THE EFFECT OF THE REACTION OF THE MEDIUM ON THE CHARACTERISTICS OF BACTERIA

I. GENERAL PRESENTATION OF THE PROBLEM, AND RESULTS OBTAINED WITH BACILLUS COLI-COMMUNIOR, SALMONELLA ENTERITIDIS AND PSEUDOMONAS PYOCYANEA

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The control of the reaction of the medium as a factor in obtaining typical responses has been recognized in the study of many characteristics of microörganisms. The mechanisms whereby changes in characteristics, due to differences in reaction, are brought about is still obscure. Most of the recorded work takes into account only one, or at best a few, of the factors involved.

As treated in this paper, the problem includes consideration of (*a*) buffer action of bacteria, (*b*) response to pH as shown by antigenic structure, (*c*) effect of pH on utilization of food substances and on metabolic products, (*d*) the effect of pH on dissociation phenomena, and (*e*) the reaction of the medium as a factor in certain specific characters of bacteria.

The pH limits of growth have been determined for many bacteria. In general it has been found that these limits vary for different strains. An excellent table of maximum, minimum and optimum values for growth of various bacterial species is given by Buchanan and Fulmer (1930). The limits of growth as well as the pH effect on growth rates have been found to depend on a number of factors, such as the character of the food supply, the temperature of incubation, adaptation to previous environment, etc.

BUFFER ACTION OF BACTERIA

The amphoteric nature of bacteria has been established both by studies of their behavior toward dyes (Stearn and Stearn, 1925) and by cataphoretic studies (Winslow, Falk and Caulfield, 1923). A mixture of two ampholytes will itself have amphoteric properties (Stearn, 1926). Such a system differs from a pure ampholyte in that, in general, it shows its maximum buffering power at or near the isoelectric point of the mixture. The analogy of the bacterial cell to this type of system has been pointed out (Stearn and Stearn, 1928). Shaughnessy and Falk (1923 and 1924) found that *B. coli* exhibits buffering action passing through a maximum about pH 6.5. The pH 5.5 had been reported by Stearn and Stearn (1925) as the isoelectric point of this organism.

Apparently living bacteria have two buffering mechanisms. Shaughnessy and Winslow (1927) found that, in solutions more alkaline than the reaction of optimum viability, *B. coli* liberates acidic substances, while in solutions more acid than this reaction the organism liberates alkaline substances.

There have been a number of reports of a final limiting pH produced by growing cultures, which was originally thought to be characteristic of the culture and regarded as a "physiological constant" of the organism (Michaelis and Marcora, 1912; Clark, 1915; Ayers, 1916; *et al.*). It was later realized that the factors controlling this final pH are numerous. It depends, according to Shunk and Wolf (1921), not only on the kind of organism, but also on (*a*) initial reaction of medium, (*b*) kind and concentration of acid or buffer used in adjusting the reaction of the medium, (*c*) kind and concentration of fermentable carbohydrate, (*d*) food accessories, and (*e*) physical state of the medium.

EFFECT OF THE REACTION OF THE MEDIUM ON THE FORMATION OF CERTAIN ANTIGENS, AND ON THE AGGLUTINABILITY OF ORGANISMS

A summary of the literature on this subject is given in table 1.

TABLE 1

A. Antigen formation

+ indicates that the specified reaction favors development of the antigen noted while − indicates retardation of its formation.

ORGANISM	REACTION			AUTHOR
	Acid	Neutral	Alkaline	
B. proteus		− (H-antigen of O-form); − (H-antigen of H-form on neutral agar containing sugar)	+ (H-antigen)	Schiff and Nathorff (1920)
B. proteus HX	− (H-antigen)		+ (H-antigen)	Olitzki (1929)
B. enteritidis		+ (H-antigen)		Olitzki (1929)
B. paratyphosus B		+ (H-antigen)		Olitzki (1929)
B. typhosus	+ (H-antigen)	− (H-antigen)	− (H-antigen)	Olitzki 1929
B. typhosus	+ (H-antigen)			Felix (1924)
B. aertrycke	− (X-antigen) (pH 5.0 to 5.5)		+ (X-antigen)	Happold (1929)

B. Mucous wall phenomenon

According to Olitzki (1929) the wall phenomenon of the paratyphoid group is dependent on the O-antigen and is an endoplasmic appearance which is suppressed by the presence of the H-substance.

ORGANISM	ACID REACTION	ALKALINE REACTION	AUTHOR
B. enteritidis (Breslau)	+ (in presence of NaCl)		Elkeles (1925–26)
B. paratyphosus B	− (if below pH 5.9)		Hohn and Becker (1927)
B. paratyphosus B		+ (in presence of lactates)	Knorr and Braun (1928)
B. enteritidis		+ (in presence of lactates)	Knorr and Braun (1928)
B. paratyphosus B		+	Brinck (1927)
B. enteritidis	+ (in extreme acidity)	+ (in extreme alkalinity)	Olitzki (1929)

TABLE 1—*Concluded*

C. Agglutinability

+, increased agglutinability on culturing in media of specified reaction; *, unchanged agglutinability on culturing in media of specified reaction; −, decreased agglutinability on culturing in media of specified reaction.

