixture then incubated at 37°. At intervals, a 4 ml sample was withdrawn and analysed for acid-precipitable and inorganic phosphorus. After 19 hours incubation, the mixture was heated on a waterbath at 100° for 15 minutes in order to deactivate any traces of phosphatase which may have been present in the ribonuclease preparation. After cooling, 20 ml of this solution were added to 5 ml of the ribonuclease-free Actinomyces phosphatase, and the mixture incubated at 37°. Samples of the solution were withdrawn at intervals and analysed as above.

The results (Table IV) show that the Actinomyces phosphatase liberates inorganic phosphorus from the acid soluble nucleotides produced by the action of the pancreatic ribonuclease on ribonucleic acid.

The action of the Actinomyces nucleases on the "enzyme-resistant cores" of nucleic acids

LORING, CARPENTER AND ROLL[11] have shown that when yeast ribonucleic acid is acted upon by pancreas ribonuclease, there remains a "core" which is resistant to further hydrolysis by the enzyme and which contains an increased amount of guanine. Thymus deoxyribonucleic acid also contains a "core" which is resistant to pancreas deoxyribonuclease (CHARGAFF AND ZAMENHOF[12]; OVEREND AND WEBB[13]). That these "resistant cores", obtained from yeast ribonucleic acid and thymus deoxyribonucleic acid, were susceptible to hydrolysis by the Actinomyces ribonuclease and deoxyribonuclease, respectively, is shown by the following experiments:

The hydrolysis of a 0.1% solution of sodium ribonucleate by a solution containing 0.5 mg/ml of pancreatic ribonuclease was followed as before until the activity curve (Fig. 8) showed that hydrolysis had reached a constant value. The addition of more ribonuclease to a sample of the solution at this stage resulted in no further hydrolysis. The addition of the Actinomyces enzyme solution to the system, however, gave complete hydrolysis of the "core". That this was due to the Actinomyces ribonuclease and not to the phosphatase follows from the results of Table IV which show that the latter enzyme alone has no action on the ribonucleic acid "core".

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TABLE IV

THE ACTION OF THE ACTINOMYCES PHOSPHATASE ON THE ACID-SOLUBLE NUCLEOTIDES PRODUCED BY THE ACTION OF PANCREATIC RIBONUCLEASE ON SODIUM RIBONUCLEATE

The formation of acid soluble and inorganic phosphorus as a result of the action of ribonuclease alone is shown in the first part of the table. After 19 hrs at 37°, the mixture was heated at 100° for 15 mins, cooled and an aliquot of the solution* added to the Actinomyces phosphatase at 37°. The liberation of inorganic phosphate by the action of the phosphatase on the acid soluble polynucleotides is apparent from the second part of the table.

Time (hours)	*Hydrolysis of ribonucleic acid (% of total P rendered acid-soluble)*	*Inorganic P (% of acid soluble P)*
0	0	0.0
2	55.3	0.0
4.5	59.3	2.8
19	77.2	10.8
19	77.2	10.8
20	—	19.4
22.5	—	25.9
26	77.2	35.6
43	77.2	57.2

* This solution, when incubated alone (*i.e.* without addition of the Actinomyces phosphatase), showed no increase in inorganic phosphorus.

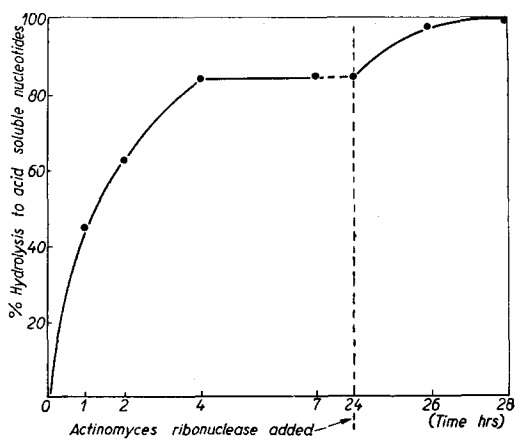

Fig. 8. The action of Actinomyces ribonuclease on the "resistant core" of ribonucleic acid

