LONG CHAIN FORMATION BY STRAINS OF GROUP A STREPTOCOCCI IN THE PRESENCE OF HOMOLOGOUS ANTISERUM: A TYPE-SPECIFIC REACTION*

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Plates 34 to 36

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In the course of studies of the bactericidal system for the detection of type-specific antibody to group A streptococci (1), a number of strains were found which grew in extremely long chains in the presence of homologous antiserum. This paper reports some preliminary experiments designed to gain further insight into the mechanism of this phenomenon. The results indicate that long chain growth in liquid media of the strains of streptococci studied is dependent in part upon the interaction of type specific antibody and the M protein of the cells.

Materials and Methods

Cultures.—A type 30 strain of group A streptococcus used in most of this work was obtained from Dr. Rebecca Lancefield and is designated in her laboratory as D-24. This was originally Griffith's strain "Quinn." It had been passed through mice repeatedly to enhance its virulence and was received as a lyophilized culture. It grew luxuriantly and resisted phagocytosis in human blood in the absence of antibody. 0.5 ml. of a 10^{-6} dilution of an 18 hour broth culture was lethal for mice by the intraperitoneal route in less than 5 days. Surface colonies on blood agar were typically large, flat, matt, with good zones of beta hemolysis after 24 hours incubation.

In contrast to the D-24 Quinn strain of type 30 streptococci, a rabbit-adapted strain of type 30, known in the literature as the "Gay" strain, was also studied.[1] While this strain was typable, and therefore contained M protein, it failed to grow in long chains in the presence of homologous antiserum. Fourteen additional strains of type 30[2] were also studied. Several strains of type 12 streptococci were screened for the long chain reaction. These included strains recently isolated from the throats of patients at Northwestern University Clinics, Great Lakes Naval Training Center, and Children's Memorial Hospital (Chicago), as well as laboratory strains. Two type 12 strains were selected for detailed study. One of these, obtained from Dr. Lancefield, and designated as SF 42, was known to produce large amounts

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[1] Kindly supplied by Dr. Beatrice Seegal.

[2] Kindly supplied by Dr. Rebecca Lancefield.

of M protein but was only moderately virulent for mice. Its virulence was enhanced by serial passage through mice nine times in our laboratory. Another strain designated in our laboratory as 19162 GL was classified as type 12 upon original isolation at Great Lakes Naval Training Center, but had subsequently dissociated following repeated subculture on artificial media and was no longer typable during the course of this investigation.

All strains were preserved by lyophilization. Grouping and typing rections were confirmed by capillary precipitin tests according to the method of Swift, Wilson, and Lancefield (2). Stock cultures were prepared by inoculation of lyophilized samples into Todd-Hewitt broth. After one or two broth passages to stimulate growth, an 18 hour broth culture was kept refrigerated at 4°C. for several weeks. Overnight growth of subcultures made from the stock culture were used for each experiment.

Bactericidal Tests and Studies of Phagocytosis.—The method employed for the bactericidal tests was a further modification of the original method of Todd (3) and the subsequent modifications of Rothbard (4) and of Kuttner and Lenert (5).[3] The method consisted of adding varying dilutions of a culture of known serologic type to fresh, heparinized normal human or rabbit whole blood, to which was added the serum to be tested for type-specific antibody. After 3 hours of incubation at 37°C. in a roller tube apparatus designed to rotate the tubes end over end, a small sample of each tube was inoculated into melted agar containing 5 per cent rabbit blood and the contents were poured into Petri dishes. After overnight incubation the number of colonies were counted as a measure of the survival of organisms in the blood-antiserum mixtures. The details of this method and a study of various factors involved in the bactericidal effect will be published elsewhere.

Studies of phagocytosis were made by examining mixtures of heparinized fresh blood and antibody in which relatively heavy inocula (10^{-2} dilution of an 18 hour culture) of organisms were incubated for 3 hours in roller tubes. Blood films prepared in the conventional manner were stained with Wright's stain. One hundred polymorphonuclear leucocytes were counted on each of two duplicate slides and the percentage containing streptococci were recorded as the phagocytic index.

