STUDIES ON THE MECHANISM OF THE LONG CHAIN PHENOMENON OF GROUP A STREPTOCOCCI

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PLATE 28

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Streptococci of Group A form long chains under a variety of conditions, the most interesting and useful of which is during growth in the presence of free homologous type-specific antibody. This phenomenon is the basis for a test for anti-streptococcal type-specific antibody, first described in 1957 (1), evaluated in 1959 (2), and improved and simplified in 1961 (3). The requirement for a metabolically active system, both for chaining and for subsequent dechaining, has been shown, but the manner by which type-specific antibody prevents dechaining is uncertain. One interpretation is that the antigen-antibody reaction somehow inhibits an enzymatic system which is otherwise continuously active in dechaining (4). Some general objections to this hypothesis as well as to the alternate one of polarized agglutination, have been given (3).

The present studies are restricted to long chaining and dechaining in the presence of or after removal of, respectively, type-specific antibody. The data indicate that a form of end-to-end agglutination offers the simplest and most likely explanation for formation of long chains by Group A streptococci grown in the presence of homologous type-specific antibody.

Materials and Methods

Cultures.—T1/155/4 (Type 1), C203 (Type 3), and D58x/11/2 (Type 3) were received from Dr. Rebecca Lancefield. Strain 59154 (Type 18) was isolated in this laboratory from a patient with a sore throat. Strain 5216 (Type 2) was received from the Diagnostic Reagents Section, Communicable Disease Center, Atlanta, Georgia. All strains were passed through mice at least once a week during the course of these studies. A single colony picked from rabbit blood agar plates of the mouse isolates was inoculated into 10 cc Todd-Hewitt broth (Difco Laboratories, Inc., Detroit) and incubated 18 hours at 37°C before use.

Acid Extracts.—The acid extracts used in the following experiments were made by extracting the sediment from 120 cc of an 18 hour Todd-Hewitt broth culture in the classic manner (5).

Antisera.—White rabbits (2 kg, NIH strain) were injected intravenously with heat-killed

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vaccines of the appropriate organisms on alternate days during the week. The rabbits were test-bled after 4 weeks of injections and the sera absorbed with heterologous type streptococci. When strong precipitin reactions were noted with homologous but not heterologous acid extracts, 50 to 60 cc of serum was collected from each animal.

Long Chain Test.—0.2 cc of the test serum was placed in 9.0 x 100 mm tubes and 0.05 cc of a 10^{-2} dilution of a 24 hour Todd-Hewitt culture of a mouse-passed strain was added. The tubes were incubated in a water bath at 37°C. The time of incubation is noted for each experiment. At the end of the period of incubation, one drop of the culture material was placed on a microscope slide, covered with a coverslip, and examined with a phase contrast microscope. Fifty chains in each preparation were counted and the mean chain length calculated.

Protein Determinations.—Determinations were performed according to the method described by Lowry (6).

Digestion of Antibody Globulins.—Peptic digestion of rabbit antibody was performed according to the method devised by Nisonoff *et al.* (7). 10 ml of rabbit anti-type 2 globulins was digested with 3x crystallized pepsin, (Sigma Chemical Company, St. Louis) at pH 4.5 in acetate buffer for 24 hours at 37°C. The amount of pepsin used was 1 per cent of the weight of globulin present. The digested material was then divided into equal portions. One part was dialyzed against phosphate-buffered saline containing 0.01 M cysteine, pH 7.5; the other against phosphate-buffered saline for 24 hours at 37°C. Both portions were then dialyzed against three 1-liter changes of phosphate-buffered saline, pH 7.5 in the cold. Papain digestion of the isolated globulin fractions was accomplished exactly as described by Porter (8).

Preparation of Fluorescein-Conjugated Globulins.—The globulin fractions of rabbit antisera were obtained by precipitation with 50 per cent ammonium sulfate at 5°C for 2 hours, solution in 0.15 M saline, and by dialysis against saline in the cold until free of ammonium sulfate. These fractions, as well as pepsin- and papain-treated fragments, were conjugated with fluorescein isothiocyanate by the method of Marshall *et al.* (9). Unbound fluorescein and buffer were removed according to Killander *et al.* (10), by passage though a 1 x 40 cm column of sephadex G-25[1] equilibrated with 0.15 M saline buffered with 0.05 M phosphate at pH 7.5.