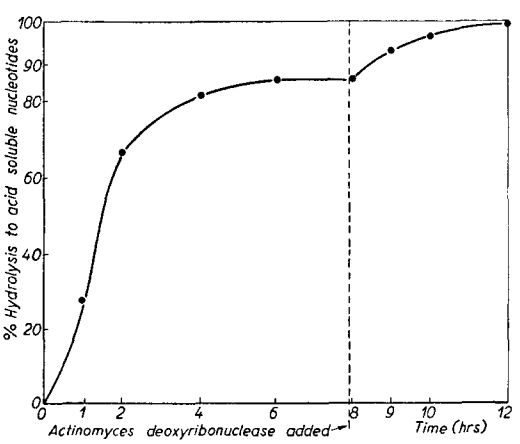

Fig. 9. The action of Actinomyces deoxyribonuclease on the "resistant core" of deoxyribonucleic acid

A similar experiment was carried out using deoxyribonucleic acid which was acted upon by pancreatic deoxyribonuclease. In this case also, the "resistant core" was hydrolysed by the Actinomyces deoxyribonuclease (Fig. 9).

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DISCUSSION

The foregoing experimental work has shown that the lysis of heat-killed Gram-positive cells by the bacteriolytic enzyme system of the Actinomyces "A" strain is dependent on the preliminary loss of the Gram-positive character of the cells by the action of the ribonuclease. The resulting Gram-negative cell-bodies are then lysed by the proteolytic enzyme system. In this respect the enzyme system studied in the present work differs from that described by WELSCH[14], which, although apparently free from detectable ribonuclease activity, lysed Gram-positive cells. It was shown in Part 2 of this series (MUGGLETON AND WEBB[2]) that the Actinomyces proteolytic enzyme system alone was inactive against killed Gram-positive cells, unless the latter were first rendered Gram-negative in various ways (*e.g.* by ribonuclease, extraction with sodium cholate, autolysis etc.). WEBB[15] has shown that certain heat-killed Gram-positive cells are rendered Gram-negative by the action of lysozyme; the change in the Gram staining reaction being accompanied by the removal of ribonucleic acid from the cells. Thus it appears possible that lysis of killed Gram-positive cells could be accomplished by a bacteriolytic enzyme system which was without ribonuclease activity, provided it contained, in addition to the proteolytic system, an enzyme of the lysozyme type. In this connection it is of interest that the presence of such an enzyme ("Actinomyces lysozyme") has apparently been detected in certain Actinomyces by KRASSILNIKOV AND KORENIAKO[16].

The Actinomyces ribonuclease is extremely labile, and is readily denatured at 60°. Little inactivation of the proteolytic enzymes occurs under the conditions necessary for complete inactivation of the ribonuclease. The importance of the latter enzyme of the bacteriolytic system in the lysis of Gram-positive cells follows from the fact that destruction of the ribonuclease by heat, renders the bacteriolytic system inactive against killed Gram-positive cells, but does not affect its activity against killed Gram-negative cells. That the conversion of Gram-positive cells to the Gram-negative state is accomplished by the breakdown or release of ribonucleic acid, follows from the experiments on the adsorption of the enzymes by heat-killed Gram-positive cells. Whether the ribonucleic acid is hydrolysed to acid soluble nucleotides before or after it is liberated from the cells remains to be determined. It is evident, however, that the nucleic acid is hydrolysed rapidly, since the level of acid precipitable nucleic acid in solution does not increase during lysis of the cells. In this respect the process differs from that of autolysis in the first stage of which ribonucleic acid and nucleoprotein are released from the cells (THOMPSON AND DUBOS[17]).

It was shown by KUNITZ[10] that the conversion of Gram-positive pneumococci to the Gram-negative state was brought about by pancreatic ribonuclease. Thus, in their action on Gram-positive cells, the Actinomyces and pancreatic ribonucleases appear to be similar. The enzymes differ, however, in their heat labilities and possibly in their mode of action, since the Actinomyces ribonuclease is able to hydrolyse the "core" of ribonucleic acid, which is resistant to the pancreatic enzyme.

