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

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ABSTRACT

HAHN, JEROME J. (National Institutes of Health, Bethesda, Md.) AND ROGER M. COLE. 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. Bacteriol. **83**:85–96. 1962—We have described a statistically controlled method for evaluating the long chain reaction of group A streptococci grown in homologous antisera. The method is based on the chi-square analysis of the frequency distribution of chain lengths found in test and control sera, and is called the Size-Class Frequency method. Useful tables for rapid application of the method are presented. It is reproducible, and is simpler, more rapid, and more sensitive than the previously described method.

Using this method, we have shown that the long-chain reaction reaches a maximum and then declines with time of incubation. The time at which the maximal response and height are attained depends directly on the initial antibody concentration and inversely on the initial antigen concentration. The inter-relationships of time, antibody concentration, total number of cocci, total number of chains, and long-chain frequency (or chain length) are presented in the discussion.

In view of the findings reported here, we suggest a re-evaluation of possible methods of "dechaining" or "chaining" among group A streptococci. Present evidence appears inadequate to explain the long-chain reaction on the basis of inhibition of a single enzyme.

The "long-chain reaction," as a test for detection of type-specific antibody to streptococci of group A, was introduced by Stollerman and Ekstedt (1957). In this test, streptococci grown in homologous-type antiserum form chains of an average length exceeding that found when the same organisms are grown simultaneously in normal serum or in heterologous-type antiserum. The test appears to depend on the presence both of M protein in the streptococci and of free antibody, specific to that type of M protein, in the serum; it correlated with results of bactericidal tests in detecting type-specific antibodies in human sera (Stollerman, Siegel, and Johnson, 1959). More recent studies by Ekstedt and Stollerman (1960*a*) suggest that chain-splitting may be an enzymatic process that is somehow inhibited by the presence, in the growth medium, of specific antibody.

Many aspects of the long-chain reaction, however, are not clear. The protein concentration of the media and the virulence of the organism have been shown to affect chain length (Stollerman and Ekstedt, 1957; Stollerman et al., 1959), but the mechanism is not understood. The extent by which the reaction is influenced by the presence, in the streptococcal cell wall, of other antigens such as T protein (Stollerman and Ekstedt, 1957) is unknown, although it has been demonstrated that antigen-antibody reactions other than M-anti-M (e.g., R_3-anti-R_3) may also result in long chains (Ekstedt and Stollerman, 1960*b*). The effects of inter-related factors of inoculum size, bacterial growth, and incubation time on the detection of different levels of antibody have not been previously reported. As a result, no adequate methods of titrating antibody have been described. Finally, the long-chain test, as previously described, is slow and cumbersome in practice. These facts stimulated us to investigate the time and concentration relationships of the reaction, and led to a revision of previous methods that simplifies reading the test and allows ready titration of antisera.

MATERIALS AND METHODS

Streptococcal strains. Most experiments were done with a strain of lyophilized group A strep-

tococcus, type 1, obtained from Rebecca Lancefield (Rockefeller Institute for Medical Research, New York), and designated as T1/155/4. This organism resisted phagocytosis and the bactericidal effect of normal human and rabbit bloods, and its acid extracts gave a 4 + precipitin reaction with several different heterologously-absorbed type 1 antisera. In experiments with mice, at least 50% were killed, usually within 3 days after intraperitoneal injection of 0.5 ml of a 10^{-6} dilution of a 4-hr broth culture of this strain. An avirulent (LD_{50} = 10^{-1}) M-variant of this strain, used in some control experiments, was derived by repeated serial passage in artificial media for several months.

To confirm the generality among group A streptococci of phenomena observed in experiments with type 1, experiments with mouse-virulent strains of types 1 (other strains than T1/155/4), 2, 3, 5, 12, 14, and 18 were successfully performed. The results of these experiments are not reported in detail in this paper (see Discussion).

Cultures from lyophilized preparations were made routinely in horse meat infusion broth containing 2% neopeptone. The inocula for long-chain tests were grown in Todd-Hewitt broth modified by use of lean horse meat instead of beef heart. Cultures in the horse meat-neopeptone broth, originally virulent, were kept at 4 C for several weeks. If shortening of chain length in homologous antiserum was observed and a bactericidal effect of normal blood occurred in a subsequent test, virulence was restored by mouse passage.

Antisera. Antisera for use in the long-chain test were prepared by intravenous injection of suspensions of heat-killed streptococci into rabbits (Lancefield, 1938). To prepare presumptive type-specific antisera, portions of the crude antisera were absorbed with group A streptococci of heterologous type until the group-precipitin reaction in capillary tubes (Swift, 1947) versus heterologous-type extracts disappeared. Specificity of the absorbed antisera was tested in the capillary tube-precipitin test (Swift, Wilson, and Lancefield, 1943) against extracts of heterologous types and of several strains of the homologous type. Early results showed no differences, in the long-chain reaction, between heated and unheated antisera; thereafter, most testing was done with unheated sera.

Antisera for the routine grouping and typing of streptococcal strains were supplied by the Diagnostic Reagents Section, Laboratory Branch, Communicable Disease Center, U. S. Public Health Service, Atlanta, Ga. These antisera, unless dialyzed free of Merthiolate, inhibit streptococcal growth and are therefore unsuitable for direct use in the long-chain test.

