STUDIES ON VIRULENCE.

II. The Increase in Virulence in Vitro of a Strain of Pneumococcus.

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This paper is the second of a series on the study of virulence of microorganisms grown *in vitro*. Paper I[1] is a preliminary investigation and has to do with the influence of virulence on microorganisms transferred during the logarithmic increase phase—the period of optimum multiplication activity. In recent years, considerable attention has been directed toward the activity of so called young microorganisms as compared to the old culture. Chesney[2] has shown that the 6 to 8 hour growth of pneumococci has greater infectivity than the culture of 18 to 24 hours. Chick,[3] who worked with *Bacillus paratyphosus*, alleges that young microorganisms are more resistant to antiseptics than old ones, while Sherman and Albus,[4] in a publication on the physiological youth of bacteria, conclude that differences exist between young and mature bacterial cells, the newly formed cells passing through a period of physiological youth. They base their views upon the varied actions of mature and young bacteria exposed to 2 per cent sodium chloride, phenol, and low temperatures, the young cell being more susceptible to these agents injurious to life. The lag in rate of growth following inoculation—an inability of organisms to continue cell division at a constant rate after being introduced into a change of environment—can be explained from the standpoint of age of the cells, for Barber[5] and McKendrick and Pai[6] have proved

[1] Felton, L. D., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 262.

[2] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

[3] Chick, H., *J. Hyg.*, 1908, viii, 92.

[4] Sherman, J. M., and Albus, W. R., *J. Bact.*, 1923, viii, 127.

[5] Barber, M. A., *J. Infect. Dis.*, 1908, v, 379.

[6] McKendrick, A. G., and Pai, M. K., *Proc. Roy. Soc. Edinburgh*, 1910–11, xxxi, 649.

that bacterial lag is absent when young cultures are used for inoculation. Young organisms may be thought then to possess greater reproductive activity than old organisms, or, perhaps, to become more easily adapted to environmental conditions inimical to the multiplication of older cells. If we may assume that a fundamental difference exists in bacteria at varying intervals of time after cell division—variation in age and function—analogous to those found in the stages of animal life (infancy, childhood, maturity, old age) a possibility is foreseen that microorganisms transferred in the vigor of their youth may adapt themselves more easily to conditions which favor an increase or decrease in virulence; that is, the microorganism, when young, may perhaps be more readily immunized, according to Welch's hypothesis, against the substances or conditions unfavorable to existence, or, to express the matter from another standpoint, be trained to live on such a food supply as will fit it for life on living animal tissue. In a previous study, it was observed that the youth of the cell is a factor in the rate of reaction to environment. This fact prompted the undertaking of a study to determine what changes, if any, in the virulence of bacterial cells might be found when transfers are made during the period of greatest growth activity; namely, youth.

Estimation of Virulence.

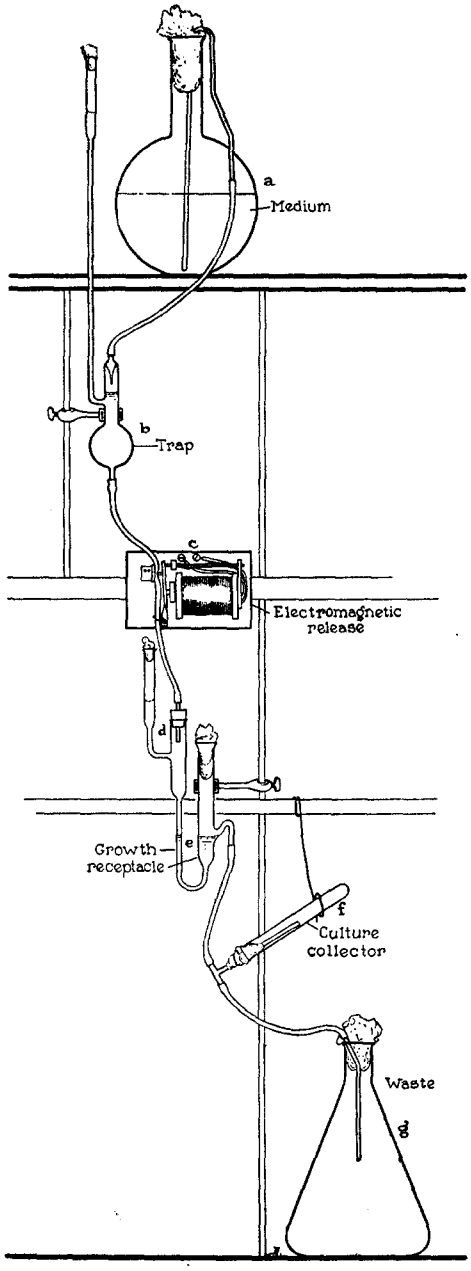
The virulence of the strains of pneumococci employed in the present work was estimated in terms of the minimum number of organisms which caused the death of a mouse in from 36 to 72 hours. The actual titration of virulence was done by injecting mice intraperitoneally (three or four for each test) with varying numbers of organisms, the successive dilutions of which represented a logarithmic series, as for example, 1:10, 1:100, 1:1,000, 1:10,000. The minimum lethal dose was assumed to be the least number of organisms that caused death consecutively in the series of mice injected. This procedure was used to minimize probable error. The irregularities in the mortality of the experimental animals that were injected with doses of organisms which differed so widely as those of a logarithmic series may be ascribed perhaps more to variations in the individual susceptibility of the animal than to irregularities in the virulence of the pneumococci. For virulence of a culture at a given time is a constant factor when dealing with the experimental infection; the variable is the animal. Whence it follows that an estimation of virulence necessitates taking into consideration the sequence and the regularity of death in the series of mice injected with graded doses of organisms. The number of organisms that were injected for the estimation of virulence of any culture was calculated by the plate method, through the use of serum agar plates (pH = 7.4) with three different dilutions.