Absorption of Conjugated Globulins and Univalent Fragments.—Group A streptococci were grown 18 hours at 37°C in 3 liter lots of beef infusion broth, washed twice with saline, and heat-killed at 56°C for 60 minutes. The killed cells were stored in the cold in $\frac{1}{10}$ the original volume of saline. For absorption, 5 to 10 cc of conjugated globulin was added to the sediment from 10 cc of the concentrated bacterial suspension, mixed, and incubated for 2 hours at 37°C. Cells were removed by centrifugation and the process repeated as necessary. Each type-specific conjugated globulin was prepared in this fashion by absorption with at least 3 different heterologous types of Group A streptococci. A globulin was considered adequately absorbed if it produced cell wall fluorescence of homologous cells and did not produce such fluorescence of streptococci of Groups C, G, and at least 15 heterologous types of Group A.

EXPERIMENTAL

Demonstration of a Metabolic System Active during Dechaining.—

Two series of tubes, each containing 0.2 cc of fluorescein-conjugated anti-type 18 globulin, were inoculated with 0.05 cc of a 10^{-2} dilution of a 24 hour Todd-Hewitt culture of a mouse-passed strain of Type 18 streptococci. After 2 hours' incubation at 37°C, 0.2 cc of a Type 18 acid extract was added to each tube in one series and 0.2 cc of a Type 1 acid extract was added to each tube of the other series. The contents of the tubes were mixed by gently in-

[1] Pharmacia, Uppsala, Sweden.

verting the tubes twice. One tube from each series was immediately placed in an ice bath and the remaining tubes kept in the water bath at 37°C. One tube from each series was removed from the water bath and placed in ice water after 5, 10, 15, and 30 minutes. Mean chain lengths were determined on a sample from each tube and recorded. The remaining tube contents were washed three times with cold saline containing 10 per cent normal rabbit serum. The washed sediments were spread on clean microscope slides, dried, heat-fixed, and examined under UV light for fluorescence.

The data presented in Table I demonstrate that at the end of the initial 2 hour period of incubation there was an average of twenty-five cocci per chain. As Stollerman and Ekstedt (4) have shown, continued incubation at 37°C following the addition of homologous acid extract resulted in fragmentation of

TABLE I

Effect of Acid Extracts on Long Chains

Time of incubation at 37°C following addition of acid extract	Mean chain length	
	Homologous extract	Heterologous extract
min.		
0	24.9	25.4
5	22.8	—
10	20.0	—
15	16.3	35.8
30	6.7	54.0

Mean chain length determined from 50 chains. Homologous extract, Type 18. Heterologous extract, Type 1.

the chains. Following the addition of heterologous acid extract, the mean chain length continued to increase during the period of observation.

The continued increase in the mean chain length following the addition of heterologous acid extract, and the decrease after the addition of homologous acid extract, is consistent with previous work (3) which has shown that free antibody must be present during growth and division in order for long chains to be produced.

The photograph in Fig. 1 *a* shows the washed sediment obtained from the tube which contained T18 bacteria, T18 fluorescein-conjugated globulin, and acid extract from the T1 organism. The brilliant fluorescence and the completeness of the outlines of the cells (Fig. 1 *a*) indicate that throughout the period of incubation, while long chains are being formed, there was sufficient unbound antibody present to combine with new antigen formed during growth and division of the bacteria.

