PRACTICAL OBSERVATIONS ON THE TITRATION AND ADJUSTMENT OF CULTURE MEDIA

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Any one who studies the methods given in the various textbooks for the titration and adjustment of culture media, must be struck by the lack of uniformity of opinion. Not only is the beginner in media preparation bewildered but even the more experienced worker may be led into error. The difficulty arises from the fact that the complex nature of the materials dealt with is by no means fully understood, even in the case of the most fundamental culture media. In addition to this the changes that occur under even slightly different conditions and treatment are most confusing. As a result each laboratory is compelled to adopt the methods best adapted to its work and requirements, and each laboratory makes changes in these methods as need arises.

The many requests constantly made for information regarding the methods employed in our laboratories for the titration and adjustment of both general and certain special culture media seem to indicate the need for a detailed account of such procedures.

In this paper we have tried to incorporate the practical information gained after a number of years of experience. In addition, we have described experiments carried on with a view of clearing up, in a systematic manner, certain points upon which little, if any, information is available.

The *Standard Method*[1] of titrating media for water and milk analyses was devised in an attempt to secure uniform preparations of media at all times so that comparable results might be obtained.

[1] Committee on Standard Methods, 1905, 1913).

The directions are as follows:

Phenolphthalein shall be the standard indicator in obtaining reaction of all media. Tumeric paper possesses similar properties and its use advised where phenolphthalein is not available. Titrations and adjustments of reactions shall be made as follows:

Put 5 cc. of media to be titrated in 45 cc. of distilled water. Boil briskly one minute. Add 1 cc. phenolphthalein solution (5 grams of commercial salt in one liter of 50 per cent alcohol.) Titrate while hot (preferably while boiling) with $\frac{N}{20}$ caustic soda. A faint but distinct pink marks the true end point. This distinct pink color may be described as a combination of 25 per cent of red (wave length approximately 658) with 75 per cent of white as shown by the disks of the color top, described under Records of Tints and Shades of Color, p. 10. (The Standard[2] color disks used in teaching optics may be used for this purpose.)

In practice, titration is continued until the pink color of alkaline phenolphthalein matches that of the fused disks. All reactions shall be expressed with reference to the phenolphthalein neutral point and be expressed in percentages of normal acid or alkaline solutions required to neutralize them.

One of the objects of this paper is to consider whether the desired results are actually obtained by the Standard method or by modifications of this method.

In our laboratory one modification, that is, titration of broth at room temperature (about 20°C.) and of agar at a temperature of about 30°C. has given good results for a number of years past.

These and other experiences have led us to investigate further the following subjects:

a. The effect of prolonged heating on meat infusions and beef extract solutions as shown by the titration curves of both adjusted and unadjusted portions. The results of boiling samples of media in the casserole for titration.

b. The adjustment of broth and agar, including the remelting of solid media.

[2] A small sized top and disks costing only a few cents may be obtained from Milton Bradley Educational Company, Springfield, Mass.

c. The reaction of peptone solutions and the effects upon them of prolonged heating.

d. The question of indicators with consideration of the significance and sensitiveness of their end points in different media—also the method of choosing the one most suitable with reference to the hydrogen electrode as a standard.

MEAT INFUSIONS

It is a well known fact that each time a medium is heated to the boiling point, or above it, the reaction changes and becomes more and more acid, depending on the length of time and the degree of heating. On this subject Eyre (1915) says:

> Meat extract [meat infusion] is acid in reaction owing to presence of acid phosphates of potassium and sodium; weak acids of the glycolic series and organic compounds in which an acid character predominates.
>
> Owing to the nature of the substances from which it derives its reaction, the total acidity of meat extracts [infusion] can only be estimated accurately when the solution is at the boiling point. Prolonged boiling [as in media preparation] causes it to undergo hydrolytic changes which increase the acidity.

He states further that meat extract [infusion] becomes stable in reaction after being heated at the boiling point for forty-five minutes so that no additional increase of acidity occurs on further heating.

To procure more definite data as to the effect of heat on the acidity of media the following work was carried out:

Preparation of the meat infusions. Chopped lean veal was soaked over night in tap[3] water in the proportion of one pound of meat to one liter of water. It was then heated at 45° to 55° C. for one hour. At this point it was brought to a boil. Then

[3] Weekly analyses of the Croton water supply shows it to contain a negligible amount of mineral matter (only about 40 parts per million, expressed as total hardness.) Where the water supply is at all "hard," it is advisable to employ distilled water exclusively.

the material (meat and watery infusion) was divided into three lots:

B[4] 30 was kept at the boiling point 30 minutes.
B 60 was kept at the boiling point 60 minutes.
B 120 was kept at the boiling point 120 minutes.
(Volumes being made up by addition of tap water.)

After being boiled, each lot was strained through cheesecloth, then filtered through paper (S. & S. "Falten" filter) and cotton (first moistened with cold water to hold back the fatty substances.)

Each lot was titrated[5] and then divided again into two parts and one series (B 30, B 60, B 120) was run in the autoclave at least six successive times, without the addition of soda.

The second series (B 30 C, B 60 C, B 120 C) was corrected with normal sodium hydroxid to 1 per cent acidity (+1) and then run in the autoclave with the other set and under the same conditions.

In all the tests made the autoclaving was done at a pressure between 15 and 17 pounds as indicated by a Bristol recording pressure gauge. The heating was carried on up to a total of eight hours and titrations performed at one-half hour intervals for the first four half-hours; then at one hour, two hour and three hour intervals.

After each autoclaving the six samples were titrated and the corrected series (B 30 C, B 60 C, etc.) was adjusted again when necessary to plus one (+ 1).

Method of titration. Freshly boiled and cooled distilled water was used for all titrations. A 5 cc. sample of meat infusion was drawn off by means of a 5 cc. pipette and added to 45 cc. of distilled water in a casserole to which 1 cc. of a 1 per cent[6] solution

[4] Preliminary titrations on samples before boiling had been labelled "A." As these had no significance they are omitted in this article.