Apparatus.

Two changes were made in the apparatus as described in the former study. Instead of a dropping device to regulate the flow of medium, an electromagnet clamp controlled by a time-clock was employed. In the growth receptacle previously used, the straight arm overflow did not permit of transfers at short intervals when media were studied in which the pneumococci grew with difficulty, because the number of organisms which remained in the growth receptacle was insufficient to reinoculate the fresh medium. For this reason, a short siphon was made to take the place of the straight arm overflow, and thus a mixing of fresh culture was assured before the overflow began. It was then found possible to make flushings of the device as often as every half hour and still maintain growth of the cocci. A typical arrangement of the apparatus is shown in Text-fig. 1.

The large siphon flask (*a*) at the top contains the medium which enters by rubber tubing into a trap (*b*) through a capillary tube of smaller bore than the outlet. This trap, used for the purpose of insuring a constant pressure head, is in turn connected by means of rubber tubing to a capillary tube (*d*) of known bore, which is held in the inlet arm of the growth receptacle by means of a rubber stopper. The spring on the electromagnet (*c*) closes the rubber tubing between the trap and the growth receptacle (*e*). From the small siphon on the growth receptacle, a rubber tubing leads to the waste flask (*g*) through the culture collector (*f*), this being a T with a long arm, around the upper part of which cotton is wound as a plug for a test-tube of usual size. In operation, the electromagnet is connected with a timing device so that at fixed intervals the spring is released and a quantity of the medium, which will depend upon the size of the capillary and length of time of release, flows into the receptacle. The fluid rises in this vessel until the siphon is started and then overflows into the waste. Organisms are obtained when desired from the growth receptacle by placing the collector so that it points downward and clamping the tubing between it and the waste; the test-tube of culture is taken off and another, after being well flamed, is put in its place. Sterilization of the apparatus is accomplished by autoclaving, either in units or as a whole. Little difficulty has been experienced with contamination. Any trouble met with was caused either by defective cotton plugs or by rubber tubing containing a spore-bearing bacillus.

The growth receptacle made from 15 mm. tubing has a capacity up to the siphon of 7 cc., to overflow of 12 cc., inlet and outlet tubes being 3 mm. in diameter. The siphon is 10 mm. high. The capillary inlet in the rubber tubing varies, of course, with the kind of medium and the length of time of release of the electromagnet spring. With the present apparatus, the 1 mm. tubing allowed 15 cc. of plain broth to pass through in 1 second, or about 14 cc. of sterilized skimmed milk.



TEXT-FIG. 1. Apparatus designed for study of virulence. A typical set-up.

Choice of Medium.

Whatever the conception of virulence, most investigators conceive a virulent organism to be one that will grow within the animal body and be capable of producing a characteristic lesion or death of the host. This may be true whether virulence is considered a state of affairs brought about by overcoming conditions found in the living host detrimental to bacterial growth, Welch's hypothesis, or as a state developed through the influence of specific food such as that contained in animal tissue. If we recall the demonstrated fact that animal passage as a rule increases virulence, and assume further that the growth of the microorganism on animal tissue is responsible for such a biological variation, it follows logically that a medium for the study of virulence *in vitro* must be made from animal tissues. Since milk contains the food elements needed for maintenance and growth in the animal body and is in addition one of the most readily available forms of animal secretions having the constituents necessary for life, it was selected as medium for this study. Unless otherwise stated, the milk was freshly pasteurized skimmed milk, delivered packed in ice from a commercial pasteurizing depot. With one exception, milk from a single batch was employed for each experiment. Sterilization was usually accomplished in an Arnold sterilizer for 55 minutes on 3 successive days. This length of time was found necessary for effective sterilization, for the reason that 30 to 35 minutes were required to heat a large flask of medium to 100°C.

Experiment 1. Effect of Interval of Transfer on Increase of Virulence.

