STUDIES ON VIRULENCE.

III. Influence of Hydrogen Ion Concentration and of Ingredients of Plain Broth on the Virulence of Pneumococci.

By LLOYD D. FELTON, M.D., and KATHARINE M. DOUGHERTY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, August 9, 1923.)

In the preceding paper[1] it has been reported that the virulence of a single strain of pneumococcus was increased several thousand fold when grown on milk in an automatic transferring device by which young cells were assured for inoculation. The degree of increase depended upon the specimen of milk, the frequency of transfer, and the state of virulence of the organisms. It was further shown that pneumococci grown upon ordinary meat extract broth at a 2 hour interval of transfer rapidly lost their virulence for mice. According to many investigators microorganisms in general lose their virulence more or less gradually on cultivation by the ordinary laboratory technique. Eyre and Washburn (1897),[2] Cotoni (1912),[3] and Wadsworth and Kirkbride (1918)[4] made a special study of pneumococci. They demonstrated a decrease in virulence of this organism when grown on plain broth. Stryker[5] also has reported the loss of this biological property as well as an alteration in agglutinability when pneumococci are grown on specific immune sera. Increase of virulence of pneumococci, though but to a slight degree, by cultivation *in vitro*, has been reported by Cotoni and also by Wadsworth and

[1] Felton, L. D., and Dougherty, K. M., *J. Exp. Med.*, 1924, xxxix, 137.

[2] Eyre, J. W., and Washburn, J. W., *J. Path. and Bact.*, 1897, iv, 394; *Lancet*, 1899, i, 19.

[3] Cotoni, L., Le virulence des pneumocoques, Thèse de Paris, 1912, No. 78.

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

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

Kirkbride. It may be assumed, therefore, from the present studies and those of others that the fact is established that pneumococci grown *in vitro* will either decrease or increase in virulence according to the condition provided. It is the purpose of the present study to report an analysis of the influence on virulence of pneumococci of transferring at varying intervals in plain broth of various H ion concentrations and of the individual ingredients of plain broth. This analysis was undertaken as a step toward the recognition of conditions and chemical substances which influence the virulence of pneumococci.

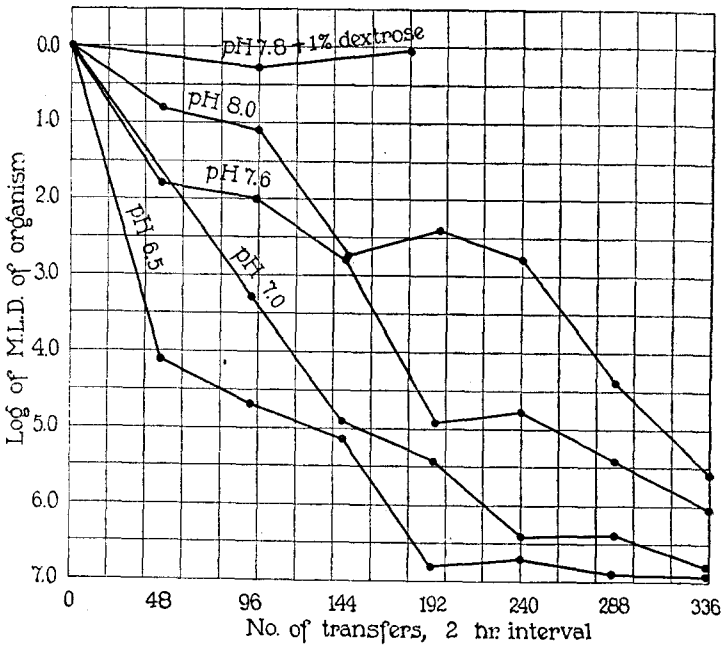
Experiment 1. Effect of H Ion Concentration on the Rate of Decrease in Virulence of Pneumococci Transferred at 2 Hour Intervals.

The apparatus and technique for this and the other experiments to be reported have been described in Paper II.[1] The strain of pneumococcus used in all experiments was the Neufeld, Type I.