Long-chain test. The test was performed essentially as described by Stollerman and Ekstedt (1957). An 18-hr culture was diluted 10^{-2} in Todd-Hewitt broth. An inoculum of 0.05 ml of the diluted culture was added to 0.2 ml of serum or serum dilution, and the stoppered tubes (9.0 × 100 mm) held stationary during incubation at 37 C in a water bath. Because lowering of protein concentration causes some long chaining (Stollerman and Ekstedt, 1957), all dilutions of antisera were made in heat-inactivated, pooled normal rabbit sera instead of broth. After incubation for the desired time, each tube was gently inverted once, a drop of culture from the underside of the rubber stopper touched to a slide, covered with a glass coverslip, and the edges sealed with Vaseline. Examination was made under oil immersion by dark phase contrast microscopy.

Numbers of cocci and chains counted, variations in inoculum size, antiserum concentration, and incubation time were the subjects of this study and are described in the Results.

RESULTS

Size-Class Frequency method of detecting long chains. The detection of homologous antibody by the long-chain reaction depends on the ability to tell when chains in a test serum are significantly longer than those grown simultaneously in normal serum. We felt that a method of detecting differences based on the frequency of occurrence of chains above and below a selected length would be simple and rapid and would reflect the mean chain length. The significance of any observed differences could then be tested by chi-square analysis.

We measured chain lengths in the population grown in normal serum by counting the number of cocci per chain in a given number (50 to 100) of chains, after which, mean and median chain lengths were determined. For the purposes of this study, we used twice the median value (2 × Me) as the class divisor. Chain populations

in any serum can then be separated into two classes by this divisor. The wavy lines labeled 2 × Me in Fig. 1 indicate the level of this divisor in normal serum, using a type 1 *Streptococcus*. The difference in distribution of the two size classes above and below this level, in normal serum as compared with homologous antiserum, is readily apparent. A large number of tests, the results of which are expressed in a scatter diagram, indicated that the frequencies of chains exceeding the class divisor in length were in general agreement with the corresponding mean chain lengths (Fig. 2).

Following determination of 2 × Me in the control serum, chains grown in any test serum are readily classified as belonging to the longer or shorter class. Obviously, once the number of cocci in a given chain is seen to exceed the selected number (e.g., > 2 × Me), no further counting of cocci withing that chain is necessary and it is simply recorded as belonging to the longer class. For example, in 39 tests of type 1 *Streptococcus* grown in normal rabbit serum, the average median value was 5.3 cocci per chain. On the average, therefore, in a type 1 system, one would not expect to count more than 11 cocci per chain to ascertain that a long chain (i.e., one exceeding 2 × Me) had been formed.

We compare the observed class frequencies in control and test sera for significant differences by

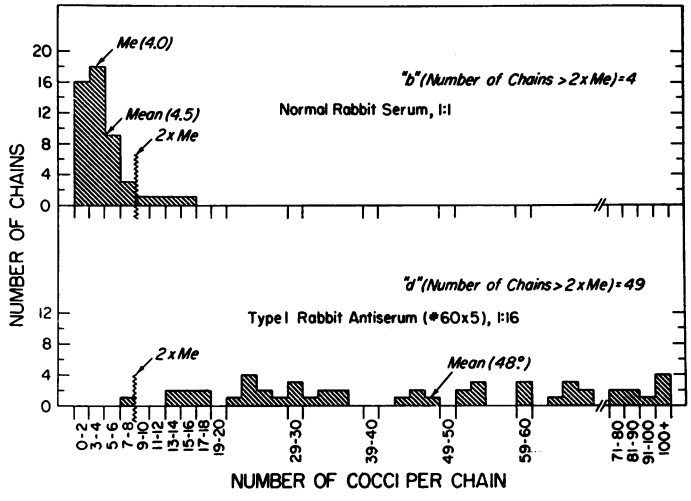

FIG. 1. *Distribution of chain lengths of type 1 Streptococcus (T1/155/4) grown in normal and in type-specific rabbit sera. Incubation time was 3.5 hr.*

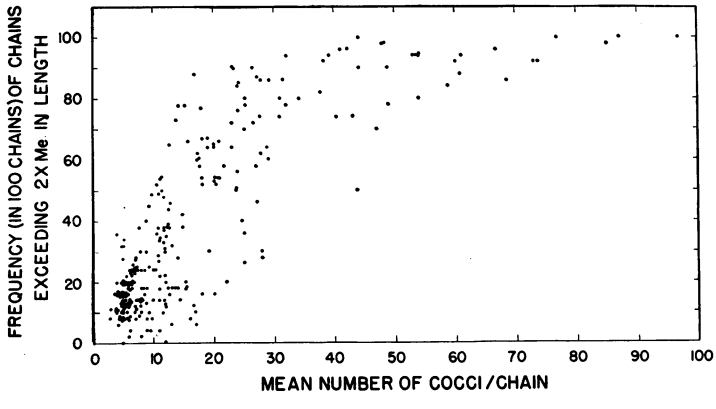

FIG. 2. *Relation of size-class frequency (> 2× Me) to mean chain length in 268 long-chain tests, taken at random, in both normal rabbit sera and in type-specific rabbit antisera.*

using fourfold tables to calculate chi-square values.