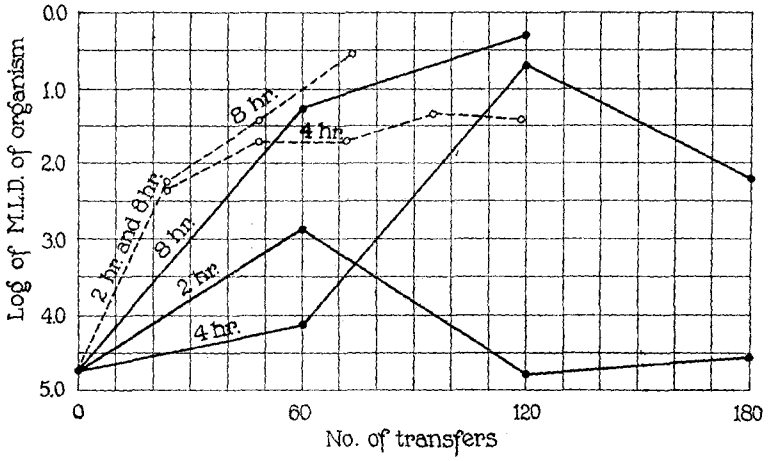
The purpose of the following experiment was to establish the optimum point of the growth curve, as determined by interval of transfer, for enhancing virulence.

Two experiments (Text-fig. 2), involving the same culture grown on two different lots of skimmed milk, were carried out with transfers made at 2, 4, and 8 hour intervals. The strain of pneumococcus used was the Neufeld, Type I, which had been made avirulent by 24 hour transfers on horse serum (Stryker;[7] Wadsworth and Kirkbride[8]). The culture grew characteristically turbid on plain broth, was bile-soluble, and true to type.

[7] Stryker, L. A., *J. Exp. Med.*, 1916, xxiv, 49.

[8] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1918, xxviii, 791.

The results as represented on the chart reveal the possibility of markedly increasing the virulence for mice of a strain of pneumococcus by growth *in vitro*, the degree of enhancement depending to some extent on the interval between transfers. In the study represented by the solid lines, the 8 hour interval is optimum and the 4 hour almost as favorable, while the 2 hour interval acts to bring about some increase of virulence with a subsequent lessening. The experiment illustrated by the dotted lines demonstrates somewhat different results; the 4 hour interval is as favorable in this case as the 8 hour interval, but the 2 hour approaches the efficacy of the 4 hour interval

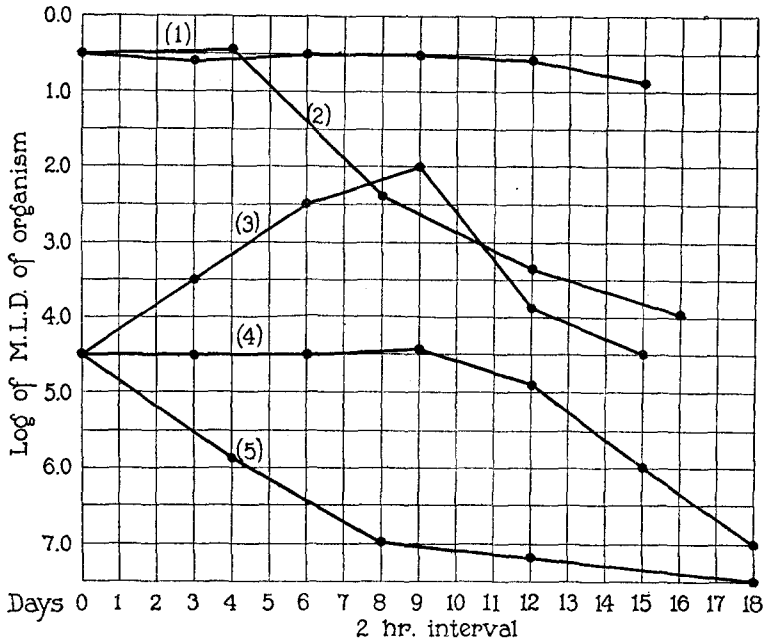


TEXT-FIG. 2. Effect of variations in interval of transfer; milk medium.

of the previous experiment. The lack of correspondence in these two experiments is rather perplexing and indicates that some factor other than the interval of transfer is operative in the increase in virulence of pneumococci in milk. The average number of organisms per cubic centimeter was approximately the same in both experiments, 4×10^5, the number in the culture of a 2 hour period being somewhat less. Among possible reasons for the irregularity in the enhancement of virulence shown in these experiments, that of variability in the character of the milk suggested itself. To ascertain whether different lots of this medium vary in suitability, Experiment 2 was carried out.

Experiment 2. Effect of Different Lots of Milk.

Four different lots of milk of the same H ion concentration (pH = 6) were studied in their influence on a highly virulent and a relatively avirulent strain of pneumococcus. The organisms used were the avirulent Neufeld, Type I, of Experiment 1 (Text-fig. 3, Nos. 3, 4, and 5), and the same strain with initial virulence maintained by occasional animal passage (Text-fig. 3, Nos. 1 and 2).