[5] See: "Method of Titration."

[6] These are also variations from the standard method of using 0.5 per cent solution of phenolphthalein and twentieth normal sodium hydroxid solution. They are, however, in accordance with the methods in use for years in this laboratory and to preserve uniformity they were adhered to.

of phenolphthalein had been added. While stirring the mixture, deci-normal[6] soda solution from a burette was run in without any heating whatever. The end point taken was the first delicate pink tinge, observable throughout, which did not disappear after stirring the solution—and should not disappear for at least one minute. The figures were then recorded. The casserole with the mixture was then set over the flame, brought to a boil and boiled one minute by the watch.

Outline of Experiments

Meat infusions First lot of veal

- B 30 = preliminary boiling of 30 min.
- B 60 = preliminary boiling of 60 min.
- B 120 = preliminary boiling of 120 min.
- B 30 C = preliminary boiling of 30 min. — Corrected to plus one according to *room temperature* titration after each autoclaving.
- B 60 C = preliminary boiling of 60 min.
- B 120 C = preliminary boiling of 120 min.
- B_1 60 = preliminary boiling of 60 min.
- B_1 120 = preliminary boiling of 120 min.
- B_1 60 C = preliminary boiling of 60 min. — Corrected to plus one according to *boiling temperature* titration after the *first* autoclaving. No further adjustments were made because of error in adding too much soda.
- B_1 120 C = preliminary boiling of 120 min.

Meat infusions Second lot of veal

- B_2 30 = preliminary boiling of 30 min.
- B_2 60 = preliminary boiling of 60 min.
- B_2 120 = preliminary boiling of 120 min.
- B_2 30 C = preliminary boiling of 30 min. — Corrected to plus one according to *boiling temperature* titration after each autoclaving.
- B_2 60 C = preliminary boiling of 60 min.
- B_2 120 C = preliminary boiling of 120 min.

Since the boiling had caused the faint pink color to disappear, the hot mixture was then promptly titrated again, the same end point being approximated[7] as closely as possible, and the figures recorded. The room temperature figure plus that obtained after boiling one minute gave a total which represents the boiling titration figure as recorded in the charts.

In the first lot of veal, (series B 30, B 60, B 120), the corrections were made to plus one at the room temperature figures. A second lot of veal infusions (B_2 30, B_2 60, B_2 120) were prepared in the same manner as above. The corrections in this lot were made to plus one at the boiling figures.

DESCRIPTION OF CHARTS

In charts 1 and 2 are shown the uncorrected portions of meat infusions titrated after successive heatings in the autoclave and plotted according to both the room temperature and the boiling temperature figures. It will be seen that in each lot of meat the room temperature titrations fall into one group and the boiling titrations into another; also that the boiling figures are the higher.

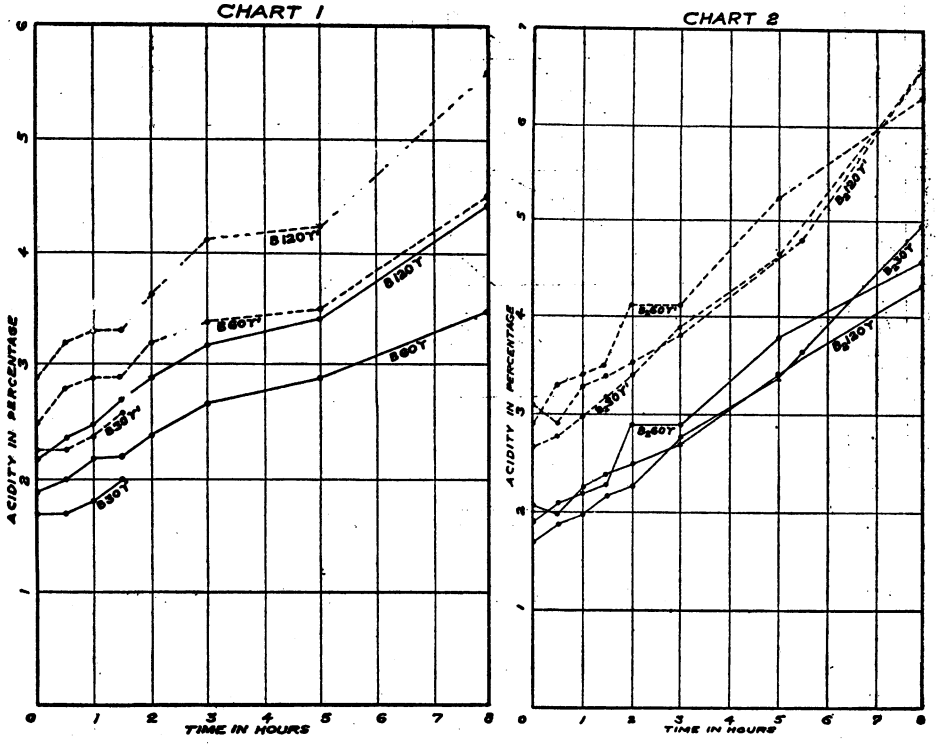
Chart 3 is a sample[8] chart showing not only the uncorrected portion of B 120 as given in Chart 1, but also the corrected portion, B 120 C. This portion was corrected to plus one (+ 1) according to the room temperature titration figures and readjusted to plus one after each autoclaving, as shown by the α line. The α' line shows the figures of this same material when boiled one minute in the casserole and titrated hot. This line is hypothetical and shows only the amount of soda that would have been needed had the boiling figures been used for adjustment to plus one.

The θ line shows the total amount of acidity produced in the corrected portion even after the addition of soda, according to the room temperature figures.