The photographs in Figs. 1 *b* to 1 *f* demonstrate the sequence of events with continued incubation following the addition of homologous acid extract to the

tubes. During this time interval the mean chain length was decreasing. The first picture (Fig. 1 *b*) represents the sample which was immediately iced after the addition of homologous extract. The completeness of the outlines of the cells and the brilliance of the fluorescence is comparable to that seen after the addition of heterologous extract and indicates there has been little if any shifting of antibody from the cell wall to the antigen in solution. The dark areas separating the stained cell walls seen in the photographs taken at subsequent time intervals (Figs. 1 *c* to 1 *f*) represent cell wall formed during incubation after free antibody has been removed from the system. The use of this technique to study the details of cell wall formation in growing cultures has been presented in detail in another communication (11). For the present, it is sufficient to note that the continued brilliance of the previously stained portions of the cell walls indicates that in the time interval concerned, there has not been any noticeable shifting of antibody from the cell wall to the antigen in solution. The absence of conjugated antibody in the areas of new cell wall formation after the addition of homologous antigen indicates that free antibody had been removed from the system and that there is no appreciable redistribution of antibody from either the old cell wall or the added soluble antigen to the antigen produced by continued growth and division of the bacteria.

Growth of Group A Streptococci in the Presence of Univalent Antibody Fragments.—

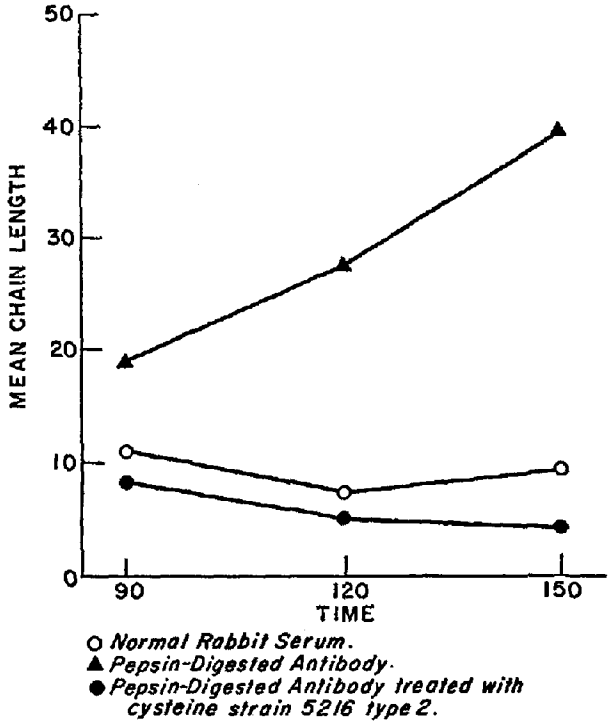
1.0 cc of the pepsin-digested Type 2 globulins and 1.0 cc of the same pepsin-digested globulins treated with cysteine were diluted serially in normal rabbit serum and used in long chain tests with strain 5216T2. The undiluted materials were also used in precipitin tests with acid extracts from homologous organisms.

The pepsin-digested material gave strong precipitin reactions with the homologous acid extract. The pepsin-digested material treated with cysteine failed to precipitate with the homologous acid extract.

The data in Text-fig. 1 show that the growth of strain 5216T2 in the presence of pepsin-digested Type 2 globulins results in the formation of long chains. The continuous increase in the mean chain length during the period of observation indicates that an excess of free antibody was present throughout the period of observation. The pepsin-digested antibody globulin treated with cysteine failed to produce long chains at any time during the experiments. Similar results were obtained with treated globulins against Types 1, 3, and 18 with their homologous cells.

Equal concentrations of protein from papain-digested and untreated globulins were diluted in normal rabbit serum. Each dilution was then used in a long chain test with homologous organisms.

The data in Table II show that long chains are produced after 2 hours' incubation of the homologous organisms in the presence of 58 μg/cc of untreated T1 globulin or in the presence of 39 μg/cc of untreated T18 globulin. Incubation



TEXT-FIG. 1. Long chain formation by pepsin-digested antibody.

TABLE II

Long Chain Formation by Papain-Digested Antibody

	Dilution of untreated globulin giving positive long chain test	Protein in highest dilution giving positive long chain test
T1	1:256	58 μg
T18	1:128	39 μg
	Concentration of papain-digested globulin tested*	**Long chain test**
T1	7500 μg/cc	Negative
T18	2500 μg/cc	Negative

* Highest concentration available for testing.

for 2 hours in the presence of 7500 μg/cc of T1 papain-digested material or 2500 μg/cc of T18 papain-digested material did not produce long chains.