ORGANISM	ACID REACTION	ALKALINE REACTION	AUTHOR
B. typhosus		− (2 strains); * (4 strains)	Kirstein (1904)
		*	Hirschbruch (1906)
		− (9 out of 10 strains)	Riemer (1913)
B. paratyphosus A		−	Riemer (1913)
B. paratyphosus B		*	Riemer (1913)
B. typhosus	+	−	Capone (1919)
B. paratyphosus	+	−	Capone (1919)
B. dysenteriae	+	−	Capone (1919)
B. dysenteriae Flexner	−	+	Olitzki (1929)
B. dysenteriae Shiga	−	+	Olitzki (1929)
B. proteus		−	Schiff and Nathorff (1920)

EFFECT ON METABOLISM

a. General

In 1895 Blumenthal reported that the quantity of metabolic products of bacteria depends on the alkalinity of the medium, while the intensity of the decomposition is only slightly, if at all, affected by the addition of alkali. Lazarus (1909) concluded that the reaction of the medium modifies the availability of the nutrient material, and Bunker (1917) pointed out that the metabolic products of *B. diphtheriae* are influenced more significantly by pH than by titratable acidity.

Cole and Lloyd (1917) found that the importance of reaction depends to a large degree on the composition of the medium. In simple media it is of great importance. The actual optimum reaction may be a mean between the optimum for enzyme action and for synthesis, and may change as growth proceeds.

Alteration in pH frequently changes entirely the nature of the predominant reaction products (Wyeth, 1919; Wolf, 1920; Brooks, 1922; Virtanen and Bärlund, 1926; Arzberger, Peterson and Fred, 1920).

b. Effect on enzyme action

Table 2 gives a survey of results reported on this point. In our present state of knowledge it would be difficult completely to explain the general effect of the reaction of the medium on

TABLE 2

pH effect on enzyme action

ENZYME	ORGANISM	OPTIMUM REACTION, pH	MATERIAL ACTED UPON	AUTHOR
Protease	*B. prodigiosus*	7.2		Meyer (1911)
	Ps. pyocyanea	7.2		Spitzer and Parfitt (1930)
Protease, trypsin and erepsin	*B. ichythyosmius*	6.0–8.0	Skimmed milk and gelatin	Spitzer and Parfitt (1930)
Proteolytic (extracellular)	*B. subtilis*	6.0–7.0	Peptone and gelatin	Dernby (1921)
	B. pyocyaneus	6.0–7.0		Dernby (1921)
	B. proteus	6.0–7.0		Dernby (1921)
	B. prodigiosus	6.0–7.0		Dernby (1921)
	B. sporogenes	6.0–7.0		Dernby (1921)
	B. histolyticus	6.0–7.0		Dernby (1921)
Peptonase and protease	Pneumococcus	7.0–7.8		Avery and Cullen (1920)
Invertase	Pneumococcus	7.0		Avery and Cullen (1920)
Amylase	Pneumococcus			Avery and Cullen (1920)
Tryptic-like enzyme	*B. tuberculosis*	Alkaline	Protein	Corper and Sweany (1918)
Erepsin-like enzyme	*B. tuberculosis*	Acid	Peptone	Corper and Sweany (1918)
Pepsin-like enzyme	*B. tuberculosis*	Acid	Proteins	Corper and Sweany (1918)
Proteolytic enzyme	*B. proteus*	Alkaline		Jones (1916 and 1920)
Gelatinase	*B. proteus*	8.0–9.0		Merrill and Clark (1928)

bacterial metabolism from the point of view of the specific effect on enzyme activity, however attractive such an explanation may appear.

THE REACTION OF THE MEDIUM AS A FACTOR IN DISSOCIATION PHENOMENA

Reported results are summarized in table 3.

TABLE 3

Reactions observed as favorable to certain dissociations

ORGANISM	REACTION*		GROWTH MEDIUM	PLATING MEDIUM	AUTHOR
	$S \rightarrow R$	$R \rightarrow S$			
B. coli	Alkaline		Plain broth	Agar	Dulaney (1928)
B. coli	6.0–7.0		Beef heart infusion broth	1.5 per cent infusion agar	Nungester and Anderson (1931)
B. coli	6.7–6.8		Broth or agar	4.5 per cent bacto-nutrient agar with 1.5 per cent dextrose	Hunter (1931)
B. tumefaciens	6.0	7.0	Beef infusion	Beef infusion agar	Quirk (1931)
B. phytophthorus	6.0	7.0	Beef infusion	Beef infusion agar	Quirk (1931)
Cornborer parasite	6.0	7.0	Beef infusion	Beef infusion agar	Quirk (1931)
Streptococci	7.8–8.0	Neutral or sl. acid	Veal infusion	Alkaline agar	Dutton (1928)
Paratyphoid bacilli	Extreme acid		Broth	Buffered agar	Olitzki (1929)
B. subtilis	Neutral or slightly acid		Beef broth	Agar	Soule (1928)
B. dysenteriae	7.0 or above		5 per cent peptone solution	Agar slants	Koser and Styron (1930)
B. dysenteriae Shiga	7.3–7.4		Hottinger bouillon	Buffered agar	Truchina and Korolewa (1930)
M. leprae	Alkaline	Acid			Reed (1931)
B. leptisepticum	8.5		Peptone broth	10 per cent rabbit serum agar	De Kruif (1922)
Colon, typhoid, dysenteriae group and capsulated bacteria	7.8				Hadley (1927)
	(Rp S)	(Sp Rp)			
B. anthracis	6.3	Alkaline	Beef infusion	2 per cent agar, 7.7–7.8	Nungester (1929)

* Numbers represent pH values.

THE REACTION OF THE MEDIA AS A FACTOR IN CERTAIN SPECIFIC CHARACTERS OF BACTERIA

a. Morphology

Morphological observations are summarized in table 4.