TEXT-FIG. 3. Influence upon virulence of different lots of milk. The numbers 1 and 3 represent the experiments made with a single sample of milk; the numbers 2, 4, and 5 represent those on three different lots.

The results depicted upon Text-fig. 3 signify that milk does not supply a uniform medium for the increase of virulence. The virulence of a strain of microorganism which was highly virulent to begin with was maintained or was decreased, while that of an avirulent strain was either increased, maintained, or decreased when it was grown on the various samples of milk. The question naturally arises as to what cause the varied results may be attributed to. Pasteurized skimmed milk has undergone certain changes caused by heat and

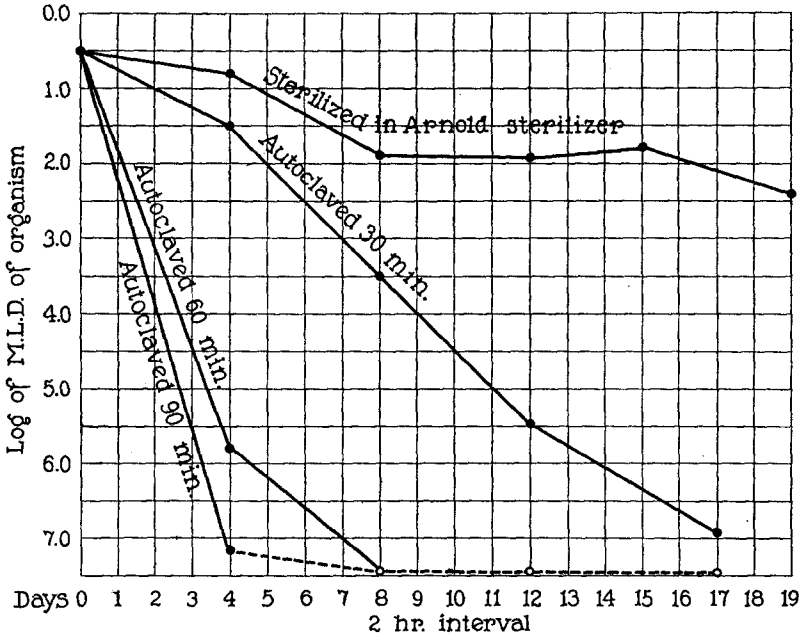
during the process of cream separation. Whole milk is difficult to handle with the apparatus used in this study, so before making an investigation of this question, the effect of varying degrees of heat on the suitability of skimmed milk for maintaining and increasing the virulence of pneumococci was studied.

Experiment 3. Effect of Heating Milk on the Ability to Influence Virulence in Vitro.

Two strains of pneumococci from one culture (Neufeld) were used, a virulent strain (Text-figs. 7 and 4) and a strain that remained avirulent in spite of all attempts to alter its virulence by either *in vivo* or *in vitro* methods. The same batch of milk was divided into four lots and each portion was sterilized by heat for varying lengths of time; one lot (Text-figs. 7 and 4) was sterilized in an Arnold sterilizer on each of 3 days for 55 minutes; one autoclaved 30 minutes at 17 pounds; one autoclaved 60 minutes at 17 pounds; and the fourth portion autoclaved 90 minutes at 17 pounds pressure. All were adjusted to pH = 7.4 after heating.

The rate of growth of the pneumococci was about the same in the four experiments, variation in numbers in the milk sterilized in the Arnold sterilizer and in milk autoclaved for 90 minutes averaging about 20 per cent, with the higher count in the former. The avirulent culture remained avirulent throughout the experiment; 1 cc. direct from the growth receptacle or from an 18 hour culture did not produce a fatal infection. The effect of varying degrees of heat influenced the suitability of the milk for maintenance of virulence. Although this particular sample of milk heated in an Arnold sterilizer did not supply conditions appropriate in a 2 hour interval of transfer for the maintenance of the original virulence of this strain of pneumococci, it is clear that the suitability of the medium for preserving virulence was decreased in proportion to the length of time it was autoclaved. Though growth occurred in all four lots of milk of this experiment, it may be concluded that milk subjected to high temperatures is so altered in its constitution that virulent pneumococci grow on it when transferred at 2 hour intervals lose their virulence for mice. The number of organisms in each portion of milk, regardless of the method of sterilization or length of time of exposure to heat, was approximately the same; the difference in the cultures was the rate at which changes in virulence took place. Not improbably, a heat-labile substance in

milk which is essential for maintaining or increasing virulence was destroyed. The avirulent strain of pneumococci remained avirulent throughout these experiments, as might be expected.