The Combining Properties of Papain-Digested Antibody.—

24-hour Todd-Hewitt broth cultures of Types 1, 3, and 18 were washed twice in saline, heat-killed (56°C for 30 minutes), washed again in saline, and resuspended to the original

volume. 0.05 cc of each bacterial suspension was added to 0.2 cc of papain-digested or untreated globulin from homologous type antisera. The tubes were incubated in the water bath at 37°C for 1 hour. 0.2 cc of fluorescein-conjugated, type-specific globulin, homologous for the cells present in the tube, was then added and incubation continued for another hour. The tube contents were then washed three times in saline containing 10 per cent normal rabbit serum, spread on slides, heat-fixed, and examined under UV light for fluorescence.

Incubation of papain-digested or untreated antibody globulin with homologous bacteria inhibits the combination of homologous type-specific, fluorescein-conjugated antibody. When incubated with heterologous bacteria, neither papain-digested nor untreated globulins inhibited the staining of the bacteria by fluorescein-conjugated antibody-specific for that organism. (See Table III.)

TABLE III

Inhibition of Type-Specific Fluorescence by Papain-Digested and Untreated Antibody Globulin

Material tested	Type cells used		
	T1	T2	T18
T1 papain*	+‡	0	0
T1 globulin§	+	0	0
T3 papain	0	+	0
T3 globulin	0	+	0
T18 papain	0	0	+
T18 globulin	0	0	+

* Papain digest.

‡ +, inhibition; 0, no inhibition.

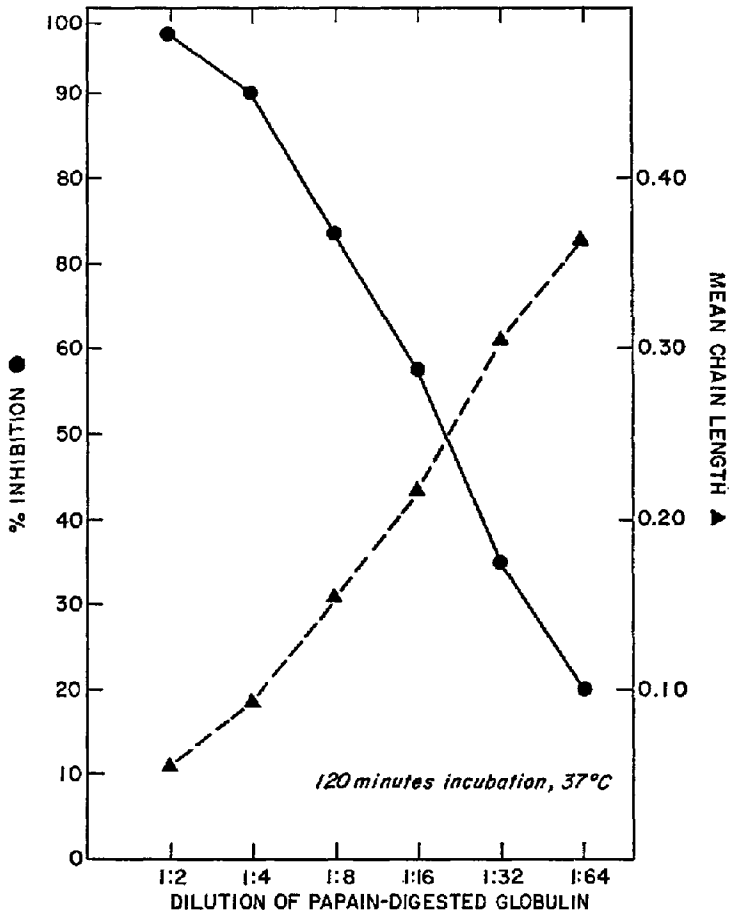
§ Untreated antibody globulin.

The brilliant fluorescence observed under these latter conditions indicates that any residual papain had not digested the M proteins.