	Number of chains		
	$< 2 \times$ Me	$> 2 \times$ Me	Total
Normal serum	a	b	n_1
Test serum	c	d	n_2
	$a + c$	$b + d$	N

Before calculating the chi-square values, however, we felt that possible errors in sampling chain lengths in normal serum should be considered. The estimate of b obtained from a single sample of 50 chains may, for example, underestimate the true value. This would tend to give results indicating the presence of long chains when, in fact, the difference is due to random sampling variations. Similar reasoning indicates that an overestimate of the true population b would give results indicating that long chains were not produced and that no antibody is present.

Errors of underestimation are more serious in determining the actual presence of long chains and should be minimized. This can be done by substituting the upper limit of an appropriate confidence interval in the fourfold table for the observed b value. From tables compiled by Mainland, Herrera, and Sutcliff (1956), we have prepared Tables 1 and 2 which show, for an observed value of b, the upper limits of the 95 and 99% confidence intervals to be substituted and the corresponding values of d required to give chi-square values significant at both the 5 and 1% levels. Table 1 gives these values when the number of chains counted in each sample (n_1 and n_2) is 50. Table 2 indicates the values when n_1 or n_2 equals 100.

In Fig. 1, b was observed to be 4. Table 1 shows the corresponding upper limit of the 99% confidence interval to be 12, and therefore the value of d, to be significant, must be at least 23 if $p = 0.01$. The observed d value is 49 and the difference between the two samples is therefore significant at either level. When significantly different frequencies of b and d occur, we assume that the presence of antibody in the test serum is responsible.

Results of testing by the Size-Class Frequency (SCF) method were compared with results obtained by analyzing the same tests by the Long-Chain Index (LCI) of Stollerman et al. (1959). The LCI is said to be significant at the 1% level if the mean chain length in antiserum exceeds the mean chain length in normal serum by a factor of 3 or more. Tests of antiserum dilutions as well as tests read after different times of incubation are included in the comparison. For each test, all cocci in each of 50 or 100 chains were counted in both test and normal sera. The means, medians, and frequency distributions were then determined from these data.

Of 82 tests, 30 were positive by a LCI of 3 or greater. These results are compared with results of the SCF method, using four different sets of criteria, in Table 3. All 30 tests positive by the LCI were also positive by SCF, with the exception of only 2 tests, when the most rigid of the criteria (99% confidence limits and $p = 0.01$ in

TABLE 1. *Values of d required for significance at two levels for each of the confidence intervals and for observed values of b to 25. The number (n_1 and n_2) of chains counted in each serum was 50*

Value (b) observed	Upper limit 99% confidence interval	Value (d) required to give X^2 significant at 1% (p = 0.01) level	Value (d) required to give X^2 significant at 5% (p = 0.05) level	Upper limit 99% confidence interval	Value (d) required to give X^2 significant at 1% (p = 0.01) level	Value (d) required to give X^2 significant at 5% (p = 0.05) level
1	7	20	17	6	18	15
2	9	22	19	7	20	17
3	10	24	20	8	21	18
4	12	26	23	10	24	20
5	13	27	24	11	25	22
6	14	28	25	12	26	23
7	16	30	27	13	27	24
8	17	31	28	15	29	26
9	18	32	29	16	30	27
10	19	33	30	17	31	28
11	20	34	31	18	32	29
12	21	35	32	19	33	30
13	22	36	33	20	34	31
14	23	37	34	21	35	32
15	25	39	36	22	36	33
16	26	40	37	23	37	34
17	27	41	38	24	38	35
18	28	41	39	25	39	36
19	29	42	40	26	40	37
20	30	43	40	27	41	38
21	31	44	41	28	41	39
22	32	44	42	29	42	40
23	32	44	42	30	43	40
24	33	45	43	31	44	41
25	34	46	44	32	44	42

TABLE 2. *Values of d required for significance at two levels for each of two confidence intervals, for observed values of b to 30. The number (n_1 and n_2) of chains counted in each serum equalled 100*

Value (*b*) observed	Upper limit 99% confidence interval	Value (*d*) required to give X^2 significant at 1% (p = 0.01) level	Value (*d*) required to give X^2 significant at 5% (p = 0.05) level	Upper limit 95% confidence interval	Value (*d*) required to give X^2 significant at 1% (p = 0.01) level	Value (*d*) required to give X^2 significant at 5% (p = 0.05) level
0	5	18	15	4	16	13
1	7	21	17	6	19	16
2	9	24	20	7	21	17
3	11	27	23	9	24	20
4	12	28	24	10	25	21
5	14	30	27	11	27	23
6	15	32	28	13	29	25
7	16	33	29	14	30	27
8	18	35	31	15	32	28
9	19	39	33	16	33	29
10	20	38	34	18	35	31
11	22	40	36	19	38	33
12	23	41	37	20	38	34
13	24	43	38	21	39	35
14	25	44	39	22	40	36
15	26	45	41	24	43	38
16	28	47	43	25	44	39
17	29	48	44	26	45	41
18	30	49	45	27	46	42
19	31	50	46	28	47	43
20	32	51	47	29	48	44
21	33	52	48	30	49	45
22	34	54	49	31	50	46
23	36	56	51	33	52	48
24	37	57	52	34	54	49
25	38	58	53	35	55	50
26	39	59	54	36	56	51
27	40	60	55	37	57	52
28	41	61	56	38	58	53
29	42	62	57	39	59	54
30	43	63	58	40	60	55

