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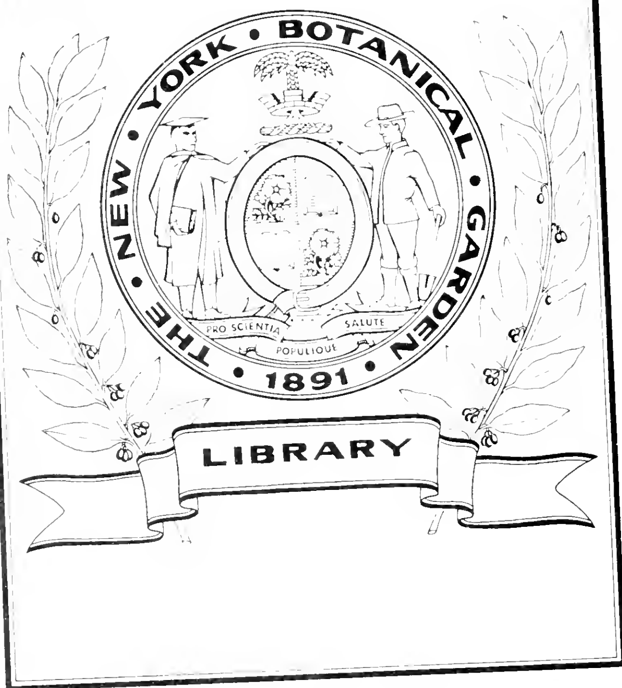

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EDITOR-IN-CHIEF
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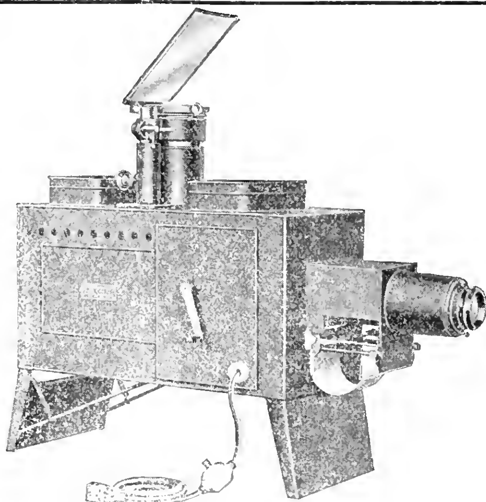

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STUDIES IN THE METABOLISM OF ACTINOMYCETES

III. NITROGEN METABOLISM

SELMAN A. WAKSMAN

Department of Soil Bacteriology, New Jersey Agricultural Experiment Station

Received for publication, July 11, 1919

The utilization of different nitrogenous compounds by actinomycetes and the transformation of these substances due to the action of the organisms will be taken up in the present paper.

Rullman (1899) stated that *Actinomyces odorifer* cannot nitrify. Beijerinck (1900) observed the reduction of nitrates to nitrites by actinomycetes. Nadson (1903) found that organisms of this type from mineral mud are able to decompose proteins very readily with the liberation of ammonia and hydrogen sulfide. Macé (1905) noted that *Cladothrix chromogena* can split blood serum, with the production of ammonia, propeptone and tyrosin crystals. Fousek (1912) found that the actinomycetes isolated from the soil assimilate nitrate, ammonia and amino-nitrogen and reduce nitrates to nitrites, but cannot assimilate atmospheric nitrogen. Münter (1912) studied seven actinomycetes isolated from the soil; scant growth or no growth at all was obtained on nitrogen free media; all organisms were able to utilize equally well nitrogen in the form of nitrates, ammonia and asparagin; hemialbumin, casein, asparagin and alanin were utilized readily and tyrosin to a smaller extent, both as sources of carbon and nitrogen; urea, thiourea and dicyanamide gave no growth at all when used as a source of both carbon and nitrogen, but were used by some organisms as sources of nitrogen. Münter (1914) has further shown that casein is ammonified well, glue and peptone to a smaller extent, and urea only very slightly; ammonia formed a good source of nitrogen, only small quantities of nitrates being produced from this substance and

no free nitrogen given off; no reduction of nitrates to ammonia could be demonstrated. Krainsky (1914) stated that the actinomycetes assimilate both organic and inorganic nitrogen; of the organic substances, the proteins and amino bodies could serve not only as sources of nitrogen, but also of carbon; casein and peptone were split by all species to ammonia. All species, with one exception, grew readily upon a solution of gelatin in water (with the addition of 0.05 per cent K_2HPO_4), *A. flavochromogenus* forming an insoluble gelatin compound. The actinomycetes assimilated ammonia, nitrite and nitrate nitrogen compounds; NH_4Cl was not always favorable, particularly in the presence of glucose. Nitrates were reduced to nitrites by most actinomycetes, this varying with the composition of the medium; with many species, the nitrite formation was absent, due to the fact that this phenomenon is so slow that all the nitrites formed are at once assimilated by the cells; in no instance could ammonia be demonstrated as a reduction product of nitrates and nitrites; *A. flavus* alone could not reduce nitrates. No urease could be demonstrated, while casein was split by means of a proteolytic enzyme.

Emerson (1917) stated that since Actinomyces colonies were formed on nitrogen free media, these organisms are able to assimilate free nitrogen. This last fact could in no instance be confirmed by the writer. The development of Actinomyces colonies on nitrogen-free agar media is due to the fact that many of these organisms can develop readily with mere traces of nitrogen, which are probably present as impurities in the agar or as traces in the laboratory air.

Waksman and Curtis (1916) found that nearly all the actinomycetes studied could liquefy gelatin, varying in degree of rapidity of liquefaction, but they were found to be rather weak as ammonifying organisms. Lutman and Cunningham (1914) have shown that the ammonia production of *A. scabies* is small compared with that of bacteria under similar circumstances.

EXPERIMENTAL

Since glycerol proved to be a very favorable source of energy (see paper II of series), it was used in the following experiments. To each liter of medium containing the following ingredients: 30 grams glycerol, 1 gram K_2HPO_4, 0.5 gram KCl, 0.5 gram $MgSO_4$, 0.01 gram $FeSO_4$, were added fibrin, casein, powdered egg-albumin, Witte peptone, asparagin, leucin, glycocoll, or urea, 5 grams each; $NaNO_3$, $NaNO_2$, $(NH_4)_2SO_4$ or $(NH_4)_2CO_3$, 2 grams each. The casein and egg-albumin were first dissolved in a dilute $NaOH(\frac{N}{10})$ solution, then added to the medium; the fibrin was added in small pieces to the individual tubes. The media were mixed, tubed, 10 to 12 cc. to each tube, and sterilized at 15 pounds pressure for fifteen minutes. Several tubes from each medium were inoculated with each of a series of representative Actinomyces and incubated at 25° for a period of fifteen to sixty days.

Growth is designated by figures as follows: 0—none, 1—scant, 2—fair, 3—good, 4—very good, 5—excellent. In studying the figures for growth one should keep in mind that they are only relative and 2 does not designate twice as much growth as 1 or half as much as 4. The scantest growth, even if only a few tiny flakes at the bottom of the tube or a few minute masses floating on the surface or distributed through the medium, was designated as 1. The most abundant growth was designated by 5, while the other figures fall between. All the cultures were compared on the same basis considering the set as a whole and not each organism separately. In describing the aerial mycelium the signs, + and + + were used, when it was present, the first to designate a thin powdery layer and the second a heavy, usually cottony cover. The ammonia was not determined quantitatively in this experiment, but merely qualitatively, by means of Nessler's reagent. The amino-nitrogen was determined by means of the micro-apparatus of Van Slyke. The hydrogen-ion concentration was obtained by means of the phenol-sulphon-phthalein series of indicators suggested by Clark and Lubs (1917); this was designated by the terminology of Sörensen, using the pH values.

TABLE 1

*The utilization of different nitrogen compounds by actinomycetes (3 per cent glycerol as a source of carbon)**

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
Fibrin														
Growth†	0	43	44	33(23)	32	23	43	13	43	22	22	31	44(12)	2.75
	0	43	44	44	43	25	43	43	45(13)	22	23	31	44(23)	3.33
Aerial mycelium‡	0	+	+	+	0	0	+	+	+	0	0	+	+	
	0	+	+	+	0	0	+	++	++	0	0	+	+	
Soluble pigment§	0	r	y	0	b	b	0	b	b-sh	0	f-y	0	b-sh	
	0	r	y	y-sh	d-b	d-b	y-sh	b	y-sh	0	f-y	0	b	
NH_2-N, milligrams in 10 cc.	0.15	1.14	1.54	0.09	0.11	0.40	–	0.42	1.14	2.14	4.71	0.18	1.37	
	0.15	–	2.85	0.29	1.22	0.85	0.72	4.20	0.30	4.56	–	0.31	2.28	
NH_3-N¶	0	0	0	1	0	1	0	0	1	1	1	1	0	
	0	0	1	2	0	3	1	0	0	1	2	1	0	
Casein														
Growth	0	43	45	44	23	13	22	34	42(13)	22	43	31(22)	–	3.1
	0	44	45	45	24	14	43	45	42(23)	23	44	31(22)	–	3.7
Aerial mycelium	0	+	++	+	0	+	0	+	+	0	++	+	–	
	0	++	++	++	0	+	0	++	++	0	++	+	–	
Soluble pigment	0	r	g	b	b	b	0	b	b	0	y	0		
	0	r	g	b	b	d-b	y-sh	d-b	b	0	y	0	–	

NH_2-N, milligrams in 10 cc.	0.45	1.37 0.60	1.37 0.63	0.24 0.94	0.12 1.10	1.06 1.54	0.42 0.66	0.56 0.97	1.42 2.20	2.14 3.08	1.57 2.85	0.36 0.60	– –	
NH_3-N	0 0	0 0	0 0	0 1	0 0	1 3	0 0	0 0	0 1	1 2	1 1	1 2		
Egg-albumin														
Growth	0 0	32(22) 22	44(24) 44	44(14) 45	21 23	22 23	43 44(24)	33 –	42(22) 44(22)	22 22	13 14	23(31) 23(31)	21 –	2.5 3.4
Aerial mycelium	0 0	+ +	+ +	++ ++	0 0	0 0	+ +	+ –	+ ++	0 0	+ +	+ +	0 –	
Soluble pigment	0 0	p-b	0 y-sh	0 b-sh	b d-b	b b	0 0	b –	b b	0 0	y-sh y-sh	0 0	0 –	
NH_2-N, milligrams in 10 cc.	0.15	0.91 0.85	0.62 0.62	0.22 0.36	0.29 0.72	0.42 0.96	0.45 0.26	0.30	0.68 0.68	1.23 1.26	1.80 2.65	0.34 0.62	0.38 –	
NH_3-N	0 0	0 0	0 0	0 0	0 0	1 2	0 0	0 –	1 0	0 0	2 1	0 1	0 –	
Witte peptone														
Growth	0 0	33 44	45 45	34 44(15)	42 44	23 24	22 44(23)	34 44	42(23) 42(24)	23 23	22 22	32(22) 32	13(44) –	3.1 3.7
Aerial mycelium	0	+ ++	++ ++	0 0	0 0	0 0	0 ++	+ ++	+ +	0 0	0 0	+ +	+ –	
Soluble pigment	0 0	0 0	g g	b b	b d-b	b b	0 0	b b	b b	0 f-b	y-sh y-sh	0 0	b –	
NH_2-N, milligrams in 10 cc.	0.86	1.34	2.08	1.18	2.11	1.58	2.40	0.90	2.37	2.77	1.90	0.93	1.01	

TABLE 1—*Continued*

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
Witte peptone—Continued														
NH_2-N, milligrams in 10 cc.	0	0.60	1.25	0.78	1.62	2.17	1.28	0.96	2.94	2.59	4.02	1.60	–	
NH_3-N	0 0	0 0	1 0	1 2	1 0	0 3	1 2	0 1	1 3	1 3	1 2	0 2	0 –	
Asparagin														
Growth	0	43(11) 44	33(13) 43	44(13) 44(22)	21 21(41)	22 22	22(42) –	43(11) 43	43 35	22 23	22 23	31(21) 31	33(12) –	2.42 2.90
Aerial mycelium	0	+ ++	+ +	+ +	0 0	0 0	0 –	+ +	+ ++	0 0	0 0	+ +	+ –	
Soluble pigment	0	r r	g g	b-sh b	0 b-sh	0 y-sh	y-sh	b-sh b	0 y-sh	0 0	0 0	0 0	b –	
NH_2-N, milligrams in 10 cc.	3.99	1.94 1.65	2.46 2.28	1.30 1.48	2.99 1.90	3.08 2.32	2.56 –	2.91 2.85	2.06 0.63	3.65 3.78	3.34 1.80	3.92 3.86	2.06 –	
NH_3-N	0	0 0	2 3	1 0	2 3	3 3	1 –	– –	– –	1 2	1 2	1 2	0 –	
Leucin														
Growth	0	33(11) 33(12)	43 43	34(13) 45	22 –	12 23	23 43	13 13(43)	43 44(13)	23 23	22 22	22 22	33(13) –	2.75 3.10
Aerial mycelium	0	+ +	+ +	++ ++	0 –	0 0	+ +	0 +	+ +	0 0	0 0	0 0	+ –	

Soluble pigment.........	0	0 r	y-sh g	0 y-sh	b —	b-sh b-sh	0 0	b d-b	f-b f-b	0 0	0 0	0 0	b-sh —	
NH_2-N, milligrams in 10 cc.	3.98	3.00 3.03	2.56 2.56	2.27 0.48	3.12 —	3.09 3.12	2.85 2.82	2.91 2.85	2.85 1.20	3.50 3.38	3.85 3.80	3.83 3.92	2.58 —	
NH_3-N................	0	0 0	0 0	0 0	0 —	0 1	0 0	0 0	0 0	0 0	1 1	1 2	0 —	
Glycocoll														
Growth................	0	33(11) 34	44 43	45(13) 45(13)	22 43	22 13	33 44	33 45	13(32) 45(13)	23 23	12 12	22(31) 22(31)	13 —	2.9 3.5
Aerial mycelium.........	0	+ +	+ +	0 +	0 0	0 0	0 0	+ ++	+ ++	0 0	0 0	+ +	+ —	
Soluble pigment.........	0	0 r	y-sh g	f-b b	0 0	0 0	y-sh y-sh	b d-b	0 gray	0 0	y-sh y-sh	0 0	b-sh —	
NH_2-N, milligrams in 10 cc..................	7.40	6.00 5.15	1.50 3.62	2.06 1.18	6.30 6.72	7.13 7.20	3.15 1.25	4.85 2.00	6.30 1.35	— 6.85	6.39 —	6.85 —	5.70 —	
NH_3-N.................	0	0 0	3 2	2 2	0 0	0 2	0 1	0 2	1 0	1 2	1 2	1 2	0 —	
Urea														
Growth................	0	31 42	21 21	21 21(0)	21 21	21 22	21 21	43(12) —	0 23	21 21	21 21	21 21	0 0	
Aerial mycelium.........	0	0 0	0 0	0 0	0 0	0 0	0 0	+ —	— 0	0 0	0 0	0 0	— —	

TABLE 1—*Continued*

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
Urea—Continued														
Soluble pigment	0	0	0	0	0	0	0	green	—	0	0	0	—	
		g	0	0	0	0	0	—	y-sh	0	0	0	—	
Acetamide														
Growth	0	12	31	32(22)	21	21	21	31	21	21	21	21	0	
	0	31(12)	31	42(22)	22	22	21	31	22	—	21	21	—	
Aerial mycelium	0	0	+	+	0	0	0	+	0	0	0	0	0	
		+	+	+	0	0	0	+	0	—	0	0	—	
Soluble pigment	0	blue	0	0	0	0	0	0	0	0	0	0	0	
		blue	0	0	0	0	0	0	y	—	0	0	—	
NH_3-N	0	0	0	0	0	0	0	0	0	0	0	0	0	
		0	0	0	0	0	0	0	0	—	0	2(?)	—	
$NaNO_3$														
Growth	0	21	21	23	21	21	21	42	22	21	21	21	21	
		31	42	45(13)	—	22	25	42	22		21	21		
Aerial mycelium		0	0	0	0	0	0	+	0	0	0	0	0	
		+	+	+	—	0	0	+	0	—	0	0		
Soluble pigment	0	0	0	0	0	0	0	green	0	0	0	0	0	
		0	g	y-sh	—	0	y-sh	green	y-sh	—	0	0		

No_2	0	5	1	1	0	0	2	2	1	1	2	0	
	0	1	2	2	0	1	3	1	1	0	3	4	
NH_3-N	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	—	0	0	0	0	—	0	0	
$NaNO_2$													
Growth	0	33(11)	21	23	0	21	21	31	21	21	21	0	21
		33	41	43(22)	0	33	21	43	21	21	—	—	—
Aerial mycelium	0	+	0	0	0	0	0	+	0	0	0	0	0
		++	+	0	0	+	0	+	0	0	—	—	—
Soluble pigment	0	p	0	0	0	—	0	b-sh	0	0	0	0	0
		p	0	y-sh	0	b-sh	0	b-sh	0	0	—	—	—
NH_3-N	0	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	—	—
$(NH_4)_2SO_4$													
Growth	0	0	0	21	0	0	0	0	22	0	0	0	0
		0	21	25	0	0	21	0	22	0	0	0	—
Aerial mycelium	0	—	—	0	—	—	—	—	0	—	—	—	—
		—	0	0	—	—	0	—	0	—	—	—	—
Soluble pigment	0	—	—	0	—	—	—	—	0	—	—	—	—
		—	0	0	—	—	0	—	y-sh	—	—	—	—
$(NH_4)_2CO_3$													
Growth	0	0	0	21	0	0	0	0	0	0	0	0	0
		0	0	22(42)	0	0	0	0	41(11)	0	0	0	—

TABLE 1—*Concluded*

SUBSTANCE USED	CONTROL	A. VIOLACEUS RUBER	A. GRISEUS	A. AUREUS	A. BOBILI	A. SCABIES	A. ALBUS	A. VIRIDO-CHROMOGENUS	ACTINOMYCES 215	A. VERNE	A. BOVIS	A. ASTEROIDES	A. RETICULI	AVERAGE
$(NH_4)_2CO_3$—*Continued*														
Aerial mycelium.........	—	—	—	0	—	—	—	—	—	—	—	—	—	
		—	—	0	—	—	—	—	+	—	—	—	—	
Soluble pigment.........	—	—	—	0	—	—	—	—	—	—	—	—	—	
		—	—	0	—	—	—	—	y-sh	—	—	—	—	

*Upper row of records was obtained for the first period of incubation, second row for the later period. The first period was fifteen and the second, thirty days for *A. aureus*, *A. bobili*, *A. scabies*, *A. albus*, *A. reticuli*, *A. viridochromogenus* and *A. verne;* the first thirty and second, sixty days for *A. bovis* and *A. asteroides*.

†1—designates faint growth, 2—fair, 3—good, 4—very good, 5—excellent; 10 designates masses (colonies) throughout medium; 20 masses or floccules on bottom of tube; 30—masses on surface of liquid; 40—heavy solid surface growth.

‡0—absent, + present, scant; ++ abundant.

§r—red, b—brown, g—golden, y—yellow, db—dark brown, b-sh—brownish, y-sh—yellowish, f-y—faint yellow, p-b—purplish blue.

¶0—designates none, 1—traces, 2—good, 3—abundant.

The chemicals used throughout the work were chemically pure, usually Kahlbaum's or Merck's. The casein was purified by the method of Hammarsten.

In glancing through table 1, one can readily see that most of the organic nitrogenous substances both proteins and amino acids, form a readily available source of nitrogen for the actinomycetes; while the amides, namely acetamide and urea used in this investigation and the inorganic nitrogenous substances form a much poorer source of nitrogen for this group of microorganisms. Nearly all the organisms studied, with very few exceptions, notably *A. asteroides*, made a fair to excellent growth upon the proteins, but grew rather poorly on the amides and on the inorganic nitrogenous substances. The quantity of growth is not absolute, but since the record was made by the same investigator under as nearly identical conditions as possible, it lends itself to comparison. The ammonium salts, both the sulfate and carbonate, were found to be the poorest sources of nitrogen under the conditions of the experiment and for the organisms studied; only *A. aureus* and *A. ruber* made a fair growth on these substances, as the only source of nitrogen, while all the others produced no growth or hardly any noticeable growth at all. This held true when these substances were studied in solution; a better growth might have been obtained on solid media. This fact has been brought out by Krainsky (1914), who stated that, with NH_4NO_3 as a source of nitrogen, the nitrate radical was used up in a comparatively short time, while the ammonia persisted for a long period. This relative inability of the actinomycetes to use ammonia compounds as a source of nitrogen may help to explain the fact that in their action upon proteins and amino acids the actinomycetes produce at first very little ammonia, especially when compared in this respect with the bacteria and molds. This brings up the whole question of the relative nitrogen metabolism of these groups of microörganisms, which will be taken up later.

Nitrates are readily used as a source of nitrogen, although the actual amount of growth, particularly on liquid media, is far inferior for most organisms to that produced on the proteins and

amino acids. The particular thing to call attention to in this respect is the reduction of nitrates to nitrites, which is greatly influenced by the source of carbon. Out of 45 species, 35 reduced nitrates in the presence of starch and only 20 in the presence of sucrose. Nitrite reactions were obtained in certain cases even when the proteins and amino acids were used as sources of nitrogen. Notably a strain of *A. californicus*, when freshly isolated from the soil, produced nitrites not only from proteins but also from ammonium sulfate. This fact recalls a paper by Joshi (1915), who reported a bacterium which could transform protein nitrogen directly into nitrites, without the nitrogenous substances undergoing the 3 stages of splitting of proteins to ammonia by one or more groups of organisms, and oxidation of ammonia to nitrites, then to nitrates by Nitrosomonas and Nitrobacter respectively. The photograph of the organism as well as its action strongly resemble that of an Actinomyces. This ability of the actinomycetes to produce nitrites out of nitrates, without any further reduction of the nitrogen compounds to ammonia or elementary nitrogen, was pointed out by Beijerinck (1900), Fousek (1912) and Krainsky (1914). The latter used plate cultures only and found that few species were able to reduce nitrates to nitrites strongly; in many cases the small quantities of nitrites produced were assimilated by the organism as soon as formed. The data on the reduction of nitrates to nitrites, affected by the source of carbon were reported in a previous paper and are summarized in table 2 for the purpose of comparison.

The nitrites were determined by the Griesz (1870) colorimetric method. First 1 cc. of sulfanilic acid solution and 1 cc. of α-Napthylamin solution were mixed in test tubes and allowed to stand a few minutes, so as to insure against the presence of any nitrites in the tubes. Then 1 cc. of the culture was added to the tube, shaken, the mixture allowed to stand in the cold three to four minutes and a reading taken. The active nitrate reducing organisms, such as *A. violaceus-ruber*, reduce with all sources of carbon; some that do not reduce nitrates readily, such as *A. violaceus-caesari*, *A. albus*, and *A. bobili* produce no nitrites

or only traces of nitrites with all sources of carbon studied; while most organisms vary in their power to reduce nitrates to nitrites with the source of carbon in the medium.

Different suggestions have been made concerning the physiological importance of nitrate reduction for the bacterial cell. A complete discussion of this subject can be found in the work of Klaeser (1914). He refuted various theories on this subject

TABLE 2

Reduction of nitrates to nitrites as affected by different sources of carbon

ORGANISMS	A. VIOLACEUS-RUBER	A. VIOLACEUS-CAESARI	A. ALBUS	A. AUREUS	A. EXFOLIATUS	A. GRISEUS	A. SCABIES	A. VIRIDOCHROMOGENUS	A. VERNE	A. DIASTATICUS	A. FRADII	A. ALBOSPOREUS	A. BOBILI	A. POOLENSIS	A. MADURAE	A. HOMINIS	A. ASTEROIDES	A. CHROMOGENUS 205	A. RETICULUS-RUBER	ACTINOMYCES 128	ACTINOMYCES 168	ACTINOMYCES 215
Arabinose	5	1	0	1	0	0	1	2	—	1	2	0	0	0	—	—	0	0	0	0	0	4
Glucose	5	0	0	1	0	2	0	2	1	0	0	1	0	0	—	—	3	0	4	0	2	1
Lactose	5	0	1	1	1	0	1	3	3	1	1	1	1	0	3	5	2	0	1	0	1	3
Maltose	5	0	0	—	3	1	0	2	2	0	0	0	0	0	1	4	4	1	4	3	3	3
Sucrose	3	1	1	0	1	0	0	2	3	0	1	0	1	1	1	0	2	0	1	0	1	1
Mannitol	5	1	1	1	3	3	1	2	4	1	1	3	0	1	—	—	1	0	4	0	2	1
Glycerol	5	0	0	1	0	1	2	3	1	1	1	0	1	1	1	3	4	1	1	0	4	1
Starch	5	1	1	1	1	2	1	2	1	1	2	3	1	3	—	—	0	1	5	0	1	1
Cellulose paper	2	0	0	0	0	0	0	1	1	0	0	0	0	0	—	—	1	1	1	0	0	1
Cellulose precipitated	3	1	0	0	0	1	—	1	1	0	0	0	0	0	1	0	1	0	1	0	0	1
Sodium acetate	5	0	0	0	0	2	0	1	—	0	0	1	0	0	1	0	2	1	2	1	0	2

and concluded that the reduction of nitrate to nitrite and ammonia by bacteria is a process by which these bacteria cover the nitrogen need in their metabolism. This conclusion of Klaeser in regard to bacteria is fully confirmed by the study of the metabolism of actinomycetes. These organisms reduce the nitrates to nitrites, in the process of utilization of this nitrogen compound, but not for any other purpose. This reduction is brought about by hydrogen, as shown by several investigators (Klaeser, 1914). The importance of this phenomenon in relation to the change of reaction of the medium will be discussed in the following paper of this series.

To return back to table 1, we find that when nitrites are present in the medium as the only source of nitrogen, the amount of growth is less than in the case of nitrates, unless the nitrates are present in very small amounts, otherwise (even in the presence of 0.2 per cent $NaNO_2$), they seem to become toxic, so that the amount of growth is very limited. In the presence of 0.2 per cent $NaNO_2$, many organisms made a small growth, limited in most cases to a few floccules on the bottom of the tube and only in the case of the organisms that reduce nitrates to nitrites vigorously (*A. violaceus-ruber*) was the growth more than fair. The question of utilization of nitrites will be discussed later.

The amides, as stated above, form a very poor source of nitrogen in comparison with the proteins and amino acids. Only a few species made more than a very limited growth, the reaction in the case of urea, either remaining unchanged, becoming slightly acid, or, in most cases, turning alkaline; this latter phenomenon is no doubt due to the rapid splitting off of ammonia from urea. In the case of acetamide, the reaction became in nearly all cases more acid, which may be explained by the fact that the NH_2 radical is used up and may be replaced by an OH radical, which may turn the medium more acid.

The utilization of proteins and amino acids can be followed up in two ways, first by observing the actual amount of growth, secondly, by estimating the splitting of the proteins, as measured by the amount of amino nitrogen produced in the case of proteins, or by the decreasing quantities of the amino nitrogen, in the case of the amino acids, using the Van Slyke micro-apparatus.

No sweeping conclusions should be made from these experiments as to the relative utilization of proteins and amino acids by the different species of Actinomyces, because it is quite possible and even probable that different conditions, such as age of culture, time since its isolation from a natural substratum, concentration of nitrogenous substances, nature of carbon compounds, etc., may result in an entirely different range of figures; but, even with all these limitations, certain definite conclusions can be drawn.

A. asteroides splits proteins and uses amino acids only to a very limited extent, although it produces an abundant growth on certain inorganic media (particularly with glucose as a source of carbon). Certain organisms, such as *A. verne,* using only small quantities of amino acids when grown on amino-acid-containing media, allow a large accumulation of amino-nitrogen-rich substances when grown on protein containing media, showing that there is no necessary correlation between the amount of growth and the protein split.

The production of amino acids and other amino-nitrogen-rich substances is not a waste resulting from the growth of the organisms, but is a definite step in the metabolism of the organisms, since in many cases these substances do not accumulate in the medium, but tend to decrease, either due to their transformation into other substances, such as ammonia (which was not the case), or to their assimilation as such or as transformation products, by the organism, as indicated by the increase in growth.

Fibrin, casein, egg-albumin and peptone allow a very good growth of nearly all the actinomycetes studied, the second and the fourth leading, as to the actual amount of growth. Small quantities of amino-nitrogen are present in the proteins; these are equivalent to one-half of the lysin nitrogen, as shown by Van Slyke and Birchard (1914). Some organisms seem to start using this lysin nitrogen at first, particularly the species which are weak proteolytically, such as *A. asteroides* on the casein medium. In most cases, the accumulation of amino-nitrogen becomes prominent only when the organism has made most of its growth. This may be due either to the fact that during the period of its active growth, the organism uses all or most of the amino-nitrogen that it can split, or to the fact that the splitting is accomplished by means of a proteolytic enzyme, which is absent in the early stages of growth or present in only minute quantities.

The amino acids studied, particularly glycocoll, were also well utilized by most organisms. The progress of growth can also be followed by the decrease in the amino-nitrogen present in the medium, either due to its assimilation by the growing organ-

isms or to its transformation into other substances, such as ammonia. It will be noticed that the organisms that made the poorest growth on certain amino acids, used the least nitrogen, while those that made a good growth caused a good deal of the amino-nitrogen to disappear from the medium. For example, in the case of asparagin, the medium containing originally 3.99 mgm. NH_2-N in 10 cc., *A. asteroides* with a faint growth caused a decrease in thirty days to 3.92 and in sixty days to 3.86; while *A. griseus* with a very good growth, changed it in fifteen days to 1.30, which seems to have accompanied the maximum growth, since in thirty days there was a slight increase in the amino nitrogen content, namely 1.48, which may be due either to an individual variability of the cultures or to some autolysis that might have set in.

The production of ammonia does not seem to be a characteristic property of this group of organisms, as was already pointed out elsewhere (Waksman and Curtis, 1916). It is possible that ammonia does not enter as a necessary step in their metabolism, as some investigators claim for other microörganisms and that it is not formed in large quantities as a waste product of metabolism, although we find individual variations between the different species and with different substrata. These organisms seem to assimilate the amino acids directly or indirectly, but not necessarily only after reduction to ammonia. The question of the function of ammonia as a protein split product in the metabolism of molds has been already discussed by the writer elsewhere (Waksman, 1918). It need only be pointed out here that the actinomycetes and molds seem to split the proteins with different results; while most of the latter reduce the proteins to ammonia very quickly and allow its accumulation in the medium, the former seem to split the proteins chiefly to the amino acid stage and only to a limited extent to ammonia. Most ammonia was produced from the peptone and asparagin, least from the egg-albumin and leucin.

The change in the hydrogen-ion concentration of the media are reported in the paper following this one.

Of the different amino acids used, leucin tends to result in a more acid medium, while asparagin and glycocoll lead to a neutral or slightly alkaline reaction. The leucin medium changed on the average for the organisms studied for the first period from pH 7.3 to pH 6.7 and 6.43, while the glycocoll media changed on the average from 7.1 to 7.23 and 7.33, allowing for the variation of the organisms. The protein and peptone media all tended to become more acid, but not to such as extent as the leucin media. It is possible that the reaction of media containing certain proteins may change either to acid or alkaline depending entirely upon whether the organism attacks one amino acid group or another in the protein molecule. The final hydrogen-ion concentration of media, upon which miroörganisms have grown in the presence of the same carbohydrate, is influenced by the nitrogen source and varies greatly, depending upon the source of nitrogen. A more detailed study of this question will be found in the following paper of this series.

The utilization of tyrosin and creatinine by actinomycetes was also studied; the results for the first are reported below in table 3, while creatinine was readily used as a source of nitrogen by all species tested, accompanied by a slightly acid reaction (pH usually changing from 7.0 to 6.4–6.8); the growth was always fair to good and consisted of colonies throughout the medium or as flakes on bottom of tube. No. 205 produced a characteristic soluble yellow pigment, *A. viridochromogenus*, and *A. pheochromogenus* a brownish and *A. violaceus-ruber* a bluish pigment, while *A. scabies*, *A. aureus* and *A. exfoliatus* produced no pigment at all.

A word should be said concerning the pigment production by the actinomycetes upon the media containing proteins and amino acids. These pigments are very characteristic of the species and seem to be stimulated by the organic nitrogen. The chromogenus species, reported in table 1 include *A. scabies*, *A. viridochromogenus*, *A. aureus* and to some extent *A. bobili*, *A. ruber* and *A. reticuli*. These species are characterized by a brown or dark brown (yellowish in few cases, particularly accompanying poor growth) pigment which slowly dissolves into the medium.

The pigment production is usually ascribed to an enzyme tyrosinase, which is able to convert tyrosin into dark colored melanins. Beijerinck (1913) has shown, however, that tyrosin is oxidized into melanin only by a symbiotic action of an *Actinomyces* with a common soil bacterium; neither organism alone can oxidize the tyrosin to the same stage. Attention was called in the previous experiments to the fact that many species of *Actinomyces* produce a yellow brown to dark brown soluble pigment even on media that do not contain any tyrosin. We must therefore conclude that the brown pigment produced by certain species on organic media is due not only to the melanins produced from tyrosin, but also to some other substances produced from amino acids besides tyrosin and from the sources of carbon.

A number of species were inoculated upon an alkaline tyrosin agar (Krainsky, 1914) and only *A. scabies* and no. 205 produced a soluble brown pigment. All the other species made a good growth upon this medium, but produced no soluble pigment. If, as Beijerinck (1913) stated, tyrosinase is a mixture of two oxidizing enzymes, one converting tyrosin into homogentisic acid and the other oxidizing the acid to melanin, then out of all the species studied, only *A. scabies* and no. 205 are able to produce both of these enzymes together. Out of four strains of *A. scabies* obtained from different sources, only two gave the tyrosinase reaction, these two being the more vigorous growers. The experiment on the utilization of tyrosin and on the production of a brown to black soluble pigment was repeated again in solution. A medium was made up containing the same constituents as the synthetic solution used (Czapek's) except that the $NaNO_3$ was replaced by 0.1 per cent of tyrosin and the sucrose by 3 per cent glycerol. The medium was tubed, sterilized, inoculated and incubated at 25° for fifteen days. The results are presented in table 3.

The previous results are confirmed; not all the species that are able to produce a brown to black soluble pigment on gelatin, potato plug and synthetic media containing organic substances, particularly proteins and peptones, are able to produce a soluble

dark pigment on tyrosin. Only some cultures of *A. scabies* and to some extent two to three other chromogenic strains are able to produce this pigment, indicating that only these species are able to form the two enzymes necessary to transform tyrosin into melanin. A detailed study of this question will be taken up in a paper on the "Enzymes of actinomycetes." At present, the fact can only be pointed out that when the actinomycetes were grown on gelatin plus 1 per cent starch, to which HCl and KI had been added only those species that are able to produce a

TABLE 3

The growth of actinomycetes on tyrosin and the production of a soluble pigment

ORGANISM	GROWTH	AERIAL MYCELIUM	SOLUBLE PIGMENT	pH
Control				6.8
A. violaceus-ruber	2*	+	Bluish	6.2–6.6
A. exfoliatus	2	0	0	6.6
A. aureus	3	+	0	6.6
A. viridochromogenus	3	+	Trace of brown	6.6
A. scabies I	2	+	Deep brown	5.4
A. scabies II	3	0	Pinkish	7.0
A. scabies III	2	+	0	6.7
A. 205	3	+	Greenish brown	6.6
A. pheochromogenus	2	+	Brownish	6.7

* The figures have the same designation as in table 1.

brown pigment on the gelatin, produced a purplish color; this indicates that an oxidase is produced by those species that color the cultures containing gelatin and other proteins brown, while they may not be able to convert tyrosin into melanin.

A more detailed study of pigment production by actinomycetes will be published later. Attention may be here called to the fact that *A. violaceus-ruber* which produced a beautiful red brown pigment changing to violet blue is a very active nitrite forming organism; the part played by the nitrites in the synthesis of the pigment may be important.

Attention was called, in connection with table 1, to the fact that ammonium salts and amides form a rather poor source of nitrogen for most actinomycetes. Whether this is true only with glycerol as a source of energy or also with glucose, one can

see from the following experiment, where to the synthetic media previously used 3 per cent of glucose was added in place of glycerol (nitrogen source 0.2 per cent).

Most of the species tested made a better growth with glucose as a source of carbon than with glycerol, using ammonium salts and urea as sources of nitrogen. The poorest growth was obtained with ammonium sulfate, due to the fact that the reaction soon became distinctly acid with this source of nitrogen, the pH values changing from 5.8 to 4.6 and even 4.2 which is a limiting reaction for the growth of actinomycetes, as will be shown in the following paper.

TABLE 4

The utilization of ammonium salts and urea as sources of nitrogen of actinomycetes, with glucose as a source of carbon

ORGANISM	$(NH_4)_2SO_4$		$(NH_4)_2CO_3$		UREA	
	Growth	Aerial mycelium	Growth	Aerial mycelium	Growth	Aerial mycelium
A. violaceus-ruber	1	0	4	—	5	—
A. viridochromogenus	1	0	3	0	4	—
A. aureus	2	—	4	—	4	—
A. reticuli	0	0	1	0	1	0
A. bobili	2	0	2	0	1	0
A. scabies	3	0	3	0	2	0
A. verne	1–2	0	3	0	1	0
A. madurae	Trace	0	0	0	1	0
A. bovis	1–2	0	2	0	2	0
A. asteroides	2	0	3	0	4	0

To test further the utilization of nitrites, different concentrations of this salt were added in place of the nitrate to the synthetic solution containing either glucose or glycerol as a source of carbon. The results are recorded in table 5.

It is obvious that the actinomycetes can assimilate nitrites readily, when these are present in small enough amounts not to exert any toxic effect. In the presence of only 50 mgm. of $NaNO_2$ per 1000 cc. of medium, all the species tested produced a fair to good growth with both glucose and glycerol as sources of carbon. With the increase of the nitrite content, the growth increases, due to the larger amount of available nitrogen, but

TABLE 5

The utilization of nitrites by actinomycetes. (NaNO$_2$ *used*)

ORGANISM	NITRITE ADDED																													
	0.005 per cent						0.01 per cent						0.05 per cent						0.2 per cent						0.5 per cent					
	Glucose			Glycerol			Glucose			Glycerol			Glucose			Glycerol			Glucose			Glycerol			Glucose			Glycerol		
	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$	Growth	pH	NO$_2$
Control		6.4	+		7.3	+		6.4	+		7.1	+		6.4	+		7.3	+		6.4	+		7.3	+		6.4	+		7.3	+
A. violaceus ruber	2	5.2–5.8	−	2	6.4–6.7	−	3	5.2–5.4	−	3	5.2	−	3–5	5.0–6.6	+	2–4	6.6–7.0	+	2	5.6	+	1	7.3	+	0	6.4	+	1	6.6	+
A. aureus	3	5.0–5.3	−	3	7.1–7.3	±	2	6.0	−	3	7.1–7.2	±	4	5.8	+	4	7.3–7.7	+	0	6.4	+	1–2	6.5	+	1	5.4	+	1–2	6.4	+
A. scabies	3	6.4–6.6	+	3	4.6–4.8	−	3	4.6	−	4	4.6–4.8	−	0–1	5.4–6.4	+	4	5.4–6.6	−	0	6.4	+	3	7.5	+	0	6.4	+	0	7.3	+
A. bobili	3	5.4	−	2	6.8	−	3	6.4	−	1	6.8	−	1	6–4	+	5	7.7	+	0	6.4	+	1	7.1	+	0	6.4	+	0	7.3	+
A. reticulus ruber	3	6.6	±	3	6.4	−	3	6.6	−	3	6.4–7.4	±	1	6.2–6.4	+	5	7.0–7.3	+	0	6.4	+	1	6.8	+	0	6.4	+	0	7.3	+

here the toxic effect of the nitrite may become apparent, particularly with some sources of carbon, so that in the presence of 0.05 per cent of $NaNO_2$, most of the species tested made a very good to excellent growth with glycerol as a source of carbon, while the same species produced only a scant growth in the presence of glucose. When the nitrite content of the medium is still further increased, the growth ceases entirely or is only very limited.

In the previous experiments on the action of the actinomycetes upon different proteins, only purified native substances were used. To study further the action of these organisms upon protein-rich substances, they were grown on gelatin (15 per cent in distilled water), with and without one per cent of starch, on milk and on glucose broth (1 per cent peptone, 0.5 per cent meat extract and 1 per cent glucose). The results are reported in table 6.

Here again no greater importance should be attached to the data presented in table 6 than to any other set of biochemical data with a group of microörganisms, where only one or two tubes for each organism are studied. But, although the results are not absolute, a comparative study of the data will be of interest. These experiments were repeated several times at different temperatures and, although a great deal of variation was obtained, the comparative results hold true as a whole; and it really makes very little difference, when such a variable group of organisms is studied, whether one species accumulates 25 or 35 mgm. of NH_2-N in 10 cc. when grown on a 15 per cent gelatin solution. All the species studied cause a splitting of gelatin, peptone and milk proteins to a greater or less extent, but the organism that produces a maximum splitting of the gelatin does not necessarily split the maximum of peptone, casein and other proteins. As we might expect, however, many species that split one protein actively, also split the others actively. The amount of ammonia accumulated in milk is relatively large, particularly for the organisms which are active proteolytically; this is due to the long incubation period at a rather high temperature; in a shorter period of incubation the

TABLE 6

*The decomposition of organic substances by actinomycetes**

ORGANISM	15 PER CENT GELATIN†	15 PER CENT GELATIN PLUS 1 PER CENT STARCH†	GLUCOSE BROTH‡	MILK §	
	NH_2-N	NH_2-N	NH_2-N	NH_2-N	NH_3-N
Control	6.41	6.35	3.08	2.65	0
A. violaceus-ruber		32.49	5.13	21.38	9.6
A. violaceus-caesari			5.21	20.80	6.5
A. scabies	15.75	15.39	4.85	12.83	9.1
A. pheochromogenus	23.94	22.80	5.70	4.85	2.2
A. viridochromogenus			4.84	17.96	4.3
A. chromogenus 205	34.20	37.05	4.34	13.12	4.3
A. aureus	31.35	23.66	4.87	14.82	7.2
A. exfoliatus			4.57	15.96	9.1
A. albus	10.83	6.56	4.10	23.66	11.0
A. griseus	38.48	23.66	4.56	33.21	13.9
A. fradii	15.96	12.83	5.73	19.95	5.3
Actinomyces (206)	48.74	31.64	5.19	28.32	13.7
A. alboflavus		12.26	6.36	26.32	7.7
A. reticuli		28.79	4.38	14.25	6.5
A. reticulus-ruber	13.68	11.97	3.77	16.18	12.7
A. rutgersensis	48.17	32.21	4.57	22.80	5.8
A. halstedii	20.82		4.52	11.69	1.9
A. albosporeus	25.37	18.53	5.42	9.41	1.9
A. diastaticus	33.06	11.97	6.21	25.94	10.1
A. poolensis	36.48	26.72	4.38	30.21	13.0
A. madurae	27.08	14.82	8.04	35.63	
A. hominis	26.51	13.97	4.52	19.95	
Actinomyces 128	29.92	22.52	3.42	25.65	12.7
Actinomyces 161	45.80	25.65	3.70	21.66	10.8
Actinomyces 96	39.33	27.65	4.16	21.95	11.8
Actinomyces 104	25.65	23.66	4.02	8.55	−2.2
Actinomyces 145	20.52	32.49	3.12	5.70	3.5
Actinomyces 154	10.83	16.53	5.19	23.66	6.7
Actinomyces 168	25.08	21.95	4.14	16.96	13.2
Actinomyces 215			6.05	15.20	
Actinomyces 216	22.80	16.82	3.12	19.95	5.3
Average	27.93	21.72			

* The data present milligrams of nitrogen per 10 cc. of medium.

† Incubated at 16° to 18° for thirty to thirty-five days.

‡ Incubated at 25° for fourteen days.

§ Incubated at 37° for forty days.

quantities of ammonia are much smaller; this again brings out the fact that, while the proteolytic bacteria and molds allow a rapid accumulation, the actinomycetes will produce only small quantities of ammonia in a short period of time, the ammonia tending to increase with the prolongation of the period of incubation.

The presence of starch in gelatin seems to exert in many instances a protective action upon the gelatin, and, though a better growth might have been obtained in the presence of starch, there was less of the gelatin split. This does not hold absolutely true for all species; in a few instances there was a greater splitting of the gelatin in the presence of starch. On the average, however, there was a greater accumulation of amino-nitrogen in the gelatin in the absence of starch than in its presence. The gelatin containing originally 6.35 to 6.41 mgm. amino nitrogen in 10 cc., was found, at the end of the period of incubation, to contain, on an average, 27.93 mgm. of NH_2-N, in the presence of 1 per cent starch, and only 21.72 mgm., in the absence of starch. This is in accord with the investigations of the writer and others which indicate that available carbohydrates exert a protective action upon the proteins.

To get a further insight as to the effect of available carbohydrates upon the splitting of proteins by actinomycetes and compare these organisms in this respect with bacteria and molds, the following experiment was made. One lot of ordinary bouillon was made up and divided into 2 portions; 1 per cent glucose was added to one half and the other half left unchanged. These media were distributed in flasks, sterilized and inoculated, as usual in duplicates, with *B. coli*, *B. proteus*, *Aspergillus niger* and 5 species of *Actinomyces*. The flasks were incubated at 37° and, at the end of seven days, the amino and ammonia-nitrogen were determined. The data are given in table 7.

The addition of 1 per cent glucose causes, in the case of all microörganisms, a lesser splitting of the proteins and peptones accompanied by a smaller ammonia accumulation. But, while the active proteolytic bacterium (*B. proteus*) and mold (*A. niger*) were greatly repressed in their action upon the proteins and in

the production of ammonia, the bacterium, which is recognized as not very active proteolytically (*B. coli*), and the actinomycetes were repressed in their action upon the proteins to a much smaller extent.

It is quite possible that here as well as in the case of higher animals we have two kinds of metabolism: endogenous and exogenous. A definite quantity of ammonia may always be produced out of protein materials, as a waste product, independent of whether an available carbohydrate is present or absent; this

TABLE 7

The action of microorganisms on peptone in presence and absence of available carbohydrates. Period of incubation seven days at 37°*

ORGANISMS	BOUILLON		BOUILLON PLUS 1 PER CENT GLUCOSE	
	NH_2-N	NH_3-N	NH_2-N	NH_3-N
Control	45.73	0	44.25	0
B. coli	50.44	9.45	47.79	7.35
B. proteus	73.75	32.55	48.08	8.40
Aspergillus niger	33.93	32.60	21.54	15.80
Act. diastaticus	83.09	11.55	67.85	9.45
Act. viridochromogenus	68.74	10.50	50.15	10.50
Act. fradii	63.43		56.64	
Act. griseus	97.35	13.65	85.24	11.55
Act. poolensis	80.83	12.60	67.85	12.60

* The data present milligrams of nitrogen per 100 cc. of medium.

ammonia may be reabsorbed, in the presence of available carbohydrates, by organisms that are able to utilize it readily as a source of nitrogen (*A. niger*). In the absence of available carbohydrates, the strongly proteolytic organisms use the proteins readily as sources of carbon, leaving large quantities of ammonia, as waste material, in the medium. The ammonia produced in the first case, will not be appreciably decreased (except when reutilized again), while the production of ammonia in the second case may be entirely prevented by a utilizable carbohydrate.

The milk data in table 5 point clearly to the fact that most of the actinomycetes can attack the milk proteins readily and split them to amino acids and ammonia. This question was dis-

cussed in detail elsewhere (Waksman, 1919). To get an insight into the rapidity of the proteolytic action of some species on the milk proteins, as affected by temperature of incubation and mode of action of the different species, the following experiment was conducted:

A series of tubes, each containing exactly 10 cc. of sterile milk, were inoculated with *A. griseus* and *A. exfoliatus*, inoculating all tubes as nearly alike as possible. Ten tubes for each species were inoculated at 25° and 10 at 37°. Observations were made daily as to the clotting and peptonization. The amino and ammonia nitrogen of the milk were determined at the end of definite intervals and results calculated back to the total amount. This experiment was repeated. The results are given in table 8.

The results reported in the table are not sufficient for plotting an exact curve, because the average error between the duplicates is greater than should be allowed for accurate work. The importance of using a definite period of incubation for all the groups is brought out clearly: while the strongly proteolytic species produce a continuous splitting of the proteins, the weaker forms may, at an early period, allow a splitting, which will not advance much further. It is also interesting to note that when the period of incubation is long enough the difference in the quantities of amino nitrogen content of the milk and ammonia accumulated approaches to zero.

The ammonia accumulation by actinomycetes is slow, but increases steadily. When these organisms are compared with molds and bacteria in their power of producing ammonia, an interesting difference is observed. The rapidly growing bacteria and molds allow a rapid accumulation of ammonia, which soon reaches its maximum as a result of the metabolism of these organisms; the slow growing actinomycetes allow only a slow accumulation of ammonia; if a long enough period of incubation is allowed, the amount of ammonia may even become as large as that produced by the active proteolytic bacteria and molds.

