STUDIES ON THE AUTOLYTIC SYSTEMS OF GRAM POSITIVE MICRO-ORGANISMS

I. THE LYTIC SYSTEM OF *STAPHYLOCOCCI*

by

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INTRODUCTION

The phenomenon of bacterial autolysis is well known and has been the subject of numerous investigations since the early observations of RETTGER (1904), but only infrequent attempts have been made to study the chemical reactions concerned in the process. Of these, detailed studies have been chiefly concerned with the pneumococcus. In the preliminary investigations with this organism, crude preparations of an intracellular protease and a lipase, as well as several carbohydrate-splitting enzymes were isolated by AVERY AND CULLEN (1920*a*, 1920*b*, 1920*c*) from autolysed broth cultures. These enzymes possessed several features in common, *e.g.*, they were absent from the culture medium during the early phase of active growth, were easily denatured by heat, had p_H optima 7.0–7.8, and were inactive at p_H 4.5. Following this it was shown (AVERY AND CULLEN, 1923) that autolysates containing the cellular substances of the pneumococci possessed an enzyme, or a group of enzymes, capable of exerting a lytic action on suspensions of heat-killed pneumococci and, to a less extent, on suspensions of the closely related *Streptococcus viridans*. The enzyme corresponded closely in p_H optimum and stability to the enzymes previously studied. It was not type specific in its action and had no action on *Staphylococcus aureus*.

Extension of these studies (GOEBEL AND AVERY, 1929) showed that autolysis of pneumococci was accompanied by a proteolysis resulting in an increase in amino and non-coagulable nitrogen and by a lipolysis which gave rise to the liberation of ether-soluble fatty acids. When autolysates containing the active intracellular enzymes were added to heat-killed pneumococci, lysis of the cells occurred and there was an increase in the non-coagulable nitrogen and amino-nitrogen comparable to the changes accompanying spontaneous autolysis. When incubated with emulsions of the alcohol-soluble lipoids of pneumococci, these active autolysates caused an increase in the ether-soluble fatty acids.

Confirmation of much of the previous work was provided by DUBOS (1937) who also made the significant observation that, before lysis of the cells occurred, the cocci first became gram negative and this change in staining properties was not necessarily associated with actual complete lysis of the cells. The importance of this observation

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was emphasized when it was shown (THOMPSON AND DUBOS, 1938) that ribonucleic acid and a nucleoprotein were present among the substances released into solution when a suspension of rough Type II *Pneumococci* was incubated at 37° until the cells became gram negative. It was also observed that by a similar process of controlled autolysis, it was possible te release from staphylococci a substance which gave the reactions of ribonucleic acid.

Recent work by HENRY AND STACEY (1946) has revealed that gram positive cells contain at the cell surface a complex which consists of carbohydrate and the magnesium salt of ribonucleic acid, the latter being linked to basic proteins of the cell "cytoskeleton". When these components were removed from the cells by extraction at 60° with 2% sodium cholate solution, the gram staining reaction became negative. The importance of ribonucleic acid in the gram complex was shown by the fact that the residual gram negative "cytoskeleton" could, after suitable reduction, be recombined with magnesium ribonucleate to restore the initial gram positive staining characteristics.

That the ribonucleic acid and carbohydrate of the gram complex are in combination has been shown (WEBB, 1948) by the fact that hydrolysis of certain carbohydrate linkages located at or near the surface of gram positive organisms by the action of lysozyme, is accompanied by the liberation of ribonucleic acid from the cell together with a change in the staining reaction from gram positive to gram negative.

In view of these observations and following suggestions by Dr H. HENRY, it occurred to us that a study of such enzyme reactions may give rise to a more complete picture of the components of the bacterial cell. In particular, it was considered that by a detailed study of autolysis and the characterization of the enzymes involved in the various stages of cellular disintegration, a more complete understanding of bacterial structure could be obtained.

In preliminary investigations with a strain of *Clostridium welchii* and *Staphylococcus citreus* (B 9) it has been shown (Dr H. HENRY, private communication; WEBB, PH. D. Thesis, University of Birmingham, 1946) that the autolytic enzyme systems of these organisms are of similar nature and are composed of several separate and distinct enzymes. A detailed study of the change in the gram staining reaction which occurs as the first stage in the disintegration of the cells revealed that this is brought about by an enzyme which has the property of hydrolysing ribonucleic acid but not deoxyribonucleic acid. This enzyme differs from the ribonucleinase of animal tissues in several respects. It is inactive under oxidising conditions, but reactivated by reducing agents; has p_H optimum at p_H 8.0 and is irreversibly inactivated at p_H 4.0. At 80° it is completely inactivated, but at 60° the degree of inactivation is dependent upon the p_H of the solution, the enzyme being most stable at p_H 8. Although these properties are possessed by the enzymes of both *Cl. welchii* and *Staph. citreus* they are species and, to some extent, strain specific in their action on heat-killed gram positive cells. Thus, the enzyme from *Cl. welchii* readily converts heat-killed gram positive, *Cl. welchii* to gram negative forms, but has no action on heat-killed, gram positive *Staph. citreus, Staph. aureus*, or *Lactobacillus helv·ticus*. Similarly, the enzyme from *Staph. citreus* exhibited no action against killed gram positive *Cl. welchii* or *Staph. aureus*, but was active against a rough variant isolated from an aged culture of a strain of *Staph. aureus*. In view of the latter observation it was suggested that the specificity of these bacterial enzymes was, in some way, determined by the specific carbohydrates of the cell. It was further suggested that the second stage of autolysis, namely the lysis of the gram negative cell bodies which remain

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intact after the action of the nucleinase, was brought about by a proteolytic enzyme.

In the present work, the studies on the autolytic enzymes of staphylococci have been extended to the enzyme system responsible for this second stage of autolysis. The lytic enzyme system has been separated, concentrated in some degree and partially fractionated to reveal the presence of two proteolytic enzymes.

Isolation of Autolytic Strains of Staphylococci

Staphylococcus citreus B. 9 had been used in the studies of the first stage of autolysis and was known to autolyse with a maximum of 80–90% lysis at p_H 7–8 within 6 days at 37°. Other strains of staphylococci were isolated in pure culture by plating out pathological specimens. The autolytic properties of such strains were determined as follows:

The cells from 48 hour cultures of the organisms on glucose-peptone-agar were removed with distilled water and the resulting homogeneous suspension suitably diluted such that the addition of 10 drops from a uniform pipette to distilled water (5 ml) gave an opacity corresponding approximately to No. 10 on MACFARLAND's standard barium sulphate opacity scale. 10 Drops of this suspension were then added to each of a series of uniform test tubes containing 1.0 ml of the appropriate buffer (p_H 4.0–10.0 in intervals of 0.5) in physiological saline (4.0 ml). At the same time, a standard opacity scale was prepared by adding 1 to 10 drops of the cell suspension to 10 uniform tubes each containing 5 ml 2% formol saline (20 ml 40% formaldehyde in 1000 ml 0.85% sodium chloride), for it was found that direct comparison of the tubes of the experimental series with such a scale gave more accurate and reproducible results than when the barium sulphate opacity scale was used.