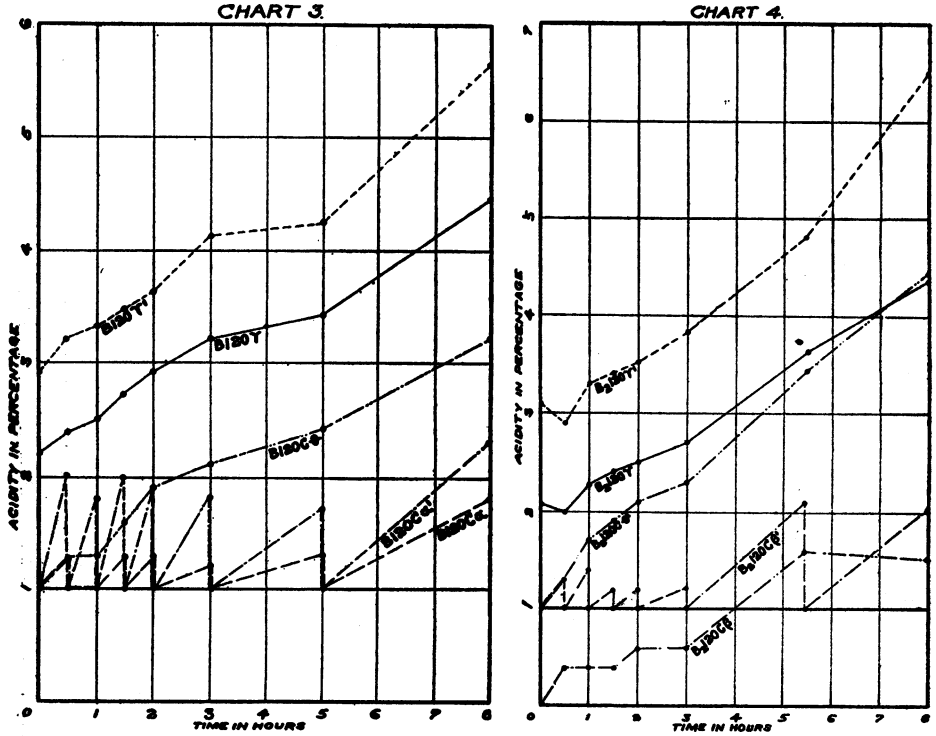
[7] The difficulty of catching the first color change of phenolphthalein in hot solutions will be discussed under "Indicators."

[8] This chart is typical also of the B 30 and B 60 sets.

KEY TO CURVE NOTATION.		
LETTER	LINE	DENOTES
α	—— —— —— ——	= corrected at room temperature
α'	—— ·— ·— ·— ——	= " " " " "(boiling figures)
β'	—— —— ·— —— ——	= " " boiling temperature
β	—— —— ·— ·— ——	" " "(room temp. figures)
γ	————————	= uncorrected (room temperature)
γ'	- - - - - - - - - - - -	= " (boiling temperature)
θ	—— ·— —— ·— ——	= Total acidity, corrected at room temp.
θ'	—— ·— ·— ·— ····	= " " " " boiling temp.



In Chart 4[9] is represented B_2 120 (second lot of veal) just as B 120 is shown in Chart 3 except that here the corrected portion (B_2 120 Cβ') was adjusted to plus one (+1) according to the boiling titration figures. The β line shows the figures obtained each time at room temperature before the sample was boiled one minute in the casserole to give the boiling titration



figures. The room temperature curve is inserted here for comparison.

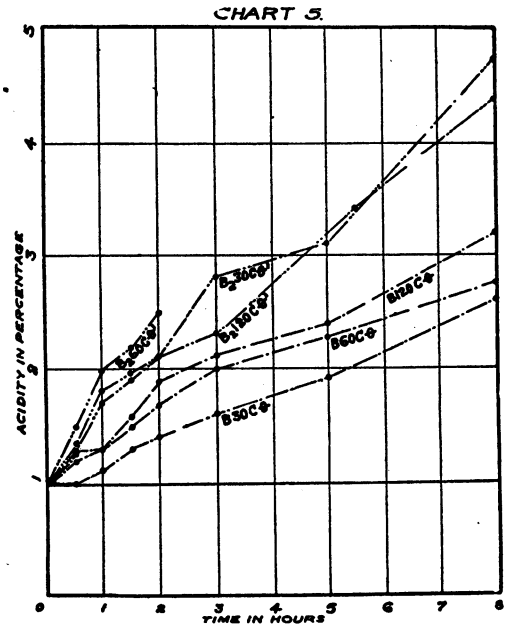
The θ' line shows the total acidity produced in the portion corrected according to the boiling titration figures.

In chart 5 all the total acidity lines of the various corrected portions are compared. As with the uncorrected portions as

[9] This chart is typical also of the B_2 30 and $B_2$60 sets.

noted in charts 1 and 2, the total acidity lines fall into two groups according to the method of titration.

If chart 5, showing the total rises in acidity of the series B 30 C, B 60 C, B 120 C and the series B_2 30 C, B_2 60 C, B_2 120 C, were applied successively to the corresponding curves of the uncorrected series in charts 1 and 2, so that the point of origin in each set were the same, it would be seen that in every instance the



total rise of acidity of the corrected curve equals or exceeds that of the uncorrected portion.

From this it is plainly evident that, in spite of successive corrections of acidity with normal sodium hydroxid, hydrolysis not only continues on the application of heat but there is produced in meat infusion media approximately as much acidity as would be developed were no correction made.