The medium consisted of the usual meat infusion, 1 pound of lean beef to a liter, to which is added 1 per cent peptone and 0.5 per cent sodium chloride. The pH was adjusted so as to be 0.2 higher than the H ion concentration ultimately desired, and the medium so titrated was sterilized by free steam for 1 hour on 3 successive days. Generally the drop in H ion concentration during the process of sterilization compensated for the excess to which the medium had been adjusted; but when this did not occur, a readjustment was made with precautions to prevent contamination. Plain broth of four different H ion concentrations was studied, pH = 8, 7.6, 7, and 6.5. In order to assure growth, the different media were seeded with a large inoculum (2 cc.) of a young culture. Throughout this experiment, the average number of pneumococci per cc. amounted to, respectively, 25 million for pH = 6.6, 55 million for pH = 7, 50 million for pH = 7.6, and 35 million for pH = 7.8. The number of organisms, as well as the H ion concentration, remained practically the same throughout the experiment.

As can be seen from Text-fig. 1, the trend of virulence is downward, regardless of the H ion concentration of the medium, a confirmation of the general experience that growth of a microorganism in plain broth results in a decrease in virulence. These observations differ from those obtained by Wadsworth and Kirkbride who demonstrated clearly that a virulent organism can maintain its virulence for mice

when grown on plain broth or blood broth with an 8 hour interval of transfer. In a subsequent experiment this point will be taken up. The effect of H ion concentration on the virulence of this strain of pneumococcus is one of rate at which decrease occurs. It varies in direct ratio to the H ion concentration; the higher the concentration, the more rapid the attenuation. With milk this was not found to be the case, as was proven in Paper II, for the growth of the same



TEXT-FIG. 1. Effect of variation in pH; plain broth.

strain of pneumococci on lots of skimmed milk adjusted to H ion concentrations of pH = 5, 6, 7, 8, and 9 resulted in a decrease in virulence only in the milk of H ion concentrations of pH = 8 and 9, with practically no decrease at pH = 5, 6, and 7. The lack of correspondence, the reversed influence, even, in the effect of H ion concentration of plain broth and skimmed milk implies that other constituents of the medium may affect the virulence of pneumococci to a greater degree than the concentration of H ions.

Experiment 2. Effect of Constantly Changing H Ion Concentration of the Media.

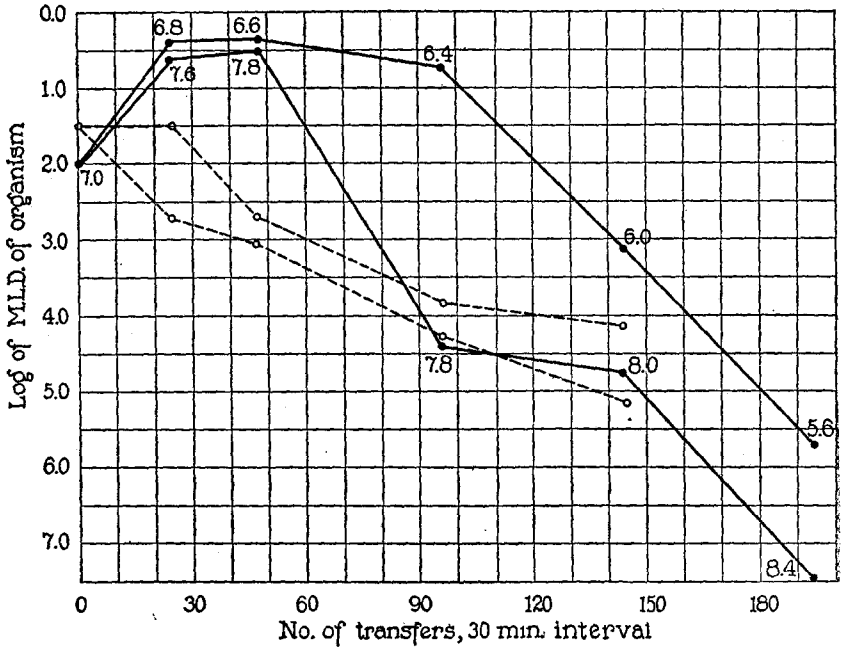
In the preceding experiment conclusions could not be drawn as to the relative influence of H ion and OH ion because of insufficient range in concentration of these ions. This fact, together with observations made during the study on milk, in which decrease in rate of virulence varied inversely with the H ion concentration, a result the opposite of that with plain broth, led us to devise an experiment in which the H ion concentration was gradually changed during the experiment, to either the acid or the alkaline side.