TABLE 3. *Comparison of 82 type 1 antisera in the long-chain reaction when evaluated by both the Long-Chain Index and the Size-Class Frequency method*

Criterion for SCF	LCI result		
	Positive (3 or above)	Negative	Total
2 × Me, p = 0.01			
99% confidence limits			
Positive	28	12	40
Negative	2	40	42
2 × Me, p = 0.01			
95% confidence limits			
Positive	30	18	48
Negative	0	24	34
2 × Me, p = 0.05			
99% confidence limits			
Positive	30	17	47
Negative	0	35	35
2 × Me, p = 0.05			
95% confidence limits			
Positive	30	21	51
Negative	0	31	31

TABLE 4. *A comparison of the LCI and SCF methods based on 82 tests*

Criterion	Positive	
	no.	%
LCI of 3 or greater, p = 0.01	30	36.5
SCF > 2 × Me, p = 0.01, 99% confidence limits	40	48.7
SCF > 2 × Me, p = 0.01, 95% confidence limits	48	58.5
SCF > 2 × Me, p = 0.05, 99% confidence limits	47	57.3
SCF > 2 × Me, p = 0.05, 95% confidence limits	51	62.1

the chi-square test) were applied. By all other criteria, the SCF method indicated more positive results than did the LCI. Results of the 82 tests, by the several criteria, are summarized in Table 4.

It appears that the SCF method detects antibody more frequently than does the LCI, when using the previously described (Stollerman et al., 1959) criteria of significance for the latter.

For convenience, we have occasionally expressed the results of the SCF method by an index designated the Size-Class Frequency Index (SCFI), which can be applied regardless of the confidence limits and level of probability selected. Because it incorporates the findings in both the test serum and the normal control serum, it enables one to determine statistical significance at a glance. It is derived by dividing the frequency in antiserum of a chosen size-class (i.e., >2 × Me) by the minimal frequency of such size-class required to give the selected level of probability. This minimal theoretical frequency is *d* after adjustment of *b* to the upper confidence limit as previously explained. It therefore depends on the frequency (*b*) of the same size-class found

in the simultaneous normal serum control and can be found, when b is known, by using Tables 1 and 2.

If, for example, b for $>2 \times$ Me in a given test is observed to be 10, its substituted value for a 95% confidence limit is 17 (Table 1); the corresponding value of d must then be 28 or greater in order for $p = 0.05$ or less. If the observed value of d in the test serum is 46, then SCFI = 46/28 = 1.6. A SCFI of 1.0 means that the observed and required values of d are the same and that p is equal to the selected probability level. An index greater than 1.0 indicates the p is less than the selected level: the height of the index above 1.0 corresponds to decreasing values of p and, therefore, to greater degrees of statistical significance. Indices below 1.0, of course, indicate no statistical significance at the chosen level of probability, and sera giving such results are recorded as negative.

Effect of incubation time on chain length. Two series of inoculated tubes, one containing normal and the other homologous antisera, were placed in a water bath at 37 C. At 30-min intervals, one tube of each series was removed from the water bath and placed in ice water. The mean chain lengths and the frequencies of chains above and below 2 × Me in each successive pair of sera were determined.

Figure 3 demonstrates the aggregate results of several experiments in which T1/155/4 was grown in pooled normal sera and in homologous antisera. Sampling the chain lengths at time intervals in normal sera indicates that the strain tends to a constant basal chain length during the period of observation. We have found this to be true of all strains of all types tested so far. Samples taken simultaneously from an antibody-containing system indicate that, with time, the chain length increases to a maximum and then decreases toward the basal length found in antibody-free sera.

Effect of antibody concentration on chain length. Series of inoculated tubes containing pooled normal rabbit sera and varying concentrations of homologous antiserum were placed in a water bath at 37 C. At successive time intervals, one tube from each series was removed from the water bath, placed in ice water, and the size-class frequencies and mean chain lengths in each sample determined.

Figure 4 shows typical results of one such test, plotted as SCFI. (Curves of similar shape are obtained whether SCF alone, mean chain length, or LCI are plotted.) The results indicate that the maximal long-chain effect in a low concentration of antibody is reached sooner and is lower than in a higher concentration of antibody. In addition, the length of time over which the long-chain effect remains statistically significant (if significance is attained in a given dilution) is longer in higher concentrations of antibody than in low.