Chart 6 shows the actual acidity of the different corrected portions after the successive adjustments and periods of heating. As can be seen, those portions adjusted according to the

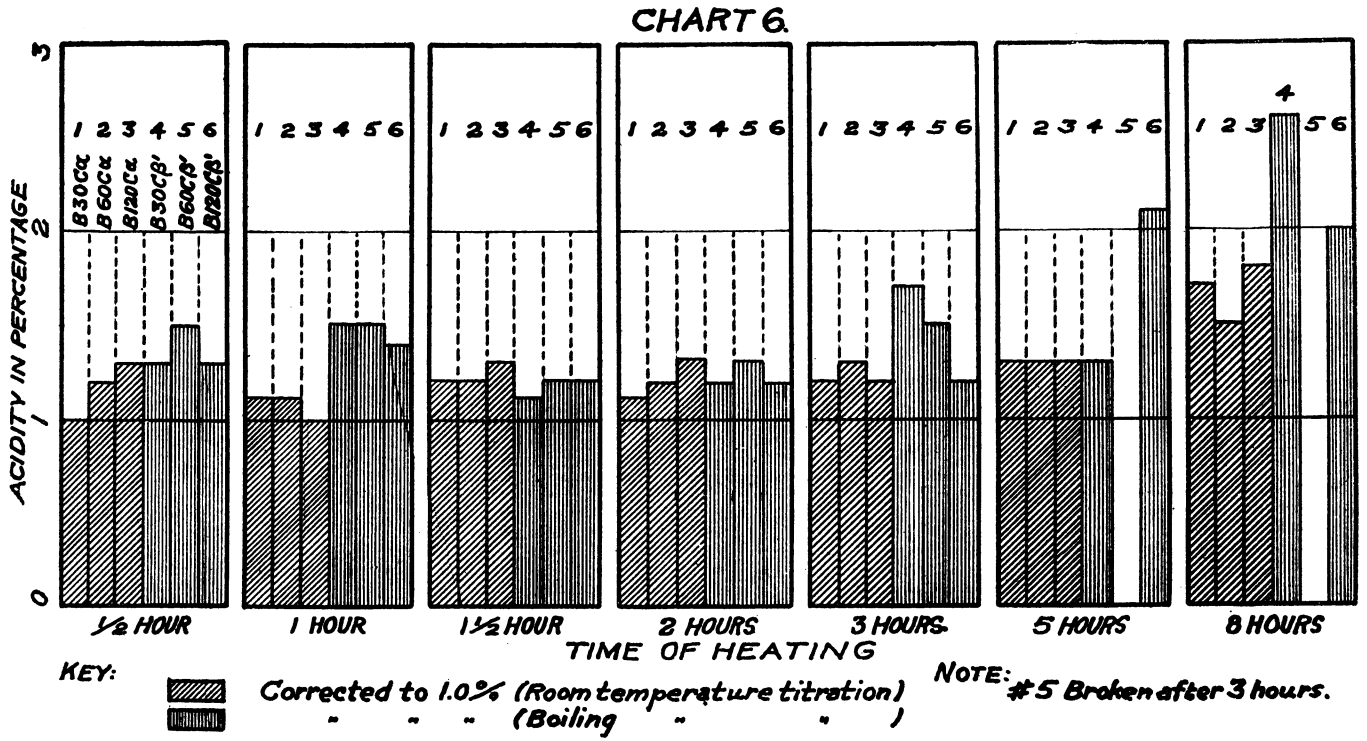
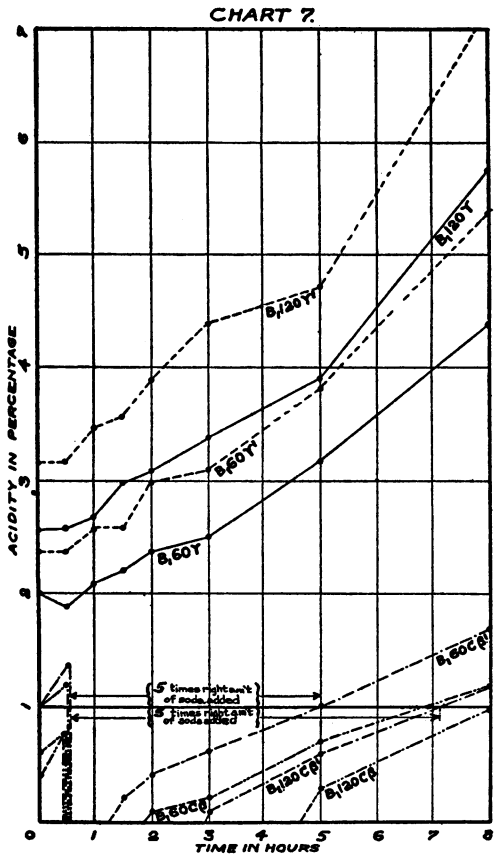


CHART 6.
ACIDITY IN PERCENTAGE
0
1
2
3
4
1 2 3 4 5 6
B30Cα
B60Cα
B120Cα
B30Cβ'
B60Cβ'
B120Cβ'
½ HOUR
1 HOUR
1½ HOUR
2 HOURS
3 HOURS
5 HOURS
8 HOURS
TIME OF HEATING
KEY:
Corrected to 1.0% (Room temperature titration)
" " " (Boiling " ")
NOTE: #5 Broken after 3 hours.

room temperature titration are in general nearer to the desired reaction of plus one (+ 1), especially in the first two half-hours—the periods of time of greatest practical interest.

In chart 7 are shown not only the uncorrected portions of B_1 60 (γ and γ') and B_1 120 (γ and γ') (duplicate material from



first lot of veal), but also the effect on the corrected portions of the addition of too much normal sodium hydroxid. By mistake after the first half hour in the autoclave and the subsequent titration, there was added to B_1 60 C β' approximately five times the amount of normal sodium hydroxid necessary to correct it to plus one, and to B_1 120 C β' also about five times

the right amount. These amounts were in accordance with the boiling titration figures in both cases. The quantities needed for correction were, in round numbers, twice as much for the latter as for the former. The relation of the quantities of alkali added may, therefore, be expressed by the numerical relation of ten to five. The portions of meat infusion (without further additions of soda) were then run as usual in the autoclave with the uncorrected portions and titrated at the same intervals.

Curiosity led us to continue these tests rather than discard them. In consequence, an interesting fact was brought out. To our surprise both over-corrected portions recovered the acidity of plus one (and more) but at different intervals of time. B_1 60 Cβ' gained plus one at the end of the fifth hour while B_1 120 β' reached plus one at about the seventh hour.

In all the tests the curves show a distinct and steady rise in acidity. This rise is due to hydrolysis caused by heat and increases continually as more heat[10] is applied. Further, it is plainly evident that a preliminary heating to the boiling point for at least forty-five minutes as advocated by Eyre (1915) does not produce a stable reaction uninfluenced by further heating.