TABLE 4

ORGANISM	ACID MEDIUM	ALKALINE MEDIUM	AUTHOR
B. coli		Filamentous	Schmidt (1892)
	Oval forms	Long rods	Escherich and Pfaundler (1903)
	Short forms		Adami, Abbott and Nicholson (1899)
	Widely pleomorphic		Hort (1917)
B. typhosus	Widely pleomorphic		Hort (1917)
B. diphtheriae	Large, irregular, solid staining	Minute, solid staining triangle forms of D2 type	Bunker (1917)
	Small, irregular with few granules	Minute triangle forms with few or no granules	Laybourn (1921)
		Coccoid forms	Yarisawa (1926)
Enterococcus	Streptococcus forms	Tetrads	Thiercelin and Jouhaud (1903)
Anaerobic bacilli		Giant forms	Hibler (1908)
B. influenzae	Long filaments, coccus, or variety of irregular forms (pH 6.5 and below)	Similar to acid forms but thicker, more waxy and irregular in staining (pH 8 to 8.5 and above)	Reed and Orr (1923)
B. tuberculosis	Thick, non-granular and short	Thin, granular and very long	Gieszczkyiewisz and Wroblewski (1927)
B. bifidus	Pleomorphic		Adam (1921)
Vibrio comma	Pleomorphic at extreme pH limits		Reed (1924)

b. Behavior toward dyes

The influence of pH both on staining reactions and on bacteriostasis has been extensively studied by Stearn and Stearn as well as by Churchman (1922), Burke (1922), Burke and Ashenfelter (1926), and others. This behavior has been discussed in detail by Stearn and Stearn (1928).

c. Motility

Table 5 summarizes observations on the effect of pH on motility.

TABLE 5

+ *denotes increased motility;* − *denotes decreased motility*

ORGANISM	ACID REACTION	ALKALINE REACTION	AUTHOR
B. prodigiosus	+		Schottelius and Wasserzug (1896)
B. dysenteriae		+	Mühlmann (1909)
B. typhosus	− (below pH 6 to 4.5)	− (above pH 8.0 to 9.0)	Reed and MacLeod (1924)
Ps. pyocyanea	− (below pH 6 to 4.5)	− (above pH 8, slight motility to pH 10)	Reed and MacLeod (1924)
B. paratyphosus B		− (pH 7.8 to 8 on media containing lactates) (loss of flagella)	Knorr and Braun (1928)
B. enteritidis		− (pH 7.8 to 8 on media containing lactates) (loss of flagella)	Knorr and Braun (1928)
Paratyphoid bacilli		Non-motile at extreme alkalinity	Olitzki (1929)

d. Spore formation

Table 6 summarizes the reports of observations on the effect of the reaction of the medium on spore formation.

e. Chromogenicity

The effect of the reaction of the medium on chromogenicity of *B. mallei* was noted by Smith (1890), on that of *Ps. pyocyanea* by Sullivan (1905), on that of *B. luteus* by Garbowski (1907), and on that of *B. prodigiosus* by Amako (1930).

f. Luminescence

B. giardi (Kruse) produces light when grown on peptone media made up with sea water when the pH is about 8.1. If the pH is not proper there will be no luminescence (Inman, 1927). Luminescent bacteria grow and luminesce best on alkaline media, pH 8 (Hill and Shoup, 1929).

g. Fluorescence

Georgia and Poe (1932) found the best pigment production by *Ps. fluorescens* or closely allied species in 0.5 per cent peptone broth at pH's between 6.8 and 7.3. Its development was retarded at pH 6.1 to 6.5 but appeared by the third day of incubation.

TABLE 6

Showing the influence of reaction on spore formation

ORGANISM	REACTION, FAVORABLE	REACTION, INHIBITORY	AUTHOR
Butyric acid bacillus	Alkaline		Grassberger and Schattenfroh (1900)
B. Welchii		Acid	Noguchi (1907)
B. Welchii	From 6.8 to alkaline limits of growth	More acid than pH 6.6	Torrey, Kahn, and Salinger (1930)
B. luteus—sporogenes	Alkaline	Acid	Garbowski (1907)
B. amylobacter	Alkaline		Bredeman (1909)
B. subtilis	pH 5 to 10		Itano and Neill (1919)
Cl. botulinum	Neutral or slightly acid, optimum about pH 6.2	Below 6.0	Leifson (1931)
Cl. tetani	Neutral or slightly acid; optimum about pH 6.2		Leifson (1931)
B. vulgatus	Neutral or slightly acid; optimum about pH 6.2	8–8.4	Leifson (1931)
B. circulans	Neutral or slightly acid; optimum about pH 6.2		Leifson (1931)

h. Viscosity of organisms

Marked increases in viscosity occur in pH zones 3 to 4 and 13 to 13.5, where bacteria show acid and alkali agglutination markedly (Falk and Harrison, 1926). Nungester (1929) found that agar at pH 7 was unfavorable for the development of the mucoid property of Rm and Sm types of *B. anthracis*. Agar of pH 7.8 was favorable.

i. Virulence

Petroff (1929) noted loss of virulence when the tubercle bacillus was cultured on veal broth with an acid reaction. Amoss (1925) grew an avirulent strain of type I pneumococcus by plating cultures from a single cell which had grown on repeated transfers in beef infusion broth at pH 6.8. Felton and Dougherty (1924) found that pneumococci lost their virulence "in direct proportion to the acidity of the culture media."

j. Production of toxin

Table 7 indicates that toxin production in the cases of the organisms studied is greatest in alkaline media.

