A STUDY OF THE VARIATIONS IN HYDROGEN-ION CONCENTRATION OF BROTH MEDIA

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Received for publication August 15, 1920

At the present time it would seem scarcely necessary to lay emphasis upon the importance to bacterial growth and metabolism of the reaction of the environmental culture medium. That different degrees of acidity and alkalinity in media may profoundly influence the morphology, rate of fermentation, pigment production, growth, or viability of bacteria has been so thoroughly recognized that in the routine preparation of culture media as carried on in every bacteriological laboratory, the proper adjustment of reaction is carefully regulated. The use of scales of reaction such as that of Fuller, based upon adjustment to a definite "degree" of titratable acidity, has permitted a certain amount of uniformity, and in general, it may be said that these old titrimetric procedures have served a very useful purpose. But with the development, during the last few years, of the newer physicochemical conception of hydrogen-ion concentration the theory of titration has undergone a fundamental change. As a consequence many of the data obtained in earlier investigations are of little value, having been based upon unsound premises.

An adequate conception of the far-reaching biological effects of hydrogen-ion concentration may best be gained through a study of the classic works of Michaelis (1914),[1] Sörensen (1912, 1909a, 1909b) and Clark and Lubs (1917a, 1917b, 1917c). The following statement from the works of the last-named investigators will serve to emphasize the importance to the science of bacteriology of this modern conception of acidity and alkalinity:

[1] Bibliography is found at the end of the third article, in this series, p. 231.

Hydrogen-ion concentration influences the condition in solution of every substance with acidic or basic properties—native proteins and their hydrolytic products, amines and amides, carboxyl, sulphonic, and phenolic compounds, even alcoholic compounds, as well as many inorganic compounds. It has a large effect on the effective solubilities and dispersion of colloids, upon determining tautomeric equilibria, and in one way or another in governing the activity of catalysts such as hydrolytic enzymes and oxidases. One or the other of these effects, induced directly or perhaps indirectly by the hydrogen-ion concentration must impress bacterial life.

That the expression of reaction in terms of titratable acid or alkali does not adequately define the true reaction of a solution has perhaps best been brought out by W. M. Clark (1915a) in his admirable paper, "The 'reaction' of bacteriologic culture media." The objections to the older procedure may be summarized in a quotation from Clark and Lubs (1917a):

> The titrimetric method, designed originally for the quantitative estimation of strong acids and bases, cannot be applied to complex mixtures of very weak acidic and basic groups such as are found in the constituents of most culture media. In so far as the method is used to determine the "free acid" or to adjust to a certain degree of "free acid" it is an absolute failure when applied to culture media. There is however, an even more fundamental reason why the titrimetric method is inappropriate. Two media adjusted to the same degree of acidity may have widely divergent hydrogen-ion concentrations as shown by Clark (1915a).

With the development of the hydrogen electrode, making possible a direct measurement of hydrogen-ion concentration, some of the experimental and mathematical difficulties involved in the older methods were obviated, but there still remained to be elaborated some simpler and more rapid procedure that would be adapted to the adjustment of culture media and to the study of reaction changes in bacterial cultures. Guided by the earlier work of Friedenthal (1904), Salm (1904), Friedenthal and Salm (1907) who were the first to give a well worked-out series of indicators, Sörensen (1909a) in 1909 published his colorimetric method for determining hydrogen-ion concentrations. Since

this time a number of modified procedures have been suggested by Levy, Rowntree, and Marriott (1915), Hurwitz, Meyer and Ostenberg (1915, 1916); McLendon (1916); Barnett and Chapman (1918); Clark and Lubs (1916a, 1917a, 1917b, 1917c); Haas (1919); so that at the present time it is a relatively simple matter to prepare and have on hand in the ordinary bacteriological laboratory a suitable set of colorimetric standards for the measurement of the hydrogen-ion concentration of media and cultures. It is to Clark and Lubs (1917b, 1916b), Lubs and Clark (1915, 1916) that we are especially indebted for several new and valuable indicator substances as well as for a careful study of the ranges and usefulness of an entire set of indicators for the examination of biological fluids.