After the addition of toluene (0.1 ml), the tubes of the experimental series were tightly corked and incubated at 37°. When there occurred no further increase in the percentage of gram negative cells, as determined by the examination of stained smears (the change requiring a period which varied from 36 hours to 5 days according to the strain of staphylococcus), the corks were removed and the tubes replaced in the incubator for a further 24 hours in order to remove completely the toluene. The percentage lysis at each p_H value was then determined by direct comparison of the experimental series with the standard opacity scale.

Of the organisms examined, a strain of *Staph. albus* (9238) was selected which underwent maximum autolysis (90% lysis) at p_H 7–8 within 48 hours (Fig. 1) with the liberation of a very active lytic enzyme system. It differed in autolytic properties from *Staph. citreus* (B 9) previously employed in that it autolysed more rapidly (48 hours compared with 5 days for the latter organism). In consequence the division of autolysis into two separate and distinct stages, namely the conversion of the gram positive cell to the gram negative state and the subsequent lysis of the gram negative cell bodies, was not so sharply defined.

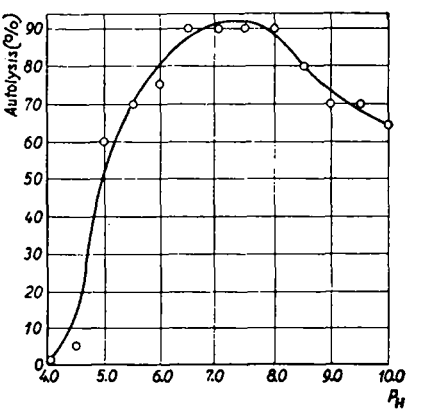

Fig. 1. Autolysis – p_H curve. *Staph. albus* (9238)

Proteolytic Changes during Autolysis

It appeared that much information concerning the mode of action of the proteolytic enzymes of the autolytic enzyme system of gram positive micro-organisms was to be obtained by examination of the type (total, non-coagulable and amino) and amount of protein and peptide nitrogen liberated from the cells at intervals during autolysis.

In such an examination carried out during the autolysis of *Staph. citreus B* 9 the total nitrogen

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was determined by means of a micro-Kjeldahl method capable of estimating 0.001–0.1 mg of nitrogen. The preliminary digestion was carried out in the usual manner with concentrated sulphuric acid, potassium bisulphate, copper sulphate and a trace of selenium as catalyst. As the potassium bisulphate and copper sulphate contained small quantities of nitrogenous matter, these substances were added in the form of a solution containing 120 mg of potassium bisulphate and 10 mg of copper sulphate per ml; 0.5 ml of this solution were used for each digestion. The nitrogen converted into ammonium sulphate was determined by distillation of the ammonia, in the apparatus described by MARKHAM (1942), into boric acid solution. The ammonium borate was then titrated with N/100. hydrochloric acid. Non-coagulable nitrogen was determined by adding 7 ml of 10% trichloroacetic to the required volume of solution diluted to 10 ml. After 1 hour the solution was filtered and an aliquot portion of the filtrate then analysed for total nitrogen by the micro-Kjeldahl method.

Amino nitrogen was estimated by the copper method of POPE AND STEVENS (1939).

It was realized, however, that the proteolytic enzymes were not the only enzymes present during autolysis which were capable of liberating nitrogen in these three forms. For example, the action of the ribonucleinase on the gram complex would result in the formation of soluble nitrogenous products, and these would be estimated in the solution. However, as has been shown in preliminary investigations, the proteolytic enzymes of the autolytic system are inhibited by reducing agents whereas the enzyme responsible for the conversion of the gram positive cell to the gram negative form is active under these conditions. Hence, by a comparison of the liberation of soluble nitrogen compounds from identical cell suspensions, one of which contained a suitable reducing agent, it was possible to obtain a measure of the proteolytic changes which accompany autolysis.

It was established that the estimation of total, non-coagulable and amino nitrogen was not affected by the presence of the reducing agent (hydrogen sulphide).

The experimental determinations were carried out as follows:

A suspension of the washed cells from 20 Roux bottles of a 48 hour culture of *Staph. citreus* B 9 on glucose-peptone agar in distilled water (250 ml) and 0.2 M phosphate buffer pH 7.5 (310 ml) was shaken homogeneous and then divided into two equal fractions. Of these, one was made up to 350 ml with distilled water, and the other adjusted to 350 ml with a saturated aqueous solution of hydrogen sulphide. Toluene (1 ml) was added to each, and the suspensions allowed to autolyse in closed vessels at 37°. Fractions of each suspension were withdrawn at suitable time intervals, centrifuged at high speed until the supernatants were completely clear, and aliquot fractions of the solutions analysed in duplicate for total, non-coagulable and amino nitrogen. The volume of the autolysate taken for analysis was decreased during the experiment from 70 ml to 10 ml.

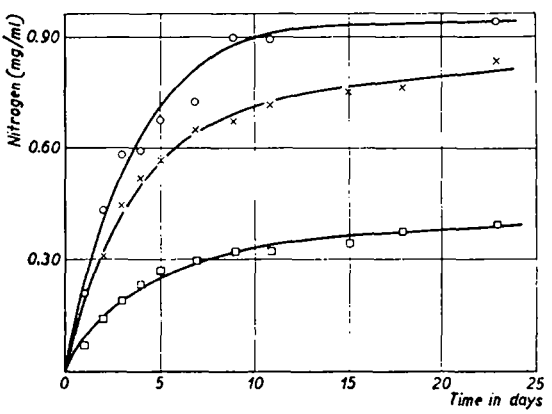

Fig. 2. Nitrogen liberated during autolysis of *Staph. citreus* (B 9). o——o total nitrogen, ×——× non-coagulable nitrogen, □——□ amino nitrogen

The results obtained with the two suspensions were similar, but, in the presence of the reducing agent, the nitrogen liberated in the above three forms (Fig. 3) was considerably less than that liberated from the cells undergoing normal autolysis (Fig. 2). A measure of the total, non-coagulable and amino nitrogen liberated from the cells by the action of the enzyme inhibited by hydrogen sulphide was provided by the difference between the results of Fig. 2 and Fig. 3 and is shown in Fig. 4.