TABLE 7

ORGANISM	REACTION FAVORABLE FOR TOXIN PRODUCTION	AUTHOR
B. botulinus	Alkaline	Van Ermengen (1897)
	Alkaline	Dickson (1915)
B. tetanus	pH 8 (initial)	Dernby and Allander (1921)
B. diphtheriae	pH 7.8 to 8.2 (final)	Bunker (1919)
	Alkaline (if not above pH 8.3)	Heeren (1930)
	pH 7.1 and above	Hazen and Heller (1932)
	pH 7.5 to 8.9	Hartley and Hartley (1922)
B. dysenteriae Shiga	pH 7.5 (for 24 hours)	Jonesco-Mihaesti and Popesco (1922)

EXPERIMENTAL

Two methods were employed to test the effect of the reaction of the medium on bacterial behavior. One was based on Quirk's technic (1931) in which three factors, dilution before and after the young growth period, the age of the culture, and the pH of the medium were controlled. Young cultures were used. Unbuffered beef infusion broth and 1.5 per cent agar were employed. Beef extract broth and agar were found to give results similar to those obtained with the above named media.

The media were adjusted to desired pH's before sterilization. After incubating for twenty-four and forty-eight hours, these

values were determined electrometrically. Little change in pH of the unbuffered broth was noted thereafter, for in tubes of broth kept at room temperature for eight days the greatest change noted was 0.1 pH.

The second method consisted of continued subculturing over an extended period of time in M/15 phosphate-buffered beef infusion broth, transplanting to agar plates at intervals.

Cultures

The organisms used for this study were: *Bacillus coli-communior, Salmonella enteritidis* and *Pseudomonas pyocyanea,* obtained from the Northwestern University Medical School, and *Bacillus subtilis* and *Bacillus cereus,* obtained from the American Type Culture Collection. The work on the last two organisms will be reported in succeeding papers.

Before starting the experimental work the organisms were planted to neutral nutrient broth, incubated for a few hours and streaked to agar plates. From these a colony was selected, planted again in broth, and the process repeated until both the appearance of the broth and microscopic examination of the plates and smears gave assurance of rapid growth and homogeneity of the culture.

Experiment 1 (changes produced by varying pH of medium). Fifteen-hour agar cultures were transferred to unbuffered beef infusion broth adjusted to pH's ranging from 5.15 to 8.2. The tubes were allowed to stand for one-half hour at room temperature, after which one loopful from each tube was transferred to another tube of corresponding pH. This second series of tubes was allowed to remain at room temperature for eighteen to twenty-four hours, after which two transfers, as in the first series, were again made. Since often no growth was obtained if transfer to agar was made at once, the last tubes were allowed to stand at room temperature for eighteen to twenty-four hours. From these tubes 1.5 per cent infusion agar plates were streaked, or a transfer was made to agar tubes of corresponding pH's for poured plate examination. These were incubated at room temperature.

Results with Bacillus coli-communior

a. Appearance of colony:

pH range	*Colony appearance*
5.15-5.60	Metallic, opaque, compact, granular (fig. 1), the thin wall surrounding the colony is iridescent; some rugose with secondary colonies (fig. 2)
5.8 - 6.1	Intermediate forms (fig. 3)
6.1 -7.6	Familiar form, glistening, moist, easily removed (fig. 4)
7.8 -8.4	Thin, with spreading grape-leaf structure, viscid, irregular

Above pH 7.1 the colonies become increasingly irregular in shape and more spreading. At pH 8.2 to 8.4 they are very thin and translucent (fig. 5). Any of the above described colonies, except those from the tubes at pH 8.2 to 8.4, when transferred to neutral broth gave the same type of growth. The 8.2 colonies gave a viscous, stringy growth.

b. Appearance of broth cultures:

pH range	*Culture appearance*
5.15-5.80	Granular sedimentary growth; easily dispersed sediment; pellicle forms within three to seven days
5.95-6.6	Cloudy, compact sediment; time for pellicle formation varies from twenty-six to forty-eight hours
6.6 -7.9	Turbid, flaky sediment; pellicle formation after twenty-four hours
8.2 -8.4	Cloudy, with gelatinous stringy growth and sediment; no pellicle forms within nine days

If the tubes at the lower pH's are allowed to stand for a week at room temperature their appearance in general tends to approach that of tubes at higher pH's. None, however, give the stringy growth obtained at the upper pH limits, 8.2 to 8.4, even after standing.

c. Appearance of agar slants (fig. 6):

pH	*Appearance*
5.2	Metallic, mealy, opaque, moist, with slight iridescence at the edges of growth at the bottom of the tube
6.0	Intermediate
7.0	Glistening, grayish-yellow, smooth
8.4	Glistening, yellowish-white, smooth, translucent, lobate

d. Appearance of suspensions in physiological salt solution. Growth of the organism from twenty-four hour agar cultures at the pH's designated gave the following results when emulsified in 0.85 per cent sodium chloride solution. Through the pH range, 5.15 to 5.8, the organism formed granular and flaky suspensions. Through the range, 6.0 to 8.0, homogeneous suspensions were formed.

e. Morphology. The following results were obtained from eighteen-to twenty-four hour broth cultures.

pH range	*Description*
5.15–5.60	Variation in size and shape from coccoid to very long rods; chains and clumps of organisms quite common
5.9 –7.4	Normal size; short and plump rods which appear singly
7.6 –8.4	Varying from coccoid to long rods

On agar, below pH 6 and above pH 7.8 the size varies from very short to long rods, though not as markedly as in broth.

f. Motility. At and below pH 5.6, motility is often absent, and non-motile chains and clumps are seen if broth cultures ranging from 5.15 to 5.6 in pH are observed during incubation periods of sixteen to thirty hours at room temperature. At all pH's from 5.7 to 8.0 motility was observed during that period. The motility seemed greatest in sixteen- and thirty-hour cultures at pH's of 7.8 and 7.9.

