Reaction of Culture Media.

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The reaction of culture media has worried every bacteriologist at some time in his career. During the past two years there have appeared several papers, in American publications, dealing with the reaction of bacteriologic culture media. Among these may be mentioned those by Clark (1), (2), (3), (4); Itano (5); Anthony and Ekroth (6). Clark and Lubs have presented papers (3) and published a series of articles entitled, "Colorimetric Determination of Hydrogen Ion Concentration and Its Applications in Bacteriology" (4). This work, as well as all papers published to date, including those presented at the 1916 meeting of the American Society of Bacteriologists shows that bacterial activities in general are greatest when the culture medium is neutral or approximately so. A simple, practically neutral medium is most desirable for general use. Anything which tends to produce or make it necessary to adjust acidity should be avoided if possible.

Evidence points to physical and chemical laws applying to culture media just as well as they do to water solutions of pure salts, the only difference being, media are more complicated and not as fully understood. Bacteriological media are of two kinds, liquid and solid. This paper is almost entirely confined to solid media. The bases of solid media are usually agar agar, gelatin or silicate jelly. Chemicals are added to these bases to furnish food for bacterial life and to make the reaction of the media such, that bacteria may thrive. More attention has been paid to the adding of chemicals for supposed food values than to the ascertaining of the reactions that take place between the chemicals themselves and the basis of the media.

Acidity or alkalinity of culture media are due to the nature of the basic substance used in making the media, and to the nature of the chemicals added to this base. The resultant equilibrium, produced by physio-chemical phenomena, notably ionization and hydrolysis, as influenced by mass action, temperature and pressure determines the reaction of the culture media.

The two principal methods now employed to determine the reaction of media are the determination of the hydrogen ion concentration by means of the hydrogen electrode, and the total titratable acid present as determined by titration. The hydrogen electrode was applied to biochemistry by Sorensen (7). Since 1912 several investigators have used the hydrogen electrode in the study of bacterial activities. Among these are Michaelis and Marcola (8); Brunn (9); Clark (1); Itano (5); and Clark and Lubs (10).

The advantages of the hydrogen electrode in bacteriological work are claimed to be that it gives the hydrogen ion concentration the bacteria are in contact with and that it can be used advantageously in colored solutions. Its disadvantages are that it can not be used in solid media and that for every grouping of chemicals there is a new electro chemical problem. Different investigators working with the hydrogen electrode, from a purely scientific point of view, have not agreed on the contact potential between 0.1 N. HCl.—0.1 N. KCl. (11).

This paper is written not to find fault with the hydrogen electrode in its applications to bacteriology but to point out some factors in the making of culture media and in the controlling of its reaction that are as important as the method by which the reaction is determined. It is (so-called) acidity due to the crude methods of making media that is discussed in the following paragraphs.

Hot Solutions.

The usual procedure followed in titrating culture media is crude. Titrations are conducted in hot solutions (12). Hydrolysis increases with temperature and titrations of culture media containing meat, peptone, gelatine, agar agar or plant extracts when made at high temperatures are much greater than they would be at lower temperatures. The difference between hot and cold titrations is often more than the titration of the media at room temperature. Clark (1) mentions a 10 per cent gelatine, 1 per cent peptone, and 5 per cent meat media titrating plus 1.0 per cent acid when hot and plus 0.5 per cent acid at room temperature.

Small Aliquots.

Too small aliquots of media are generally used. Aliquots are pipetted or poured out from graduated cylinders. These methods of taking

aliquots allow errors as great as 10 per cent of the 5 cc. aliquot taken. An error of .5 cc., which is easily made with a graduate, means an error of 10 cc. per 100 cc. of media. Again an error of .05 cc. (one drop) of N/10 alkali in titrating means an error of plus or minus 0.1 per cent in the calculated acidity.

Indicator.