Inhibition of Long Chain Formation by Papain-Digested Antibody Globulins.—

Solutions of papain-digested antibody globulins were diluted with saline to the same protein concentration as papain-digested normal rabbit globulins. Each was then serially diluted with normal rabbit serum. 0.1 cc of a dilution of untreated antibody globulin which would produce long chains after 2 hours incubation with homologous streptococci was added to 0.1 cc of each dilution of the papain-digested antibody globulins and the papain-digested normal rabbit globulins. The mixtures were inoculated with 0.05 cc of a 10^{-2} dilution of a 24 hour Todd-Hewitt culture and incubated for 2 hours at 37°C. The tubes were taken from the water bath, immediately iced, and mean chain lengths determined from a sample of each tube. These experiments were performed with strains T1/155/4 (Type 1), and 59154 (Type 18), and their respective untreated and papain-digested globulins.

Text-fig. 2 presents the effect of papain-digested material (Type 18) in terms of per cent inhibition as well as in terms of the absolute mean chain lengths. These experiments show that papain-digested antibody will inhibit the formation of long chains when mixed with untreated antibody globulin of homologous type.



TEXT-FIG. 2. Long chain inhibition by papain-digested antibody.

DISCUSSION

The chain length of streptococci has been studied by many investigators and the various factors affecting the chain length have been tabulated. Genetic variation has been suggested to result in changes in the mode of cellular division as well as the length of the chains (12). Environmental conditions have been

shown to alter both the morphology and composition of the streptococci (13). The studies of Stollerman and Ekstedt (1) and later of Hahn and Cole (3) indicated that the long chains produced by growth of Group A streptococci in the presence of homologous antisera are not due to a genetic mutation inasmuch as the long chains so produced will fragment with continued growth following the removal of antibody. It would appear then that the production of long chains in type-specific antibody may be explained in terms of environmental conditions.

Because we are dealing with a biologic test, it is not yet possible to define every factor operating in the system and it is therefore necessary to define rigorously the conditions of experimentation. For the purposes of these experiments and this discussion we have arbitrarily defined the normal chain length as that which is found after growth in normal serum. Numerous experiments in this laboratory have found that the average length remains constant throughout growth in normal serum. The main question to be answered, then, is how does the presence of type-specific antibody during growth of the organism maintain chain integrity and so produce long chains?

Ekstedt and Stollerman (4) suggested that an active metabolic system was involved in the destruction of long chains formed by growth in type-specific antibody. They postulated that the addition of excess antigen to the system might result in the redistribution of antibody from the chains to the soluble antigen and thereby allow the chains to fragment. Long chains formed by growth in type-specific antibody did fragment with continued incubation after the addition of excess antigen, and the rate of dechaining could be altered by changing the pH, temperature, and by adding various protoplasmic poisons to the system. These studies led the authors to suggest the active metabolic system may be an enzyme or group of enzymes responsible for dechaining and which are inhibited by the combination of type-specific antibody with its antigen.

The studies with fluorescein-conjugated antisera presented in this paper have shown that while the chains are fragmenting, following the addition of excess homologous antigen, growth and division continue. They have also shown that there is no demonstrable shifting of antibody from the cell walls during the period of observation. Although recent studies by Grey (14) have shown that dissociation of M anti-M does occur and can be quantitated, the degree and rapidity of dissociation was found to depend in part on the length of immunization of the animal from which the antibody was derived. His results indicate a relatively slow process of dissociation which would therefore be incompatible with the hypothesis set forth by Ekstedt and Stollerman (4).

An alternative model, involving end-to-end agglutination would be consistent with all experimental observations collected thus far. The agglutination model for the long chain phenomenon in type-specific antibody postulates that

the valencies of the bivalent antibody molecule combine with antigenic sites on adjacent cell walls. The increased strength due to the bridging maintains the integrity of the chains as growth and division occur. When antibody is depleted, by addition of excess antigen or continued growth and division, completion of division or new cell division results in the formation of weak spots, not bridged by antibody, which are broken by physical forces in the system.

Porter (8) has demonstrated that bivalent antibody can be split into univalent fragments by digestion with papain. Nisonoff and associates (7) have shown that digestion of antibody with pepsin splits the molecule into two parts, one of which contains both antibody-combining sites. The bivalent fragments can be further degraded by reaction with agents which reduce labile disulfide bonds and split the bivalent fragments into univalent fragments. The specificity and avidity of the antibody sites are not altered by such treatment (15).