TABLE 8

*The effect of temperature on the action of actinomycetes upon milk**

PERIOD OF INCUBATION	A. GRISEUS						A. EXFOLIATUS					
	25°			37°			25°			37°		
	Action on milk	NH_2	NH_3	Action on milk	NH_2	NH_3	Action on milk	NH_2	NH_3	Action on milk	NH_2	NH_3
days												
3	None clotted	3.9	4.8	Five clotted	5.3	1.6	None	2.8	4.2	Five clotted		0.8
5	Four tubes clotted, digestion definite	3.9	3.4	Nine clotted	5.7	4.8		4.0	3.6	All clotted	6.9	2.4
7	All, but one, clotted digestion begins	6.5	4.8	Two completely digested	10.8	7.0	Thickening of milk in tubes	5.8	4.8	Digestion rapid		4.8
9	All clotted		3.2	Five digested	16.0	13.2	All clotted	7.2	5.0		7.2	5.6
12	Milk half digested	9.5	7.8	All digested	19.7	8.4	Digestion begins	6.9	7.2		7.7	6.4
15		11.7			19.3	12.0		7.4			9.1	
18	One tube all digested		8.5		23.9	8.6		11.4	5.8		12.0	10.6
23		18.2	9.6		23.8	14.9		11.2	6.4			7.6
28		18.2	10.3		24.6	14.5			6.7		11.2	

*NH_2 and NH_3 designate milligrams of amino and ammonia nitrogen in 10 cc.

SUMMARY

1. The actinomycetes do not fix any atmospheric nitrogen, although some colonies will develop on routine nitrogen free media.

2. Most species are able to reduce nitrates to nitrites with the proper source of carbon, a few species are able to reduce nitrates to nitrites actively with nearly all sources of carbon studied, while a few others give no reduction or only traces with nearly all sources of carbon.

3. The proteins and amino acids studied were found to form the best sources of nitrogen for this group of organisms. Amides are used only to a very small extent. Nitrates are used fairly well in the presence of the proper source of carbon. Nitrites present in small quantities in the medium are utilized well by most species, particularly by those that reduce nitrates actively. Ammonium salts form the poorest sources of nitrogen, with glycerol as a source of carbon; with glucose as a source of carbon both amides and ammonium salts are utilized well as sources of nitrogen, if the reaction of the medium does not tend to become too acid.

4. Most actinomycetes split proteins actively as indicated by an increase of the amino-nitrogen content of the medium. The organisms that produce only a small amount of growth split proteins only to a very limited extent and use up only small quantities of the amino acids.

5. The production of ammonia from proteins and amino acids is not characteristic of this group, although, on continued incubation, considerable quantities of ammonia may accumulate in the medium, as indicated by the growth of the organisms in milk or on pure proteins added to sterilized soil.

6. Many species produce soluble yellow, brown to dark brown pigments in media containing proteins and amino acids, the production of a brown pigment being due, in most cases, not to a tyrosinase reaction. Only some strains of *A. scabies* and a few other chromogenus species are able to produce a soluble brown pigment from tyrosin; most of the species that produce

brown pigments on protein media, even if they do not give the tyrosinase reaction, produce an oxidase.

7. For comparative cultural purposes, a definite incubation period is very important, since two organisms will show a different relationship in their metabolism (splitting of milk in this case) at different periods of incubation. With the prolongation of the period of incubation the difference in the quantity of the products obtained from the splitting of milk will greatly decrease and may, in some cases, almost disappear.

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STUDIES IN THE METABOLISM OF ACTINOMYCETES

IV. CHANGES IN REACTION AS A RESULT OF THE GROWTH OF ACTINOMYCETES UPON CULTURE MEDIA

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We have a great deal of information on the influence of reaction upon the growth of microörganisms as well as on the changes of reaction of the medium which result from the growth of these organisms. But, unfortunately, the largest amount of work along this line has been done by merely titrating the medium, using a single indicator, with acid or alkali solutions. Very few attempts have been made actually to measure the concentration of the hydrogen-ions in solution and study the effect of the buffers upon these changes in reaction and thus obtain more definite data upon the changes in reaction produced by microörganisms. This has been recently pointed out by Clark and Lubs (1917) in a series of investigations. Another point to be considered is the fact that most of the studies of the changes of reaction of culture media produced by microörganisms were conducted with protein and peptone rich media, where the buffer effect of the substances is very important, an effect which was usually not taken into consideration.

Michaelis and Marcora (1912) stated that *B. coli* will produce in lactose bouillon, independent of the lactose content and initial alkalinity of the solution, a concentration of lactic acid equivalent to pH 5.0 and thus approach a maximum acidity for the organism.

Clark (1915) confirmed the conclusion of Michaelis and Marcora (1912) that the final hydrogen-ion concentrations are a physiological constant for *B. coli;* the greater the buffer effect of the

medium the lower the final hydrogen-ion concentration attained. Ayers (1916) has shown that streptococci reach a more or less definite hydrogen-ion concentration.

Itano (1916) found that the minimum exponent (pH) for *B. subtilis* was between 4 and 5 (4.82) and the maximum between 9 and 10 (9.43). The pH of the media was altered in such a way that the different exponents were brought toward the optimum pH (7.5–8.5). Boas and Leberle (1918) recently reviewed the acid production by molds and yeasts and stated that acidity depends upon the splitting of the nitrogenous substances, when the action hereby produced represses the production of acid from carbohydrates; that the acidity is due chiefly to the decomposition of the carbohydrates, when the nitrogen source does not leave an injurious acid as in the case of ammonium salts of strong acids; thus the acidity may vary with both carbon and nitrogen sources in the medium. Malfitano (1900) has pointed out that with the age of cultures of molds proteolysis takes place resulting in a decrease in acidity. Boas and Leberle (1918) found that the final H-ion concentration obtained lies within wide limits, for *A. fumigatus* between pH 1.56 and 5.7 and for yeasts between pH 2.94 and 3.80, tending in each case towards a maximum. When a maximum is reached, a slow decrease in actual acidity takes place, particularly during a long period of incubation, due partly to utilization of the acid and partly to its destruction. The largest quantities of acid are produced by molds and yeasts from the carbon sources; this acid production is repressed by the acid radical of ammonium salts of strong acids used as sources of nitrogen. The carbohydrate induces sooner or later an enzymatic splitting of protein reacting substances from the nitrogen source, chiefly ammonia, which modifies the reaction.

Davis (1918) and others observed that *B. diphtheriae* grown in a nutrient bouillon produces an initial increase in the hydrogen ion concentration followed by a steady decrease until apparently a limited alkaline reaction is attained; this may finally change to a secondary acidity, the latter never reaching the high point of the first acidity.

Hoagland (1918) stated that nutrient solutions with an acid reaction reach an approximately neutral reaction after contact with plant roots for various periods of time. Gainey (1918) has shown that the occurrence of Azotobacter in the soil depends on the hydrogen-ion concentration of the soil, the organisms being present when the concentration of the hydrogen-ion is not more than 10^{-6} (pH value not less than 6.0). Waksman (1918) confirmed these observations by showing that cranberry soils having an hydrogen-ion concentration of more than 10^{-6} (pH 6.0) do not contain any Azotobacter cells. Fred and Loomis (1917) found that alfalfa bacteria bring about changes in the reaction of the medium which are favorable for their reproduction.

Münter (1913) reported that actinomycetes are very susceptible to acids. Lutman and Cunningham (1914) have shown that *A. scabies* produces no acid, or only very seldom produces any acid, on organic media; the best growth of this organism is obtained in slightly acid or neutral media; the organism is quite sensitive to larger quantities of either acids or alkalies.

Gillespie (1918) has shown that there is a limiting acidity (as measured by the hydrogen-ion concentration) for the growth of *A. scabies* (*chromogenus*); the limiting exponent (pH value) is between 4.8 and 5.2; the organisms will grow at a reaction having a higher exponent, but not a lower; the growth is accompanied by a marked decrease of acidity and the manner of growth gives reason to doubt whether more than a poor growth can occur at the limiting exponent.

The investigations reported in the following paper will deal with the changes in reaction of the culture medium as affected by the growth of actinomycetes as well as the effect of the initial reaction of the medium upon the growth of these organisms.

The medium used most extensively in previous investigations, namely a modified Czapek's solution, was also used in this work. To a liter of distilled water containing 1 gram K_2HPO_4, 0.5 gram $MgSO_4$, 0.5 gram KCl, 0.01 gram $FeSO_4$, were added the different carbon and nitrogen compounds. The solutions were distributed in tubes (or Erlenmeyer flasks), plugged with cotton and sterilized at 15 pounds steam pressure for fifteen minutes. The

gelatin media and those containing glucose were sterilized in flowing steam for thirty minutes on three consecutive days. Inoculation was usually made from agar slants, by introducing a small piece of growth into each tube. The cultures were then incubated at 25° for fifteen days, unless otherwise stated. At the end of the incubation period, the cultures were taken out from the incubator and the H-ion concentration of the solution determined by the use of the phenol-disulphonic acid series of indicators of Clark and Lubs (1917). The hydrogen-ion exponent, the pH of Sörensen, rather than the concentration of the hydrogen-ions, is used in this work. The data on the growth and transformation of nitrogen compounds of the studies given in tables 1 and 2 were reported elsewhere (papers II and III of this series). Two grams of $NaNO_3$ and 30 grams of each source of carbon were used per liter of medium in the first series of experiments, reported in table 1.

The data in table 1 clearly bring out the fact that the actinomycetes are not acid producers as so many bacteria are. The source of nitrogen was such that it could not stimulate the production of alkaline substances as would proteins through the formation of ammonia and other basic substances; the carbohydrates present were easily fermentable and readily convertible into acids, but notwithstanding these two factors, the reaction tended to change rather towards alkalinity than towards acidity. The data in table 1 do not warrant us to say that any one actinomyces produces always a change towards acidity of alkalinity or that any one source of carbon is always changed in either direction. It was pointed out in paper II of this series that a greater change in reaction (usually towards alkalinity) is observed with a larger amount of growth and that the change in reaction seems to coincide with the amount of nutrients (source of nitrogen rather than that of carbon) removed by the organism from the solution. It was suggested that the change in reaction towards alkaline (increase in the value of the exponent designating the hydrogen-ion concentration) may be due to the fact that the organisms remove the nitrate ions but not the sodium ions. But this explanation will not hold in the light of the data brought

TABLE 1

The influence of different sources of carbon upon the reaction changes as affected by the growth of actinomycetes (pH *values of culture solutions*, $NaNO_3$ *serving as a source of nitrogen*)

CARBON SOURCE	ORGANISM																										
	Control	*A. violaceus-ruber*	*A. violaceus-caesari*	*A. albus*	*A. aureus*	*A. exfoliatus*	*A. griseus*	*A. scabies*	*A. verne*	*A. lipmanii*	*A. viridochromogenus*	*A. bobili*	*A. poolensis*	*A. diastaticus*	*A. fradii*	*A. albosporeus*	*A. madurae*	*A. hominis*	*A. bovis*	*A. asteroides*	*A. reticulus-ruber*	*104*	*128*	*154*	*168*	*A. ruber* (215)	*A. chromogenus* 205
Glucose	6.3	7.0	6.4	6.4	6.5	7.0	6.9	6.4	6.3	7.0	7.1	6.5	6.4	7.1	6.5	6.8				6.7	6.9	6.4	6.4	6.4	7.2	6.4	6.3
Lactose	6.2	7.1	6.3	6.9	6.4	6.8	7.1	6.9	6.4	7.0	6.9	6.7	6.3	6.8	6.8	6.3		7.5	6.8	7.0	6.3	6.3	6.4	6.9	6.9	6.9	6.3
Sucrose	7.0	7.0	6.9	6.9	7.4	7.1	7.4	6.9	6.8	6.8		6.9	7.0	7.4	7.3	7.0	7.6	7.3	7.2	6.8	7.6	7.6	6.9	7.0	7.0	7.2	6.8
Maltose	6.2	7.2	5.6	6.0		6.2	7.6	6.0	6.2		5.6	6.2	6.4	8.0	6.2	6.6	6.2	7.4	6.5	6.2	7.1	6.0	6.2	6.2	6.6	7.0	7.2
Mannitol	7.2	7.2	7.2	7.1	7.2	7.0	7.7	7.6	7.2	7.4	7.3	7.1	7.2	7.4	7.3	7.4				6.4	7.0	7.0	7.1	7.3	7.5	7.2	7.1
Glycerol	7.1	6.7	7.4	7.1	7.0	7.0	7.2	7.4	7.0	7.5	7.3	7.4	7.2	7.4	7.2	7.4	7.2	7.6	7.2	6.7	7.3	7.0	7.1	7.2	7.5	7.3	7.0
Starch	7.0	7.0	7.0	7.2	7.1	7.3	7.4	7.4	6.9	7.3	7.5	7.1	7.3	7.6	7.1	7.5				6.9	7.0	6.8	6.8	7.1	7.2	7.6	7.3
Inulin	6.8	8.0				6.6	7.7				8.0			6.8	7.7		7.6		7.6		7.9					7.7	8.1
Sodium acetate	7.3	7.8	7.8	7.7	7.0	7.1	8.0	7.0			7.5	7.4	7.3		7.3	7.3	7.4	7.5	7.4	7.6	8.0	7.7	7.3	7.4	7.4	8.0	7.9

forth in table 2. If the alkalinity of the medium is due to the utilization of the nitrate-ion, while the sodium ion is left in the medium, the same should hold true when $NaNO_2$ is present as the only source of nitrogen; here the nitrite ion would be removed from the medium as the source of nitrogen. But just the opposite holds true, the nitrite solution becoming in nearly all cases more acid. We must look, therefore, for the difference in change of reaction not to the using up of the acid ion by the organism, but to another factor. It is a commonly accepted fact that the addition of $NaNO_3$, as a fertilizer, to the soil will leave the soil more alkaline, and the tendency is to suggest the previous explanation. But the fact has not been established as yet whether the $NaNO_3$ is assimilated by the plants and microörganisms as such or only in the form of ions; in the latter case the explanation would stand. It cannot, however, explain the difference in behavior of the same organisms upon $NaNO_3$ and $NaNO_2$ by which the first medium is left alkaline and the second acid.

It was pointed out in paper II of this series that the change of reaction towards alkaline usually coincided with the amount of growth of a particular species and the nitrate reduction. Those sources of carbon which offered a better source of energy would allow a greater change in reaction which would be accompanied in many cases by the presence of larger quantities of nitrites. In the process of reduction of a nitrate to a nitrite molecule, one atom of oxygen is split off and this may either combine with one atom of hydrogen producing an hydroxyl ion (which may not be possible at all) or with a molecule of hydrogen giving a molecule of water. In either case the hydrogen tension of the medium would be reduced which would explain the decrease of the hydrogen ion concentration of the medium or increase in alkalinity, in the case of the nitrate, but not of the nitrite medium.

The following experiments were carried out, with the purpose of finding the effect of the source of nitrogen upon the change in reaction; 30 grams of glycerol were used in all cases as a source of carbon, and 2 to 5 grams of each source of nitrogen per liter. Glycerol was used in this last series, since it proved to be a favorable source of carbon for nearly all actinomycetes, and since it

did not change appreciably the reaction of the medium on sterilization, thus eliminating the source of carbon as a factor in the change in reaction (many nitrogen sources, namely the proteins and amino acids, could also serve as sources of carbon). The concentration of the sources of nitrogen in the experiment reported in table 2 was as follows: all the inorganic nitrogen sources 0.2 per cent, organic substances (proteins, amino acids and amides) 0.5 per cent.

There were two periods of incubation of the cultures, the first period for most organisms was fifteen to twenty days and the second period thirty to thirty-five days; *A. bovis* and *A. asteroides*, which are slower growing forms (at 25°) were incubated for thirty and sixty days. The upper line (early) always designates the earlier period of incubation, and lower line (late) the later period of incubation for the same organism.

The data given in table 2 clearly bring out the fact that the change in reaction depends entirely upon the nitrogen source, varying greatly in this respect with the different organisms. Some of these data have been discussed in paper III of this series, where the question of the utilization of different nitrogen compounds by actinomycetes was taken up. It was pointed out that certain compounds, such as leucin, seem to result, in nearly all cases, in a more acid reaction and the possibility was suggested that the change in reaction of a culture medium containing proteins, as a result of the growth of microörganisms, may be due to the fact that the particular organism may attack one or another amino acid group in the protein molecule.

The different species certainly do not behave alike in their action upon the different sources of nitrogen. *A. scabies*, for example, produced in nearly all cases, with different nitrogen sources, a change in reaction towards alkalinity, having a larger exponent, while *A. asteroides*, almost invariably, with few exceptions, changed the reaction towards acidity. The fact that the organisms utilize the various amino acids in a different degree may throw some light upon this question. But a great deal more information on this subject is needed, before we can draw any definite conclusions.

TABLE 2

The influence of different sources of nitrogen upon the change in reaction (pH *values*) *in culture solution produced by Actinomycetes**

NITROGEN SOURCE		ORGANISM												
		Control	*A. violaceus-ruber*	*A. griseus*	*A. aureus*	*A. bobili*	*A. scabies*	*A. albus*	*A. viridochromogenus*	*Actinomyces 215*	*A. verne*	*A. bovis*	*A. asteroides*	*A. reticuli*
Fibrin	Early	7.3	6.6	6.6	6.6	7.0	7.6	6.7	6.6	7.4	7.4	8.4	6.4	6.9
	Late	7.3	5.6	6.8	6.2	6.6	8.1	6.2	6.2	7.2	7.6	7.7	6.5	6.6
Casein	Early	7.4	6.8	7.0	6.9	7.4	7.7	7.2	6.7	7.5	7.5	8.6	6.6	
	Late	7.4	6.2	6.6	6.6	7.6	8.4	7.3	6.4	7.4	7.8	8.2	6.4	
Egg-albumin	Early	7.4	6.6	7.4	6.8	7.4	7.7	6.8	6.6	7.5	7.5	8.2	7.6	7.6
	Late	7.4	6.6	7.4	6.6	7.4	8.2	6.6		7.4	8.0	8.2	6.8	
Peptone	Early	7.2	6.6	7.2	7.2	7.2	7.4	7.4	6.6	7.7	7.5	8.2	6.4	7.0
	Late	7.2	5.8	6.4	6.6	6.2	8.4	7.7	5.8	7.7	7.6	8.2	5.8	
Asparagin	Early	7.2		7.7	6.6	7.7	8.2	7.0	6.5	7.0	7.4	7.7	6.6	7.0
	Late	7.2	6.4	7.7	6.2	8.2	8.2		6.6	7.5	7.7	8.2	5.8	
Leucin	Early	7.3	6.4	6.6	6.4	7.1	7.2	6.4	5.8	6.7	7.0	7.3	6.5	7.0
	Late	7.3	5.6	6.6	6.0		7.1	5.8	5.6	6.6	6.8	7.6	6.6	
Glycocoll	Early	7.1	6.4	8.2	6.8	7.2	7.4	6.7	6.6	7.5	7.7	8.7	6.4	7.1
	Late	7.1	5.6	7.5	7.1	6.9	7.9	7.6	8.6	6.8	8.0	8.2	6.4	
Urea	Early	8.0	7.8	8.6	8.0	8.0	8.0	8.0	7.7		8.0	8.0	8.0	
	Late	8.0	8.6	8.0	7.8	7.8	8.4	8.1		7.8	8.0	8.4	7.8	
Acetamide	Early	7.7	7.0	8.0	7.1	7.4	7.4	7.5	7.1	7.5	7.3	7.5	8.0	7.6
	Late	7.7	6.6	7.5	7.2	7.2	7.6	7.4	6.6	7.3		8.0	8.0	
$NaNO_3$	Early	7.1	7.0	7.2	7.2	7.0	7.4	7.0	7.7	7.1	7.1	7.4	6.5	7.1
	Late	7.1	6.0	7.8	7.7		7.2	8.4	7.8	7.1		7.2	5.8	
$NaNO_2$	Early	7.3	6.6	7.4	7.1	7.3	7.1	7.1	7.1	7.1	7.3	7.4	6.8	7.1
	Late	7.3	6.2	7.5	6.4	7.3	7.3	7.1	6.6	7.1	6.8	7.9	6.4	
$(NH_4)_2SO_4$	Early	6.7			6.7									
	Late	6.7		6.4	6.4			5.8		6.3				

* The data on growth and utilization of the different compounds can be found in table 1 of paper III of this series.

Only one ammonium salt was included in table 2; the data obtained do not tell us much about the change in reaction produced with ammonium sulfate as the only source of nitrogen, since this salt was used by most species to only a very limited extent. This limited utilization of the ammonium salt may possibly be due to the fact that glycerol as a source of carbon is not readily available with this source of nitrogen. The same may hold true about urea.

Glucose was found to be a superior source of carbon to glycerol for most actinomycetes. This source of carbon was therefore

TABLE 3

The change in reaction produced by Actinomycetes in a synthetic medium, with glucose as a source of carbon

ORGANISM	AMMONIUM SULFATE	AMMONIUM CARBONATE	UREA
	pH	pH	pH
Control	5.8	6.8	7.4
A. violaceus-ruber	4.4	4.8	8.4
A. aureus	4.6	4.4	7.7
A. viridochromogenus	4.2	5.2	6.6
A. scabies	4.6	5.6	8.6
A. bobili	4.4	5.8	8.6
A. reticuli	4.6	7.1	8.1
A. verne	4.4	6.4	8.4
A. madurae	4.6	7.0	7.7
A. bovis	4.6	7.5	8.6
A. asteroides	4.4	5.6	8.6

used (3 per cent) with ammonium sulfate, ammonium carbonate and urea (0.2 per cent each), as sources of nitrogen. The cultures were incubated at 25° for 24 days. A much better growth was obtained on these nitrogen compounds, in the presence of glucose, than with glycerol. *A. madurae*, *A. bovis*, *A. bobili*, *A. reticuli*, and *A. verne* made a scant to fair growth on these nitrogen sources. *A. scabies*, *A. aureus*, *A. asteroides*, *A. viridochromogenus* and *A. violaceus-ruber* made a good to very good growth on urea and ammonium carbonate and a fair to good growth on ammonium sulfate. The changes in reaction are recorded in table 3.

All the species turned the reaction of the medium containing ammonium sulfate more acid and most of them did so also in the media containing the ammonium carbonate, except *A. reticuli*, *A. madurae* and *A. bovis* which changed the reaction to alkaline. In the case of urea, we find that all the organisms, with the exception of *A. viridochromogenus*, caused an increase in the exponent of the medium. This change of reaction is readily explained in the case of the ammonium salts, since the organisms use up the ammonium-ions as a source of nitrogen, leaving the sulfate-ion in the medium and this will tend to make the medium acid. In the case of the three species that changed the reaction of the medium containing the carbonate to alkaline, we assume that perhaps the anion is utilized here as well as the cation. The increase in the value of the exponent in the urea medium is no doubt due to the production of ammonium carbonate out of the urea, while the *A. viridichromogenus* that changes the reaction of the urea medium to acid made an abundant growth upon the medium and probably used up the alkaline radical.

The increase in acidity in the ammonium sulfate medium was so great that most species made a very poor growth, because the limiting hydrogen-ion concentration was promptly reached.

When ammonium salts of organic acids are present in the medium both as sources of nitrogen and carbon, the medium not only does not become more acid, but may become strongly alkaline. When ammonium malate replaces the sucrose and nitrate in the synthetic solution most Actinomyces cultures grow very readily, the reaction of the medium changing in all cases to distinctly alkaline. This is due to the fact that the malate-ion is used as a source of carbon to a much greater extent than the ammonium-ion as a source of nitrogen, since the energy-need of the organism is greater than the nitrogen-need.

Four liquid media commonly used in bacteriological work were next studied as to their change in reaction resulting from the growth of Actinomycetes. These media were prepared as follows: 15 per cent of Gold Label Gelatin in distilled water as well as 15 per cent of gelatin, with the addition of 1 per cent of starch, were made up in the usual way, tubed and sterilized; the reaction

was left unadjusted; glucose broth containing 1 per cent peptone, 0.5 per cent Liebig's meat extract, 0.5 per cent of NaCl and 1 per cent of glucose, reaction adjusted to pH 8.0; milk to which brom cresol purple was added as an indicator, as suggested by Clark and Lubs (1917). The gelatin cultures were incubated at 18° for thirty-five days and pH determinations made on the liquefied portion of the gelatin; the glucose broth cultures were incubated at 25° for 15 days and the milk at 37° for fifteen to twenty days. No pH determination could be made with the milk cultures, for very obvious reasons, but the change in reaction was designated as follows: 0 no change, + faint alkalinity, ++ fair, +++ good, ++++ highest alkalinity.

The data presented in table 4 again bear out the fact that an Actinomyces may produce acid in one medium, while another medium will be made alkaline; the change in reaction seems to depend entirely on the source of nitrogen. While there was usually very little difference between the gelatin cultures containing starch and those not containing any available carbohydrate, the difference between the change in reaction produced in gelatin, glucose broth and the milk was often quite distinct. In several cases the organisms acted alike on all media: *A. violaceus-ruber*, *A. poolensis*, *96*, *A. griseus*, *206*, *161*, and others left all the media distinctly alkaline; *A. scabies*, which, as was pointed out in table 2, has a tendency to turn the medium always alkaline behaved in a similar way in these experiments. *A. reticulus-ruber*, 145, and 154 left all the media distinctly acid (milk unchanged, gelatin changed from acid to slightly less acid). Other organisms, however, such as *A. lavendulae*, *A. exfoliatus*, *A. alboflavus*, *A. albosporeus* and others changed one medium to acid and another medium to alkaline.

The addition of 1 per cent starch to the gelatin had, in most instances, no effect on the change of reaction and even resulted in few cases in a more alkaline reaction (*A. scabies*, *A. pheochromogenus*, *A. rutgersensis*), although, where there was a change in reaction, it was usually towards acidity. The addition of starch resulted in a number of cases in leaving the medium more acid (lower pH value or greater hydrogen-ion concentrations are

TABLE 4

*Change in reaction (pH values) produced by the growth of Actinomycetes in culture solutions containing different proteins**

ORGANISM	15 PER CENT GELATIN	15 PER CENT GELATIN AND 1 PER CENT STARCH	GLUCOSE BROTH	MILK
Control	6.2	6.2	7.9	0
A. violaceus-ruber	8.0	8.0	6.6	+++
A. fradii	6.3	5.8	6.8	+
A. scabies	7.9	8.2	7.8	++
A. pheochromogenus	7.7	8.4	8.0	++
206	7.7	7.7	8.2	++
A. chromogenus 205	7.8	7.4	7.6	+++
A. aureus	7.4	7.6	5.6	0
161	7.6	7.6	8.2	+++
168	8.0	8.0	8.1	++
A. albus	7.9	6.6	7.1	++++
A. albosporeus	7.8	7.1	7.5	0–+
A. alboflavus		6.4	7.2	+++
A. exfoliatus	5.8	5.8	7.2	++
A. griseus	7.8	7.6	8.4	++++
A. rutgersensis	7.6	8.0	7.8	++
A. reticuli		7.6	7.3	0
A. reticulus-ruber	6.5	6.6	5.2	0
A. lavendulae	5.8	5.6		++++
A. halstedii	6.2	5.6	7.6	++
A. diastaticus	7.5	6.4	7.5	+
A. poolensis		7.6	8.0	++++
A. madurae	7.9	6.4	8.0	++
A. hominis	7.9	7.7	5.2	+++
96	8.0	8.0	8.3	+++
104	6.5	6.4	8.6	++
128	8.5	7.6	8.6	++
145	5.8	6.2	6.4	0
154	5.8	5.8	7.4	0
216	6.4	6.0	5.8	++++

* The gelatin cultures were incubated for thirty days at 18°, glucose broth at 25° and milk at 37° for fifteen to twenty days.

obtained) than in the corresponding culture on gelatin where the starch was absent. But no hasty conclusion should be drawn that these organisms produce acid out of the starch; there was probably no acid produced, but the presence of starch resulted in a change in the utilization of the gelatin, which produced a

different reaction. When the utilization and splitting of the gelatin in the absence and presence of starch are studied (data reported in paper III of series), we find that, in the presence of starch, the gelatin was often attacked to a lesser extent than in the absence of starch and since the reaction in that case was usually more acid, we can conclude that the alkalinity is produced as a result of the splitting of the gelatin molecules and a greater acidity is due not to the production of acid from the starch, but to the smaller amount of gelatin split. The available carbohydrate thus exerts a protective action upon the protein (selective metabolism) as in the case of bacteria and molds, and the difference in reaction in this instance is due to the difference in the nitrogen and carbon metabolism of the organism.

To study further the effect of an available carbohydrate upon the change in hydrogen-ion concentration of protein media as a result of the growth of actinomycetes and compare it with the effect produced upon bacteria, the following experiment was outlined. Plain bouillon was made up according to the routine bacteriological methods and divided into 2 portions; 1 per cent glucose was added to one portion, but not to the other. The media were tubed and sterilized, then incubated with a known acid producing bacterium (*B. coli*), a known protein splitting bacterium (*B. proteus*), a common mold (*Aspergillus niger*) and several actinomycetes. The cultures were incubated at 37° and, at the end of three and seven days, the reaction was determined.

B. coli, in the absence of glucose, changed the reaction at first to slightly acid (acting probably on the sugar present in the meat infusion), but soon turned it to alkaliné; in the presence of glucose, the medium changed to a distinct acid, the acidity increasing with the period of incubation. *B. proteus* changed the medium, free from glucose, to a distinct alkaline and, in the presence of glucose, to a distinct acid. *Aspergillus niger*, which rapidly attacks both proteins and carbohydrates left the glucose-free and the glucose media more alkaline than did both bacteria, also, however, producing a distinct acidity in the glucose medium. The actinomycetes changed the glucose-free medium in nearly all cases to alkaline (except *A. poolensis*) and

the glucose medium to a slight acid, but the increase in acidity was a good deal less than that produced by the bacteria or even by *Aspergillus niger.* It may be quite possible that some actinomyces produce certain acids from carbohydrates, but to explain the change in reaction produced by these organisms in the medium, we must look rather towards their action upon the proteins. The carbohydrates affect the change in reaction by affecting the protein metabolism of the actinomycetes.

Gillespie (1918), has pointed out that *A. scabies* will not develop in a medium that has a lower exponent than pH – 4.8 to 5.2.

TABLE 5

The effect of glucose upon the change in reaction produced by microörganisms in the medium

ORGANISM	NO GLUCOSE		1 PER CENT GLUCOSE	
	Three days	Seven days	Three days	Seven days
	pH	pH	pH	pH
Control	8.2	8.2	7.8	7.8
B. coli	7.5	8.8	6.0	4.8
B. proteus	8.1	8.8	6.6	5.2
Aspergillus niger	8.4	9.0	7.4	7.1
A. diastaticus	8.4	8.3	7.6	7.5
A. viridochromogenus	8.6	8.8	7.8	7.3
A. fradii		8.3		7.5
A. griseus	8.6	8.5	7.7	7.7
A. poolensis		7.8		7.2
A. scabies	7.8	7.7	7.6	7.3

The effect of reaction upon the growth of actinomycetes was studied by us both in synthetic and organic media.

A series of synthetic media were made up having different hydrogen-ion concentrations adjusted by the use of buffers. Each medium contained 30 grams glycerol, 0.5 gram $MgSO_4$, 0.5 gram KCl, 0.01 gram $FeSO_4$ and 5 grams asparagin per liter. The reaction was adjusted by the use of phosphates and carbonates.

The cultures were inoculated in duplicate, as usual, from the same mother culture, and incubated at 25°. At the end of fifteen days, the cultures were examined and pH values of solutions determined.

TABLE 6

*The influence of the hydrogen-ion concentration of the medium upon the growth of Actinomycetes, using synthetic media**

NAME OF ORGANISM		INITIAL REACTION OF MEDIUM (pH VALUE)									
		4.4	4.6	4.8	5.0	6.2	6.6	7.1	8.2	8.5	8.7
A. violaceus ruber	Growth	0	0	0	0–1	2	3	3	3–4	2–3	3–0
	Final pH	4.4	4.6	4.8	5.0	6.6	6.6	7.2	8.0	7.2	8.6
A. scabies	Growth	0	0	0	0–1	2	3	2	1	0	0
	Final pH	4.4	4.6	4.8	5.0	8.0	8.1	7.4	8.2	8.5	8.5
A. griseus	Growth	0	0	0	0–1	3	3	3	3	3	3
	Final pH	4.4	4.6	4.8	5.0–7.5	7.9	8.0	7.8	8.2	8.4	8.5
A. reticulus ruber	Growth	0	0	0	1–2	3	3	3–4	0	0	0
	Final pH	4.4	4.6	4.8	6.6–7.1	7.3	7.0	7.2	8.2	8.5	8.7

* Where two values are given, each value stands for a different tube.

TABLE 7

Growth of Actinomycetes on gelatin media of different hydrogen-ion concentrations and the change in reaction resulting

NAME OF ORGANISM		INITIAL pH VALUE							
		4.6	5.0	5.4	6.6	6.8	7.3	8.2	8.6
A. violaceus ruber	Growth	0	3	3	3	3	3	3	3
	Final pH	4.6	7.7	7.4	7.8	7.7	7.7	8.0	8.4
A. scabies	Growth	0	3	3	3	3	3	3	3
	Final pH	4.6	8.2	7.6		8.0	8.0	8.0	8.0
A. lavendulae	Growth	1	3	4	4	4	4	4	3
	Final pH	4.8	7.1	7.8	7.8	7.7	8.0	8.0	8.0
A. madurae	Growth	0	0	2	4	4	4	4	3
	Final pH	4.6		6.6	8.6	8.2	8.2	8.6	8.2
A. exfoliatus	Growth	0	0	2	3	3	3	3	0
	Final pH	4.6		6.4	7.6	7.6	7.7	7.8	
A. 215	Growth	0	0	4	4	4	4	4	3
	Final pH	4.6	5.2	7.6	7.6	7.6	7.7	7.7	7.7
A. 168	Growth	0	3	4	4	4	4	4	2
	Final pH	4.6	8.2	7.7	7.8	8.1	8.0	8.0	8.2

The same species were also inoculated upon a series of gelatin tubes (15 per cent in distilled water) adjusted to different hydrogen-ion concentrations. These were incubated at 18 to 20° for thirty days. The reaction of the liquefied portion was then determined, using the phenol-sulphonephthalein series of indicators and comparator tubes. The results of these few experiments are given in tables 6 and 7.

The results reported in the two tables are interesting; they point out clearly how little trust we should lay upon the change of reaction produced in the medium if the initial reaction is not known. It is quite evident that the reaction tends to a final point for most species, this being the optimum reaction for the growth of the actinomycetes, if the initial reaction is not beyond the limits where any growth may take place. When the reaction of the medium is too acid it tends to become less acid or more alkaline as a result of the growth of the Actinomyces species; when the reaction of the medium is too alkaline, it tends to become less alkaline as a result of the growth of the organisms. As far as the reaction is concerned, most actinomycetes are able to develop on the medium for a long period of time since the reaction thus tends to reach an optimum value.

SUMMARY

1. The Actinomycetes are not able to produce any appreciable quantities of acid from the carbohydrates studied. The change in reaction of the medium is due to the source of nitrogen.

2. With different sources of carbon and $NaNO_3$ as a source of nitrogen the reaction of the medium tends to become alkaline. When $NaNO_3$ is replaced by $NaNO_2$, those organisms that can grow on the latter source of nitrogen, change the medium to acid rather than to alkaline. This difference in behavior of the organism upon those two sources of nitrogen is explained as follows: The Actinomycetes reduce the nitrate to nitrite more or less actively; the oxygen split off from the nitrate molecule is united with the hydrogen or other reducing substances of the medium, thus tending to reduce the hydrogen tension of the medium.

3. When ammonium salts of strong acids are present as the only source of nitrogen, the medium tends to become distinctly acid, due to the fact that the cation is used up by the organism and the anion is left in the medium.

4. With proteins and amino acids the reaction may be unchanged, or may become acid or alkaline, depending on the species, source of carbon and original hydrogen ion concentration of the medium. Certain species seem to change the reaction of the protein and amino acid media always to alkaline, others always to acid. Leucin, as the only source of nitrogen, nearly always favors a distinct acidity of the medium.

5. The presence of an available carbohydrate in a protein medium seems to favor the change of reaction to a more acid one. This is explained by the effect of the carbohydrate upon the nitrogen metabolism of the organisms and not by a direct acid production.

6. With media of different hydrogen-ion concentrations, the reaction tends to an optimum; the more acid media tend to become less acid and the more alkaline media (above the optimum) less alkaline.

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THE STERILIZATION OF OILS BY MEANS OF ULTRA VIOLET RAYS

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The interesting fact that ultra violet rays have the power of destroying bacteria has been known for some time and has received extensive application in a variety of ways. These rays, which are emitted by nearly every source of light to some extent, and by electric discharges between electrodes of easily volatilizable metals in particular, are of wave lengths shorter than those perceptible to the eye. Ultra violet radiations are very active chemically, among their noticeable chemical reactions being the formation of nitrous acid and of ozone, the decomposition of silver and mercury salts and the decomposition of certain organic compounds. Some organic substances tend to become polymerized when exposed to the action of ultra violet rays. Thus, Pribram and Franke found that recently distilled formaldehyde after being exposed to ultra violet rays for some time contained a substance which was identified as glycolyl aldehyde. Similarly, Ostromisslenski (1912) showed that vinyl bromide is polymerized in a few hours to cauprene bromide. Numerous other investigators, Kailau, Thiele, Lesure, Berthelot and Gaudechon, have demonstrated the effect of these rays in causing polymerization or decomposition.

Ultra violet rays have been employed chiefly as a bactericidal agent and the reason for this is not far to seek. Sterilization by light affords a very convenient mode of application and does not introduce any objectionable substance into the material acted upon. The method is limited, practically, by the fact

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that turbid and colloidal solutions are difficult to sterilize, although in small quantities the presence of a colloidal substance does not interfere seriously. The particles present in a turbid solution cast shadows and consequently cut down the effectiveness of the rays (Oker-Blom, 1913). Other limitations imposed are due to the thickness of the liquid films and the distance from the source of the light. The presence of coloring matter does not decrease the effectiveness of the rays to any great extent (Houghton and Davis, 1914).

The intense activity of ultra violet rays has also been shown in a number of other ways. It has been shown, for instance, that the amoebae in a water supply, whether motile or encysted, are killed by a comparatively short exposure to ultra violet rays (Chamberlain and Vedder, 1911), hemolytic amboceptors diluted 1:100 and exposed for 10 minutes are destroyed (Stiner and Abelin, 1914), diphtheria toxin is weakened by exposure (Hartoch, Schürmann and Stiner, 1914), amylase and invertin are sensitive to ultra violet rays, and albumin is coagulated by their action (Chaulpecky, 1912–13).

The activity of ultra violet rays as a bactericide is indicated by the fact that exposure of a fraction of a second (in some cases one-twentieth) close to the lamp will result in the death of microbes (von Recklinghausen, 1914). The abiotic power diminishes about as the square of the distance from the lamp. It has been found that the relative sensitivity of different germs does not vary as much as in the case of heat and disinfectants. For instance, spores are often twenty times as resistant as the unprotected germs against chemicals, while against ultra violet rays they are only 1.5 to 5 times as resistant as ordinary unprotected water bacteria.

The abiotic action of ultra violet rays is independent of the temperature between 0 and 55°C. Thus, it has been found that the effect is the same in clearly frozen ice as in water. It is scarcely probable that by the action of the rays during the short time necessary to kill the bacteria the entire bacterial cell should be chemically changed, coagulated or otherwise modified. The opinion has been ventured (von Recklinghausen, 1914) that

probably some ferment or similar product contained in the cell is modified by the rays and that thereby the cell is poisoned.

With the innovation of lipovaccines, the question of an adequate method of sterilizing the oil which serves as a vehicle for the antigen has taken new form. Apparently it is difficult to sterilize such oils as olive or cotton seed oil contaminated with spore forming organisms without carrying the oil to a higher temperature than is usually employed for sterilization. Again, such oils heated for a time at high temperatures are found to be irritating when injected subcutaneously (Le Morgine and Pinoy, 1916), probably due to the toxicity of the fatty acids and aldehydes split from the oil in the process of sterilization. Free fatty acids (from butyric acid and upwards in molecular weight), in so far as they are soluble in water, and aldehydes, are poisonous.

Because of the bactericidal properties of ultra violet rays, the experiments detailed herein were made, with the view of testing the applicability of rays of this type to the sterilization of certain oils. The oils employed in these experiments were (1) sweet almond oil, (2) cottonseed oil of a grade known as Wesson Oil, and (3) olive oil (Lucca Cream). These oils as received were usually very slightly opaque and contained a small amount of water. As an emulsion of oil and water is opaque to ultra violet rays, and as the presence of water in the oil is objectionable in the preparation of lipovaccines since it causes autolysis of the bacteria, the oils were dried and filtered. The principal drying agents used were calcium chloride and anhydrous sodium sulphate. Of these two the latter is quite satisfactory for routine work.

The dried oil after filtration was purposely inoculated with various spore forming bacteria or molds and exposed to the ultra violet rays. Portions of inoculated oil exposed in petri dishes at various distances from the source of ultra violet radiation and for various lengths of time established the fact that oil could be completely sterilized by an exposure of 3–5 minutes at a distance of 10 cm. The inoculated oil was chilled throughout the exposure by floating the Petri dish on ice water.

The source of ultra violet light was an R.U.V. straight tube quartz mercury vapor lamp operating at 175 volts and 3.8 amperes when at its maximum. This lamp furnished a very constant source of ultra violet radiation, having once attained its maximum brilliancy. The straight tube lamp is especially advantageous in securing uniformity of exposure, since the rays are distributed throughout its length rather than concentrated at one point.

TABLE 1

Exposure at 5 cm. distance

NUMBER	AMOUNT	TIME	RESULTS	
			Plate	Glucose agar tube
		B. Coli		
	cc.	*minutes*		
1	0.3	3	No growth	No gas
2	0.3	10	No growth	No gas
3	0.3	15	1 colony	No gas
4	0.3	3	No growth	No gas
5	0.3	10	No growth	No gas
6	0.3	15	No growth	No gas
		B. subtilis		
1	0.3	3	2 colonies	
2	0.3	10	2 colonies	
3	0.3	15	No growth	
4	0.3	3	No growth	
5	0.3	10	No growth	
6	0.3	15	No growth	

The samples of inoculated oil were prepared by grinding up the dried bacteria or mold spores with oil, so as to form an even emulsion and then filtering through coarse filter paper in order to free the oil from the larger particles. Control experiments made with this inoculated oil by plating it on agar always yielded a large number of colonies. In order to test the effect of the ultra violet rays on this inoculated oil, portions of it were exposed in open dishes at a fixed distance from the lamp and for varying lengths of time. The preliminary results thus obtained are shown in table 1.

In another series of experiments, ten tests were made with 0.3 cc. of dried oil containing *B. subtilis*. The time of exposure was two minutes in one case, four minutes in one case, five minutes in one case and three minutes in the other seven cases. Agar plates made after treatment were all sterile while control plates were crowded.

In these experiments the inoculated oil after exposure to the ultra violet radiation was emulsified with beef infusion agar, plated and incubated for periods up to forty hours. These results indicated that three minutes exposure at a distance of five centimeters was sufficient to sterilize the oil effectively. Further experiments were made with *B. subtilis*, in order to test the effect

TABLE 2

B. subtilis in dried and filtered oil

NUMBER	TIME	DISTANCE	VOLTAGE	RESULT
	minutes	*cm.*		
1	1	5	110	No growth
2	2	5	110	No growth
3	3	5	Light subdued	20 colonies
4	3	25	110	No growth
5	4	25	110	No growth
6	5	25	110	No growth
Control				Crowded

of the ultra violet rays upon the spores, since this is an important test of the efficiency of the method of sterilizing oils. The results were as follows, exposure of the oil inoculated with *B. subtilis* being always for three minutes; in eighteen tests made with one emulsion (the voltage being 60 in six cases and 90 in twelve cases) and in twelve tests made with a second emulsion (the voltage being 60 in three cases and 90 in nine cases) all plates were sterile after 16 to 40 hours.

The results of another series of tests in which dried and filtered oil was used are shown in table 2.

The results obtained above in sterilizing oil that had been inoculated with *B. subtilis* indicated that an exposure of the oil for three minutes at a distance of five centimeters was sufficient

to kill both the vegetative form and the spore. Further experiments were made in which both the time of exposure and the distance from the source of light were varied. These are set forth in the following table. Apparently *Aspergillus niger* and *B. coli* in oil emulsion are somewhat more susceptible to ultra violet radiation than *B. subtilis*.

TABLE 3

TIME	B. SUBTILIS	ASPERGILLUS NIGER	B. COLI	
			Agar plate	Glucose agar tube
	Emulsion I. Distance from lamp 10 cm.			
minutes				
5	No growth	No growth	No growth	No gas
4	No growth	No growth	No growth	No gas
3	No growth	No growth	No growth	No gas
2	2 colonies	No growth	No growth	No gas
1	17 colonies	Growth		
$\frac{3}{4}$		Growth		
$\frac{1}{2}$	35 colonies	Growth		
$\frac{1}{4}$	Crowded	Growth		
Control	Crowded	Growth	Growth	Gas
	Emulsion II. Distance from lamp 5 cm.			
3	No growth	No growth	No growth	No gas
2	Colonies	No growth	No growth	No gas
1	Colonies	Colonies	No growth	No gas
$\frac{3}{4}$	Colonies	Colonies	No growth	No gas
$\frac{1}{2}$	Colonies	Colonies	No growth	No gas
$\frac{1}{4}$	Colonies	Colonies	3 colonies	No gas
Control	Colonies	Colonies	3 colonies	Gas

It will be noted in this connection that spore bearing molds are quite as susceptible to ultra violet rays as *B. subtilis*, an important point, since raw olive oil in particular was frequently found to be contaminated with various molds. The effect of ultra violet rays upon oil emulsions of Mucor and Penicillium was also noted. The results obtained were similar in all respects to those obtained with *Aspergillus niger*. With reference to the above, it is interesting to note that in the case of aqueous suspensions of different species of Penicillium, Asper-

gillus and Mucor, Haughton and Davis (1914) noted only a relatively small destructive effect of ultra violet radiation—in no case greater than 20 per cent. They concluded, therefore, that molds cannot be killed by ultra violet light except when present in very small amounts.

The method described above of exposing oil in open dishes to the ultra violet radiation in order to produce sterility is not applicable practically. Numerous means suggest themselves of sterilizing a liquid in thin films, such as flowing the oil over a quartz plate placed directly over the source of ultra violet energy, flowing the oil in open troughs beneath the lamp, etc. all of which are limited by the fact that the oil is thus exposed to contamination by dust, or likely to be heated unduly. The method of exposure finally adopted was that of flowing the oil through a spiral tube made of transparent quartz having a bore of 3 to 3.5 mm. and extending the length of the mercury vapor lamp. The oil within the spiral was kept cool by enclosing the spiral in a gauze jacket over which ice water flowed from a number of fine jets. With this arrangement, the oil at the point of delivery never exceeded 60° in temperature, although it was exposed at a distance of only one centimeter from the lamp. Oil was fed into the spiral by gravity and the lower end of the spiral extended into a sterile flask so that the oil from the spiral could be collected under sterile conditions. One and a half liters per hour of cotton seed oil can be sterilized with this arrangement. The apparatus set up for use is shown in figure 1.

With the above arrangement and with the mercury lamp working at 48 to 60 volts, samples of olive oil emulsions of *B. subtilis* were run through the spiral, the time of exposure being varied over several minutes. These experiments indicated that eight minutes at a voltage of 48, or three and a half minutes at a voltage of 60, secured effective sterilization. These results indicate that the voltage at which the lamp is operating considerably influences its effectiveness. The efficiency of the lamp was therefore increased by insulating the cathode with asbestos. By means of this arrangement the voltage across the terminals was increased to 150.

The method of determining sterility adopted in the earlier part of this work, i.e., emulsifying the oil directly with beef infusion agar and plating, is open to the objection that incomplete contact is secured between the oil and the agar, so that no

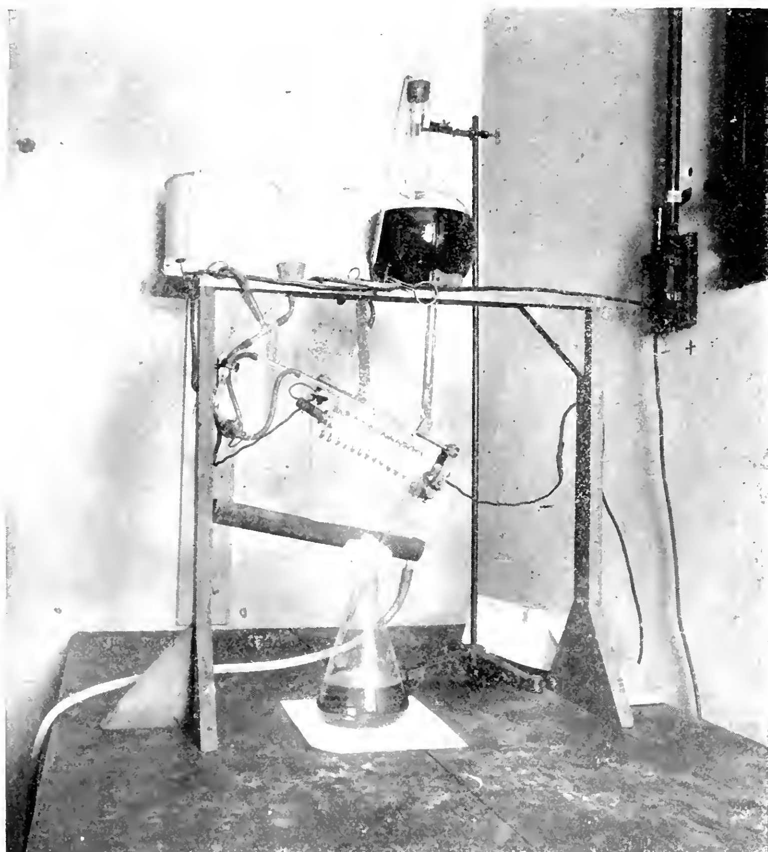

Fig. 1

growth is not conclusive proof of sterility. As a consequence, a method which gives a far more accurate index of sterility was adopted. This consists in shaking samples of the oil with sterile beef broth contained in small glass bottles together with a few glass beads and incubating for twenty-four hours. At the end

of the twenty-four hour period, 1 cc. samples of the broth were plated out in beef infusion agar. While this increased the chance of contamination somewhat, it afforded a much more delicate test of sterility. The effectiveness of the mercury lamp when operating at higher voltages in causing sterility is indicated by the fact that twenty tests of *B. subtilis* suspended in olive oil in the quartz tube for four minutes (voltage 140 in ten tests, 150 in the other ten) showed no growth in any case after twenty-four hours incubation in broth and twenty-four hours cultivation on the plate, although control plates were crowded with colonies. The same result was obtained in another series of seven tests made by four minute exposure to 150 volts and of five tests made by two minute exposure to only 130 volts.