The procedure was as follows: medium of the siphon flask which emptied into the growth receptacle was replenished through a siphon connection to another flask containing medium of different H ion concentration, thus insuring a more or less gradual change in the pH of the medium supplied to the organism of the growth receptacle. Two experiments, the one involving a change to the acid and the other to the alkaline side of neutrality, were conducted, media of pH = 5 and pH = 9, respectively, being siphoned into media of pH = 7. The rate of change in H ion concentration of the medium and also the effect on virulence of such conditions is shown in Text-fig. 2. A 30 minute interval of transfer was used because it was thought that this short interval of transfer would render more evident the detection of the influence of the H ion or the OH ion. The Neufeld, Type I, strain which had lost some of its virulence by the ordinary stock method of transfer once a week on 10 per cent normal horse blood broth (24 hour incubation with subsequent storage 6 days in the ice box) was the one chosen for this experiment.

The results of the experiment (Text-fig. 2) are interesting from the standpoint of both virulence and agglutinability. The first 48 transfers, regardless of changes in the H ion concentration between pH = 6.6 and 7.8, revealed a noticeable increase in virulence with a final decrease in both series of tests. Here, as in the experiment reported on the influence of H ion concentration in milk, the OH ion is more detrimental to virulence than the H ion. The organisms in the growth receptacle were not counted as routine because growth was so slight that it was necessary to make inoculations from the machines into rabbit blood broth in order to have a sufficient number of organisms to estimate virulence. At the end of the experiment, however, counts were made of the number of organisms in the

respective growth receptacles, and there were found in the alkaline media 4,500,000 per cc. and in acid media 2,400,000 per cc.

How far the process of adaptation permitted growth in media of such wide variation in H ion concentration was not determined, nor was the alteration of the organisms, with the single exception of their reaction toward immune sera. This was taken up for the reason that in a 24 hour culture, the pneumococcus became auto-agglutinable as do hemolytic streptococci, an 8 hour culture, however,

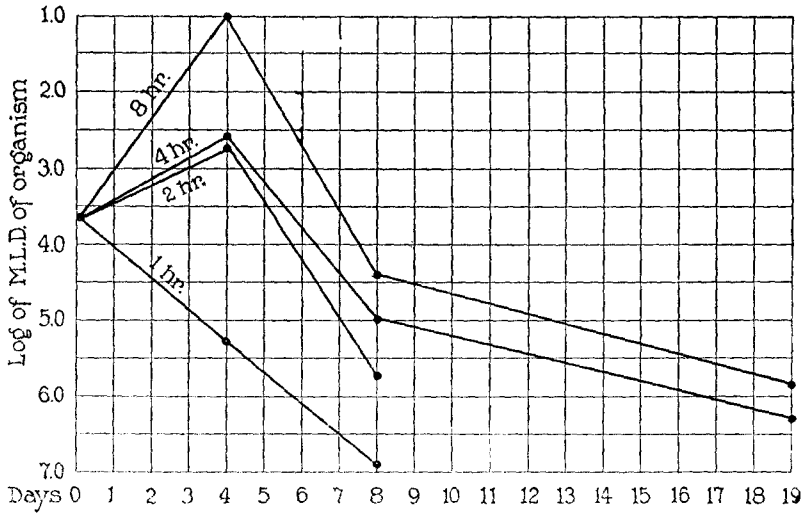


TEXT-FIG. 2. Effect of constantly changing pH in plain broth.

still remaining evenly turbid. The dotted lines on Text-fig. 2 represent logarithmically the titer of agglutination in Type I serum as determined by tests from time to time. The organism in the beginning of the experiment agglutinated at 1:50. The alkaline medium produced an immediate change in sensitivity toward agglutinins, which continued gradually until agglutination occurred in a dilution of immune sera of 1:200,000. The change in acid media was not so sudden and the organisms did not become so agglutinable, clumping in an approximate dilution of 1:20,000.

Experiment 3. Effect of Different Intervals of Transfer on the Virulence of a Somewhat Virulent Culture.