On agar slants kept at 37°, motility was observed at pH's ranging from 5.4 to 8.0. At room temperature the agar culture at pH 5.6 showed no motility. When transferred to neutral broth tubes all strains showed motility, though those from tubes of pH below 5.6 had many non-motile forms.

g. Cultural characters. In this study cultures at pH's 5.5, 6.8 and 8.0 were used. Needle transfers were made into the culture media from twelve-hour broth cultures, eight-hour and twenty-four hour agar cultures, and all were incubated at 37°C.

No differences were noted, with one exception, in the fermentation of glucose, maltose, lactose, sucrose and mannitol, acid and gas being formed. In the strain at pH 5.5 gas production was delayed, being negligible and sometimes entirely absent in maltose broth even when incubated for ninety-six hours.

Using 0.2 per cent soluble starch in broth, a test for erythrodextrin was obtained in the cases of cultures at pH's 6.8 and 8.0, but no test was obtained from the pH 5.5 culture during an eleven-day incubation period at 37°.

Gelatin was not liquified.

No differences were observed in the growth on potato slants.

Results with Salmonella enteritidis

a. Appearance of colony:

pH range	*Colony appearance*
5.15–6.0	Oqaque, discrete, granular, iridescent, with raised centers which flatten and appear lysed and in which secondary colonies form (fig. 7), with toothed margins
6.1 –6.3	Circular, glistening, white, moist, smooth and entire (fig. 8)
6.3 –7.6	Normal translucent colony form (fig. 7)
7.8 –8.4	Very thin, spreading, irregular, translucent, viscid (fig. 10)
8.2 –8.4	Very thin, translucent and spreading

Agar cultures transferred to broth all appear the same, except that the cultures at the extreme pH limits give granular sediments.

b. Appearance of broth cultures (twenty-four to thirty hours at room temperature):

pH range	*Appearance*
5.15–5.40	Finely granular sediment; broth becomes clear
5.45–5.8	Granular and some flocculating sediment; a thin pellicle may form after forty-eight hours
5.95–8.0	Turbid, with thin pellicle and sediment
8.2 –8.4	Turbid, with gelatinous sediment, no pellicle, but a small ring forms

c. Agar slants:

pH range	*Description*
5.2	Metallic, dull, echinulate (fig. 11)
6.0	Intermediate
7.0 and 8.4	Smooth, glistening, undulate, surrounded by a thin wall (fig. 12)

d. Suspensions of agar slant cultures in physiological salt. The cultures at pH 5.15 to 5.6 gave granular suspensions. These, however, when once dispersed, did not flocculate.

The cultures through the pH range 5.8 to 8.0 gave homogeneous suspensions.

e. Morphology. Broth cultures in the pH range 5.15 to 5.6 show, during the first twenty-four hour incubation period, chains and clumps. The organisms vary greatly in size from coccoid forms to long curved rods. Agar cultures in the same pH range give organisms similar in size to these, and staining deeply with safranin. At pH's 5.6 to 7.0 in both broth and agar cultures the organisms are generally short and fat with marked bipolar staining. In the pH range 7.0 to 8.4 there is an increasing tendency to form evenly-staining and longer, more slender rods, which also tend to be Gram amphophyle.

f. Motility (sixteen to thirty hours at room temperature). Broth cultures below pH 6 show decided loss of motility. Occasionally a few motile cells are seen. When subcultured to neutral broth, motility is regained. From pH 6 to 8 the broth cultures are motile. Agar cultures at corresponding pH's give results similar to the broth cultures except that subcultures from agar at pH's 7.8 to 8.0 to neutral broth remain non-motile.

g. Cultural characters. The method of study here was the same as in the case of the *B. coli-communior.*

Four cultures at pH's 5.5, 6.8, 7.3 and 8.0 all gave the same results in this study which were as follows:

Acid and gas were produced from glucose, maltose and mannitol, but there was no fermentation of lactose or sucrose.

Gelatin was not liquified.

The culture at pH 5.5 did not hydrolyze starch, but erythrodextrin was produced by the other cultures.

No differences were observed on potato slants—the growth being abundant, moist and yellowish-brown.

Results with Pseudomonas pyocyanea

a. Appearance of colony:

pH range	*Description*
5.15–5.6	Metallic, opaque, granular, moist, with purple and green iridescence but no green pigment, lytic area but no secondary colony formation
5.8 –6.0	Intermediate forms showing plaques of green pigment and diffusion into agar
6.1 –7.9	Large, spreading, glistening, with translucent edge and diffusion of green pigment
8.0 –8.2	Very thin, translucent, spreading, with deep green pigment

All the agar cultures when transferred to neutral broth gave the same kind of growth.

b. Appearance of broth cultures:

pH range	*Description*
5.15–5.60	Cloudy and somewhat granular, sedimentary, with surface flakes, remaining colorless during six days at room temperature
5.7 –6.6	Cloudy, pale green at top, pellicle within twenty-six hours. (Tubes at pH as high as 6.13 did not form green pigment though kept for two weeks. At the end of this period they were very alkaline)
6.8 –8.2	Markedly turbid and deep green, with thick pellicle and heavy viscous sediment

If broth cultures in the pH range 5.15 to 5.60 are streaked to neutral agar the colonies have raised metallic or white centers with spreading edges. No pigment is formed during incubation for a week to ten days.

c. Agar slants:

pH	*Description*
5.2	Grayish-white or metallic, slightly raised irregular margins, purple-green fluorescence but no green pigment diffusing into the medium
6.0	Intermediate, with decided fluorescence but negligible pigment formation
7.0 and 8.4	Usual even yellowish layer, confluent and glistening, with the medium turning green

d. Suspension in physiological salt. The cultures at pH 5.2 to 5.8 give granular and flaky suspensions which, if once dispersed, do not flocculate.