These and subsequent experiments have amply demonstrated that oils of the nature described can be sterilized at the rate of $1\frac{1}{2}$ liters an hour with a small ultra violet ray equipment.

THE ACTION OF ULTRA VIOLET RAYS ON LIPASE

In connection with the action of ultra violet rays on bacteria it is of interest to note also their effect on the lipolytic enzymes present in raw vegetable oils. The presence of lipases in oil used in the preparation of lipo vaccines would be somewhat objectionable owing to the more rapid formation of free fatty acids after injection. As an instance of this phenomenon, raw cotton seed oil which had been incubated at 37° for eighteen hours showed an increase in acid value from 0.11 to 0.78.

That ultra violet rays definitely affect enzymes has been shown in a number of cases. Agulhon (1912) found that sucrase, amylase, catalase, lactase, and peroxidase were all rendered inactive by these radiations. Mazouc found that the inactivation due to ultra violet light rays is extremely rapid at the beginning upon amylase and invertin. In a mixture of the two, the amylase, because of its greater sensitiveness to the rays, is weakened much more rapidly than the invertin. Bruge, Fischer and Weill (1916) found that secretin, pepsin, trypsin, ptyalin, amylopsin and trypsinogen were destroyed by exposure to ultra violet radiation, the rate of destruction being proportional to

the amount of energy applied. The following study shows that lipases suspended in oil are greatly weakened, if not destroyed, by exposure to ultra violet rays.

To obtain a suspension of lipolytic enzymes in oil, 0.5 gram of pancreas extract (Fairchild Bros. & Foster) was emulsified in 500 cc. of dried and filtered cotton seed oil. Portions of this emulsion were exposed at a distance of 5 cm. from the quartz mercury vapor lamp for different lengths of time. The temperature was kept below 40° by exposing the emulsion in thin layers in open dishes floated on ice water. 10 cc. of the emulsion so exposed were emulsified with 10 cc. of distilled water and incubated at 37° for eighteen hours. No great increase in acidity was observed in samples so incubated over those incubated for a few hours only. After incubation the samples of emulsion were titrated with N/100 sodium hydroxide, using phenolphthalein as an indicator.

Similar experiments were made using a fresh suspension of the pancreatic extract, but flowing the suspension around the lamp by means of the quartz spiral and at the same time chilling the oil. Small quantities could thus be handled at a speed allowing an exposure of one minute. By returning the oily suspension through the spiral, exposures of from one to ten minutes could be made with the one sample. Portions were drawn off for each minute of exposure, emulsified with 10 cc. of distilled water and incubated for eighteen hours at 37° in flat dishes. The purpose of this technique was to insure as large a surface of contact between the oil suspension and the water as possible. After incubation, the dishes were washed out with water and the emulsion titrated as before. The results are collected in the tables below and shown graphically in figure 2.

The variation between the values for the incubated lipase suspensions may perhaps be due to variation in the enzyme content of different portions of the pancreatic extract. In any case, however, these values indicate that the lipolytic action of the enzyme is weakened or inhibited by the action of the ultra violet rays and that the greatest change in the enzyme occurs within the first few minutes of exposure, i.e., during the period

of effective sterilization of the oil. The amount of enzyme present in the raw oil must be small, since the amount of acid present

TABLE 4

Lipase suspension in cotton seed oil

CONDITION	EXPOSURE	INCUBATION PERIOD	N/100 NaOH REQUIRED
	minutes	*hours*	*cc.*
Raw oil	0	0	0.2
Dried and filtered oil	0	0	0.2
	0	18	1.4
Dried, filtered suspension	0	0	4.0
	0	18	50.0
	3.5	18	13.0
	5.0	18	9.1
	10.0	18	5.4

TABLE 5

Lipase suspension in cotton seed oil

CONDITION	EXPOSURE	INCUBATION PERIOD	N/100 NaOH REQUIRED
	minutes	*hours*	*cc.*
Dried and filtered oil	0	18	1.0
Lipase suspension	0	0	1.6
	0	18	27.9
	1	18	4.4
	2	18	3.9
	3	18	3.6
	4	18	3.2
	5	18	2.7
	6	18	2.3
	7	18	1.9
	8	18	1.6
	9	18	1.9
	10	18	2.3

after eighteen hours incubation with distilled water serves to increase the amount of N/100 NaOH required from 0.2 cc. to 1.0–1.4 cc. only.

The pancreatic lipase preparation is not wholly satisfactory to work with in this case, as we are more particularly interested in the action of ultra violet rays upon the vegetable lipases occurring in the oil naturally. Accordingly similar experiments were made with a lipase from a vegetable source (castor bean lipase). The lipolytic activity of the castor bean lipase was about one

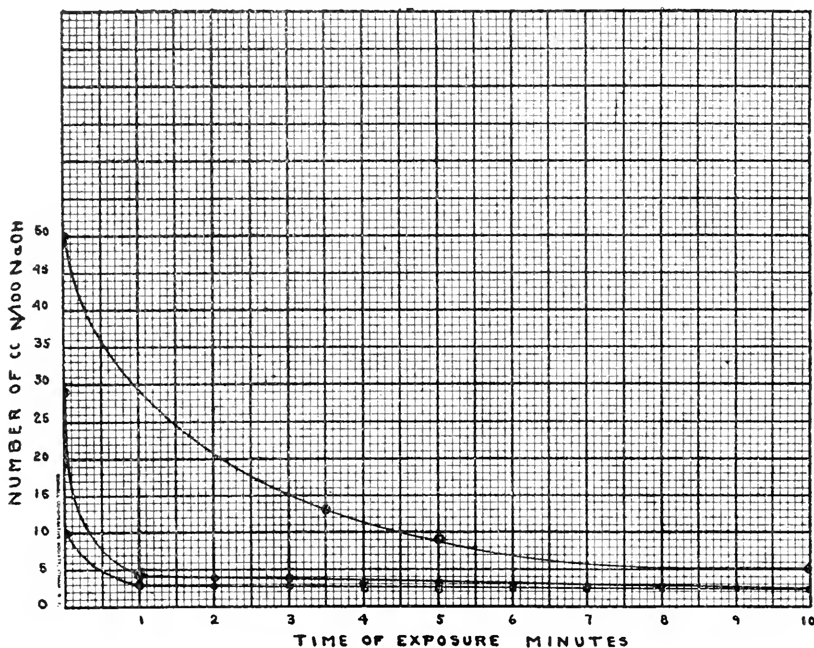

Fig. 2

third that of the pancreatic lipase, but relatively the same effect was obtained on exposing the lipase suspensions in oil to ultra violet rays, namely an effective decrease in enzyme action after exposure.

THE RATE OF STERILIZATION OF OIL

In order to obtain an approximation of the rate of sterilization of oil by means of ultra violet rays, portions of an emulsion of *B. subtilis* in olive oil were exposed for different periods of time.

Samples of 2 cc. were exposed in glass dishes under the lamp at a distance of 10 cm. After exposure, 1 cc. of each sample was emulsified in 5 cc. of beef broth, plated in agar and incubated for

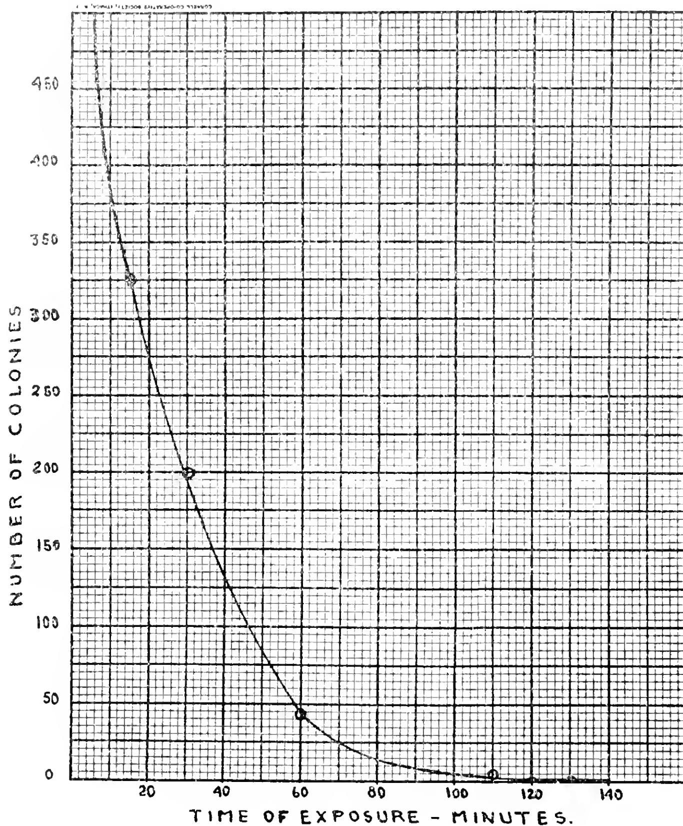

Fig. 3

twenty-four hours at 37°. The results of these experiments are shown in the curve, figure 3, and indicate that by far the greatest amount of sterilization is secured during the first sixty seconds of exposure.

THE PENETRABILITY OF OIL TO ULTRA VIOLET RAYS

In connection with the sterilization of oils, a measure of their penetrability to ultra violet rays is of some practical importance. In order to determine this, agar plates were painted with a saline suspension of a staphylococcus and exposed to ultra violet rays filtered through layers of oil of various thicknesses. The oil was contained in a cylindrical cell provided with a flat transparent quartz bottom. The cell was so fixed in the cover of a light-tight box that a portion of the agar plate within the box was exposed to ultra violet rays filtered through the oil films. After incubation, the area exposed, in cases where the sterilization was effective, was clearly marked as a circle in which no colonies were visible. The results are given in table 6.

TABLE 6

The penetrability of oil to ultra violet rays

DEPTH OF OIL	FIVE MINUTES EXPOSURE	THREE MINUTES EXPOSURE	ONE MINUTE EXPOSURE
mm.			
5	Developed	Developed	Developed
4	No growth	Developed	Developed
3	No growth	Developed	Developed
2	No growth	Developed	Developed
1	No growth	No growth	Developed
Control	Developed	Developed	Developed

In these experiments (table 6) the agar plates were at a distance of 6 cm. from the lamp and the quartz plate was 2 mm. thick. From the results obtained it is apparent that layers of oil 4 mm. in thickness may be penetrated by ultra violet rays in sufficient amount to effect sterilization provided the time of exposure is sufficient. In this connection it is interesting to note that Vallet (1910) found that with aqueous salt solutions the ultra violet rays were held back and sterilization did not occur when the light had to penetrate a few drops of oil. In the latter case the opacity was doubtless due to the oil-water emulsion at the surface of contact, a condition quite different from that presented by an oil film itself.

CHEMICAL CHANGES PRODUCED IN THE OIL BY EXPOSURE TO ULTRA VIOLET RAYS

The effect of the ultra violet rays on the oil itself was carefully studied with the view of detecting the extent of the changes produced. The oils used were almond, cotton seed and olive. The quantitative examination included the determination of the acid value, the iodine value, Reichert-Meissl value, saponification value, soluble acids, density, viscosity and refractive index. These analytical values were obtained in each case with (*a*) the raw oil, (*b*) oil that had been dried and filtered, and (*c*) dried and filtered oil that had been exposed to ultra violet rays sufficiently long to ensure sterilization.

No significant change was noted in any of the above cases. The two constants that would most likely be affected by ultra violet rays are the iodine absorption number and the acid number. If there were any tendency towards polymerization or resinification, as is found to be the case with some unsaturated organic compounds under the action of light, the iodine number would be decreased. Oxidation would produce the same effect. If the ultra violet rays affected the glycerol esters, causing them to split into free fatty acids and glycerol, the acid value would be markedly increased. As a matter of fact no significant change in the oil could be detected by means of any of the constants determined. In spite of these results, however, it is not reasonable to say that ultra violet rays are wholly without action on the oil. There is always a slight bleaching action and oil that has suffered long exposure to ultra violet rays acquires a rancid odor and taste. The bleaching action is probably due to the action of the rays on carotin, the unsaturated hydrocarbon to which certain oils owe their color (Gill, 1917). We can only state that this action is without measurable effect when the oils are exposed for the short periods of time necessary for their sterilization. For all practical purposes the oil has all the properties of the raw oil in the case of almond, cottonseed and olive oils after this length of exposure.

It was found that oil exposed in the open air to ultra violet light acquires the property of liberating iodine when shaken with an aqueous solution of potassium iodide. This is probably due to the presence of a small quantity of an ozonide of oleic acid formed during the exposure. According to Molinari one molecule of ozone is assimilated with the formation of oleic acid ozonide, $C_{18}H_{34}O_5$, when ozone or ozonized air is passed through oleic acid. Experiments were carried out in which oils were exposed in an atmosphere of carbon dioxide in a small sealed tube of transparent quartz surrounded by a larger quartz tube through which ice water was flowed. Oil so exposed showed no greater oxidizing power than the raw oil. That this substance (oleic acid ozonide) plays no important rôle in the sterilization of the oil was proved by exposing samples of the oil under an atmosphere of carbon dioxide in a quartz tube and testing for sterility. The oil so exposed was readily sterilized indicating that the specific effect is produced by the ultra violet rays alone.

The chemical changes produced in oils exposed to ultra violet rays for long periods of time have been studied in a few cases. Lesure (1910) found that olive oil was bleached but not noticeably changed in exposures of less than one half hour. Exposure of olive oil to the rays for an hour caused an increase of more than 5 per cent in acidity (Lesure), 1910. Romer and Sames (1910) found that butter fat when exposed to ultra violet rays for one and three fourths hours showed a decrease of 7 in its iodine number.

A number of determinations of the change in acid value of olive oil following relatively long periods of exposure was made with a view of estimating the magnitude of this effect. Samples of oil exposed for the same period of time both in the open air and in an atmosphere of carbon dioxide showed no significant variation in acid content. The results of these experiments are shown graphically in figure 4. It will be noted that the oil does increase in acidity with exposure and that this increase is directly proportional to the length of time. In curve (1) the oil was 4 cm. distant from the lamp, while in curve (2) the distance was 1 cm. only. In the latter case the fat splitting property is more pronounced

because the distance from the source of ultra violet light is less. While a definite increase in acidity is noticeable in periods extending over four hours, the increase in acidity is not noticeable in the short time of two to three minutes, so that the oil after sterilization by ultra violet rays, aside from a slight bleaching, may be said to be unaltered from a chemical and physical standpoint.

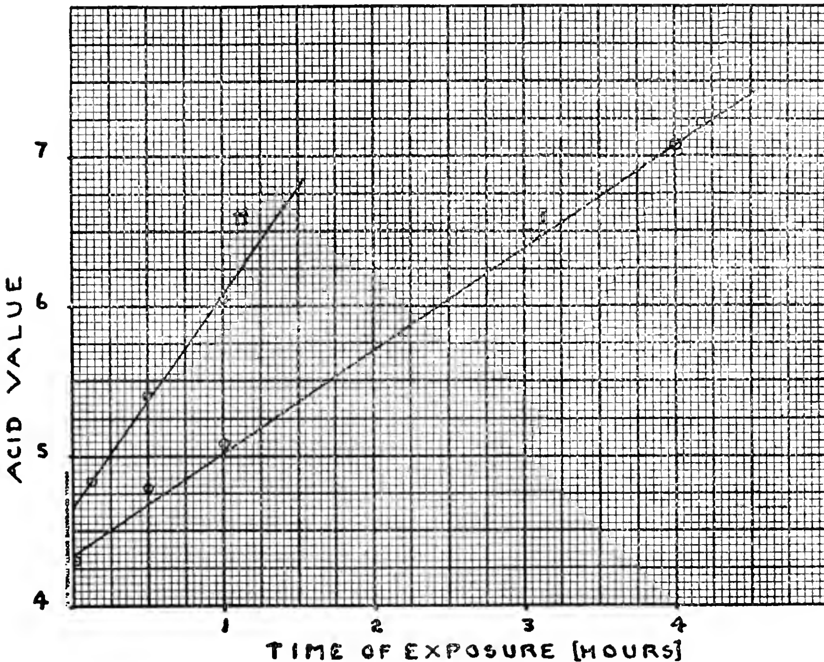

Fig. 4

SUMMARY

Certain oils, such as olive, cotton seed and almond oils, can be effectively sterilized by means of relatively short exposure to ultra violet rays. The abiotic power of the ultra violet rays is not restricted to vegetative bacterial cell alone but extends to the spores as well as to certain molds such as Penicillium, Aspergillus and Mucor. Lipolytic enzymes in oil are sensitive to, and their action is inhibited by, exposure to ultra violet rays. Ex-

cept for a slight bleaching, the oil is unchanged physically and chemically by this exposure. The sterilizing effect of ultraviolet rays is still apparent after they have been filtered through layers of oil 4 mm. in thickness. Olive oil when exposed for long periods of time shows an increase in acidity and this increase is directly proportional to the length of exposure.

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ON THE BACILLUS OF MORGAN NO. I[1]—A META-COLON-BACILLUS

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INTRODUCTION

The bacillus of Morgan was first described in the years 1905 and 1906, when Morgan found it in stools from cases of summer diarrhoea in children. The investigations of Morgan were carried on by Morgan and Ledingham (1909), who consider this bacillus one of the main causes of the diarrhoea of infants.

According to the description of the English authors the bacillus of Morgan has the following characters:

It is a Gram negative bacillus of the size of a dysentery or colon bacillus. Ordinarily it is motile, but can occasionally show immobility. It produces acid and gas in media containing glucose, but does not ferment the other ordinarily used sugar media, such as lactose, maltose, sucrose and mannitol. It produces indol in beef broth, but does not coagulate milk or liquefy gelatine.

The production of gas in glucose may be so slight that it can only be observed in agar deep cultures and there may even be no production of gas at all.

Serologically, it was impossible to make a convenient classification of strains of this organism, as the English authors found heterologous strains inagglutinable in a serum produced with a typical strain. In sera from patients they occasionally found agglutination of the homologous strains in the dilution 1:40.

[1] Partly published in Medicinish Revue, no. 4, 1918.

The microbe was found fairly pathogenic to animals. Rats and monkeys acquired a fatal diarrhoea after feeding on agar slope cultures of the microbe.

The bacillus of Morgan has also been described from Denmark, where first Bahr and Øhrum, and later Bahr and Thomsen found it in stools from diarrhoea of infants. The Danish authors report certain characteristics which differ from the description of Morgan and Ledingham, such as frequent failure to produce indol; yet in spite of these smaller differences there is no doubt as to the identity of the English bacillus of Morgan and the Danish so-called Metacoli.

The Danish authors however classify the microbe in four subgroups according to the growth of the various strains in media not used by the English investigators (galactose, mannose, xylose, arabinose and adonitol). There is however the greatest probability that these subgroups are not of any great importance.

Later on the microbe is mentioned by Tribondeau and Fichet (1916) who found it in stools from clinical dysentery from the Dardanelles. The authors never found the various strains agglutinable in sera from the patients, from whom the strains had been isolated.

Logan (1916) found the bacillus in stools from children suffering from acute diarrhoea and in the stools of 2 out of 21 healthy children.

D'Herelle (1917) reported this microbe as the cause of dysentery, while German authors (Seligman, 1917, Jungmann and Neisser, 1917, Kindberg, 1917) found "atypical dysentery strains," that seem to be identical with the bacillus of Morgan.

Lastly this bacillus has been studied by Bang in Copenhagen.

SOURCE OF MATERIAL STUDIED IN THE PRESENT INVESTIGATION

The technique used in the isolation and study of the following strains has been the one used in the author's work on dysentery in Norway (Thjøtta, 1919).

Immediately after isolation the strains have been examined as to motility, growth in the various sugar media, production of

indol and agglutination in sera from rabbits immunized against typhoid, paratyphoid A and B and dysentery bacilli of groups I, II and III. Then sera were produced against the seven first of the Morgan strains, and each strain tested as to agglutination and complement-fixation in the homologous and heterologous sera.

It will be convenient to give a short résumé of the symptomatology of the patients, from whom the strains were isolated.

I. Acute diarrhoea. Stool fluid without mucous or blood. On plates many colonies of Morgan bacilli.

II. Man, forty-four years old. During the last years occasionally diarrhoea with blood in stool.

Some days before examination of stool, admitted to hospital, suffering from an intense attack of diarrhoea. Abdomen meteoristic. On examination in rectoromanoscop the mucous membrane of the colon appeared swollen, hyperemic and bleeding.

Plates showed numerous colonies of Morgan bacilli.

III. Man, thirty years old. The day before admittance to hospital severe pains in abdomen, diarrhoea, vomiting and cramps. Disease lasted one month, all the time showing a gastrointestinal or intestinal character. Stools of broth consistency, containing mucus.

Plates showed only few colonies of the typical Morgan bacilli.

IV. Woman, thirty years old. Had suffered from chronic diarrhoea for a long time. Discharged 6 to 7 bloody and mucous stools a day. Plates showed numerous colonies of Morgan bacilli. On examination later on, when the stool had become fecal and only contained traces of glassy mucus, there were no colonies of Morgan bacilli to be found in stool.

Patient died six months later from cancer of the colon. Cultures from the colon showed no Morgan bacilli.

V. Woman, forty-five years old. Chronic diarrhoea for two months. Numerous colonies of Morgan bacilli.

VI. Man, sixty-eight years old. In the last six months before admittance to hospital continual diarrhoea; 6 to 7 stools a day, containing big lumps of mucus and pus. Had grown very lean.

Diarrhoea continued in spite of treatment, and the patient died twenty-five days after admittance to the hospital.

Post-mortem examination: As only the examination of the intestine showed anything of interest only this part of the protocol will be cited.

All over the mucous membrane of the colon from coecum to rectum were found extended and numerous ulcers. The ulcers were in the main situated across the intestine, of a rather longish shape. They were ordinarily sharply contoured and had a clean bottom. All ulcers stretched down into the submucous membrane. Only the ulcerations in the rectum had the character of erosions. The healthy parts of mucous membrane between the ulcers were swollen, pale and oedematous. No considerable swelling of the glands.

From the stool and from the ulcers growth of numerous colonies of the typical Morgan bacilli.

VII. Man, twenty-one years old. Suddenly taken ill with severe abdominal pains, tenesmi and stools, containing blood and mucus ten to fifteen times a day. Often hemorrhages pr. rectum. Excessive prostration. Stools at last chocolate coloured, stinking, containing big necrotic fibres of tissue. Temperature about 38°C.

Death in emaciated condition on the eleventh day of disease.

Post-mortem examination: On opening the abdominal cavity the lower parts of the omentum were found discolored, slightly covered with pus and attached to the paries of the pelvis. In the small pelvic cavum were found 50 cc. of pus.

The outside of the small intestine was a little hyperemic in the lower parts, while the serosa of the colon was considerably injected, and fragile. In the mesenterium several swollen, excessive injected glands.

After opening the colon there were found considerable alterations of this intestine. Nearly all the mucous membrane had been dejected and in its place was found an immense ulcer interrupted here and there by small isles of membrane, that was blood coloured and excessively oedematous. The ulcers in many places bared the muscle of the intestine and even perforated it so that only the serosa remained. Very often the process was necrotic, but there were no veritable perforations of the intestine.

Often there were thick brown necrotic fibres (dejected mucous membrane) attached to the ulcerating intestine; or the bottom of the ulcers was filled with a purulent gangrenous and smeary covering.

From the feces and the ulcers were isolated numerous colonies of the typical Morgan bacilli and a strain of proteus.

VIII. Man, forty years old. Suddenly taken ill with numerous stools consisting of mucus, blood and pus. Temperature between 38 and 39°. After the first week of disease the stools became purulent, as if a large ulcer was continually discharging pus.

After one month of disease stools grew solid, and patient recovered.

During the whole disease there might always be cultivated typical Morgan bacilli from the feces and there were never found any other pathogenic microbes in the stools.

The Morgan bacilli disappeared when the stools grew solid.

IX. Man, twenty years old. Suddenly taken ill with severe attack of acute colitis. Stools numerous consisting mostly of pus and mucus.

Recovered after two weeks of illness.

Cultures from stools regularly showed masses of colonies of the Morgan bacilli.

MORPHOLOGICAL CHARACTERS OF BACILLI ISOLATED

All these strains were Gram negative bacilli. As to motility a disagreement was apparent between the first examination and an examination made a year and a half after the isolation from stools.

On the first examination strain I was found immobile, all the others distinctly motile. On the second examination, however, only one, strain III, of the first seven strains showed motility, while the others had lost this faculty completely.

The two last strains being isolated after that examination were only examined once and were found mobile.

CULTURAL CHARACTER OF BACILLI

All these strains were culturally typical Morgan strains immediately after the isolation. It must, however, be stated that strain IV showed itself atypical in as far as it produced slight acid in mannitol and sucrose after a growth of three days and did not produce indol in beefbroth. It did, however, ferment glucose with production of gas and acid and could serologically not be placed in any other known class of microbes. It was therefore considered as an atypical Morgan strain and studied together with the typical ones.

After the first examination the strains (with the exception of VIII and IX) were grown on artificial media up to one and a half years and then examined as to growth again.

It will be seen from table 1 that five of these strains continually fermented the sugars like typical Morgan strains, not counting a late fermentation of sucrose in strains I and V. Two strains, however (II and IV), had altered their characters so much, that they no longer could be considered Morgan strains, having lost the production of gas in glucose and acquired the faculty of producing acid in mannitol and sucrose. Culturally they had accordingly to be considered as dysentery-strains, a classification that was so much more natural as both these strains on the second examination had lost their motility as well.

TABLE 1

Result of second cultural examination

STRAINS	MANNITOL	MALTOSE	GLUCOSE	SUCROSE	LITMUS WHEY	INDOL
I	0^{1-25}	0^{1-25}	$A + G^{1-25}$	0^{1-13} A^{14-25}	0^{1-25}	+
II	0^{1-3} A^{4-25}	0^{1-25}	A^{1-25}	0^{1-3} A^{4-25}	0^{1-25}	+
III	0^{1-25}	0^{1-25}	$A + G^{1-25}$	0^{1-25}	0^{1-25}	+
IV	A^{1-25}	0^{1-25}	A^{1-25}	0^{1-7} A^{8-25}	0^{1-2} A^{3-25}	+
V	0^{1-25}	0^{1-25}	$A + G^{1-25}$	0^{1-5} A^{6-25}	0^{1-25}	+
VI	0^{1-25}	0^{1-25}	$A + G^{1-25}$	0^{1-25}	0^{1-25}	+
VII	0^{1-25}	0^{1-25}	$A + G^{1-25}$	0^{1-25}	0^{1-25}	+

0, no fermentation.
A, acid.
A + G, acid and gas.
Figures signify the day of the examination.

In litmus whey all the typical strains grew without production of acid, while one of the two dysentery like strains produced acid in this medium. All the strains produced indol in beefbroth on the second examination.

Thus it is obvious that the greater part of the Morgan strains have kept their fermenting faculties unaltered after one and a half years of growth on artificial media, while one typical strain had reverted culturally to the characters of the dysentery bacilli.

AGGLUTINATION TESTS

Immediately after isolation of the Morgan strains agglutination tests had been carried out in serum from patients III, IV, VI, VII, VIII, and IX with the homologous Morgan strain and with *B. typhi*, *B. paratyphi* A and B dysentery I, II and III and always with a negative result.

Sera had been produced by injection in rabbits of heated agar slope cultures emulsified in saline solution, and these sera had been tried against the homologous and heterologous strains.

TABLE 2

Agglutination tests

Lowest dilution; in first examination 1: 50; in second 1: 5

STRAIN	IMMUNE SERUM PRODUCED AGAINST STRAIN						
	I	II	III	IV	V	VI	VII
I	1600–1600	0–0	0–0	0–0	0–0	0–0	0–0
II	0–0	6400–6400	0–0	0–0	0–5	0–0	0–0
III	0–0	0–0	3200–3200	0–0	0–0	0–0	0–0
IV	0–5	0–5	0–5	6400–6400	0–0	0–0	0–0
V	0–0	0–10	0–0	0–0	6400–6400	800–0	0–0
VI	0–40	0–20	0–20	0–0	0–0	3200–3200	0–0
VII	0–0	0–0	0–0	0–0	0–0	0–0	1600–1600
VIII	0	0	0	0	0	0	0
IX	0	0	0	0	0	0	0

Seven months later the agglutination test was repeated to find out whether the growth on artificial media had altered the reaction or not.

The result of these tests are shown in table 2.

The first column of figures gives the result of the first examination, the second column that of the second one.

It will be noted that all the strains tested show a high agglutination titre in the homologous sera, but none or very low ones in the heterologous sera. Only strain V shows some degree of agglutination in a heterologous serum from strain VI, but this reaction is completely extinguished seven months later.

One year after this examination there were produced new sera, living bacilli being used for the injection. The result of this

third examination was exactly the same as that of the second test.

Thus it is obvious that the bacillus of Morgan neither causes the production of agglutinins in the serum of patients, in whose intestines the bacillus lives, nor are the various strains of this bacillus effected by the agglutinins of other similar strains.

FIXATION OF COMPLEMENT

Corresponding to the agglutination test cross examinations of the fixation of complement by the various strains and sera have been carried out.

The result is given in table 3.

TABLE 3

Fixation of complement

STRAIN	IMMUNE SERUM PRODUCED AGAINST STRAIN						
	I	II	III	IV	V	VI	VII
I	0.0001	0	0	0	0	0	0.1
II	0	0.0032	0	0	0	0	0.2
III	0.05	0	0.0001	0	0	0	0.1
IV	0	0	0	0.0001	0	0−	0.05
V	0	0	0	0	0.0032	0	0
VI	0	0	0	0.0016	0	0.0002	0.0125
VII	0	0	0	0	0	0	0.0016

The titres of complement fixation in homologous strains and sera are always high, while the heterologous strains and sera usually do not cause any absorption of complement. Only one strain, VI, is found to give a fairly high titre when tried with a heterologous serum from strain IV. Titres such as 0.2 and 0.1 are too low to be counted and can hardly be taken as a significance of any relation between strains.

RÉSUMÉ AND DISCUSSION

Nine cases of diarrhoea or dysentery-form colitis are discussed. Two of these ended fatally and the post-mortem examination showed the existence of a severe colitis.

From these cases of intestinal disturbance typical strains of the bacillus no. I of Morgan were isolated, while it was impossible to discover the presence of other pathogenic germs.

Seven of these strains have been studied immediately after isolation as well as after one and a half years of growth on artificial media, and most of them altered their characters considerably during this time. Thus while only one strain was immobile on isolation, only one had kept its faculty of motility at the end of the examination. Further, two strains had lost their power of producing gas in glucose and had achieved the faculty of producing acid in mannitol and sucrose. As these strains had also lost their motility they could culturally no longer be recognized as belonging to the Morgan group, but had to be looked upon as dysentery strains.

Serologically there could not be detected any relation between these strains and other pathogenic microbes such as typhoid, paratyphoid A and B, dysentery I, II and III or colon bacilli. Neither was it possible to find any considerable relation between the various strains themselves, nor did any of the strains show the slightest agglutination in the serum from the patients.

The bacillus no. I of Morgan has hitherto as mentioned before been found and described by a few authors; but it has never been given the place in the bacteriological system where it really belongs.

In considering the question of the identity of this microbe we must first put another question: Is the bacillus of Morgan a microbe *sui generis* or does it consist of several biologically different microbes that only show the same cultural character? It is obvious, that this microbe is not such a well defined germ as the typhoid bacillus, and its characters are in a way not so fixed and stable, since they may alter during the saprophytic growth on artificial media. Yet the fermentative reactions of most of the strains are fairly characteristic even after a long period of saprophytic growth. If therefore one should consider only the cultural characters it is likely that one would look upon the bacillus of Morgan as a special kind of bacillus and put it somewhere in the neighborhood of the colon and the

dysentery bacilli. But if it be so it is a peculiar thing that it is impossible to find any serological connections between strains of the same kind, such as is the case with all the other pathogenic microbes of the intestinal tract. This point is of great importance.

Agglutination is of very little value when it comes to connecting different strains of the colon bacilli. As a rule there is no clear serological connection between colon bacilli from different persons, even if strains are tested that are culturally absolutely alike.

In a very large collection of colon bacilli from calves Christiansen finds that strains of one fermenting type are seldom affected by agglutinating sera produced with other strains from the same fermenting type and that the serological connections are as often found between strains from different fermenting types as within the same cultural type.

Thus it is the rule of the colon bacilli to behave as we have found the Morgan bacilli do. It is therefore natural to conclude that the Morgan bacillus is simply a *Bacterium coli* of a certain fermenting type. Consequently it would be better to give it the Danish name metacolon organism as this name points to the large group of the colon bacilli, while the name of Morgan bacillus gives the idea of a microbe of a certain special type.

The next question to deal with is this:

Is the metacolon bacillus pathogenic and does it cause such severe cases of colitis as those related in this paper? Or does it only occur as a simple saprophyte while the real cause is another, undetected microbe. If we consider the metacolon a pathogenic microbe able to produce a dysentery-form colitis it should be expected that the affected organism would produce antibodies against the homologous strain, especially since the rabbits on being treated with parenteral injections of the microbe very easily produce agglutinins and complement absorbing antibodies. This has, however, never been found to be the case in this investigation, and we cannot therefore bring forth any certain evidence of the pathogenicity of the microbe. It is

not enough that we find the microbe in cases of colitis, as there is nothing to prove that the intestinal flora may not alter considerably under the pathological conditions that take place in the intestines under a severe attack of colitis.

If there be pathogenic as well as non pathogenic specimens of this microbe we have owing to Christiansen's work on the colon bacilli no methods of detecting the pathogenic ones except the direct experiment as to pathogenicity. As the pathogenic colon bacilli show a high degree of diarrhoea producing effect in young calves, it is not unlikely that calves would be the best object for experiments as to the pathogenic action of the metacolon organisms. Such experiments have never been carried out, and for the time being there is nothing to prove that the metacolon organism is more than a saprophytic colon bacillus, that thrives especially well in an inflamed intestine.

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ON METHODS OF ISOLATION AND IDENTIFICATION OF THE MEMBERS OF THE COLON-TYPHOID GROUP OF BACTERIA

STUDY OF THE BACTERICIDAL ACTION OF CR INDICATOR[1]

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In the course of a study of the fermentation of carbohydrates by the members of the colon-typhoid group, we have tried to find an indicator which could be added to the medium to permit direct reading of the hydrogen ion concentration during the progress of fermentation, without necessitating the destruction of the culture. A mixture of China blue and Rosolic acid (CR) seemed to answer this purpose.

Since the time of our first publication on this subject, we have successfully continued to use CR as indicator in our bacteriological studies, and have made several observations some of which are of sufficient importance to deserve special mention. In our original description of this indicator (Bronfenbrenner, 1918) it was stated that its great tinctorial powers permit the use of very dilute solutions of the dyes in the medium, and for this reason the bactericidal properties of the dyes can be reduced to a negligible quantity. So long as we continued working with the bacteria of the colon-typhoid group we found this to be the

[1] This work is a part of the investigation of food poisoning, conducted under the Direction of Dr. M. J. Rosenau, Professor of Preventive Medicine and Hygiene, Harvard Medical School. The investigations were made under the auspices of the Advisory Committee of the National Research Council on the Toxicity of Preserved Foods, and under a grant to Harvard University from the National Canners Association.

ORGANISMS TESTED†	CHINA BLUE				ROSOLIC ACID										CR MIXTURE									
Amount of the stock solution of the dyes in cubic centimeters used per each 100 cc. of medium*	0.25	1.25	2.50	6.25	0.0050	0.0125	0.0250	0.0500	0.1000	0.1500	0.2500	1.2500	2.5000	6.2500	0.0050	0.0125	0.0250	0.0500	0.1000	0.1500	0.2500	1.2500	2.5000	6.2500
Final concentration of the dyes (solid) in grams per each 100 cc. of medium.†	0.0025	0.0125	0.0250	0.0625	0.00010	0.00025	0.00050	0.00100	0.00200	0.00300	0.00500	0.02500	0.05000	0 12500										
1. *B. typhosus*	+	+	+	+							+	+	+	+							+	+	+	+
2. *B. typhosus* MNH 140	+	+	+	+							+	+	+	+							+	+	+	+
3. *B. typhosus* MNH 330	+	+	+	+							+	+	+	+							+	+	+	+
4. *B. typhosus* MNH 438	+	+	+	+							+	+	+	+							+	+	+	+
5. *B. typhosus* MNH 607	+	+	+	+							+	+	+	+							+	+	+	+
6. *B. typhosus* MNH 683	+	+	+	+							+	+	+	+							+	+	+	+
7. *B. typhosus* MNH	+	+	+	+							+	+	+	+							+	+	+	+
8. *B. typhosus*	+	+	+	+							+	+	+	+							+	+	+	+
9. *B. typhosus*	+	+	+	+							+	+	+	+							+	+	+	+
10. *B. typhosus*	+	+	+	+							+	+	+	+							+	+	+	+
11. *B. paratyphosus*	+	+	+	+							+	+	+	+							+	+	+	+
12. *B. paratyphosus* A MNH 294	+	+	+	+							+	+	+	+							+	+	+	+
13. *B. paratyphosus* A MNH 295	+	+	+	+							+	+	+	+							+	+	+	+
14. *B. paratyphosus* A MNH 322	+	+	+	+							+	+	+	+							+	+	+	+
15. *B. paratyphosus* B MNH 18	+	+	+	+							+	+	+	+							+	+	+	+
16. *B. paratyphosus* B MNH 22	+	+	+	+							+	+	+	+							+	+	+	+
17. *B. paratyphosus* B MNH 323	+	+	+	+							+	+	+	+							+	+	+	+
18. *B. paratyphosus* B	+	+	+	+							+	+	+	+							+	+	+	+
19. *B. paratyphosus* B	+	+	+	+							+	+	+	+							+	+	+	+

20. *B. enteriditis* 9	+	+	+	+							+	+	+	0							+	+	+	0
21. *B. enteritidis* 4	+	+	+	+							+	0	0	0							+	0	0	0
22. *B. enteritidis* MNH 234	+	+	+	+					+	+	0	0	0	0							0	0	0	0
23. *B. enteritidis* Dr. O. Teague 1	+	+	+	+							+	+	+	+							+	+	+	+
24. *B. enteritidis* Dr. O. Teague 2	+	+	+	<+							+	+	<+	0							+	+	0	0
25. *B. enteritidis* Dr. O. Teague 3	+	+	+	+							+	+	+	<+							+	+	+	0
26. *B. enteritidis* Dr. O. Teague 5	+	+	+	+							+	+	+	+							+	+	+	+
27. *B. enteritidis* Dr. O. Teague 6	+	+	+	+							+	+	+	+							+	+	+	+
28. *B. enteritidis* Dr. O. Teague 7	+	+	+	+							+	+	+	+							+	+	+	+
29. *B. enteritidis* Dr. O. Teague 10	+	+	+	+							+	+	+	+							+	+	+	+
30. *B. enteritidis* MNH 18	+	+	+	+							+	+	+	+							+	+	+	+
31. *B. enteritidis* MNH 25	+	+	+	+							+	+	+	+							+	+	+	+
32. *B. enteritidis* MNH 26	+	+	+	+							+	+	+	+							+	+	+	+
33. *B. enteritidis* MNH 132	+	+	+	+							+	+	+	+							+	+	+	+
34. *B. enteritidis* Sch	+	+	+	+							+	+	+	+							+	+	+	+
35. *B. suipestifer* PD 48	+	+	+	+					+	+	0	0	0	0							0	0	0	0
36. *B. suipestifer* PD 51	+	+	+	+							+	+	+	+							+	+	+	+
37. *B. suipestifer* PD 72	+	+	+	+							+	+	+	+							+	+	+	+
38. *B. suipestifer* PD 74	+	+	+	+							+	+	+	+							+	+	+	+
39. *B. suipestifer* PD 78	+	+	+	+							+	+	+	+							+	+	+	+
40. *B. cholerae*	+	+	+	+							+	+	<+	0							+	+	0	0
41. *B. dysenteriae* Shiga R. I.	+	+	+	+							+	+	+	+							+	+	+	+
42. *B. dysenteriae* Kitasato Institute	+	+	+	+							+	+	+	+							+	+	+	+
43. *B. dysenteriae* R. I. 84	+	+	+	+							+	+	+	+							+	+	+	+
44. *B. dysenteriae* Flexner R. I.	+	+	+	+							+	+	+	+							+	+	+	+
45. *B. dysenteriae* Flexner Hiss R. I.	+	+	+	+							+	+	+	+							+	+	+	+
46. *B. dysenteriae* Flexner Strong R. I.	+	+	+	+							+	+	+	+							+	+	+	+
47. *B. paracoli* Al 2	+	+	+	+							+	+	+	+							+	+	+	+
48. *B. paracoli* B 6	+	+	+	+							+	+	+	+							+	+	+	+
49. *B. cloacae*	+	+	+	+							+	+	+	0							+	+	+	0

ORGANISMS TESTED†	CHINA BLUE				ROSOLIC ACID										CR MIXTURE									
Amount of the stock solution of the dyes in cubic centimeters used per each 100 cc. of medium*	0.25	1.25	2.50	6.25	0.0050	0.0125	0.0250	0.0500	0.1000	0.1500	0.2500	1.2500	2.5000	6.2500	0.0050	0.0125	0.0250	0.0500	0.1000	0.1500	0.2500	1.2500	2.5000	6.2500
Final concentration of the dyes (solid) in grams per each 100 cc. of medium†	0.0025	0.0135	0.0250	0.0625	0.00010	0.00025	0.00050	0.00100	0.00200	0.00300	0.00500	0.02500	9.05000	0.12500										
50. *B. acidi-lactici*	+	+	0	0			+	+	0	0	0	0	0	0		+	+	+	0	0	0	0	0	0
51. *B. aerogenes*	+	+	+	+					+	+	+	+	0	0		+	+	+	+	+	+	+	0	0
52. *B. proteus*	+	+	+	+							+	+	+	+							+	+	+	+
53. *B. alcaligenes*	+	+	+	+							+	+	0	0							+	+	0	0
54. *B. fluorescens*	+	+	+	+							+	+	+	+							++	+	+	+
55. *B.* X 197	+	+	+	+							+	+	+	+							++	+	+	+
56. *B.* X 407	+	+	+	+							+	+	+	+							+	+	+	+
57. *B.* X 611	+	+	+	+							+	+	+	+							+	+	+	+
58. *B. mesentericus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	<+	0	0	0	0	0
59. *B. subtilis*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	<+	0	0	0	0	0
60. *B. vulgatus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	<+	0	0	0	0	0
61. *B. xerosis*	+	+	+	+	+	+	+	0	0	0	0	0	0	0	+	<+	<+	0	0	0	0	0	0	0
62. *B. pneumoniae*	+	+	+	+					0	0	0	0	0	0	+	+	+	+	<+	0	0	0	0	0
63. *M. flavus*	+	+	+	0				+	+	0	0	0	0	0			+	+	+	0	0	0	0	0
64. *M. tetragenus*	+	+	+	+	+	0	0	0	0	0	0	0	0	0										
65. *Staphylococcus albus*	+	+	+	+	+	+	+	+	+	<+	0	0	0	0			+	+	+	0	0	0	0	0
66. *Staphylococcus albus*	+	+	+	+	+	+	+	+	+	0	0	0	0	0	+	+	+	0	0	0	0	0	0	0
67. *Staphylococcus albus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	<+	0	0	0	0	0	0
68. *Staphylococcus albus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	<+	0	0	0	0	0	0

69. *Staphylococcus albus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
70. *Staphylococcus albus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
71. *Staphylococcus albus*	+	+	+	+	+	+	+	+	<+	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
72. *Streptococcus*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
73. *Streptococcus fecalis*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
74. *Streptococcus fecalis*	+	+	+	+	+	+	+	+	<+	0	0	0	0	0	+	+	+	+	0	0	0	0	0	0
75. *Sarcina lutea*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	0	0	0	0	0	0	0
76. *Sarcina lutea*	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	+	+	0	0	0	0	0	0	0
77. *Staphylococcus aureus*	+	+	+	+	+	+	+	+	+	<+	0	0	0	0	+	+	+	+	+	0	0	0	0	0
78. *Staphylococcus aureus*	+	+	+	+	+	+	+	+	+	0	0	0	0	0	+	+	+	+	+	0	0	0	0	0

* The stock solutions of the dyes used for this experiment contained respectively 1 per cent of China blue in 50 per cent alcohol, 2 per cent rosolic acid in 50 per cent alcohol, and equal volume of double concentration of these dyes in the CR mixture. The concentration of the dyes recommended for the preparation of the ordinary media for fermentation tests is placed between the heavy lines.

† Strains of bacteria marked "MNH" were obtained from the Museum of Natural History, New York. Those marked "PD" were obtained from Parke, Davis and Company, Detroit, and those marked "RI" were obtained from the Rockefeller Institute, New York.

case. As soon, however, as we attempted to use this indicator in connection with other bacteria, we found that it exhibited decided antiseptic action for some of them, even when used in lower concentration than that which was found to be non-inhibitory for the colon group.

This difference in the relative sensitiveness of various bacteria to the antiseptic action of CR at once suggested the necessity of further study of this indicator if it is to be incorporated in the culture medium.

METHOD OF STUDY

With this end in view, a number of bacteria were tested against various concentrations of China blue, Rosolic acid and CR (which is a mixture of the two), in order to determine the respective concentrations affecting growth. In all 78 strains were tested. Of these, 43 were pathogenic members of the Colon-Typhoid-Dysentery group; *B. typhosus*, *B. paratyphosus* A and B, *B. dysentereae* Shiga and Flexner, *B. enteritidis* and *B. suipestifer*, a number of strains of each being tested. The rest of the cultures, 35 in number, were cocci of various types, Gram positive spore bearers, *B. coli*, *B. proteus*, *B. cloacae*, *B. fluorescens*, *B. alkaligenes*, and several strains of an unidentified Gram negative bacillus. Most of these non-pathogenic strains were freshly isolated by us especially for this study, from feces and other sources, and have not been definitely identified. A few were obtained from the Museum of Natural History.

The China blue used was manufactured by Grubler and the Rosolic acid by Merck. Stock solutions were made up in 50 per cent alcohol, and contained respectively 1 per cent China blue, 2 per cent Rosolic acid and in the CR mixture 1 per cent China blue plus 2 per cent Rosolic acid.

The medium used was the same in all cases, a solution of 1 per cent Difco peptone and 0.5 per cent chemically pure sodium chloride in distilled water. The dyes in alcoholic solution were added in appropriate amounts to this medium which was then placed in

test tubes and autoclaved.[2] After incubating over night to test for sterility, each tube was inoculated by means of a sterile capillary with two drops of a well-grown peptone water culture of the organism to be tested. Incubation was carried on at 37.5° for a maximum of four days. A control tube of the same medium without dye was also inoculated at the same time, and served as a standard by means of which comparative amounts of growth could be estimated.

The composition and method of preparation of this medium is exactly the same as that of the stock media used in this laboratory for the determination of carbohydrate fermentation, with the exception that in this case carbohydrate was not added.

The media containing China blue were perfectly clear. The media with 0.125 per cent and 0.05 per cent concentrations of Rosolic acid were turbid at times, and a slight precipitate was often present. In these cases the percentage of dye actually dissolved in the medium is of course not definitely known.

RESULTS

China blue, in the highest concentration used, 0.0625 per cent, had no inhibiting action on the majority of the organisms which we tested. *Bacillus acidi-lactici* was, however, completely inhibited by 0.025 per cent, and the *Micrococcus flavus* by a 0.0625 per cent concentration of this dye. A few of the other organisms were slightly inhibited by a concentration of 0.0625 per cent.

[2] The media with the greatest concentration of the dye (0.0625 per cent) contained about 3 per cent of alcohol by volume, and it was suspected that the alcohol might have some effect upon bacterial growth. However, practically all the organisms which we tested grew as well on media containing China blue and 3 per cent of alcohol as on media without the dye or the alcohol. There was, therefore, practically no inhibition from the alcohol. According to Kligler (1918) 4 per cent alcohol is required to inhibit the growth of B. dysenteriae, a greater concentration being required to inhibit the other bacteria which he tested. In our work, the media containing the second highest concentration of dye (0.025 per cent) contained a little more than 1 per cent of alcohol. It is probable that the effect of the alcohol present may be neglected for our purposes.

Rosolic acid, however, even in a concentration of 0.005 per cent was markedly inhibitory for many of the organisms tested. Furthermore, this inhibition was almost strictly selective. None of the Gram positive organisms which we tested grew in peptone water containing 0.005 per cent of this dye, and only a few grew to a slight extent when the concentration was one-half of this amount. On the other hand, all the Gram negative organisms which we tested, with the exception of two strains: namely, *B. enteritidis* "M. N. H. 234" and *B. suipestifer* "P. D. 48" grew well on a medium containing 0.005 per cent Rosolic acid.[3] The great majority of these forms grew on a medium containing 0.05 per cent, and most grew on that containing 0.125 per cent of this dye. Fifteen other strains of *B. enteritidis* tested by us grew on a medium containing 0.005 per cent of Rosolic acid. Of these, fourteen grew on 0.05 per cent and twelve on 0.125 per cent of Rosolic acid. Four cultures of *B. suipestifer* grew on 0.125 per cent of Rosolic acid.