The examination of stained smears during the course of the determinations showed that in both suspensions the cells became completely gram negative after 5 days. At the end of this period, no further

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lysis of the cells occurred in the suspension containing the reducing agent. In the second suspension, disintegration of the cells, as evidenced by the appearance of gram negative cell debris in the stained smears and by the clearing of the suspension, occurred. The results of Fig. 4, which show that little total nitrogen was liberated from the cells by the action of the lytic enzyme (*i.e.*, the enzyme inhibited by the reducing agent) during the first 3–4 days of the experiment, would indicate that this enzyme does not play any part in autolysis until the cells have become gram negative. Furthermore, the liberation of non-coagulable nitrogen was not appreciable until 4–5 days after the commencement of autolysis. The liberation of total nitrogen by the action of the enzyme inhibited with hydrogen sulphide is not in itself proof that this enzyme is a proteinase (endopeptidase). However, as the total nitrogen liberated during the first 8 days was mainly insoluble in trichloroacetic acid it is considered that this could only result from one type of proteolytic enzyme, namely a proteinase.

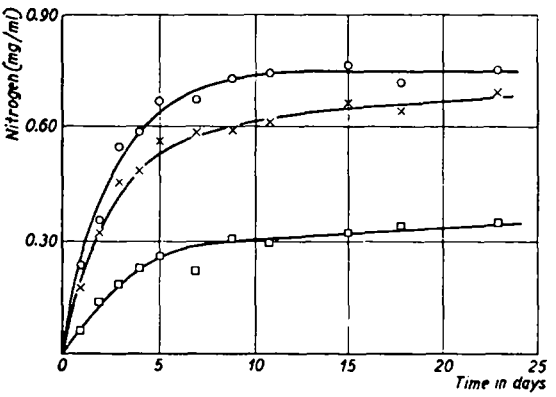

Fig. 3. Nitrogen liberated during autolysis of *Staph. citreus* (B 9) in the presence of hydrogen sulphide. o——o total nitrogen, ×——× non-coagulable nitrogen, □——□ amino nitrogen

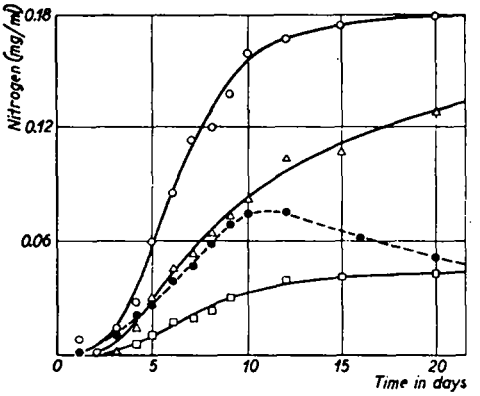

Fig. 4. Nitrogen liberated from autolysing *Staph. citreus* (B 9) by the action of the enzymes inhibited by hydrogen sulphide. o——o total nitrogen, △——△ non-coagulable nitrogen, •——• coagulable nitrogen, □——□ amino nitrogen

Fig. 4 shows that the non-coagulable nitrogen liberated continued to rise after the total nitrogen had reached a maximum value. The liberated coagulable nitrogen attained appreciable values after 3–4 days autolysis, reached a maximum after 8–10 days, and then decreased. From these results it was concluded that protein material was first liberated from the autolysing cells in a form insoluble in trichloroacetic acid solution and then underwent subsequent enzymatic hydrolysis into smaller units soluble in trichloroacetic acid. The fact that the amino nitrogen was but slowly increasing during this period showed that these units were polypeptides of relatively high molecular weight and were, therefore, produced by the hydrolysis of peptide bonds in the middle of polypeptide chains. That is, by the action of a proteinase (endopeptidase).

That the suspension of gram negative cells in which lysis had been inhibited by the presence of the reducing agent, still contain an active lytic enzyme was shown as follows:

The washed centrifuged suspension was suspended in 0.05 M citrate buffer pH 5.0 and a current of air drawn through for 6 hours to remove the last traces of hydrogen sulphide and to ensure oxidising conditions. The suspension was then centrifuged and the cells washed twice with distilled

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water. 10 Drops of a suspension of the cells in distilled water were added from a pipette to each of a series of uniform test-tubes containing physiological saline (4.0 ml) and 1.0 ml of the appropriate buffer (p_H range 4.0–10.0). The series was incubated at 37° for 24 hours and the degree of lysis in each tube then determined by comparison with a standard opacity scale prepared from the gram negative cells.

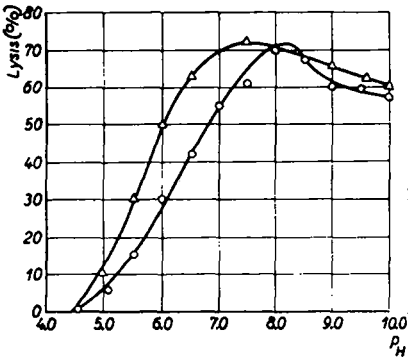

Fig. 5. p_H optimum for the lysis of the gram negative cytoskeletons of *Staph. citreus* (B 9) by the lytic enzyme of the autolytic system (o——o) and by trypsin (△—△)

The results (Fig. 5) show that the lytic enzyme of the autolytic system was not inactivated by contact with the reducing agent and that it had optimal activity at p_H 8.0–8.5. A similar p_H optimum was found when the gram negative cells were heated at 80° for 30 minutes and then lysed with trypsin (Fig. 5).

The Proteolytic Enzymes of the Autolytic System of Staphylococci

The activities of subsequent enzyme preparations were determined against 5% casein and 10% Witte peptone solutions adjusted to p_H 7.5. 1 ml of the substrate was added to the enzyme preparation and distilled water added to 5 ml. After the addition of toluene (0.1 ml) the solution was incubated at 37°. At convenient time intervals, fractions (1 ml) of the solutions were withdrawn and the hydrolysis determined by NORTHROP's (1926) modification of the formol titration. Control solutions containing enzyme preparations which had been heated at 80° for 30 minutes showed that no autohydrolysis of the substrates occurred under the conditions of the experiments and that the proteolytic enzymes were deactivated at 80°. Further, no appreciable self-hydrolysis was observed when the enzyme solutions were incubated alone.