Those at pH 6.0 to 8.0 give homogeneous suspensions. The alkaline strains flocculate in physiological salt.

e. Morphology. In twenty-hour broth cultures at pH's 5.15 to 6.0, chains and clumps form. The rods vary greatly in size appearing about twice the normal diameter and as long as two or three normal rods. They stain deeply with safranin. In the pH range 6.0 to 7.6 one finds slender, delicate rods. When agar cultures are examined, those below pH 6 show the same increase in size noted above. After transfer to neutral media no morphological differences are observed.

f. Motility. At pH's 5.15, 5.38 and 5.45 broth cultures show either no motility or only a few motile organisms during a twenty-hour incubation period. Subsequently, apparently when some adjustment of the reaction has taken place, the cultures show distinct motility. During the first twenty-four hours agar cultures show no motility up to pH 5.6. When transplanted to neutral broth these cultures show a large percentage of motile organisms. All the cultures when so treated showed good motility even up to pH 8.

g. Cultural characters. Using the same methods as in the cases of the other organisms the following results were noted:

All of the cultures (pH's 5.4, 5.5, 6.8, 7.3 and 8.4) produced a small amount of acid from glucose, but maltose, lactose, sucrose and mannitol were not attacked. Starch was not hydrolyzed. Gelatin was liquified more rapidly by the cultures at pH 7.3 and 8.4, the fluid becoming green in all of them. Growth on potato slants was luxuriant, with the formation of a brownish pigment.

Experiment 2 (a study of the degree of permanence of changes produced by prolonged cultivation at different pH values). Starting with the same original culture of *B. coli* and *B. enteritidis*, transplants were made every twenty-four hours from one buffered infusion-broth tube to another of the same pH. The broth was buffered by means of phosphate mixtures of total phosphate concentration M/15. Thirty-six consecutive transfers were made. The study was limited to the pH's 5.6 to 5.8, 6.8 to 7.0 and 7.8 to 8.0. The culture obtained from broth kept at pH 5.6 to 5.8 will be hereafter referred to as the acid strain, the one from the 6.8 to 7.0 tubes, as the neutral strain and the other as the alkaline strain. In all cases incubation was at 37°C.

Changes observed in the broth cultures and agar plates duplicated those reported under experiment 1, and are therefore not again described. Those reported below are for cultures after 36 transplants.

Results with Bacillus coli-communior

All three strains, i.e. the acid, the neutral and the alkaline, produce the same colony type when streaked to neutral agar.

In neutral broth the alkaline strain shows a greater tendency to form flakes, and early pellicle formation, but otherwise the strains appear alike. Motility and morphology are the same on neutral media. Physiological salt suspensions of twenty-four-hour neutral agar cultures are homogeneous for the acid and neutral strains, the alkaline strain flocculating during twenty-four hours.

Staining and sensitivity to gentian violet. Broth cultures were streaked to neutral agar, twenty-four and forty-eight-hour cultures being used. When the three strains are smeared on the same slide and stained by the Gram technic the acid strain always

TABLE 8

Sensitivity to gentian violet in beef extract broth

DYE DILUTION	ACID STRAIN			NEUTRAL STRAIN			ALKALINE STRAIN		
	pH 5.2	pH 6.2	pH 7.7	pH 5.2	pH 6.2	pH 7.7	pH 5.2	pH 6.2	pH 7.7
1:10,000	+	−	−	−	−	−	−	−	−
1:20,000	+	−	−	+	−	−	+	−	−
1:40,000	+	+	−	+	+	−	+	+	−

Sensitivity to gentian violet on 2 per cent meat infusion agar plates

DYE DILUTION	ACID STRAIN	NEUTRAL STRAIN	ALKALINE STRAIN
1: 8,750	+	+	−
1:17,500	+	+	−
1:50,000	+	+	±

stains most deeply with safranin. This led to an investigation of isoelectric ranges. The method of Tolstoouhov (1929) was used. The acid strain was pink at pH 3.3 and blue at 3.7; the neutral and alkaline strains were pink at pH 2.7 and blue at pH 3.0, showing a distinct shift in the isoelectric range.

The change in sensitivity to gentian violet was studied by inoculating one loopful of broth cultures into buffered gentian violet broth, or streaking to plates. Table 8 gives results from twenty- to twenty-four-hour cultures.

Any changes in sensitivity noted were at once lost when the organisms were first transplanted to neutral media and then inoculated into the gentian violet media.

Results with Salmonella enteritidis

All three strains when transferred to neutral agar produce the same colony type, but that from the alkaline strain is more spreading and translucent. In neutral broth all three strains appear about the same but the alkaline strain is more flocculent. Motility in the acid and alkaline strains is not restored on the first subculture to neutral media. All three give homogeneous suspensions in physiological salt solution.

Staining and sensitivity to gentian violet. If smears of the three strains are made on the same slide and stained by the Gram technic, the acid strain stains deeply with safranin, the neutral strain less so, while the alkaline strain is Gram amphophyle. In determining the isoelectric ranges of the strains it was noted that the acid strain was pink at pH 1.8 and blue at pH 4.3, while the other two strains were violet at pH 3.0 and blue at pH 4.3.

To test for changes in sensitivity to gentian violet, the same procedure as in the case of the *B. coli* was followed. In the range of dye dilutions studied, the broth cultures showed no differences in sensitivity. On agar, the differences in the dye dilutions studied (1:100,000 to 1:1,000,000) were negligible, and disappeared if a slightly older culture was used or if the strains were first transplanted to neutral broth.