The addition to the medium of both dyes, composing the indicator CR, gave practically the same results as those given by Rosolic acid in the same concentration, as might be expected from the inertness of China blue alone.

These results correspond rather closely to those obtained by Churchman (1912, 1913), and Krumwiede and Pratt (1914) and Kligler (1918) who used various dyes of the triphenylmethane group, such as brilliant green, methyl and crystal violet, etc. However, these workers found that the growth of *Bacillus dysenteriae* was almost as readily inhibited by the dyes tested as that of Gram-positive bacteria. Rosolic acid is quite different from the other dyes in that the various strains of *Bacillus dysenteriae*

[3] The *B. enteritidis* strains which we used are apparently identical with those used by Churchman (1912, 1913) and Krumwiede and Pratt (1914) in their work on gentian violet and on various green dyes respectively. It is interesting to note that strain "M.N.H. 234" which persistently failed to grow on gentian violet and on the green dyes also failed to grow on Rosolic acid.

are quite as resistant to its action as are the other Gram-negative bacteria.[4]

CONCLUSIONS

The bactericidal power of CR (China blue-Rosolic acid) is due entirely to the action of Rosolic acid. Moreover, the inhibition of growth seems to be directed only against Gram-positive bacteria. Almost all Gram-negative bacteria tested grow readily on a medium containing twenty-five times the amount of Rosolic acid which is inhibitive for Gram-positive organisms. This apparent selective action of Rosolic acid, coupled with its failure to inhibit the growth of *B. dysenteriae* render this dye particularly suitable for the preparation of selective media to be used for the isolation of intestinal bacteria.

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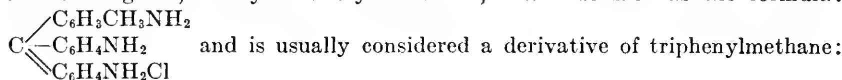
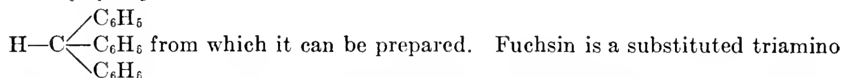
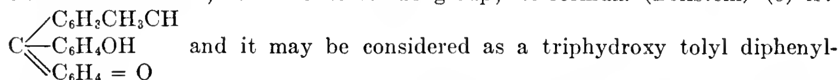
[4] In this connection it is of interest to compare the chemical constitution of rosolic acid with that of fuchsin, which is closely related to brilliant green, malachite green, methyl and crystal violet, etc. Fuchsin has the formula:



and is usually considered a derivative of triphenylmethane:



from which it can be prepared. Fuchsin is a substituted triamino triphenylmethane, namely, a triamino tolyl diphenylmethane (5). Rosolic acid, on the other hand, contains no amino group; its formula (Beilstein) (6) is:



and it may be considered as a triphydroxy tolyl diphenylmethane. It differs from fuchsin in containing hydroxy radicals in place of amino radicals. It would seem, therefore, that this substitution is responsible for the difference in action of the respective dyes upon the Dysentery bacillus.

EXTRACTS OF PURE DRY YEAST FOR CULTURE MEDIA

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Dry yeast has been extensively used for the preparation of media in these laboratories for the past five years with excellent results; but has received little if any attention from other bacteriologists in this country.

Eldredge and Rogers (1914) in 1913 used an extract from dry yeast with peptone and phosphate for studying the fermentations of cheese cultures because they desired a sugar free broth in which their cultures would grow readily. For the same reason yeast peptone broth was used by one of the authors (Ayers, 1916) during 1915 and is still being used in studying the fermentations of the streptococci. This sugar free broth was found to be of particular value in supporting the growth of pathogenic streptococci which would not grow in extract broth or meat infusion broth from which sugar had been removed by fermentation. Yeast peptone broth was also used by Miss Evans (1918) in a study of the streptococci concerned in cheese ripening.

Extracts from dry yeast have been used so successfully in other bacteriological work in the Research Laboratories of the Dairy Division that it is believed special attention should be given to their possibilities. It is, therefore, the purpose of the writers to mention briefly in this paper some of the ways in which yeast extracts have been used in recent experiments.

DRY FRESH YEAST

In the work previously noted, an imported dry yeast preparation was used about which nothing was known as to the methods of manufacture. The fact that, with peptone, it gave a broth

which was sugar free and supported the growth of bacteria, among which were pathogenic streptococci, made it very useful until the preparation was found in the year of 1918 to contain starch which was evidently added during the process of manufacture. The usefulness of extracts of this preparation for fermentation tests was, therefore, ended although for general bacteriological purposes it was still valuable.

A dry yeast was then obtained through the kindness of a manufacturer[1] in this city which has given valuable and interesting results. The dry yeast consists of pure washed fresh yeast and was found to contain about 6.0 per cent of moisture.

In our experiments, the value of yeast extracts for replacing meat extract have been given most attention, therefore they have been used largely in combination with peptone. Various types of streptococci were used to test the value of the yeast medium, since they are among the most difficult of the bacteria to grow in culture media. Many other organisms were found to grow more readily than the streptococci.

The yeast extract was prepared by mixing 1 per cent of dry fresh yeast with cold distilled water and after being allowed to stand 10 minutes was steamed in an Arnold sterilizer for thirty minutes, and then filtered. Considerable difficulty was encountered in obtaining a clear filtrate, the liquid always appearing hazy. The haziness could be removed by the addition of kieselguhr and a second filtration. Probably Merck's dialyzed iron could be used to advantage for clearing the extract as has been mentioned by Eberson (1919), who pointed out the value of a yeast medium for prolonging the life of the meningococcus.

It was found that the difficulty in obtaining a clear extract could be overcome by heating the dried yeast before use at 105°C. for four or five hours. After the first steaming, the extract appeared cloudy upon filtration but when the reaction was adjusted to about pH 7.7 and the extract steamed a second time, a precipitate was produced which could be readily filtered out leaving a clear solution.

[1] This opportunity is taken to express our thanks to R. L. Corby and Miss Glasgow of the Corby Company, for supplying us with preparations of dry yeast.

To the extract from 1 per cent of dry yeast, 1 per cent peptone was added, the reaction adjusted to pH 7.5, and the medium then heated and filtered, tubed and sterilized.

Most of the streptococci grew readily in this medium and some, vigorously. It was observed, however, that the hydrogen ion concentration changed from pH 7.5 to from pH 6.0 to 6.3 and it was evident that the dried fresh yeast contained some fermentable material.

This preparation of dry fresh yeast could not be used for fermentation tests because of the change in hydrogen ion concentration which occurred without the presence of a test sugar or other similar substance. The yeast peptone medium described above is lightly buffered for this is necessary in studying fer-

TABLE 1

	TOTAL NITROGEN	AMINO NITROGEN	PROTEIN OTHER THAN AMINO ACIDS	REDUCING SUGAR	pH
	per cent	*per cent*	*per cent*	*per cent*	
One per cent extract of dry fresh yeast*	0.0209	0.0040	0.1078	0.008	6.3

* Moisture in dry yeast 5.9 per cent.

mentations, at least with the streptococci; and this is probably true of other bacteria. It has been found that the limiting hydrogen ion concentration of some of the streptococci is from about pH 6.2 to 6.5 so it is at once evident that a medium cannot be used in which the hydrogen ion concentration without a test sugar may reach the pH values mentioned. The yeast peptone medium could be easily buffered so as to take care of the increase in acidity but then the fermentations of such organisms as those previously mentioned would be entirely missed.

The extract of 1 per cent of dry fresh yeast contained nitrogenous material including amino acids, some reducing sugar, and other fermentable material as is shown in table 1.

Although a 1 per cent yeast extract made from dry fresh yeast contained a relatively small amount of amino acid it evidently contained something valuable to support the growth of deli-

cately growing streptococci. The amount of reducing sugar found would not give sufficient acid to account for the change in pH previously noted so there was doubtless other fermentable material present not determined as a reducing sugar.

The yeast extract from dry fresh yeast made a good medium for many bacteria but was of most value when used with peptone. It was not, however, satisfactory for fermentation tests on account of the fermentable material present. In this connection, it should be mentioned that both pressed yeast and yeast extracts have been found by Ickert (1918) to be a cheap and suitable substitute for meat extract in ordinary media.

DRY AUTOLIZED YEAST

It is well known that yeast readily undergoes autolysis which as Vansteenberge (1917) pointed out may operate in two ways. The nitrogenous material undergoes proteolysis in which peptones, amino acids, and other products are formed, while the hydrocarbon material, principally glycogen, is transformed into glucose, then into CO_2 and alcohol.

Vansteenberge also showed that an extract from autolized yeast contained much more nitrogenous material than a similar extract from fresh yeast and that such an extract was a suitable medium for the growth of yeast and lactic acid bacteria. An extract of autolized yeast has been found by Dienert and Guillerd (1919) to be a cheap and satisfactory medium for the growth of *B. coli.*

Kligler (1919) has recently advocated the use of yeast autolysate as a culture medium. He used brewer's yeast which he autolized in the laboratory. The use of brewer's yeast as he has suggested presents two serious difficulties. First, it is difficult to obtain fresh brewer's yeast in many parts of the country and second, brewer's yeast undergoes autolysis so rapidly that if it was shipped in a moist condition it would arrive at its destination in various states of decomposition depending upon the time and temperature during transit. It would doubtless be impossible to make uniform media in different laboratories with such mate-

rial. The use of dry fresh yeast seems to overcome these difficulties.

Our object in obtaining an autolized yeast was not so much to increase the protein content of the extract as to eliminate the fermentable material. A dry autolized yeast was prepared by one of the yeast manufacturers in accordance with our suggestions. Fresh yeast was autolized for twenty-three hours at a temperature of from 45° to 50°C., then dried at a low temperature. A medium made of a 1 per cent extract of this dry autolized yeast and 1 per cent peptone adjusted to pH 7.4 proved to be satisfactory for growth of many streptococci and the change in the hydrogen ion concentration was from pH 7.4 (control) to pH 7.0. This slight change in acidity showed that there was

TABLE 2

	TOTAL NITROGEN	AMINO NITROGEN	PROTEIN OTHER THAN AMINO ACIDS	pH
	per cent	*per cent*	*per cent*	
One per cent extract in dry autolized yeast*.	0.0616	0.0274	0.2192	5.6

* Moisture in dry autolized yeast 5.96 per cent.

some fermentable material left after autolysis but not enough to interfere in any way with fermentation tests. The figures in table 2 show an analysis of the extract.

From a comparison of the figures in tables 1 and 2, it is apparent that the 1 per cent extract from autolized yeast contained about twice as much protein material (not including amino acids) and about seven times as much amino nitrogen as the 1 per cent extract from dry fresh yeast.

To us, the most interesting and valuable feature was the reduction in fermentable material. No figures on reducing sugar are given in table 2 because of substances present in the extract from autolized yeast which interfered with the sugar test. The changes in pH by bacterial growth, however, showed conclusively that fermentable material was greatly reduced, in fact, to a negligible amount.

Doubtless, the increase in amino acids and other nitrogenous material makes autolized yeast a more valuable medium than dried fresh yeast for many purposes. This is, of course, true when fermentations are being studied. However, in combination with peptone when abundant growth alone is desired, the extract of dried fresh yeast and peptone medium showed more vigorous growth with all organisms tested.

As far as our experiments are concerned, the value of an autolized yeast peptone broth lies in the fact that it gives a sugar free medium in which streptococci, particularly the pathogenic types, will grow readily. As most of our pathogenic streptococci would not grow in the ordinary extract peptone broth, the yeast medium proved to be of great value in fermentation tests.

Since dried *autolized* yeast could not be obtained commercially, experiments were conducted to determine if the dry fresh yeast could be autolized in the laboratory. Ten grams of dry fresh yeast were mixed with 200 cc. of distilled water and incubated at 42°C. for twenty-three hours. The dry yeast used had not been heated to 105°C. but was in the condition received from the manufacturer. After incubation this yeast was heated to 100°C. for one-half hour then cooled and filtered. The filtrate which was turbid was then cleared by the addition of kieselguhr and a second filtration. The extract was then made up to the proper amount to represent 1 per cent of dried yeast. One per cent peptone was added and the reaction corrected to pH 7.5 as in the media previously described. Analysis showed that the extract from the autolized yeast was practically identical with that made from the dried autolized yeast mentioned above and it acted the same in cultural tests.

It is evident that the dried yeast preparation used in this work can be readily autolized in the laboratory and can be so handled as to give uniform results.

Yeast manufacturers are urged to give attention to the preparation of autolized yeast for use in bacteriological work. Dernby (1918) has been able to show that in yeast cells there must be at least three different groups of proteolytic enzymes acting at different hydrogen ion concentrations: pepsin, splitting proteins

into peptones at about pH 4; tryptase, splitting peptones into peptides at about pH 7.0; and ereptase, splitting peptides into amino acids at about pH 7.8. It appears, therefore, that it should be possible to control the process of yeast autolysis so that dried preparations would contain various amounts of peptones, peptides, and amino acids, according to the method of autolysis. Such preparations should be valuable in studying the nutrition of bacteria and should be relatively inexpensive.

YEAST DIGESTED WITH ACID

Other methods of obtained extracts from dried fresh yeast were tried as, for example, heating with acid and alkali. The extract of yeast treated with acid gave the best results and will be the

TABLE 3

	TOTAL NITROGEN IN EXTRACT	AMINO NITROGEN IN EXTRACT	PROTEIN OTHER THAN AMINO ACIDS	FERMENTABLE MATERIAL
	per cent	*per cent*	*per cent*	
One per cent yeast extract from:				
Dried fresh yeast	0.0209	0.0040	0.1078	Small amount
Dried autolized yeast	0.0616	0.0274	0.2192	Trace
Dried fresh yeast treated with acid	0.0491	0.0069	0.2692	More than in extract from dried fresh yeast

only one discussed. To obtain the extract, 10 grams of dried fresh yeast were added to 400 cc. of distilled water together with 50 cc. *N* HCL. This was then heated in an autoclave at 14 lbs. pressure for 30 minutes. After heating, 50 cc. of *N* NaOH was added to the yeast extract which was allowed to cool before filtering. A clear filtrate was obtained. The reaction which was slightly above pH 7.0 was adjusted to pH 7.5. This yeast extract was made up to 1000 cc. with distilled water to make a 1 per cent solution. The extract when used with 1 per cent peptone showed luxuriant growth with cultures of streptococci, but from the change in pH 7.5 to about pH 5.7 it was evident that

the acid treatment had increased the fermentable material. This was also indicated by the sugar analysis which, however, did not give consistent results, due to interfering substances. Analysis further showed that the extract made from acid treated yeast was somewhat different from the extract from either the dry fresh yeast or the dry autolized yeast. The difference is shown in table 3.

The most noticeable difference between the three yeast extracts was the increase of amino acids and total nitrogen in the autolized yeast extract over the fresh yeast extract and the increase in protein (other than amino acids) in the acid treated yeast extract without much increase in amino nitrogen.

Extracts made from yeast treated with acid may give good results in many lines of bacteriological work where the presence of fermentable material is not undesirable.

YEAST DIGESTED WITH PEPSIN

An extract was also made with dry fresh yeast which was partly digested with pepsin. Ten grams of dry yeast was added to 100 cc. of distilled water with 0.1 gram of pepsin. Sufficient HCL was then added to bring the reaction to about pH 4.4. The mixture was then incubated at 40°C. for twenty-four hours, steamed for thirty minutes, filtered, diluted to 100 cc. with distilled water, and the reaction adjusted to pH 7.5. This extract without peptone supported the growth of delicately growing streptococci so well that the value of this kind of yeast extract appears promising for many purposes other than fermentation tests.

SUMMARY AND CONCLUSIONS

The value is emphasized of using extracts made from dried pure yeast, that is, yeast which has been washed and then dried at a low temperature without the addition of starch or other fillers. This extract may be used alone or as a basis for more complicated media when necessary.

Extracts of pure yeast contain, besides amino acids and other proteins, fermentable material in small amounts, probably pres-

ent in the yeast cell, which makes them valuable for general bacteriological purposes. The fermentable material probably stimulates growth but renders the extract valueless as an ingredient of media for fermentation tests.

For use in a medium for fermentation tests, the dry fresh yeast, at least the preparation used in our work, may be autolized in the laboratory. In this process all but a trace of the fermentable material is destroyed and the small amount left does not interfere in any way with the determination of the fermentation of test substances even in a lightly buffered medium.

Yeast extracts can be made by treatment with acid or alkali or by peptic or tryptic digestion. The value of extracts made by digestion with HCL and pepsin are only indicated by the experiments reported but the results are promising.

Dry fresh yeast can be readily obtained and when prepared under definite conditions should be uniform in composition. Its keeping quality and ease with which it can be procured apparently make it much more valuable than ordinary undried brewer's yeast. Whether extracts from fresh brewer's yeast possess any advantages over extracts from dry yeast as a culture medium remains to be determined.

Dry autolized yeast has been prepared for us by a yeast manufacturer which in combination with peptone proved to be an excellent medium for the growth of streptococci, particularly pathogenic types, which would not grow in other sugar free media. This medium was practically free of fermentable material and for this reason was valuable for fermentation tests.

It is not the purpose of this paper to convey the idea that the yeast extracts can be used to replace all other ingredients in media. While they can be used to advantage both alone and as a basis for more complicated media, it has been found that they are more useful with some organisms than with others. However fragmentary our knowledge of the value of yeast extracts for culture media, the results of other investigators and our own experience clearly point to the desirability of giving much more attention to what appears to be a very valuable subject.

Since yeast has been shown to contain some of the vitamines highly essential in the nutrition of the higher forms of animal life, it is obvious that the use of yeast extracts may offer a fertile field for the study of vitamines in bacterial nutrition.

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A MODIFIED PROCEDURE FOR THE PREPARATION OF TESTICULAR INFUSION AGAR

GUY W. CLARK

From the Research Department of the Cutter Biological Laboratories, Berkeley, California

Received for publication, September 16, 1919

Different workers in the culture media department of this laboratory have experienced some difficulty in preparing, according to Hall's (1916) method, testicular infusion agar that would regularly provide a medium suitable for the growth of the gonococcus in the production of vaccines. Without any effort to try other media, experimental work was carried out with the view of so modifying the above mentioned method that a satisfactory medium could be regularly obtained.

From the results obtained with nine strains of gonococci (some rather old laboratory cultures, others recently isolated), upon several lots of testicular infusion agar, the following procedure is suggested:

Remove and discard the *tunica vaginalis* from fresh beef testicles, rinse in running water and grind.

Mix 500 grams of ground testicle and 500 cc. of distilled water and infuse overnight at room temperature.

The following morning heat to 50°C. and keep warm for one hour (the value of this step is questionable), heat in a steam kettle or double boiler, stirring constantly, until the proteins are completely coagulated and tend to collect in large flocculi. This material should never be heated over a bare flame as it scorches easily. Strain through coarse cloth and, if necessary, add distilled water to bring the volume to 750 cc. To the warm liquid add 20 grams of Parke, Davis and Company's peptone and 3 grams of monobasic sodium phosphate ($NaH_2PO_4.H_2O$), stir to dissolve and while warm (not more than 40°C.), adjust to pH 7.4 to 7.8, using the phosphate mixtures of Sörensen (1912) or Clark and Lubs (1916) as comparative standards of pH, with phenol red as an in-

dicator. The titration tube and comparator of Hurwitz, Meyer and Ostenberg (1915, 1916) have been found very convenient, using 3 to 5 cc. of media and 0.05 normal NaOH. From the results of this titration is calculated the amount of 2 normal NaOH necessary to give the desired pH to the entire lot of medium. After heating to boiling the reaction is again determined and more precisely adjusted.

Now add 25 to 30 grams of agar which has been melted in 250 cc. of distilled water (soak the finely chopped agar in the water and autoclave just long enough to melt it before adding to the infusion). Finally add 5 grams of glucose, mix thoroughly and distribute into desired containers.

Autoclave tubes at 15 pounds for twenty minutes, being sure to displace the air in the autoclave. Before slanting, rotate the tubes to make the contents uniform.

The procedure just outlined differs essentially from that of Hall (1916) in the following points:

1. The medium is titrated and adjusted to a definite hydrogen ion concentration instead of the old method of titrating to "degrees of acidity" with phenolphthalein as an indicator. Several writers (Hurwitz, Meyer and Ostenberg, 1915, 1916; Clark, 1915) have recently shown that the last method of adjusting the reaction of culture media is not a reliable procedure. For a detailed discussion of this subject reference is made to the article by Clark (1915).

2. The glucose is added last. This is done in order to minimize the caramelizing effects of a hot, alkaline solution. Even though the final concentration of hydroxyl ions is small, considerable caramelization may result when strong NaOH is added in adjusting the reaction.

3. Reduction of the time of sterilization which is of general value to all media and especially those containing carbohydrates. Twenty minutes was found to be the minimum for different sized tubes of this medium. Now and then a lot came through grossly contaminated which is to be attributed to infected testicles. On one occasion a sporulating organism was encountered which resisted two of the twenty minute periods in the autoclave. Since it is not easy to detect these infected testicles it seems better to make up small lots (not more than 5 to 7 liters) of this medium.

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DESCRIPTION OF AN APPARATUS FOR OBTAINING SAMPLES OF WATER AT DIFFERENT DEPTHS FOR BACTERIOLOGICAL ANALYSIS

FRANK C. WILSON

From the Biological Laboratory of the Wisconsin Geological and Natural History Survey, Madison, Wisconsin

Received for publication October 23, 1919

A number of years ago a biological survey of the waters of Lake Mendota, Wisconsin, was begun by the Wisconsin Geological and Natural History Survey. In this survey it was planned to include a study of the number and types of bacteria. The samples were to be taken at various depths and in all sorts of weather. It was realized that an apparatus which would enable the analyst to collect the samples with the least expenditure of time and trouble would be a great aid in the work.

A review of the literature shows that many forms of deep water samplers have been used. In general these may be divided into two groups: first, the vacuum bulb type; second, the bottle type which consists of a bottle from which the stopper is lifted and the water allowed to run in after the apparatus has been lowered to the desired depth.

The commonest type of the exhausted bulb sampler consists of a thinly blown bulb such as a retort bulb. The bulb is partially exhausted and sealed at the neck. By means of a mechanical device the constricted neck is broken when the apparatus is at the desired depth in the water and the sample of water allowed to pass into the bulb. This type of sampler is unsatisfactory because the bulb must be blown especially for the sampler. Where a considerable number of samples are to be taken it would be difficult to keep a supply of bulbs on hand.

Another type of sampler used, consists of a metal frame-work inclosing a bottle with a ground glass stopper (Abbott, 1915).

A spring clamp is attached to the stopper which is operated by a string so that when the apparatus is at the desired depth in the water the stopper may be lifted. When the bottle is filled, the string is released and the stopper is forced back into position. The disadvantage of this type of sampler is the possibility of contaminating the bottle and stopper near the neck while it is being placed in the metal frame. It is also inconvenient to manipulate two connecting lines—one for lowering the apparatus and one for lifting the stopper. In addition to these points, it is difficult to fasten the stoppered bottle in the metal frame.

An inexpensive substitute for this type of sampler is sometimes used for shallow water work. A glass stoppered bottle is lowered in the water by means of a string and when the desired depth is reached the stopper is lifted slightly by a second string. When the bottle is filled, the string is released and the stopper allowed to slide back into place. The sample may then be drawn up.

For deep-sea work a special apparatus is required. An excellent sampler for deep-sea work is described by Matthews (1913). The apparatus works on a different principle from any of the samplers previously described. The container for the sample consists of a strong glass cylinder which is closed at each end by thick rubber washers secured by metal plates. The cylinder is filled with 95 per cent alcohol before lowering in the water. The alcohol is supposed to kill any bacteria that may be in the sampler and at the same time to prevent the entrance of water before the ends are opened. At the desired depth, the ends of the cylinder are opened by dropping a messenger on the line. The alcohol, being of lower specific gravity than the water, diffuses out almost instantaneously, causing an upward flow of water through the cylinder, after which the second messenger is sent down to close the ends of the cylinder. The sample is drawn up, and the water is siphoned into sterilized bottles before they are brought to the laboratory for analysis. For deep-sea water investigations this apparatus is very useful, but for work on a shallow body of water a less complicated apparatus may be used.

The sampler here described is the one used by the Wisconsin Geological and Natural History Survey in the work on Lake Men-

dota. It is a modification of the apparatus used by Russell (1892) in his investigation of sea water. In the original form, the sampler required two lines for manipulation, but was later changed by Russell so that it required only one. In the survey of Lake Mendota the Russell sampler was tried, but, the frame being of wood,

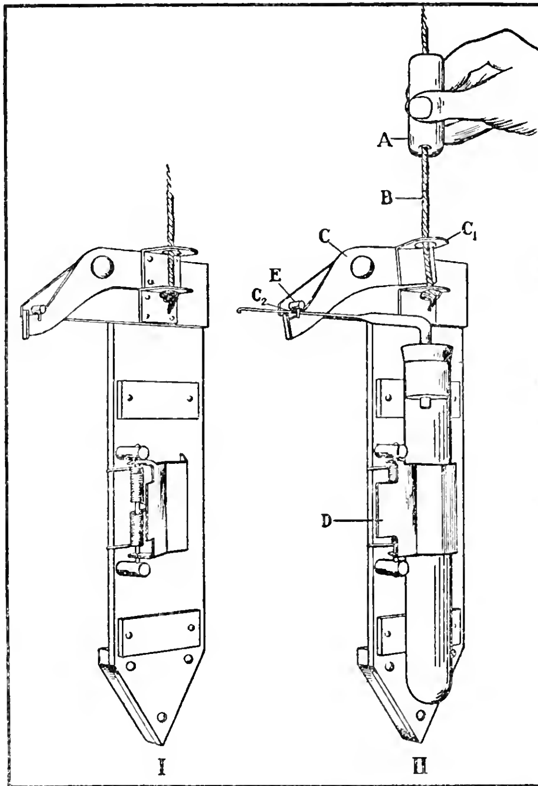

Fig. 1

it required a large weight which made it cumbersome to handle. The modification of this sampler is simple and overcomes many of the disadvantages of the older type. This apparatus with the exception of an exhausted glass tube is made entirely of heavy sheet brass (see fig. I). The sterilized, exhausted tube is attached to the holder by means of a rubber covered spring clamp, *D* (see II, fig. 1,). A brass arm, *C*, operates between the connecting line,

B, at C_1 and the finely drawn-out glass tube at C_2. When the apparatus has been lowered to a desired depth, a small brass messenger, A, is sent down on the connecting line of the sampler. It strikes the lever arm at C_1 and the force is transmitted to the constricted glass tube at C_2, which is broken against the breaking pin, E, and the vacuum destroyed. The sample tube fills rapidly.

The sample tubes are ordinary 2.5 by 20 cm. (100 cc. volume) hard glass test tubes, fitted with a one-hole rubber stopper. A small glass tube bent at a right angle is inserted in the opening in the stopper. The lower end of the glass tube should project through the rubber stopper about half an inch. The other end is heated and drawn out at a certain point, the point being determined by the size of the holder, to a diameter of about 2 mm. so that it may be sealed easily. It is best, of course, to draw out the tube before inserting it in the stopper. The sample tube together with the rubber stopper containing the small glass tube is sterilized in the autoclave. When sufficiently cool, the stoppers should be fitted tightly into the tubes. If the same stoppers are used repeatedly, they should be coated with a mixture of rosin and paraffin after they are sterilized to prevent loss of vacuum. A partial vacuum is produced in the tubes by attaching the filling tube to a vacuum pump. The tube is sealed at the constriction by a flame at the time the air is being exhausted. It is preferable to have only a partial vacuum in the sample tube so that it will not fill entirely with water, as the space left in this way permits the shaking of the water sample before the dilutions are made.

This type of sampler offers several advantages. The tubes are easily and quickly clamped in the frame. They fill quickly when the tip of the filling tube is broken off. The rapidity of operation is shown by the fact that it requires only about ten minutes to draw samples of water from seven different depths—at the surface, 1, 5, 10, 15, 20, and 23 meters. There is no special container required for carrying the samples to the laboratory as they are left in the same tubes in which they are collected. The operation of placing the tubes in the frame may be carried out without touching the tips of the filling tubes with the hands,

thus preventing contamination. With the small opening and the bend in the filling tube, contamination of the sample is almost impossible either from the water while the sample is being drawn or from the air after it leaves the water. Several thorough tests have been made with a tube which has the tip seal broken. Such a tube was lowered to 22 meters in the water and pulled rapidly up and down in an attempt to force water in, but when the apparatus was drawn up there was no water in the sample tube and only a drop in the tip of the filling tube. We may therefore be sure that samples collected in this way contain only the organisms from the water at the desired depths.

The use of one line to lower the apparatus and to break the seal is a decided advantage which the majority of samplers do not possess. The greatest inconvenience we have found with this sampler is the preparation of the tubes. It requires time to prepare the tubes and unless they are handled carefully the tips are easily broken.

In the work on Lake Mendota this sampler has only been used to a depth of 23 meters, but without doubt it could be used to a much greater depth without any influence of hydrostatic pressure. Its use indicates that it is safe up to a depth of 23 meters.

SUMMARY

In this article there has been described a bacteriological water sampler, together with the method for its use. This sampler furnishes a convenient and rapid means for drawing samples of lake water for bacteriological analysis.

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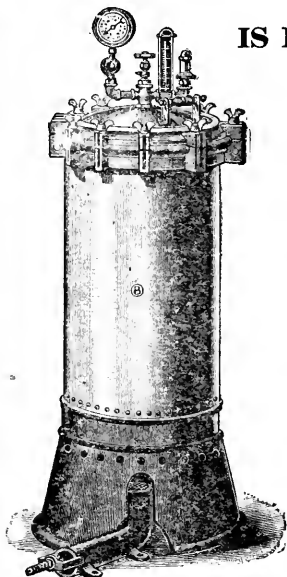

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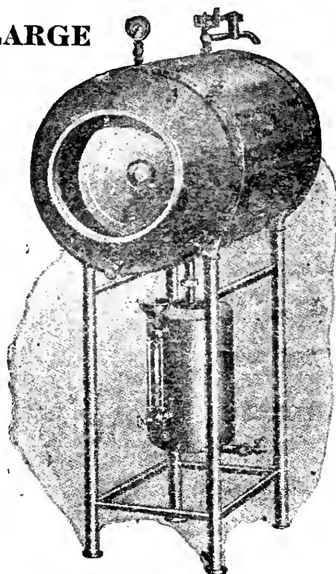

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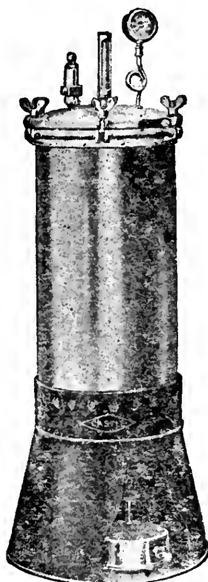

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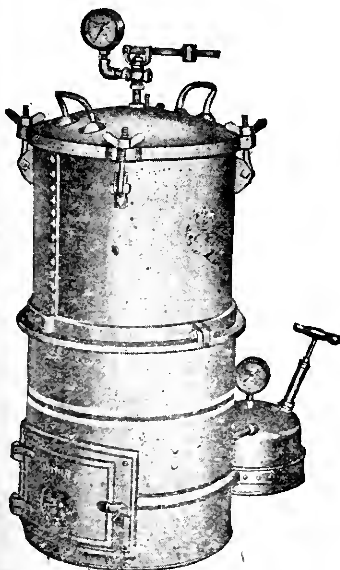

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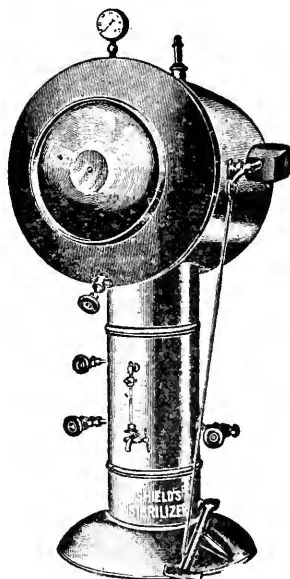

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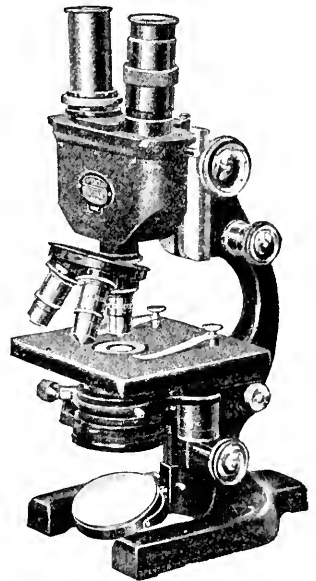

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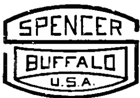

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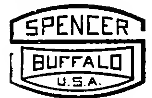

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One of the most striking phenomena of the world war period—through which we have, we hope, now happily passed—is the great stimulation which has taken place in all branches of pure and applied science. While during this half-decade discovery and invention have been especially directed in many scientific fields to the development of more terrible methods of destruction of human life and property, the energies of those in our own profession have been largely turned toward the constructive side, in the alleviation of pain and disease, the control of infections, and the development of means to promote rather than retard the welfare of the individual and the race. We have witnessed the tremendous expansion which has taken place in chemistry, in aeronautics, in war engineering of all kinds, and we have also seen and participated in the vast but perhaps less spectacular developments in our own field of bacteriology—developments which will attain their complete flower and fruition as arts of peace rather than of war. It is only necessary to review the work of the past two or three years, as evidenced by our own former programs, to observe the tremendous impetus that the war has given to medical and public health bacteriology. The somewhat more restricted field of industrial bacteriology and its applications, as in fermentation, soil bacteriology and the preservation and conservation of foods, while less conspicuous, has, however, shared in the general growth of the science that

[1] Address of the President of the Society of American Bacteriologists delivered at twenty-first annual meeting, Boston, December 29, 1919.

has taken place, and gives promise of an even greater development as a result of the demands of commerce and industry. Processes which five years ago were thought of merely as interesting possibilities, such as the production of glycerin and acetone by fermentation, have now been so perfected that they may compete with purely chemical methods. The manufacture of numerous organic bodies is now possible, and agriculture seems likely to receive its next great impetus as a result of advances which will be made in the bacteriological investigations of soils. Food conservation has never occupied so important a position, from the standpoint of world welfare, and here, too, advances of great import have been made. It is of one phase of this subject that I wish especially to speak at this time.

In days of peace, with new countries opening up and rapidly extending their agriculture, with larger crops, better knowledge of utilization of fertilizers, improved methods of food handling and better transportation facilities, we have been inclined to regard the dangers of general food shortage or starvation except in restricted localities as imaginary and impossible, and to believe that our system of crop production and marketing, storage and conservation, while not fully utilizing the scientific knowledge available, would at least sufficiently safeguard the world from hunger or serious economic disturbances. War conditions have modified our points of view very greatly, however, and we have been awakened to the importance of the principles expressed by Malthus in 1798, or at least a modification of his thesis, pertaining to the relation of food supply to population. Now we are more appreciative than ever before of the dangers which may and actually do threaten us because of waste, unscientific methods of production and conservation, and improperly coordinated regulation of supply and demand. The war years through which we have just passed have caused us to give serious attention to the problems of food supply and food control. It has fallen to this country to be the storehouse from which enormous supplies of foods have been withdrawn for the use of the fighting forces and civilian populations of Europe. While this demand has been largely for meats and cereals, and especially for wheat, it has

also affected almost every type of food substance. Because of the necessity for sending these enormous quantities of breadstuffs abroad it has been especially desirable that the problems of food conservation in this country should be studied with especial care, to utilize as fully as possible all those methods of food treatment which would make it possible to lessen waste and build up a reserve against another period of shortage, bad crops, or unusual exportation. These investigations had their first fruits in the movement for war gardens and home canning and preserving, and their later and more permanent results in the stimulation of renewed interest in the great industrial methods of food preservation, and particularly in the impetus given to drying as a means of protecting foods against spoilage, and preserving them for future use. Since the problem of spoilage is essentially a microbiological problem, it follows that the investigation of dehydration has manifold bacteriological aspects.

Dehydration, desiccation, or drying as applied to foods, may be defined as the process of removal of the surplus water from the food substance in such a way as to prevent destruction of the cellular tissues, maintain the energy values, and preserve potentially the color, flavor and physical condition of the food. Since practically all foods, with exception of the ripe seeds of cereals, legumes, and a few other plants, contain a large percentage of water and as a result have relatively short storage periods in the fresh state, it is clear that the rapid, regulated drying of such foods will, if it can be successfully accomplished, add greatly to the world's stock in a form which is capable of easy storage, favorable transportation and universal employment. Moreover, when we appreciate that approximately 50 per cent of the total food produced in America in a single year never reaches the consumer because of poor crop management, bad market conditions, distance from means of transportation or losses en route and in the city markets, the great possibilities of dehydration begin to be apparent. Much could be said on the economic side of this subject, but at this time I desire to emphasize especially those phases which are of direct interest to us as bacteriologists, physicians or sanitarians.

Dehydration however is not a new art, for drying has been known for hundreds of years, and is probably the oldest method of food preservation which the human race has employed. It was undoubtedly known to the ancients, in fact it must have been practised as soon as man became a food-producing and food-storing animal. In arid regions this could be accomplished with little difficulty. The Indians of the plains and later the early settlers, dried their beef or buffalo meat by cutting it into thin strips and hanging them up for the sun and wind to remove the excess moisture and sear the surface with a protective coating which would prevent infection and spoilage. The material so dried was known as jerked beef. Our early Massachusetts colonists dried corn after it had been cooked, the product being known as samp. Native fruits and berries were dried in considerable quantity, especially the apple. Along the coast fish drying became an important industry, and throughout New England to the present time will be found the application of this process of food preservation as a sort of local or primitive industry, with little exact knowledge of the underlying principles. Similarly, in other parts of the country other fruits, vegetables and meat products have been dried. Peas and sweet corn may be mentioned as examples of the former, while along the Pacific coast the long sunny dry period lends itself particularly to the drying of prunes, raisins and other sweet fruits. That the results have now always been highly satisfactory cannot be gainsaid, for local differences in method, and differences in season have not infrequently been the cause of losses, spoilage and inferior products.

Dried or evaporated fruits have been in use in America for many years and have become an important part of our food products. On the other hand, dried vegetables have, until recently, been prepared only on a small scale, although the drying of potatoes was done on a semi-commercial scale in 1886 by A. F. Spawn, an American, resident at that time in Australia. The history of commercial and semi-commercial drying of vegetables in America is an interesting one, but the industry has not until the war period been a success financially, probably owing to the abundance of fresh materials, and to the prejudices which are

prone to develop in the public mind when new food products are put upon the market. The war, however, has changed the attitude of the public in these matters, and it is probable that there will soon be a considerable expansion of the dehydration industry in this country if the products can fulfill the commercial, nutritional and sanitary requirements.

Although somewhat aside from the main line which I wish to discuss it may be of interest from the standpoint of general information to observe how dehydration has been utilized in other countries. Little has been done in England or France. In Switzerland there are a few small concerns engaged in the drying of vegetables. In Germany dehydration of foods has had a great expansion since 1900 and to this process may be ascribed at least a portion of the success that country had in meeting the food situation during the war. In 1898 there were three small plants with an insignificant output, largely potatoes and other root vegetables. In 1906 the number of plants in operation had increased to 39, in 1909 to 199, and in 1914, at the outbreak of the war, to 488; in 1916 there were 841 drying plants and in addition about 2000 breweries were using some portion of their equipment in the drying of food materials. By 1917 the number of drying plants had again more than doubled, and the quantity of potatoes alone dehydrated in Germany was more than three times the total crop of the United States. This fact alone suggests one of the reasons why Germany was able to maintain her food supplies during the war. It also by inference supports the claims that have been made that drying does not seriously impair the nutritive qualities of foods, a point to which I may later return, although the bearing is not essentially bacteriological.

Let us now turn to some of the biological questions involved in this process of food preservation, considering first the relation of the water content to the growth of microörganisms. Although it has long been recognized that most microörganisms thrive but poorly in substances of low water content, even if those substances are rich in nutrients, definite data on this point are surprisingly difficult to secure. The general impression of the bacteriologist who is familiar with the general literature is that there is much

information on this subject. Search reveals relatively few papers, however, in which the studies of the growth of bacteria in food substances of high concentration have been reported, and even fewer dealing with the development of yeasts and molds. A few of these may be mentioned.

Eschenhagen found that as the concentration of the substratum increased fungi growing thereon modified their vegetative character, and especially developed swollen tips in the young hyphae.

Rasciboski observed wall thickenings, giant cells, and multiplication of nuclei.

Puriewitsch, in investigations on the growth of *Aspergillus pseudo-clavatus* in concentrated sugar solutions found that the walls became swollen, and the filaments developed the appearance of a string of pearl beads.

Klebs, in investigations on *Aspergillus repens*, found the limits of concentration which the organisms could withstand were 95 per cent for grape sugar and 57 per cent for glycerin. Similar results have been obtained by other investigators using fungi as the organisms studied. Perhaps the most interesting and illuminating of the investigations on this subject have been done by our fellow members, the Kopeloffs, in their studies of deterioration of cane sugar. They have found that in sugars containing as little as 1 per cent of water slight decomposition by fungi could take place. Other investigations which need not be enumerated in detail here have shown that many fungi have the ability to develop in or upon substances almost devoid of water, and our common experience with food substances and fabrics bears witness on this point.

With yeasts and bacteria, high concentration is apparently much more inhibitive than with the thread fungi, although these organisms too, can remain alive and sometimes increase slowly in and upon substances containing small amounts of water. Obviously, the character of the substance, its reaction and chemical composition have much to do with the ability of the organisms to remain viable.

Laurent found that certain yeasts would grow in and ferment 60 per cent sucrose solution. I have personally observed similar phenomena.

Working with bacteria, Zopf found that *Bact. vernicosum* would produce fermentation of 70 per cent sucrose in a mixture with cottonseed meal, and that *Staphylococcus aureus* destroyed cane sugar gelatin in a concentration of 48 per cent.

Grafenhan showed that certain bacteria will grow on 70 per cent cane sugar and dextrine.

The Kopeloffs, again, have confirmed or even exceeded these figures.

It is interesting to note on this point that cane sugar, or sugars, have generally been the subject which have been used for tests.

Although bacteria generally tend to disappear or at least to become inactive when the moisture falls below 40 per cent, there are many instances known when percentages of water much smaller than this will still permit growth. The ordinary evaporated fruits, such as apples, peaches and apricots contain in general about 20 to 24 per cent moisture. While usually this high concentration is sufficient to inhibit development, it sometimes requires but a small addition of moisture to permit growth and consequent deterioration by molds and yeasts or their enzymes. It must especially be borne in mind that the enzymes of these microörganisms, as Kopeloff has quantitatively proved, may be active even in the presence of minute moisture percentages, and that they may bring about marked deteriorations. This we have also found to be true in certain dehydrated products.

Resistance to drying and ability to increase in the presence of small percentages of water *seems* to be particularly characteristic of soil bacteria and those native to vegetables themselves while most microörganisms of pathogenic character, accustomed to the fluids of the body have fortunately much less endurance to dryness. The importance of this fact (if it proves to be a fact) in the preparation of dehydrated food substances cannot be overestimated.

BACTERIOLOGY OF DEHYDRATED VEGETABLES

It was my good fortune, during the period of the war, when I was in the Food Division of the Surgeon General's Office, to have assigned to me a certain part of the investigation work on new

food materials and the various food compounds and substances which were brought to the Surgeon General's Office for examination. Among these were the dehydrated vegetables which were later purchased in considerable quantity and used by our forces on the other side. Although the quantity actually used was small as compared with the total food supply, you may be interested to known that something like forty thousand tons were sent over. In connection with investigations on this subject, I thought it desirable to make certain bacteriological studies of these foods, which I wish to report upon at this time.

Dehydrated vegetables generally contain less than 10 per cent of water. They have often been subjected to a pre-treatment by hot water or steam and to more or less thorough washing. The drying temperatures vary from 130° to 185°F. (55° to 85°C.) with different processes, and the heating period is of 3 to 24 hours' duration, although rapid evaporation may considerably reduce this during a portion of the time.

It may be further noted that these dehydrated substances will, when soaked in water, reabsorb the water which has been lost and assume the normal condition of the tissues. On the central table there are some specimens of dehydrated products which have been restored by simply adding water, and at the end of this meeting I should be very glad to have you examine the dried and restored substances as they appear there.

The presence of bacteria in or upon dehydrated vegetables may therefore be of some interest. They may be:

1. Organisms originally on food which have survived the process (and perhaps increased).

2. Organisms gaining access from wash water, handling in raw state, or utensils.

3. Organisms deposited on surfaces during packing, handling in dried state, or utensils.

Studies on a large number of samples of dried foods have shown very few, if any, of them to be sterile, although often the numbers of bacteria present were very small. Moreover, long continued keeping under suitable conditions causes the number to decrease gradually. Mold spores were commonly present, but yeasts

have invariably been absent. The methods of quantitative determinations are at best imperfect because of the character of the materials examined. It was found necessary therefore to make multiple examinations, and derive the "normal" figure from the results obtained. The nature of the materials studied rendered it impossible to extract, plate and count all the organisms present, but it was possible to secure a result which showed the general order of magnitude of the microbic population. The method found most satisfactory was as follows:

Ten grams of the sample, well broken up, with aseptic precautions, were weighed out into a tared sterile dish and later transferred to an Erlenmeyer flask containing 200 cc. of sterile tap water. After thorough mixing in order to get all the particles completely wetted, the sample was placed in a 37° incubator for two hours to permit absorption of water, return of tissues to as near normal as possible and easy separation of bacteria. At the end of this time the sample was again thoroughly agitated and aliquot portions removed, dilutions prepared and plates made as rapidly as possible. The two hour period of infusion was determined upon as a result of preliminary studies. Soaking is necessary to make it possible to remove the bacteria from the surfaces of the particles, but too long continued infusion permits the growth of bacteria which have been restored from the plasmolysed condition.

The media which were found most satisfactory for quantitative studies were plain agar and glucose agar or litmus glucose agar for the bacteria, and Czapek's medium for fungi. Plating on beer wort gelatine or agar was carried out for a time for the purpose of enumerating yeasts, but the results were so uniformly negative that this process was discontinued.

Incubation at 37° for forty to forty-eight hours with plates inverted was found to be most satisfactory. Counts were made with a reading glass, the dish being placed on a black ruled plate. Examination of each plate was made for predominant types of colonies, and these were fished and later identified in order to determine as fully as possible the source and kinds of organisms.

Products from a number of different manufacturers were examined, and these varied greatly in results, in part because of the different methods of treatment and in part, no doubt, because of differences in sanitary conditions of plant and methods of handling. The chart here shown may serve to show the general character of the numerical results obtained.

NUMBER	MATERIAL	MOISTURE	BACTERIA PER GRAM			MOLDS PER GRAM
			Total P. agar	Gluc. agar	Acid	
		per cent				
1	Potato	3.14	9,400	9,800	3,800	60
2	Carrot	4.42	23,000	24,000	8,600	20
3	Cabbage	5.93	32,000	340,000	240,000	600
4	Turnip	5.62	220,000	300,000	20,000	20
5	Mixed vegetables	5.60	1,600,000	2,000,000	2,000,000	80
11	Turnip	5.00	3,800	7,000	3,000	
12	Cabbage	6.24	7,000	8,000	2,800	200
13	Onion	5.73	2,200	2,400	1,000	>20
14	Potato	2.78	6,800	6,900	1,500	>20
16	Tomato	3.32	26,000	5,000	1,000	>20
21	Beet	3.65	700	800		60
22	Banana	4.72	600	600		>20
23	Sweet potato	4.42	100	900		100
24	String beans	4.27	600	800		>20
27	Peaches	2.79	800	200		40
31	Carrots	5.85	80	200		>20
32	Onions	4.03	400	6,000	2,000	400
33	Parsnips	4.09	4,400	18,000	10,000	60
34	Turnip	7.08	60	180		>20
36	Potato	2.69	400	1,200		440

> means less than.

The foregoing table serves to show the variations found in the total numbers of organisms occurring on dehydrated products. Study of the types predominating has given no evidence of objectionable organisms, practically all the species found having their habitat in soil or water. The following species were identified: *B. vaculatus, B. denitrificans, B. Lustigi, B. cuticularis, B. weichselbanini, Ps. cohaerea, Ps. sinuosa, B. subflavus, B. tenacatis, B. ginglymus, Bact. mycoides, M. radiatus, Ps. viridescens, Ps. ambigua.*

The molds isolated were identified as of the following genera: Aspergillus, Mucor, Penicillium, Spicaria, Sporotrichum, Trichoderma, Herpocladiella.

At least two species of Mucor, two of Penicillium and five of Aspergillus were isolated. These are all organisms which may be found on fresh vegetables or fruits. Probably the spores survive all pretreatment and dehydrating processes.

From the standpoint of numbers and types of bacteria, these dehydrated products have the same characteristics as raw fruits and vegetables, except that the number of organisms has been greatly reduced by the processes to which the foods have been subjected.

STORAGE OF DEHYDRATED PRODUCTS AND EFFECT OF STORAGE ON BACTERIAL CONTENT

The practicability of dehydrated foods must depend largely on whether they may be held in storage for long periods without undergoing any deteriorations. If, as has been claimed, they may be kept under all kinds of conditions if properly sealed, their great usefulness is unquestioned, but the type of container becomes of supreme importance. To determine this point an extended study has been made during the past two years to determine the effects of various conditions of storage on the quality of the product.