The digested antibody globulins used in this work have been shown to combine specifically with their antigens, inhibit the combination of undigested antibody with its antigen, and lack precipitating ability, and therefore are presumably univalent fragments. The absence of long chain formation during growth of streptococci in homologous univalent fragments indicates that the long chain phenomenon is not due solely to the covering of certain antigenic sites with antibody but depends on the bivalent nature of the antibody. This dependence on the bivalent nature of the antibody, we believe, is strong evidence in favor of the agglutination model outlined above.

However, an explanation for the lack of generalized agglutination in long chain tests with intact or bivalent antibody is required and is not readily apparent. In the first place, it is difficult to ascertain agglutination of living streptococci. When chains of growing streptococci in the long chain test become sufficiently long, intertwining, clumping, and falling out of suspension occur: this effect often cannot be distinguished from true agglutination. On the other hand, nearly all tests of streptococcal agglutination have been performed with killed, heated, or trypsinized suspensions, and information derived from such tests is not necessarily applicable to living systems. Even in such tests, the roles of various antigens are obscure. In analogy with the enteric bacteria, streptococcal antigens have been called somatic (O) and envelope (K) antigens by Thulin (16), but their identify with known streptococcal antigens is not clear. When living streptococcal agglutinogens were used (16) to describe a so-called L antigen, test conditions (2 hours at 37°C in serum dilutions; overnight at 4°C) were such that clumping due to long chaining may have been confused with agglutination.

Even assuming that a true agglutination of living streptococci in type-specific antibody may occur under some conditions, there is no information concerning the possible roles, or even existence, of superficial or blocking anti-

gens. A familiar example is the O inagglutinability of Vi-producing strains of *Salmonella typhosa*. Similar antigens which would block the M anti-M agglutination of Group A streptococci are not known, although such antigens, or even the non-antigenic hyaluronic acid capsule, might cause similar inhibition in a living system while being ineffective or absent in killed or treated suspensions. The capsule, for example, does not prevent access of type-specific antibody to the cell wall, as amply shown repeatedly in this laboratory by direct specific immunofluorescence of many strains and types of Group A streptococci growing directly in labeled specific antisera (11). (Although not so stated in reference 11, all strains growing in serum or antiserum were capsulated, especially those of Types 18 and 19.)

It is therefore tempting to speculate that, in a long chain test in type-specific antibody, the presence of the capsule may prevent agglutination of streptococcal chains. Some strains, both in our experience and that of others (1), do indeed appear to agglutinate in long chain tests, but most do not. This phenomenon, however, has not been correlated with chain length, degree of capsulation, time of occurrence, presence of various antigens, or other characteristics. It appears theoretically possible that whole or bivalent antibody, which in the long chain test is continously binding to M protein as this antigen forms, may be able to bridge adjacent cells in a chain by both valences because of the physical proximity and manner of formation of new cell wall (11). The common envelope of capsular material may prevent the unattached valences of antibody from reaching M antigen sites on capsule-covered cocci of adjacent chains, thus preventing general or side-by-side agglutination. In practice, however, we have not been able to reproducibly counter this theoretical effect by continuous decapsulation with added testicular hyaluronidase during the long chain test and to thereby produce agglutination. These results, while preliminary and still under investigation, suggest that other and unknown blocking antigens, of variable distribution among streptococcal strains, may be responsible; or that factors of time, temperature, mixing, pH, and the like, are not optimal in the usual long chain test for demonstration of true generalized agglutination of living Group A streptococci.

SUMMARY

The formation and destruction of long chains by growth of Group A streptococci in the presence of type-specific antibody have been studied with the fluorescent antibody technique. Long chain formation has been shown to depend on the presence of free antibody during the growth of the bacteria. Destruction of long chains has been shown to depend on the continued growth and division of the bacteria in the absence of free antibody.

Univalent antibody fragments formed by proteolytic digestion of antibody globulin have been shown to have the combining properties of untreated antibody but do not result in the production of long chains.