Effect of inoculum size on chain length. A 24-hr

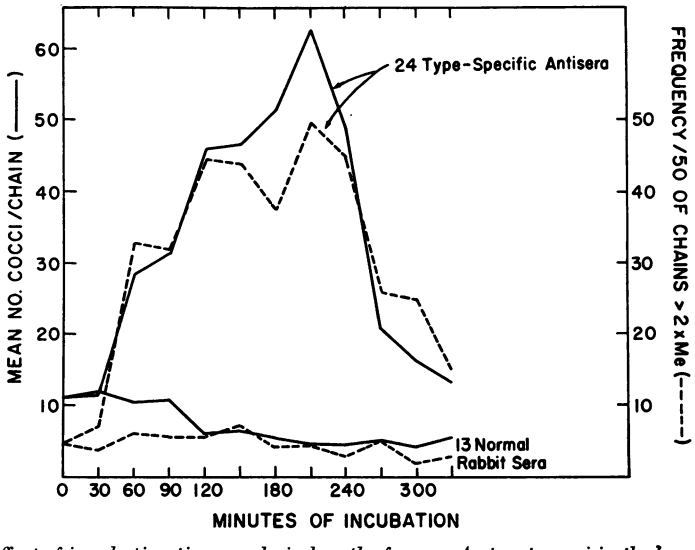

FIG. 3. *Effect of incubation time on chain length of group A streptococci in the long-chain test*

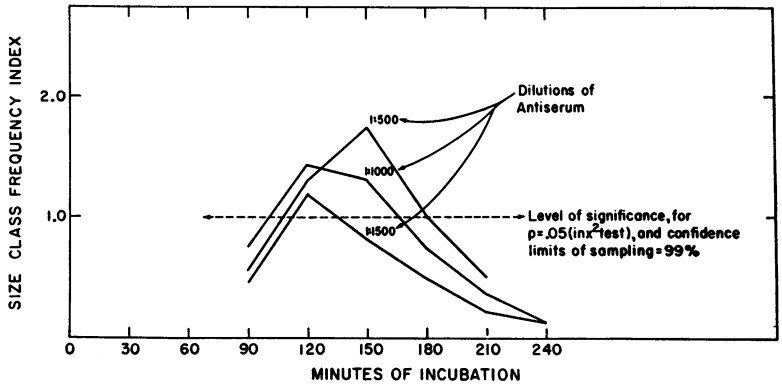

FIG. 4. *Effect of antibody dilution + incubation time: long-chain reaction of type 1 streptococci (T1/155/4) grown in type-specific antiserum.*

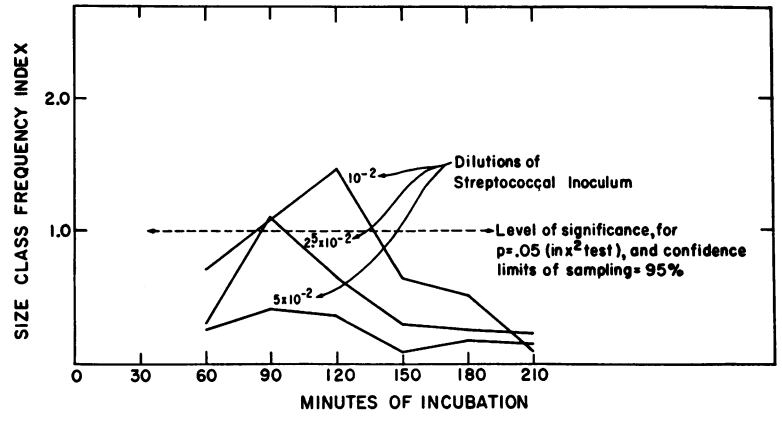

FIG. 5. *Effect of inoculum size + incubation time: long-chain reaction of type 1 streptococci (T1/155/4) grown in type-specific antiserum.*

Todd-Hewitt culture of T1/155/4 was diluted 5.0×10^{-2}, 2.5×10^{-2}, and 10^{-2} in broth. The standard volume (0.05 ml) of each concentration of streptococci was added to tubes containing either pooled normal rabbit sera or a constant amount of antiserum. At 30-min intervals a pair of tubes from each series was taken from the water bath, iced, and the mean chain lengths and size frequencies determined.

The results (Fig. 5) indicate that the effect of increasing inoculum size is inverse to that of increasing antibody concentration, i.e., the maximal long-chain effect (measured either as mean chain length or as frequency of the larger size-class) is reached sooner and is lower when inocula are large than when they are small, provided that the amount of antibody at the start of the test is constant.

Effect of antibody on growth. A 24-hr culture of T1/155/4 was diluted 10^{-2} in broth. Long-chain tests in normal rabbit serum and in type-specific rabbit antiserum were set up in the usual fashion, using 0.05-ml inocula of this culture dilution. The tests were placed in a water bath at 37 C to incubate for 2.5 hr, and the dilution used for inocula was then immediately placed in ice water. Samples of the dilution were then treated, to reduce chain length, first by standardized to-and-fro pipetting, ten times, in a 10-ml pipette, and then by exposure in sterile 2-ml lusteroid tubes to sonic oscillation (1-min exposure, Raytheon sonic oscillator, Model DF 101, 10 kc, at 1.25 amp output current) as suggested by Slade and Slamp (1956). The lusteroid tubes were immersed through a one-hole rubber stopper in water filling the oscillator cup, which was cooled by circulating ice water. Following each pro-

cedure, serial dilutions were made and pour plates for colony counts prepared.