Deeleman (1897) in 1897, using the titration procedure, noted that media underwent certain changes in reaction during sterilization and sought to avoid such variation through the addition of proper amounts of sterile acid or alkali to the autoclaved material. Hesse (1904) used the same procedure in the adjustment of his media and further emphasized the fact that only that type of glassware which yields no alkali should be employed for containers, to prevent the increase in alkalinity that otherwise might occur. According to Sörensen (1909a) however, such factors as alkalinity from glassware and CO_2 from the atmosphere exert only slight effects if the medium in question is properly buffered. Using the titration method, Anthony and Ekroth (1916) attempted to bring media to a stable reaction by repeatedly alkalinizing and autoclaving, but were unable to produce such a stabilized condition even after many additions of alkali, supplemented by a total of fourteen hours autoclaving. They explain the change as due to the formation of acid principles through hydrolysis. In one case five times the quantity of base needed was added through an error, with the result that after several sterilizations the reaction of the broth fell to the required level. Wright (1917) has suggested that the amount of alkali indicated by titration is never sufficient to bring about a complete neutralization of the medium, it being always necessary to add a considerable excess over the amount indicated. On the other hand, Noyes

(1916) states that properly prepared media do not increase appreciably in acidity when the length of sterilization is increased or when repeated autoclavings are carried out. It is a known fact that many proteins may exist in solution only between certain limits of hydrogen-ion concentration and that slight changes, at or near the critical zones, cause the formation of precipitates. This phenomenon occurs in peptone solutions and as Kligler (1917) has shown it is possible to establish the limits of P_H which determine precipitation for each brand of peptone.[2] Cook and Lefevre (1918) showed that as much as 12 per cent of peptone may be lost through precipitation depending on whether this material were added previous to coagulation and filtration or subsequently. That a change in P_H accompanies such a precipitation in media has been found by Clark (1915a) who reported a fall in P_H of 0.80 (from 8.52 to 7.72) in an infusion broth containing 0.5 per cent K_2HPO_4. Itano (1916a) using the hydrogen electrode in his P_H measurements, was able to establish a rough correlation between the changes in P_H of an extract broth upon autoclaving and the increase in COOH groups as determined by the formol titration of Sörensen. Strangely enough the changes in P_H reported by Itano were always of the nature of an increase in alkalinity, and with this there appeared an increase in formol-titrating nitrogen, indicating that hydrolysis had occurred. As a result of boiling the broth for forty-five minutes this observer found that the material became stable as regards further changes in P_H. This last experiment, however, was tried only on media adjusted between P_H 5.45 and 6.88. By sterilizing the constituents of his media separately it was possible to adjust to the desired P_H and obtain values which remained fairly constant throughout the entire experiment. Norton (1919) has reported that appreciable changes in the reaction of neutral and alkaline media, but little variation in the acid range, result from sterilization. Davis (1920), in recognition of the possibility of a change in the P_H of media adjusted in the alkaline range, has suggested that for the proper preparation of a glucose broth of P_H 8.0–8.2 reaction it is well to

[2] The symbol P_H of Sörensen is used throughout to designate the hydrogen-ion concentration.

bring the material to an initial P_H of 8.6. Davis also emphasizes the superiority of the autoclave over the Arnold for media sterilization pointing out that prolonged heating is always to be avoided in order that the vitamine or hormone content may not undergo destruction. On the other hand, Fennel and Fisher (1919) report that in the preparation of over one hundred lots of beef infusion broth the initial P_H of 7.8 did not show variation as a result of autoclaving. In connection with his study of the effect of initial reaction of a medium upon *Corynebacterium diphtheriae*, Bunker (1919) noted certain reaction changes in his media upon sterilization. The variations appeared almost entirely on the alkaline side and were always noted as increases in acidity.[3] Very recently, Grace and Highberger (1920b) have carried out experiments with extract broth which seem to indicate that changes in reaction upon sterilization may not be of any greater order than are the changes which a medium may undergo simply upon standing, following autoclaving. The variations of greatest magnitude occurred in the alkaline range and all changes were toward a more acid reaction. No consistent tendencies could be detected, therefore it was not possible to come to definite conclusions as to the reasons for the observed changes. However, the possibilities of the influence of glass and atmospheric CO_2, as well as of slow hydrolysis, were suggested.

Early in the present investigation it was noted that culture media (broth) adjusted to definite P_H levels underwent changes in reaction upon autoclaving, thus rendering difficult the preparation of broth of desired reaction. Consequently it was considered important to investigate these changes with a hope of finding an explanation and perhaps of discovering some means of avoiding them.