Large amounts of indicator are used. In the literature and in the standard methods (12) 1 cc. of a ½ per cent solution of phenolphthalein is specified. In accurate chemical work the amount the mass of indicator affects the accuracy of the determination is taken into consideration. One or two drops of indicator have proven sufficient. Anthony and Ekroth (6) give a list of shades of color called suitable or correct end-points with phenolphthalein. The colors listed vary from "first trace of pink" to "brilliant red." Clark (1) presents a table showing that the variations in acidity of a 1 and a 5 per cent peptone media when these media were titrated by four chemists and four bacteriologists. The acidities calculated from the titrations of the different workers varied from 0.58 cc. to 1.40 cc. N/40 alkali for the 1 per cent and from 2.68 cc. to 7.40 cc. N/40 alkali for the 5 per cent media.

Clark and Lubs (4) describe indicators which undergo rapid color changes at certain definite hydrogen ion concentrations. They give Brom thymol blue as undergoing color changes between P_H 6.0 and P_H 7.6. These indicators are new and have been manufactured (and there, almost under protest) by only one chemical supply house. Their stability and the exactness with which they can be used under the crude conditions phenolphthalein has been used are unknown. At the present time it is fair to assume that these new indicators will come into general use, but as long as different investigators do not agree on a definite value for the contact potential between 0.1 N. HCl. and 0.1 N. KCl. phenolphthalein is not to be discarded for use under exactly defined and proper conditions.

A further evidence that phenolphthalein (properly used) is satisfactory for determining neutrality of media is found in Itano's work on the proteolysis brought about by certain bacteria when put under known initial hydrogen ion concentration. The reaction of all the media (19)

changes to very close to the hydrogen ion concentration at which phenolphthalein changes from colorless to pink.

The last report (13) of the committee on standard methods for bacteriological analysis of milk makes no recommendation as to the adjusting of the reaction of the media. This is taken as an indication of a growing realization by this committee that proper selection of materials in making media gives a media near to neutral in reaction. Other evidence that most bacteria will thrive when media are somewhere near neutral is brought out in the fact that most ensymes function when kept close to neutral.

Carbon Dioxide.

Usually some carbon dioxide is present in the alkali used, and many bacteriologists consider freshly distilled water carbon dioxide free. Carbon dioxide has affected the accuracy of some titrations, for we have reference to where investigators advise against titrating the media to a low per cent of aciditiy for fear of volatilizing ammonia from the ammonium salt used in making the media, (14). Ammonia is not easily volatilized from acid solutions but is slowly evolved by alkaline solutions even at low temperatures (40° C.), therefore these investigators are making their media nearer neutral than they think. Slightly alkaline media saturated with carbon dioxide is acid to phenolphthalein.

Apparatus supply houses are advertising water stills which, according to the advertisements, give pure distilled water. Quoting from the advertisement of one of the leading firms, we have "water of the highest purity—free from ammonia and all gaseous and organic impurities." These stills, as shown by the titrations given in the following table do not give carbon dioxide free water where the water used in them is hard. Freshly distilled water made from the same local hard water supply with different stills gave the following titrations with N/10 carbonate free alkali and phenolphthalein.*

* The water from which distilled water is prepared in many localities is as hard or harder than that in this locality.

TABLE I.

Carbon Dioxide in Freshly Distilled Water.

Titrated at room temperature 22°C.

Make of Still.	cc. N/10 Alkali per 100 cc. H_2O.	
	Water from Collecting Vessels.	Water Direct from Still Outlet.
Stokes stills—		
No. 1	0.40	.45
No. 2	0.05	.50
Barnstead still	0.08	.35
Large local plant	0.65	2.10

All yield water containing carbon dioxide and the amount of carbon dioxide varied with the same make as well as different makes of stills.