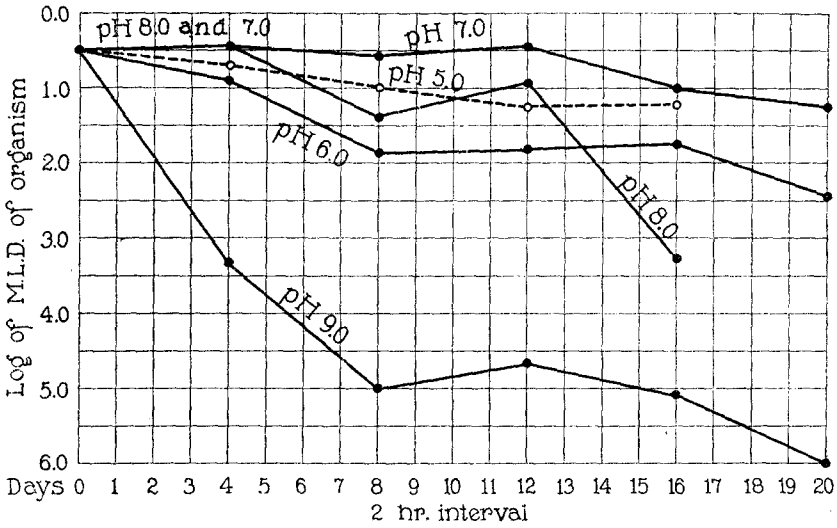
TEXT-FIG. 4. Effect of heat upon the suitability of milk to increase virulence.

Experiment 4. Effect of Hydrogen Ion Concentration.

The same strains of virulent and of avirulent organisms were used in this experiment as in the preceding one. Adjustment of the milk to various H ion concentrations was carried out after sterilization. Duplicate flasks of five different H ion concentrations, pH = 5, 6, 7, 8, and 9, were prepared from a single batch of milk. No attempt was made to estimate the pH nearer than 0.2. In a study of the effect on virulence of H ion concentration of plain broth, to be reported later, it was found that growth of pneumococci occurred at an H ion concentration as low as pH = 5. Inasmuch, however, as Avery and Cullen[9] have shown that the optimum H ion concentration for the initiation of the growth of pneumococci in plain broth is pH = 7.8, a large number of organisms, 5 cc. of a young broth culture, was used to assure inoculation of media of such wide variation

[9] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 359.

in H ion concentration as those between pH = 5 and pH = 9. Growth began immediately in them, although some variation in the number of organisms occurred in the milk of the various H ion concentrations. The flask of pH = 5 averaged 4 × 10^6, pH = 6 averaged 4.5 × 10^6, pH = 7 averaged 5.8 × 10^6, pH = 8 averaged 2.5 × 10^6, and pH = 9 averaged 2.2 × 10^6. The optimum H ion concentration for multiplication was thus found in the medium of pH = 7, the pH = 6 producing a little less growth. While the media titrated to pH = 5, 8, and 9 all supported good growth, there was a gradual falling off in numbers in the last two (pH = 8 and 9) after the 8th day. On the average, the final H ion concentrations of the cultures in the growth receptacles were as follows: pH = 5 remained 5.0; pH = 6 dropped to 5.8; pH = 7 dropped to 6.4; pH = 8 dropped to 7.2; pH = 9 dropped to 7.8.



TEXT-FIG. 5. Effect of the H ion concentration of milk on virulence.

The results in regard to virulence (Text-fig. 5) are definite and surprising. Virulence was maintained at a practically constant level in milk varying as widely in H ion concentration as pH=5, 6, and 7 for 192 transfers; at pH=8 in the same number of transfers a tenfold decrease in virulence occurred; while with the pH=9 flask, the decrease in virulence began immediately and continued throughout 20 to 30 times to be less than that of the organisms of the other flasks of milk. Obviously, under the conditions of this experiment, the H ion concentration of milk between pH=5 and 7 exerted little

influence detrimental to virulence of pneumococci. On the other hand, milk of pH = 8 and 9 caused a definite decrease in the virulence of this organism. The fact that no increase in virulence was obtained with the avirulent culture implies that this culture, which was granular, bile-soluble, and typical as far as could be determined, was in such a state that no development of virulence was possible by the method employed.