METHODS AND TECHNIC

Standard solutions

All solutions were prepared according to the methods outlined by Clark and Lubs (1916a, 1917a) from boric acid and salts which

[3] The term acidity in the present paper signifies true acidity as expressed in terms of P_H.

had been recrystallized three to five times. Triple distilled water served as solvent. The stock solutions, as well as the standard buffer mixtures, were kept in heavily paraffined, glass-stoppered bottles. Check determinations on the mixtures at the outset and after a period of seven months showed that the standard buffers, from bottles in which the paraffin was not broken, had remained constant in P_H in spite of the fact that molds had developed in some of the liquids. Sörensen (1909a) reported a similar observation on solutions after nine months standing. The desired P_H ranges and the solutions used in their preparation are given below:

Solutions	P_H
M/5 Potassium acid phthalate, M/5 NaOH	4.0–5.8
M/5 KH_2PO_4, M/5 NaOH	5.8–7.6
M/5 H_3BO_3, M/5 KCl, M/5 NaOH	7.8–9.0

Indicators

The indicator solutions were the following:

CHEMICAL NAME	COMMON NAME	CONCENTRATION IN 50 PER CENT C_2H_5OH	RANGE P_H
		per cent	
Ortho carboxy benzene azo di-methyl aniline	Methyl red	0.02	4.4–6.0
Di brom ortho cresol sulphon phthalein	Brom cresol purple	0.04	5.2–6.8
Phenol sulphon phthalein	Phenol red	0.02	6.8–8.4
Thymol sulphon phthalein (alkaline range)	Thymol blue	0.04	8.0–9.6

Color standards

Color standards were prepared by adding 0.3 cc. of the required indicator solution to 5 cc. of the buffer mixture. Tubes of colorless glass and uniform bore, 4 by $\frac{3}{8}$ inches were used for the color standards as well as for the test liquids. Fresh standards were made up each week, as fading is apt to occur if the solutions are allowed to stand for a longer period. This is most pronounced in the methyl red series and least noticeable in the brom cresol purple series.

Colorimetric determination of hydrogen-ion concentration

In properly buffered solutions it is possible partially to eliminate such factors as color and turbidity by diluting the test fluid with water. Preliminary tests showed that with broth and cultures it was possible to dilute 1cc. of the material with 4 cc. of distilled water without altering the hydrogen-ion concentration. Accordingly this technic was employed in all the determinations. Freshly boiled and cooled distilled water was used for diluting as preliminary tests had shown that unboiled water gave slightly lower P_H readings. The P_H of the water itself was usually found to rise from 4.8 to 6.8 upon boiling, probably due to liberation of carbon dioxide. To eliminate factors of color and turbidity more completely Walpole's (1911) method of superposition was used by employing the comparator block described by Dernby and Avery (1918). All determinations were carried out at room temperature. The limit of error in the readings was 0.1 P_H.

The adjustment of broth media

One cubic centimeter of the broth was diluted with distilled water (freshly boiled and cooled). Two acid solutions and two basic solutions were kept on hand. They were N/1 HCl and an exact 1:10 dilution of the same; N/1 NaOH and an exact 1:10 dilution. A specially made micro burette, of 1 cc. capacity and graduated to 0.01, contained the diluted acid or base. This was added to the tube containing the medium, water, and 0.3 cc. of the proper indicator solution until the color produced therein exactly matched that of the color standard of desired P_H. The reading on the micro burette was then taken and by calculation the amount of stock acid or base needed to adjust the total amount of broth was determined. Following the addition of the acid or base to the entire lot of medium a check determination was always carried out. The broth was autoclaved at 15 pounds for twenty minutes. In case this caused the formation of a precipitate the medium was filtered and subjected to a second autoclaving for twenty to thirty minutes at 10 pounds pressure. The low pressure prevents a second precipitation of the medium. The

P_H should always be taken on the broth following the final autoclaving as well as at the outset of any given experiment. The reason for this will appear in the experiments about to be described.

In case the broth was to contain a sterile sugar this was added aseptically in 10 per cent solution to the sterile medium to avoid any possibility of splitting the sugar through heating. This procedure is especially important if the broth is adjusted in the acid or alkaline range as it is a known fact that glucose and other sugars are altered by heating with even small amounts of acid or base (Mathews, 1916). Furthermore, Mudge (1917) has observed an increased titratable acidity when sugars, at least disaccharides, are autoclaved with media. By adding the sugar aseptically in concentrated solution no change in reaction was ever noted.