This experiment was prompted by the results of the preceding experiment, showing an initial rise of virulence followed by a decrease, regardless of H ion concentration, as further by the success of Wadsworth and Kirkbride in maintaining the virulence of pneumococci by transfer at an 8 hour interval, and by our own desire to determine in what way the age of the culture as judged by interval of transfer might influence virulence. The same strain of pneumococci used in the



TEXT-FIG. 3. Effect of varying the interval of transfer; plain broth pH 7.4.

previous experiment, but now in still less virulent condition, was employed. The intervals of transfer were four; namely, 1, 2, 4, and 8 hours. From Text-fig. 3 it can be seen that the virulence was affected differently when transfers were made at various intervals, 1 to 8 hours. The organism transferred every hour underwent a steady decrease in virulence in this medium, while at 2, 4, and 8 hour intervals of transfer, an initial increase in virulence occurred, followed by a decrease. These comparisons are somewhat misleading, inasmuch as tests for virulence were made with reference to days and not to number of transfers. Although no experiment

has been carried out to establish the fact, it has been observed in other studies that the initial rise in virulence on plain broth seemingly is dependent upon the number of transfers and independent of intervals of transfer from 1 to 8 hours. For example, in the experiment just described, in which a 30 minute interval of transfer was employed, a rise of virulence was noted after 48 transfers. It is possible that had the organism in the 1 hour machine been tested for virulence after 48 transfers, an initial increase of virulence might have been found. Although there was an initial increase in virulence with intervals of transfer of 2, 4, and 8 hours, the fact that virulence of the pneumococci eventually decreased irrespective of the interval of transfer makes it difficult to reconcile the present work with that of Wadsworth and Kirkbride who found it possible to maintain the virulence of several strains of pneumococci by transfers every 8 hours either on plain broth or rabbit blood broth. Inasmuch as our results have been amply confirmed and those obtained by Wadsworth and Kirkbride are equally definite, the disparity can, no doubt, be assigned to differences in technique.

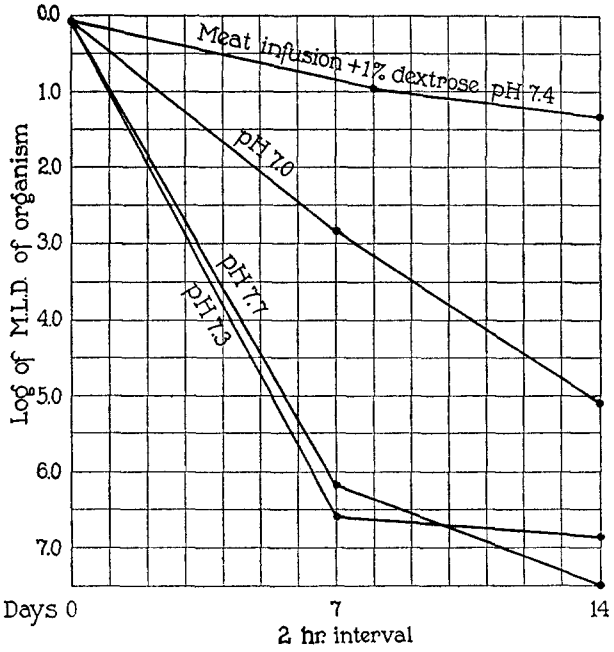
Experiment 4. Effect of Meat Infusion on the Virulence of Pneumococci Transferred at 2 Hour Intervals.

It is apparent at this point in our study that plain broth of a wide range of H ion concentration is not a suitable medium for maintenance of virulence, even when transfers are made while the organism is young. This is true despite the initial rise in virulence observed in the experiment. The unsuitability of meat infusion to maintain, much less increase permanently the virulence of the pneumococcus led us to undertake an analysis of the ingredients of plain broth to determine effects of meat infusion or of peptone on virulence.

The meat infusion used in this experiment consisted of 1 pound of lean beef to a liter, infused in the ice box overnight, with the addition of 0.5 per cent sodium chloride and 0.2 per cent disodium phosphate. Portions of this medium were adjusted to various H ion concentrations, pH = 7, 7.3, and 7.7. The Neufeld, Type I, pneumococcus, made virulent by animal passage, was employed. Owing to the press of other studies, estimation of virulence at frequent intervals

was impossible; however, it was found that the virulence of the pneumococcus was decreased (Text-fig. 4), the rate of decrease here again, as in milk, following in inverse ratio to the H ion concentration.

Meat infusion under the conditions of the experiment does not supply the proper food elements or environmental condition for the maintenance of virulence of pneumococci; but growth was abundant, so it may be assumed either that the meat infusion is lacking in some



TEXT-FIG. 4. Effect of variation in pH; meat infusion.

substance or condition which is necessary for pneumococci to retain their virulence, or that substances or conditions are present which rob them of their antibiotic faculty. An experiment was carried out to test whether or not a carbohydrate, dextrose, which is split by pneumococci, would provide the means to maintain virulence. This was done because of work carried out by one of us on metabolized media[6] in which it was shown that media exhausted by growth of

[6] Felton, L. D., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 313.