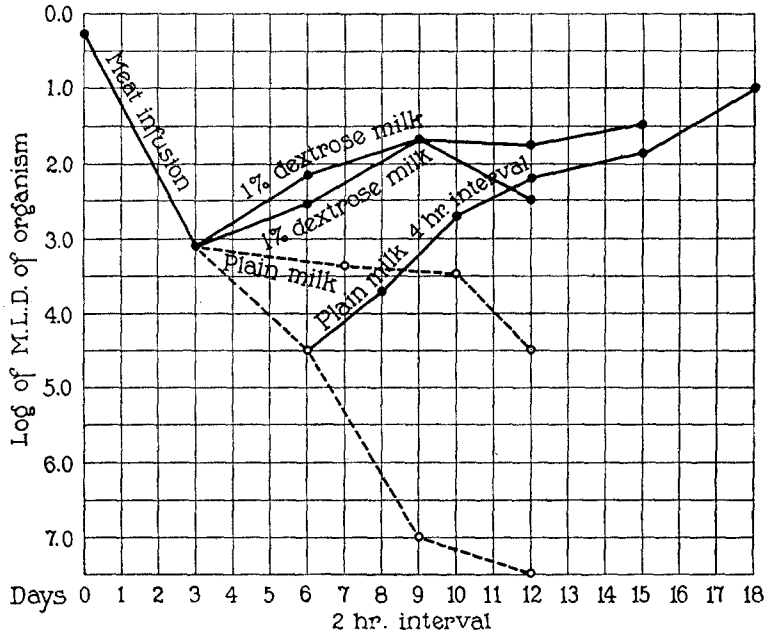
Experiment 5. Pneumococci Attenuated in Meat Infusion Again Become Virulent on Milk.

In another study[10] it has been shown that virulent pneumococci grown on meat infusion or plain broth suffer a rapid decrease in virulence so that 1×10^7 or 1×10^8 organisms were not fatal for mice in this state of attenuation. All efforts to restore the lost virulence of the culture failed. To determine just when a virulent pneumococcus grown on meat infusion loses its virulence to a degree at which it can no longer be restored, an experiment with meat infusion was planned involving the inoculation of this medium with a virulent organism and transfers from time to time to milk in an attempt to restore the lessened virulence before it had been entirely lost.

A culture was taken from the meat infusion at a time when 1,000 organisms were required to cause the death of a mouse and transferred to three automatic machines, two of which contained 1 per cent dextrose milk and one plain milk, all with the same interval of transfer as the meat infusion (2 hour). The plain milk (Text-fig. 6) arrested the decrease in virulence for a time, while the dextrose milk produced a condition favorable for increase. Again, when the virulence of the organisms grown on meat infusion had decreased so that not less than 50,000 organisms proved fatal for a mouse, the culture was inoculated into plain milk medium with 4 hour intervals of transfer. In 2 days of transfers, a definite increase of virulence was noted which continued without a break for 12 days, when the experiment was terminated. No other trials to increase virulence were made until the culture grown on meat infusion had become so avirulent that 10,000,000 organisms failed to produce a noticeable effect on a mouse. When in this state of attenuation, growth of the organism on dextrose milk with periods of transfer of 2, 4, 8, and 12 hours failed to increase virulence.

[10] Felton, L. D., *J. Exp. Med.*, 1924, xxxix, 155.

In association with change in virulence, the strain of organism grown in meat infusion had developed at the end of the experiment peculiar characteristics; in its avirulent state the capsule was absent, it sedimented during growth like a hemolytic streptococcus, and grew i n long chains. It retained, however, the ability to produce methemoglobin with rabbit blood corpuscles and was bile-soluble. Its behavior toward immune sera is noteworthy (Table I). Whereas the usua agglutination titer for the original strain of Neufeld pneumococcus



TEXT-FIG. 6. Restoration of lost virulence by growth on milk.

with the corresponding immune sera was 1:64 with no agglutination occurring in Type II or Type III sera, it was now flocculated by a 1:32,000 dilution of Type I serum and by dilutions of 1:4,000 and 1:1,600, respectively, of Type II and Type III sera. Apparently the specificity is retained in high dilution, but the organism is much more sensitive to agglutination. The work of De Kruif[11] describing

[11] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxvi, 309.

two types of organism in a culture of bacillus of rabbit septicemia, virulent and avirulent, with varying morphological characteristics, having a different isoelectric point, led us to perform an experiment similar to his with this avirulent organism. The difference in the behavior between the avirulent pneumococcus and the original culture was similar to that of the two types of organism described by De Kruif. The Neufeld virulent strain grew evenly turbid, while the avirulent one settled to the bottom of the culture tube as did, respectively, Types D and G of De Kruif. In a single experiment with the phosphate buffer series, the isoelectric point of the virulent pneumococcus culture and that of the same culture when rendered avirulent differed significantly. Other strains

TABLE I.

Changes in the Behavior of a Pneumococcus with Immune Sera on Becoming Avirulent in Meat Infusion Media.

Types of sera.	Dilution of immune sera.						
	1:500	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000
I	+++	+++	+++	+++	+++	+++	+++
II	+++	+++	+++	++			
III	+++	+++	++				

Dilutions were made by halving the concentration from 1:2 in series to 1:32,000; agglutination occurred in all types of sera alike up to 1:500.

of organisms in various stages of virulence have behaved in a like fashion; details of this work will be published elsewhere.

Experiment 6. Increase of Virulence by a Pure Line Avirulent Pneumococcus.