As the preservation of dehydrated vegetables depends upon the inhibitive effect of their low moisture content upon any destructive bacteria or other organisms which may be present, it is obvious that the greatest difficulty of preservation would occur where the products were exposed to a high relative humidity, permitting them to absorb moisture and at the same time exposed to a temperature which was most favorable to the growth of organisms after the inhibition of a low moisture content was removed. Consequently, in order to subject samples of the dehydrated foods to the severest conditions they would be likely to encounter in transporation, storage, or as merchandise, a series of investigations was carried out with the purpose of determining the effect

of the environment on the bacteriological character of the foods themselves. Four types of storage conditions were employed. The first is called "*severe*," since the chamber used was kept at 37°C. and the relative humidity at 100 per cent. The second was "*moderate*" in character, the temperature being 20° to 25°C. with a relative humidity of about 70 per cent. Third, "*favorable*" storage, with chamber maintained in one case at ordinary temperature and in another at 37°C. but with no artificial humidification. In both, the relative humidity was approximately 50 per cent. This condition represents fairly well the conditions which may be met in ordinary transportation and commercial handling. The fourth condition employed was "*cold storage*" at 0°C. and about 95 per cent relative humidity.

Six weeks was taken as the maximum period of storage under the various conditions with the first set of products. Examination of the products was made at the end of each two weeks.

Since this study is a combination of the sanitary and the commerical or industrial phases of the subject, we have deemed it desirable to use as containers for the material under investigation a variety of the newer types of treated paper cans or cylinders as well as tinplate and glass. It is obvious that in tightly stoppered glass containers and in tin cans sealed or tightly closed by friction tops, there is no noteworthy opportunity for additional moisture to gain entrance. Any changes in the food product must therefore be the result of bacterial or enzymic action taking place at low moisture percentage. With the paper containers, on the other hand, the exclusion of moisture is far from perfect, even in those holders treated by paraffin, waxes, etc., to secure impermeability. Statements are often made that ordinary paper bags will serve to preserve dried vegetables and fruits, and while this is often true if storage conditions are dry and temperature low, it is on the other hand, often fallacious, especially in damp climates and with vegetables containing sugars or other hygroscopic ingredients.

Without going minutely into the methods of experimentation which have been followed, I wish to place before you the general results obtained.

Vegetables of the same kind and lot, thoroughly mixed, were subjected in the different types of containers to the four different sets of conditions which I have described. Initial determinations of moisture and microbic content were made, and the samples properly sealed, were left undisturbed for two, four and six weeks. At the end of this period new sets of determinations were made and the material subjected to careful examination for evidence of discoloration, loss of flavor and enzymic activity. Any deviation from normal appearance was recorded. While very small increases in moisture may not readily be detected microscopically, any really appreciable increase is evidenced by the changed appearance and texture of the material. Bacterial changes cannot be easily detected unless the increase is enormous, but comparatively slight development of mold is at once apparent.

Our data may be summarized as follows: Materials stored in tin and glass containers under all conditions of temperature and moisture remained practically constant in their moisture content and mold content. The bacterial counts generally diminished, sometimes rapidly, sometimes gradually. In other words, bacteria with proper storage conditions die off slowly.

Samples stored at 37°C. and high humidity in paper containers, even though they were heavily treated with paraffin showed little or no increase in moisture at the end of two weeks, and often had not doubled their percentage of water in four weeks, whereas in six weeks, the amount of moisture often increased by 400 per cent or even in some instances by 1000 per cent. It is a strange fact, but apparently a fact, that atmospheric moisture will penetrate very rapidly through the paraffin containers if exposed four to six weeks, while some containers could be immersed in water and opened at the end of that time without finding any visible water inside the container. The rate of absorption of atmospheric moisture by dehydrated vegetables when directly exposed to moist air is very moderate at first. The acceleration of absorption may be due to heightened permeability of the cell membranes as a result of slight swelling and increased conductivity of the fibrovascular bundles and other types of cellular structures during the early stages of absorption of water.

This increase in moisture content was quickly followed by changes in the flora of the samples. At first the bacterial count dropped somewhat and the fungus (mold) count remained practically constant, although the incipient development of mold mycelium was frequently apparent. At the end of four weeks the bacterial count was generally still low, although showing an upward trend in many instances, and the mold count (spores) had not greatly increased. Mycelium was, however, more marked. At the end of six weeks the bacterial count and the mold count had both increased greatly, although sometimes an interesting antagonism was exhibited, the bacteria falling off rapidly in numbers as the molds developed to large numbers and produced their characteristic cleavage and growth products.

It is apparent from the data obtained that ordinary paper containers do not protect dehydrated foods sufficiently at high temperatures and high humidity. Interestingly enough, similar results were obtained in cold storage (0°, 95 per cent humidity). The moisture content of the vegetables increased in six weeks by 400 per cent, the bacterial counts trebled while the molds remained nearly constant.

The samples stored in paper containers at ordinary temperatures and humidity showed little if any increase in moisture during the first four weeks, but at the end of six weeks it had somewhat increased. This was not sufficient to revive the dried up bacterial cells, or cause mold spores to germinate, and there was therefore no important increase in either of these groups of organisms. In this connection it may be stated that for certain molds and some bacteria, limiting moisture minima were observed, it being found that marked development did not ensue until the moisture of the sample had reached a definite percentage. These limiting minima vary from 22 to 32 per cent with different organisms.

Storage at 37°C. in a "dry" atmosphere differs slightly from that at 20°C. In general, there was a consistent decrease in moisture unless the material stored had been very thoroughly "dehydrated." By the end of six weeks the moisture had generally fallen to, or below, half the percentage of water in the original

sample. As would be expected, there was a decrease in the number of bacteria. The mold spores remained practically constant.

The practical result of this somewhat extended investigation was to prove beyond doubt that paper or wood pulp containers of good quality may be used with safety under the ordinary conditions pertaining in the temperate zones. Their cheapness and availability will help to establish the industry commercially. No invasion of organisms from outside takes place, and the variations in temperature and humidity of the outside are accompanied by a corresponding but perhaps smaller rise and fall in moisture in the food materials. Eventually an equilibrium with the air is reached. It is doubtful if the moisture would ever reach the point sufficient to bring about bacterial decomposition. On the other hand, these containers do not appear to be suitable for use in hot moist conditions for the reasons that have been pointed out through the results I have given.

DANGER OF FOOD POISONING OUTBREAKS THROUGH USE OF DEHYDRATED FOODS

Our attention has been directed during recent years to a number of cases of so-called food poisoning. These are now generally recognized as infections with some one or more of a number of organisms, among which may be named *B. paratyphosus A* and *B*, *B. enteritidis*, *B. welchii*, *B. suipestifer* and *B. botulinus*. The fact that dehydrated foods are not cooked or sterilized as they are prepared and packed for sale, brings up a question as to possible dangers from this source. Moreover, the methods of gardening employed in the production of vegetables for dehydration in some parts of the country, as in California, where Japanese gardeners produce them in large quantities, make it conceivable that the vegetables may reach the dehydration establishment infected with some of these bacteria or with *B. coli* or *B. typhosus*.

This matter seemed to be of sufficient practical importance to warrant a careful study of the effect of drying on these organisms. Two sets of experiments were conducted in the laboratory and one small investigation has been carried out in a dehydrating

plant under actual commercial conditions. In the laboratory tests stringless beans, parsnips, tomatoes and spinach were used. These vegetables were inoculated with cultures of the following organisms: *B. coli*, *B. typhosus*, *B. paratyphosus A* and *B*, *B. enteritidis*, *B. paracoli*, *B. subtilis* and *B. welchii*. The vegetables were dried in air in an oven at 80°C. for four hours, after which examinations were made to discover surviving organisms.

Of the organisms employed, *B. subtilis* was recovered in typical culture. Organisms resembling *B. typhosus* and the paratyphoid forms in some respects, but not typical, were found, but these have not yet been tested by serum reactions. All others were destroyed.

A second series has also recently been studied imperfectly in which *B. coli*, *B. paracoli*, *B. enteritidis*, the paratyphosus A and B strains, *Danysz bacillus*, *B. suipestifer* and *Microspira protea* and *B. diphtheriae* were used. The treatment given was the same as in the first test, four hours at 80° in an air oven. The only organisms recovered were atypical *B. typhosus* and atypical *B. suipestifer* and these have yet to be proved by serum reactions.

These experiments can only be regarded as preliminary, but so far as they go they are reassuring. It should here be stated that many vegetables contain on their surfaces organisms markedly simulating members of the colon typhoid group in gross appearance and in many reactions, as well as microscopically. Serum tests and animal tests may therefore prove the surviving types to be entirely different from the organisms used.

To make a more satisfactory investigation on this point, I have recently conducted in Chicago a set of experiments under actual working conditions in a dehydrating plant using a method which we had found to be highly satisfactory. The vegetables employed were carrots, cabbage and potato. The organisms used were: *B. typhosus*, *B. paratyphosus A*, *B. paratyphosus B*, *B. suipestifer*, *B. of Danysz* (*B. murisepticum*), *B. enteritidis*, *B. coli*, *B. paracoli*, *B. welchii*, *B. botulinus*, *Microspira protea* and *Micrococcus pyogenes aureus*. Heavy inoculations were made from rich broth or agar shake cultures, and the vegetables then submitted to the regular processes of pretreatment and dehydration.

The results of this work were most reassuring, since all the organisms, even *B. subtilis*, were destroyed, or at least so greatly reduced that they could not be discovered by subsequent culture examinations. These results must be regarded as preliminary and an extended study on the subject is projected for the near future.

There are many nutritional and commercial phases of dehydration, such as the effect on the so-called vitamines, which are of great interest, but would be out of place in a paper of this kind. I hope, however, that I have been fortunate enough to bring clearly before you the importance of this new-old method of food preservation, and to convince you that the products are not only excellent in appearance and appetizing in flavor, but are also safe and wholesome and free from the dangers of infections which may give rise to serious illness or death. The great advantages of fresh foods at all times and in all parts of the world, combined with the sanitary quality and safety which may be secured, should go far to promote this industry, while the economic advantages alone should make it worth while to foster its development along scientific lines. There are many studies yet to be conducted on the bacteriological side, and I trust that at some future meeting of this Society I may have an opportunity to present the results of later researches.

REPORT OF THE COMMITTEE ON THE DESCRIPTIVE CHART FOR 1919

H. J. CONN, *Chairman*, H. A. HARDING, I. J. KLIGLER, W. D. FROST, M. J. PRUCHA, AND H. N. ATKINS

Presented at the Boston Meeting of the Society of American Bacteriologists, December 29, 1919

PART I. METHODS OF PURE CULTURE STUDY. REVISED

The 1917 report of this committee contained a description of the methods recommended to be used with the Descriptive Chart. Copies of the report have been on sale by the Society and the first edition is now practically exhausted. Opportunity is therefore taken to revise the report, printing it in the JOURNAL OF BACTERIOLOGY to call it to the attention of the members of the Society, the reprints to be placed on sale as before. As soon as these reprints are available, one copy will be furnished with every order for Descriptive Charts, and additional copies may be obtained for 15 cents apiece postpaid, or if five or more copies are ordered, for 10 cents each, transportation not postpaid. Orders for these reports, as well as for the Descriptive Charts, are to be sent to the chairman of the committee (address Geneva, N. Y.).

Methods are given in this report for all the determinations listed on the 1917 chart (recommended for instruction) but methods have not yet been prepared for all the determinations called for by the older chart. As the chart designed for instruction is now in most general use, it has seemed wisest to give further attention to these few methods rather than to do work on some of the other, less frequently used, determinations. The methods given here are not to be considered official. They are merely the best that have come to the attention of the committee at the present time. Criticisms and suggestions are at all times welcome, in order that future editions of this report may be brought up-to-date.

PREPARATION OF MEDIA

Beef-extract broth shall have the following composition:

Beef-extract	3 grams
Peptone	5 grams
Distilled water	1000 cc.

Beef-extract agar shall be of the same composition plus the addition of 12 grams of oven-dried agar or 15 grams of commercial agar. *Beef-extract gelatin* shall be of the same composition, with the addition of 120 grams of Gold Label gelatin, or 100 grams of some brand of gelatin (such as "Bacto-gelatin," or United States Glue Company gelatin) having greater jellying power. These media are to be made up according to the directions given by the committees of the American Public Health Association on water analysis and on milk analysis (1916, 1917), except that white of egg may be used for clarification if desired. It is recommended, however, that instead of using phenolphthalein in adjusting the reaction of these media the simpler and more accurate procedure be adopted of adjusting to the neutral point of brom thymol blue.[1] Bring the media to such an acidity as to turn this indicator a distinct grass-green (neither yellow green nor blue green). This color indicates approximately "true neutrality," i.e., a hydrogen-ion concentration between $pH = 6.6$ and $pH = 7.4$. Another equally satisfactory method of adjusting media to this hydrogen-ion concentration is to bring them to such an acidity as to cause the first faint trace of permanent pink to appear with phenol red.[2]

Sugar broths. Just before sterilization 1 per cent of the required carbohydrate is to be added to beef-extract broth. Otherwise proceed as for sugar-free broth. Adjust reaction with brom thymol blue or phenol red.

Plain gelatin. Proceed as for beef-extract gelatin, but omit beef-extract and peptone. Clarify with white of egg.

Nitrate broth. For routine work add 0.1 per cent KNO_3 to the above formula for beef-extract broth. Routine *nitrate agar* should contain 0.1 per cent KNO_3 added to the ordinary formula

[1] Use a 0.04 per cent solution of brom thymol blue in 95 per cent alcohol.

[2] Use a 0.02 per cent solution of phenol red in 95 per cent alcohol.

for beef-extract agar. Modification of these routine formula is often necessary, as explained below (see p. 130).

Starch agar. Add 0.2 per cent of water-soluble starch to the ordinary beef-extract agar.

Indicator media. Saccharine media with some indicator to show acid production are frequently used. Litmus is the most common indicator, enough of which should be added in saturated aqueous solution to give the medium a distinct blue color (taking care that the litmus solution used is not so alkaline as to alter, appreciably, the reaction of the medium). Litmus, however, does not give accurate results in terms of hydrogen ion concentration; so except for certain special purposes (see p. 130) it is recommended that brom cresol purple be used to detect increase in acidity, and cresol red to detect increase in alkalinity. It is convenient to keep these indicators in concentrated alcoholic solutions of such strength that 1 cc. will be sufficient for each litre of medium. For this purpose a 1.6 per cent solution of either indicator in 95 per cent alcohol is recommended. Brom cresol purple is purple in neutral or alkaline media and is yellow in acid media. Cresol red is yellow at neutrality and in acid media, turning red under alkaline conditions. Under certain circumstances it is desirable to have an indicator that will show a change in either direction from neutrality. Brom thymol blue does this, but is not satisfactory in media because its range is too short to distinguish differences between different kinds of bacteria. Very satisfactory results, however, may be obtained with a mixture of brom cresol purple and cresol red (0.5 cc. each of 1.6 per cent alcoholic solution of the two dyes to the litre of medium.) This mixture changes very slowly from purple to yellow through a long range (from about pH = 8.0 to about pH = 5.0) extending to a considerable distance on both sides of neutrality. By comparing with a blank tube of the neutral medium, it is very easy to detect an increase in either acidity or alkalinity.

Recently certain other combinations of indicators have been recommended for this same purpose. Bronfenbrenner (1919) recommends a combination of china blue with rosolic acid or preferably its sodium salt, and Morishima (1920) a combination

of china blue with phenol red. Both of these combinations are colorless or nearly so at pH = 7.0, turning blue as the reaction becomes acid and red as it becomes alkaline. Rosolic acid has the advantage over phenol red of having a more alkaline range (pH = 7.3 to pH = 9) than phenol red, hence giving the Bronfenbrenner combination a sensitive range from pH = 5 to pH = 9. Rosolic acid is insoluble in water, but it is possible to keep it in concentrated alcoholic solution (as above recommended for brom cresol purple and cresol red) so that only 1 cc. of alcohol is added to a liter of medium. The concentration of china blue in the medium should be 0.0025 per cent, that of rosolic acid or its sodium salt 0.005 per cent, while in combination with china blue a 0.001 per cent solution of phenol red is recommended. Either of these combinations should have distinct advantage over the combination of brom cresol purple with cresol red; but there has been as yet no opportunity to compare them.

Variations of these media. For certain organisms the above formulae are not the best—many pathogenic bacteria, for instance require more peptone than is provided in the above formula for broth, while some organisms are best studied in media of a hydrogen-ion concentration different from that recommended above. In such cases the individual investigator is free to modify the media to suit his own purposes; but whenever other than these routine formulae are used, the fact should be stated on the chart. In employing a reaction other than that of neutrality it is recommended that instead of using the titrimetric method, the reaction be adjusted to some definite shade of brom cresol purple, if a more acid reaction is desired, or of phenol red if it is to be more alkaline.

Optional media. In many laboratories other media than those specifically mentioned on the chart are in general use, such as potato, blood serum, agar stabs, and so forth. Blank spaces are left on the chart for recording characteristics on any optional media.

INVIGORATION OF CULTURES

Provided a medium can be found upon which the organism to be studied grows vigorously, it should always be invigorated before study, even though freshly isolated from its natural habitat. The procedure to employ is as follows:

Prepare duplicate sub-cultures in standard glucose broth, and on standard agar slopes, placing cultures of each at 37° and 25°C. On the basis of the resulting growth the organism falls into one of the following series:

Series I. Organisms which produce good growth (surface growth, distinct turbidity, or heavy precipitate) in twenty-four hours at 37° in glucose broth.

Series II. Organisms which do not produce good growth in twenty-four hours as above, but do in forty-eight hours at 25° in glucose broth.

Series III. Organisms which do not grow well in glucose broth but do produce good growth on the surface of agar in twenty-four hours at 37°.

Series IV. Organisms excluded from the above groups but which produce good growth on the surface of agar in forty-eight hours at 25°.

Record the series number on the chart at the proper place and proceed with the invigoration by inoculating into another tube of glucose broth for organisms of series I and II, or of standard agar for organisms of series III and IV. Incubate this tube at the temperature, and for the time, called for by the series in which it belongs; then transfer from this tube to a third tube and incubate as before. From this third culture make a gelatin or agar plate and incubate at the temperature previously used until colonies of sufficient size for isolation are obtained. Transfer from a typical colony to one or more agar slants and incubate for one day at 37° or for two days at 25° according to the temperature relation of the organism studied.

In case the organism does not produce vigorous growth on either of these media at either temperature, it should be invigorated with any medium and at any temperature known to be

adapted to its growth. Under such circumstances invigorate by the procedure just outlined but using the medium and temperature found most favorable for the organism in question, recording on the chart the method of invigoration adopted. If no conditions are known under which the organism in question produces vigorous growth, it should be studied without preliminary cultivation as soon as possible after isolation from its natural habitat. Such an organism is not likely to give good growth on any ordinary media, and the results of the study called for by the chart will have little significance.

STUDY OF MORPHOLOGY

The routine study of morphology should be from dried preparations, stained with fuchsin, methylen blue, or gentian violet. Preparations to show the vegetative cells should be made, preferably, from agar slant cultures, from a few hours to two days old, according to the rapidity of growth. The medium and temperature used and the age of the culture should be recorded.

Motility. Hanging-drop preparations of young broth or agar cultures should be examined for motility. If motile, microscopic preparations should be made to show the arrangement of the flagella, using any of the ordinary methods of flagella staining with which the student can obtain good success. Even if motility is not observed in hanging-drop, it is wise to attempt a flagella stain, because motile organisms often lose their motility under the conditions of observation. Even negative results from both hanging-drop preparation and flagella stain do not absolutely prove that the organism is immotile.

Presence of spores. Routine examinations for spores should be made on stained, dried preparations from agar slant cultures a week old. Stain with methylen blue. Vegetative forms take the stain, but spores do not. In most cases there will be no trouble in finding spores if the organism produces them. All rather large rods however, (0.8 micron or more in diameter) should be regarded as possible spore-producers, even though microscopic examination does not show spores. Such bacteria should be

mixed with sterile water and heated to 85°C. for ten minutes; if still alive, spores may be regarded as unquestionably present. Also make repeated transfers of the culture onto agar and examine at various ages. A culture of a large rod should not be recorded as a non-spore-former unless all these tests are negative.

Capsules. An organism should not be recorded as having capsules unless they have been actually stained by one of the methods of capsule-staining described in bacteriological text books.

Irregular forms. Forms that differ from the typical shape for the organism (i.e., "involution forms," etc.) such as branching forms, clubs, spindles or filaments should be noted and sketched.

Special stains. Of these the Gram stain has been given particular attention by the committee and at present the Stirling modification is recommended. Further work is being done at present upon an improved method of making this stain, which will be discussed in the next number of the JOURNAL. The Stirling modification is carried out as follows:

Prepare gentian violet solution by grinding 5 grams in 10 cc. of 95 per cent alcohol in a mortar. Add 2 cc. anilin oil, distilled water 88 cc. Filter.

Iodine solution is as usual: 1 gram iodine, 2 grams potassium iodide, 300 cc. water.

The procedure recommended in the 1918 report was as follows: one minute in stain; wash in water; one minute in iodine solution; wash in water; two minutes in absolute alcohol; wash in water; thirty seconds in counter stain (10 cc. saturated alcoholic safranin in 90 cc. water). This procedure gives very good results; but recent work undertaken in the army cantonments shows that equally good results can be obtained by the following rapid method: one to five seconds in stain; wash in water; five to ten seconds in iodine solution; wash in water; ten to twenty seconds in absolute alcohol; wash in water; five to ten seconds in counterstain.

We are informed that the Stirling solution of gentian violet can be made more stable by mixing normal hydrochloric acid with the anilin oil before dissolving in water. (Add 0.5 cc. normal HCl to 2 cc. anilin oil; dissolve in 88 cc. water and filter;

mix with 10 cc. of alcoholic gentian violet prepared as above.) The committee has not yet tested out this procedure.

Sketches. Drawings of all the morphological characteristics should be made on the blank spaces on the chart to the right of the descriptions. Both typical and atypical forms should be sketched, using care to designate which are typical.

CULTURAL CHARACTERISTICS

Cultures for the study of cultural characteristics should be incubated at 37°C. in case of organisms of series I and III, and at 25° in case of organisms of series II and IV, except that gelatin cultures should be incubated at 20°. Room temperature may be used in place of 25° at certain seasons of the year; but if a minimum thermometer shows that the temperature falls below 22° during the course of the work, note should be made of the fact. On the day when good growth first appears the proper descriptive terms on the card should be underlined; after subsequent study, the changes should be noted in the space provided, and sketches of the different stages should be made.

PHYSIOLOGY

Liquefaction of gelatin. Old method. The method in most common use is to hold gelatin stab cultures six weeks at 20°C. Plain gelatin should be used.

Provisional method. It is recommended that the following method proposed by Rothberg (1917) be put in provisional use until experience shows its value. It is designed to distinguish "true liquefiers" (organisms producing ecto-enzymes) from the organisms that produce endo-enzymes of proteolytic action that are released from the cell after death and cause liquefaction of the gelatin if incubated for the long period mentioned above. The method is to give the organism a preliminary cultivation for eighteen to forty-eight hours (according to its rapidity of growth) in a 1 per cent solution of gelatin at 25° or 37° according to its temperature relations; then inoculate on surface of gelatin in test tube and incubate fifteen days at 20°.

Relation to free oxygen. Provisional method. Determine by noting the presence or absence of growth in open and closed arm, respectively, of fermentation tubes containing glucose broth. Care must be taken to use fermentation tubes from which the dissolved oxygen has been recently driven off by heating. In case of gas production, this test is of comparatively little value, because bubbles of gas may carry the sediment up with them; hence if an organism produces gas from glucose, the test should, if possible, be made in the presence of some other sugar which it attacks (acidifies) without gas-formation. It must be remembered, however, that even anaerobes do not grow in the absence of free oxygen except in the presence of a chemical substance (such as carbohydrate) which they are able to reduce and use as a source of oxygen.

Fermentation of sugars and glycerol. This is normally to be studied in fermentation tubes. Ordinarily use beef-extract broth containing 1 per cent of the substance investigated; but if the organism does not grow well in such broth and some medium is known in which it does grow well, the latter may be used. Generally speaking, organisms of series I and II should be studied in broth, organisms of series III and IV in some other medium. Incubate organisms of series I and III at 37°, organisms of series II and IV at 25°. Test ordinarily on 1st, 3rd, and 7th days, although the best days for testing will depend upon the rapidity of growth of the culture. Hence on the chart, although space is given for recording reaction on three separate days, blanks are left for the individual student to fill in with the days upon which the tests are actually made. Inoculations should always be made at least in triplicate.

To test for acid, it is recommended that in place of the illogical titrimetric method, determinations of hydrogen-ion concentration be made by the colorimetric method described by Clark and Lubs (1917 a). In accurate research work the exact shade of the indicator should be compared with that obtained in standard "buffer" solutions, and results recorded in terms of pH. In laboratories where these standard solutions cannot be obtained, it is better to record results simply as + or −, according to the

reaction of the culture to litmus, than to use the titration method. Under such conditions it is possible, however, to obtain a rough idea of the hydrogen-ion concentration by the use of Clark and

TABLE 1

Degrees of acidity easily recognized in clear media

ACIDITY	INDICATOR REACTIONS	APPROXIMATE pH-VALUE
"Neutral"	Blue or green to brom thymol blue*	Over 6.2
"Weak"	Yellow to brom thymol blue Purple to brom cresol purple*	5.2–6.0
"Moderate"	Yellow to brom cresol purple Orange to methyl red†	4.6–5.0
"Strong"	Maximum red to methyl red Blue or green to brom phenol blue*	3.2–4.4
"Very strong"	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.
† Use a 0.02 per cent alcoholic solution.

TABLE 2

Degrees of acidity easily recognized in milk

ACIDITY	INDICATOR REACTION, ETC.	APPROXIMATE pH-VALUE
"Neutral"	Same color with brom cresol purple* as sterile milk; i.e., blue to gray green	6.2–6.8
"Weak"	Color with brom cresol purple lighter than in sterile milk; i.e., gray-green to greenish yellow	5.2–6.0
"Moderate"	Yellow with brom cresol purple. Not curdled	4.7–6.0
"Strong"	Curdled. Blue or green to brom phenol blue*	3.2–4.6
"Very strong"	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.

Lubs' series of indicators without making accurate determinations of pH. Four different degrees of acidity can be easily distinguished by this simple method in sugar broth with an initial reaction of neutrality. The indicator reactions for these differ-

ent degrees of acidity are listed in table 1, together with the approximate range of pH to which each corresponds. In the absence of accurate determinations, these degrees of acidity may be recorded by the indefinite terms, "weak," "moderate," "strong and "very strong," or by the symbols +, ++, +++, and ++++. If the student desires to record increase in alkalinity in the same table on the chart, he can use the symbol 0 for neutrality and – for an alkaline reaction.

Gas production is ordinarily measured in percentage of gas in the closed arm, and the ratio of H: CO_2 by means of absorption with NaOH, using the methods described in laboratory manuals (filling open arm with 4 per cent NaOH, allowing gas to enter open arm, shaking and returning gas to closed arm). As this method is far from accurate, it is recommended for provisional use only.

The fermentation test is ordinarily of no significance for organisms of series III and IV because of their poor growth in broth. Sometimes these organisms can be studied in some other liquid medium in which they do give good growth; but generally it is preferable to use agar slants. In such a case, use a sugar agar containing brom cresol purple, china blue, or a mixture of indicators as suggested on page 129. Increase in acidity can be detected by fading of the purple color of the brom cresol purple or by the appearance of blue if china blue is used. Gas-production can usually be detected in agar cultures by the presence of cracks and air bubbles, but as a test for gas, agar slants are not as reliable as fermentation tubes.

A more detailed discussion of hydrogen-ion concentration and of methods for determining acid-production is given in the 1918 report of this committee, copies of which can be obtained from the chairman.

Milk. Acid production in milk can be detected by adding brom cresol purple to the culture and comparing with the color obtained by adding the same proportionate quantity of indicator to sterile milk. (Brom thymol blue does not give satisfactory results in milk.) Four degrees of acidity that can be simply recognized in milk are listed in table 2. They correspond closely

to those listed in table 1, differing only in that brom cresol purple is used instead of brom thymol blue to show "neutrality" and that the curdling point (pH = 4.7) is used to separate between "moderate" and "strong" acidity instead of the less definite point of maximum red to methyl red. The same methods of expression used in recording acidity in clear media should be used in recording that of milk.

Litmus milk often gives valuable information, showing not only the production of acid, but also decolorization of the litmus by organisms that are able to reduce it. More accurate results as to acidity can be obtained by using brom cresol purple, as shown by Clark and Lubs (1917 b). This indicator, however, does not show the reduction phenomena which are sometimes of diagnostic value in litmus milk cultures; its substitution for litmus is not, therefore, always to be recommended.

Reduction of nitrates. The following procedure is recommended: Inoculate first into nitrate broth and onto slants of nitrate agar, the media having the composition given on p. 128. Test the cultures on various days as indicated on the chart. On these days examine first for gas as shown by foam in the broth or by cracks in the agar. Then test for nitrite with the following reagents:

(1) Dissolve 8 grams sulphanilic acid in 1 litre of 5 N acetic acid (1 part glacial acetic acid to 2.5 parts of water), or in 1 litre of dilute sulphuric acid (1 part concentrated acid to 20 parts water).

(2) Dissolve 5 grams α-naphthylamine in 1 litre of 5 N acetic acid or of very dilute sulphuric acid (1 part concentrated acid to 125 parts water).

Put a few drops of each of these reagents in each broth culture to be tested, and on the surface of each agar slant. A distinct pink or red in the broth or agar indicates the presence of nitrite. It is well to test a sterile check which has been kept under the same conditions, to guard against errors due to absorption of nitrite from the air. Presence of nitrite or of gas shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary, as follows:

In case the fault seems to lie in poor growth, search should be made for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increasing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite or gas in any nitrate medium whatever should be recorded as nitrate-reduction. Unless the routine formula is used, the exact composition of the medium must always be given.

If the organism grows well and yet produces no nitrite or gas, the determination must be recorded as doubtful unless the organism can grow well in some synthetic medium containing no nitrogen except nitrate. It is recommended that such an organism be tested in a medium containing small quantities of phosphate, calcium, chlorine, etc., with KNO_3 as a source of nitrogen and sucrose as a source of energy and of carbon.[3] Such a medium generally allows good growth with an organism capable of utilizing nitrate and sucrose. Unfortunately neither glucose nor lactose can be used in this medium as a source of carbon and energy, for the ordinary "c.p." preparations of these sugars contain much ammonia. If the organism in question grows (even but slightly) on a synthetic medium of this sort, it should be tested for nitrite by the usual method and for ammonia by means of Nessler's reagent (comparing with an uninoculated tube as a check). The presence of nitrite, of ammonia (i.e., a more pronounced ammonia reaction than in check tube), or of gas indicates nitrate-reduction.

The production of gas (free N) from nitrate is not a very common one; but a considerable number of soil organisms have this power, and one should be on the lookout for it in studying soil bacteria. The agar slant test is ordinarily a sufficiently delicate test; but, if liquid media are used, more reliable results may be obtained by the use of fermentation tubes.

Chromogenesis. Color production should be recorded if observed in broth, on beef-extract agar, gelatin or potato, or if

[3] An illustration of such a medium which has proved satisfactory for some bacteria is: K_2HPO_4, 0.5 gram, $CaCl_2$, 0.5 gram, KNO_3, 1 gram, sucrose 10 grams, agar 12 grams, water 1000 cc.

noticed to a striking extent on any other medium. In the group number, the point devoted to chromogenesis refers to the color produced on beef-extract agar.

Diastatic action on starch. Provisional method. Use beef-extract agar containing 0.2 per cent of soluble starch. Pour into a petri dish, and after hardening make a streak inoculation on its surface. Incubate at 37° for organisms of series I and III, at 25° for organisms of series II and IV. Determinations for the group number shall be based upon results obtained on the seventh day. To make the test, flood the surface of the petri dishes with a saturated solution of iodine in 50 per cent alcohol. The breadth of the clear zone outside of the area of growth indicates the extent of diastatic action. If over 2 mm. in width on the seventh day it shall be recorded as "strong;" if under 2 mm. as "feeble;" if no clear zone is present, as "absent."

This method requires some modification, e.g., reducing the amount of peptone in the medium, for organisms that grow so rapidly as to cover the entire surface of the plate in seven days, thus leaving no room for a clear zone outside.

The group-number is a brief means of recording the salient features of the organism. It is primarily a summary of the physiological characteristics just discussed. As each of the determinations is made, the proper figure for that place in the group number is to be checked or underscored. After completing the determinations, the entire group number is to be written in at the place left for it on the chart. The genus symbol should precede the group number. The present group number, adopted by the Society in 1907, was intended for use with the generic names of Migula. As Migula's genera are not in such general use today as they were ten years ago, a revision of the group number on some other basis is now being undertaken by the committee, and will be discussed in Part II of this report.

Brief characterization. On the right hand margin of page one of the chart is a place for recording by a + or − sign other important characteristics of the organism (primarily cultural)

not included in the group number. This margin together with the group number constitute a brief characterization of the organism—a summary of the tests outlined above.

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Glossary of Terms Used on the Chart

Adherent, Applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen; strictly **aerobic**, growing *only* in the presence of free oxygen.

Amorphous, without visible differentiation in structure.

Anaerobic, growing in the absence of free oxygen; **strictly anaerobic**, growing *only* in the presence of free oxygen; **facultative anaerobic**, growing both in presence and in absence of free oxygen.

Arborescent, branched, tree-like growth.

Beaded, (in stab or stroke culture) disjointed or semi-confluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Ciliate, having fine, hair-like extensions, resembling cilia, sometimes not visible to the naked eye.

Clavate, club-shaped.

Coagulation, the separation of casein from whey in milk.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Convex, surface the segment of a sphere.

Crateriform, a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.
Diastatic action, conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.
Echinulate, a growth along line of inoculation with toothed or pointed margins.
Effuse, growth thin, veily, unusually spreading.
Endospores, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis*.
Entire, with an even margin.
Erose, border irregularly toothed.
Filaments, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented, to be distinguished from chains (q.v.) by the absence of constrictions between the segments.
Filamentous, growth composed of long, irregularly placed or interwoven threads.
Filiform, in stroke or stab cultures, a uniform growth along line of inoculation.
Flocculent, containing small adherent masses of bacteria of various shapes floating in the culture fluid.
Fluorescent, having one color by transmitted light and another by reflected light.
Granular, composed of small granules.
Infundibuliform, form of a funnel or inverted cone.
Iridescent, exhibiting changing rainbow colors in reflected light.
Lobate, having the margin deeply undulate, producing lobes (see *undulate*).
Luminous, glowing in the dark, phosphorescent.
Maximum temperature, temperature above which growth does not take place.
Membranous, growth thin, coherent, like a membrane.
Minimum temperature, temperature below which growth does not take place.
Mycelioid, colonies having the radiately filamentous appearance of mold colonies.
Napiform, liquefaction in form of a turnip.
Opalescent, resembling the color of an opal.
Optimum temperature, temperature at which growth is most rapid.
Papillate, growth beset with small nipple-like processes.
Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.
Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.
Peritrichiate, covered with flagella over the entire surface.
Persistent, lasting many weeks or months.
Plumose, a fleecy or feathery growth.
Polar, at the end or pole of the bacterial cell.
Pulvinate, decidedly convex, in the form of a cushion.
Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.
Radiate, showing ray-structure.
Raised, growth thick, with abrupt or terraced edges.
Reduction, removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen, and to the decolorization of litmus.
Rhizoid, growth of an irregular branched or root-like character, as in *B. mycoides*.
Ring, growth at the upper margin of a liquid culture, adhering to the glass.
Rapid, developing in twenty-four to forty-eight hours.

Rugose, wrinkled.

Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.

Slow, requiring five or six days for development.

Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called **clostridia**.

Sporangia, cells containing endospores.

Spreading, growth extending much beyond the line of inoculation, i.e., several millimeters or more.

Stratiform, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.

Transient, lasting a few days.

Truncate, ends abrupt, square.

Turbid, cloudy with flocculent particles; i.e., cloudy plus flocculence.

Umbonate, having a button-like, raised center.

Undulate, border wavy, with shallow sinuses.

Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

NOTES ON THE CLASSIFICATION OF THE WHITE AND ORANGE STAPHYLOCOCCI

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INTRODUCTION

The white and orange cocci of the skin were first isolated in pure culture by Rosenbach (1884) and described as var. *aureus* and var. *albus* of a species which he named *Staphylococcus pyogenes*. In later years such systematic bacteriologists as Migula (1900) and Chester (1901) discarded the generic name suggested by Rosenbach and grouped these organisms along with the large series of yellow and red cocci under the genus, *Micrococcus*. For some time all thought of any important differences correlated with variations in pigment production appeared to be abandoned; and yellow, as well as white and orange, cocci found upon the skin were commonly classed by medical observers as varieties of a single species.

In 1908 the Winslows showed that, in the case of the yellow and red chromogens at least, the type of pigment production was associated with other characteristics of fundamental systematic importance. They made it clear that the white and orange cocci belong to a series of cocci, including the streptococci and diplococci, which are essentially parasitic in nature, Gram positive and active in fermentative power, while the yellow and red forms (including the sarcinae) are normally found in environments outside the human body, are Gram-negative and exhibit a much more restricted fermentative activity.

The Gram-positive, acid-forming parasitic cocci, which occur in irregular growth masses rather than in pairs or chains, and which produce a fairly abundant growth on media, were again

subdivided by the Winslows into two genera—*Aurococcus* including the orange pigment producers, and *Albococcus* including the forms which produce a more abundant growth, of porcelain white color. Buchanan (1915) has pointed out that in spite of the neglect of the generic term *Staphylococcus* by recent systematic writers, the name has perfectly valid standing according to the accepted rules of biological nomenclature and should be used in place of *Aurococcus* for the orange pigment formers. The Committee on Classification (1917) of the Society of American Bacteriologists accepted this view and in its first report recognized the genera, *Staphylococcus* (orange pigment formers) and *Albococcus* (white pigment formers).

In the discussions which followed the report of the Committee on Classification the more fundamental question was raised as to whether—aside from any questions of terminology—the separation of the orange and white staphylococci into two distinct genera was justified.

The Winslows based the distinction primarily on the difference in pigment production, less heavy growth on media and more vigorous liquefaction of gelatin by the orange forms. Both orange and white groups included some non-liquefiers but among those which did produce liquefaction the orange forms were twice as active. Earlier results of Dudgeon (1908) were cited as suggesting that the orange chromogens differ from the white forms in exerting a more powerful reducing action on neutral red and a more active fermentation of mannitol, glycerol and raffinose. Dudgeon, himself, however, finally concluded that the white and orange staphylococci were all varieties of a single species. The Winslows reviewed the results obtained by various other observers in regard to the variability of chromogenic power among the staphylococci but finally, in spite of such observations, recognized the white and orange forms as constituting different genera. Kligler (1913) from a study of the strains of staphylococci in the collection of the American Museum of Natural History concluded that the difference in rate of gelatin liquefaction between the white and orange cocci was a valid one.

Both these American systematic studies of the staphylococci were based on a rather small series of strains. The Winslows had 181 orange forms in their series, of which 126 were liquefiers, but only 23 white forms including 14 liquefiers. Kligler studied only 15 orange and 12 white strains. In order to throw further light on the relationships of these organisms, it seemed worth while to collect a larger series of strains and to submit them to a more exhaustive series of quantitative tests. This was the purpose of the present study.

SOURCE OF CULTURES

Our aim in collecting the cultures for this investigation was to obtain 100 strains, each, of white and orange cocci, of which about half should be from the human body and about half from air, dust, water and other environmental sources.

We finally obtained a total of 185 strains, of which 5 were discarded for reasons to be noted later; and our complete studies were conducted on 180 different strains. Of these, 104 were from pathological conditions in men and animals, 22 were isolated from the hands and 54 were isolated from air, dust or water.

The 104 pathogenic strains were courteously furnished for our use by Parke, Davis and Company, H. K. Mulford Company, The Abbott Laboratories and the Lederle Laboratories, and they came from a widely diversified series of conditions of which the following were the most important; abscesses, acne, boils, cellulitis, conjunctivitis, coryza, endometritis, furunculosis, gonorrhea, impetigo, osteomyelitis, otitis media, pharyngitis, pyorrhea, prostatitis, sarcoma, septicemia, sinus infection, tonsillitis, tuberculosis, ulcers, urethritis, whooping cough and wound infections in man; abscesses, distempers, equine influenza, mange, nasal discharge and septicemia in the horse. Whether the organisms sent to us bore any etiological relation to these diseased conditions is of course uncertain, but they were in all cases isolated from tissues in which an active disease process was in progress.

The 22 cultures isolated from the hands were obtained by rinsing the surface of the hands in sterile water and plating on glucose agar, from which characteristic white and orange colonies were fished.

Of the 54 strains which came from sources other than the human body 39 were obtained from the air by exposing glucose agar plates in the offices and workrooms of the American Museum, in the New York Subway, and in the streets and parks of New York City. Thirteen were isolated from dust collected in the same general localities, one strain was isolated from a sample of butter and one from water.

In analyzing our results we have divided our strains from the standpoint of source into these three main groups, 104 isolated from pathogenic conditions, 22 from the hands and 54 from sources outside the human body.

CHROMOGENESIS

The chromogenic power of our 185 strains was determined as follows: Each strain was cultivated on a nutrient agar streak for fourteen days at 20°C. A portion of the growth was then spread with a platinum loop over white paper (Whatman No. 2) and allowed to dry in the air. The hue and tint were then matched against the frontispiece of the Systematic Relationships of the Coccaceae (by C.-E.A. and A. R. Winslow). The determinations of chromogenesis were made for the whole series on two different occasions about six months apart.

Four strains originally isolated as orange pigment producers proved on examination to belong to the group of the red chromogenic cocci (*Rhodococcus*) and were excluded from subsequent consideration. One strain gave inconsistent results on the two series of tests being recorded as orange on the first occasion and white on the second. This strain was also excluded from our series leaving 180 strains for detailed study.

These 180 strains divided themselves naturally into two very clearly marked groups, as did the cocci studied by the Winslows. One hundred of them were of the *Albococcus* type giving a pigment which could be matched in the Light Lemon Yellow,

Light Cadmium Yellow and Medium Cadmium Yellow of the Winslow's chart, usually in the three lightest chromas. The other 80 were of the *Aurococcus* type, their pigment being matched by the Orange Yellow and Cadmium Orange hues, usually in the darker chromas.

So far as pigment production alone is concerned, these two types are clearly and definitely to be distinguished from each other.

Of the 104 strains isolated from pathological conditions 53 were of the white and 51 of the orange type. Of the cultures from the hands 16 were white and 6 orange and of the air and dust strains, 31 were white and 23 orange. No correlation appears, therefore, to exist between chromogenesis and habitat.

GRAM STAIN

The Gram stain was made by the method described in the Systematic Relationships of the Coccaceae. Agar cultures which had been incubated for twenty-four to forty-eight hours at 20° and 37°C. respectively were treated with anilin-oil-gentian violet for one and one-half minutes; with Gram's solution for one and one-half minutes; with 95 per cent alcohol for three minutes; and counterstained with Bismarck Brown for one-half minute. Altogether we repeated the whole series five separate times.

The cocci exhibit a somewhat variable reaction to the Gram stain but of our 180 strains 136 were consistently positive on all five occasions, 39 were generally positive but gave negative results on 1 or 2 out of the five trials and 5 were consistently negative. These 5 strains had no other special characteristics in common except that all were gelatin liquefiers.

LIQUEFACTION OF GELATIN

The property of gelatin liquefaction was studied in the following manner. Each strain was first grown for twenty-four hours at 37°C. in nutrient broth containing 1 per cent gelatin and one loopful of this culture was then spread over the surface

of ordinary nutrient gelatin in tubes of $\frac{5}{8}$ inch diameter. These gelatin tubes were incubated at 20°C. for thirty days and the depth of the gelatin liquefied was recorded at frequent intervals by measuring down to the surface of the solid portion from a line drawn with a glass pencil around the tube at the original level of the medium.

Of the total of 180 strains studied, 101 liquefied gelatin to some degree within thirty days. Of 100 white strains, 47 (47 per cent) were liquefiers; of 80 orange strains 54 or 67 per cent were liquefiers, indicating a slightly higher tendency to attack gelatin within this color group as noted in the Systematic Relationships of the Coccaceae. It was also suggested by the

TABLE 1

Average depth of gelatin liquefaction in centimeters after various intervals (at 20°C.)

	DAYS											
	1	3	5	7	9	11	14	16	18	21	25	30
	cm.	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*
Orange forms	0	0.1	0.2	0.6	1.0	1.2	1.2	1.7	2.0	2.4	2.8	3.1
White forms	0	0.1	0.2	0.2	0.6	1.0	1.1	1.4	1.5	1.8	2.1	2.5

Winslows that the orange forms when they do liquefy act more rapidly and more vigorously than the white cocci. We find that there is indeed a slight difference of this kind as indicated by table 1, which, however, shows the differences observed by us to be much slighter than those recorded in earlier investigations. The Winslows report an average liquefaction after thirty days of 2.2 cm. for the orange forms and 1.1 cm. for the white strains; while Kligler cites figures of 3.5 cm. for the orange and 1.4 cm. for the white forms. The Winslows studied only 16 white liquefying strains and Kligler only 3; and their results are probably not typical. The fact that the absolute values recorded in the present investigation are so much higher than those reported by the Winslows may in part be attributed to the preliminary cultivation in gelatin broth; but it seems clear from the fairly large series of strains studied by us that the difference in rate of gelatin liquefaction between the white and orange staphylococci is only a relative and a rather slight one.

FERMENTATION OF CARBOHYDRATE MEDIA

In view of the comparatvely slight difference in gelatinolytic power between the white and orange cocci, it seemed important to study as many other biochemical properties as possible in order to determine whether the two chromogenic types were really deserving of generic rank; and in view of the important light thrown by the study of fermentative reactions upon the systematic relationships of the colon-typhoid group we have devoted considerable attention to this point. Very little previous work has been done on the power of the staphylococci to ferment various carbohydrate media. It is well known of course that they usually attack the more familiar sugars with the formation of acid, but no gas. Gordon (1906) studied the action of the white staphylococci on lactose, maltose, glycerol and mannitol. Dudgeon (1908) used a considerable series of carbohydrates but his work was not quantitative. The Winslows studied glucose and lactose only while Kligler (1913) added sucrose. In both cases generally positive results were reported.

In our own study we have used not only glucose, lactose and sucrose but also maltose, raffinose, mannitol, dulcitol, salicin and inulin. Two different media were employed in this study, the dehydrated bacto nutrient broth prepared by the Digestive Ferments Company and the peptone medium of Clark and Lubs.

The dehydrated medium contained 3 parts of bacto beef extract and 5 parts of bacto peptone and when dissolved (8 grams to a liter of distilled water) and sterilized for twenty minutes at 15 pounds, it had a pH value between 6.7 and 6.8. The peptone medium contained 0.5 per cent K_2HPO_4 and 0.5 per cent Witte's peptone; and was adjusted to a pH value of 7.4.

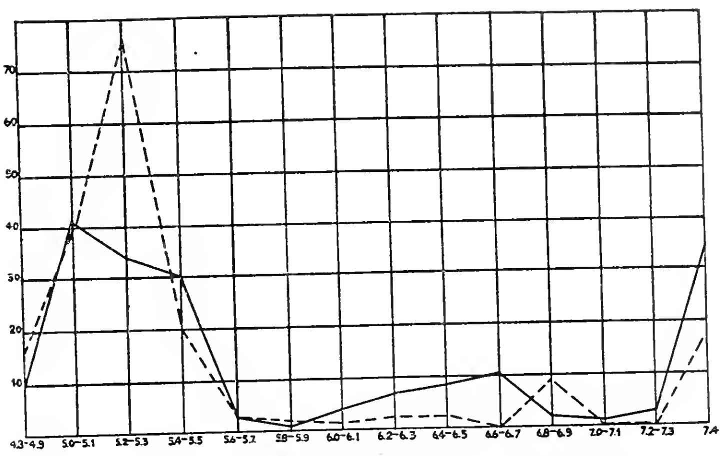
To each medium was added 2.7 per cent of a 0.04 per cent alcoholic solution of bromcresol purple before sterilization (as suggested by Bronfenbrenner, 1918) and 0.5 per cent of sterile carbohydrate after sterilization. The pH value was determined after 1, 3, 5 and 7 days of incubation at 30°C. by matching the tubes against the standards of Clark and Lubs (1917).

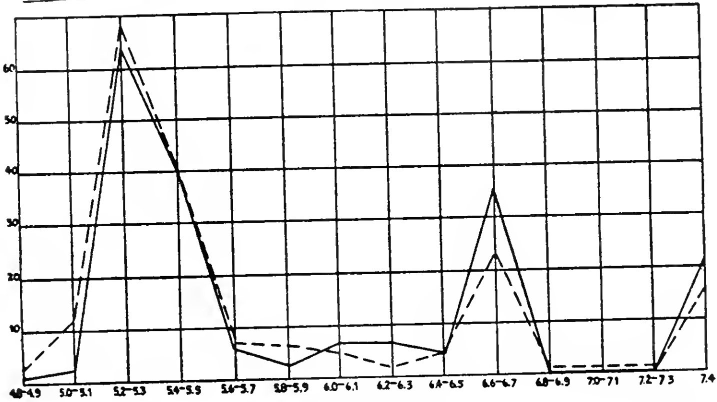
The bromcresol purple indicator gives a reasonably sharp color change between pH values of 5.2 and 6.8 but above 6.8 the color change is slight and the adjustment of the final endpoint of the peptone medium was therefore somewhat uncertain. It was always, however, over 6.8.