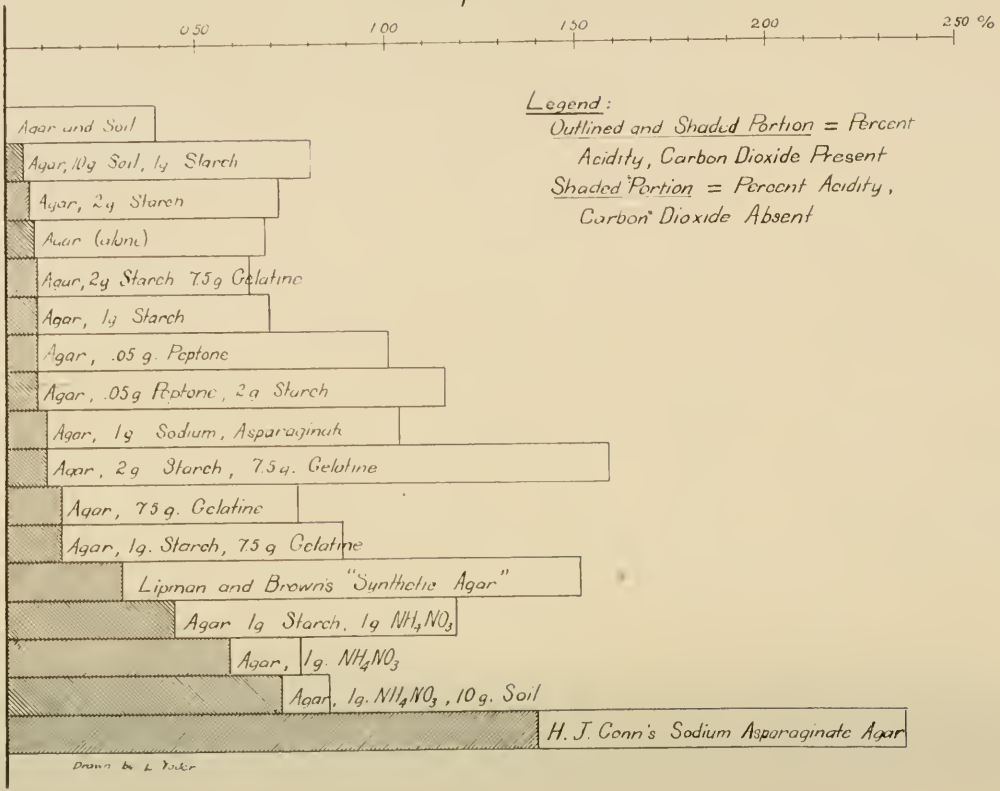
Test of Effect of Carbon Dioxide on Media Titrations.

The results reported in Table II were an attempt to find out how much the titration of media would be affected by the carbon dioxide present in distilled water from one of the above stills. The point under investigation being to determine the effect of carbon dioxide, the water was prepared and titrations were made at about 70°C. so that it would be evident that the results were not due to carbon dioxide being absorbed by the media or water from the air of the room while cooling to room temperature. Two two-liter flasks which had previously been proven to be made of non-soluble glass were filled with distilled water. The water in one flask was boiled for about five minutes to remove the carbon dioxide present while that in the other flask was heated to 75°C.

Duplicate twenty-five cc. aliquots of each media were weighed into clean, carbon dioxide free, erlenmeyer flasks; 100 cc. of the hot carbon dioxide free water was added to one of each of the duplicate aliquots of media and 100 cc. of the hot yet unboiled water added to the other flask of each set of duplicates. Two drops of phenolphthalein were added to each flask after they had been shaken until the contents appeared homogenous. Titrations were made with carbonate free N/10 sodium hydroxide* and the faintest discernible, yet permanent pink coloration

* Make a solution of the alkali (sodium) so strong that the carbonate contained will be precipitated. Add the clear supernatant liquid which is carbonate free to carbon dioxide free water and standardize.