Experiment I. The extent of the changes in hydrogen-ion concentration which broth media adjusted to different initial P_H levels undergo upon autoclaving and standing

The unadjusted broth was divided into portions of 75 cc. which were brought to values ranging from P_H 5.0 to 9.0 at intervals of 0.4. Five cubic centimeter amounts were then tubed and autoclaved at 15 pounds for fifteen minutes after which they were allowed to cool and P_H readings taken. The tubes comprising each lot were divided into three sets, one of which was allowed to remain at room temperature, another was placed in the ice chest, while the third was incubated at 37°. After standing at these temperatures for intervals of two, seven, and fourteen days tubes were removed and P_H determinations made.

Five series were carried through and the data obtained are to be found in tables 1 to 5.

SERIES	COMPOSITION OF BROTH	RESULTS IN TABLE
I	Beef infusion	1
II	Beef extract	2
III	Bacto beef	3
IV	Beef infusion (repetition of I)	4
V	Beef extract (repetition of II)	5

Reference to tables 1 and 4, containing data for the two beef infusion series, reveals differences in P_H changes as a result of autoclaving. Whereas every tube of series I showed an increased acidity upon sterilization, the tubes of series IV from 5.0 to 5.8 inclusive exhibited a decrease in acidity; those of P_H 6.1–7.3 suffered no alteration in reaction, while those lying in the 7.8–8.9 range showed a definite increase in acidity. Upon standing, the greatest changes in both series are manifest in the 8.6 and 9.0

TABLE 1

Experiment I. Changes in reaction upon autoclaving and standing. (Beef infusion broth)

Composition:

Distilled water	1000 cc.
Chopped lean beef	300 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0	4.8	4.8	4.4	4.7*	4.8	4.4	4.7*	4.8	4.4	4.7*
5.3	5.0	4.9	4.8	5.0*	4.9	4.8	5.0*	5.0	4.8	5.0*
5.8	5.6	5.5	5.4	5.3	5.5	5.4	5.3	5.5	5.4	5.3
6.2	5.9	5.8	6.0	5.8	5.8	6.0	5.9	5.8	6.0	5.9
6.5	6.2	6.2	6.4	6.2	6.1	6.3	6.2	6.2	6.4	6.2
7.1	6.9	6.8	7.0	7.0	6.8	6.8	7.0	6.7	6.9	7.0
7.3	7.1	7.1	7.2	7.3	7.1	7.2	7.3	7.1	7.3	
7.8	7.3	7.3	7.6	7.6	7.3	7.5	7.6	7.4	7.6	7.6
8.1	7.8	7.7	7.9	7.8	7.7	7.8	7.8	7.7	7.9	7.8
8.6	8.4	8.4	8.4	8.1	8.4	8.4	8.1	8.4	8.4	8.1
9.0	8.6	8.6	8.5	8.2	8.5	8.5	8.2	8.6	8.6	8.3

* The unexpected increase in alkalinity may have been more apparent than real due to a fading of the standard buffer mixtures of the methyl red series.

tubes. These changes are in the nature of increases in acidity and are as great in magnitude as those produced by autoclaving. A possibility of this sort has apparently been overlooked by many observers. No differences worthy of mention appear as a result of storing the broth under different conditions of temperature.

Passing to the two beef extract series (tables 2 and 5) a remarkably small number of alterations are notable in one case (V). An increase in acidity of 0.2 P_H occurred in the two lots of highest

P_H, namely the 8.6 and 9.0 tubes. These two lots were practically the only ones to exhibit changes upon standing, the 9.0 registering an acidity change of 0.7 P_H after fourteen days standing. In series II (table 2) decreases in acidity are noted in the acid and alkaline ranges upon autoclaving while within the range 6.6–7.3 the broth remained unchanged. In every lot of this series the acidity increased upon standing, the greatest changes occurring in the

TABLE 2

Experiment I. Changes in reaction upon autoclaving and standing. (Beef extract broth)

Composition:

Distilled water	1000 cc.
Liebig's beef extract	3 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0*	5.2*	5.3	4.6	4.7	5.2	5.1	4.8	5.3	4.8	4.8
5.3*	5.4*	5.6	4.7	4.8	5.5	5.1	4.8	5.5	4.8	4.8
5.8	6.0	6.1	5.2	5.2	6.1	5.2	5.2	6.1	5.2	5.2
6.2	6.4	6.3	5.6	5.6	6.3	5.4	5.6	6.3	5.6	5.6
6.6	6.6	6.6	6.2	6.3	6.6	6.3	6.4	6.6	6.5	
7.0	7.0	7.0	6.8	6.8	7.0	6.8	6.8	7.0		
7.3	7.3	7.3	7.0	7.0	7.3	7.0	7.0	7.3	7.0	7.0
7.7	7.9	7.9	7.5	7.8	7.9	7.4	7.6	7.9	7.4	7.8
8.0	8.3	8.4	7.8	7.9	8.4	7.7	7.9	8.4	7.6	7.9
8.7*	8.9*	8.5	8.4	8.6†	8.6	8.4	8.6†	8.6	8.6	8.7†
9.0*	9.2*	8.8	8.6	8.8†	9.0	8.4	8.6†	9.0	8.6	9.0†

* Precipitate.