The results of the preceding experiment raise the question as to the mechanism involved in the increase of virulence on growth *in vitro* or *in vivo*. Is the process one of adaptation of the bacterial cell to the condition supplied by animal life, or is virulence a more or less fixed characteristic of certain cells which enables them to live in or on living animal tissue, the avirulent organisms being destroyed while the virulent ones survive, with cell division furnishing a new genera-

tion, the offspring of which possesses both weak and strong cells? It is not our intention to do more than inquire into the mechanism of increase of virulence by the method described in this paper.

To obtain a pure line strain of pneumococcus, the Barber pipette, used so successfully by the originator to pick single microorganisms,[12] was resorted to. A strain of Neufeld pneumococcus, rendered so nearly avirulent by transferring it after the ordinary fashion in horse serum broth medium that 1×10^3 cocci

TABLE II.

Virulence of Pure Line Strains of Pneumococci Picked from One Mother Strain.

Culture.	Duration of life.							
	Dilution of a 16 hr. culture.							
	0	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
N-1	S.*	S.	S.	S.	S.	S.	S.	S.
			days					
N-2	36	48	7	"	"	96	"	"
			hrs.					
N-3	36	48	48	48	48	S.	"	"
			days					
N-4	36	S.	6	S.	S.	"	"	"
			hrs.					
N-5	S.	"	48	"	"	"	"	"
N-6	24	36	48	48	48	48	48	60
N-7	36	48	48	S.	S.	S.	S.	S.
N-8	S.	S.	S.	72	"	"	"	"
N-9	"	"	"	S.	"	"	"	"
N-10	36	48	48	48	48	48	48	48
N-11	S.	S.	S.	S.	S.	S.	S.	S.

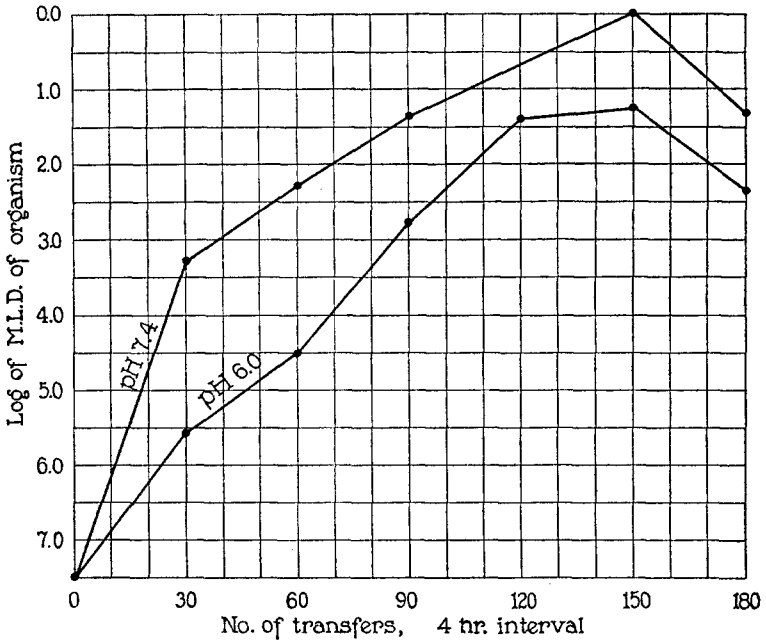
S. indicates survived.

were needed to kill a mouse, was used as the source from which to pick; picking was done from a 1:100,000 dilution of an 18 hour broth culture of the organism. Eleven takes (Table II) out of seventeen were obtained. The virulence for mice was determined for each pure line strain in a manner shown in Table II. Eight mice were used for each strain, the number of organisms injected into each animal representing a logarithmic series of dilutions beginning with an undiluted 18 hour culture.

[12] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

The outstanding fact from this part of the experiment is the demonstration of the existence of cells of varying virulence in the same culture. The variation is great, ranging from that of a strain of which 1 cc. of a 16 hour culture failed to cause the death of a mouse, to one of such a degree of virulence that 10 to 100 organisms were sufficient to cause the death of an animal.

Culture N-1 was chosen as a suitable, avirulent pure line strain for the purpose at hand. Although H ion concentration of milk influenced but slightly the virulence of a strain of pneumococci, it was thought advisable to use this medium unadjusted (pH = 6) and also adjusted to pH = 7.4.



TEXT-FIG. 7. Increase of virulence by a pure line avirulent strain in milk medium.

The results of these experiments are shown in Text-fig. 7. The virulence of the avirulent pure line strain progressively increased in milk adjusted to a pH = 7.4 until it reached its maximum, one diplococcus as determined by plating producing a fatal infection of a mouse. With the unadjusted milk, the rise in virulence was perhaps not so marked. The difference in the rate of increase in virulence in the two experiments is not sufficiently great to permit of any conclusion as to

the optimum H ion concentration of milk necessary to enhance virulence. The significant fact is that a pure line avirulent strain produced from a single diplococcus underwent a maximum increase in virulence when grown *in vitro*. To our knowledge, such an enhancement of virulence by *in vitro* methods has never been obtained prior to this study. The results indicate that the selection of fit cells is not the only mechanism operative in the increase or maintenance of virulence, and that perhaps adaptation is just as important as selection for change in this bacterial function.