After 2.5 hr of incubation (in sterile 2-ml lusteroid tubes), the tubes containing streptococci with normal sera and with antisera were immediately placed in ice water. One drop of each culture was used to determine mean chain length in 100 chains. Chains were then fragmented by each of the two methods described above. Mean chain lengths were then again determined, and 0.1 ml of each test mixture was appropriately diluted (usually three doubling dilutions, starting at 1:8,000) in sterile iced Trypticase soy broth for pour plates. Plates were poured from 0.1 ml of each dilution in 10 to 12 ml of 5% sheep-blood agar. Colony counts were made after 24 hr incubation at 37 C. The count of each dilution was adjusted to the number of colonies per milliliter of the incubated serum-streptococcal mixtures. The counts for all dilutions of each serum-streptococcal mixture were then averaged.

The results (Table 5) indicated that growth (total cocci produced in a given time) in normal rabbit serum and in type-specific rabbit antiserum was comparable, and that the comparability could be shown by either method of chain fragmentation (assuming throughout that one chain, of whatever length, gives rise to one colony). Sonic treatment, as expected, was more effective than pipetting in fragmenting chains; it produced mean chain lengths approximately half as long as those found after pipetting. In conformity, colony counts after sonic treatment were approximately twice those following use of the pipette method.

TABLE 5. *Results, after chain fragmentation, of total streptococcal growth in normal rabbit serum and in type-specific rabbit antiserum determined by two different methods**

Method of fragmentation	Colonies/ml (in millions)	Mean no. cocci per chain	Total cocci/ml (in millions)
Pipetting			
Normal serum	17.1	3.5	59.85
Antiserum	14.1	4.2	59.22
Sonic treatment			
Normal serum	34.3	1.6	54.88
Antiserum	35.5	1.8	63.90

* Inocula were measured as 3.3 million colonies per ml (pipetting method) and 3.1 million colonies per ml (sonic method). Type 1 antiserum, normal rabbit serum, and type 1 streptococcus (T1/155/4) were used in all tests. All were incubated 2.5 hr in a water bath at 37 C.

TABLE 6. *Reproducibility of long-chain test in two methods, tested in triplicate on two successive days*

Dilution of type 1 antiserum	Experiment on day 1		Experiment on day 2	
	SCF*	LCI†	SCF*	LCI†
1:1200	+	+	+	0
	+	0	+	0
	+	+	+	+
1:1600	+	0	+	0
	+	0	+	0
	+	+	+	0
1:2400	0	0	0	0
	0	0	0	0
	0	0	0	0

* Criteria for SCF positive: class divisor = 2 × Me; $p = 0.01$, 99% confidence limits.

† Criteria for LCI positive: index, 3 or greater; $p = 0.01$.

Reproducibility of results. Long-chain tests, using T1/155/4 streptococci and dilutions of a single homologous antiserum, were performed in the usual fashion. The tests were set up in triplicate and performed on two separate days. The summarized results are presented in Table 6.

Observed chain lengths, measured as either the mean or the number exceeding 2 × Me, varied somewhat from day to day or from one replicate test to another. In relation to the paired control sera, however, the observed values analyzed by the SCF method gave reproducible results throughout in detecting long chains at the selected level of significance. At the same level, detection by the LCI method was variable in replicate and nonreproducible from day to day.

DISCUSSION

The SCF method, as employed in this paper, is more sensitive than the previously described LCI (Stollerman et al., 1959) in detecting a long-chain reaction in homologous antiserum. Had confidence limits been similarly applied to data for the LCI, the difference in sensitivity of the two methods would be even greater. It is, therefore, possible that the long-chain test, analyzed by the SCF method, may prove at least as sensitive as the bactericidal test—even under the conditions of low antibody concentration,

in which the latter test was described as more sensitive by Ekstedt and Stollerman (1960*b*). Preliminary tests indicate that the SCF method is indeed more sensitive than the bactericidal test, but we have not completed extensive comparisons.

In addition to the greater sensitivity and better statistical control, the SCF method is simpler and more rapid than the LCI. There are, however, certain precautions to be taken in its use and interpretation. It is obvious, for example, that selection of the level of the class divisor may influence the test sensitivity according to whether one selects twice the median value in normal serum, three times the median, or some other figure. In addition, sensitivity varies with the combination of confidence limits and significance levels selected from Tables 1A and 1B. Also, it is well to emphasize that the use of chi-square values tests only the heterogeneity of distribution of chain lengths in normal and test sera compared, and does not test a degree of response. Therefore, a greater value of chi-square for one test serum-control serum pair as compared to another does not indicate that there is more antibody present in the test serum. The best measure of antibody level is determination of the highest dilution of a serum which produces a long-chain effect according to the criteria selected for significance in the SCF test. Such end points in different sera may be compared if the same criteria, conditions, and controls are employed.

Our results show, however, that antibody titration by dilution requires consideration of several factors. Chief among these is the time of incubation prior to examination for long chains. Because of the parabolic nature of the long-chain response with time (Fig. 3, 4, and 5), readings made too early or (in particular) too late may fail to detect the long-chain effect (e.g.: in Fig. 4, a sample at 120 min indicates the presence of antibody at 1:1,500 dilution, whereas a sample of the same dilution at any other 30-min interval fails to do so).