The accuracy of the formol titration in the determination of proteinase action has been questioned. NORTHROP AND KUNITZ (1932) considered that the method was not a direct measure of the initial hydrolysis of the protein, but only determined th changes which occurred after the latter had been partially hydrolysed by the proteinase. However, in the present work it was considered that the use of NORTHROP's modification of the formol titration was justified for it was found that using 5 ml quantities of solution titrated with 0.01 N sodium hydroxide, 1% of the maximum attainable hydrolysis of casein could be easily measured. Thus, it was possible to detect the hydrolysis of one peptide bond in every hundred. Hydrolysis of this order would result in the production of polypeptides containing on average 100 amino acid residues and of average molecular weight, 11500. The hydrolysis of the casein substrate produced by the enzyme from *Staph. citreus* (B 9) never exceeded 5% of the value obtained by acid hydrolysis according to the method of VAN SLYKE (1912) and which therefore, represented the production of polypeptides of an average molecular weight of 2300. That the production of polypeptides of such molecular weight is due almost entirely to the action of a proteinase is evident from the work of WINNICK (1944) in which it was shown that the products remaining after the action of crystalline proteolytic enzymes on casein ranged from pentapeptides (molecular weight about 600) to heptapeptides (molecular weight about 800).

The presence of proteolytic enzyme in Staphylococcus autolysates

a) *Staph. citreus* (B 9). The experiments already described indicate that the proteolytic enzymes of the autolytic system do not come into operation until most of the cells have become gram negative. Therefore, in order to obtain an enzyme preparation relatively free from the enzyme responsible for the conversion of the gram positive

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cell to the gram negative state, autolysis was allowed to proceed at p_H 7 until the cells were gram negative. The suspension was then centrifuged, the cells resuspended at p_H 8 and allowed to autolyse further until examination of stained smears revealed marked disintegration of the gram negative cells.

The experimental procedure was as follows:

Cells from 10 ROUX bottles of a 48 hour culture of *Staph. citreus* B 9 on EVANS' peptone-glucose-agar were suspended in water (80 ml) and 0.2 M phosphate buffer p_H 7.0 (40 ml). Toluene (6 ml) was added and the suspension then allowed to autolyse at 37°. When the cells were uniformly gram negative (4–5 days) the suspension was centrifuged. The supernatant which contained the nucleinase of the autolytic enzyme system and, in some cases, weak proteolytic activity, was decanted and the cells resuspended in water (80 ml) and 0.2 M phosphate buffer p_H 8.0 (40 ml). Toluene (1 ml) was added and the suspension incubated at 37° until cellular disintegration occurred (4–7 days). The suspension was centrifuged at high speed and the turbid supernatant clarified by filtration through the SEITZ filter.

The hydrolytic activities of autolysates (4 ml) obtained in this manner against casein and peptone are shown in Table I.

TABLE I

HYDROLYSIS OF CASEIN AND PEPTONE BY AUTOLYSATES OF *Staph. citreus* B 9

Time (hours)	*Staph. citreus* B 9 autolysate. Preparation:					
	R_1		R_2		R_3	
	Increase in formol titration (ml 0.01 N NaOH)					
	Casein	Peptone	Casein	Peptone	Casein	Peptone
2.0			0.17	0.17	0.15	0.15
8.0			0.27	0.42		
16.5	0.41	1.52				
20	0.43	1.67			0.38	0.35
24			0.65	0.88		
41	0.89					
44					0.42	0.61
48			0.93	1.11		

b) *Staph. albus* (9238). Because of the rapidity with which autolysis of suspensions of this organism took place it was not possible to separate the enzymes of the autolytic system into the two distinct stages observed in the case of *Staph. citreus* B 9. Autolysis was allowed to proceed as follows:

Cells from 10 ROUX bottles of a 48 hours culture of *Staph. albus* (9238) on peptone-glucose-agar were suspended in distilled water (120 ml) and 0.2 M phosphate buffer p_H 7.5 (60 ml). The suspension was allowed to autolyse at 37° in the presence of toluene (2 ml). After 48 hours marked clearing had taken place and microscopic examination of stained smears showed that considerable disintegration of the cells had occurred. The suspension was centrifuged and the supernatant (solution F.A.) decanted. The deposit was suspended in distilled water (10 ml) and 0.2 M phosphate buffer p_H 7.5 (20 ml) and incubated at 37°. After 10 days the suspension was centrifuged at high speed until the supernatant (S.A.) was completely clear.

The hydrolytic activities of such autolysates (4 ml) against the chemical substrates are shown in Table II.

The results recorded in Table II show that the main proteolytic activity was contained in solution F.A. These solutions were, therefore, employed in subsequent work. The fact that with the second solution (S.A.) the ratio hydrolysis of peptone.

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hydrolysis of casein was, in general, greater than with solution F.A. suggested the presence in the autolysate of at least two proteolytic enzymes.

TABLE II

HYDROLYSIS OF CASEIN AND WITTE PEPTONE BY AUTOLYSATES OF *Staph. albus* 9238

Preparation	Time (hours) at 37°	Peptone	Casein
		Increase in formol titration ml 0.01 N NaOH	
F.A.1	43.0	1.45	1.32
S.A.1	41.5	0.65	0.53
F.A.2	45.5	1.85	3.86
S.A.2	20.0	0.47	0.50
F.A.3	46.5	2.15	3.53
S.A.4	17.5	0.00	0.00
F.A.5	47.5	1.95	2.50
S.A.5	46.5	1.13	1.05
F.A.6	16.0	1.64	2.51
F.A.8	20.0	1.45	2.07

The Lytic Activity of Autolysates on Killed Cells

The substrate of gram negative cells was prepared by re-heating a suspension of heat-killed staphylococci (B 9 or 9238), which had been rendered gram negative by the action of their own nucleinase at 80° for 30 minutes. After cooling, the suspension was centrifuged and the cells resuspended in a small volume of distilled water. 10 Drops of a uniform suspension of the substrate were added to each of a series of uniform tubes containing the crude enzyme solution (1 ml), 0.2 M buffer (p_H range 4.0–9.5, 1.0 ml) and physiological saline (3.0 ml). After the addition of toluene (0.1 ml), the tubes were corked and incubated at 37°. After three days the corks were removed and the tubes replaced in the incubator for a further 24 hours in order to completely remove the toluene. The degree of lysis in each suspension was then determined as described above.

Such experiments showed that the autolysates were capable of bringing about the lysis of the gram negative cell bodies with p_H optima which agreed closely with those obtained from the study of the spontaneous autolysis of *Staph. citreus* B 9 and *Staph. albus* 9238 respectively.