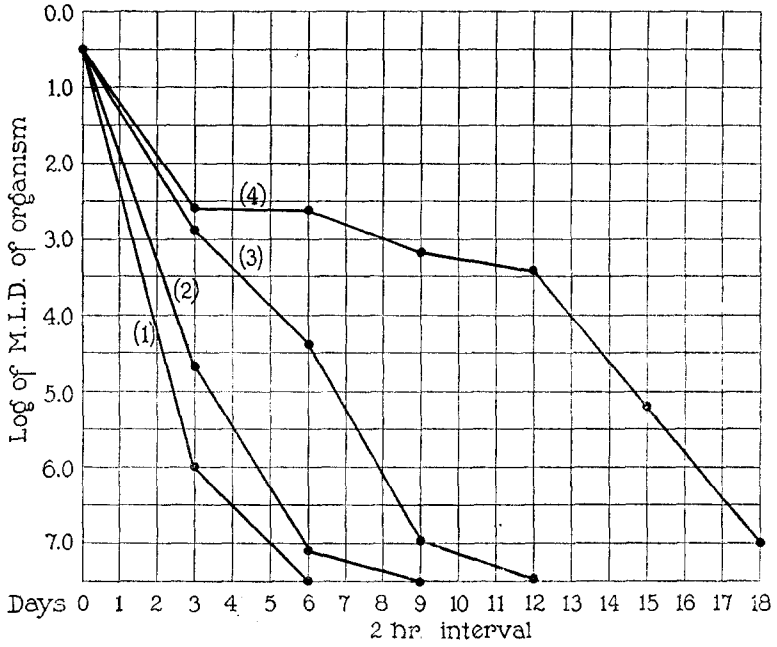
an organism could be revived, so to speak, by the addition of a carbohydrate normally utilized by the microorganism. Accordingly, dextrose was added to meat infusion and also to plain broth to make a 1 per cent solution, and these media placed in the usual automatic transfer apparatus were inoculated with highly virulent pneumococci. The results indicate (Text-figs. 1 and 4) that dextrose, added both to plain broth and meat infusion, so alters the condition of these media that the virulence of pneumococci grown thereon is maintained for a considerable length of time. It is possible that lack of carbohydrate in meat infusion will account for the failure to maintain virulence of pneumococci. On the other hand, since a maximal growth occurred in broth and meat infusion without dextrose, it seems possible that some chemical substance antagonistic to the virulence of the organism may be present in the media.

Experiment 5. Effect of Concentration of Meat Infusion on the Virulence of Organisms Transferred at 2 Hour Intervals.

The assumption that meat infusion in the usual concentration (1 pound to a liter) either lacks a substance or condition necessary for maintenance of virulence, or that there exists in it something detrimental to the virulence of this organism is open to experimental test. Dextrose added to plain broth or meat infusion, as recorded in the previous experiment, does away, at least partially, with the effect of these media to diminish virulence. If it be true that the infusion lacks some substance necessary to sustain virulence, meat infusion of a high concentration ought not to be any more destructive to this form of bacterial activity than is ordinary infusion, and perhaps not so destructive. On the other hand, should a substance antagonistic to virulence be present in the meat infusion, one would expect that the higher the concentration the more pronounced the action. With this question in mind, the following experiment was planned and carried out (Text-fig. 5).

Four lots of beef infusion of different concentrations were prepared: 3 pounds to a liter, 2 pounds to a liter, 1 pound to a liter, and $\frac{1}{2}$ pound to a liter, all adjusted to pH = 7.4. The number of organisms growing in these media varied from time to time. No. 1 ($\frac{1}{2}$ pound to a liter) averaged 30 million, No. 2 (1 pound to a liter) averaged 40

million, No. 3 (2 pounds to a liter) averaged 44 million, and No. 4 (3 pounds to a liter) averaged 44 million. Decrease in virulence was found to vary with the concentration of the meat infusion; the more dilute the infusion, the more rapid the loss. That meat infusion in the usual concentration lacks some essential for maintenance of virulence is suggested from the fact that a high concentration furnishes more suitable media than a lower concentration.