Graph 1


050
100
150
200
250 %
Legend:
Outlined and Shaded Portion = Percent Acidity, Carbon Dioxide Present
Shaded Portion = Percent Acidity, Carbon Dioxide Absent
Agar and Soil
Agar, 10g Soil, 1g Starch
Agar, 2g Starch
Agar (alone)
Agar, 2g Starch 7.5g Gelatine
Agar, 1g Starch
Agar, .05 g. Peptone
Agar, .05g Peptone, 2g Starch
Agar, 1g Sodium, Asparaginate
Agar, 2g Starch, 7.5g. Gelatine
Agar, 7.5g. Gelatine
Agar, 1g. Starch, 7.5g Gelatine
Lipman and Brown's "Synthetic Agar"
Agar 1g Starch, 1g NH_4NO_3
Agar, 1g. NH_4NO_3
Agar, 1g. NH_4NO_3, 10g. Soil
H. J. Conn's Sodium Asparaginate Agar
Drawn by L Yoder

was taken as the end point. The results of these tests with 23 lots of media are shown in Table II and Graph I.

TABLE II.

Acidity of Media (*Calculated in Per Cent.) as Affected by Carbon Dioxide in Distilled Water.

	(1) CO_2 Present in Dilution Water.	(2) CO_2 Free Dilution Water.	(3) Acidity Due to CO_2 in (1).	(4) Actual Acidity if Corrected to .80 by (1).	(5) Actual Acidity if Corrected to .50 by (1).
Agar† (alone)	.68%	.07%	.61 (c)	.19	alk.
Agar and 1 gm. starch	.69	.08	.61	.19	alk.
Agar and 2 gm. starch	.72	.06	.66	.14	alk.
Agar and 10 gm. soil	.39	.00—	.39+	—.41	alk
Agar and ammonium nitrate	.76	.58	.18	.62	acid.
Agar and 7.5 gms. gelatine	.72 (a)	.12	.60	.20	alk.
	.80	.16	.64	.16	alk.
Agar and .05 gms. peptone	1.00	.08	.92	—.12	alk
Agar and 1.0 gms. sodium asparaginate	1.03	.10	.93	—.13	alk.
Agar, 1 gm. starch and 10 gms. soil	.80	.04	.76	.04	alk
Agar, 1 gm. starch and 1 gm. (ammon. nitrate (b))	1.18	.44	.74	.06	alk.
Agar, 1 gm. starch and 7.5 gelatine	.66	.11	.55	.25	alk.
	1.10	.16	.94	—.14	alk
Agar, 2 gm. starch and 7.5 gelatine	1.58	.10	1.48	—.68	alk.
Agar, 2 gm. starch and .05 gm. peptone	1.15	.08	1.07	—.27	alk.
Agar, 10 gms. soil and 1 gm. (ammon. nitrate)	.84	.72	.12	.68	acid.
Agar, 10 gms. soil, 2 gms. starch and 7.5 gelatine	.64	.08	.56	.24	alk.
Lipman and Brown's "synthetic agar"	1.25	.22	1.03	—.23	alk.
	1.40	.28	1.12	—.32	alk.
	1.48	.32	1.16	—.36	alk.
	1.87	.36	1.51	—.69	alk.
H. J. Conn's (sodium asparaginate agar)	2.24	1.38	.86	—.06	alk.
	2.46	1.40	1.06	—.26	alk.

*1.00% would mean the requirement of 1 cc. normal alkali for neutralization of 100 cc. of media.

†Fifteen grams of air dry agar basis of all media.

(a) Each figure given represents one lot of media. No two lots of same media were made on same date.

(b) Phenolphthalein is not the most desirable indicator to use when ammonium salts are present.

(c) Distilled water prepared from soft water is often practically free from carbon dioxide.

The table shows—

(1) That the carbon dioxide normally present in distilled water prepared from hard water by a modern still affects the titration of media.

(2) That the titration, due to carbon dioxide present in diluting water may be much greater than the total titration of the acidity of the media itself.

(3) That the carbon dioxide does not affect the acidity of all media in the same proportion.

(4) Media adjusted by results of titrations made of aliquots diluted

with water containing carbon dioxide are always less acid than desired, in fact some media are alkaline, note columns headed (4) and (5).