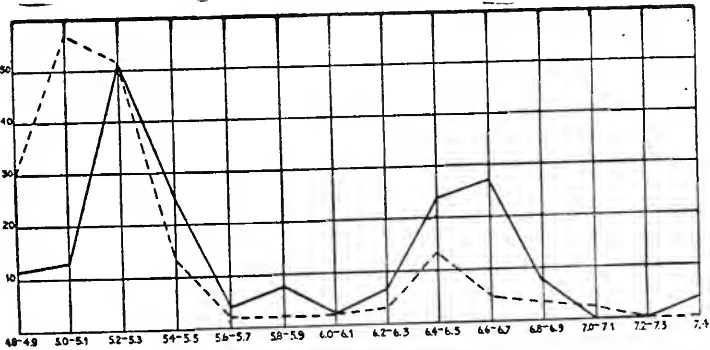
In general we found that glucose, lactose, maltose and sucrose were fermented by a large majority of the cultures studied while raffinose, mannitol, dulcitol, salicin and inulin were but rarely attacked. The analysis of the results was materially complicated, however, by the presence of three distinct modal points of acidity and by the variation between the results obtained in the two different media studied.

In figure 1 we have presented the distribution of the pH values recorded after seven days of incubation in the two types of broth and with the four carbohydrates which were most readily attacked. It will be noted that in nearly all the curves there are three more or less distinct modes, one at a pH value between 5.0 and 5.3, indicating vigorous fermentation, a second at a pH figure between 6.4 and 6.9, and a third at 7.4. In the succeeding discussion we shall designate these three reactions as acid (+), neutral (±), and alkaline (−). A neutral reaction indicates no change in the case of the dehydrated medium (which started at a pH of 6.8) while it implies a slight production of acid in the case of the peptone medium which started at 7.4. A neutral reaction in the dehydrated medium and an alkaline reaction in the peptone medium alike indicate no change in reaction while an alkaline reaction in the dehydrated medium implies a real decrease in acidity.

In general, as might be expected, the dehydrated broth with its slightly lower initial alkalinity, showed a higher final acidity than did the peptone broth with its slightly higher initial alkalinity. Out of 180 strains tested in glucose, 116 were ultimately acid in both media, 29 were acid in dehydrated and neutral in peptone broth, 8 were acid in dehydrated and alkaline (indicating no change) in peptone broth, 12 were neutral in dehydrated and alkaline in peptone broth (indicating no change in either case) and 16 were alkaline in both (indicating slight alkali pro-







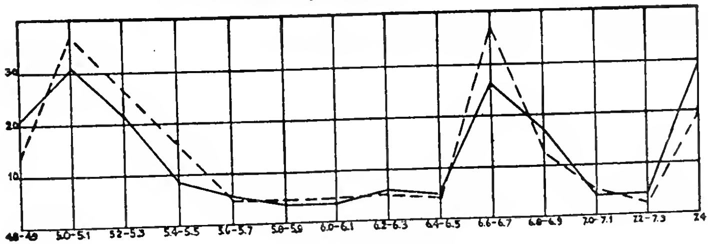


Fig. 1. Acid Production by Staphylococci Ordinates, Per Cent of Strains, Abscissae P_H Values

duction in dehydrated broth). The average reactions for these groups after various periods of incubation are indicated below.

The 116 cultures which ultimately became acid in both cases (class A) changed more slowly in the peptone medium, either because of its greater initial alkalinity, greater buffer action, or lower nutritive value, but by the fifth day at 30° they reached about the same point. Members of class B changed more slowly in the dehydrated medium than did those of class A but reached the same final end point, while in the peptone medium they never passed beyond the neutral point. Class C behaved like class B in the dehydrated medium, but in the peptone medium showed no

TABLE 2

Average pH *values in dehydrated and peptone media*

CLASS	DEHYDRATED MEDIUM					PEPTONE MEDIUM				
	Final reaction	pH value after				Final reaction	pH value after			
		1 day	3 days	5 days	7 days		1 day	3 days	5 days	7 days
A	Acid	6.1	5.3	5.2	5.2	Acid	7.2	5.8	6.3	5.2
B	Acid	6.4	5.9	5.4	5.3	Neutral	7.3	7.0	6.6	6.4
C	Acid	6.9	5.7	5.3	5.2	Alkaline	7.4	7.4	7.3	7.3
D	Neutral	7.2	6.9	6.7	6.7	Alkaline	7.4	7.4	7.1	7.3
E	Alkaline	7.3	7.4	7.4	7.4	Alkaline	7.3	7.4	7.4	7.4

change. Class D and class E showed no change in the peptone medium but slight, transitory or permanent, production of alkali in the dehydrated medium. In view of the difficulty in making accurate readings over 6.8 these last two groups may best be considered as one.

We may therefore consider the strains studied by us as divided into two main groups, Classes A, B and C which strongly acidify the dehydrated broth and classes D and E which fail to form acid in either medium. The first group, however, includes 116 strains which show an equally strong acid production in the peptone broth, 29 which show a slower acid production in the peptone broth and 8 which show no change in peptone broth.

It appears evident that we are dealing here with a group of organisms which generally attacks glucose but which contains a

considerable proportion of strains that fail to do so if the composition of the medium is not altogether favorable.

The distribution of results in broth containing maltose, sucrose or lactose is generally similar except that the differential effect of the peptone broth seems to be less marked in the case of maltose and lactose.

Throughout all the tests, however, the dehydrated broth is characterized by a higher acidity than the peptone medium.

TABLE 3

Final reactions (after seven days at 30°)

CARBOHYDRATE	PERCENTAGE OF CULTURES IN EACH CLASS					
	Peptone broth			Dehydrated broth		
	Acid	Neutral	Alkaline	Acid	Neutral	Alkaline
Glucose	68	15	16	84	7	9
Maltose	63	26	10	75	16	8
Sucrose	61	35	3	83	14	2
Lactose	49	33	18	54	33	12
Salicin	5	89	5			
Inulin	1	91	7			
Raffinose	1	78	21			
Dulcitol	0	92	8			
Mannitol	0	87	12			

The proportion of acid, neutral and alkaline reactions for each of the carbohydrates studied is indicated in the table above. Raffinose, mannitol, dulcitol, salicin and inulin were studied only in the peptone medium.

It may be of interest to compare with the results presented in table 3 the data obtained by Dudgeon (1908) using the change in the color of litmus as the sole measure of acid production. We have calculated the percentage of positive results obtained by him for his two chief groups of 46 orange strains and 71 white strains in table 4.

Glucose, maltose, sucrose and lactose appear most readily fermentable in either case, but the use of litmus (which is a highly inaccurate indicator), with no study of the progressive change in reaction, led Dudgeon to report a large number of positive results

with mannitol, raffinose, inulin and salicin (and perhaps with glycerol and erythritol) which as our observations suggest were probably erroneous.

From our detailed quantitative studies with the new indicators it seems evident that salicin, inulin, raffinose, dulcitol and mannitol are attacked by the staphylococci so rarely as to be of no serious diagnostic value; that glucose, maltose and sucrose are most readily attacked and with about equal frequency, and that lactose is slightly, though distinctly, less available than glucose, maltose and sucrose. It is of interest to note that the indicated metabolic gradient is different from that which occurs in the

TABLE 4

Fermentation results reported by Dudgeon (1908) on basis of change in color of litmus

MEDIUM	ORANGE	WHITE
	per cent positive	*per cent positive*
Glucose	100	100
Lactose	100	96
Mannitol	89	49
Maltose	100	96
Glycerol	73	54
Sucrose	98	93
Raffinose	64	42
Erythritol	40	21
Salicin	46	41
Inulin	33	28

colon group or among the streptococci. In both the latter groups lactose is more readily attacked than sucrose and among the colon bacilli, at least, sucrose and raffinose (the two ketonic sugars) are fermented with equal frequency. Among the staphylococci the fermentative processes involved must be distinctly different since sucrose is more readily fermented than lactose while raffinose is not attacked at all.

The action of the staphylococci upon glucose, maltose, sucrose, and lactose would seem to offer a possible basis for classification, although the marked differences due to the effect of the medium indicated in figure 1 would suggest that the use of this property as a differential test might prove of doubtful value.

For simplicity of analysis we have used in the following comparison only the results obtained in dehydrated broth, which yielded the clearest differentiation between fermenting and non-fermenting forms, and have assumed that in this medium all pH values above 6.0 indicated lack of fermentative power.

Of the 180 cultures studied 90 or 50 per cent produced a distinct acid reaction (pH 5.9 or below) in all four sugars; 41 or 23 per cent produced an acid reaction in glucose, maltose and sucrose but not in lactose; 11 or 6 per cent fermented glucose, maltose and sucrose but not lactose; 11 or 6 per cent fermented glucose and sucrose only; 23 or 13 per cent attacked none of the sugars; while 15 or 8 per cent showed special variations not fitting into either group (glucose-lactose-sucrose, glucose-lactose-maltose, maltose-sucrose, glucose-lactose, lactose-sucrose, glucose alone, sucrose alone, maltose alone).

In view of the marked variations shown in figure 1 it would seem unsafe to lay stress upon any of the smaller groups indicated by this analysis; and for comparison with other characteristics of the organisms in question we have therefore divided them into three main groups, group I those fermenting all four sugars; group II those fermenting glucose, maltose and sucrose, but not lactose; and group III, including all the rest of the strains.

Group III is a highly heterogeneous agglomeration; but the forms which fail to ferment lactose (group II) seem to constitute a fairly well defined group.

Correlations between fermentative power, on the one hand, and habitat, chromogenesis and liquefaction of gelatin, on the other, are indicated in table 5.

There is a marked tendency for the strains isolated from pathogenic conditions to ferment rather strongly, 76 (73 per cent) of all such strains attacking all four carbohydrates; while of the 54 strains from dust and air only 7 (13 per cent) attacked all four sugars and 30 (56 per cent) belonged to the heterogeneous feebly fermenting, group III.

Gelatin liquefaction was also slightly but distinctly more common among the active fermenters (60 per cent) while the members of group III were predominantly non-liquefiers (only 37 per cent showing liquefaction).

White and orange pigments on the other hand were fairly evenly divided among the various fermentative groups with a slightly greater preponderance of vigorous fermenters in the orange than in the white groups.

TABLE 5

General characteristics of 180 strains

	GROUP I. GLUCOSE, MALTOSE, SUCROSE, LACTOSE FERMENTED	GROUP II. GLUCOSE, MALTOSE AND SUCROSE FERMENTED	GROUP III. ALL OTHER STRAINS
Habitat			
Pathogenic strains	76	17	11
Strains from hands	7	7	8
Strains from air, dust and other external habitats	7	17	30
Chromogenesis			
White	43	28	29
Orange	47	13	20
Gelatin			
Liquefied	64	19	18
Not liquefied	26	22	31

REACTIONS IN MILK

In addition to the study of the sugar broths the reactions of the complete series in litmus milk have been determined with the following results:

Seventy-five strains acidified and clotted the milk and subsequently liquefied the clot.

Sixty strains acidified the milk, generally with clotting, but showed no subsequent liquefaction.

Seven strains showed no appreciable change in reaction.

Twenty-two strains turned the milk alkaline and liquefied the casein.

Sixteen strains turned the milk alkaline without liquefaction.

Dudgeon (1908) obtained somewhat lower results, 6 per cent of his orange and 48 per cent of his white strains clotting milk and 2 and 7 per cent respectively of the two groups peptonizing the clot.

Acid production in milk was, as might be expected, closely correlated with acid production in lactose broth as shown by table 6.

TABLE 6

Reactions in milk compared with reactions in lactose broth

LACTOSE BROTH	ACID	NEUTRAL	ALKALINE	TOTAL
(+) Acid	94	2	1	97
(±) Neutral	29	5	27	61
(−) Alkaline	12	—	10	22
Total	135	7	38	180

TABLE 7

Gelatin liquefaction compared with liquefaction of casein

LIQUEFACTION OF CASEIN	+	−	TOTAL
+	68	29	97
−	33	50	83
Total	101	79	180

TABLE 8

General grouping of staphylococci studied

FERMENTATIVE POWERS	ACTION IN GELATIN	NUMBER OF STRAINS IN EACH CLASS: Chromogenesis, White	NUMBER OF STRAINS IN EACH CLASS: Chromogenesis, Orange
Glucose maltose, sucrose and lactose all fermented	Liquefied	23	41
	Not liquefied	20	6
Glucose, maltose and sucrose positive lactose negative	Liquefied	10	9
	Not liquefied	18	4
Miscellaneous results, not falling in above groups	Liquefied	13	5
	Not liquefied	15	15

The liquefaction of casein was correlated to some extent, but not very closely, with the liquefaction of gelatin as shown in table 7.

Combining the results of tests for fermentation, gelatin liquefaction and chromogenesis we obtain the subdivision of our strains shown in table 8.

PRODUCTION OF INDOL, AMMONIA AND NITRITES

Indol production was studied in a medium containing 1 per cent peptone and 0.5 per cent K_2HPO_4 incubated at 20° for ten days and tested by the para-dimethyl-amido-benzaldehyde method. All tests were negative.

Ammonia production was determined in a medium containing 1 per cent peptone, 0.5 per cent NaCl, 0.05 per cent K_2HPO_4 and 0.01 per cent Na_2CO_3 incubated at 30° for seven days and tested with Nessler reagent. This test was suggested by Kligler (1913) as perhaps of special differential value, but in the present study all but 11 strains gave positive results.

The reduction of nitrates was first tested in a medium containing 1 per cent peptone, 0.5 per cent K_2HPO_4 and 1 per cent KNO_3. Incubation periods of seven days, fourteen days, and incubation temperatures of 30° and 37°, gave almost uniformly positive results. At 30°, 8 strains only out of 180 were consistently negative, while 19 more gave variable or doubtful readings.

This last result seemed somewhat surprising since the senior author in the Systematic Relationships of the Coccaceae reported only 21 per cent of the orange cocci studied and 13 per cent of the white strains as reducing nitrates. In 1908 when this earlier work was done the standard method of testing for nitrate reduction prescribed by the Committee on Standard Methods of Water Analysis of the American Public Health Association involved the use of a medium containing only 0.1 per cent peptone and 0.02 per cent nitrate. In order to see if this difference would explain the conflicting results we repeated our tests with the old medium but even here we found after seven days at 20° 132 strains clearly positive, 38 clearly negative and 10 doubtful or variable.

Gordon (1906) in his exhaustive study of the white staphylococci based one of his types on failure to reduce nitrates and the Winslows attributed this property to *Aurococcus aureus*, *Auro-*

coccus aurantiacus, and *Albococcus pyogenes*. In view of the results here reported we are inclined to suspect that these earlier results were perhaps due to the imperfections in technique which Conn and Breed (1919) have shown to have been so common in the past. In the small series of laboratory strains studied by Kligler only 4 out of 11 orange strains failed to reduce nitrates although 11 out of 12 results with white strains were negative.

CONCLUSIONS IN REGARD TO THE JUSTIFICATION OF A GENERIC DISTINCTION BETWEEN THE ORANGE AND WHITE STAPHYLOCOCCI

In view of the general results of this study of 180 strains of staphylococci we are forced to conclude that the generic distinction between the white and orange staphylococci previously suggested by the senior author is of doubtful validity. There is indeed a slight difference in liquefying power between the two chromogenic groups and of course, as is well known, a considerable difference in pathogenic power. In view, however, of the similarity between the orange and white pigment formers in all the other characteristics studied it seems on the whole most reasonable to consider them as belonging to a single generic group which, according to the citations of Buchanan (1915) should obviously bear the name *Staphylococcus*. This genus may be defined as follows:

Genus, *Staphylococcus* (Rosenbach). Parasitic cocci. Cells in groups and short chains. Gram positive. Produce on agar good growth of orange color or abundant growth of porcelain white color. Glucose, maltose and sucrose generally, and lactose frequently, fermented without production of gas.

CONCLUSIONS IN REGARD TO THE SPECIFIC TYPES TO BE INCLUDED IN THE GENUS STAPHYLOCOCCUS

Gordon (1906) classified his white staphylococci on the basis of nine tests which included liquefaction of gelatin, coagulation and peptonization of milk, reduction of nitrates and neutral red and fermentation of lactose, maltose, glycerol and mannitol.

His commonest type found on the skin coagulated milk but did not peptonize it, liquefied gelatin, reduced nitrates and neutral red, and fermented lactose, maltose and glycerol but not mannitol. A second type common on the scalp was the opposite of the first in every respect except that it also reduced nitrates. A third type fermented maltose and lactose but gave negative reactions to the other seven tests.

The characteristics of these three types of Gordon's (which are of importance from the fact that they were based on the study of 300 strains) are indicated in table 9.

TABLE 9

Characteristics of Gordon's three types of white staphylococci

	COMMONEST FORM ON SKIN ST. EPIDERMIDIS-ALBUS	COMMONEST FORM ON SCALP	SECOND FORM IN ABUNDANCE ON SKIN
Gelatin	+	−	−
Milk, coagulation	+	−	−
Milk, peptonization	−	+	−
Nitrate reduction	+	+	−
Neutral red, reduction	+	−	−
Lactose	+	−	+
Maltose	+	−	+
Glycerol	+	−	−
Mannitol	−	+	−

The Winslows used the liquefaction of gelatin and the reduction of nitrates for establishing their specific types in both the orange and white series, *Aur. aureus* being defined as liquefying and non-reducing, *Aur. aurantiacus* as non-liquefying and non-reducing, and *Aur. mollis* as reducing; *Alb. pyogenes* as liquefying and non-reducing, *Alb. epidermidis* as liquefying and reducing and *Alb. candidus* as non-liquefying.

If our practically universal positive results in regard to nitrate reduction are correct, the differentiation based on this characteristic in the earlier investigations must be considered as of doubtful value. There remain chromogenesis, liquefaction of gelatin and fermentation of lactose as the chief differential characters available for classification, with coagulation of milk closely cor-

related with lactose fermentation and peptonization of the clot, much less closely correlated with liquefaction of gelatin.

The use of the three primary characteristics listed above for specific differentiation would seem to be justified by the fact that all of them show a distinct bimodal curve, as shown by the Winslows for chromogenesis and gelatin liquefaction, and in the present study, for lactose fermentation.

TABLE 10

Reactions of twelve groups of Staphylococci

GROUP	PER CENT OF EACH GROUP POSITIVE													NAME
	Orange pigment	Glucose	Maltose	Sucrose	Lactose	Salicin	Gelatin	Gram	NO_2	Clotted milk	Liquefy milk	Pathological habitat	Number strains	
1	0	100	100	100	100	13	100	96	100	70	22	70	23	St. epidermidis (also perhaps St. ureae)
2	0	100	100	100	100	5	0	100	95	68	31	81	20	St. candidus (also St. tetragenus)
3	0	100	100	100	0	40	100	90	90	20	20	60	10	
4	0	100	100	100	0	0	0	100	52	16	26	21	18	St. candicans
5	0	63	8	63	31	0	100	92	70	23	23	48	13	
6	0	69	12	62	12	6	0	100	52	31	31	25	16	
7	100	100	100	100	100	0	100	95	100	36	61	90	41	St. aureus
8	100	100	100	100	100	0	0	100	100	83	16	100	6	St. aurantiacus
9	100	100	100	100	0	11	100	100	100	44	44	77	9	
10	100	100	100	100	0	0	0	100	75	25	50	0	4	
11	100	40	0	20	20	0	100	100	40	20	60	20	5	
12	100	6	13	6	6	0	0	100	94	20	26	0	15	

The use of these three characteristics would give us the eight distinct groups shown in the upper two thirds of table 8 and the four groups in the lower third of the table which are characterized by more restricted and highly variable fermentative power. The detailed reactions of these 12 groups are presented in table 10.

In establishing types among bacteria it is obviously absurd to give a specific name to every combination of characters which may occasionally be met with and we must give proper weight to the frequency with which a given type is found in nature.

From this standpoint it is evident that by far the commonest homogeneous type among the staphylococci is that which includes the orange, liquefying, actively fermenting strains. Forty-one of our 180 cultures were of this type and as shown by the detailed characteristics presented in group 7 of table 10 these 41 strains were remarkably uniform in all their properties except the clotting and liquefaction of casein in milk. Of the 203 strains of staphylococci studied by the Winslows 126 were of this type (if we ignore the distinction based on reduction of nitrates). It seems quite evident that this organism is the most abundant form among the staphylococci and the natural center about which all the other types are grouped. Assuming the distinction between *Aur. aureus* and *Aur. mollis* on the basis of nitrate reduction to be unwarranted, this central type of the staphylococcus group should certainly bear the name *Staphylococcus aureus;* and all the other types of the genus may be assumed to have been derived from this one by the loss of one or more of the characteristics of the primitive form.

Among the types which have retained the orange pigment production but which fail either to liquefy gelatin or to ferment lactose, none has occurred with sufficient frequency in the present series of strains to appear deserving of specific rank (see table 8 and groups 8 to 12 in table 10). Dudgeon's orange chromogens were also practically all alike in liquefying gelatin and fermenting lactose. On the other hand the Winslows found 49 strains in their series which produced orange pigment but failed to liquefy gelatin or ferment lactose identifying the type as *Aur. aurantiacus.* We may perhaps recognize this species (group 8, table 10) as *Staphylococcus aurantiacus* leaving the other two types, characterized respectively by gelatin liquefaction and failure to ferment lactose, and by lactose fermentation and failure to liquefy gelatin, (as well as the groups characterized by miscellaneous fermentative reactions) without specific names.

The series of white pigment formers appears from all the investigations to be much more variable in its reactions. Of the six groups represented in the third column of table 8 three are represented by 18 or more strains out of the 100 white strains

studied by us; and it is significant that these three types correspond to the three types of white staphylococci described by Gordon (compare table 9).

The most abundant form in each investigation was the type which fermented lactose and liquefied gelatin, called by Gordon *Staphylococcus epidermidis albus* and by the Winslows *Alb. epidermidis* and *Alb. pyogenes* (according as nitrates were or were not reduced). This type (group I, table 10) may best perhaps be known as *Staph. epidermidis* since it was Gordon's work which first made its characteristics clear. Our forms of this series agree with those of Gordon in generally coagulating milk and generally failing to peptonize the clot; though the agreement is by no means very close. Several cultures which came to us as *St. ureae* belong in this group. They acidify milk and produce ammonia.

The second type in abundance in our study, and the type found most commonly on the skin after *St. epidermidis* by Gordon, is the form which ferments lactose but fails to liquefy gelatin, identified by the Winslows as *Alb. candidus* and now to be called *St. candidus* (group 2 of table 10). Our strains, however, reduced nitrate and generally clotted milk which Gordon's type did not. Three strains sent in to the Museum collection as *M. tetragenus* all belong to this group. None of them reduce nitrates and results are variable in milk and in regard to ammonia production.

Finally the third form found commonly in the present study resembles Gordon's form characteristic of the scalp, which neither ferments lactose nor liquefies gelatin. This is the type for which Kligler suggested the name *Albococcus candicans,* and which should be called *St. candicans* (group 4 of table 10). Our strains however differed from Gordon's in failing to peptonize casein or ferment mannitol and in fermenting maltose.

Two of the strains in this group came to us originally as *M. neoformans,* both being alkaline in milk with variable results in regard to nitrate reduction.

The lactose-negative gelatin-positive type of white pigment producers appears in our study, as in that of Gordon, to be a

rarer one and this form, as well as the forms which exhibit miscellaneous fermentative reactions (see table 8) may best be left for the present without specific names.

The relations of the five principal types of staphylococci discussed above may be summarized as follows:

- A. Orange Pigment
  - a. Lactose fermented, gelatin liquefied
    - *St. aureus* Rosenbach, Type, No. 4 of American Museum collection
  - b. Lactose not fermented, gelatin not liquefied
    - *St. aurantiacus* Schröter, Type, No. 348 of American Museum collection
- B. White pigment
  - a. Lactose fermented, gelatin liquefied
    - *St. epidermidis* Gordon, Type, No. 25 of American Museum collection. *St. ureae* Cohn, No. 464 of the American Museum collection is apparently identical with *St. epidermidis*
  - b. Lactose fermented, gelatin not liquefied
    - *St. candidus* Cohn, Type, No. 49 of American Museum collection
    - *St. tetragenus* Gaffky which may be differentiated from *St. candidus* by characteristic grouping of cells in fours and by viscid growth belongs in the same group, (No. 209 of American Museum collection)
  - c. Lactose not fermented. Gelatin not liquefied
    - *St. candicans* Flügge, Type, No. 526 of American Museum collection

In a group so variable as the staphylococci, it is clear that the conception of species has only a limited value, although it is convenient to have names associated with the types of most frequent occurrence. Fundamentally we are inclined to agree with Dudgeon in considering the whole group a reasonably homogeneous one; and it seems clear that the central type of the whole genus is the orange-pigment-forming, vigorously-fermenting, gelatin-liquefying, somewhat actively pathogenic *St. aureus*. As we depart from this type there is a progressive weakening of the various biochemical activities of this more vigorous form. The loss

of one characteristic of the *St. aureus* type tends in some degree to be associated with the loss of others. Thus the white chromogens are less actively pathogenic than the orange forms, less actively gelatinolytic, and slightly less vigorous in fermentative action. The forms which fail to liquefy gelatin also tend to be less active fermenters than the liquefiers (table 5).

Finally in considering the significance of these relationships, it must be pointed out that while the more typical and more vigorous orange-pigment producing gelatin-liquefying, lactose-fermenting staphylococci are the characteristic forms found associated with pathological conditions the types of weaker biochemical powers are the ones most frequently isolated from air and dust and other sources outside the human body. This correlation is brought out in Table 5 and is made clear by an inspection of the individual data presented in Table 10. It may plausibly be explained on the assumption that the loss of the bio-chemical powers characteristic of the typical *St. aureus* is promoted by the unfavorable conditions of life outside the human body.

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A SPECTROPHOTOMETRIC STUDY OF THE "SALT EFFECTS" OF PHOSPHATES UPON THE COLOR OF PHENOLSULFONPHTHALEIN SALTS AND SOME BIOLOGICAL APPLICATIONS[1]

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INTRODUCTION

This study was undertaken in connection with the investigation of the physiological, physical and chemical factors governing the growth of fungi and bacteria on culture media, trees, pulp wood, lumber, wood pulp and other cellulosic materials. In order to study the nature and habits of the fungi and bacteria causing the decay of wood and pulp and to devise and put into application methods of control, it is necessary to have accurate data on each organism concerned. Not only must the organisms be identified in pure cultures, but the maximum, optimum and minimum values of each essential physical, chemical and physiological factor must be established. For example, each organism living on an aqueous medium, and therefore in contact with hydrogen and hydroxylions (and other chemicals), will die if the concentration of the hydrogen ions (or other chemicals) is too large or too small, and will have its optimum growth when the hydrogen ion concentration is somewhere between the maximum and minimum values of tolerance, this statement, of course, presupposing that all other essential factors are constant

[1] This article is one that we are publishing in coöperation with Dr. Haven Metcalf, in charge of Forest Pathology, Bureau of Plant Industry, Department of Agriculture, on the quantitative studies of the various physical and chemical factors governing the growth of fungi and bacteria on culture media and cellulosic materials.

or optimum in value. We have already established some of these limiting values of ionic concentrations for several wood destroying fungi[2] and have begun to put the results into practical application in culturing the fungi, and in preventing the decay of chestnut trees[3] and of wood pulp,[4] for example.

The concentration of the hydrogen ions is a very important physiological and chemical factor governing the growth of wood destroying fungi. It is therefore essential to devise methods for measuring this factor accurately, especially as a very great effect on the growth is produced by small changes in hydrogen ion concentration in the regions of maximum and minimum values of tolerance. This is well illustrated in figure 1, which gives one of the many sets of experiments worked out by Meacham which will be reported later.

The hydrogen electrode[5] and sulfonphthalein indicators[6] were developed by Desha, Loomis, Myers, W. F. Clark, Slagle, White, Lubs, W. M. Clark, Guy, Birge, Hopfield and the writers for measuring hydrogen ion concentrations in solutions of weak and strong acids and bases, in the presence of salts, and in general under conditions such as we find in culture media. These methods are probably the best known for measuring the hydrogen ion concentration, but the presence of certain salts has an appreciable effect on the apparent values obtained by both methods. As the electrometric and colorimetric methods give different results, it is necessary to learn the magnitude of the errors in-

[2] See preliminary report: Meacham, Science, **48**, 499.

[3] Ibid. and several reports appearing later.

[4] See address by Acree before Buffalo Convention of Tech. Assn. Pulp and Paper Industry, published in *Paper*, June, 1919.

[5] Science, **30**, 624. Loomis and Acree: Am. Chem. J., **46**, 585, 621. J. Am. Chem. Soc., **38**, 2391. Desha and Acree: Am. Chem. J., **46**, 638. Myers and Acree: Ibid, **50**, 396. Clark, Myers and Acree: J. Phys. Chem., **20**, 243.

[6] Acree: Am. Chem. J., **37**, 72; **39**, 155, 528. Slagle and Acree: Ibid, **42**, 115, and unpublished data on phenolsulfonphthalein. White and Acree: Science, **42**, 1101; J. Am. Chem. Soc., **39**, 648; **40**, 1092; **41**, 1190. White and Davis: Jour. of Urology, **2**, 107. Lubs and Acree: J. Am. Chem. Soc., **38**, 2773, Lubs and Clark: J. Wash. Acad. Sci., **5**, 609; **6**, 483. J. Bact., **2**, 1, 109, 137. Birge and Acree: J. Am. Chem. Soc., **41**, 1031. Brightman, Hopfield, Meacham and Acree: Ibid, **40**, 1940.

herent in each, and to devise means of expressing the true hydrogen ion concentration, or activity, in terms of the results given by the two methods. We have therefore begun an investigation of these so-called "salt effects" by the use of a number of methods,[7] and as phosphates are cheap and efficient chemicals for regulating hydrogen ion concentrations, we are presenting here some data showing that increasing concentrations of such phosphates pro-

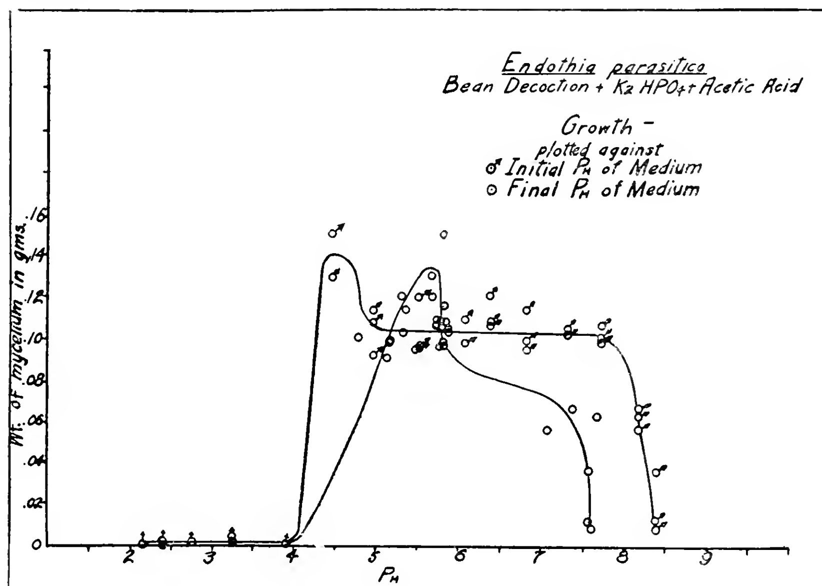

FIG. 1

duce increasing differences between the hydrogen ion concentrations indicated by the hydrogen electrode readings and by the colors of the indicators. These and other more extensive meas-

[7] Brunel and Acree: Am. Chem. J., **36**, 120. Acree: Ibid, **41**, 457. Loomis and Acree: Ibid, **46**, 5861, 632. Loomis, Meacham and Essex: J. Am. Chem. Soc., **38**, 2310; **39**, 1133. Acree and Slagle: Am. Chem. J., **42**, 130. White and Acree: J. Am. Chem. Soc., **40**, 1094; **41**, 1212. Lubs, Cloukey and Acree: Ibid, **38**, 2773, 2784. Mayer and Hantzsch: Ber. d. chem. Ges., **40**, 3479; **41**, 2568. Szyszkowski: Z. Physik. Chem., **58**, 420; **63**, 421; **73**, 269. Arrhenius: Ibid, **1**, 110; **11**, 823; **31**, 197. Harned: J. Am. Chem. Soc., **38**, 1916, and many other excellent papers. See also many papers in Amer. Chem. J. and J. Am. Chem. Soc., by Acree and coworkers on salt effects in reaction velocities.

urements now in progress show that reliable data on hydrogen ion concentrations will be secured only by using the isohydric principle, conductivity methods, the hydrogen electrode and the spectrophotometer with solutions of buffer salts and indicators having varying concentrations and increasing per cent of neutralization up to 100 per cent for both the indicator and buffer salts.

As such a study is very comprehensive and as other workers may be interested in knowing whether, and to what extent, phosphates show "salt effects" with phenolsulfonphthalein, we shall present here a provisional graphical method for showing the magnitude of the "salt effect" in a few cases and its influence on the apparent hydrogen ion concentration and ionization constant of this indicator. The application of these results to investigations of soils, wood extracts, biological fluids of all kinds, foods, industrial liquids and solutions of every description, will naturally occur to those interested.

EXPERIMENTAL WORK

When a tautomeric monobasic indicator is ionized[8] we have the following expression $K = \frac{H \times \alpha}{1 - \alpha}$ where K is the apparent ionization constant of the indicator acting as an acid, α is the concentration of the colored anions, H is the hydrogen ion concentration and $(1-\alpha)$ is the concentration of the non-ionized molecules.

If we assume that the color of the indicator solution is a measure of the amount of the indicator which has been ionized, then it is possible to determine the value of $(1-\alpha)/\alpha$ colorimetrically. For example, if we use monosodium phenolsulfonphthalein as a monobasic indicator, we find that it shows an absorption band with a maximum for light of wave length $\gamma = 0.5557\ \mu$. In the future we shall refer to this band as the green band.

If light of intensity I_0 falls upon a cell containing a solution of the indicator, and the intensity of the transmitted light is I, then

[8] For fuller discussion of the quinone-phenolate theory applying to the sulfonphthaleins see references 6 and 7.

the ratio of the two intensities can be expressed by $\frac{I}{I_0} = 10^{-N}$. The term N we shall call the absorption index. If the absorption index for the middle of the band, when the indicator is partly ionized, is N_α, and the index when the indicator is completely ionized is N, and we assume the absorption to be proportional to the ionization, then $(1 - \alpha)/\alpha = (N - N_\alpha)/N_\alpha$ and if the hydrogen ion concentration (H) were known then $K = \frac{H \times N_\alpha}{N - N_\alpha}$. It is well known[9] that the presence of neutral salts affects the color of some indicators. It is then impossible to determine K directly if disturbing neutral salts are present. It is possible, however, to make solutions of approximately the same hydrogen ion concentration and yet vary the concentration of the salt. By determining the value of $(1 - \alpha)/\alpha$ due to the successive concentrations of the salt we can extrapolate and find the value that $(1 - \alpha)/\alpha$ would have were there no salt present, and, from that and the known hydrogen ion concentration, we can find the value of the ionization constant of the indicator and its variation with changing concentration of salt.

The method adopted was as follows: Three solutions were made up of sodium phosphate and alkali in such a manner that the amount of phosphate salt in each was the same (N/2). The concentration of the phenolsulfonphthalein was N/5000, but the values of the hydrogen ion concentration were different in each solution. These solutions were called A, B and C. To 100 cc. of these solutions 50 cc. of N/2500 phenolsulfonphthalein were added and the whole made up to 200 cc. with conductivity water. These solutions were denoted by A_2, B_2, and C_2, and are N/4 in concentration. To 100 cc. of these solutions 25 cc. of N/2500 phenolsulfonphthalein were added and the whole made up to 150 cc. with conductivity water. These were denoted by A_3, B_3, and C_3, and are N/6 in concentration.

The absorption index at the middle of the green band for each of these solutions was then measured by means of a Brace spec-

[9] For literature see reference 7.

trophotometer. The measurements are therefore more accurate than ordinary colorimetric data. The actual data and calculations involved are of course very extensive. We shall not give all these numerical values in this paper but state how the final figures given in the tables were obtained. The method was to measure the absorption for several wave lengths in the band. A smooth curve was drawn with wave lengths as abscissae and absorption indices as ordinates. The ordinate with the maximum value on this curve was taken as the absorption index for the middle of the green band. This index would change, of course, for different depths of cell and for various concentrations of the indicator. In order, therefore, that Birge's work might be utilized,[10] the value of the index was reduced, by Beer's law, to the value it would have were the cell 6 cm. deep and the concentration of the phenolsulfonphthalein N/1000. The values of the index (N_α) thus obtained are given in table 1, column 3.

Birge found in his work that, with different dilutions, the value of the absorption index for the green band when the indicator was wholly transformed showed some slight variation which will not appreciably modify the results in this paper. The value given for N/5000 concentration was 225. This was then taken provisionally for our value of N but will be measured for each concentration of the different indicators and of the phosphate and other buffers. Column 4 in the table gives the values of $(1 - \alpha)/\alpha$ as computed from the values $(N - N_\alpha/N_\alpha$.

The hydrogen ion concentration (H) of each solution was measured by the Loomis-Acree method[11] involving the N/10 KCl-HgCl electrode, 4.1NKCl as a connection link and the hydrogen electrode, and recorded in column 5 of the table. Column 6 gives the value of the ionization constant as computed from the values of $(1 - \alpha)/\alpha$ and (H). It will be seen that the value of K

[10] Unpublished work by Birge, Brightman, Meacham, Hopfield and Acree. See also Birge and Acree: J. Am. Chem. Soc., **41**, 1031, Brightman, Hopfield, Meacham and Acree, Ibid., **40**, 1940.

[11] Loomis and Acree: Am. Chem. J., **46**, 585, 621, Myers and Acree: Ibid, **50**, 396. Clark, Myers and Acree, J. Phys. Chem. **20**, 243. The errors involved in the Bjerrum method, and in the Walpole modification, which was used by Lubs and Clark, will be discussed in another article.

is far from constant and increases with increasing concentration of the salt, but averages about 2.65×10^{-8}.

In order to better appreciate this relation the points were plotted on coordinate paper, using values of *H* as abscissae and corresponding values of $(1 - \alpha)/\alpha$ as ordinates (fig. 2). Then

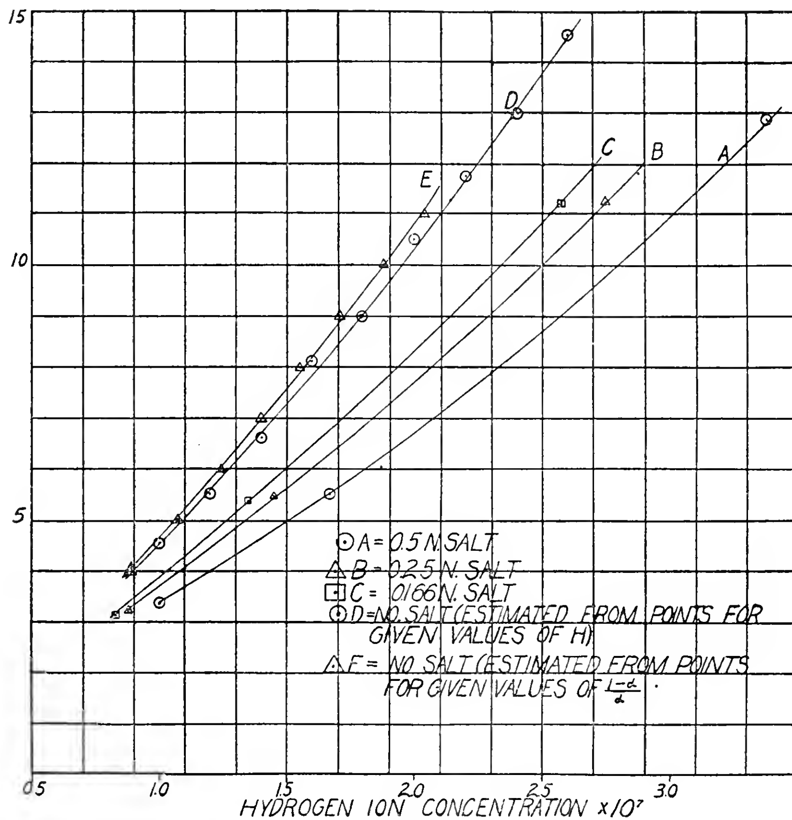

Fig. 2

the smooth curves *A*, *B*, and *C*, were drawn through the points which represented the same concentration of salt in the solution. These curves were then treated as follows: for values of $(1 - \alpha)/\alpha$ equal to 4, 5, 6, 7, 8, 9, 10 and 11 the change in hydrogen ion concentration for changes in concentration of salt from 0.5 N to 0.25 N and from 0.25 N to 0.166 N, were measured on the curves.

The average ratio of these two values was computed, and from this ratio we computed the value of the change in hydrogen ion concentration when the salt concentration changes from 0.166 N to 0. These changes were measured off on the curve sheet, the points being indicated by Δ, and a curve E was drawn through these points. The slope of this line can be called the ionization constant of the phenolsulfonphthalein. The value as determined from this curve is 1.93×10^{-8} (table 2). In the same way Curve D was obtained by taking given values of hydrogen ion concen-

TABLE 1

(1) SOLUTION	(2) SALT CONCENTRATION	(3) N_α	(4) $\frac{N-N_\alpha}{N_\alpha}$	(5) $H \times 10^7$	(6) $K \times 10^8$
A_1	0.5 N	16.2	12.88	3.39	2.63
A_2	0.5 N	18.4	11.23	2.75	2.44
A_3	0.5 N	18.4	11.23	2.57	2.28
B_1	0.25 N	34.2	5.54	1.67	3.02
B_2	0.25 N	34.8	5.46	1.46	2.67
B_3	0.25 N	35.2	5.39	1.35	2.50
C_1	0.16 N	51.5	3.37	1.00	2.96
C_2	0.16 N	53.3	3.22	0.88	2.73
C_3	0.16 N	54.0	3.16	0.83	2.63
Average value					2.65

tration between 10^{-7} and 2.6×10^{-7} and using the changes in $(1 - \alpha)/\alpha$ freed from "salt effects." The ionization constant obtained from curve D is 1.98×10^{-8} (table 3).

If this graphical method is approximately correct, curves D and E should coincide and the corresponding ionization constants should be identical. That such is found to be approximately true gives confidence in this method as a provisional one. The decrease in ionization constant is about that expected from our theory of these indicators and justifies the use of the simplified equations given above in place of the more complicated ones actually applying to the dibasic sulfonphthalein series. The decrease in ionization constant with decrease in sodium and other

applications of the isohydric principle and "salt effects," etc., will be studied in the future.

TABLE 2

(*Curve E*)

$(1-\alpha)/\alpha$	$H \times 10^7$	K
4	.89	2.21
5	1.08	2.16
6	1.24	2.06
7	1.41	1.98
8	1.56	1.95
9	1.61	1.79
10	1.69	1.69
11	1.74	1.58
Average value..........		1.93

TABLE 3

(*Curve D*)

$H \times 10^7$	$(1-\alpha)/\alpha$	K
1	4.6	2.17
1.2	5.5	2.16
1.4	6.62	2.12
1.6	8.15	1.96
1.8	9.00	2.00
2.0	10.5	1.90
2.2	11.75	1.87
2.4	13.00	1.84
2.6	14.55	1.79
Average value..........		1.98

CORRECTIONS FOR "SALT EFFECTS"

The bacteriologist and botanist, as well as the chemist, are interested in knowing the error in measurement of hydrogen ion concentration caused by these "salt effects." It will be observed that in the above curves the indicator is changed into the deeply colored salt to the extent of from 8 to 30 per cent, which covers the best range for accurate colorimetric work. As stated above, the entire range will be given later. The percentage change in hydrogen ion concentration necessary to produce the same color intensity in the different concentrations of the phosphate solutions is practically independent of the color intensity in this range. In other words, between about 8 and 30 per cent change of the indicator into the colored salt, the deviation in P_H with changing phosphate concentration, but constant color intensity is practically independent of the value $(N - N\alpha)/N\alpha$ or per cent of indicator present as colored salt.

Another way of stating this is to say that for a given hydrogen ion concentration more phosphate produces more color in the solution or a greater positive "salt effect," while more sodium produces less color. Now the magnitude of this "salt effect" is

not large and the "corrections" are small. If there is any change in this "correction," it fortunately becomes smaller with increasing color. These facts have been known *qualitatively* to Acree from preliminary work ever since this study of the sulfonphthalein series was suggested by him and undertaken by Slogle, White, Lubs, Clark, Guy, Birge, Hopfield and the present authors; it was one of the chief reasons, along with the intense color[12] of these indicators and the lack of "fading" in excess[13] of alkali, for choosing this as the most promising series of indicators to develop for colorimetric work. Bacteriologists not familiar with the history of the work are referred to the articles in the Journal of the American Chemical Society. Now that we have the present study completed, we can express *quantitatively* in the following table the "corrections" which workers must use to compare their P_H values for the same color in different phosphate concentrations. Any worker can calculate the "correction" to be applied to his own data in order to compare his results with those for another concentration of phosphate or even for cases where no phosphate is present. The table gives the corrections in P_H which must be *added* to the observed values in order to give the P_H in solutions free from phosphates and "salt effects" and having the same indicator transformation. The corrections are calculated from the above curves and the unpublished ones and can be considered accurate within the usual experimental errors. There may be some changes for the dilute solutions. As it requires careful work to determine hydrogen ion concentrations within 0.02–0.03 P_H it is clear that the "salt effects correction" for these phosphates below N/10 or N/25 concentrations is within the usual experimental error. As the writers have obtained excellent results in growing fungi on media buffered with M/25 and M/50 solutions of phosphates mixed with asparaginates, acetates and phthalates we recommend in general the use of the more dilute buffers in order to obviate these "salt" errors. Of course the concentration and "buffer range" required depends not only

[12] See the articles by White, Lubs, Birge and Acree, loc. cit., for the relation of the color intensity to the tautomeric equilibrium and ionization constants.

[13] See Brightman, Hopfield, Meacham and Acree, loc. cit.

on the limiting and optimum hydrogen ion concentration used by the organism, but also upon the concentration and ionization constant of the acid or base generated by the organism, and the proper selection can be made only after the preliminary study of the generated acid or base by (a) successive titrations with suitable indicators covering the P_H ranges involved or by (b) making a titration curve for the resulting medium with the aid of a hydrogen electrode. A fuller discussion of these points will be given in another article now completed.

TABLE 4

P_H *Correction to be added to colorimetric* P_H *for different concentrations of phosphate in order to obtain the electrometric* P_H

COLORED INDICATOR SALT	N/2 PHOSPHATE	N/4 PHOSPHATE	N/6 PHOSPHATE	N/10 PHOSPHATE	N/25 PHOSPHATE	N/50 PHOSPHATE	N/100 PHOSPHATE
per cent							
8.0	0.16	0.12	0.070	0.05	0.02	0.01	0.00
10.0	0.16	0.10	0.075	0.05	0.02	0.01	0.00
16.6	0.15	0.10	0.068	0.05	0.02	0.01	0.00
20.0	0.13	0.08	0.063	0.05	0.02	0.01	0.00
Rounded average	0.15	0.10	0.07	0.05	0.02	0.01	0.00

CONCLUSIONS

1. It is shown that phosphate solutions varying in concentration from N/2 downward and showing the same hydrogen ion concentration by the hydrogen electrode have marked influence on the color of phenolsulfonphthalein salts. This "salt effect" becomes small in phosphate solutions having low concentrations from 0.05 N down.

2. The apparent ionization constant of the phenolic group of the phenolsulfonphthaleins varies with the concentration of the phosphate present and averages about 2.65×10^{-8} when uncorrected for "salt effect."

3. When a graphical method is used for calculating the ionization constant freed from "salt effects" of phosphates, the value is lowered to about 1.95×10^{-8}.

4. In a more comprehensive study now in progress, the "salt effect" is under investigation for (a) all degrees of neutralization of several sulfonphthalein indicators and buffer salts from 0 to 100 per cent, and for (b) different concentrations of the indicators and buffer salts.

5. When measuring the limits of tolerance for hydrogen, or other ions, or molecules by organisms, care should be taken to state the concentration of chemicals and buffers used and possibly to free the published constants of these "salt effects."

A MODIFICATION OF LOEFFLER'S FLAGELLA STAIN

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Although the presence of flagella on motile bacteria was determined somewhat earlier, Robert Koch, in 1877, was the first to demonstrate their presence by the use of stains. He succeeded by using an aqueous solution of haematoxylin, and dilute chromic acid as a mordant. About 1889 Loeffler, by an improved method, succeeded in staining the flagella on a number of organisms whose flagella had previously not been demonstrated.

As it has been found that the arrangement of the flagella on bacteria is one of the characters which varies perhaps less than any other morphological characters, this arrangement furnishes an easy method for classification. Certain genera are separated on this one point alone as in the separation of the genus *Pseudomonas* from the genus *Bacillus* according to Migula's classification. Since the arrangement of flagella is thus used in establishing genera, it is necessary to determine definitely the character of the flagella on bacteria, and hence the need of reliable and simple flagella stains.

Loeffler, in his process, allowed the cover-glass preparation to dry, fixed it by passing through the flame, and then used the following solutions:

No. 1. Mordant

	parts
Solution of tannic acid (20 per cent aqueous)	10
Saturated aqueous solution (cold) of ferrous sulphate	5
Saturated alcoholic solution of basic fuchsin	1

No. 2. Stain

Carbol-fuchsin.

No. 3. Corrective solutions

A 1 per cent solution of caustic soda.