DISCUSSION.

The method used does not permit of a comparison between the results thus far obtained and those reported by other investigators who made use of the usual bacteriological technique. In our work, warm medium is supplied to a large number of bacteria during a period with a nearly constant multiplication rate, in contrast to the ordinary transfer procedure of introducing relatively few organisms of varying growth activity into a new supply of medium, with the sudden environmental change entailed thereby. The number of pneumococci which remain after flushing the growth receptacle with the amount of medium equal to twice its capacity is about one-third of the number present just before flushing, a fact established both by counting the organisms before and after flushing of the growth receptacle and by introducing a dye, the color intensity of which could be readily estimated. It can thus be seen that the present method of study is to be thought of as representing a feeding procedure at different hours rather than one involving transfers at various intervals. How far the factor of large inoculum of young organisms and of residual medium after flushing the growth receptacle have influenced the results can only be speculated on until work now in progress is finished.

The impossibility of general deductions is apparent for the reason that the work was limited to one strain of organism owing to the length of time required to obtain significant results in each experiment. In subsequent work, however, other strains of pneumococci of different serological types have been found to act similarly to the one reported in this study, some cultures increasing in virulence maximally and others not at all. We believe that the negative results

obtained with certain strains of avirulent organisms do not imply a fixed avirulent state, but are indicative that the method of study does not furnish proper environmental conditions to restore lost virulence. It should also be borne in mind that a single species of animal host has been used. Although it is probable that facts established concerning the virulence for mice of pneumococci may be applied to other animal species, such a view involves the problem of animal specificity and pathogenicity, a field of investigation, which, however tempting and important, is foreign to the present study.

Whatever future work may develop, it has been established by the present study that the virulence of a single avirulent pure line strain of pneumococcus has been enhanced maximally by growth outside the animal body. This holds also for the mother culture which had become avirulent under the conditions of ordinary laboratory technique. The fact that individual diplococci, when propagated separately in an 18 hour culture were found to possess highly varying degrees of virulence raises the question as to the mechanism of the increase of virulence of pneumococci *in vitro* or *in vivo*; of whether the process is one of adaptation, or selection, or both. The development of virulence in a pure line avirulent pneumococcus *in vitro* implies an adaptative phenomenon, whether or not cognizance is taken of the rate of development of virulence or the finished virulent state. In the experiment represented in Text-fig. 7, it is seen that the virulence of a pure line avirulent strain developed slowly, which suggests a gradual alteration in the bacterial cell. However, if it is assumed that hereditary influence plays a rôle in the character of bacteria, it is possible to explain this experiment as either an adaptative or a selective process, the existence of the bacterial cell being the important issue, its environment, viewed from the standpoint of selection, determining whether the selected organism be virulent or avirulent, or, from the standpoint of adaptation, whether or not the organism be so altered that it becomes antibiotic or saprophytic in nature. A study of the exact mechanism of the change in virulence is, perhaps, not so important as investigations of causes of this change.

SUMMARY AND CONCLUSIONS.

1. An automatic apparatus is described by which a new supply of food can be furnished actively growing organisms at any desired interval of time—an automatic transferring device.

2. A single strain of pneumococcus, Type I, Neufeld, which had become avirulent for mice, acquired virulence of maximal degree when grown in the described apparatus with skimmed milk sterilized in an Arnold sterilizer as the medium. Transfers were made at intervals of 2, 4, and 8 hours. It is difficult to determine the best interval of transfer, but the 8 hour interval apparently is most suitable, with the 4 and 2 hour following in preference in the order named.

3. Pasteurized skimmed milk from a single dairy, but obtained on different days, when used as medium at a 2 hour interval, varied in effect on the virulence of pneumococci, the results showing that the virulence might be either increased, decreased, or maintained.

4. Skimmed milk heated for varying lengths of time, 30 minutes at 17 pounds pressure, or 60 or 90 minutes at the same pressure, lost its suitability for maintaining virulence of a pneumococcus when transferred every 2 hours, the effect being in direct proportion to the length of time the milk was heated.

5. The H ion concentration of milk had slight effect on virulence. A virulent strain of pneumococcus was grown in milk adjusted to pH = 5, 6, 7, 8, and 9, transfers being made every 2 hours. Virulence was maintained to a like degree on milk titrated to pH = 5, 6, and 7, but when the organism was grown in milk of pH = 8 and 9, virulence decreased, more rapidly at pH = 9 than at pH = 8.

6. A pure line practically avirulent strain of pneumococcus picked by the Barber method, when grown in milk at a 4 hour interval of transfer, increased in virulence 10 million fold; that is to say, until one diplococcus would kill a mouse. Prior to this study, no record has been found in which the virulence of any microorganism has been increased to such a degree by an *in vitro* method.