Antisera.—Antisera against various types of streptococci were prepared by injecting rabbits intravenously with suspensions of heat-killed streptococci as described by Lancefield (6). Some additional antisera that were tested were prepared in other laboratories.[4] Assays for type-specific antibody in these sera were made by the bactericidal test described above, by capillary precipitin tests (2) and, in some instances, by mouse protection tests (7). Type-specific antisera were made by absorption of crude rabbit antisera with equal volumes of sedimented organisms from cultures of heterologous types. This procedure was repeated, as necessary, to remove traces of cross-precipitating antibody until sera reacted only with extracts of homologous types.

The Long Chain Reaction.—After the original observations of long chain growth in the bactericidal system described above, it was found that long chain formation would take place in Todd-Hewitt broth to which had been added homologous antiserum. The technique finally adopted for the study of the long chain phenomenon consisted of adding to 0.3 ml. of Todd-Hewitt broth, in a 9.0 × 100 mm. (outside diameter) test tube, 0.05 ml. of antiserum and 0.1 ml. of a 10^{-2} dilution in fresh Todd-Hewitt broth of an overnight culture. Tubes containing normal rabbit serum instead of antiserum were included in all experiments as controls. The tubes were closed with sterile rubber stoppers and rotated end over end 8 times per minute at 37° C. for 4 hours. At the end of this time hanging drop preparations were made and the number of cocci in 50 chains were counted.

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[3] We are indebted to Dr. Rebecca Lancefield for suggestions and help in the use of this method.

[4] Kindly supplied by Dr. Elaine Updyke and by Dr. Rebecca Lancefield.

RESULTS

Morphology.—The initial observations of the formation of long chains of the D-24 strain of type 30 streptococci were made when blood films were examined to study phagocytosis. Although phagocytosis was extremely active in the blood cultures containing homologous antibody, the inoculum employed was large enough so that many extracellular chains of streptococci could be examined. These chains appeared extremely long, approximately 50 to 100 cocci in each unit, compared with chains 4 to 12 cocci in length observed in the control mixtures containing normal rabbit serum rather than homologous antibody (Figs. 1 and 2).

An unusually prominent capsule of striking size and morphology surrounded young cultures of this strain with or without the addition of antibody, but the long chain growth induced by the presence of antibody made the morphological features of the capsule particularly evident (Figs. 1 to 3). The capsules were identical with those described by Seastone (8) in an earlier study of certain strains of group A streptococci that form large capsules in young cultures grown in blood or serum. The capsular material stained variably in different preparations but most often appeared either as a granular eosinophilic material (Fig. 3) or as a fine fibrillar structure. Within the capsular material deeply basophilic granules appeared parallel to the chain of cocci (Fig. 4). In some preparations fine basophilic fibrils appeared to form septa between the cocci.

The individual cocci often appeared flattened at each end and seemed to be dividing in the manner described by Bisset (9–11) as characteristic of "rough" variants of certain bacteria that spontaneously grow in long chains on solid media. Capsules were readily demonstrated by India ink preparations and could also be stained with Giemsa and other polychrome stains but not with methylene blue or Gram stains. Long chains of streptococci grown in homologous antibody were examined under the phase contrast microscope in view of Tomcsik's (12, 13) use of this method for the demonstration of capsular changes induced by antibody to certain strains of bacilli. Phase contrast microscopy did not reveal visible capsular changes in the D-24 streptococcus grown in antiserum.

Conditions for the Long Chain Growth.—A number of observations were made with regard to the factors influencing long chain growth in the presence of homologous antiserum. No difference in chain length was noted when cultures were held stationary rather than rotated during incubation. Maximal chain length was reached by 3 to 4 hours. Longer intervals of incubation up to 24 hours did not produce longer chains, and in some cases the chains began to break up after 7 to 8 hours' incubation.

The long chains of streptococci were quite delicate, and preparations of smears by usual bacteriological methods or by the blood smear technique often resulted in fragmentation of the chains. Table I presents the results of a typical experiment comparing the chain length of the D-24 strain in the presence of

homologous rabbit antiserum and normal rabbit serum. In most experiments the mean chain length in preparations containing antibody was about 100 cocci with a standard deviation of 50. In the control samples grown in normal serum the mean length was usually 6 to 12 cocci with a standard deviation of 7.