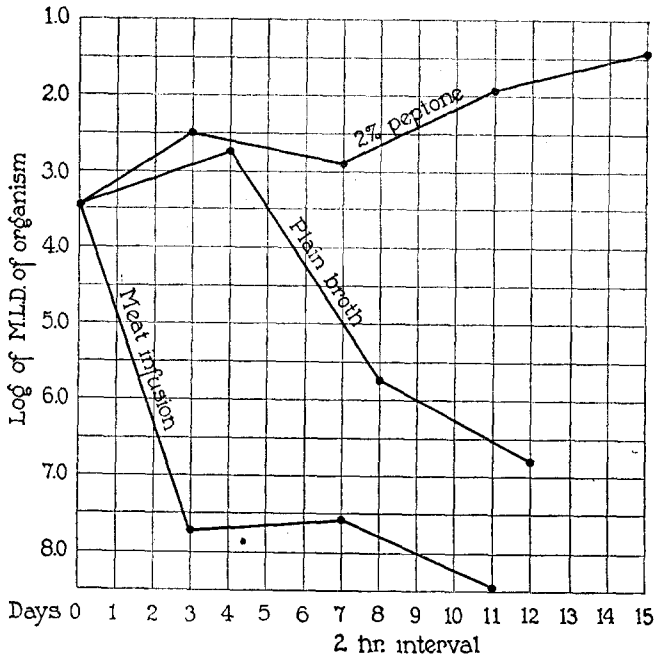


TEXT-FIG. 5. Effect upon virulence of variation in concentration of meat infusion.

Experiment 6. Effect of Peptone on Virulence of Pneumococci Transferred at 2 Hour Intervals.

This experiment was planned to test the effect of peptone on the virulence of pneumococci and to serve also as a check upon the results obtained in the previous experiments with regard to the influence of plain broth (beef infusion plus 1 per cent peptone) and of beef infusion.

The ingredients were uniform with these and the other conditions as nearly identical as possible. Since it had been found that pneumococci would grow to a slight extent on 2 per cent peptone with the addition of 0.5 per cent sodium chloride, these concentrations were used both in the test for the effect of peptone on virulence and for the control, plain broth, made by adding peptone to meat infusion (1 pound to a liter). All media were adjusted to pH = 7.4. The same strain of pneumococcus, somewhat avirulent, was used as in the previous experiments.



TEXT-FIG. 6. Effect of different ingredients of plain broth pH 7.4.

The results of this experiment (Text-fig. 6) are striking and to us surprising; the virulence of pneumococci grown on 2 per cent peptone during a period of 15 days, transferred every 2 hours, remained constant or even increased; on meat infusion, a rapid decrease occurred; while on plain broth, containing a mixture of ingredients favorable and unfavorable for maintenance of virulence, a decrease resulted and at a slower rate than occurred on meat infusion alone. Peptone neutralized to some extent the unfavorable action of meat

infusion, as did dextrose, described above. The outcome with peptone presents an interesting issue, for one could scarcely expect commercial peptone, a mixture of albumoses, peptones, and amino-acids, to be capable of furnishing conditions suitable at least for the preservation of the virulence of a fastidious organism like the pneumococcus. Although a reason cannot be assigned for the unexpected results with peptone, they may lead to simplification of present knowledge of the problem of virulence. The limited character of the studies with peptone, which, however, have since been substantiated, deter us from generalization. However, this much may be said. The virulence of the pneumococcus, as of other microorganisms, may be thought to be, perhaps, just as dependent upon definite chemical substances as the proliferation of the organism is upon certain food elements. One of the chief circumstances which determine virulence evidently is the presence or absence of certain chemical compounds. Life of the cells is possible with many different food elements, but virulence only with certain chemical substances, and these perhaps under special physicochemical conditions.

DISCUSSION.

Several inferences seem justified from the results presented. The H ion concentration of plain broth has a definite influence on the maintenance of virulence of pneumococci, but at no H ion concentration of this medium are conditions suitable for the permanent increase of virulence. Although an H ion concentration of the blood (pH = 7.4) was maintained in several experiments for a considerable length of time, the virulence of the pneumococci decreased, thus demonstrating that the H ion concentration alone of the blood does not insure the preservation of virulence even in young cells, much less fulfill conditions fit for the increase of this form of bacterial activity. Under the conditions of the experiments, regardless of the H ion concentration of plain broth or meat infusion, the virulence of the pneumococci was lost, the rate of loss depending upon the H ion concentration. In plain broth (Experiment 1) the more acid the media, the more rapid was the decrease; while in meat infusion, the reverse effect is to be noted. Seemingly, the OH ion is more destructive to virulence (Experiment 2) than the H ion. The