A model involving end-to-end agglutination during growth of Group A streptococci has been presented to explain the mechanism of production of long chains by growth of Group A streptococci in the presence of type-specific antibody.

The skilled technical assistance of Richard Whitt is gratefully acknowledged.

BIBLIOGRAPHY

1. Stollerman, G. H., and Ekstedt, R. D., Long chain formation by strains of group A streptococci in the presence of homologous antiserum: A type-specific reaction, *J. Exp. Med.*, 1957, **106**, 345.
2. Stollerman, G. H., Siegel, A. C., and Johnson, E. E., Evaluation of the "long chain reaction" as a means for detecting type-specific antibody to Group A streptococci in human sera, *J. Exp. Med.*, 1959, **110**, 887.
3. Hahn, J. J., and Cole, R. M., Time and concentration relationships in the long-chain reaction of group A streptococci in homologous antiserum, and an improved method for evaluation of test results, *J. Bact.*, 1962, **83**, 85.
4. Ekstedt, R. D., and Stollerman, G. H., Factors affecting the chain length of Group A streptococci. I. Demonstration of a metabolically active chain-splitting system, *J. Exp. Med.*, 1960, **112**, 671.
5. Lancefield, R. C., Antigenic complex of *Streptococcus haemolyticus*: demonstration of type-specific substance in extracts of *Streptococcus haemolyticus*, *J. Exp. Med.*, 1928, **47**, 91.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193**, 265.
7. Nisonoff, A., Wissler, F. C., Lipman, L. N., and Woernley, D. L., Separation of univalent fragments from bivalent antibody molecules by reduction of disulphide bonds, *Arch. Biochem. and Biophysics*, 1960, **89**, 230.
8. Porter, R. R., The hydrolysis of rabbit-gamma-globulin and antibodies with crystalline papain, *Biochem. J.*, 1959, **73**, 119.
9. Marshall, J. D., Eveland, W. C., and Smith, C. W., Superiority of fluorescein isothiocyanate for fluorescent-antibody technique, with a modification of its application, *Proc. Soc. Exp. Biol. and Med.*, 1958, **98**, 898.
10. Killander, J., Ponten, J., and Roden, L., Rapid preparation of fluorescent antibodies using gel-filtration, *Nature*, 1961, **192**, 182.
11. Cole, R. M., and Hahn, J. J., Cell wall replication in *Streptococcus pyogenes*, *Science*, 1962, **135**, 722.
12. Bisset, K. A., The cytology of the Gram-positive cocci, *J. Gen. Microbiol.*, 1948, **2**, 126.
13. Gooder, H., and Maxted, W. R., External factors influencing structure and activities of *Streptococcus pyogenes*, *Symp. Soc. Gen. Microbiol.*, 1961, **11**, 151.

14. Grey, H. M., Studies on the binding between streptococcal M protein and antibody, *J. Exp. Med.*, 1962, **115**, 671.
15. Nisonoff, A., Wissler, F. C., and Woernley, D. L., Properties of univalent fragments of rabbit antibody isolated by specific adsorption, *Arch. Biochem. and Biophysics*, 1960, **88**, 241.
16. Thulin, E. K., Serological aspects of hemolytic streptococci, with special reference to the occurrence of O, K, and L antigens, and clinical applications, *Acta Path. et Microbiol. Scand.*, 1948, suppl. 75.

EXPLANATION OF PLATE 28

FIG. 1 *a*. Sediment from the tube which contained Type 18 streptococci and Type 18 conjugated globulin immediately after the addition of an acid extract from heterologous streptococci (Type 1). × 3000.