Distilled water is believed *by so many* to be carbon dioxide free, no matter whether the water from which it is made is hard or soft, that, as a rule bacteriologic culture media has been adjusted to a less degree of acidity than planned for. Litmus is not sensitive to carbonic acid, thus it seems fair to assume that acidities of culture media, observed with phenolphthalein, but which do not prove out with litmus may be partly due to the carbon dioxide present in the dilution water added to the aliquot titrated. Anthony and Ekroth (6) make statements concerning the work of MacNeal, Muir and Ritchie, Stilt, and others concerning comparisons between litmus and phenolphthalein titrations. Titrations with phenolphthalein carried out near the boiling point of the media are unreliable, due to the increased hydrolysis of the media and to the fact that phenolphthalein is more sensitive in cold solutions (15).

Hot and Cool Titrations With Especially Prepared Media.

An experiment was conducted to find out the effect of temperature on acidity titrations when agar agar plus gelatin were present with salts that undergo changes in hydrolysis with increasing temperature. The agar agar and gelatin used were selected because of their small changes in acidity when autoclaved or heated. They were selected by a procedure described by the author (16) in another article. Unfiltered water solutions of the agar and gelatin used were free from precipitates and thus by themselves did not even need filtering.

Two basic media were made up according to the following procedure:

Agar agar Media.—Thirty grams of agar agar were dissolved in the inner part of a double boiler in 2,000 cc. of carbon dioxide free distilled water. When solution was complete distilled water (carbon dioxide free) was added to make the weight of agar and water up to 2,000 gms.

Agar plus Gelatin Media.—This was made up exactly as the agar media except that 7.5 grams of gelatin were added per 1,000 grams of media.

Fifty gram aliquots of each media were weighed out into clean 250 cc. erlenmeyer flasks. Thirty-four aliquots of each media were taken. The chemicals were previously prepared by making water solu-

tions of them of such concentration that they contained .05 grams of salt per cc. of solution. One cc. aliquots of the proper solutions were added to aliquots of the media using a 1 cc. pipette graduated to .01 cc. This was to give a concentration of the salt of 1.0 gram per liter of media.

The flasks were tightly plugged with cotton and autoclaved for different lengths of time under 17 pounds pressure of live steam. It was assumed from previous tests that the one cc. of water added with the salt would be lost in the autoclaving. As soon as autoclaved approximately 100 cc. of boiling carbon dioxide free distilled water was added to each flask. Titrations were made at the temperatures specified using 2 drops of 0.5 per cent solution of phenolphthalein as indicator and N/10 *carbonate free* sodium hydroxide. The results are given in Table III.

TABLE III.

ACIDITY OF AGAR AGAR AND AGAR PLUS GELATINE MEDIA AS AFFECTED BY SALTS AND LENGTH OF TIME OF STERILIZATION.

(Figures express cc. normal alkali needed to neutralize 100 cc.)

	Hot 90°.	40° to 45°.	Increase 90° Over 40°.	(1) Increase Due to Salts. 90° 40°.	(2) Increase Due to Gelatin. 90° 40°.
Potassium nitrate (3)	.03	.01	.02		
Ammonium nitrate (3)	.30	.30	.50		
Aluminum nitrate (3)	.84	.80	.04		
Agar—					
Autoclaved 0.0 hours	.03	.01	.02		
Autoclaved 0.5 hours	.04	.03	.01		
Autoclaved 1.0 hours	.04	.03	.01		
Autoclaved 2.0 hours	.03	.03	.00		
Autoclaved 4.0 hours	.03	.03	.00		
Average			.008		
Agar and KNO_3—					
Autoclaved 0.5 hours	.05	.03	.02	.01 .00	
Autoclaved 1.0 hours	.05	.03	.02	.01 .00	
Autoclaved 2.0 hours	.05	.04	.01	.02 .01	
Autoclaved 4.0 hours	.03	.03	.00	.00 .00	
Averages			.013	.01 .003	
Agar and NH_4NO_3—					
Autoclaved 0.5 hours	.36	.21	.15	.32 .18	
Autoclaved 1.0 hours	.38	.20	.18	.34 .17	
Autoclaved 2.0 hours	.35	.20	.15	.32 .17	
Autoclaved 4.0 hours	.40	.18	.22	.37 .15	
Averages			.175	.338 .168	

TABLE III—Continued.