A solution of sulphuric acid of such strength that 1 cc. will neutralize 1 cc. of the soda solution.

For certain organisms Loeffler claimed that no. 1 was sufficient, while in other cases varying amounts of the corrective solutions had to be added, according to the organism studied.

Since this method was devised by Loeffler, a number of modifications have come into use, one of the first being the abandonment of the corrective solutions, as their use was found to be unnecessary. Loewit (1896) modified the process by using a copper sulphate-tannic acid mordant instead of the ferrous sulphate-tannic acid. Another modification was made by Bunge (1894) who used ferric chloride in the mordant instead of ferrous sulphate. The use of ferric chloride in the mordant, in part, in my new modification is an application of Bunge's modification.

As a usual rule, flagella staining has been a more or less hit and miss process. In text-books on bacteriology it is in substance so acknowledged, as by Park and Williams (1914), who state that with the method advised "frequently the flagella appear well stained but often the process has to be repeated a number of times." Giltner (1916) states that "failure to make a good flagella stain with any method is no sign that the student is not a good workman, nor is success the sign of a good bacteriologist."

I have believed that at least a part of the difficulty of staining flagella has been due to difficulties in fixing the bacteria on the cover-glass. In most processes it is advised to heat the cover-glass preparation by passing it through the flame or by holding it over a flame, but it is sometimes added that care must be taken not to overheat. It is a difficult matter always to heat to the same extent by passing a cover-glass through a flame, and furthermore the flagella of certain bacteria might be overheated more readily than those of certain others.

As the fixing by heat has been one of the doubtful procedures, I have experimented with several different chemical solutions in order to accomplish the fixing without heat. Two solutions especially have been used, both of which have given good results, the preference being perhaps for the first, although both are effective. These solutions are made up as follows:

No. 1

Commercial formalin (40 per cent formaldehyde)	1
Distilled water	1

No. 2

Chromic acid (5 per cent. aqueous solution)	2
Acetic acid (10 per cent aqueous solution)	1

As soon as the cover-glass preparation, made as described later, has dried in the air, the cover-glass is flooded with either of these solutions, and after a minute and a half or two minutes it is washed off with water. Although I have had good results with both of these solutions, I have found that the mordant used by me is sufficient without previously using a special fixative so that it simplifies the process to dispense entirely with any fixing solutions.

It was my aim besides doing away with the heating of mordants and stains, so that the entire process is performed at room temperatures, to use only those solutions which will keep well, so as to avoid making them up freshly every time.

One of the first requisites for success in flagella staining is to have cover-glasses which are absolutely clean and free from every trace of oil or grease. I have found that they may be satisfactorily cleaned in the following manner: I drop them, one at a time, into a small stender dish containing a solution prepared as follows:

	parts
Water	1000
Potassium bichromate	60
Sulphuric acid (commercial)	60

After allowing the cover-glasses to stand in this cleaning solution for ten minutes, or longer, the solution is washed out by running clear tap water into the dish until every trace of color has disappeared. I then pour off the water and cover the cover-glasses with 95 per cent alcohol, using about 20 to 25 cc. of alcohol, and add about 15 drops of concentrated ammonium hydroxide. A few minutes later the cover-glasses are picked out, one at a time, with a pair of forceps, and dried carefully by wiping them with lens paper. It is best to use only one side of the paper,

as otherwise grease from the fingers may get on the cover-glasses which should never be touched with the fingers except around the edges. When wiped, the cover-glasses are placed in a well cleaned Petri dish and heated in an oven at a temperature of about 275 to 300°C. for one hour. In addition to these precautions I frequently flame the cover-glasses a little just before using.

In obtaining cultures to stain, it is usually necessary to have a culture eighteen to twenty hours old. However, in the case of certain organisms which multiply very rapidly, and which begin to form spores in a few hours, cultures not more than twelve hours old are sometimes desirable, as for example in the case of *Bacillus subtilis*. Certain other organisms which develop only at relatively low temperatures, and grow slowly, may give good results from much older cultures, even a week or more, as for example *Pseudomonas janthina*. For all of my work which has given favorable results, cultures on slanted agar-agar have been used.

The tube of slanted agar-agar is inoculated, and incubated at 37° if it will grow under those conditions; otherwise it is allowed to remain at room temperatures. When the culture is ten to twenty hours old or older, depending upon the species, some of the bacteria are carefully removed from the surface of the agar-agar, and placed in a test tube containing 2 or 3 cc. of sterilized tap water. Care is taken to remove none of the culture media while transferring the bacteria. It is best to have the tap water at the same temperature as the culture, and to accomplish this I usually place the tubes of sterile water in the incubator while the cultures are developing. After the transfer to the water, the tube is allowed to stand fifteen to twenty minutes, or longer, at room temperatures, or preferably in the incubator, in order to allow the bacteria to diffuse through the water, and to permit of some slight development.

Several small drops of this water are then placed on a clean cover-glass with a very small platinum loop, and without any attempt at spreading them, the drops are allowed to dry in the air. If the drops are small they will dry more rapidly. Good

results may be obtained from fairly large drops, but ordinarily they should not be over 2 or 3 mm. in diameter. As soon as the drops are dry, the preparation is ready for the mordant which is prepared as follows:

Solution A

	parts
Ferric chloride (1-20 aqueous solution)	1
Saturated aqueous solution tannic acid	3

This solution improves with age, and should be at least a week or two old. It should be kept made up in stock but filtered before using, although when using at frequent intervals, it need not be filtered except every few days.

Solution B

Anilin-oil	1
95 per cent. alcohol	4

About eight drops of solution A are placed on the cover-glass, this is followed immediately by one drop of solution B, and the combination is allowed to act at room temperatures for two minutes, then washed off with water, and the water drained off by touching the edge of the cover-glass to a piece of filter paper. The preparation should not be blotted as there is danger of scratching the film. Then the cover-glass is flooded with the stain which may be carbol-fuchsin, 1 per cent safranin in 50 per cent alcohol, anilin-oil-gentian-violet, or Loeffler's alkaline methylene blue. While I have had success with all of these stains, the stain which I prefer, and use most frequently is a special methylene blue prepared as follows:

	parts
Saturated alcoholic methylene blue	30
Potassium hydroxide (1:10,000)	100
Solution B of mordant	13

It is my practice to make up this stain by adding to 30 cc. of Loeffler's methylene blue, 3 cc. of mordant solution B. This stain is immediately ready for use and keeps well. The blue gives a color which is easier on the eye than the red of fuchsin or safranin.

The stain is allowed to act for two or three minutes at room temperatures, then the cover-glass is washed thoroughly with water, allowed to dry, and mounted in balsam.

The addition of the drop of mordant solution B to the eight drops of ferric tannate in solution A, gives a very fine precipitate which is largely responsible for the success of this method. Although I have been unable to prevent the precipitate from clinging to the glass to a greater or less extent, I have been able to demonstrate flagella with a remarkable regularity. In certain species it is usual to get flagella to show on practically every slide, and they are usually quite heavily stained. It is frequently possible to note the presence of flagella by the use of the 16 mm. objective of the microscope.

This process has given very good results in the hands of students who are doing only their third laboratory period's work on staining of any kind. Most of the class are able to get flagella in their first attempt, without any previous experience in flagella staining at all. I have found it possible, by shortening the time of allowing the mordant and stain to act, to demonstrate flagella, mounted in balsam, in a little less than five minutes from the time the bacteria were placed on the cover-glass.

I have modified the process slightly to see if it were possible to color the flagella differently from the cell wall. After allowing the action of the chrom-acetic fixing solution, and of the mordant, the preparation was stained with either Loeffler's methylene blue, or the special methylene blue for four or five minutes, then 1 per cent safranin in 50 per cent alcohol was applied for from two to four minutes, and the cover-glass preparation was washed, dried, cleared with cedar oil, and mounted in balsam. This modification of the process was tried mainly with *Bacillus vulgaris*, and gave fairly good results at times. The flagella appear red, the cell wall bluish, and the color of the protoplast is about the same color as that of the flagella. This gives further support to the contention that the flagella are not appendages of the cell wall, as they were formerly supposed to be, but rather continuations of the protoplast.

SUMMARY

This modification of Loeffler's method differs from previous modifications chiefly in the use of a solution of anilin-oil in alcohol (1:4) in connection with the ferric-chloride tannic acid

mordant which was Bunge's modification. By using one drop of this solution with about eight drops of the ferric tannate solution, applied together on the cover-glass, all necessity of heating the mordant or the stain is obviated.

The stain recommended is a special methylene blue made by adding an alcoholic solution of anilin-oil (1: 4) to Loeffler's methylene blue in the ratio of 1: 10.

The chief advantages of the process are (1) its simplicity, (2) the use of solutions that keep well, (3) the use of all solutions at room temperatures, and (4) the high percentage of successful attempts, even in the hands of inexperienced students.

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BOUILLON CUBES AS A SUBSTITUTE FOR BEEF EXTRACT OR MEAT IN NUTRIENT MEDIA

ZAE NORTHRUP WYANT

From the Bacteriological Laboratory of the Michigan Agricultural College, East Lansing, Michigan

Received for publication, December 1, 1919

During the war the high price of meat and the frequent scarcity of meat extract seriously hampered the preparation of ordinary nutrient media in large quantities, and ordinary bouillon cubes were therefore tested out as a possible substitute. As these cubes contain, besides beef extract, certain vegetable extracts, their use in media for some organisms might be of doubtful value, while for others the growth would be more or less favored. It was to determine this point that the following experiments were carried out. Bovim and Steero bouillon cubes were used in all the tests. Comparisons were made of cultures grown in similar broths made from each kind of cube.

Two lots of 500 cc. each of broth were prepared using Bovim and Steero cubes respectively in the proportions of one cube to 250, 500, 750, 1000 and 1500 cc. of water.

The cubes were dissolved in boiling tap water, the peptone added and dissolved; then the liquid was cooled to coagulate the fats present, and filtered cold, after which it was tubed and sterilized at 15 pounds in the autoclave.

Subsequent to sterilization two tubes of each dilution both with and without peptone were inoculated with the following organisms, *B. typhosus*, *B. prodigiosus*, *B. subtilis*, *B. coli* and *B. botulinus*. Results proved entirely satisfactory.

Since these experiment were concluded (nearly a year) the use of bouillon cubes as a substitute for meat extract in ordinary media has been general and has given entire satisfaction to all our laboratory staff. As a substitute for meat it is as satis-

factory if not more so, than the ordinary meat extracts. Our laboratory stock cultures, pathogens and all, with but few exceptions, are at present growing very well indeed on this bouillon cube medium. One or one and a half cubes (about 3 or 5 grams) are used per liter at a much decreased cost, with an increased ease in preparation, and the titer of the medium does not have to be changed unless so desired.

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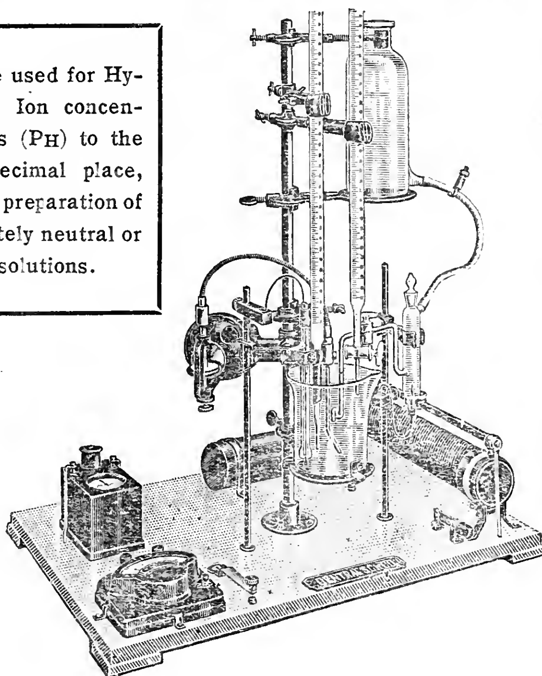

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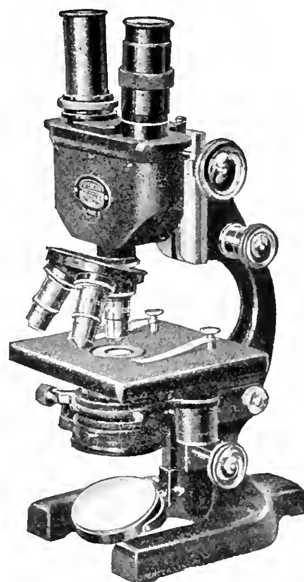

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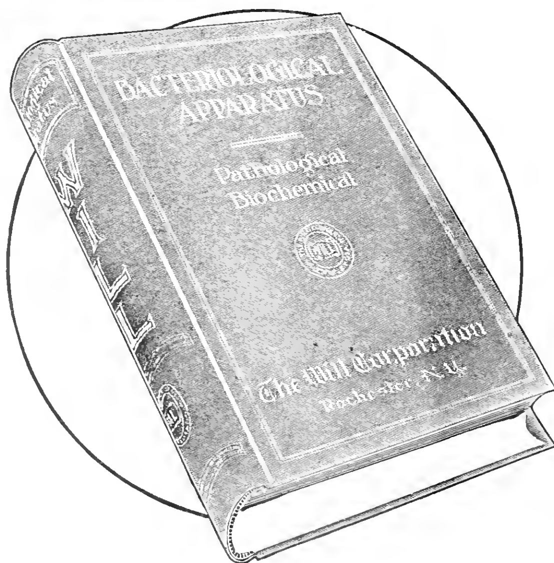

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THE FAMILIES AND GENERA OF THE BACTERIA

FINAL REPORT OF THE COMMITTEE OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS ON CHARACTERIZATION AND CLASSIFICATION OF BACTERIAL TYPES

C.-E. A. WINSLOW, *Chairman*, JEAN BROADHURST, R. E. BUCHANAN, CHARLES KRUMWIEDE JR., L. A. ROGERS, AND G. H. SMITH[1]

I. INTRODUCTION

The first report of the Committee on Characterization and Classification of Bacterial Types was published in the Journal of Bacteriology for September, 1917, vol. 2, p. 505, and was discussed in some detail at the succeeding meeting of the Society.

In this preliminary report the committee reviewed briefly the historical development of systematic bacteriology from Ehrenberg to Orla-Jensen, discussed the principles of botanical nomenclature with extensive citations from the International Rules of Botanical Nomenclature, and presented a tentative system of classification of the Schizomycetes into families and genera.

The detailed classification presented was criticised in certain respects in the full discussion which followed, and in later correspondence between the committee and other members of the Society, and it was felt desirable that the scheme presented in 1917 should be revised in certain particulars and made more definite and if possible supplemented by an index of genera showing where the commoner bacterial species should be placed.

It was found impossible to complete this task and to prepare a list of approved genera for the 1918 meeting of the Society, but the task set before the committee has been at last completed and the committee is ready to make its final report at this time.

[1] Prof. R. S. Breed has coöperated actively in the work of the committee during the past two years, but has not felt that he could accept formal membership on the committee.

JUL 6 1920

In discussions which have taken place between the members of the committee during the past two years the first question to be decided was whether the committee should simply present a list of approved genera for adoption by the Society, or should also prepare a revision of the general scheme of classification presented in 1917.

It was felt by some that the presentation by the committee of any scheme of classification would tend to give such a scheme undue authority and to impose arbitrary limits upon the development of the changing science of systematic bacteriology. It should be recognized most clearly that the limits of biological groups must always be subject to change with the growth of knowledge. The classification presented at this time seems to the committee the most reasonable outline for true biological relations among the bacteria which can be drawn up in the state of present knowledge, but this outline will necessarily be modified with the progress of investigations by individual systematists of the future. It is exceedingly improbable that any member of the committee would present the same classification in 1925 that is presented today. Indeed as to the position of certain genera the committee is itself in serious doubt at the present time.

In spite of these facts the Committee was of the opinion that it would be helpful to the members of the Society of American Bacteriologists to have the best judgment of the members of the committee as to the most natural method of classification at present available, particularly in view of the desirability of correcting certain errors in the earlier report already in type. Your committee has therefore prepared a modified arrangement of the families and genera of the Actinomycetales and Eubacteriales, which is presented as section III of this report. The sequence of families and genera has no special significance, and in some cases it is doubtful in which families certain genera should be placed. The 38 genera themselves that are here presented are however believed by the committee to represent for the most part real biological groups.

The second general problem, which had to be met by the committee, concerned the method of defining bacterial genera. A

recent report of the committee on generic types of the Botanical Society of America, published in Science for April 4, 1919, has urged that the application of generic names should be determined by type species rather than by attempts at generic characterization; and with this point of view the members of the committee on characterization and classification of bacterial types are in accord. The committee was agreed that type species with proper literature references should be included in all the genera listed, but that it was also desirable to include brief characterizations of the genera themselves. The situation with which we deal in attempting to classify the bacteria is somewhat different from that which exists among the higher plants. In the latter case actual type species have been deposited in herbaria and are available for reference; while among the bacteria this is not the case except for a few type species which have recently been deposited in the collection of the American Museum of Natural History in New York. In consideration of the uncertainty which surrounds the description of many bacterial species it was felt that it would be helpful to furnish at least tentative characterizations of the genera presented, and this policy has therefore been pursued in the preparation of section III of this report.

It will perhaps be convenient to indicate briefly the general changes in classification which distinguish the present report from that of 1917. The main departures are as follows:

The group recognized in 1917 as the family Mycobacteriaceae has now been elevated to the rank of an order Actinomycetales, and divided into two families, Actinomycetaceae and Mycobacteriaceae. To the former family we have added the genera *Actinobacillus* and *Erysipelothrix*, and we have omitted *Nocardia*, which Breed (1919) has recently shown should be combined for the present with *Actinomyces*. To the second family we have added the genus *Pfeifferella*.

The Nitrobacteriaceae have been divided into two tribes, the Nitrobactereae and Azotobactereae, and the definition of the family has been modified to permit the inclusion of *Rhizobium* which recent investigations have shown to possess peritrichous flagella, but whose general characteristics ally it clearly with

Azotobacter. The name *Acetobacter* has been substituted for *Mycoderma* to characterize the vinegar organisms.

Among the Coccaceae a new tribe was created for the genus *Neisseria*. The genus *Albococcus* is united with *Staphylococcus* and the new genera *Diplococcus* and *Leuconostoc* are added.

The Bacteriaceae are divided into seven tribes: Chromobactereae, Erwineae, Bactereae, Lactobacilleae, Pasteurelleae, Hemophileae, and Zopfeae, and the new genera *Erythrobacillus, Chromobacterium, Zopfius,* and *Proteus* are added. The Lactobacillaceae, originally recognized as a distinct family, are thus classed as a tribe of the Bacteriaceae.

For the convenience, particularly of students, we have prepared in section IV of this report an artificial key to the families and genera of the Actinomycetales and the Eubacteriales, which we hope may be of value. It should be possible to place a key of this kind in the hands of a student and enable him at least to determine the general generic group to which any organism belongs. In the case of certain genera the specific types can be easily identified by reference to monographs such as those of Wenner and Rettger (1919) on Proteus, Ford (1916) on Bacillus, Winslow, Rothberg and Parsons (1920) on Staphylococcus, and Winslow, Kligler and Rothberg (1919) on Bacterium.

Finally in section V of this report we have presented a generic index of the commoner species of bacteria with the names ordinarily used in the texts and with the new nomenclature indicated by the proposed classification. This list has been prepared by Miss Dorothy F. Holland of the Department of Public Health of the Yale School of Medicine. It is not intended to be exhaustive or to deal in any sense with problems of *specific* identity, but merely to serve as an index of generic reference for the more familiar types.

II. SPECIFIC RECOMMENDATIONS

The classification presented by the committee, the key and the generic index, as stated above, represent merely the consensus of opinion of the members of the committee as corresponding to the most natural system of classification indicated by present knowl-

edge. They are in no sense presented as official or binding. On the other hand in order that stability of nomenclature may be assured it is essential that certain generic names should be formally adopted by the Society, and where necessary established in the future by an International Botanical Congress, as genera conservanda. Such genera are provided for in the International Rules of Botanical Nomenclature in cases where a strict application of the rules of nomenclature, and especially the principle of priority starting from a certain date, would produce confusing and disadvantageous changes. In our own case it seems desirable to preserve in this way a number of generic names which have come into such general use that their abandonment would cause confusion, particularly in dealing with the large number of medical bacteriologists who are not familiar with the principles of botanical taxonomy. It is essential to proceed in a somewhat conservative fashion if any influence for good is to be exerted upon general practice in this field.

The following names are recommended for adoption as approved genera:

Acetobacter Fuhrmann
Actinomyces Harz
Bacillus Cohn
Bacterium Ehrenberg
Chromobacterium Bergonzini
Clostridium Prazmowski
Erythrobacillus Fortineau
Leptotrichia Trevisan
Leuconostoc Van Tieghem
Micrococcus Cohn
Rhizobium Frank
Sarcina Goodsir
Spirillum Ehrenberg
Staphylococcus Rosenbach
Streptococcus Rosenbach
Vibrio Mueller

Its work so far as possible being completed, we recommend that the Committee on Characterization and Classification of Bacterial Types be discharged and that a new Committee on Bacterial Taxonomy be appointed (1) to study and report to the Society from time to time in regard to problems of nomenclature, including such revisions of the nomenclature in the present report as may seem necessary; and (2) to take the proper steps to secure action at the next International Botanical Congress leading to the general ends contemplated in the 1916 recommendations of the Society:

(a) That French, English or German may be substituted for Latin in the diagnosis of bacterial species.

(b) That the date of publication of the third edition of Zopf's Spaltpilze (1883) be considered the beginning of bacterial nomenclature for the purpose of determining priority, with the exception of a definite list of genera conservanda.

(c) That such of the approved generic names specified above as may be found to require such action be recognized as genera conservanda in bacterial taxonomy.[2]

III. SUGGESTED OUTLINE OF BACTERIAL CLASSIFICATION

THE CLASS SCHIZOMYCETES

Minute, one-celled, chlorophyll-free, colorless, rarely violet-red or green-colored plants, which typically multiply by dividing in one, two or three directions of space. The cells thus formed are usually spherical, cylindrical, comma-shaped, spiral or filamen tous and are often united into filamentous, flat, or cubical aggregates. Filamentous species often surrounded by a common sheath. The cell plasma generally homogeneous without a morphologically differentiated nucleus. Reproduction by simple fission. In many species resting bodies are produced, either endospores or gonidia. Cells may be motile by means of flagella.

A. ORDER MYXOBACTERIALES[3]

Cells united during the vegetative stage into a pseudoplasmodium which passes over into a highly-developed cyst-producing resting stage.

B. ORDER THIOBACTERIALES[3]

Cells free or united in elongated filaments. Typically water forms, not cultivable on ordinary media. Life energy derived mainly from oxidative processes. Cells typically containing either granules of free sulphur or bacterio-purpurin or both, usually growing best in the presence of hydrogen sulphide.

[2] The Society of American Bacteriologists took favorable action on these resolutions at its meeting December 29, 1919.

[3] These first three orders are included briefly to give the complete setting of the fourth and fifth with which we are primarily concerned.

C. ORDER CHLAMYDOBACTERIALES[3]

Cells normally united in elongated filaments, often showing false but never true branching. Typically water forms. Sulphur and bacterio-purpurin are absent. Iron often present and usually a well-marked sheath.

D. ORDER ACTINOMYCETALES Buchanan 1917a, p. 162

Cells usually elongated, frequently filamentous and with a decided tendency to the development of branches, in some genera giving rise to the formation of a definite branched mycelium. Cells frequently show swellings, clubbed or irregular shapes. No pseudo-plasmodium. No deposits of free sulphur or iron. No bacteriopurpurin. Endospores not produced, but conidia developed in some genera. Usually Gram-positive. Non-motile. Some species are parasitic in animals or plants. Not water forms. Complex proteins frequently required. As a rule strongly aerobic, (except for some species of *Actinomyces* and the genera *Fusiformis* and *Leptotrichia*) and oxidative. Growth on culture media often slow; some genera show mold-like colonies.

Family 1. Actinomycetaceae Buchanan 1918a, p. 403

Filamentous forms often branched and sometimes forming mycelia. Conidia sometimes present. Some species parasitic.

Genus 1. *Actinobacillus* Brumpt, 1900, p. 849

Filament formation, resembling streptobacilli. In lesions no mycelium formed, but at peripheries finger shaped branched cells are visible. Gram negative. Not acid fast.

Type species, *Act. Lignieresi* Brumpt.

Genus 2. *Leptotrichia* Trevisan 1879, p. 138

Synonyms: Leptothrix Robin 1847, not *Leptothrix* Keutzing 1843; not *Leptothrix* Zopf 1885; *Rasmussenia* Trevisan 1889.

Thick, long, straight or curved threads, unbranched, frequently clubbed at one end and tapering to the other. Gram positive when young. Threads fragment into short, thick rods. Anaerobic or facultative. Non-motile. Filaments sometimes granular. No aerial hyphae or conidia. Parasites or facultative parasites.

The type species is *Leptotrichia buccalis* (Robin 1847) Trevisan.

Genus 3. Actinomyces Harz 1877, p. 125

Synonyms: Streptothrix Cohn 1875, not *Streptothrix* Corda 1839; *Discomyces* Rivolta 1879; *Nocardia,* Trevisan 1889; *Micromyces* Gruber 1891, not *Micromyces* Dangeard 1888; *Oospora* Sauvageau and Radais 1892; not *Oospora* Wallroth 1833; *Thermoactinomyces* Tsilinsky 1899; *Cohnistreptothrix* Pinoy 1913.

Organism growing in form of a much-branched mycelium, which may break up into segments that function as conidia. Sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in animal body. Some species are microaerophilic or anaerobic. Non-motile.

The type species is *Actinomyces bovis* Harz.

Genus 4. *Erysipelothrix* Rosenbach, 1909, p. 367

Rod-shaped organisms with a tendency to the formation of long filaments which may show branching. The filaments may also thicken and show characteristic granules. No spores. Non-motile. Gram-positive. Do not produce acid. Microaerophilic. Usually parasitic.

The type species is *Erysipelothrix rhusiopathiae* (*Bacillus rhusiopathiae suis* Kitt 1893; *Mycobacterium rhusiopathiae* Chester 1901; *Erysipelothrix porci* Rosenbach 1909), the causal organism of swine erysipelas.

Family II. Mycobacteriaceae Chester 1897, p. 63

Parasitic forms. Rod shaped, frequently irregular in form but rarely filamentous and with only slight and occasional branching. Often stain unevenly (showing variations in staining reaction within the cell). No conidia.

Genus 1. *Mycobacterium* Lehmann and Neumann, 1896a, p. 363

Synonyms: Coccothrix Lutz 1886; *Sclerothrix* Metschnikoff 1888, not *Sclerothrix* Kuetzing 1849; *Mycomonas* Jensen 1909.

Slender rods which are stained with difficulty, but when once stained are acid-fast. Cells sometimes show swollen, clavate or cuneate forms, and occasionally even branched cells. Non-motile, Gram-positive. No endospores. Growth on media slow. Aerobic. Several species pathogenic to animals.

The type species is *Mycobacterium tuberculosis* (Koch 1882) Lehmann and Neumann.

Genus 2. *Corynebacterium* Lehmann and Neumann 1896b, p. 350

Synonyms: Corynemonas Jensen 1909; *Corynethrix* Bongert 1901.

Slender, often slightly curved, rods with tendency to club and pointed forms, branching cells reported in old cultures. Barred uneven staining. Not acid fast. Gram-positive. Non-motile. Aerobic. No endospores. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when cells divide.

The type species is *Corynebacterium diphtheriae* (Loeffler 1884) Lehmann and Neumann.

Genus 3. *Fusiformis* Hoelling 1910, p. 240

Synonym: Mantegazzaea Vuillemin 1913, not *Mantegazzaea* Trevisan 1879.

Obligate parasites. Anaerobic or microaerophilic. Cells frequently elongate and fusiform, staining somewhat unevenly. Filaments sometimes formed; non-branching. Non-motile. No spores. Growth in laboratory media feeble.

The type species is *Fusiformis termitidis* Hoelling.

Non-fusiform types like *B. acne* and the anaerobic types found in Hodgkin's disease may for the present be tentatively left in this genus.

Genus 4. *Pfeifferella* Buchanan 1918b, p. 54

Non-motile rods, slender, Gram-negative, staining poorly, sometimes forming threads and showing a tendency toward branching. Gelatin may be slowly liquefied. Do not ferment carbohydrates. Growth on potato characteristically honey-like.

Type species, *Pfeifferella mallei* (Loeffler 1886) Buchanan (the glanders bacillus)

The real lines of demarcation between the genera *Actinobacillus*, *Erysipelothrix*, *Fusiformis* and *Pfeifferella* and their relations to *Actinomyces* on the one hand and to *Mycobacterium* on the other seem very obscure and the above arrangement can be considered as only tentative.

E. ORDER EUBACTERIALES Buchanan 1917b, p. 162

The order Eubacteriales includes the forms usually termed the true bacteria, that is, those forms which are considered least differentiated and least specialized. The cell metabolism is not primarily bound up with hydrogen sulphide or other sulphur compounds, the cells in consequence containing neither sulphur granules nor bacterio-purpurin. The cells apparently do not possess a well-organized or well-differentiated nucleus. These organisms are usually minute and spherical, rod-shaped or spiral, in most genera not producing true filaments; and rarely branching. The cells may occur singly, in chains or other groupings. They may be motile by means of flagella, or non-motile; but are never *notably* flexuous. Cell multiplication occurs always by transverse, never by longitudinal fission. Some genera produce endospores, particularly the rod-shaped types. Conidia not observed. Chlorophyll is absent, though the cells may be pigmented. The cells may be united into gelatinous masses, but never form motile pseudoplasmodia nor develop a highly specialized cyst-producing fruiting stage, such as is characteristic of the *Myxobacteriales*.

Family I. NITROBACTERIACEAE Buchanan, 1917c, p. 349

Organisms usually rod-shaped (sometimes nearly spherical in *Nitrosomonas* and possibly in *Azotobacter*.) Cells motile or non-motile. Branched involution forms in *Rhizobium* and *Aceto-*

bacter. Endospores never formed. Obligate aerobes, capable of securing growth energy by the direct oxidation of carbon, hydrogen or nitrogen or of simple compounds of these. Non-parasitic (except in genus Rhizobium)—usually water or earth forms.

Tribe I. NITROBACTEREAE

Organisms deriving their life energy from oxidation of simple compounds of carbon and nitrogen (or of alcohol).

Genus 1. *Hydrogenomonas* Orla-Jensen 1909, p. 311

Monotrichic short rods capable of growing in the absence of organic matter, and securing growth energy by the oxidation of hydrogen (forming water). Kaserer (1905) who first described the organism states that his species will also grow well on a variety of organic substances.

The type species is *Hydrogenomonas pantotropha* (Kaserer 1906) Orla-Jensen. Nikleuski (1910) described two additional species, *H. vitrea* and *H. flava*.

Genus 2. *Methanomonas* Ora-Jensen 1909, p. 311

Monotrichic short rods capable of growing in the absence of organic matter and securing growth energy by the oxidation of methane (forming carbon dioxide and water). The type species is *Methanomonas methanica* (Söhngen 1906) Orla-Jensen.

Genus 3. *Carboxydomonas* Orla-Jensen 1909, p. 311

Autotrophic rod-shaped cells capable of securing growth energy by the oxidation of carbon monoxide (forming carbon dioxide). The type species, *Carboxydomonas oligocarbophila* (Beijerinck and van Delden 1903) Orla-Jensen, is described as non-motile.

Genus 4. *Acetobacter* Fuhrmann 1905, p. 8

Synonyms: Mycoderma Persoon 1822; *Ulvina* Kuetzing 1837; *Umbina* Naegeli 1849; *Bacteriopsis?* Trevisan 1885; *Gliacoccus* Maggi 1886; *Acetimonas* Jensen 1909.

Cells rod-shaped, frequently in chains, non-motile. Cells grow usually on the surface of alcoholic solutions as obligate aerobes, securing growth energy by the oxidation of alcohol to acetic acid. Also capable of utilizing certain other carbonaceous compounds, as sugar and acetic acid. Elongated, filamentous, club-shaped, swollen and even branched cells may occur as involution forms.

The type species is *Acetobacter aceti* (Thomson 1852), Committee.

Genus 5. *Nitrosomonas* Winogradsky 1892a, p. 127

Includes *Nitrosococcus* Winogradsky 1892

Cells rod-shaped or spherical, motile or non-motile, if motile with polar flagella. Capable of securing growth energy by the oxidation of ammonia to nitrites. Growth on media containing organic substances scanty or absent.

The type species is *Nitrosomonas europoea* Winogradsky.

Genus 6. Nitrobacter Winogradsky 1892b, p. 87

Synonym: Nitrosobacterium? Rullmann 1897.

Cells rod-shaped, non-motile, not growing readily on organic media or in the presence of ammonia. Cells capable of securing growth energy by the oxidation of nitrites to nitrates.

The type species is *Nitrobacter Winogradskyi*, Committee 1917a, p. 552.

Tribe 2. Azotobactereae

Nitrogen-fixing organisms

Genus 7. *Azotobacter* Beijerinck 1901a, p. 561

Synonyms: Parachromatium Beijerinck 1903; *Azotomonas* Jensen 1909.

Relatively large rods, or even cocci, sometimes almost yeast-like in appearance, dependent primarily for growth energy upon the oxidation of carbohydrates. Motile or non-motile; when

motile, with tuft of polar flagella. Obligate aerobes usually growing in a film upon the surface of the culture medium. Capable of fixing atmospheric nitrogen when grown in solutions containing carbohydrates and deficient in combined nitrogen.

The type species is *Azotobacter chroococcum* Beijerinck.

Genus 8. *Rhizobium* Frank, 1889, p. 338.

Synonyms: Phytomyxa Schroeter 1886; *Cladochytrium* Vuillemin 1888; *Rhizobacterium* Kirchner 1895; *Pseudorhizobium* Hartleb 1900; *Rhizomonas* Jensen 1909.

Comment. *Phytomyxa* Schroeter has priority over *Rhizobium*, but because of the confusion which would arise from the substitution of the older correct name for the better known term *Rhizobium*, the committee recommends the adoption of the latter.

Minute rods, motile when young. Involution forms abundant and characteristic when grown under suitable conditions. Obligate aerobes, capable of fixing atmospheric nitrogen when grown in the presence of carbohydrates in the absence of compounds of nitrogen. Produce nodules upon the roots of leguminous plants.

The type species is *Rhizobium leguminosarum* Frank.

Family II. Pseudomonadaceae, Committee 1917b, p. 555

Rod-shaped, short, usually motile by means of polar flagella or rarely non-motile. Aerobic and facultative. Frequently gelatin liquefiers and active ammonifiers. No endospores. Gram stain variable, though usually negative. Fermentation of carbohydrates as a rule not active. Frequently produce a water-soluble pigment which diffuses through the medium as green, blue, purple, brown, etc. In some cases a non-diffusible yellow pigment is formed. Many yellow species are plant parasites.

Genus 1. *Pseudomonas* Migula 1894, p. 237, emended

Synonyms: Bacterium Ehrenberg emended Cohn 1872; *Bactrillum* Fischer 1895; *Arthrobactrinium* Fischer 1895; *Arthrobactrillum* Fischer 1895; *Eupseudomonas* Migula 1895; *Bactrinius* Kendall 1902; *Bactril-*

lius Kendall 1902; *Bacterium* Ehrenberg emended E. F. Smith 1905; *Denitromonas* Jensen 1909; *Liquidomonas* Jensen 1909.

Characters, those of family.

Type species, *Ps. aeruginosa* (Schroeter) Frost?

FAMILY III. SPIRILLACEAE. Migula 1894, p. 237

Cells elongate, more or less spirally curved. Cell division always transverse, never longitudinal. Cells non-flexuous. Usually without endospores. As a rule motile by means of polar flagella, sometimes non-motile. Typically water forms, though some species are intestinal parasites.

Genus 1. *Vibrio* Mueller 1786, p. 39, emended E. F. Smith 1905

Synonyms: Pacinia Trevisan 1885; *Microspira* Schroeter 1886; *Pseudospira* De Toni and Trevisan 1889; *Liquidovibrio* Jensen 1909; *Solidovibrio* Jensen 1909; *Photobacterium?* Beijerinck 1889.

Cells short bent rods, rigid, single or united into spirals. Motile by means of a single (rarely two or three) polar flagellum, which is usually relatively short. Many species liquefy gelatin and are active ammonifiers. Aerobic and anaerobic. No endospores. Usually Gram-negative. Water forms, a few parasites.

The type species is *Vibrio comma* (Koch 1884) Schroeter 1886.

Genus 2. *Spirillum* Ehrenberg 1830, p. 38 emended Migula 1894, p. 237

Synonyms: Spirobacillus? Metschnikoff 1889; *Spirosoma* Migula 1894; *Sporospirillum?* Jensen 1909.

Cells, rigid rods of various thicknesses, length, and pitch of the spiral, forming either long screws or portions of a turn. Usually motile by means of a tuft of polar flagella (5 to 20) which are mostly half circular, rarely wavy-bent. These flagella occur on one or both poles; their number varies greatly and is difficult to determine; since in stained preparations several are often united into a common strand. Endospore formation has been reported in some species. Habitat: water or putrid infusions.

Type species *Spirillum undula* (Mueller 1786) Ehrenberg.

Family IV. Coccaceae Zopf 1884, p. 45, emended Migula 1894

Synonyms: Sphaerobacteria Cohn 1872; *Coccaceen* Zopf 1884; *Coccogenae* Trevisan 1885; *Coccacei* Schroeter 1886; *Coccobacteria* Schroeter 1886; *Sphaerobacteries* Maggi 1886; *Kokkaceen* Hueppe 1886; *Coccacees* Mace 1897.

Cells in their free conditions, spherical; during division somewhat elliptical. Division in one, two or three planes. If the cells remain in contact after division they are frequently flattened in the plane of division, and form chains, packets or irregular masses. Motility rare. Endospores absent. Metabolism complex, usually involving the utilization of amino-acids or carbohydrates. Pigment often produced.

Tribe A. Neissereae, Nov. Trib.

Strict parasites, failing to grow or growing very poorly on artificial media. Cells normally in pairs. Gram-negative. Growth fairly abundant on serum media.

Genus 1. *Neisseria* Trevisan 1885, p. 105

Synonyms: Diplococcus Weichselbaum 1886 in part; *Gonococcus?* Neisser? 1879; *Merismopedia* Zopf 1885; not *Merismopedia* Meyen 1839.

Characters, those of tribe.

Type species, *N. gonorrhoeae* Trevisan.

Tribe B. Streptococceae Trevisan, 1889a, p. 1051 emended

Parasites (thriving only or best on or in the animal body) except genus *Leuconostoc*. Grow well under anaerobic conditions. Many forms grow with difficulty on serum-free media, none very abundantly. Planes of fission usually parallel, producing pairs or short or long chains, never packets. Generally stain by Gram. Produce acid but no gas in glucose and generally in lactose broth. Pigment, if any, white or orange.

Genus 2. *Diplococcus* Weichselbaum 1886, p. 506 emended

Synonyms: Klebsiella Trevisan 1885, in part; *Hyalococcus* Schroeter 1886; *Pseudodiplococcus* Bonome, 1888; *Pneumococcus?* Schmidlechner 1905.

Parasites, growing poorly, or not at all, on artificial media. Cells usually in pairs of somewhat elongated cells, often capsulated, sometimes in chains. Gram positive. Fermentative powers high, most strains forming acid in glucose, lactose, sucrose and inulin.

Type species, *D. pneumoniae* Weichselbaum.

Genus 3. *Leuconostoc* Van Tieghem 1878, p. 198, emended

Synonyms: Ascococcus Cienkowski 1878; not *Ascococcus* Cohn 1875; *Leucocystis?* Schroeter 1886.

Saprophytes, usually growing in cane sugar solutions. Cells in chains or pairs, united in large zoogleal masses. Some types at least Gram negative.

Type species, *L. mesenteroides* (Cienkowski) Van Tieghem.

Genus 4. *Streptococcus* Rosenbach 1884a, p. 22, emended Winslow and Rogers 1905, p. 669

Synonyms: Sphaerococcus Marpmann 1885, not *Sphaerococcus* Agardh 1821; *Arthrostreptokokkus* Hueppe 1886; *Perroncitoa* Trevisan 1889; *Babesia?* Trevisan 1889; *Schuetzia* Trevisan 1889; *Lactococcus* Beijerinck 1901; *Hypnococcus* Bettencourt et al. 1904; *Myxokokkus* Gonnermann 1907, not *Myxococcus* Thaxter 1892; *Melococcus?* Amiradzibi 1907; *Diplostreptococcus* Lingelsheim 1912

Chiefly parasites. Cells normally in short or long chains (under unfavorable conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. Capsules rarely present, no zoogleal masses. On agar streak, effused translucent growth, often with isolated colonies. In stab culture, little surface growth. Many sugars fermented with formation of large amount of acid, but inulin is rarely attacked. Generally fail to liquefy gelatin or reduce nitrates.

Type species is *Streptococcus pyogenes* Rosenbach.

Genus 5. *Staphylococcus* Rosenbach 1884b, p. 19

Synonyms: Micrococcus Cohn 1872 em. Migula 1894; *Botryomyces* Bollinger 1888; *Botryococcus* Kitt 1888, not *Botryococcus* Kuetzing 1849; *Galactococcus* Guillebeau; *Bollingera* Trevisan 1889; *Gaffkya* Trevisan 1885; *Pyococcus* Ludwig 1892; *Carphococcus* Hohl 1902; *Albococcus* Winslow and Rogers 1906; *Aurococcus* Winslow and Rogers 1906; *Indolococcus* Jensen 1909; *Liquidococcus* Jensen 1909; *Peptonococcus* Jensen 1909; *Enterococcus?* (Thiercelin) Rougentzoff 1914.

Parasites. Cells in groups and short chains, very rarely in packets. Generally stain by Gram. On agar streak good growth, of white or orange color. Glucose, maltose, sucrose and often lactose, fermented with formation of moderate amount of acid. Gelatin often liquefied very actively.

Type species is *Staphylococcus aureus* Rosenbach.

Tribe C. MICROCOCCEAE Trevisan, 1889b, p. 1067, emended (as METACOCCACEAE) Winslow and Rogers 1905, p. 669

Facultative parasites or saprophytes. Thrive best under aerobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cell aggregates in groups, packets or zoogleal masses. Generally decolorize by Gram. Pigment yellow or red.

Genus 6. *Micrococcus* Cohn 1872 a, p. 153, emended Winslow and Rogers, 1905, p. 669

Synonyms: Microsphaera Cohn 1872, not *Microsphaera* Leveille 1851; *Ascococcus* Cohn, 1875; *Pediococcus* Balcke 1884; *Merista* Van Tieghem 1884, not *Merista* (Banks and Soland) Cunningham 1839; *Planococcus* Migula 1894; *Urococcus* Miquel 1879; not *Urococcus* Kuetzing 1849; *Carphococcus* Hohl 1902; *Pedioplana* Wolff 1907; *Tetradiplococcus?* Bartoszewicz and Schwarzwasser 1906; *Solidococcus* Jensen 1909; *Planomerista* Vuillemin 1913.

Facultative parasites or saprophytes. Cells in plates or irregular masses (never in long chains or packets). Generally decolorize by Gram. Growth on agar abundant, with formation

of yellow pigment. Glucose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied, but not rapidly.

The type species is *Micrococcus luteus* (Schroeter) 1872b, Cohn.

Genus 7. *Sarcina* Goodsir 1842, p. 432, emended Winslow and Rogers 1905, p. 359

Synonyms: Urosarcina Miquel 1879; *Planosarcina* Migula 1894; *Pseudosarcina?* Maze 1903; *Tetradiplococcus?* Bartoszewicz and Schwarzwasser 1908; *Lactosarcina* Beijerinck 1908; *Sporosarcina?* Jensen 1909.

Sarcina differs from *Micrococcus* solely in the fact that cell division occurs under favorable conditions in three planes, forming regular packets.

The type species is *Sarcina ventriculi* Goodsir.

Genus 8. Rhodococcus Zopf 1891, p. 28, emended Winslow and Rogers 1906, p. 546

Synonyms: Not *Rhodococcus* Molisch 1907.

Saprophytes. Cells in groups or regular packets. Generally decolorize by Gram. Growth on agar abundant with formation of red pigment. Glucose broth slightly acid, lactose broth neutral. Gelatin rarely liquefied. Nitrates generally reduced.

Type species, *Rhodococcus rhodochrous* Zopf.

Family V. Bacteriaceae Cohn 1872b, p. 231 Emended Committee 1917c, p. 560

Rod-shaped cells without endospores. Usually Gram-negative. Flagella when present peritrichic. Metabolism complex, amino-acids being utilized, and generally carbohydrates.

Tribe 1. *Chromobactereae*, Nov. Trib.

Water bacteria producing a red or violet pigment.

Genus 1. *Erythrobacillus*, Fortineau 1905, p. 104

Synonyms: Zaogalactina Sette 1824; *Serratia* Bizio, 1825; *Bacillus*, in part, of many authors.

Small aerobic bacteria, producing a red or pink pigment, usually a lipochrome. Gram stain variable. It is possible that related yellow and orange chromogens should be included here as well.

Type species, *Erythrobacillus prodigiosus* (Ehrenberg) Committee.

Genus 2. *Chromobacterium* Bergonzini 1881, p. 153

Synonyms: The name was spelled *Cromobacterium* by Bergonzini and corrected by Zimmerman 1881.

Aerobic bacteria, producing a violet chromoparous pigment, soluble in alcohol but not in chloroform. Motility and Gram reaction variable.

Type species, *Chr. violaceum* Bergonzini.

Tribe 2. Erwineae, Nov. Trib.

Plant pathogens. Growth usually whitish, often slimy. Indol generally not produced. Acid usually formed in certain carbohydrate media, but as a rule no gas.

Genus 3. *Erwinia* Committee 1917d, p. 560.

Characters those of the tribe.

Type species, *E. amylovora* (Burrill 1883, p. 319; Trevisan 1889, p. 19) Committee 1917.

Tribe 3. Zopfeae, Nov. Trib.

Gram positive rods, growing freely on artificial media. Not attacking carbohydrates.

Genus 4. *Zopfius*, Wenner and Rettger, 1919, p. 334

Synonyms: Bacterium Ehrenberg 1828 in part; *Bacillus* Cohn, 1872 in part; *Proteus* Hauser 1885, in part.

Long rods occurring in evenly curved chains. Gram positive. Motile. Proteus-like growth on media. Facultative anaerobes. Carbohydrates and gelatin not attacked, hydrogen sulphide not formed.

Type species, *Z. zopfii* (Kurth) Wenner and Rettger.

Tribe 4. Bactereae, Nov. Trib.

Gram negative rods growing freely on artificial media. Generally forming acid from carbohydrates and often gas composed of CO_2 and H_2.

Genus 5. *Proteus* Hauser 1885, p. 1

Synonyms: Spirulina Hueppe 1886; not *Spirulina* Turpin 1827; *Liquidobacterium* Jensen 1909.

Highly pleomorphic rods, filaments and curved cells being common as involution forms. Gram negative. Actively motile. Characteristic amoeboid colonies on moist media. Liquefy gelatin rapidly and produce vigorous decomposition of proteins. Ferment glucose and sucrose (but usually not lactose), with formation of acid and gas (the latter being CO_2 only).

Type species, *P. vulgaris* Hauser.

Genus 6. *Bacterium* Ehrenberg 1828, emended Orla-Jensen 1909, p. 315

Synonyms: Tyrothrix Duclaux 1879; *Actinobacter* Duclaux 1882 in part; *Klebsiella* Trevisan 1885 in part; *Kurthia?* Trevisan 1885; *Gliscrobacterium* Malerba and Sanna Salaris 1888; *Pneumobacillus?* Arloing 1889; *Aerobacter* Beijerinck 1900; *Salmonella* Lignieres 1900; *Pyobacillus* Koppanyi 1907.

Gram negative, evenly staining rods. Often motile, with peritrichic flagella. Easily cultivable, forming grape-vine leaf or convex whitish surface colonies. Liquefy gelatin rarely. All forms except *B. alcaligenes* and the *B. abortus* group attack the hexoses and most species ferment a large series of carbohydrates. Acid formed by all, gas (CO_2 and H_2) only by one series. Typi-

cally intestinal parasites of man and the higher animals although several species may occur on plants and one (*B. aerogenes*) is widely distributed in nature. Many species pathogenic.

Type species, *B. coli* Escherich 1885, p. 518.

Tribe 5. Lactobacilleae, Nov. Trib.

Rods, often long and slender, Gram-positive, non-motile, without endospores. Usually produce acid from carbohydrates, as a rule lactic. When gas is formed, it is CO_2 without H_2. The organisms are usually somewhat thermophilic. As a rule microaerophilic; surface growth on media poor.

Genus 7. *Lactobacillus* Beijerinck 1901b, p. 214

Synonyms: Dispora? Kern 1882; *Tyrothrix?* Duclaux 1882 in part; *Saccharobacillus?* van Laer 1889; *Lactobacter* Beijerinck 1901; *Streptobacillus* Rest and Khoury 1902; *Brachybacterium* Troili-Petersson 1903; *Caseobacterium* Jensen 1909.

Generic characters those of the tribe.

The type species is *Lactobacillus caucasicus* (Kern?) Beijerinck.

Tribe 6. *Pasteurelleae*, Nov. Trib.

Gram negative rods, showing bipolar staining. Parasitic forms of slight fermentative power.

Genus 8. *Pasteurella* Trevisan 1888, p. 7.

Synonyms: Octopsis? Trevisan 1885; *Coccobacillus* Gamaleia 1888, not *Coccobacillus* Leube 1885; *Dicoccia?* Trevisan 1889; *Diplobacillus?* Weichselbaum 1887.