TABLE I

MEAT INFUSION	REACTION BEFORE AUTOCLAVING		AFTER 8 HOURS IN AUTOCLAVE		AFTER 14 HOURS IN AUTOCLAVE	
	R. T.*	B. T.†	R. T.	B. T.	R. T.	B. T.
$B_2$30	1.7	2.7	4.9	6.5	7.4	10.5
$B_2$60	1.9	2.9	4.6	6.3	7.3	10.4
$B_2$120	2.1	3.1	4.3	6.5	6.9	9.3

*R. T. = room temperature titration.
*B. T. = boiling temperature titration.

In the above tests we have gone outside the limits of interest from the practical standpoint. We were led to this, however, in an attempt to locate the point of complete hydrolysis or maximum acidity of meat infusions. This goal was not reached,

[10] Three of the uncorrected portions, B_2 30, B_2 60, B_2 120 were run an additional six hours, making fourteen hours all told in the autoclave at 15 pounds pressure. The results are shown in last columns of Table I.

as stated above, even after fourteen hours autoclaving. We are continuing this work.

In the usual preparation of media from meat the total amount of heating in the autoclave varies from one half hour, at about 15 pounds pressure, for sterilization of ordinary broth, to two and one-half hours in the preparation and sterilization of agar.

It developed in the tests made on the corrected meat infusions that the change in acidity in the first half hour in the autoclave (the usual time for the sterilization of finished media) varied from nothing to 0.3 per cent at the room temperature figures, while in boiling temperature figures the change in acidity was 0.3 to 0.4 per cent (see chart 6).

The nature of the acid products which are formed as a result of the hydrolytic decomposition on boiling with sodium hydroxid may be different from those produced by hydrolysis alone when boiling unadjusted media. Therefore, while the reaction may be adjusted in each to the same point of acidity, the behavior of the media toward the various organisms may not be the same.

Similar tests to those above were made on Liebig's beef extract dissolved in tap water, filtered and titrated in a similar fashion. These tests showed no change in the reaction of the uncorrected series even after eight hours heating. Although a beef extract solution is undoubtedly quite stable,[11] as compared with meat infusions, a sample corrected[12] to neutral, after two hours in the autoclave, rose 0.2 per cent in acidity. This was corrected to neutral once more and did not change again although heated four hours longer.

While the above change is almost negligible, the addition of peptone to this as to other media, raises the change in acidity further. This must be taken into consideration in the preparation of media with beef extract when delicate end points are desired.

[11] This stability is due probably to very prolonged heating in the preparation of the beef extract itself.

[12] Correction based on room temperature titration.

BROTH[13]

The tests on the meat infusions were carried out before any peptone or salt had been added. In the preparation of broth the choice of titration methods must of course be governed by the manner of preparing the meat juice in the preliminary steps. The relative merits of pressing out the meat juice before or after heating the soaked meat must be determined by experiment in the kinds of work for which the media are destined.

For those workers whose needs and experience lead them to express the meat juice in the cold state and then dissolve the peptone and salt with very little preliminary heating, the use of the boiling titration for correction is essential, in order to approximate the future conditions due to further heating and sterilization after the reaction of the batch of medium has been set.

In this laboratory the best toxin production has seemed to be obtained when the meat juice is pressed out after heating large amounts (20 liters) for one hour at 45° to 55°C. and then boiling up strongly until the meat coagulates. The meat infusion[14] is then strained through cheese-cloth. After the requisite amount of peptone and salt have been dissolved by further heating up to the boiling point and the mixture boiled one half hour, the reaction is set according to a room temperature titration.

If the specimen is boiled[15] in the casserole before titration, it no longer represents the lot of broth in the kettle but has risen somewhat in acidity. Consequently, if the large lot be adjusted according to the boiling titration a false correction is made. To be sure, after adding the normal soda solution,

[13] Broth = meat infusion plus peptone and, usually, salt.

[14] This method proves useful in other lines of work for at this point the infusion can be filtered, sterilized and stored. It is ready for further use on the addition of any suitable peptone and may be set at any desired reaction; or it is ready as a basis for making agar.

[15] At this point may be mentioned the length of time recommended for boiling by different authors. Heinemann (1911) heats "to boiling." Jordan (1914) MacNeal (1914) and the Standard Method (1913) boil one minute. Park and Williams (1914) boil two minutes, Abbott (1915), Abel (1914), Hiss and Zinsser (1914), Mallory and Wright (1915), Swithinbank and Newman (1903) boil for three minutes.

any further boiling of the large lot, together with the final sterilization raises the acidity but not to just the desired point as shown in the tests on meat infusions (chart 6). For example, the final reaction of + 1.2 in the case of diphtheria toxin broth, is found to be uniformly obtained by titrating at room temperature and setting the reaction to + 1. This allows 0.2 rise due to heating if the broth is to be sterilized at 15 pounds pressure (121.6°C.) for one half hour. If the sterilization is to be carried on at only 5 pounds pressure (108.8°C.) for one hour on three successive days, or in the Arnold sterilizer, streaming steam (100°C.), for the same length of time, the reaction is set at + 1.1 as the more moderate heating raises the broth only about 0.1 per cent in acidity, making the finished product + 1.2 in reaction. The use of this method for the diphtheria and tetanus toxin broths for a number of years has shown fully its value and it is the method still employed in our laboratory.

On the other hand Eyre (1915) states that the correct estimation of acidity present can be made only by titration at the boiling point. Judged from our results as shown in chart 4 by the β' and β curves, this statement is erroneous. The β' curve shows the reactions and successive adjustments to plus one (+ 1) based upon the *boiling temperature* figures. The reactions at room temperature of this material are shown by the β curve. This latter is far below the β' curve (from 0.5 to 1 per cent), and gives the actual reaction of the medium at a temperature nearer that of the incubator (37.5°C.)[16]

To make this point clear, let us assume for example that a medium is to be adjusted to a definite acidity of + 1, according to the boiling titration, as stated in the Standard Method. The real reaction at which the bacteria will then be grown in the incubator is not that indicated by the boiling titration figure but a reaction which is lower in acidity to an extent of about one per cent—in other words, almost neutral.