	Hot 90°.	40° to 45°.	Increase 90° Over 40°.	(1) Increase Due to Salts. 90°	40°.	(2) Increase Due to Gelatin. 90°	40°.
Agar and Al $(NO_3)_3$—							
Autoclaved 0.5 hours	.71	.62	.09	.67	.59		
Autoclaved 1.0 hours	.73	.63	.10	.69	.60		
Autoclaved 2.0 hours	.75	.60	.15	.72	.57		
Autoclaved 4.0 hours	.78	.68	.10	.75	65		
Averages			.110	.708	.602		
Agar plus Gelatin—							
Autoclaved 0.0 hours	.11	.07	.04			.08	.06
Autoclaved 0.5 hours	.12	.10	.02			.08	.07
Autoclaved 1.0 hours	.13	.10	.03			.09	.07
Autoclaved 2.0 hours	.13	.08	.05			.10	.05
Autoclaved 4.0 hours	.13	.10	.03			.10	.07
Averages			.034			.09	.064
Agar plus Gelatine and KNO_3—							
Autoclaved 0.5 hours	.11	10	.01	−.01	.00	.06	.07
Autoclaved 1.0 hours	.10	10	.00	−.03	.00	.65	.07
Autoclaved 2.0 hours	.13	.10	.03	.00	.02	.08	.06
Autoclaved 4.0 hours	.13	.10	.03	.00	.00	.10	.07
Averages			.018	−.010	.005	.073	.068
Agar plus Gelatin and NH_4NO_3*—							
Autoclaved 0.5 hours	.53	.29	.24	.41	.19	.17	.08
Autoclaved 1.0 hours	.53	28	.25	.40	18	.15	08
Autoclaved 2.0 hours	.53	18	.35	.10	.10	.18—	.02
Autoclaved 4.0 hours	.58	.38	.20	.45	.28	.18	.20
Averages			.26	.413	.188	.17	.085
Agar plus Gelatin and Al $(NO_3)_3$*—							
Autoclaved 0.5 hours	1.03	.99	.04	.91	.89	.32	.37
Autoclaved 1.0 hours	1.03	.98	.05	.90	.88	.30	.35
Autoclaved 2.0 hours	1.03	.91	.12	.90	.83	.28	.31
Autoclaved 4.0 hours	1.13	.88	.25	1.00	.78	.35	.20
Averages			.115	.928	.845	.313	.308

*Precipitation occurred in all aliquots of this series.

(1) Figures in this column are difference between the media without and with added salt.

(2) Figures in this column are difference between corresponding media containing no gelatin.

(3) These salts were used because they are typical of classes of salts that vary in hydrolysis.

Table III brings out the following:

(1) The temperature of the media affects the titration.

(2) The effect of temperature on titration varies with the bases of the media and the chemicals used in making the media.

(3) Increasing length of time of autoclaving does not appreciably change the acidity of the media.

(4) Potassium nitrate did not appreciably change the acidity of the agar or the agar plus gelatin media.

(5) The effect of the nitrates used seemed to be due more to the hydrolysis of the nitrates themselves rather than to reactions taking place between them and the agar and gelatin.

(6) Reaction of media should be adjusted by titrations made at the temperature at which they are to be used.

The results of this test lead one to presume that if proper care was used in selecting the chemicals to be used in culture media, the acidity of bacteriologic culture media would rarely have to be neutralized.

Evidence Drawn From Literature in Support of Contention That Hydrolyzable Substances Should Be Avoided.