Serial dilution of the antibody was associated with progressive diminution in the length of the chains (Table II). The effect was no longer apparent in broth cultures containing less than 0.25 per cent antiserum. Heating the antiserum for 1 hour at 56°C. did not destroy its long chain promoting effect. This propperty was considered therefore to be independent of complement, properdin or other heat-labile serum factors.

Type Specificity of Long Chain Formation.—Addition to the media of rabbit antisera to heterologous types of streptococci (types 6, 12, and 19 antisera were

TABLE I

Chain Length of Group A Streptococcus Type 30 (D-24 Quinn) Grown in Serum-Broth Mixtures

	Average No. cocci per chain in 3 hr. cultures*			
	With specific antiserum		*With normal serum*	
	mean‡	*standard deviation*	*mean*	*standard deviation*
Experiment 1	93	±59	12	±7.0
Experiment 2	101	±55	13	±7.4

* Counts made in wet, hanging drop preparations.

‡ Average of 100 units counted.

studied) failed to produce long chain growth. The long chain effect was also abolished when type 30 antisera were absorbed with organisms of homologous type. The removal of type-specific antibody following absorption was confirmed by capillary precipitin tests and by bactericidal tests for type-specific antibody.

Antisera specific for type 30 organisms were prepared by absorption with streptococci of heterologous types. Following absorption such sera were tested by capillary precipitin and bactericidal tests and the type specificity of the reactions confirmed. The type specific antisera thus prepared produced long chain growth of the D-24 strain (Table III).

Several other lots of specific absorbed antisera prepared in other laboratories by immunization of rabbits with a variety of strains of type 30[5] were tested and similarly produced long chain growth.

The type 30 Gay strain was also used to absorb a crude homologous antiserum. Although this strain does not grow in long chains in the presence of homologous antiserum, it was capable of absorbing the long chain–forming

[5] Kindly supplied by Dr. Elaine Updyke and Dr. Rebecca Lancefield.

property from the serum. The antiserum thus absorbed did not induce long chain growth of the D-24 type 30 strain.

In a further series of experiments to show the dependence of long chain formation upon the M protein of the cells, the D-24 strain was grown in Todd-Hewitt broth containing 0.05 per cent crystalline trypsin (Armour). This

TABLE II

Effect of Dilution of Antiserum in Broth on Chain Length of Group A Streptococcus Type 30 (D-24 Quinn) after 3 Hour Incubation

Specific antiserum	No. cocci per chain*
per cent	*mean‡*
25.0	101.4
2.5	45.0
0.25	16.8
0.025	10.9
0.0025	10.9
0.00000	12.8

* Counts made in wet, hanging drop preparations.

‡ Average of 100 units counted.

TABLE III

Type Specificity of Long Chain Formation Induced by Antibody to Group A Streptococcus Type 30 (D-24 Quinn)

Antiserum	Chain length*	
	mean‡	*standard deviation*
T30 crude	45.7	±22
T30 absorbed with T6	50.3	±18
T30 absorbed with T30	6.9	± 3
T6 crude	8.0	± 3
T12 crude	7.4	± 3
Normal serum	8.0	± 4

* Counts made on stained dry films.

‡ Average of 100 units counted.

procedure has been shown by Lancefield (14) to destroy M protein without affecting the viability of the cells. Extracts from organisms grown in this way were shown to be free of demonstrable M protein by precipitin tests with specific antiserum. When these trypsinized, M protein–free cells were subcultured into trypsin broth containing excess homologous antiserum they failed to exhibit long chain growth (Table IV). The supernatant trypsin broth containing antisera was heated at 60°C. for 30 minutes to inactivate the trypsin and then reinoculated with normal D-24 cells. Long chain growth was again observed,

indicating that the trypsin had not completely destroyed the antiserum. The presence of excess antibody was also confirmed by precipitin tests.