On the other hand in chart 3 (α' curve), there is shown a similar meat infusion, adjusted to plus (+ 1) at *room temperature*

[16] It is only after four hours' autoclaving that the room temperature reaction of plus one (+1) is reached.

titration. The boiling titration figures are plotted as curve α'. The room temperature titration curve (α) comes so near the desired reaction of plus one (+1) that even a mere glance will suffice to convince one that the room temperature titration approaches more closely the one per cent line, which is the acidity we set out to secure.

This was not surprising to us as our practical experience for several years past had indicated such a condition. The modification, (page 212), devised at that time and now further substantiated by these experiments, has proved to be so very useful in its results that it is employed in these laboratories for nearly all the routine preparation of some fifty different kinds of media, aggregating over 8000 liters per year.

STERILE SODA

In order to avoid the complications of further hydrolysis and precipitation after the addition of soda to a medium which must later be sterilized, it has been suggested that sterilization be done first and that sterile soda be carefully added afterward according to the titration of samples withdrawn under sterile conditions. This has been practised by some workers, apparently with success. So far, in our laboratory, it has shown no advantages in the production of diphtheria toxin broth. Further work in this line is contemplated.

AGAR

When the need arose of supplying large amounts of neutral veal agar for the growing of the gonococcus, streptococcus and other organisms in bulk for antigens, difficulty was experienced. To grow these organisms in large lots with unfailing success is not always easy. Our trouble seemed due chiefly to the reaction of the medium. Finally the modified titration method was adopted for agar also.

Since agar solidifies at a little below 40°C. the room temperature titration was not suitable. At first any temperature between

40° and 50°C. was used. This was reduced later to about 30°C.[17] Five cubic centimeters of the hot agar are added by means of a pipet to 45 cc. of distilled water—temperature about 30°C. (verified with a thermometer). One cubic centimeter of phenolphthalein (1 per cent solution in 50 per cent alcohol) is added and the titration performed at once.

It is now our custom to titrate each batch of neutral veal agar at least twice during its preparation making the necessary adjustments of reaction. Here, as with the broth, allowance must be made for further heating in the autoclave. Experience shows that agar made from meat infusion rises in acidity usually about 0.3 to 0.4 per cent. at 15 pounds pressure during one and one-half to two hours.[18] Therefore, 3 to 4 cc. of normal soda should be added per liter in excess of the amount required to secure the phenolphthalein neutral point at the time of the first titration. The second titration is made just after filtration, before tubing and sterilizing. If the amount of soda needed does not exceed 0.2 per cent, little if any precipitate occurs on heating further. If more than the above amount is needed in adjusting the reaction, the medium should be heated in the Arnold for half an hour and the precipitate filtered out, before tubing and sterilizing.

In very careful work the medium is also titrated a third time as it comes from the autoclave. For the last test a tube or small bottle of neutralized glassware should be used in order that the reaction of the agar may be unaffected by its container. This sample is tested before it hardens—as remelting would raise its acidity further. On the addition of phenolphthalein, it should show a very delicate shade of pink if it is "neutral."

REMELTING OF SOLID MEDIA

An important factor to be considered in the adjustment of media is the remelting of solid media for the addition of sterile substances such as blood, serum, etc., or for the purpose of

[17] This slight difference of temperature had no noticeable effect on the results of the titration.

[18] This time is necessary for melting (and clearing with egg) of large batches of agar (5 to 12 liters).

immediate use in plating. If, for example, the whole is to be neutral to phenolphthalein when entirely finished an over neutralization is necessary to allow for the acid changes during the re-heating, as in making Bordet Gengou medium.

Since, in spite of the addition of soda for the correction of a medium further hydrolysis occurs when heat is applied, especially in the autoclave, it is impossible to know the exact reaction a medium will have when sterilization is complete or when the medium is re-melted. In practical work, however, it has been found that an over neutralization of 0.1 to 0.3 per cent has given good results when the titration is performed at 30°C.

The *re-sterilization* of media without suitable correction to allow for the effects of heating is to be avoided if a very definite end point is desired.

PEPTONES

The present necessity of finding substitutes for Witte's peptone, so long the standard in bacteriological work, has led us to test the reaction of various peptones on the market. A 1 per cent solution of each in distilled water was boiled one minute and then filtered through cotton and filter paper. When cool, each was titrated at room temperature and then again after the same sample had been boiled one minute, that is, the same procedure was followed as in titrating the meat infusions (page 214).

The following table shows the reactions of eight peptones, including Witte's.

TABLE II

Reaction of peptones (titrated with phenolphthalein)

KIND	ROOM TEMPERATURE FIGURE (AT 20 °C.)	RISE AFTER BEING BOILED ONE MINUTE IN CASSEROLE	BOILING TEMPERATURE FIGURE
Armour	+0.6	+0.4	+1.0
Atkinson	+0.4	+0.2	+0.6
Difco	+0.6	+0.2	+0.8
Eimer & Amend	+1.0	+0.4	+1.4
Fairchild culture	+0.7	+0.4	+1.1
Leitz	+0.4	+0.3	+0.7
Squibb	+0.3	+0.1	+0.4
Witte	+0.3	+0.1	+0.4

The peptone solutions were then divided into two sets (as with the meat infusions), one corrected and the other uncorrected. These were given successive treatments in the autoclave and titrated at the same intervals. As with the meat infusions there was a steady increase in acidity though not in so marked a degree. Three uncorrected portions of peptone solutions (Fairchild's, Eimer and Amend, Squibbs) were also run an additional six hours, making fourteen hours in all in the autoclave. As with the meat infusions the limit of hydrolysis was not reached.