Anthony and Ekroth (6) give a table which shows the reaction of different peptones when titrated at room and boiling temperatures with phenolphthalein as indicator. The results show that the variations in acidity of the different peptones are large but that the peptone having the lowest acidity at room temperature also has the lowest at boiling temperature. Witte's peptone has been almost universally agreed upon as the best and is it not fair to suppose that this is due to its freedom from hydrolyzable material?

The same authors found that "Leibig's Extract of Beef" does not undergo the hydrolysis that homemade extracts do. They say, "This stability is due probably to very prolonged heating in the preparation of the beef extract itself." In other words the more stable the extract the more reason for its use.

Itano (5) working with the hydrogen electrode finally, after experimentation, decided on a medium containing both "Leibig's extract" and Witte's peptone. He found that if these constituents were sterilized before mixing, i. e., if they were stabilized, "the medium prepared from them maintained the figured P_H fairly constantly."

Fellers (17) finds that soil bacteria prefer a very slightly acid, a neutral or just alkaline media.

Summarizing the results obtained by these recent workers and realizing that the standard method of titrating media (12) gives too high titrations and thereby low acidity of adjusted media, it seems probable that bacteriologic media in most cases should be very slightly acid or neutral in reaction.

The following procedure which is based on results reported in Tables

I, II and III, has proven satisfactory for the titration of media: Twenty-five gram aliquots of the hot media are weighed out into 350 cc. erlenmeyer flasks (Jena, pryex or non-sol), which have just been rinsed with carbon dioxide free water. Approximately 250 cc. of hot, carbon dioxide free distilled water is added to each flask and the flasks are shaken until after the mixture of water and media appear homogeneous. They are then loosely stoppered and set to one side until they attain room temperature. Titrations are then made with N/10 carbonate free alkali and two drops of a ½ per cent solution of phenolphthalein. The end point is reached on the appearance of the faintest, yet permanent pink color. The fainter the color one is able to titrate to, the more accurate the titration.

Summary.

(A) Ideal media for routine bacteriological work, if rightly prepared from selected agar agar from stabilized peptone, from stabilized meat extracts and from chemicals which hydrolize but little, does not need to be adjusted in reaction unless the chemicals inter-react (which should lead to a choice of other chemicals).

(B) It is fairly well established that most bacteria will thrive in a neutral medium. The standard methods (12) have allowed media to be adjusted to nearer neutral than the figures would indicate.

(1) Titrations have been carried out in hot solutions where hydrolysis is great and media corrected to certain standards by these titrations is always nearer neutral when at blood heat or a lower temperature.

(2) Many have used alkali and water containing carbon dioxide and the errors resulting have caused media to be adjusted to lower acidity than desired.

(C) Hydrolyzable chemicals have been used and their use has made results uncertain.

(D) Meat infusions, peptones, and other extracts have been found to vary greatly in reaction. Those extracts and peptones giving best results happen to be those that are most stabilized.

(E) Some organizims tolerate more acidity than others (3) and the hydrogen ion concentration must be determined if classifications are to be made on the basis of tolerance to H and OH ion concentrations.

(F) Workers in physical chemistry have determined that for each acid there is a dilution beyond which the per cent ionized remains constant. When 25 cc. of media that is, at most, only slightly acid is further diluted with carbon dioxide free water (as must be done to titrate at room temperature) the per cent acid ionized has reached its limit. The difference between the value obtained with the hydrogen electrode and that obtained by titration under proper conditions is thus small or negligible.

(G) Itano (5) (19) has found that proteolysis is optimum when the hydrogen ion concentration of media is in or at the range where phenolphthalein titrations properly carried out would indicate neutrality.

Different investigators have suggested brom thymol blue and phenol red for phenolphthalein. This has not been done because the paper is intended to bring out errors in making media which must be corrected if any indicator is used. The values used at present for the contact potential prevent one from adopting any shade of any indicator as absolute neutrality.

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