The effects of initial concentration of streptococci and of antibody in the test must also be considered. Their effects are inverse to one another (Fig. 4 and 5) and each must be related to the time of incubation. Even though the streptococcal inoculum be standardized as is usual, the height of the long-chain effect, the time at which it can be detected at a predetermined level of significance, and the time of maximal response decrease with increasing serum dilution. Figure 6 represents schematically the inter-relationships of initial concentration of antigen (streptococcal inoculum), initial concentration of antibody, and time of incubation. These relationships explain the original inability of Stollerman and Ekstedt (1957) to detect low

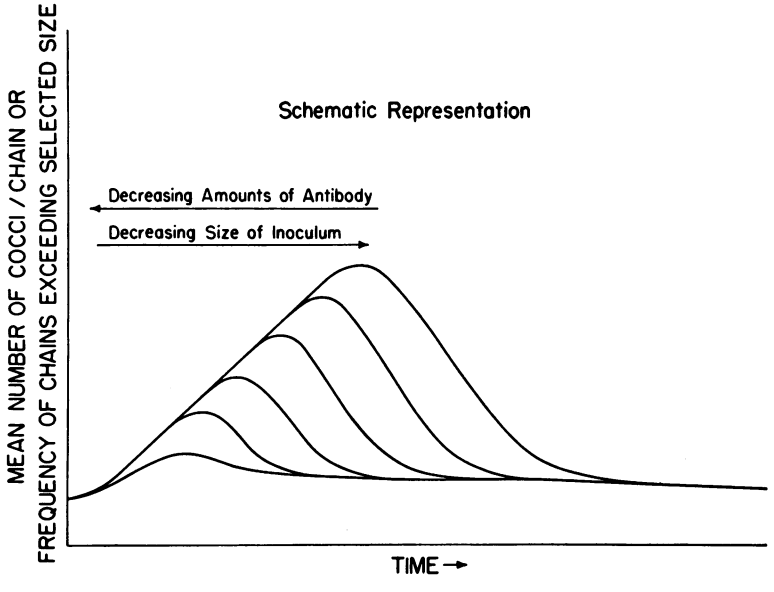

FIG. 6. *Effects of antibody content and inoculum size on the long-chain reaction.*

levels of anti-M antibody by use of certain M-rich strains, and their later success (1960*b*) when M-poor derived variants or higher antibody levels were employed. Thus, they recognized also that the initial concentration of antigen is determined not only by the numbers of streptococci in the inoculum, but also by the amount of M protein present on each cell wall. It is likely that the use of the SCF method with time-interval sampling could have detected long-chain formation in such weak antisera. There is, obviously, a lower limit of antibody concentration below which no test will be positive, because of the presence of cell wall-bound M in relative excess. No reduction in inoculum to overcome this effect appears feasible for reasons discussed below.

In performing tests for detection of the long-chain reaction, certain mechanical and sampling difficulties occur. For example, it is not practicable to make several readings in time on each of a large number of sera or serum dilutions, and yet, as we have shown, no single reading is reliable in detecting the long-chain reaction in every dilution. If samples are taken too early, there may be insufficient time for cell division to produce long chains. In addition, even though long chains may be present, the sparsity of chains in an early sample makes the microscopy a long process. This difficulty cannot be overcome entirely by increasing inoculum size because this results in an immediate binding of what may be an already low level of free antibody; the long-chain effect will then fail to take place (Fig. 5).

If samples are taken too late, free antibody may have been depleted by binding to the multiplicity of new cocci produced and the long-chain effect has ceased. Also, the plethora of chains in a late sample may fill each oil immersion field examined; such a field may not be random nor representative, and if the particular test be based on classifying the requisite 50 to 100 chains in only one such crowded field, the sampling efficiency may be very poor. On the other hand, attempting to obviate this by classifying chains in only a certain area of a field, or by classifying a given number of chains in each of many fields may also result in bias by unconscious selection of short or long chains.

To minimize the mechanical difficulties of too few or too many chains per microscopic field, we recommend the following procedure. A series of four normal sera is set up with each group of test sera. Starting at 1 hr incubation, a normal serum is examined microscopically at each 30-min interval. The test sera are removed from the water bath and iced when the normal sera reveal one to two chains per oil-immersion field. The number of longer chains per field in positive test sera is then usually less, so that each chain seen may be size-graded and a large area of the drop examined to establish the size-class frequency distribution within the required number of chains. Bias by selection in a chain-crowded field is thus avoided. This procedure may still result in sampling too late to detect the early and transient long-chain effect of a very low antibody concentration, but will minimize false negatives caused by later sampling when chain length (or long-chain frequency) is decreasing because of continued coccal growth in the absence of free antibody.