In the corrected portions the total amount of acidity, developed by heating after successive additions of normal soda, again paralleled closely the rise in acidity of the corresponding uncorrected portions.

From the above it is apparent that the introduction of a peptone into a medium will affect the reaction to some extent.

INDICATORS

The shade of phenolphthalein suitable for a correct end point varies greatly in the opinion of different authors.

Miur and Ritchie (1913) give "the first trace of pink."

Hiss and Zinsser (1914)—"faint but clear and distinct pink."

Stitt (1913)—"a delicate pink (hot titration) a purplish violet color (cold titration)."

Jordan (1914)—"Faint but distinct pink color."

Park and Williams (1914)—"Faint, but distinct pink which remains on re-heating."

Heinemann (1911)—"Faint but decided and stable pink."

Abel (1912)—"Brilliant red." (Translation.)

Abbott (1915)—"Pink color."

MacNeal (1914)—"Faint but distinct and permanent pink."

Swithinbank and Newman (1903)—"Clear bright pink color."

Mallory and Wright (1915)—"Bright pink color" not "the pinkish darkening of the fluid which preceeds it."

Eyre (1915) uses a "pinkish tinge" or "a faint rose-pink which cooled to 30° or 20°C., becomes more distinct and decidedly deeper and brighter" resembling a "deep magenta color."

These shades given differ more or less from the Standard Method (1913).

That no two people seem to titrate to exactly the same shade has often been shown in our laboratory when a different worker in the media room has attempted to set the final reaction of some special medium. If the method of titrating is to be at all accurate, it is necessary to *assume* a shade of pink for phenolphthalein. This necessity is brought out by such great discrepancies between different workers titrating the same substance as are given by Clark (1915).[19] As a help, a practical color scale may be of aid in determining the most suitable shade for a certain kind of work and approximating it as closely as possible. This should eliminate the personal factor to some extent.

As stated before, in our opinion, the correct shade for a delicate end point in pale broth or other solutions with little color is the first most delicate pink tinge observable throughout, remaining at least one minute.

With us, when titrating agar, a mixture of 5/20 red, 3/20 orange and 12/20 white on the color-top (see page 210) has proved to be a desirable shade for the first and second titrations of neutral agar;[20] while the third titration, when the medium comes out of the autoclave, should give (on the addition of phenolphthalein) a shade consisting of 3/20 red, 3/20 orange and 14/20 white on the color-top. These shades differ from the ones given above both in the Standard Method of titrating at boiling point (page 210) and our own definition (page 213) but they have yielded very good results. However, it may be as difficult to decide on an end point by means of a color-top or scale as to imagine an end point from the descriptions of the various writers.

LITMUS

Since any medium with meat infusion as a basis and peptone added is a most complex mixture, no one indicator shows all

[19] (16) page 117. Such relatively great discrepancies are surprising especially among the chemists.

[20] The deeper color of agar as compared with the usual color of broth makes necessary the use of some orange in this scale.

of its varying acid constituents. In the last 10 or 15 years phenolphthalein has been largely employed, yet it cannot be relied upon in every instance.

Park and Williams[21] state:

Different indicators differ not only in delicacy but in the substances to which they react. A medium alkaline to litmus is acid to phenolphthalein showing that there are present substances possessing a character which litmus does not detect, weak organic acids and organic compounds, theoretically amphoteric but in which an acid character predominates.

Thus a liter of bouillon becomes, on the addition of 1 per cent of peptone, more alkaline to litmus but decidedly more acid to phenolphthalein; 1000 cc. of water with 1 per cent peptone is acid to phenolphthalein to such an extent that 3.5 cc. of deci-normal NaOH is required to neutralize it. To litmus it is alkaline and requires 3.4 cc. of deci-normal HCl. Two per cent peptone doubles the difference. The same figures hold approximately true for peptone broth.

Eyre (1915) states that although meat infusion is always acid to phenolphthalein it may react neutral or even alkaline to litmus; again, if rendered exactly neutral to litmus, it still reacts acid to phenolphthalein; that this is due to the facts:

(1) Litmus is insensitive to weak organic acids whose presence is readily indicated by phenolphthalein.

(2) Dibasic sodium phosphate which is formed during process of neutralization is a salt which reacts alkaline to litmus but neutral to phenolphthalein.

On the other hand, MacNeal (1914) considers litmus the more useful:

The neutral point indicated by litmus is very nearly the actual point in respect to acidity and alkalinity, and this point is not appreciably displaced in either direction by the addition of a neutral mixture of a feebly dissociated acid and its salts to the solution. The end reaction indicated by phenolphthalein when it turns pink is actually a point at which there is a slight excess of alkali. This is so nearly the neutral point in inorganic solutions, when electrolytic dissociation

[21] Third and fourth editions—1908 and 1910.

is marked, that the error is not appreciable. In solutions of organic substances, especially when considerable amounts of feebly dissociated substances such as are contained in peptone or gelatin, are present, this error becomes very appreciable. The discrepancy between the end point for litmus and for phenolphthalein will vary for different lots of media.

Naturally those media which contain litmus as an indicator to show acid production by the growth of bacteria, must be alkaline to litmus yet not too alkaline or the indicator is rendered useless. The testing of such media by the use of litmus paper is an unsatisfactory and crude method useful for only the roughest work. The use of a litmus solution (Merck's purified in 5 per cent aqueous solution) is far more satisfactory.

"Neutral to litmus" is "so and so" acid to phenolphthalein, the figure given varying with the writer. Muir and Ritchie place it at + 2.5, Stitt at + 1.5 boiling titration and about + 0.7 with the cold titration. Abbott gives + 2.5, Abel + 1.5 to 2.5 and Heinemann + 2, all depending on the shade of pink considered by the worker as suitable and the length of time the sample is boiled in the casserole.

In the modified method used in our laboratory, the figure is as low as + 0.6 or + 0.7 with media prepared with 1 per cent peptone. (This figure rises to + 1 when the boiling titration is used.) A 1 per cent peptone (Witte) solution in water is about + 0.2 to + 0.3 with phenolphthalein at room temperature and about + 0.4 at boiling figure.