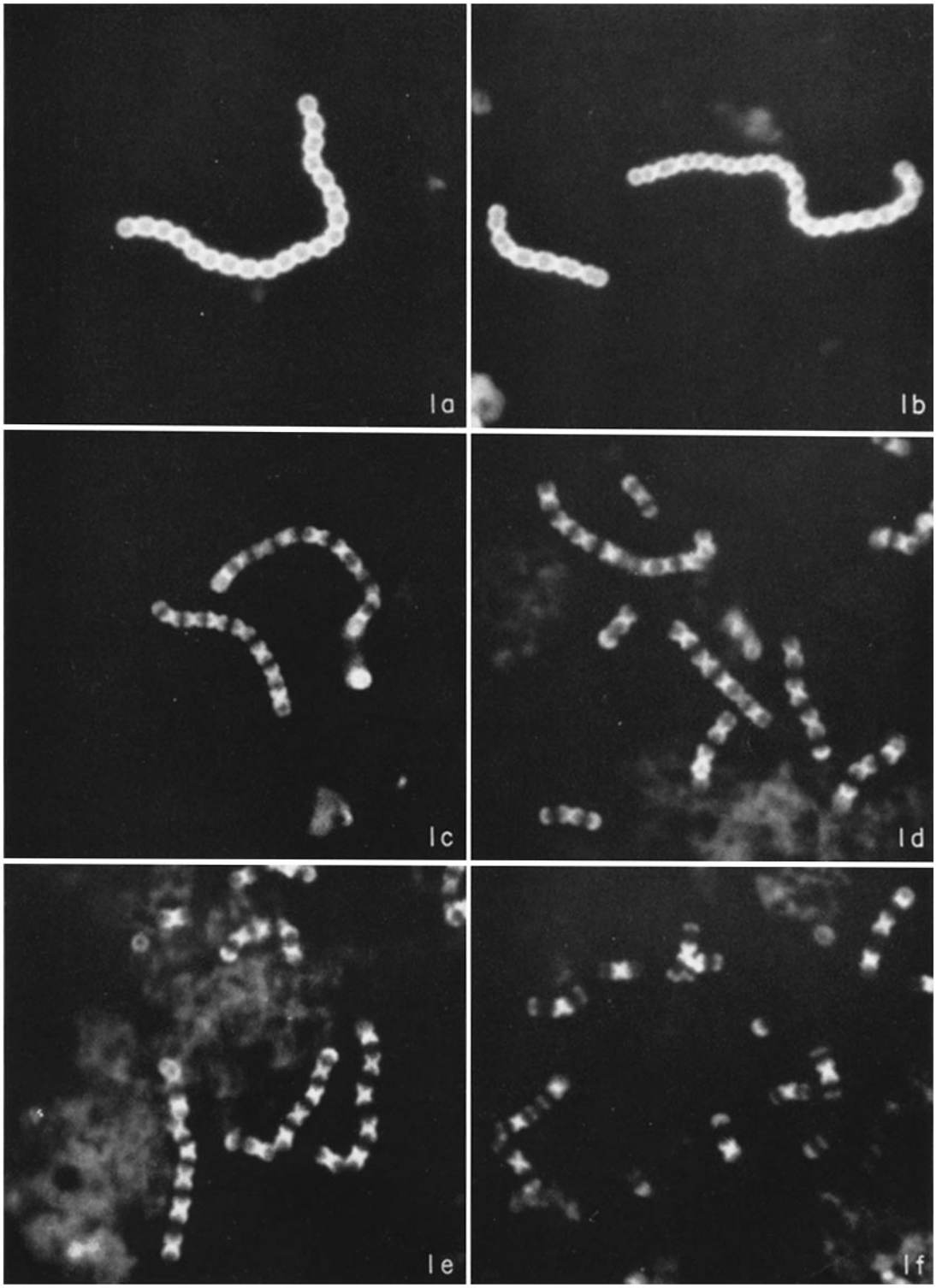
FIG. 1 *b*. Sediment from the tube which contained Type 18 streptococci and Type 18 conjugated globulin immediately after the addition of homologous acid extract.

FIG. 1 *c*. 5 minutes' incubation at 37°C following the addition of homologous acid extract.

FIG. 1 *d*. 10 minutes' incubation at 37°C following the addition of homologous acid extract.

FIG. 1 *e*. 15 minutes' incubation at 37°C following the addition of homologous acid extract.

FIG. 1 *f*. 30 minutes' incubation at 37°C following the addition of homologous acid extract.



(Hahn and Cole: Long chain phenomenon of Group A streptococci)