Is Long Chain Growth a Form of Dissociation Produced by Antibody?.—Todd and Lancefield (15) have previously demonstrated that certain strains of streptococci can be made to dissociate from matt colonies rich in M protein to glossy colonies free of M protein by repeated passage in cultures containing homologous antibody. Rothbard and Watson (16) have also demonstrated a similar dissociation from M-rich to M-poor organisms occurring in the throats of patients during convalescence from streptococcal pharyngitis. The following

TABLE IV

Effect of Tryptic Digestion of Group A Streptococcus Type 30 (D-24 Quinn) on Long Chain Growth

A Primary culture (18 hrs. at 37°C.)		B Subculture of A‡ (3 hrs. at 37°C.)		
Media	Anti-M precipitin*	Media	Serum added	Chain length
1. Trypsin-broth	0	1. Trypsin-broth	Anti-30	20.0
2. Trypsin-broth	0	2. Trypsin-broth	Normal	10.5
3. Trypsin-broth	0	3. Plain broth	Anti-30	104.2
4. Trypsin-broth	0	4. Plain broth	Normal	5.2
5. Plain broth	++++	5. Plain broth	Anti-30	95.8
6. Plain broth	++++	6. Plain broth	Normal	5.4

* Precipitin tests made by testing extracts of overnight growth of organisms against type-specific antiserum.

‡ Subcultures in media listed in B were made from primary culture of the corresponding number listed in A. Chains counted after 3 hours incubation in subculture.

experiments were performed to determine whether long chain growth of the D-24 strain is a form of dissociation induced by homologous antibody.

The D-24 strain was inoculated into antiserum-broth mixtures and grown for 3 hours. Growth of long chains occurred. Subcultures were made in fresh Todd-Hewitt broth containing normal rabbit serum. The organisms now grew in short chains (Table V).

The sediment from a culture of D-24 grown in Todd-Hewitt broth was suspended in an equal volume of homologous antiserum for 1 hour, chilled in an ice bath, and washed with saline several times in the cold. Following this absorption of homologous antibody by the cocci, these organisms were inoculated into fresh broth containing normal rabbit serum. After 3 hours' incubation the organisms were in short chains. Apparently long chains were formed only in the constant presence of antibody in growing cultures.

The D-24 strain grew on the surface of blood agar with the formation of large, flat, disc-shaped colonies with a rough "matt" surface. After several

passages through mice, organisms isolated from the spleen formed mucoid colonies on blood agar. After further incubation on the same plates these colonies reverted to flat, matt forms. After incubation of broth cultures of D-24 in the presence of antibody, subcultures on blood agar showed a change in colony form to a dome-shaped, smooth surfaced colony with opaque white centers. However, subcultures of such colonies into fresh broth did not reveal long chain growth unless antibody was added to the media. Similarly when the D-24 strain was grown on blood agar containing 5 per cent antiserum, and the colonies were subcultured in fresh broth, long chain growth did not occur unless antiserum was added to the broth (Table V).

No difference in the amount of extractable M protein could be detected in the D-24 strain when it was grown overnight in Todd-Hewitt broth containing 50 per cent homologous antiserum and in Todd-Hewitt broth containing 50 per cent normal rabbit serum.

TABLE V

Dependence of Long Chain Formation upon the Constant Presence of Antibody in the Medium

Primary Culture		Subculture	
Media	Chain length	Media	Chain length
Antiserum–broth	95.8	Plain broth	6.1
Normal serum–broth	5.4	Plain broth	5.4
Antiserum–blood agar	—	Plain broth	7.7
Normal serum–blood agar	—	Plain broth	8.2

From these observations, it appears that the long chain form of growth of the type described is not a dissociation in the usual sense but rather depends upon some surface effect of the union of antibody with M protein which apparently inhibits fragmentation of the chains during growth.

Long Chain Formation by Other Strains and Types of Streptococci in the Presence of Homologous Antisera.—Fourteen additional strains of type 30 streptococci were also studied for the long chain effect. Long chain formation occurred with all the strains tested except the Gay strain (Table VI).

The two type 12 strains, SF 42 and 19162 GL, were studied in some detail for the long chain reaction. While the SF 42 strain was mouse-virulent, gave good typing reactions, and grew in long chains in the presence of homologous antiserum, the 19162 GL strain had dissociated to a glossy non-typable strain, was not mouse-virulent, and failed to form long chains when growing in the presence of homologous antiserum (Table VII). This lends further weight to the belief that the reaction is dependent upon the presence of M protein in the cells. Strains rich in M protein, or made so by repeated mouse passage, most often gave the long chain reaction with homologous rabbit antiserum.