In contrast to the enzymes of the autolytic system responsible for the conversion of the gram positive cell to the gram negative state, the lytic fraction of the autolytic system was not species specific in nature. Thus, the lytic enzymes of *Staph. albus* 9238 or *Staph. citreus* B 9 was active against the gram negative forms of all gram positive organisms studied and readily lysed suspensions of the heat-killed, gram negative organism, *Bact. lactis aerogenes*.

Concentration of the Lytic Enzyme System

Preliminary purification of the lytic system was obtained by fractional precipitation with ammonium sulphate.

a) The lytic system of Staph. citreus (B 9). The enzymes were completely precipitated at 0.75 saturation of ammonium sulphate. An equal volume of a saturated aqueous solution of ammonium sulphate was added to the autolysate at 5°. The small precipitate (precipitate a) which slowly separated was centrifuged off after several hours, dissolved in distilled water and the solution dialysed against distilled water at 5° until free from ammonium sulphate. The dialysed solution was then diluted with distilled water to

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a volume equivalent to one tenth of the initial autolysate. The supernatant liquid was adjusted to 0.75 saturation of ammonium sulphate and the resulting precipitate (b) collected at the centrifuge after 4 hours at 0°, dissolved in distilled water and dialysed. In no case was any hydrolytic activity against either casein or peptone associated with this second precipitate. The activities of certain preparations of "precipitate a" (1 ml), determined against substrates of peptone and casein as previously described, are recorded in Table III.

TABLE III

PROTEOLYTIC ACTIVITY OF THE LYTIC ENZYME SYSTEM OF *Staph. Citreus* (B 9) CONCENTRATED BY AMMONIUM SULPHATE PRECIPITATION

Time (hours at 37°)	Enzyme Preparation									
	R1a				R2a				R3a	
	Peptone substrate		Casein substrate		Peptone substrate		Casein substrate		Peptone substrate	
	F	H	F	H	F	H	F	H	F	H
1.5	0.35	2.8								
2.5			0.18	0.7						
4.0	0.80	6.4			0.37	3.0	0.27	1.0		
4.5			0.28	1.1						
6.0	0.87	6.9	0.33	1.25			0.29	1.1		
21.5									0.91	7.3
23.0	1.70	13.9	9.85	3.15	0.84	6.7	0.76	2.9		
28.0									1.31	10.5
47.0	2.34	18.7	1.11	4.2	1.07	8.6	1.17	4.4	1.51	12.1

F = Increase in formol titration ml 0.01 N NaOH
H = % hydrolysis of substrate

These results reveal that the major part of the proteolytic activity (Table I) of the autolysates was concentrated in these precipitates and, therefore, that fractionation was accompanied by little loss in activity.

Such preparations were active in causing the lysis of killed, gram negative *Staph. citreus* B 9 with p_H optima 7.0–7.5 when examined according to the method previously described, but exhibited no activity against substrates of heat-killed gram positive cells.

b) *Staph. albus* 9238. Active autolysates were concentrated by precipitation with ammonium sulphate as in the case of the *Staph. citreus* autolysates. In contrast to *Staph. citreus*, the main bulk of the proteolytic activity was associated with the precipitate which separated at a concentration of 0.75 saturation of the salt (precipitate b). It is not, however, considered that this is due to any essential difference in the nature of the autolytic system of the two organisms, but to differences in such factors as the presence of inert material, concentration of the enzymes and duration of the initial autolysis.

After removing the ammonium sulphate by dialysis, the active precipitate was dissolved in a volume of distilled water equivalent to 1/6th the volume of the initial autolysate. The hydrolytic activities against peptone and casein of a series of such preparations (1 ml) are recorded in Table IV. Considerable variation was apparent in the ratio hydrolysis of casein: hydrolysis of peptone observed for the different enzyme preparations. Since the ratio of the rate of hydrolysis of one substrate to the rate of

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hydrolysis of a second substrate is constant for a given enzyme under similar conditions (IRVING, FRUTON, AND BERGMANN, 1941), such observations were taken as further indication that at least two proteolytic enzymes were associated with the staphylococcus lytic system.

TABLE IV

PROTEOLYTIC ACTIVITY OF THE LYTIC ENZYME SYSTEM OF *Staph. albus* (9238) CONCENTRATED BY AMMONIUM SULPHATE PRECIPITATION

Enzyme preparation	Time (hours at 37°)	Peptone substrate		Casein substrate	
		Increase in formol titration ml 0.01 N NaOH	% Hydrolysis	Increase in formol titration ml 0.01 N NaOH	% Hydrolysis
A 3 b	18.0	1.85	37.1	2.58	24.4
A 5 b	22.0	2.22	44.5	2.85	27.0
A 6 b	22.5	2.28	45.7	3.65	34.5
A 7 b	23.5	0.83	20.9	0.90	8.5
A 8 b	19.5	1.35	27.0	0.63	15.6
A 9 b	19.5	2.90	58.1	3.45	32.8
A 10 b	17.5	2.55	51.1	2.60	24.7
A 11 b	19.0	1.80	36.1	2.00	19.0

As further experiments revealed that the purification of the active fractions was not achieved by refractionation with ammonium sulphate, alternative fractionation procedures were investigated.

Concentration of Enzyme Activity by Ethanol Precipitation

The proteolytic activity of the *Staph. citreus* autolysate (R 3) was concentrated by precipitation with ammonium sulphate. After dialysis, the active solution (R 3 a. 20 ml) was cooled to 0° and an equal volume of ethanol, cooled to −15° slowly added. During the addition of the ethanol, the temperature of the enzyme solution was brought to −15°. The flocculent precipitate (R. 3a. I) which separated was collected at the centrifuge after 30 minutes and dissolved in distilled water (5 ml). The second precipitate (R. 3a. II) which separated when ethanol (40 ml), cooled to −15°, was added to the supernatant at the same temperature was collected after 30 minutes and dissolved in distilled water (5 ml). It was found that these solutions (R. 3a. I and R. 3a. II) possessed approximately the same proteolytic activity against the chemical substrates. Thus, although on the basis of total nitrogen content (R. 3a. I., 0.15 mg N/ml; R.3a. II., 0.26 mg N/ml.) it may be considered that solution R. 3a. I contained the greater proportion of the enzyme, the distribution of the activity between the two precipitates together with the relatively small quantities of the enzymes involved, led to the conclusion that the limit where fractional precipitation may be used as a method of purification had been realized.