It has been shown by Hildebrand (1913) and others (Clark, 1915; Bovie, 1915) that an indicator does not indicate the point of actual neutrality but merely a definite degree of hydrogen ion concentration. Where these two points coincide, i.e., where the hydrogen ion concentration at which the indicator changes color, is within the zone of absolute neutrality for a particular mixture of substances, this change of color in an indicator will, of course, be of significance. It would therefore seem desirable to select a specific indicator for each class of media. This could be accomplished only by determining the effect of added

alkali upon the electrical conductivity of the particular medium. Such measurement should be made by means of the hydrogen electrode. The measurements of potential are usually expressed in terms of hydrogen ion concentration, that is, acidity.

For an apparatus as described by Bovie (1915) the details of manipulation are as follows: A mixture of the medium with water in the same proportions as used for ordinary titrations is placed in a beaker kept at 30°C., and the precaution of excluding CO_2 observed. The indicator is added and the standard electrodes are immersed. Successive portions of decinormal sodium hydroxid solution are then added. After each addition of the alkali the potential of the mixture is observed and recorded.

The point at which the indicator gives its first change of color is marked. The additions of alkali should be continued and the potentials further noted until the curve changes its shape—that is, from concave to convex or vice versa. The actual point at which this change takes place is known as the point of inflection and is a true neutral point. The nearness of the indicator's point of change to this point of inflection determines its suitability for this particular class of media. The indicator in which the change comes nearest to this point should be selected for practical use. Work in choosing such indicators according to this method is planned.

When titrations are performed under ordinary circumstances at the boiling point, it is noticeable that making a decision as to the correct end point to phenolphthalein is much more difficult than when the temperature of 20° to 30° C. is used. This is due to the changes of ionization in the mixture caused by this considerable change in temperature.

Besides this, the constant presence of colloidal substances[22] in peptones,[23] phosphates and sugars in all media gives rise to

[22] Hildebrand (1913), Clark (1915) and others have shown that colloidal substances affect the sharpness of indicator end points.

[23] In the titration of the peptone solutions it was very difficult to get a sharp end point even at 20°C. The indicator (phenolphthalein) seemed to "flare" suddenly from the colorless state to a deeper pink than the one showing a really delicate end point.

a further depression of ionization, especially when these substances are decomposed by boiling.

The meagre results from the few investigations conducted in this field lead us to suspect that the presence of sugars in media may have a considerable influence on the effective acidity, that is, the dissociated acid principles (Hildebrand 1913). Work in these lines is to be continued.

SUMMARY

Marked and continued hydrolysis, resulting in the formation of acid principles, occurred on successive heatings of meat infusions in the autoclave. The state of complete hydrolysis, i.e., the point at which no further acidity is produced, was not reached with these meat infusions (which had been subjected previously to boiling over the open flame for one to two hours) even after prolonged autoclaving at fifteen pounds pressure for eight[24] hours.

Hydrolysis did not occur in solutions of Liebig's beef extract subjected to similar heatings. In the portion which had been adjusted to the neutral point, however, slight hydrolysis did take place.

Those portions of meat infusion in which the natural acidity had been adjusted with normal sodium hydroxid to plus one (phenolphthalein) showed that hydrolysis occurred on application of heat and continued to do so after successive adjustments and autoclavings. There was produced in these portions as much acidity, approximately, as was developed on heating the corresponding uncorrected portions.

The further production of acidity after the addition of sodium hydroxid is due to the hydrolytic effect of heat in the presence of water, upon these portions of the medium unbound by alkali.

The fact that hydrolysis is promoted by heat makes inaccurate the estimation of acid ions in a batch of medium when there is

[24] With three of the meat infusions the time was extended from eight to fourteen hours.

taken as an index a sample[25] titrated at the boiling point with phenolphthalein as the indicator. Boiling has also a marked effect on the ionic concentration in media mixtures, hence the boiling titration called for in the Standard Method and followed more or less closely by many workers and recommended in the various text books, is subject to greater error than titrations performed at a temperature of 20° to 30° C.

In the adjustment of culture media not only is the desired end point more closely approximated by titration at 20° to 30° C. (see chart 6), but the misleading information of the boiling titration is avoided (see chart 4). Although according to Eyre (see page 211) certain acids are detected only at the boiling point, the *actual* reaction of media at the temperatures at which they are *used* is the object of vital importance. The conditions prevailing at these temperatures (incubator, 37.5°C., for some organisms and room temperature for others) are more closely indicated by titrations conducted at room temperature.

As shown in chart 4 the actual reaction of a medium titrated by the boiling method is really from 0.5 to 1 per cent lower than is indicated by the boiling titration figures.

The tests made on the reaction towards phenolphthalein of the various peptones on the market showed them to differ greatly in acidity. The effect on peptones of prolonged heating in tests similar to those on the meat infusions showed that hydrolysis occurred on the application of heat. The development of acidity took place after successive adjustments of reaction with sodium hydroxid, as with the meat infusions and the total amount of hydrolysis approximated closely that of the uncorrected portions. Here, too, prolonged heating of fourteen hours in the autoclave did not give complete hydrolysis.

In the usual titration methods, no one indicator gives all the

[25] That is, if the titration is performed after a preliminary boiling of the whole batch of meat infusion. When a titration is made on meat juice pressed out in the cold and containing added peptone dissolved at a low temperature, the boiling of the sample in the casserole is necessary to approximate future conditions after the boiling of the whole batch.

evidence desired in every case, e.g., the differences between litmus and phenolphthalein.

The necessary dependence on the change in color of an indicator to show the reaction of a solution, especially in the case of culture media, gives rise to error. This is due partly to the depressing effect of colloids, phosphates and some sugars which affect the sensitiveness of indicators; and also to variations of judgment with different workers as to the correct shade of color for an end point. Where it is desirable to avoid these influences titrations may be made by measurement of the electrical potential.

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