Fresh human antisera, sterile and free of preservatives, were not available to study the effect of human type-specific antibody upon long chain formation. Attempts to determine the suitability and sensitivity of this system for detecting type-specific antibody in human sera are in progress.

TABLE VI

Comparison of Chain Length of 16 Strains of Type 30 Streptococci Growing in the Presence of Homologous, Heterologous, and Normal Serum

	Average No. cocci per chain		
Strain of T-30	T-30 antiserum	T-12 antiserum	Normal serum
D-24 Quinn	67.8	8.2	8.4
T-30 Gay	9.9	8.9	9.9
2RSC 26	47.5	6.0	8.2
6RSC 25	49.6	8.1	7.0
D 53 A	62.6	4.9	5.6
D 53 D	67.4	7.3	7.4
C 600	54.9	6.2	8.9
C 601	97.8	10.2	8.0
C 602	51.0	9.8	7.5
C 603	140.0	9.8	8.3
C 604	56.0	11.0	8.1
C 605	46.2	7.0	5.8
C 576	152.0	15.9	12.3
C 577	70.8	8.4	5.9
C 578	53.0	6.0	7.2
932 Oswald	108.1	8.5	6.9

TABLE VII

Chain Length of M Protein Rich and M Protein Poor Strains of Type 12 Streptococci Grown in the Presence of Homologous, Heterologous, and Normal Serum

	Average No. cocci per chain			
Strain of T-12	T-12 antiserum	T-30 antiserum	Normal serum	Precipitin* test
SF42‡	93.0	14.2	13.2	++++
19162GL§	5.4	5.8	3.1	0

* Type specific antisera used.

‡ SF42 M protein rich strain.

§ 19162GL M protein poor strain.

DISCUSSION

Chain length of various bacteria has been studied previously from several aspects. Bisset (9–11) has differentiated two types of chain division by certain species, including *Streptococcus pyogenes*, grown on solid media. Variants that form long chains have heavy cell wall septa between the dividing individual

protoplasts (rods or cocci) with no tendency for the cell wall to constrict and "pinch off" smaller units. Variants which grow in short chains, on the other hand, show indentation and constriction of the cell wall between protoplasts which results in fragmentation. In general, long chain variants grow in "rough" colonies on solid media and impression smears of these colonies reveal a "medusa head" appearance due to the long threads in the colony.

The appearance of long chains of streptococci growing in the presence of homologous antibody is quite similar morphologically to Bisset's demonstration of the division of long chain variants. It is unlikely, however, that the long chain growth in the presence of antibody is due to a dissociation into a variant growing in the manner described by Bisset inasmuch as the organism reverts promptly to short chain growth when antibody is absent from the medium.

It would appear from our experiments that the long chains are a result of a physical change in the cell wall following combination of M protein with its specific antibody and that this in some manner prevents fragmentation of the chain. Tomcsik (13) has demonstrated physical changes in the cell walls and capsules of organisms exposed to specific antibodies by phase contrast microscopy or by special staining procedures. He points out that the familiar "quellung" phenomenon of encapsulated pneumococci exposed to type-specific antibody is not due to an actual swelling of capsular material but to a change in its properties which makes it visible with ordinary light. He has demonstrated similar changes in the capsules of Gram-positive rods which are apparent only under the phase contrast microscope and which reveal alterations not only in the capsular material but in the cell walls and the septa dividing cell units.

Unfortunately, we were unable to observe similar changes in the D-24 strain of streptococcus when it was exposed to its specific antibody and examined under the phase contrast microscope. It was our impression, however, that the capsular and cell wall structures stained more prominently in the presence of antibody than in its absence, even if allowances were made for the fact that the increased chain length might in itself create this impression.

Increased chain length has also been produced in certain species by growth in media deficient in magnesium ions (17). The failure of division under these conditions, however, is not simply failure of fragmentation of the chain's cell wall but a complete distortion of growth and division producing pleomorphic, thread-like forms, similar to the effects of x-ray, ultraviolet, or penicillin. This factor did not, therefore, seem pertinent to the mechanism of the long chain formation we have described.