Concentration of Enzyme Activity by Precipitation with Safranin

ROBERTSON (1906) found that a precipitate was formed when solutions of safranin and trypsin were mixed together and he concluded that trypsin, acting as a weak acid, combined with the basic dye. It was shown by HOLZBERG (1913) that this precipitate possessed proteolytic activity of the same order as the original solution, but that a

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considerable quantity of inert material was not precipitated by the safranin. It was not, however, found possible to purify trypsin by this method since all attempts to dissociate the trypsin-safranin complex resulted in the deactivation of the enzyme. Further studies by MARSTON (1923) showed that safranin or other azine bases precipitated 70% of the proteolytic activity of a pancreas extract leaving the remaining solution completely inactive. The enzyme-dye complex was dissociated, with considerable deactivation, by 0.2% hydrochloric acid. Extension of safranin precipitation to bacterial enzymes showed that the proteases of whole cultures of *Cl. histolyticum* and *Cl. sporogenes* were completely precipitated by the dye (BLANC AND POZERSKI, 1920) whereas the proteolytic enzymes of fungi were not precipitated (WAKSMANN, 1918).

When an equal volume of 0.5% aqueous safranin solution was added either to the crude staphylococcus autolysate or to an enzyme preparation concentrated by ammonium sulphate fractionation, the proteolytic activity was completely precipitated. The enzyme-dye precipitates possessed considerable hydrolytic activity against the chemical substrates, casein and peptone, whereas the residual supernatant solutions exhibited no action on these substances. In these experiments, the hydrolysis of the substrates was followed by measuring the increase in amino nitrogen by the copper method of POPE AND STEVENS (1939), for owing to the presence of the safranin, it was not possible to measure the proteolysis by means of the formol titration.

It was not however, possible to dissociate the enzyme dye precipitate over the p_H range 4–10. Below p_H 4, complete loss of activity occurred.

Properties of the Proteolytic Enzymes of the Staphylococcus Autolytic System

1. *p_H optimum*

The hydrolysis of Witte peptone and casein produced after 48 hours at different p_H values (p_H 4.5–9.5) by a preparation (A3b) of the lytic system of *Staph. albus* 9238 was determined as follows:

A solution of the substrate (1 ml)and buffer (2 ml) with distilled water (4 ml) was approximately adjusted to the desired p_H (glass electrode) by the cautious addition of either 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. The enzyme solution (1 ml) was added, the solution adjusted to a volume of 10 ml with distilled water and the p_H determined. The amino nitrogen content of these solutions (1 ml) was determined by the formol titration immediately after their preparation and again after 48 hours at 37°.

The optimum p_H for the action of the proteolytic enzymes on both casein and peptone, as determined by this method was p_H 7.5 in each case.

2. *Stability*

The enzyme preparation (A5b, 5 ml), adjusted to the given p_H (glass electrode) and diluted to a volume of 10 ml, was immersed in a thermostat at 50°. At intervals of 30 minutes, aliquot fractions of the solution were withdrawn, adjusted to p_H 7.0 and examined for hydrolytic activity against casein and Witte peptone, the extent of proteolysis being taken as the increase in the formol titration value after 48 hours at 37°.

The results showed that the lytic system was most stable towards denaturation by heat at p_H 8.0. The concept previously put forward that at least two proteolytic enzymes are associated with the lytic fraction of the autolytic system, was substantiated by these results in that a parallel destruction of the casein and peptone activities was not observed. For example, when the enzyme solution was heated at acid p_H values the activity against peptone was decreased to a greater extent than that against casein.

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In particular, when the enzyme was heated at 50° and p_H 6.0 for 1 hour the peptone activity was almost completely removed, whereas the solution still exhibited 40% of its initial activity against casein. Furthermore, it was found that when preparations of the enzyme were allowed to stand at room temperature and p_H 6.5 for several days the hydrolytic activity against peptone was almost completely destroyed though much of the casein activity was retained (Table V).

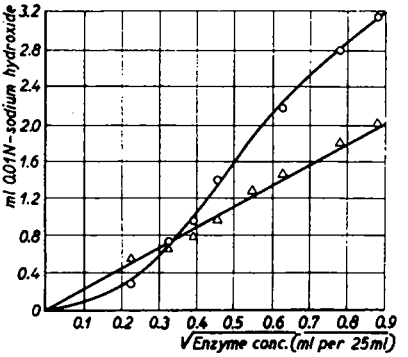

Fig. 6. Activity $\sqrt{\text{concentration}}$ curves for the action of the proteases of the autolytic system of *Staph. albus* (9238). o——o Activity against casein, △——△ Activity against Witte peptone.

TABLE V

PROTEOLYTIC ACTIVITY OF *Staph. albus* LYTIC ENZYME (PREPARATION A 3b) AFTER 5 DAYS AT ROOM TEMPERATURE

Time (hours at 37°)	Increase in formol titration ml 0.01 N NaOH	
	Peptone substrate	Casein substrate
23.0	0.00	0.75
31.0	0.00	0.94
48.0	0.19	1.30

3. *The Effect of Enzyme Concentration on the Hydrolysis of Casein and Peptone*

The hydrolysis of the casein and peptone substrates (1 ml) produced, at p_H 7.5 (0.2 M phosphate buffer 0.5 ml), by increasing concentrations of the enzyme (preparation diluted to 1.0 ml with distilled water) was determined by measuring the increase in the formol titration value after incubation at 37° for 48 hours. Such experiments showed that the activity against peptone was proportional to $\sqrt{\text{concentration}}$ (Fig. 6) as is the case with a single proteolytic enzyme, whereas the activity against casein was more complex, the hydrolysis of the substrate being greater at high concentrations than would be expected for a single enzyme. It is, therefore, concluded that the peptone-hydrolysing enzyme, itself unable to cause the hydrolysis of casein, brings about the hydrolysis of the split products resulting from the action of the second enzyme on this substrate.

4. *The Effect of Enzyme Concentration on the Lysis of Heat-killed Gram Negative Staph. albus 9238*

The lytic activity of increasing concentrations of the enzyme (preparation A6b) at p_H 7.5 determined against a substrate of the gram negative forms of heat-killed *Staph. albus* 9238 revealed that the lysis was not directly proportional to the enzyme concentration (Fig. 7).

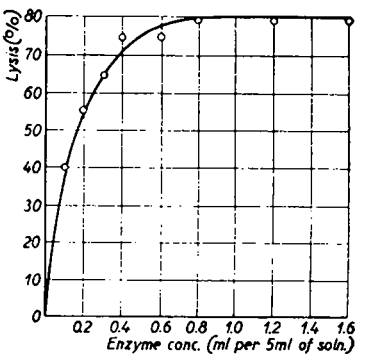

Fig. 7. Activity-concentration curve for the action of the lytic enzyme of the autolytic system of *Staph. albus* (9238) on the gram negative cytoskeletons of heat-killed, *Staph. albus*.