† A slight fading of the standard buffer mixtures of the thymol blue series may have occurred thus accounting for the apparent increase in alkalinity.

more acid and alkaline ranges. Here, as previously mentioned in the case of beef infusion broth, the changes on standing seem to be independent of the environmental temperature.

The results in the bacto-beef series (table 3) are similar to those noted in the case of beef infusion. A decreased acidity in general appears in the range, 5.0–6.2, the 6.6–8.2 tubes remain practically unchanged, while the most alkaline members, 8.6 and 9.0 show increases in acidity upon autoclaving. Upon standing at

the three different temperatures the same general tendencies as have been observed in series IV may be noted.

It appears that there is no marked consistency in the variations which a given type of broth medium may exhibit as a result of autoclaving and standing. The same conclusions have been reached by Grace and Highberger (1920b) working with beef extract broth. Itano (1916a) however, reported only decreases in acidity in lots of extract broth adjusted throughout a wide range

TABLE 3

Experiment I. Changes in reaction upon autoclaving and standing. (Bacto-beef broth)

Composition:

Distilled water	1000 cc.
Bacto-beef	50 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0*	5.4*	5.5	5.4	5.6	5.5	5.4	5.7	5.4	5.5	5.7
5.4*	5.2*	5.7	5.8	5.6	5.7	5.7	5.5	5.8	5.7	5.7
5.8*	6.3*	6.1	6.0	5.9	6.3	6.0	5.9	6.1	5.9	5.8
6.2	6.6	6.6	6.3	6.2	6.6	6.5	6.4	6.4	6.3	6.2
6.6	6.6	6.6	6.5	6.4	6.6	6.5	6.4	6.6	6.5	6.5
6.9	7.0	7.0	6.9	6.8	7.0	6.9	6.8	7.0	7.0	6.8
7.3	7.5	7.4	7.4	7.3	7.5	7.1	7.5	7.4	7.4	7.5
7.8	7.8	8.0	7.9	7.8	7.9	7.8	7.7	7.9	7.9	7.8
8.2	8.2*	8.2	8.1	7.9	8.2	8.0	7.9	8.2	8.1	7.9
8.6	8.4*	8.3	8.3	8.2	8.4	8.2	8.2	8.4	8.4	8.3
8.9*	8.6*	8.5	8.2	8.3	8.5	8.2	8.2	8.6	8.3	8.6

* Precipitate.

of P_H. His medium contained 2 per cent peptone which, as is well known, acts as a strong buffer. By sterilizing the components separately he was able to avoid anything more than slight alterations in reaction. No data were collected relative to the possibility of changes upon standing. The discrepancies appearing in the present beef infusion series were not so unexpected when it is considered that two different lots of beef were employed in their preparation, but the lack of uniformity in the changes

registered by the two beef extract series is not explainable upon such a basis for the same components were used in the preparation of each.

The remainder of the work has consisted of attempts to determine the causative factors in these reaction changes in order that some procedure might be devised to obviate the effects produced.

Although certain investigators have pointed out that the glassware employed may exert an effect upon the reaction of the con-

TABLE 4

Experiment I. Changes in reaction upon autoclaving and standing. (Beef infusion broth)

Composition:

Distilled water	1000 cc.
Chopped lean beef	300 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
5.0*	5.3*	5.2	5.3	5.3	5.2	5.3	5.3	5.2	5.3	5.3
5.4*	5.6*	5.5	5.6	5.5	5.5	5.6	5.4	5.4	5.5	5.4
5.6	5.8	5.6	5.8	5.8	5.6	5.8	5.8	5.6	5.8	5.8
5.8	5.9	5.9	6.0	6.0	5.9	6.0	6.0	5.9	6.0	6.0
6.1	6.1	6.1	6.2	6.2	6.1	6.2	6.2	6.1	6.2	6.2
6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5
7.0	7.0	6.9	7.1	7.1	6.9	7.0	7.1	6.9	7.0	7.1
7.3	7.3*	7.2	7.4	7.3	7.2	7.3	7.3	7.2	7.3	7.3
7.8	7.6*	7.5	7.6	7.6	7.4	7.5	7.6	7.4	7.5	7.6
8.0	7.9*	7.8	7.9	7.9	7.7	7.9	7.9	7.8	7.9	7.9
8.6*	8.5*	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3
8.9*	8.6*	8.4	8.4	8.3	8.4	8.4	8.3	8.4	8.4	8.3

* Precipitate.

tained media, experience in this laboratory has not borne out these contentions. In the course of the present work it has almost invariably been found that P_H determinations on a given medium distributed in different tubes check closely. Consequently this factor has at no time been seriously considered as even partially contributory to the reaction changes encountered.

It has been emphasized that in the very large majority of cases the reaction change was in the direction of an acidity increase

and further that the degree of variation upon standing was usually as great as upon autoclaving. In view of those findings the possibility of an absorption of sufficient CO_2 from the atmosphere to account for the changes noted was considered. Experiments II and III were carried out to decide this point.

TABLE 5

Experiment I. Changes in reaction upon autoclaving and standing. (Beef extract broth)

Composition:

Distilled water	1000 cc.
Liebig's beef extract	3 grams
Peptone (Parke, Davis & Co.)	10 grams
NaCl	5 grams

BEFORE AUTOCLAVING	AFTER AUTOCLAVING	ROOM TEMPERATURE AFTER DAYS			ICE CHEST AFTER DAYS			INCUBATOR AFTER DAYS		
		2	7	14	2	7	14	2	7	14
4.8*	4.8*	4.8	4.8	5.0	4.8	4.8	5.0	4.8	4.8	4.9
5.0*	5.0*	5.1	5.2	5.1	5.1	5.2	5.1	5.1	5.2	5.2
5.6	5.6	5.6	5.6	5.5	5.6	5.6	5.5	5.6	5.5	5.5
5.8	5.9	5.9	5.8	5.8	5.9	5.8	5.8			
6.1	6.2	6.1	6.0	6.4	6.1	6.0	6.0			
6.5	6.5	6.5	6.3	6.4	6.5	6.3	6.4	6.5		
6.9	7.0	7.0	7.0	6.9	7.0	7.0	6.9	7.0	6.9	
7.3	7.3	7.2	7.2	7.2	7.2	7.2	7.2	7.3	7.2	7.2
7.8	7.8	7.6	7.4	7.4	7.6	7.4	7.4	7.6	7.5	7.4
8.1	8.0	8.0	7.9	7.8	8.0	7.9	7.8	7.9	7.9	7.9
8.6	8.4*	8.2	8.2	8.0	8.2	8.1	8.0	8.2	8.0	
9.0	8.8*	8.3	8.2	8.1	8.3	8.2	8.2	8.4	8.3	

* Precipitate.

Experiment II. The effect of exposure in an atmosphere of CO_2 upon the reaction of broth

Beef infusion broth, prepared and adjusted as outlined in the preceding experiment, was tubed, autoclaved, and treated as follows: (1) Control-beginning. (2) Exposed twenty-four hours in plugged tubes to an atmosphere of CO_2. (3) Control after twenty-four hours.

The results shown in table 6 indicate that direct exposure of broth to CO_2 causes very decided increases in acidity, the amount of increase becoming greater as the alkalinity of the broth

increases. That such a condition is abnormal is, of course, quite obvious, but the experiment serves to indicate that CO_2 may not be ruled out as a factor in causing acidity increases in media upon standing.

TABLE 6

Experiment II

P_H after autoclaving	5.3	5.6	5.8	6.0	6.2	6.5	7.1	7.4	7.6	7.9	8.3	8.4	
P_H after exposure to CO_2 for 24 hours	5.3	5.4	5.5	5.6	5.7	5.8	5.9	6.1	6.2	6.2	6.3	6.4	
P_H (control) after 24 hours	5.3	5.6	5.8	6.0	6.2	6.5	7.1	7.4	7.6	7.9	8.3	8.4	

Experiment III. The effect of exposure of sterilized broth to an atmosphere free from CO_2

Tubes of the medium prepared in the preceding experiment were autoclaved and treated as follows: (1) Control, allowed to stand at room temperature. (2) Placed in a CO_2-free atmosphere. P_H readings were made at the outset, after seven days, and after fourteen days. To obtain atmosphere free from CO_2 air was drawn through a train of Woulff bottles containing concentrated NaOH, 20 per cent $Ba(OH)_2$, and $CaCl_2$ into a large Navy jar containing the tubes of media.