It seems clear that although the long chain growth of strains of streptococci in the presence of homologous antisera depends upon a type-specific reaction involving the M protein of the cells, other factors are involved which are not understood at present. The type 30 "Gay" strain which gives strong typing reactions and produces good amounts of extractable M protein, nevertheless

fails to exhibit long chain growth in the presence of homologous antiserum. This strain has been rabbit-adapted and is lethal for rabbits by the intrapleural route. In spite of its failure to grow in long chains in the presence of homologous antiserum, this strain, when used to absorb homologous antisera, removes the long chain–forming property of the serum. It remains to be determined whether the inability to form long chains by a strain which contains M protein is due to some peculiar structural configuration of the cell walls.

Another known difference between the D-24 and the Gay strain is the absence of T-antigen in the former. Of the fourteen additional type 30 strains tested which produce the long chain effect, 4 were known to lack T antigen (18). The presence or absence of this antigen in the remaining strains producing long chains has not yet been determined.

No other characteristic studied so far distinguishes the Gay strain from the D-24 strain. Both strains conform precisely to the fermentation reactions described in Bergey's Manual of Determinative Bacteriology for *Streptococcus pyogenes*. Further studies of the effect of hyaluronidase, lysozyme, and other enzymes affecting the capsular and cell wall structures, upon the chaining phenomenon are in progress.

In experiments with some strains there was a tendency for agglutination to occur in broth cultures to which serum was added. This was not a common occurrence, however, with those strains producing the most striking long chain reactions and did not appear to influence the results.

So far most strains that have been studied which are rich in M protein produce the long chain reaction. This is particularly true after repeated mouse passage. This suggests that indicator strains of other serological types might be found, and that the long chain phenomenon might serve as a useful tool for detecting type-specific antibody to a wide variety of serological types. Its potential usefulness in the detection of human type-specific antibody remains to be explored.

SUMMARY

Strains of group A streptococci, types 30 and 12, were observed to grow in extremely long chains in liquid media to which homologous antiserum was added. Addition to the media of antisera to heterologous types of streptococci failed to produce long chain growth. The long chain effect was destroyed by absorption of the antiserum with organisms of homologous type but was unaffected by absorption with organisms of heterologous types.

The reaction disappeared at concentrations of antisera smaller than 0.25 per cent and was independent of complement or other heat-labile serum factors. Addition of trypsin to the culture to remove M protein from cells prevented long chain formation. The long chain effect depended upon the constant presence of antibody to the media. In its absence, the organisms promptly reverted to short chain growth.

The phenomenon appears to have general applicability to those strains rich in M protein with only an occasional strain not responding as described. Further studies are in progress to determine the cause of this atypical response. The applicability of this phenomenon in detecting type-specific antibody using indicator strains of a variety of streptococcal types is discussed.

The technical assistance of Jesse Ortiz and Irene Worrell is gratefully acknowledged.