All of the effects described appear to depend on growth and division of individual cocci, which explains the time requirement. If growth and division occur in the continued presence of homologous antibody, the number of cocci per chain increases. In heterologous or normal serum, however, the chain tends to a constant and short basal length. This basal length varies with the strain or type of streptococcus and may be related to capsule formation and virulence (Ekstedt and Stollerman, 1960*a*). The total number of chains, however, theoretically increases in normal serum but not in antiserum. The presence of homologous antibody, as shown (Table 4), does not affect streptococcal growth as compared with growth in normal serum. Once antibody is exhausted, as time and growth continue, its effect in maintaining chain lengths ceases and the mean chain length (and frequency of long chains) decreases (Fig. 7).

The long-chain reaction, as previously described (Stollerman and Ekstedt, 1957; Stollerman et al., 1959) and presently accepted, may be defined as maintenance of chain integrity during and after streptococcal division as long as free homologous antibody is present. Although anti-M antibody usually appears responsible for the reaction, some studies (Ekstedt and Stollerman, 1960*b*) have shown that a type 3 R-anti-R system may also produce long chains. No investigations have yet ruled out participation of anti-T antibody. The latter must be present in many so-called type-specific anti-M antisera because of the manner of their preparation by absorption with heterologous types of group A streptococci. The

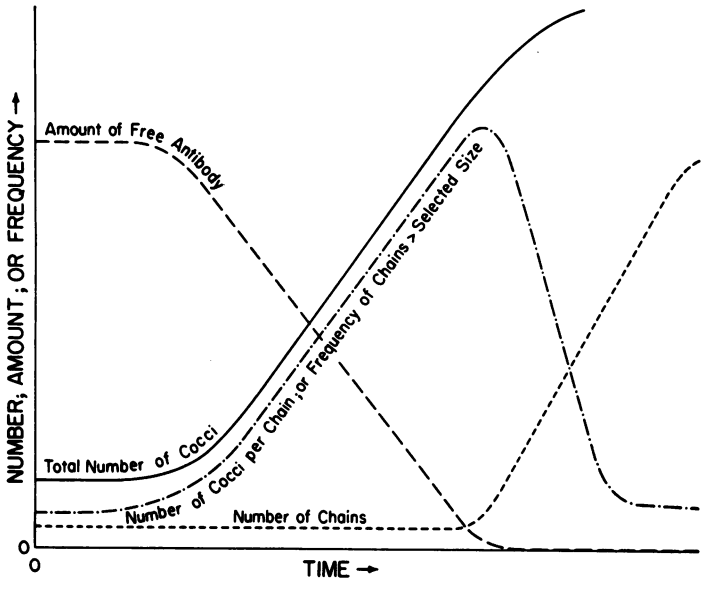

FIG. 7. *Theoretical sequence of events in positive long-chain test*

absorbing cells do not necessarily, nor even usually, contain the same T antigen as that of the immunizing M-type organisms; anti-T antibody therefore remains in the serum where it may participate in formation of long chains of the homologous organism.

Regardless of specificity, the mechanism by which homologous antibody acts to produce long chains remains obscure. Because other factors, such as protein deficiency, cultural conditions, and strain variation, may result in long chains, it is not clear whether long or short chains may be considered the "normal" state and whether, therefore, one must postulate inhibition of a dechaining mechanism to make chains long, or inhibition of a chaining mechanism to make chains short. The former mechanism has been favored by Ekstedt and Stollerman (1960*a*), who suggest that a chain-splitting enzyme exists. Their report on inhibition of dechaining by bactericidal substances and changes in pH and in temperature may be interpreted, however, according to the known deleterious effects of these substances or conditions on bacterial metabolism, growth, and division, and not necessarily dependence on a specific dechaining enzyme. If dechaining is caused by an enzyme, one must assume that it is inhibited by the above-mentioned factors as well as by antibody, and that antibody either blocks it specifically (indicating that M, R, or whatever antigen involved is itself the antigenic enzyme) or nonspecifically, perhaps by some sort of steric hindrance. If there is no enzymatic dechaining and the linkage of adjacent cocci in antiserum is simply by antibody union (as suggested for enteric bacteria by Pfaundler, 1898), one must explain why it is polarized (end-to-end) and does not necessarily result in general agglutination and why chaining occurs under other circumstances when antibody is absent. At present, in our opinion, there is no satisfactorily inclusive explanation of the mechanism of the long-chain phenomenon.

In the course of this work, many long-chain tests have been performed with a variety of types of group A streptococci, using many heterologous and homologous antisera. The phenomena related to incubation time, antigen concentration, and antiserum concentration have been observed repeatedly with all types and their homologous antisera tested, but strains representing all known M-types have not been examined. Suggestions of long chaining due to antigen-antibody combinations other than M-anti-M, as previously described by Ekstedt and Stollerman (1960*b*), have been noted. Such experiments are not reported in detail here because the only purpose of this paper is to describe a rapid and sensitive method for evaluating long-chain formation, by which previously neglected parameters of long-chain testing have been revealed and which influence results obtained in such testing. We hope

that the simple method described will stimulate and facilitate more investigations of the specificity and mechanism of the long-chain reaction of streptococci. The potential usefulness of the test in epidemiological investigation has been suggested by Stollerman and Ekstedt (1957) and Stollerman et al. (1959) and is emphasized by the availability of a more rapid, sensitive, and statistically controllable method.

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