5. *The Effect of Oxidation and Reduction on the Proteolytic Enzymes*

A solution of the lytic system of *Staph. albus* 9238 (Preparation A3b, 2 ml) was added

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to (a) a saturated aqueous solution of hydrogen sulphide adjusted to p_H 7.5 (2.0 ml) and (b) 3% hydrogen peroxide (2 ml). The solutions were allowed to stand at 0° for 24 hours and then examined for hydrolytic activity against peptone and casein. The results (Table VI) in conjunction with those of Table V showed that the lytic system of the staphylococcus autolysate was composed of at least two proteolytic enzymes. The fact that the enzyme solution in the presence of hydrogen sulphide was capable of hydrolysing casein, but exhibited no action against peptone, revealed that one of these enzymes was mainly responsible for the hydrolysis of protein fragments (such as peptones).

TABLE VI

THE EFFECT OF OXIDISING AND REDUCING AGENTS ON THE ACTIVITIES OF THE PROTEOLYTIC ENZYMES OF *Staph. albus* (9238)

Time (hours at 37°)	Enzyme A 3a with hydrogen sulphide		Enzyme A 3a with hydrogen peroxide	
	Increase in formol titration ml 0.01 N NaOH			
	Peptone substrate	Casein substrate	Peptone substrate	Casein substrate
23.0	0.00	0.00	0.20	0.80
31.0	0.00	0.22	0.20	0.95
48.0	0.00	0.33	0.40	1.30

Correlation of the proteolytic activity of the enzyme preparation with the lytic activity against the gram negative forms of *Staph. albus* was provided by the fact that both activities were inhibited by reducing conditions. Identical results were obtained with further preparations of the lytic system of *Staph. albus* 9238 and of *Staph. citreus* (B 9).

6. *The Effect of Concentration of Reducing Agent on the Action of the Enzyme System*

a) On the lysis of gram negative forms of staphylococci. The effect of increasing concentrations of 0.1 M sodium thioglycollate (0.00 to 1.0 ml) on the activity of the enzyme preparation (A 12 b, 0.5 ml) (at p_H 7.0) against the gram negative forms of *Staph. albus* (9238) was determined by the standard procedure. The results (Fig. 8) show that the inhibition of lysis increased with increasing concentration of sodium thioglycollate, but that complete inhibition was not realized.

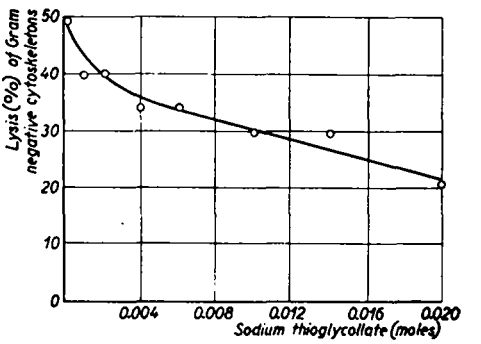

Fig. 8. Inhibition of the lytic enzyme of the autolytic system of *Staph. albus* (9238) with sodium thioglycollate

b) On the hydrolysis of Casein and Peptone

0.1 M sodium thioglycollate was added in increasing concentration to each of two series of tubes containing 1.5 ml of the enzymes preparation (A 12 b) and distilled water to 4.0 ml. After 24 hours at 0° 5% Witte peptone (1 ml) was added to each tube of one series and 10% casein (1 ml) to each tube of the other. 1 ml quantities of each solution of the two series were withdrawn initially and after 48 hours at 37° and analysed for amino nitrogen by the formol titration method.

The results (Fig. 9) revealed that, whereas the proteolytic activity against both

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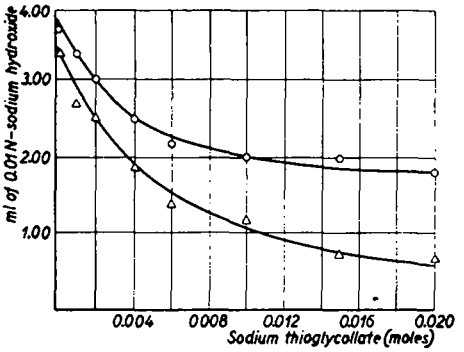

Fig. 9. Inhibition of the proteolytic enzymes of the autolytic system of *Staph. albus* (9238) with sodium thioglycollate. o——o Casein substrate, △—— △ Peptone substrate

Witte peptone and casein was considerably inhibited by sodium thioglycollate, the inhibition was not proportional to the concentration of the reducing agent and that complete inhibition was not obtained.

Separation of the proteolytic activities

Adsorption. Both enzymes were partially or completely adsorbed according to the concentration of the adsorbent, on Cγ alumina at all p_H values and 0°, but the adsorbed enzymes could not be eluted by either acid or alkaline reagents.

p_H adjustment. It was observed that when solutions of the staphylococcus lytic enzyme system were adjusted to acid p_H values, turbidity resulted and a precipitate tended to separate.

The precipitate which separated when preparation (A 8 b, 8 ml) of the lytic system of *Staph. albus* (9238) was adjusted to p_H 6.0 and allowed to stand for 24 hours, was collected at the high-speed centrifuge. The precipitate (A 8b, 6) was dissolved in distilled water (8 ml) and the supernatant adjusted to p_H 5.0. After 2 hours at 0° the latter solution was centrifuged and the precipitate (A 8b, 5) dissolved in distilled water (8 ml). After adjusting the final supernatant (A 8b, P) to neutrality, the three solutions were examined for activity against the two substrates casein and peptone. The results (Table VII) show that although the ratio of the two proteolytic activities was different in each solution, complete separation, was not achieved and that the enzymes were only partially precipitated at the two p_H values.