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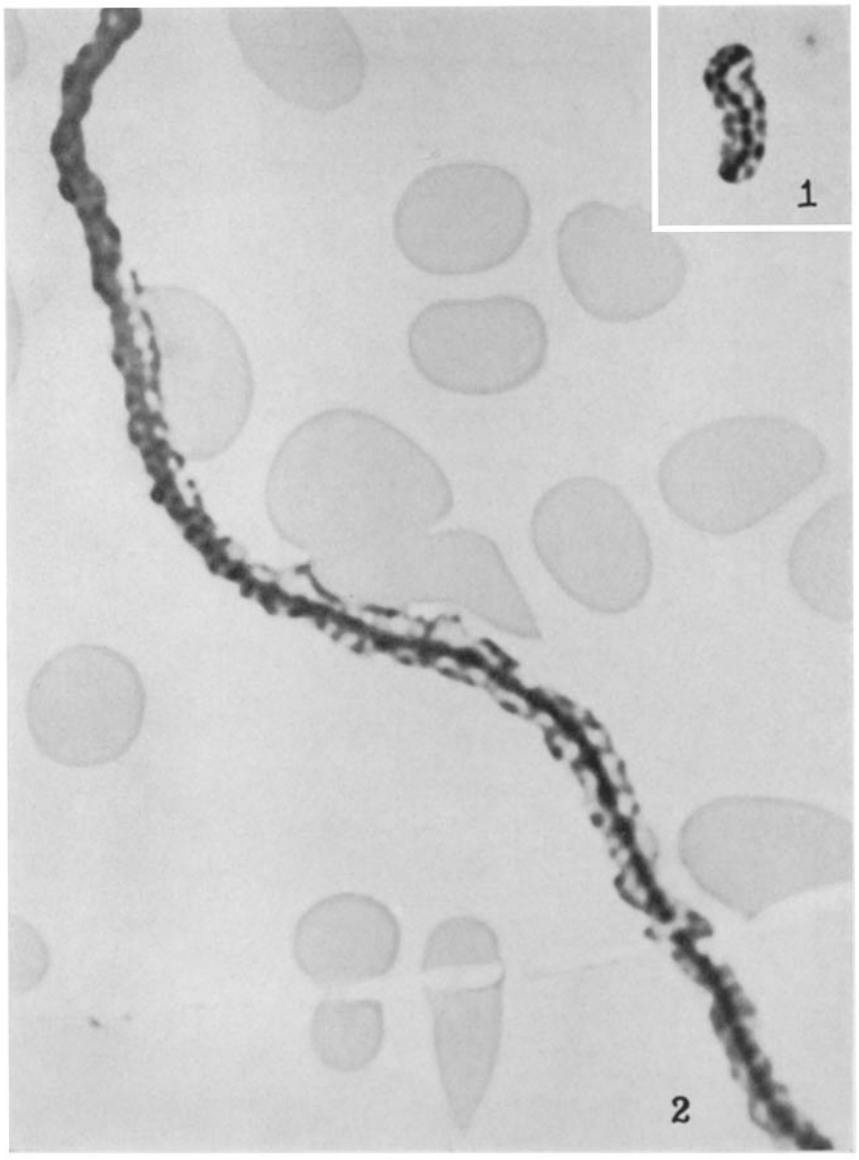
EXPLANATION OF PLATES

PLATE 34

FIG. 1. Streptococci growing in normal rabbit blood. × 1200. Wright stain.

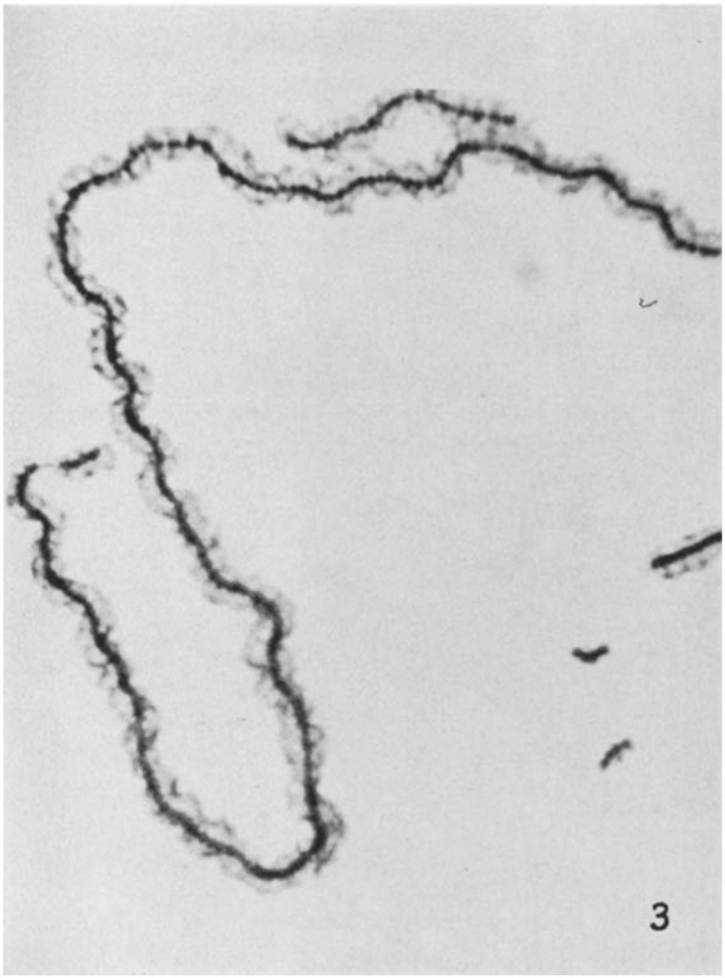
FIG. 2. Streptococci growing in rabbit blood containing homologous antiserum. × 3200. Wright stain.