results reported in Paper II[1] show this fact in a definite fashion. When a virulent strain of pneumococcus was grown on milk adjusted to various H ion concentrations, that is pH = 5, 6, 7, 8, and 9, its virulence was maintained for over 100 transfers at a pH = 7 or below, while milk adjusted to pH = 8 or 9 caused in the same length of time a decrease in virulence, the more alkaline fluid having the more pronounced effect. To make a similar deduction from the experiments with plain broth is not possible for the reason that the virulence of pneumococci is lessened at all H ion concentrations studied. However, if the rate of decrease is used as the criterion, there is sufficient evidence to indicate that the OH ion in a constant medium possesses an action more antagonistic to virulence than the H ion. Other factors, without doubt, influence the virulence of pneumococci in ordinary media more than the H ion concentration.

No decision can be made at present as to what factors in plain broth media other than the H ion concentration play a rôle in determining whether a strain of pneumococcus grown thereon will be virulent or avirulent. However, the results obtained by a study of the effect on virulence of the two ingredients of plain broth, peptone and meat infusion, are worthy of comment. For it has been shown for one strain of pneumococcus that while the virulence of this organism decreased when it was grown on plain broth or on meat infusion—and with greater rapidity on the latter medium—in peptone solution virulence was not decreased, perhaps even increased. It is the meat infusion, then, not the peptone in ordinary plain broth, that has an unfavorable action on virulence. Furthermore, it has been shown that peptone neutralizes to a certain extent the unfavorable action of the meat infusion, as did dextrose. The question arises as to the mechanism of the action of the meat infusion, whether or not it is lacking in some substance necessary for the maintenance of virulence or contains some substance which has a specific action against virulence. Little can be said, now, on this point. If we take into consideration the effect of the concentration of meat infusion, which shows that the greater the concentration the less active this action, and also the fact that dextrose or peptone lessens it to some extent, we would be apt to conclude that the influence on virulence of meat infusion is due to a lack of some food element and

that it does not contain a substance antagonistic to virulence. On the other hand, if we consider that peptone alone furnishes the necessary conditions for the maintenance of virulence and that the addition of meat infusion destroys the favorable condition, a logical inference is that meat infusion contains a substance which diminishes the virulence of pneumococci.

SUMMARY.

1. A highly virulent strain of pneumococcus, Type I, Neufeld, when grown in an automatic transferring device at 2 hour intervals on different lots of plain broth adjusted respectively to pH = 6.5, 7, 7.5, or 7.8, lost its virulence at a rate in direct ratio to the H ion concentration of the media—the more acid the media, the more rapid the decrease.

2. Growth of a virulent pneumococcus upon plain broth of an H ion concentration changing gradually either to the acid or the alkaline side of neutrality was accompanied by an initial rise in virulence with a subsequent fall, the change being more rapid in the alkaline than in the acid medium. The organism under these conditions underwent an alteration in its behavior toward agglutinating sera; although still specific, its agglutinability became much greater than that of the original organism. This change was more pronounced in the alkaline than in the acid medium.

3. The virulence of a relatively virulent culture of a pneumococcus grown on plain broth underwent different alterations when the organism was transferred at intervals of 1, 2, 4, or 8 hours. With the 1 hour interval, there occurred seemingly an immediate decrease in virulence, while at intervals of 2, 4, and 8 hours, there occurred first a rise and then a fall, the rise being greatest with the 8 hour interval of transfer and least with the 2 hour.

4. Meat infusion adjusted to various H ion concentrations—pH = 7, 7.3, and 7.7—and made with different amounts of meat furnished conditions which caused a decrease in virulence, the unfavorable action varying in inverse proportion to the concentration of the infusion. Dextrose added to both plain broth and to meat infusion neutralized to a marked degree the unfavorable action of these media.

5. The growth of pneumococci on the different ingredients of plain broth, with plain broth made from the same supply as a control, revealed the fact that peptone in 2 per cent solution maintained and even increased the virulence of the strain of pneumococcus studied, while meat infusion caused the usual decrease. The rate of decrease with plain broth was slower than with meat infusion, showing that the 2 per cent peptone neutralized to some extent the unfavorable action of the infusion.