By inspecting table 7, it will be noted at once that practically the same changes in P_H occurred in both sets of tubes. This would seem to dispose of atmospheric CO_2 as a factor operative in causing the increases in acidity so frequently noted in the previous experiments.

TABLE 7

Experiment III

P_H after autoclaving	5.1	5.3	5.8	6.1	6.3	6.6	7.0	7.2	7.6	8.0	8.5	8.7	
P_H after 7 days in atmosphere	5.2	5.5	5.8	6.1	6.4	6.6	7.1	7.4	7.8	8.0	8.3	8.6	
P_H after 7 days in CO_2-free air	5.1	5.4	5.8	6.0	6.4	6.6	7.1	7.4	7.8	8.0	8.3	8.6	
P_H after 14 days in atmosphere	5.0	5.4	5.8	6.1	6.4	6.6	7.0	7.3	7.8	8.0	8.4	8.6	
P_H after 14 days in CO_2-free air	5.0	5.4	5.7	6.0	6.3	6.6	6.9	7.3		7.9	8.3	8.4	

Assuming that the external factors of glassware and atmospheric CO_2 are not sources of change in reaction of broth media it will be necessary next to examine the internal factors, namely, the possibility of chemical changes in the medium itself. The organic

components of broth media are in themselves complex compounds, which in some cases, are relatively unstable and reactive. It has long been noted that in the preparation of media precipitates occur when certain amounts of acid or base are added. In some cases precipitation occurs as soon as the acid or base is added, in other cases autoclaving seems to be required to bring down the material. Kligler (1917) has established certain zones of hydrogen-ion concentration for aqueous solutions of peptone within which precipitation occurs, and has investigated the nature of the precipitates themselves. In the acid range he believes that the material arises largely from protein substances as upon redissolving it gives reactions of proteoses and peptones, whereas in the alkaline range it is made up largely of phosphates. It is rather significant that the ranges of P_H in which we find the greatest change in reaction upon sterilization and standing are those within which precipitation is apt to occur during adjustment of the media.

The rôle of peptone in media is two-fold. It furnishes nitrogenous food in the form of protein split products (peptones, proteoses, peptides, amino acids) and through its property of combining with acids and bases acts as a buffer. According to Rettger, Berman, and Sturges (1916) and Davis (1917) American peptones are lower in albumoses and higher in amino acids than Witte's, some of those examined by the latter having two or three times the content of amino acids.

It seems quite certain that during autoclaving of culture media the higher nitrogenous complexes are hydrolyzed to lower split products. This would be particularly true in media adjusted in the acid or alkaline ranges, inasmuch as acids and bases act as positive catalyzers of a protein hydrolysis. During the splitting of a protein by hydrolysis there occur marked changes in the acidity or alkalinity of the solution in which the change takes place. Sörensen (1912) has reported an experiment in which the digestion of peptone by trypsin was carried out, measurements of hydrogen-ion concentration and determinations of the increase in formol-titrating material being made at intervals. The increase in hydrogen-ion concentration did not stand in relation to

the increase in COOH groups and Sörensen concluded that the increased base-binding power was due to the formation of peptides. T. B. Robertson (1918) has studied rather intensively the changes in hydrogen-ion concentration which take place during the hydrolysis of certain proteins and concludes that the power to bind acids and bases resides in the –COHN– groups, inasmuch as the protein molecule does not contain a sufficient number of terminal –COOH and $-NH_2$ groups to account for its high combining capacity for acids and alkalies. While bound up in the protein molecule these groups do not assist in the neutralization of acids and bases but during hydrolysis the bonds are opened and the binding capacity is increased.

Itano (1916a) has reported an increase in formol-titrating nitrogen in media upon sterilization and has apparently shown that at least a rough proportionality exists between the change in P_H (increase) and the increase in amino acids as measured by the method of Sörensen.

With the view to ascertaining whether or not the changes in P_H found in the experiments described could be correlated with an increase in COOH groups produced through hydrolysis of the peptone or protein of the broth the following experiments were carried out:

Experiment IV. The relationship between P_H changes in media and changes in formol-titrating nitrogen

Five lots of beef infusion broth were adjusted to P_H values ranging from 5.2 to 9.2 and each lot distributed in three 30 cc. portions. The P_H and formol number were determined: (1) before autoclaving; (2) after autoclaving; (3) after seven days standing at room temperature.