DISCUSSION

As pointed out by Kligler (1917) peptone has two precipitating zones. The acid precipitate, formed between pH 5.0 and 5.4, is organic, and when redissolved gives reactions characteristic of peptones and proteoses. The alkaline precipitate, formed between pH's 8.2 and 9.0, appears to consist largely of phosphates with some organic constituents. The concentration of available food in the present study is thus seen to vary, especially at the pH limits covered. The precipitate formed in broth during its preparation was removed in most of these studies.

Another factor should be pointed out. In 1917 Redfield noted that the acidity of the agar, before being used in the preparation of media, was inversely proportional to its jelly strength. He stated that the appearance of colonies was in no way affected

by the jelly strength of the agar media for the four substances tested.

Nungester (1929) observed that differences of 10 units in hardness of nutrient agar affected the colony form of the Rs type of *B. anthracis.*

Agar, at pH's below 6, is decidedly less hard than it is at higher pH's. In interpreting the results here presented these factors must be taken into consideration. It may be that iridescence of colonies, observed in at least six different organisms, may depend not only on the degree of acidity but also on the hardness of the agar.

Since, in a final paper of this series, certain theoretical considerations will be presented with a general discussion not only of these results but also of analogous ones obtained with other organisms, it seems expedient to limit the present discussion to a few comments.

Various strains of *B. coli,* for example, have been reported as possessing different pH growth limits. Reference to the table of results given by Buchanan and Fulmer (1930) reveals variations in the lower pH limit of growth ranging from 4.3 to 5.0, and in the upper limit from 7.8 to 9.6. Several optimum ranges of growth are given in the literature for various strains of this organism which have been studied, as, for example, 5.2 to 8.42, 6.0 to 7.0, 5.7 to 7.0. The same table reveals that two strains of *Pseudomonas pyocyanea* show differences in their pH growth range, the one being reported as having the range 5.6 to 7.0, and the other the range 4.4 to 8.8.

Divergences such as these must be taken into account before generalizations can be made as to the effect of pH on bacterial characters. Not only does the pH range of growth depend on environmental factors but also on the constitution of the organism as determined by its previous history. The pH at which differences in cultural appearance are observed is thus not likely to be a fixed one for any given type.

The organisms used in this study grew readily through the specified pH range. The changes observed cannot be dismissed as due to retarded growth.

SUMMARY

1. The appearance of growth of *Bacillus coli-communior Salmonella enteritidis* and *Pseudomonas pyocyanea,* in broth and on agar is decidedly different at different pH's.

2. Though many of the changes noted are no longer observed when the strains are subcultured to a neutral medium, there are some which seem to denote more than merely a temporary adaptation.

3. The scattered observations in the literature on the effect of the reaction of the medium on the characteristics of bacteria are systematized.

4. The nature of the changes observed will be discussed in forthcoming papers.

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PLATE 1

FIG. 1. *B. coli*, after twenty-four hours at 37° and twenty-four hours at room temperature on beef infusion 1.5 per cent agar of pH 5.15 (colonies iridescent changing to a bronze-like appearance; depressed areas appear lysed). 4×.

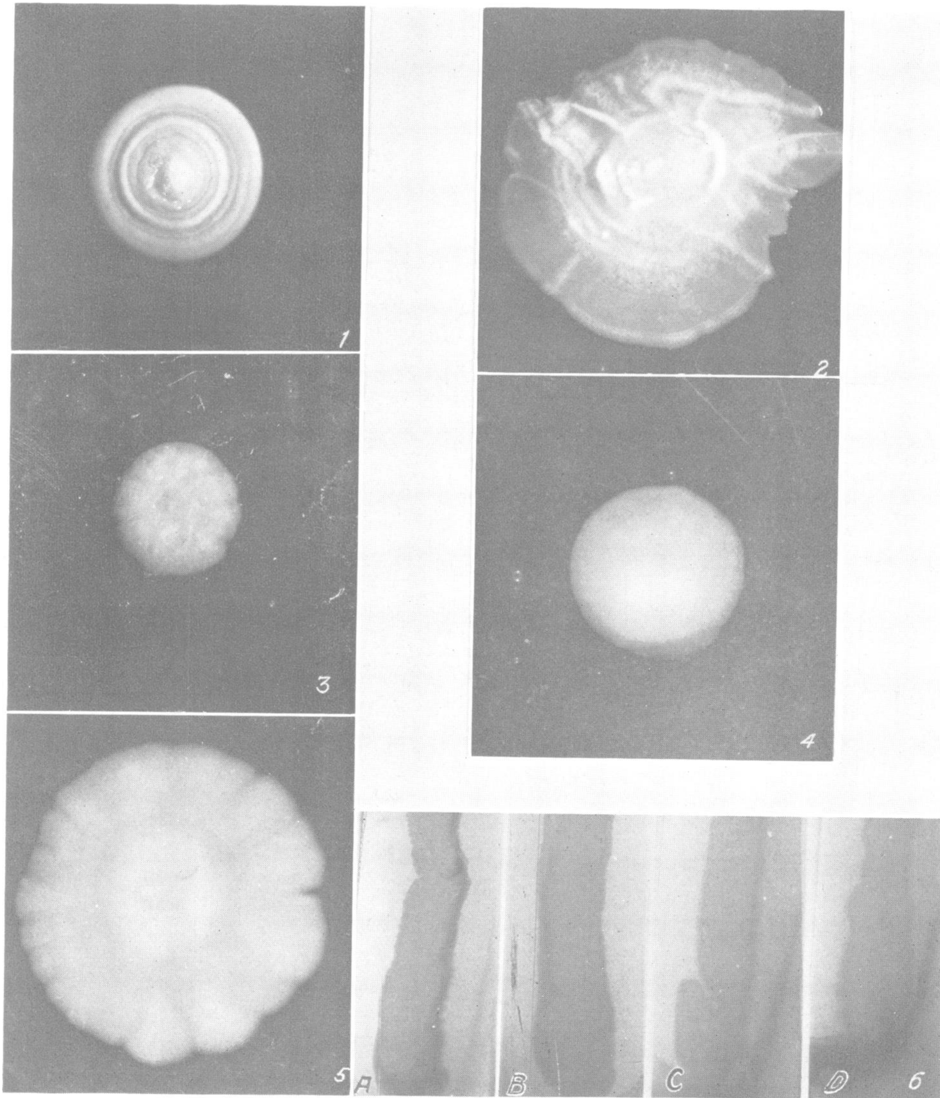
FIG. 2. *B. coli*, after twenty-four hours at 37° and twenty-four hours at room temperature on beef infusion 1.5 per cent agar of pH 5.5. 4×.