The action of the Actinomyces phosphatase on the acid soluble nucleotides formed by the action of the ribonuclease does not appear to be of any significance in regard to lysis of the cells. It is of interest to note, however, that the phosphatase has no action on sodium ribonucleate and, in this respect, differs from intestinal phosphatase (SCHMIDT AND THANNHAUSER[18]) and from the phosphatase of dog faeces (KING AND

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DELORY[19]). The latter enzyme is active against yeast ribonucleic acid, but has no action on heat-killed Gram-positive cells.

The presence of a deoxyribonuclease in the Actinomyces culture filtrates is of considerable interest, although there is no evidence that it plays any direct part in bacteriolysis. That the deoxyribonuclease and ribonuclease are different enzymes and not a single, non-specific nuclease, is shown by their different rates of production by the growing cultures (Fig. 3).

In conclusion, the lysis of killed Gram-positive cells by the Actinomyces enzyme system may be summarized as follows: The cells are first rendered Gram-negative by the action of the ribonuclease on the ribonucleic acid of the Gram complex, and the resulting polynucleotides are then further degraded by the phosphatase. Lysis of the Gram-negative cytoskeletons follows by the action of the enzymes of the proteolytic enzyme system.

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SUMMARY

The lysis of heat-killed Gram-positive cells by the bacteriolytic system of the Actinomyces (Strain A) is dependent upon the presence of an enzyme of the ribonuclease type, which renders the cells Gram-negative and therefore susceptible to lysis by the proteolytic enzymes. This is shown by the fact that the proteolytic system, freed from ribonuclease activity by preferential heat-inactivation, lyses killed Gram-negative cells, but has no action on killed Gram-positive cells.

The acid soluble nucleotides resulting from the initial action of the ribonuclease on killed Gram-positive cells are shown to be degraded further, with the liberation of inorganic phosphate, by a non-specific phosphatase.

Studies on the production and properties of the ribonuclease, and of a deoxyribonuclease also present in the culture filtrates, are described.

RÉSUMÉ

La lyse par le système bactériolytique d'Actinomyces (souche A) de cellules Gram-positives tuées à la chaleur dépend de la présence d'une enzyme du type ribonucléase qui rend les cellules Gram-négatives et par conséquent susceptibles à la lyse par les enzymes protéolytiques. Ceci est démontré par le fait que le système protéolytique, libéré d'activité ribonucléasique par inactivation préférentielle à la chaleur, dissout des cellules Gram-négatives tuées, mais n'a aucune action sur les cellules Gram-positives tuées.

Les auteurs ont montré que les nucléotides solubles dans les acides résultant de l'action initiale de la ribonucléase sur les cellules Gram-positives tuées sont dégradés d'avantage avec libération de phosphate inorganique par une phosphatase non-spécifique.

En outre, les auteurs décrivent des études concernant la production et les propriétés de la ribonucléase et de la désoxyribonucléase également présente dans les filtrats de cultures.

ZUSAMMENFASSUNG

Die Lyse von "hitze-getöteten" Gram-positiven Zellen durch das bakteriolytische System von Actinomyces (Stamm A) hängt von dem Vorhandensein eines Enzyms vom Ribonuclease-Typus ab, welches die Zellen Gram-negativ und daher deren Lyse durch die proteolytischen Enzyme möglich

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macht. Dies wird durch die Tatsache gezeigt, dass das proteolytische System, durch besondere Hitze-Inaktivierung von seiner Ribonuclease-Aktivität befreit, abgetötete Gram-negative Zellen auflöst, aber auf abgetötete Gram-positive Zelle keinerlei Wirkung zusübt.

Es wurde gezeigt dass die säurelöslichen Nucleotide, welche durch die anfängliche Wirkung der Ribonuclease auf abgetötete Gram-positive Zellen hervorgehen, durch nicht spezifische Phosphatase unter Freiwerden von anorganischem Phosphat weiter abgebaut werden.

Untersuchungen über Bildung und Eigenschaften der Ribonuclease und der ebenfalls in den Filtraten vorkommenden Desoxyribonuclease werden beschrieben.

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