TABLE VII

FRACTIONATION OF THE PROTEOLYTIC ENZYMES OF *Staph. albus* (9238) BY ALTERATION OF pH

Enzyme	Increase in formol titration (ml 0.01 N NaOH) after 20 hours at 37°	
	Peptone substrate	Casein substrate
A 8 b.6	0.45	0.13
A 8 b.5	0.15	0.30
A 8 b.P	0.80	1.05

Acetone fractionation. The enzyme solution (A 10 b, 35 ml) cooled to 0°, was brought to a concentration of 30% acetone by the addition of acetone (15 ml) cooled to −15°. The resulting precipitate was collected (centrifuge) and dissolved in water (5 ml). In a similar manner, precipitates were collected at acetone concentrations of 50 and 80% and separately dissolved in distilled water (5 ml). These solutions (1 ml) were examined for hydrolytic activity against peptone and casein in the usual way. It was found (Table VIII) that the precipitates which separated at acetone concentration of 30 and 50% respectively were active in causing the hydrolysis of peptone but exhibited no activity against casein. Since it has previously been shown (Tables V and VI) that

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an enzyme solution may be obtained which hydrolyses casein only, it is concluded that the staphylococcus lytic system contains two proteolytic enzymes one of which hydrolyses casein, but not peptone and the other peptone, but not casein. Although the precipitates which separated at acetone concentrations of 30% and 50% were without activity against the casein substrate (Table VIII) it was not possible to separate

TABLE VIII

FRACTIONATION OF THE PROTEOLYTIC ACTIVITY OF THE STAPHYLOCOCCUS LYTIC ENZYME SYSTEM BY ACETONE PRECIPITATION

Enzyme activity precipitated at acetone concentration of (%)	Increase in formol titration (ml 0.01 N NaOH) after 20 hours at 37°	
	Peptone substrate	Casein substrate
30	0.57	0.00
50	0.52	0.00
80	1.20	1.75

the peptone hydrolysing enzyme by direct precipitation at the latter concentration for the addition of an equal volume of acetone to the concentrated lytic system resulted in the separation of a precipitate which was capable of hydrolysing both substrates. By further fractionation of this precipitate with acetone at concentrations of 50, 60, 70 and 80% it was possible to obtain the peptone hydrolysing enzyme free from activity against the casein substrate but in no case was the casein activity completely freed from activity against peptone. However, as the separated peptone hydrolysing enzyme was not capable of bringing about the lysis of a suspension of the gram negative forms of staphylococci whereas the enzyme solution containing the casein hydrolysing enzyme together with some peptone activity, caused almost complete lysis of a cell suspension at p_H 7.5 after 72 hours at 37°, it is concluded that the casein hydrolysing enzyme of the autolytic system is responsible for the initial lysis of the gram negative cell bodies. The protein fragments which result from the action of this enzyme on the cells are considered to be further hydrolysed by the action of the peptone hydrolysing enzyme.

DISCUSSION

The results of the foregoing experimental work reveal that the lytic fraction of the staphylococcus autolytic system contains two proteolytic enzymes, one of which is presumably adapted to the hydrolysis of the complex high molecular weight cell proteins, to smaller units. These units are then further broken down by the action of the second proteolytic enzyme which, alone, has no action on the intact gram negative cytoskeleton. These enzymes have no action on the corresponding strain of heat-killed, gram positive staphylococcus, and only play their part in spontaneous autolysis when cells have become gram negative by the action of the, as yet, undetermined species specific factor and the nucleinase. Since ribonucleinase-free proteolytic enzymes, such as trypsin, of animal origin are unable to lyse suspensions of heat-killed gram positive cells, but readily dissolve the gram negative forms of these organisms, it is concluded that the final disintegration of the gram negative cell bodies which occurs in autolysis, is a direct result of the action of the proteolytic enzymes.

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The fact that the proteolytic enzymes of the staphylococcus lytic system are inhibited by hydrogen sulphide and sodium thioglycollate is of interest since most proteinases of the higher animals, plants and many bacteria are activated by compounds containing -SH groups. Bacterial proteolytic enzymes which are active under oxidising conditions, but which are inhibited by reducing agents, have, however, been described by MASCHMANN (1938) and WEIL, KOCHALATY, AND SMITH (1939). Furthermore, the results of GOEBEL AND AVERY (1929) revealed that the liberation of non-coagulable nitrogen from suspensions of autolysing pneumococci was much greater under aerobic than under anaerobic conditions and would indicate that the enzyme responsible was inhibited by the lack of oxygen.

It is fully realized that a complete picture of bacterial autolysis cannot be obtained from the evidence thus far obtained and it is intended to extend these investigations to other enzymes of the autolytic system.

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SUMMARY

The first major stage of the autolysis of certain strains of staphylococci involves the removal of ribonucleic acid from the cell by essentially the action of bacterial ribonucleinase.

The second stage of the autolysis, involving the dissolution of the residual gram negative cytoskeletons, is brought about by a proteolytic enzyme system. Fractionation of the components of this system has revealed the presence of two proteolytic enzymes one of which hydrolyses casein but not peptone, and another which hydrolyses peptone but not casein. The latter enzyme is without action upon the gram negative staphylococcal cytoskeletons which are readily dissolved by the combined action of the two enzymes. It is suggested that the gram negative cytoskeletons are degraded by the casein-hydrolysing enzyme and that the resulting split products are further broken down by the action of the peptone-hydrolysing enzyme.

RÉSUMÉ

La première phase de l'autolyse de quelques souches des staphylocoques comprend l'élimination de l'acide ribonucléique de la cellule essentiellement sous l'action de la ribonucléinase bactérienne.

La seconde phase d'autolyse qui renferme la dissolution des cytosquelettes gram-négatifs résiduels se produit sous l'action d'un système d'enzymes protéolytiques. La séparation des composés du système a révélé la présence de deux enzymes protéolytiques, l'une hydrolysant la caséine et l'autre hydrolysant la peptone. Cette dernière enzyme est sans effet sur les cytosquelettes gram-négatifs des staphylocoques qui se dissolvent facilement sous l'action combinée des deux enzymes. On suggère ici que les cytosquelettes gram-négatifs sont dégradés par l'action de l'enzyme hydrolysant la caséine et, par suite, les produits de dégradation sont encore dégradés davantage par l'action de l'enzyme hydrolysant la peptone.

ZUSAMMENFASSUNG

Der erste Schritt in der Autolyse gewisser Stämme von Staphylokokken ist die Abspaltung von Ribonukleinsäure von der Zelle, hauptsächlich durch die Wirkung von bakterieller Ribonukleinase.

Der zweite Schritt in der Autolyse, in dem die restlichen Gram negativen Cytoskelette in Lösung gehen, wird durch ein proteolytisches Enzym bewerkstelligt. Fraktionierung der Komponenten dieses Systems deutet auf die Gegenwart von zwei proteolytischen Enzymen. Das eine hydrolysiert Casein, aber nicht Pepton, das andere hydrolysiert Pepton, aber nicht Casein. Das letztere reagiert nicht mit Cytoskeletten der Gram negativen staphylokokken die bei der vereinten Wirkung beider Enzyme leicht löslich sind. Es wird vorgeschlagen, dass die Gram negativen Cytoskelete durch das Casein hydrolysierende Enzym abgebaut werden und die erhaltenen Abbauprodukte durch das Pepton hydrolysierende Enzym weiter abgebaut werden.

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