FIG. 3. *B. coli*, after twenty-four hours at 37° and twenty-four hours at room temperature on beef infusion 1.5 per cent agar of pH 5.95. 4×.

FIG. 4. *B. coli*, after twenty-four hours at 37° and twenty-four hours at room temperature on beef infusion 1.5 per cent agar at pH 6.8. 4×.

FIG. 5. *B. coli*, after five days at room temperature on beef infusion 1.5 per cent agar of pH 7.8. 4×.

FIG. 6. *B. coli*, after forty-eight hours at 37° on agar slants. Tube A, pH 5.2. Tube B, pH 6.0. Tube C, pH 7.0. Tube D, pH 8.4.



(Esther Wagner Stearn and Allen E. Stearn: Effect of reaction of medium on bacteria.)

PLATE 2

FIG. 7. *Salmonella enteritidis*, after twenty-four hours at 37° and twenty-four hours at room temperature on 1.5 per cent nutrient agar of pH 5.15. 4×.

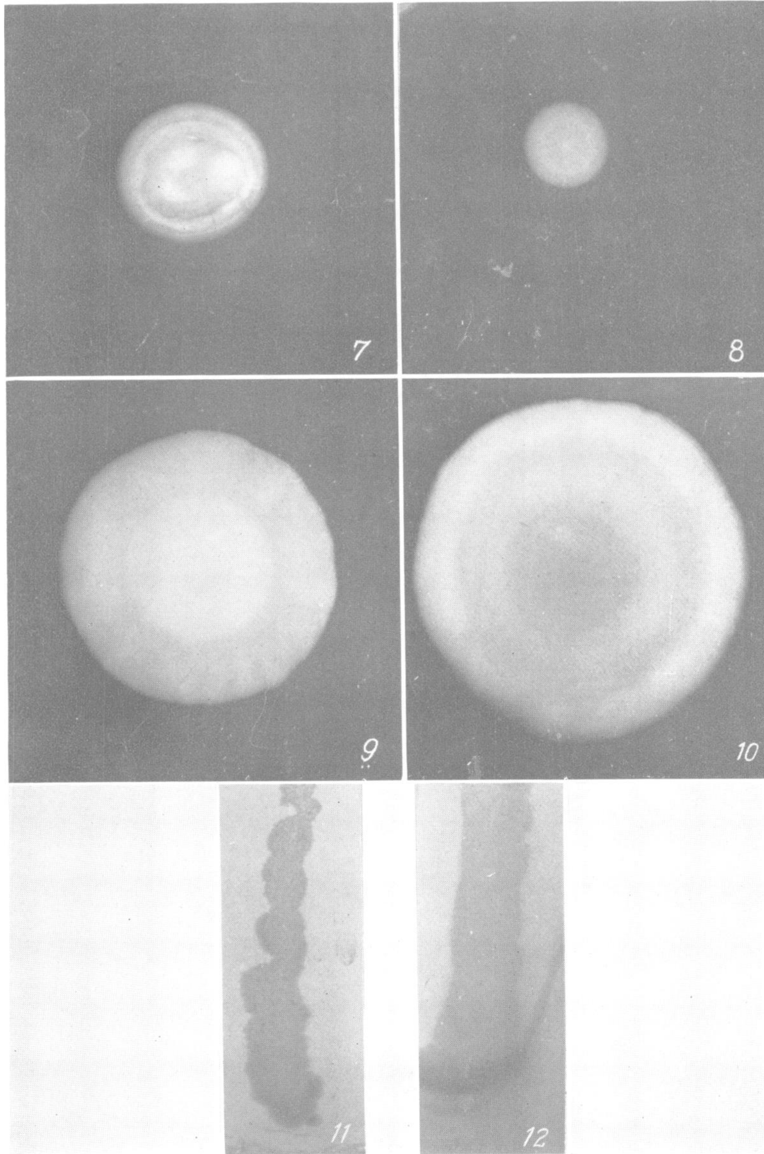
FIG. 8. *Salmonella enteritidis*, after twenty-four hours at 37° and twenty-four hours at room temperature on 1.5 per cent nutrient agar of pH 5.95. 4×.

FIG. 9. *Salmonella enteritidis*, after five days at room temperature on 1.5 per cent nutrient agar of pH 6.8. 4×.

FIG. 10. *Salmonella enteritidis*, after five days at room temperature on 1.5 per cent nutrient agar of pH 7.9. 4×.

FIG. 11. *Salmonella enteritidis*, after forty-eight hours at 37° on 1.5 per cent nutrient agar of pH 5.2. 1×.

FIG. 12. *Salmonella enteritidis*, after forty-eight hours at 37° on 1.5 per cent nutrient agar of pH 8.4. 1×.



(Esther Wagner Stearn and Allen E. Stearn: Effect of reaction of medium on bacteria.)