Technic of formol titration, Kendall, Day, and Walker (1913): Five cubic centimeters of the broth was diluted with 50 cc. of distilled water and 1 cc. of phenolphthalein (1 per cent alcoholic solution) was added. The material was titrated to a faint pink with N/20 NaOH or N/20 HCl. Five cubic centimeters of neutral formalin were then added and the mixture allowed to stand for thirty minutes after which it was again titrated with

N/20 NaOH. From the last figure, the formol number was obtained.

Formol number (*F. N.*): Milligrams of formol-titrating N per 100 cc. of culture.

The results of the experiment are contained in the following table:

TABLE 8

Experiment IV

BEFORE AUTOCLAVING		AFTER AUTOCLAVING		AFTER SEVEN DAYS	
P_H	F.N.	P_H	F.N.	P_H	F.N.
5.2	40.4	5.3	44.8	5.3	44.8
6.2	42.0	6.3	47.6	6.3	47.6
7.2	42.0	7.2	47.6	7.2	47.6
8.2	43.4	8.0	44.8	8.0	44.8
9.2	43.4	8.8	43.4	8.8	47.6

As a result of autoclaving, slight increases in formol-titrating nitrogen are manifest in every lot of broth excepting that adjusted to P_H 9.2 which was the only flask to show any appreciable change in P_H. The greatest increases in formol number are seen in the lots which showed little or no reaction change. No change in formol-titrating nitrogen occurs during the first seven days following autoclaving except in the 9.2 lot. Here a small increase occurred. From the results of this one experiment it must be concluded that changes in the P_H of broth as a result of autoclaving and standing bear no relationship to changes in formol-titrating nitrogen. The results are at variance with those reported by Itano (1916a) in which decreases in the hydrogen-ion concentration of broth upon autoclaving appeared to be roughly correlated with increases in formol-titrating nitrogen. It perhaps should be noted that fewer changes in P_H were recorded in experiment IV than were apparent in the earlier experiments.

At present, the most logical explanation of acidity increase noted in the various experiments would rest upon the observation of Robertson that as the hydrolysis of a protein proceeds the base-binding capacity of the material increases through the opening up of the –COHN– group of the protein molecule.

SUMMARY AND CONCLUSIONS

1. Broth (beef infusion, beef extract, "bacto-beef") adjusted to P_H values ranging from 5.0 to 9.0 undergoes a change in hydrogen-ion concentration upon autoclaving. This change is most marked in media adjusted in the alkaline range (7.8–9.0), less great in the acid range (5.0–6.2), and is usually inappreciable in the neutral range (6.6–7.4). The maximum change is about 0.4 P_H and in the majority of cases is not over 0.2 P_H.

2. The change is usually an increased acidity (decrease in P_H). Decreases in acidity have been observed in a few instances but these are exceptional.

3. In media of the same composition the reaction changes are not necessarily uniform in different experiments.

4. Autoclaved broth undergoes changes in hydrogen-ion concentration upon standing; the degree of change is not influenced by the environmental temperature within the limits, 10°C. (ice chest) and 37°C. (incubator).

5. The reaction changes upon standing, as in the case of autoclaving, are most noticeable in the alkaline range, less marked in the acid range, and least in the neutral range. Neutral media usually do not change at all upon standing.

6. The change upon standing is almost invariably in the direction of an increase in acidity.

7. Broth adjusted to various P_H levels ranging from 5.0 to 9.0 and exposed in tubes plugged with cotton to an atmosphere of CO_2 for twenty-four hours shows marked alterations in reaction. The change is always an increase in acidity, as would be expected. The greatest change occurs in the alkaline range.

8. Upon allowing broth adjusted to various P_H levels to stand in a CO_2-free atmosphere the same reaction changes were noted as in duplicate lots of broth allowed to stand in the air of the laboratory. The increases in acidity exhibited by broth upon standing do not seem to be due to an absorption of atmospheric CO_2.

9. Reaction changes in media of P_H 5.2 to 9.2 do not appear to stand in relation to changes in formol-titrating nitrogen.

10. The possibility of an increase in acidity of broth through the opening up of –COHN– groups during hydrolysis of the protein constituents suggested by Robertson remains a plausible one.