(Stollerman and Ekstedt: Long chain formation by strains of streptococci)

PLATE 35

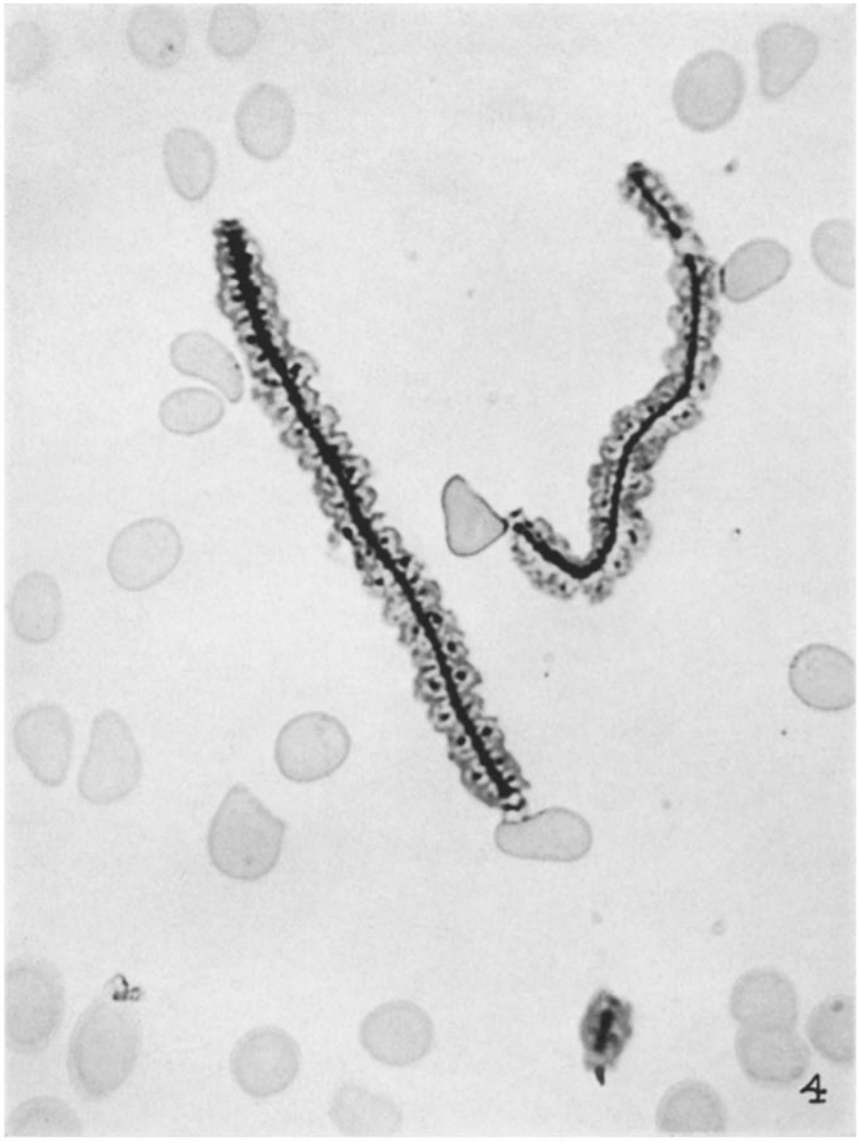
FIG. 3. Streptococci growing in Todd-Hewitt broth containing homologous antiserum. × 1200. Wright stain.



(Stollerman and Ekstedt: Long chain formation by strains of streptococci)

PLATE 36

FIG. 4. Streptococci showing capsular granules when growing in rabbit blood containing homologous antiserum. × 1430. Wright stain.



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