Aerobic and facultative. Powers of carbohydrate fermentation slight; no gas produced. Gelatin not liquefied. Parasitic, frequently pathogenic, producing plague in man and hemorrhagic septicemia in the lower animals.

The type species is *Pasteurella cholerae-gallinarum* (Flügge 1886) Trevisan.

Tribe 7. Hemophilaeae, Nov. Trib.

Minute parasitic forms growing only in presence of hemoglobin, ascitic fluid or other body fluids.

Genus 9. *Hemophilus* Committee 1917c, p. 561

Synonyms: Pyobacillus? Koppanyi 1907; *Diplobacillus* Morex 1896, not *Diplobacillus* Weichselbaum 1887.

Minute rod-shaped cells, sometimes thread forming and pleomorphic, nonmotile, without spores, strict parasites, growing best (or only) in the presence of hemoglobin, and in general requiring blood serum or ascitic fluid. Gram negative.

The type species is *Hemophilus influenzae* (Pfeiffer 1893, p. 357) Committee 1917.

Family VII. Bacillaceae Fischer 1895, p. 139

Rods producing endospores, usually Gram-positive. Flagella when present peritrichic. Often decompose protein media actively through the agency of enzymes.

Genus 1. *Bacillus* Cohn, 1872c, p. 174

Synonyms: Bactrella? Morren 1830; *Metallacter?* Perty 1852; *Bacetridium* Davaine 1868 in part; *Urobacillus* Miquel 1879; *Pollendera* Trevisan 1884; *Zopfiella* Trevisan 1885; *Streptobacter* Schroeter 1886; *Cornilia* Trevisan 1889 in part; *Bacterium* Ehrenberg, emended Migula 1894 in part; *Bactridium* Fischer 1895, not *Bactridium* Wallroth 1832; *Bactrinium* Fischer 1895; *Bactrillum* Fischer 1895; *Endobacterium* Lehmann and Neumann 1896; *Astasia* Meyer 1898; *Fenobacter* Beijerinck 1900; *Bacterius* Kendall 1902 in part; *Aplanobacter* E. F. Smith 1905 in part; *Semiclostridium* Maassen 1905; *Plennbakertum* Gonnermann 1907; *Myxobacillus* Gonnermann 1907; *Thermobacillus* Jensen 1909; *Serratia* Vuillemin 1913 in part, not *Serratia* Bizio 1823.

Aerobic forms. Mostly saprophytes. Liquefy gelatin. Often occur in long threads and form rhizoid colonies. Form of rod usually not greatly changed at sporulation.

The type species is *Bacillus subtilis* Cohn.

Genus 2. *Clostridium* Prazmowski 1880, p. 23

Synonyms: Amylobacter Trecul 1865; *Cornilia* Trevisan 1889 in part; *Granulobacter* Beijerinck 1893; *Clostrillum* Fischer 1895; *Clostrinium* Fischer 1895; *Paracloster* Fischer 1895; *Semiclostridium* Maassen 1905 *Botulobacillus* Jensen 1909; *Butyribacillus* Jensen 1909; *Cellulobacillus* Jensen 1909; *Putribacillus* Jensen 1909.

Anaerobes or micro-aerophiles. Often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms.

The type species is *Clostridium butyricum* Prazmowski.

IV. ARTIFICIAL KEY TO THE FAMILIES AND GENERA OF THE ACTINOMYCETALES AND EUBACTERIALES

A. Typically filamentous forms. *Actinomycetaceae*
  B. Mycelium and conidia formed.............................. *Actinomyces*
  BB. No true mycelium
    C. Cells show branching
      D. Gram negative.............................. *Actinobacillus*
      DD. Gram positive.............................. *Erysipelothrix*
    CC. Cells never branch. Gram positive threads later fragmenting into rods.............................. *Leptotrichia*
AA. Typically unicellular forms (although chains of cells may occur)
  B. Spherical cells. *Coccaceae*
    C. Parasitic forms. Cells in pairs, chains or irregular groups, never in packets. Generally active fermenters
      D. Cells in flattened coffee-bean-like pairs Gram negative.............................. *Neisseria.*
      DD. Cells not as above. Gram positive
        E. Cells in lanceolate pairs or chains Growth on media not abundant
          F. Cells in lanceolate pairs. Inulin generally fermented.. *Diplococcus*
          FF. Cells in chains. Inulin generally not fermented.... *Streptococcus*
        EE. Cells in irregular groups. Growth on media fairly vigorous. White or orange pigment.............................. *Staphylococcus*
    CC. Saprophytic forms. Chains occurring in zoogleal masses in sugar solutions.............................. *Leuconostoc*
    CCC. Saprophytic forms. Cells in irregular groups or packets, not in chains. Fermentative powers low
      D. Packets formed.............................. *Sarcina*
      DD. No packets
        E. Yellow pigment.............................. *Micrococcus*
        EE. Red pigment.............................. *Rhodococcus*

BB. Rods
C. Curved rods. *Spirillaceae*
D. Short comma-like rods. One-three short flagella.............. *Vibrio*
DD. Long spirals, five-twenty flagella......................... *Spirillum*
CC. Straight rods
D. No endospores
E. Rods of irregular shape or showing branched or filamentous involution forms.
F. Animal parasites. Cells of irregular shape. Staining unevenly.
G. Acid fast.. *Mycobacterium*
GG. Not acid fast
H. Cells elongate, fusiform.......................... *Fusiformis*
HH. Cells not fusiform, sometimes branching
I. Gram positive. Slender, sometimes clubbed rods.................................... *Corynebacterium*
II. Gram negative. Rods sometimes form threads. Characteristic honey-like growth on potato.. *Pfeifferella*
FF. Not animal parasites. Cells staining unevenly and with branched or filamentous forms at certain stages. Never acid fast.
G. Metabolism simple, growth processes involving oxidation of alcohol or fixation of atmospheric nitrogen (latter in symbiosis with green plants).
H. Cells minute, symbiotes in roots of leguminous plants... *Rhizobium*
HH. Oxidizing alcohol, branching forms common.... *Acetobacter*
GG. Not as above. Proteus-like colonies.
H. Not attacking carbohydrates. Gram +............. *Zopfius*
HH. Fermenting glucose and sucrose. Gram − .... *Proteus*
EE. Regularly formed rods
F. Metabolism simple, growth processes involving oxidation of carbon, hydrogen or their simple compounds or the fixation of atmospheric nitrogen. *Nitrobacteriaceae*
G. Fixing nitrogen or oxidizing its compounds
H. Fixing nitrogen
Cells large; in soil............................. *Azotobacter*
HH. Oxidizing nitrogen compounds
I. Oxidizing ammonia.......................... *Nitrosomonas*
II. Oxidizing nitrites............................ *Nitrobacter*
GG. Not as above
H. Oxidizing hydrogen........................ *Hydrogenomonas*
HH. Not as above, using simpler carbon compounds
I. Oxidizing CO............................ *Carboxydomonas*
II. Oxidizing CH_4.......................... *Methanomonas*
FF. Not as above
G. Flagella usually present, polar.
Pseudomonadaceae............................. *Pseudomonas*
GG. Flagella when present peritrichic.
Bacteriaceae

H. Parasitic forms showing bipolar staining .... *Pasteurella*
HH. Not as above
I. Strict parasites growing only in presence of hemoglobin or ascitic fluid .... *Hemophilus*
II. Not as above
J. Water forms producing red or violet pigment
K. Pigment red.......................... *Erythrobacillus*
KK. Pigment violet..................... *Chromobacterium*
JJ. Not as above
K. Plant pathogens............................ *Erwinia*
KK. Not as above
L. Gram positive, forming large amount of acid from carbohydrates and sometimes CO_2 but no H_2.............................. *Lactobacillus*
LL. Gram negative, forming H_2 as well as CO_2 if gas is produced...................... *Bacterium*
DD. Endospores present, *Bacillaceae.*
E. Aerobes.. *Bacillus*
EE. Anaerobes... *Clostridium*

V. GENERIC INDEX OF THE COMMONER FORMS OF BACTERIA

Prepared for the Committee by Dorothy F. Holland, Department of Public Health, Yale School of Medicine

The following index is not intended to be exhaustive or to deal in any sense with problems of *specific* identity. The literature has not been comprehensively studied and no attempt has been made to arrive at conclusions in regard to the priority of specific names. The list is simply an index of reference to show how the *names* of *species* commonly found in the literature should be changed to correspond with the generic classification suggested by the committee. Many of the specific names quoted are known to be synonyms, and the list is therefore in no sense a check list of valid bacterial species. Our hope is that those who wish to use the committee classification can by reference to this list easily replace the older form of any specific name by the newer one which it should bear in accord with the generic arrangement presented above; and in particular that it will facilitate the breaking up of the absurdly incongruous aggregates massed together under the older names Bacillus and Bacterium.

Acetobacter aceti (Thomson)
  xylinum (Brown)

Actinobacillus Lignieresi Brumpt

Actinomyces
  albido-flavus Rossi-Doria
  alboflavus Waksman & Curtis
  albosporeus (Krainsky?) Waksman & Curtis
  albus (Krainsky?) Waksman & Curtis
  asteroides (Eppinger) Gasperini
  aurantiacus (Rossi-Doria) Gasperini
  aureus Waksman & Curtis
  bobili Waksman & Curtis
  bovis Harz
  Californicus Waksman & Curtis
  carneus (Rossi-Doria) Gasperini
  chromogenus Gasperini
  citreus (Krainsky?) Waksman & Curtis
  diastaticus (Krainsky?) Waksman & Curtis
  exfoliatus Waksman & Curtis
  farcinicus (Trev. and de Toni) Gasperini
  flavus Sanfelice
  Foersteri (Cohn) Gasperini
  fradii Waksman & Curtis
  griseus (Krainsky?) Waksman & Curtis
  Halstedii Waksman & Curtis
  Hofmanni (Gruber) Gasperini
  Isreali Kruse
  invulnerabilis (Acosta-Grande-Rossi) Kruse
  Krausei
  lavendulae Waksman & Curtis
  Lipmanii Waksman
  madurae (Vincent) Leh. and Neu.

Actinomyces—*Continued.*
  melanocyclus Krainsky
  melanosporeus Krainsky
  necrophorus Loeffler
  pheochromogenus Conn
  pleuricolor
  Poolensis Taubenhaus
  pulmonalis
  Rosenbachii Kruse
  roseus (Krainsky?) Waksman & Curtis
  rubidaureus (Thiry) Lachner
  rubrus Kruse
  Rutgersensis Waksman & Curtis
  scabies (Thaxter) Güssow
  thermophilus Gilbert
  verne Waksman & Curtis
  violaceus (Rossi-Doria) Gasperini
  violaceus-ruber Waksman & Curtis
  violaceus-acesari Waksman & Curtis

Azotobacter
  agile Beijerinck
  Beijerinckii Lipman
  chroococcum Beijerinck
  Vinelandii Lipman
  Woodstownii Lipman

Bacillus
  abortivius (*see* Bact. abortivium)
  abortus Bang[4] (*see* Bact. abortum)
  abortus-equi (*see* Bact. abortum-equi)
  acidi-lactici Grotenfelt (*see* Bact. acidi-lactici)
  acidificans-longissimus (*see* Lactobacillus acidificans-longissimus)
  acidophil-aerogenes Torrey-Rahe (*see* Lactobacillus acidophil-aerogenes)

[4] B. bronchisepticus, B. abortus, B. melitensis according to Evans (Further Studies on Bact. abortus and Related Bacteria) J. Infect. Dis., Vol. 22, No. 6, p. 580) are related morphologically, culturally, biochemically, and serologically. They are Gram negative, do not form spores, "do not attack the sugars nor the other commonly used fermentable test substances." These organisms should probably constitute a distinct new genus, but we have hesitated to add new generic names at this time without further study of our own.

Bacillus—*Continued.*
acidophilus Moro (*see* Lactobacillus acidophilus)
acnes (*see* Fusiformis acnes)
adhaerens Ford
aerogenes Escherich (*see* Bact. aerogenes)
aerogenes-capsulatus Welch & Nuttall (*see* Clostridium aerogenes-capsulatum)
aeruginosus Schröter (*see* Pseudomonas aeruginosa)
agri Ford
albolactus Migula
alcaligenes Petruschky (*see* Bact. alcaligenes)
amethystinus Eisenberg (*see* Chromobacterium amethystinum)
amylobacter van Tieghem (*see* Clostridium amylobacter)
amyloruber Hefferan (*see* Erythrobacillus amyloruber)
amylovorus (Burrill-Trev.) *see* Erwinia amylovora)
anaerogenes Lembke (*see* Bact. anaerogenes)
anthracis Koch-Cohn
anthracis-symptomatici Kruse (*see* Clostridium anthracis-symptomatici)
anthracoides Hueppe-Wood
aquatilis Tataroff (*see* Pseudomonas aquatilis)
arborescens Frankland (*see* Erythrobacillus arborescens)
astheniae Dawson (*see* Bact. astheniae)
asterosporus (Meyer) Migula
aterrimus Leh. and Neu.
avisepticus Kitt (*see* Pasteurella avi-septica)
bibulus McBeth & Scales (*see* Bacterium bibulum)
bifidus Tissier (*see* Lactobacillus bifidus)
botulinus van Ermengem (*see* Clostridium botulinum)

Bacillus—*Continued.*
bovisepticus Kruse (*see* Pasteurella boviseptica)
brevis Migula
bronchicanis Ferry (*see* Bact. bronchicanis)
bronchisepticus Ferry[4] (*see* Bact. bronchisepticum)
buccalis Robin (*see* Leptotrichia buccalis)
bulgaricus Massol (*see* Lactobacillus bulgaricus)
Bütschlii
butyricus Hueppe
butyricus Botkin (*see* Clostridium butyricum)
campestris Pammel (*see* Pseudomonas campestris)
capsulatus Sternberg (*see* Bact. capsulatum)
capsulatus-mucosus Fasching (*see* Bact. mucosum-capsulatum)
carotovorus Jones (*see* Erwinia carotovora)
caucasicus Flügge (*see* Lactobacillus caucasicus)
centrosporus Ford
cerasus Griffin (*see* Pseudomonas cerasa)
cereuleus Voges (*see* Pseudomonas cereulea)
cereus Frankland
chauvei Arloing-Cornevin-Thomas (*see* Clostridium chauvei)
cholerae Koch (*see* Vibrio cholerae)
cholerae-gallinarum Flügge (*see* Pasteurella cholerae-gallinarum)
cholerae-suis Salmon-Smith (*see* Bact. cholerae-suis)
circulans Jordan
citri Hasse (*see* Pseudomonas citri)
cloacae Jordan (*see* Bact. cloacae)
cohaerens Gottheil
coli Escherich (*see* Bact. coli)
coli-communior Durham (*see* Bact. coli communior)
coli-communis Escherich (*see* Bact. coli-communis)

Bacillus—*Continued.*
- comma Koch (*see* Vibrio comma)
- coscoroba Trétrop (*see* Bact. coscoroba)
- cuniculicida (Gaffky) Flügge (*see* Pasteurella cuniculicida)
- cyanogenes Flügge (*see* Pseudomonas cyanogenes)
- cypripedii Hori (*see* Erwinia cypripedii)
- cytaseus McBeth & Scales (*see* Bact. cytaseum)
- Danysz (*see* Bact. Danysz)
- Delbrücki (*see* Lactobacillus Delbrücki)
- diphtheriae Klebs-Loeffler (*see* Corynebacterium diphtheriae)
- dysenteriae Flexner (*see* Bact. dysenteriae)
- dysenteriae Shiga (*see* Bact. dysenteriae or Bact. Shigae)
- edematis Koch (*see* Clostridium edematis)
- Ellenbachensis α Stutzer-Hartleb
- enteritidis Gärtner (*see* Bact. enteritidis)
- enteritidis-sporogenes Klein (*see* Clostridium enteritidis-sporogenes)
- erysipelatos-suis (Löffler) Migula (*see* Erysipelothrix erysipelatos-suis)
- erythrogenes Grotenfelt (*see* Erythrobacillus erythrogenes)
- fecalis-alcaligenes Petruschky (*see* Bact. fecalis-alcaligenes)
- feseri (Trev.) Kitt (*see* Clostridium feseri)
- fimi McBeth & Scales (*see* Bact. fimi)
- flavidus Morse (*see* Corynebacterium flavidum)
- fluorescens liquefaciens Flügge (*see* Pseudomonas fluorescens)
- Friedmanii (*see* Mycobacterium Friedmanii)
- Frostii (*see* Pseudomonas Frostii)
- fuchsinus Boekhout-de Vries (*see* Erythrobacillus fuchsinus)
- fusiformis Gottheil

Bacillus—*Continued.*
- fusiformis Veillon and Zuber? (*see* Fusiformis)
- gallinarum Klein (*see* Bact. gallinarum)
- globigii Migula
- graveolens Meyer and Gottheil
- Havaniensis Sternberg (*see* Erythrobacillus Havaniensis)
- Hoagii Morse (*see* Corynebacterium Hoagii)
- Hoffmanii Loeffler (*see* Corynebacterium Hoffmanii)
- hyacinthi Wakker? (*see* Pseudomonas hyacinthi)
- icteroides Sanarelli (*see* Bact. icteroides)
- indicus Koch (*see* Erythrobacillus indicus)
- influenzae Pfeiffer (*see* Hemophilus influenzae)
- juglandis Pierce (*see* Pseudomonas juglandis)
- Kiliensis Fischer and Breunig (*see* Erythrobacillus Kiliensis)
- lachrymans Erw. Smith and Bryan (*see* Pseudomonas lachrymans)
- lactici-acidi Grotenfelt (*see* Bact. acidi-lactici)
- lactis Flügge
- lactis-acidi Leichmann (*see* Lactobacillusl actis-acidi or Streptococcus lacticus)
- (lactis) aerogenes Escherich (*see* Bact. aerogenes)
- (lactis) erythrogenes Grotenfelt (*see* Erythrobacillus erythrogenes)
- lactis-viscosus Adametz (*see* Bact. lactis-viscosus)
- lacunatus Morax and Axenfeld (*see* Hemophilus lacunatus)
- laterosporus Ford
- lathyri Manns and Taubenhaus (*see* Erwinia lathyri)
- leprae Hansen (*see* Mycobacterium leprae)
- levans Lehmann-Wolffin (*see* Bact. levans)

Bacillus—*Continued.*
liodermos (Flügge) Leh. and Neu.
lividus Flüge and Proskauer (*see* Chromobacterium lividum)
mallei Loefler and Schütz (*see* Pfeifferella mallei)
malvacearum Erw. Smith (*see* Pseudomonas malvacearum)
medicaginis Sackett (*see* Pseudomonas medicaginis)
megatherium de Bary
melitensis (Bruce)[4] (*see* Bact. melitensis)
melonis Giddings (*see* Erwinia melonis)
mesentericus (Flügge) Migula
miniaceus Zimmermann (*see* Erythrobacillus miniaceus)
mirabilis Migula (*see* Proteus mirabilis)
mori Boyer and Lambert (*see* Pseudomonas mori)
mortiferus Harris (*see* Fusiformis mortiferus)
mucosus Zimmermann
murisepticus Flügge (*see* Bact. murisepticum)
murium Loeffler (*see* Bact. murium)
mycoides Flügge
mycoides-roseus Scholl (*see* Erythrobacillus mycoides-roseus)
neapolitanus Fraenkel (*see* Bact. neapolitanum)
niger Migula
of Achalme (*see* Clostridium Welchii)
of Boas-Oppler (*see* Lactobacillus bulgaricus)
of Bordet-Gengou (*see* Hemophilus pertussis)
of Ducrey (*see* genus Hemophilus)
of Gärtner (*see* Bact. enteritidis)
of Klebs-Loeffler (*see* Corynebacterium diphtheriae)
of Koch-Weeks(*see* genus Hemophilus)
of Morax-Axenfeld (*see* Hemophilus lacunatus)
of Morgan (*see* Bact. Morgani)

Bacillus—*Continued.*
of Schottmüller (*see* Bact. Schottmülleri)
of Shiga (*see* Bact. Shigae)
of Sternberg (*see* Bact. Sternbergii)
oleae (Arcangeli) Trev. (*see* Pseudomonas oleae)
oleraceae Harrison (*see* Erwinia oleraceae)
oligocarbophilus Beijerinck and van Delden (*see* Carboxydomonas oligocarbophila)
ozaenae (Abel) Leh. and Neu, (*see* Bact. ozaenae)
panis Migula
pantotrophus Kaserer (*see* Hydrogenomonas pantotropha)
paracoli Widal & Nobecourt (*see* Bact. paracoli)
paradysenteriae (*see* Bact. paradysenteriae)
paralyticans Ford-Robertson (*see* Corynebacterium paralyticans)
paratyphi Schottmüller (*see* Bact. paratyphi)
paratyphosus A Schottmüller (*see* Bact. paratyphosum A)
paratyphosus B Schottmüller (*see* Bact. paratyphosum B)
perfringens Veillon & Zuber (*see* Clostridium perfringens)
pertussis Bordet and Gengou (*see* Hemophilus pertussis)
pestis Kitasato and Yersin (*see* Pasteurella pestis)
pestis-caviae (*see* Pasteurella pestis-caviae)
petasites Gottheil
phaseoli Smith (*see* Pseudomonas phaseoli)
phlegmones-emphysematosae Fraenkel (*see* Clostridium phlegmones-emphysematosae)
phosphorescens Fischer (*see* Vibrio phosphorescens)
phytophthorus Appel (*see* Erwinia phytophthora

Bacillus—*Continued.*
plicatus Frankland (*see* Pseudomonas plicata)
Plymouthensis Fischer (*see* Erythrobacillus Plymouthensis)
pneumoniae Friedländer (*see* Bact. pneumoniae)
Prausnitzii Trevisan
prodigiosus (Ehrenberg) (*see* Erythrobacillus prodigiosus)
proteus-fluorescens Jaeger (*see* Pseudomonas protea-fluorescens)
proteus-mirabilis (Hauser) (*see* Proteus mirabilis)
proteus-vulgaris (Hauser) (*see* Proteus vulgaris)
proteus-Zenkeri (Hauser) (*see* Zopfius Zenkeri)
pruni Erw. Smith (*see* Pseudomonas pruni)
pseudo-anthracis Burri
pseudodiphtheriae Loeffler (*see* Corynebacterium pseudodiphtheriae)
pseudo-tetanicus (Kruse) Migula
psittacosis Nocard (*see* Bact. psittacosis)
pullorum Rettger (*see* Bact. pullorum)
putrificus Flügge (*see* Clostridium putrificum)
pyocyaneus Gessard (*see* Pseudomonas pyocyanea)
pyogenes-foetidus Passet (*see* Bact. pyogenes-foetidum)
radicicola Beijerinck (*see* Rhizobium radicicola)
ramosus Frankland
rhinoscleromatis v. Frisch (*see* Bact. rhinoscleromatis)
rosaceus Migula (*see* Erythrobacillus rosaceus)
ruber Miquel (*see* Erythrobacillus ruber)
ruber Zimmermann (*see* Erythrobacillus ruber)
rubricus Hefferan (*see* Erythrobacillus rubricus)
ruminatus Gottheil

Bacillus—*Continued.*
rutilescens Hefferan (see Erythrobacillus rutilescens)
rutilus Hefferan (*see* Erythrobacillus rutilus)
salmoneus Dyar (*see* Erythrobacillus salmoneus)
sanguinarium Moore (*see* Bact. sanguinarium)
Savastanoi Erw. Smith (*see* Pseudomonas Savastanoi)
segmentosus (*see* Corynebacterium segmentosum)
Shigae Chester (*see* Bact. Shigae)
simplex Gottheil
smegmatis Alvarez-Tavel (*see* Mycobacterium smegmatis)
solanacearum Erw. Smith (*see* Erwinia solanacearum)
solanisaprus Harrison (*see* Erwinia solanisapra)
sporogenes Klein (*see* Clostridium sporogenes)
Sternbergii (*see* Bact. Sternbergii)
Stewarti Erw. Smith (*see* Pseudomonas Stewarti)
subtilis Cohn
subtilis-viscosus Chester
subviscorum Migula (*see* Bact. subviscorum)
suipestifer Kruse (*see* Bact. suipestifer)
suisepticus Kruse (*see* Pasteurella suiseptica)
synxanthus Ehrenberg (*see* Pseudomonas synxantha)
terminalis Migula
tetani Nicolaier (*see* Clostridium tetani)
tracheiphilus Erw. Smith (*see* Erwinia tracheiphila)
tuberculosis Koch (*see* Mycobacterium tuberculosis)
tumefaciens Erw. Smith and Townsend (*see* Pseudomonas tumefaciens)
tumescens Zopf
typhi (*see* Bact. typhi)

Bacillus—*Continued.*
typhi-exanthematici Plotz (*see* Fusiformis typhi-exanthematici)
typhi-murium Loeffler (*see* Bact. typhi-murium)
typhi-suis (*see* Bact. typhi-suis)
typhosus Eberth-Gaffky (*see* Bact. typhosum)
vascularum Cobb-Erw. Smith (*see* Pseudomonas vascularum)
violaceus (Schröter) Migula (*see* Pseudomonas violacea)
vulgaris (Hauser) (*see* Proteus vulgaris)
vulgatus (Flügge) Trevisan
Welchii Migula (*see* Clostridium Welchii)
x Sternberg (*see* Bact. Sternbergii)
xerosis Kuschbert-Neisser (*see* Corynebacterium xerosis)
xylinus Brown (*see* Acetobacter xylinum)
Zenkeri (Hauser) (*see* Zopfius Zenkeri)
Zopfii Kurth (*see* Zopfius Zopfii)

Bacterium abortivium
abortum (Bang)[4]
abortum-equi
acidi-lactici (Grotenfelt)
aërogenes (Escherich)
alcaligenes (Petruschky)
anaerogenes (Lembke)
angulatum Fromme (*see* Pseudomonas angulata)
astheniae (Dawson)
bibulum (McBeth & Scales)
bronchicanis
bronchisepticum (Ferry)[4]
campestris Pammel (*see* Pseudomonas campestris)
capsulatum (Sternberg)
casei α Orla-Jensen (*see* Lactobacillus casei)
casei ϵ Orla-Jensen (*see* Lactobacillus helveticus)
cholerae-suis (Salmon-Smith)
cloacae (Jordan)

Bacterium—*Continued.*
coli (Escherich)
coli-communior Durham
coli-communis Escherich
communior (Durham)
coscoroba (Trétrop)
Danysz
dysenteriae (Flexner)
dysenteriae (Shiga)
enteritidis (Gaertner)
fecalis-alcaligenes Petruschky
fimi (McBeth & Scales)
gallinarum (Klein)
hyacinthi Wakker (*see* Pseudomonas hyacinthi)
icteroides (Sanarelli)
lactis acidi (Leichmann) (*see* Lactobacillus lactis acidi or Streptococcus lacticus)
lactis viscosus
lepisepticum Ferry (*see* Pasteurella lepiseptica)
levans (Lehmann-Wolffin)
melitensis (Bruce)[4]
Morgani (Winslow-Rottenberg-Parsons)
mucosum capsulatum (Fasching)
murisepticum (Flügge)
neapolitanum (Fraenkel)
ozaenae [(Abel) Leh. and Neu.]
paracoli (Widal & Nobecourt)
paradysenteriae
paratyphi
paratyphosum A (Schottmüller)
paratyphosum B (Schottmüller)
pestis (Kitasato and Yersin) (*see* Pasteurella pestis)
phaseoli Erw. Smith (*see* Pseudomonas phaseoli)
psittacosis (Nocard)
pullorum (Rettger)
putidum Flügge (*see* Pseudomonas fluorescens var. non liquefaciens)
pyogenes foetidum (Passet)
rhinoscleromatis (v. Frisch)
sanguinarium (Moore)
Savastanoi Erw. Smith (*see* Pseudomonas Savastanoi)

Bacterium—*Continued.*
- Schottmülleri (Winslow-Rottenberg-Parsons)
- Shigae (Winslow-Rottenberg-Parsons)
- Sternbergii
- Stewarti Erw. Smith (*see* Pseudomonas Stewarti)
- suipestifer (Kruse)
- tularense McCoy and Chapin
- typhi-exanthematici (Plotz) (*see* Fusiformis typhi-exanthematici)
- typhi-murium (Loeffler)
- typhi-suis
- typhosum (Eberth-Gaffky)
- vulgare (Hauser) (*see* Proteus vulgaris)
- xylinum (Brown) (*see* Acetobacter xylinum)
- Zopfii Kurth (*see* Zopfius Zopfii)

Betacoccus arabinosaceus Orla-Jensen (*see* Leuconostoc arabinosaceus)
- bovis Orla-Jensen (*see* Leuconostoc bovis)

Carboxydomonas
- oligocarbophila Beijerinck and van Delden

Chromobacterium
- amethystinum-(Eisenberg)
- janthinum (Zopf)
- lividum (Flügge-Proskauer)
- violaceum Bergonzini

Clostridium
- aerogenes-capsulatum (Welch & Nuttall)
- amylobacter (van Tieghem)
- anthracis-symptomatici (Kruse)
- botulinum (van Ermengem)
- butyricum (Botkin)
- butyricum Prazmowski
- chauvei (Arloing-Cornevin-Thomas)
- edematis (Koch)
- enteritidis sporogenes
- feseri (Trev.-Kitt)
- pasteurianium (Winogradsky)
- perfringens

Clostridium—*Continued.*
- phlegmones emphysematosae (Fraenkel)
- putrificum (Flügge)
- sporogenes Klein
- tetani (Nicolaier)
- Welchii (Migula)

Corynebacterium
- diphtheriae (Klebs-Loeffler) Leh. and Neu.
- Hoagii (Morse)
- Hoffmanii (Loeffler-Hoffman-Wellenhoff)
- segmentosum
- xerosis (Kuschbert-Neisser)

Diplococcus flavus Flügge (*see* Micrococcus flavus)
- gonorrheae Neissser (*see* Neisseria gonorrheae)
- intracellularis-meningitidis Weichselbaum (*see* Neisseria intracellularis-meningitidis)
- involutus Kurth
- lanceolatus Foa-Bordoni-Uffreduzzi
- mucosus (Schottmüller)
- pneumoniae Weichselbaum
- Weichselbaumii (*see* Neisseria Weichselbaumii)

Erwinia
- amylovora (Burrill-Trev.) Committee 1917
- aroideae (Townsend)
- carotovora (Jones)
- lathyri (Manns and Taubenhaus)
- melonis (Giddings)
- oleraceae (Harrison)
- phytophthora (Appel)
- solanacearum (Erw. Smith)
- solanisapra (Harrison)
- teutlia (Metcalf)
- tracheiphila (Erw. Smith)

Erysipelothrix
- erysipelatos-suis
- rhusiopathiae Kitt

Erythrobacillus
amyloruber (Hefferan)
erythrogenes (Grotenfelt)[5]
fuchsinus (Boekhout & de Vries)
havaniensis (Sternberg)
indicus (Koch)
Kiliensis (Fischer & Breunig)
(lactis) erythrogenes (Grotenfelt)[5]
miniaceus (Zimmermann)
mycoides-roseus (Scholl)
Plymouthensis (Fischer)
prodigiosus (Ehrenberg)
rubefaciens (Zimmermann)
ruber (Miquel)
ruber (Zimmermann)
rubricus (Hefferan)
rufus (Hefferan)
rutilescens (Hefferan)[5]
rutilus (Hefferan)

Fusiformis acnes
Hodgkini
termitidis (Hoelling)
typhi-exanthematici (Plotz)

Hemophilus of Ducrey[6]
influenzae (Pfeiffer)
of Koch-Weeks[6]
lacunatus (Morax-Axenfeld)
pertussis (Bordet-Gengou)

Hydrogenomonas
pantotropha Kaserer

Lactobacillus
acidificans-longissimus
acidophil-aerogenes (Torrey-Rahe)
acidophilus (Moro)
bifidus (Tissier)
bulgaricus (Massol)
casei (Orla-Jensen)

Lactobacillus—*Continued.*
caucasicus Flügge
cereale (Orla-Jensen)
Delbrücki
helveticus (Orla-Jensen)
jugurt (Orla-Jensen)
lactis (Orla-Jensen)
lactis-acidi Leichmann
planticus (Orla-Jensen)

Leptotrichia buccalis Robin

Leuconostoc arabinosaceus (Orla-Jensen)
bovis (Orla-Jensen)
mesenteroides (Cienkowski) van Tieghem

Methanomonas methanica Söhngen

Micrococcus acne (*see* Staphylococcus acne)
agilis Ali-Cohen (*see* Rhodococcus agilis)
candicans Flügge (*see* Staphylococcus candicans)
candidus Cohn (*see* Staphylococcus candidus)
casei
catarrhalis Pfeiffer (*see* Neisseria catarrhalis)
cinnabareus Flügge (*see* Rhodococcus cinnabareus)
citreus Dyar
citreus Passet
flavus (Flügge) Migula
gonorrheae (Trevisan) (*see* Neisseria gonorrheae)
intracellularis Weichselbaum (*see* Neisseria intracellularis)
lanceolatus Foa-Bordoni-Uffreduzzi (*see* Diplococcus)

[5] These organisms contain a water-soluble red pigment in contrast to the lipochrome of the organisms of the true prodigiosus group. They are placed here provisionally, but may call for separate generic classification.

[6] The bacillus of Ducrey, and of Koch-Weeks have not been given specific names. They seen to belong to this genus and are placed here awaiting specific names which would be less unwieldly than a genitive form of Ducrey and the combination Koch-Weeks.

Micrococcus—*Continued.*
- liquefaciens
- luteus (Schroeter) Cöhn
- mastitidis
- melitensis Bruce (*see* Bact. Melitensis
- meningitidis Weichselbaum (*see* Neisseria meningitidis)
- mycodermatus
- mollis Dyar (*see* Staphylococcus mollis)
- nigrofaciens Northrup
- ochraceus Rosenthal
- pharyngis-siccus Lingelsheim (*see* Neisseria pharyngis-sicci)
- rheumaticus Poynton & Paine (*see* Streptococcus rheumaticus)
- rhodochrous Zopf (*see* Rhodococcus rhodochrous)
- rosaceus Frankland (*see* Rhodococcus rosaceus)
- roseus Flügge (*see* Rhodococcus roseus)
- ruber = M. (tetragenus) ruber Bujwid (*see* Rhodococcus ruber)
- tetragenus Gaffky (*see* Staphylococcus tetragenus)
- ureae Cohn Flügge (*see* Staphylococcus ureae)
- varians Dyar
- zymogenes (*see* Streptococcus gracilis)

Mycobacterium
- Friedmanii
- leprae Hansen
- Moelleri
- phlei (Moeller) Leh. & Neu.
- rhusiopathiae Chester (*see* Erysipelothrix rhusiopathiae)
- smegmatis Alvarez-Tavel
- tuberculosis (Koch) Leh. & Neu.

Neisseria
- catarrhalis (Pfeiffer)
- gonorrheae Trevisan
- intracellularis Weichselbaum
- intracellularis-meningitidis Weichselbaum

Neisseria—*Continued.*
- meningitidis (Weichselbaum)
- pharyngis-sicci (Lingelsheim)
- Weichselbaumii

Nitrobacter Winogradskyi

Nitrosomonas europaea-Winogradsky
- javaniensis Winogradsky

Pasteurella
- aviseptica (Kitt)
- boviseptica (Kruse)
- cholerae-gallinarum (Flügge) Trevisan
- cuniculicida (Gaffky-Flügge)
- lepiseptica (Ferry)
- pestis (Kitasato-Yersin)
- suiseptica (Kruse)

Pfeifferella mallei (Loeffler) Buchanan

Proteus
- fluorescens Jaeger (*see* Pseudomonas protea fluorescens)
- mirabilis Hauser
- vulgaris Hauser
- Zenkeri Hauser (*see* Zopfius Zenkeri)

Pseudomonas
- aeruginosa (Schroeter) Frost?
- angulata (Fromme)
- aquatilis (Tataroff)
- beticola (Smith)
- campestris (Pammel)
- cerasa (Griffin)
- cereulea (Voges)
- citri (Hasse)
- cyanogenes
- fluorescens (Flügge) Migula
- Frostii
- hyacinthi (Wakker?)
- juglandis (Pierce)
- lachrymans (Erw. Smith and Bryan)
- malvacearum (Erw. Smith)
- medicaginis (Sackett)
- mori (Boyer-Lambert)
- oleae (Arcangeli-Trev.)
- phaseoli (Erw. Smith)
- pisi Sackett

Pseudomonas—*Continued.*
protea Frost
protea-fluorescens (Jaeger)
pruni (Erw. Smith)
pyocyanea Gessard
Savastanoi (Erw. Smith)
Stewarti (Erw. Smith)
syncyanea Ehrenberg
synxantha (Ehrenberg) Cohn
tumefaciens (Erw. Smith and Townsend)
vascularum (Cobb-Erw. Smith)
viridilivida Brown

Rhizobium leguminosarum Frank
radicicola (Beijerinck)

Rhodococcus agilis (Ali-Cohen)
cinnabareus (Flügge)
fulvus Cohn
incarnatus Gruber
rhodochrous Zopf
rosaceus (Frankland)
roseus (Flügge)
ruber (Bujwid)

Sarcina
aurantiaca (Schröter-Cohn)
flava De Bary
lutea Schröter
rosea Schröter (*see* Rhod. roseus)
subflava Ravenel
ventriculi Goodsir

Spirillum
cholerae-asiaticae Zopf (*see* Vibrio cholerae-asiaticae)
concentricum Kitasato
danubicum Heider (*see* Vibrio danubicus)
desulfuricans Beijerinck (*see* Vibrio desulfuricans)
Ghinda Kruse (*see* Vibrio Ghinda)
Massowah Pasquale-Pfeiffer (*see* Vibrio Massowah)
Metchnikovi Gamaleia (*see* Vibrio Metchnikovi)
Milleri (*see* Vibrio Milleri)
of Deneke (*see* Vibrio tyrogenus)

Spirillum—*Continued.*
of Finkler-Prior (*see* Vibrio Finkleri)
phosphorescens Fischer (*see* Vibrio phosphorescens)
rubrum v. Esmarch
serpens Müller-Zettnow
tyrogenum Deneke (*see* Vibrio tyrogenus)
undula Ehrenberg
volutans Ehrenberg

Staphylococcus
acne
albus
aurantiacus-Schröter-Cohn (*see* Sarcina aurantiaca)
aureus Rosenbach
candicans (Flügge)
candidus
canescens Migula
cereus-albus Passet
cereus-flavus Passet
epidermidis
mollis (Dyar)
pyogenes-albus Rosenbach
pyogenes-aureus Rosenbach
tetragenus (Gaffky)
ureae (Cohn-Flügge)

Streptobacterium casei (*see* Lactobacillus casei)

Streptobacterium plantarum (*see* Lactobacillus plantarum)

Streptococcus
bovis Orla-Jensen
conglomeratus Kurth
cremoris Orla-Jensen
endocarditis
epidemicus Davis
erysipelatos Fehleisen
faecium Orla-Jensen
glycerinaceus Orla-Jensen
gracilis Escherich
hemolyticus Rolly
inulinaceus Orla-Jensen
lacticus Kruse
lacticus-mastitidis Orla-Jensen

Streptococcus—*Continued.*
- lactis Orla-Jensen
- liquefaciens Orla-Jensen
- mucosus Schottmüller (*see* Diplococcus mucosus)
- pneumoniae Weichselbaum (*see* Diplococcus pneumoniae)
- pyogenes Rosenbach
- rheumaticus (Poynton & Paine)
- thermophilus Orla-Jensen
- viridans Schottmüller
- zymogenes

Tetracoccus liquefaciens (*see* Micrococcus liquefaciens)
- casei (*see* Micrococcus casei)
- mastitidis (*see* Micrococcus mastitidis)
- mycodermatus (*see* Micrococcus mycodermatus)

Thermobacterium
- cereale Orla-Jensen (*see* Lactobacillus cereale)
- lactis Orla-Jensen (*see* Lactobacillus lactis)
- bulgaricum Orla-Jensen (*see* Lactobacillus bulgaricus)

Thermobacterium—*Continued.*
- helveticum Orla-Jensen (*see* Lactobacillus helveticus)
- jugurt Orla-Jensen (*see* Lactobacillus jugurt)

Vibrio
- aquatilis Günther
- berolinensis Migula
- cholerae-asiaticae (Zopf)
- comma Schröter
- danubicus (Heider)
- desulfuricans (Beijerinck)
- Dunbari
- fetus Th. Smith
- Finkleri (Schröter)
- Ghindae (Kruse)
- Massowah (Pasquale-Pfeiffer)
- Metchnikovi (Gamaleia)
- Milleri
- phosphorescens (Fischer)
- proteus Buchner
- Schuylkilliensis Abbott
- tyrogenus (Deneke)

Zopfius
- Zenkeri (Hauser)
- Zopfii (Kurth)

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THE PRODUCTION OF HYDROGEN SULPHIDE BY BACTERIA

JOHN T. MYERS

From the Department of Hygiene and Bacteriology, the University of Chicago

Received for publication, December 11, 1919

Hydrogen sulphide formation in sewage is supposed to be due to two distinct processes, the splitting of protein by certain organisms and the reduction of inorganic sulphates by others.

ERRATUM

J. Bact. **5**, 231. The Production of Hydrogen Sulphide by Bacteria. John T. Myers. From the Department of Hygiene and Bacteriology, the University of Chicago.

A portion of the work recorded in this paper was done in the laboratories of the University of Nebraska, College of Medicine, Department of Pathology and Bacteriology, Omaha, Nebraska.

... formation, and thinks it probable that the action may be due to some unknown specific organism similar to the *Spirillum desulphuricans* of Beijerinck (1895).

Several workers have observed the formation of hydrogen sulphide in large quantities in the effluent of sewage disposal plants. Barr and Buchanan (1912) report the isolation of a specific organism in one such instance. Clark (1913) and others have reported the reduction of inorganic sulphates in sewages.

Sulphur metabolism may also play a beneficial rôle in sewage disposal in that certain species of bacteria may cause the oxidation of sulphur and of hydrogen sulphide to sulphates thus reducing the amount of odor. This is a well known property of such organisms as Beggiatoa and Thiothrix (Jordan, 1918).

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Hydrogen sulphide formation in sewage is supposed to be due to two distinct processes, the splitting of protein by certain organisms and the reduction of inorganic sulphates by others. Lederer (1913) concludes that more hydrogen sulphide is formed under anaerobic than under aerobic conditions. He thinks that the formation of hydrogen sulphide from protein is a selective process and that it depends a great deal on the position of the sulphur radical in the protein molecule. At any rate other factors than reduction are involved in hydrogen sulphide formation. An organism may be a very strong nitrate reducer and at the same time a weak hydrogen sulphide former, although both reactions are reducing processes. Lederer is of the opinion that the reduction of inorganic sulphates may however be an important factor in hydrogen sulphide formation, and thinks it probable that the action may be due to some unknown specific organism similar to the *Spirillum desulphuricans* of Beijerinck (1895).

Several workers have observed the formation of hydrogen sulphide in large quantities in the effluent of sewage disposal plants. Barr and Buchanan (1912) report the isolation of a specific organism in one such instance. Clark (1913) and others have reported the reduction of inorganic sulphates in sewages.

Sulphur metabolism may also play a beneficial rôle in sewage disposal in that certain species of bacteria may cause the oxidation of sulphur and of hydrogen sulphide to sulphates thus reducing the amount of odor. This is a well known property of such organisms as Beggiatoa and Thiothrix (Jordan, 1918).

Another field in which bacterial sulphur metabolism may be of importance is in soil bacteriology. The part played by sulphur here is little understood. Lipman, McLean and Lent (1916) suggest that sulphur oxidation in soils may have an effect on the availability of mineral phosphates as plant food.

Again a number of workers have attempted to apply hydrogen sulphide formation to water analysis, the assumption being that the amount of hydrogen sulphide produced when water is planted in a suitable medium is proportional to the degree of pollution. Schardinger (1894) seems to have first suggested this possibility. He observed that when water polluted with fecal material was added to peptone solution and incubated, it produced a characteristic odor, and that it blackened a strip of lead acetate paper which was suspended over the liquid. Dunham in 1897 suggested the following method for the detection of polluted water. Sterilize 10 cc. of an aqueous solution of 10 per cent peptone and 5 per cent sodium chloride in a plugged Erlenmeyer flask. To this flask add 90 cc. of the water under examination, suspend a strip of filter paper impregnated with lead carbonate over the mixture, and incubate for twenty-four hours at 37°C. Dunham maintained that the colon bacillus and the organisms of putrefaction readily multiply and cause the production of hydrogen sulphide which discolors the lead carbonate paper.

Redfield (1912) studied the effect upon the speed and amount of hydrogen sulphide production when different factors were varied. Among the conditions investigated were the effect of using filtered and unfiltered peptone solutions, the effect of the concentration of the peptone, of the type of inorganic salts added to the peptone solution, of the concentration of various salts added to the medium, of the kind of bases and acids used in adjusting the initial reaction of the medium, and of the relation of the final reaction of the culture to the amount of hydrogen sulphide production. As a result of his work Redfield suggests the following method for the detection of polluted water:

Bring 700 cc. of tap water to a boil and add 300 grams of Witte peptone and 75 grams of potassium chloride. Maintain a gentle heat and

stir constantly until as much of the peptone as will do so has gone into solution. Cool rapidly and make up to 1 liter with tap water. Transfer to a flask, heat to boiling again, plug, cool rapidly, and place in the ice box for at least twenty-four hours. Filter cold through paper and distribute in 10 cc. amounts among the special flasks suggested. Sterilize the flasks in the autoclave at one atmosphere for fifteen minutes. When cool, place in the tube of each flask a strip of bibulous paper 25 mm. in length, which had previously been impregnated with lead acetate.

The top of the plugged tube was then closed by wrapping with a strip of tin foil. The flasks alluded to were of a special design. They were shaped like a Sohxlet extraction flask, were graduated at 90 cc. and 100 cc. and a ground glass cap ending in a narrow tube was fitted over the neck of each flask.

Redfield tested this method by means of an artificial sewage, prepared from human feces. This sewage was quite dilute, since it contained only twenty colon bacilli per cubic centimeter and had a total bacterial count of twenty-eight hundred. He reports a gradual increase in the amount of hydrogen sulphide produced, and in the speed of its production as the concentration of sewage increases. The same result is reported with a considerable number of untreated waters from a variety of sources. He concludes that there is a uniform relationship between the degree of pollution of a water and the amount of lead acetate paper blackened when this method is employed.

Redfield also made some quantitative determinations of the amount of hydrogen sulphide produced by sewage organisms. He compared a number of methods for the quantitative determination of sulphur in peptone solutions. He also investigated the hydrogen sulphide producing powers of several species of bacteria, and concluded that the proteolytic organisms rather than *B. coli* are responsible for its formation.

Burnet and Weissenbach (1915) suggest another use for the hydrogen sulphide producing powers of bacteria. They found that colonies of *B. paratyphosus* B, became black when grown on agar which contained a small amount of lead acetate, while colonies of *B. paratyphosus* A did not. They consider this an accurate method for the differentiation of the two organisms.

Maymone (1917) suggests a somewhat similar method for the differentiation of *B. paratyphosus* A, and *B. paratyphosus* B based on biological characteristics and the appearance of cultures on lead acetate media.

Jordan and Victorson (1917) employed a like method for B. *paratyphosus* B, and *B. paratyphosus* A. Agar tubes containing a small amount of lead acetate were inoculated between the medium and the side of the tube. All typical strains of *B. paratyphosus* B blackened the needle track. The typical *B. paratyphosus* A strains produced no blackening. Kligler (1917) suggests a simple method for the differentiation of *B. paratyphosus* A and B, *B. typhosus*, and *B. dysenteriae*, based on a double sugar medium similar to that of Russell, containing lead acetate.

Bacterial action probably plays a rôle in the formation of intestinal gases. Hydrogen sulphide is always present in intestines and is probably formed from cystin. Senator described a case in which an intoxication with hydrogen sulphide of intestinal origin occurred, but this is apparently the only case reported. (Wells 1918). Hydrogen sulphide formation by bacteria may be concerned in some of the conditions included in that vague term, autointoxication.

The formation of hydrogen sulphide by bacteria is also of interest, because of the light it may throw on the metabolism of bacteria. Sasati and Otsuka (1912) carried out some experiments with a few organisms as to the formation of hydrogen sulphide with cystin, taurin, sulphur, sodium, sulphate, and sodium sulphite. Burger (1914) compared cystin and peptone as a source of hydrogen sulphide.

Tanner (1918) published interesting data on various sulphur compounds as a source of hydrogen sulphide when acted on by bacteria. He (1918) has also contributed a valuable article on the formation of hydrogen sulphide by yeasts.

EXPERIMENTAL WORK

The application of hydrogen sulphide formation to water analysis

Experiments were undertaken to ascertain the delicacy and reliability of the hydrogen sulphide test used by Redfield to determine the potability of water.

The medium used was that suggested by Redfield with minor modifications. Sodium chloride was substituted for potassium chloride, and instead of the special flasks which Redfield employed, Erlenmeyer flasks of 150 cc. capacity and made of Pyrex glass were used. One-half per cent sodium chloride was added to all media containing peptone because it seemed to produce a clearer medium. Difco peptone made by the Digestive Ferments Company was substituted for Witte peptone since the latter is not now obtainable. Ten cubic centimeters of the medium were placed in each flask and autoclaved for ten minutes at 15 pounds pressure. A strip of filter paper 50 mm. long and 3 mm in width was suspended in the mouth of the flask in such a manner that approximately 30 mm. of the strip was exposed. The filter paper had been moistened in a 10 per cent solution of neutral lead acetate, and sterilized in the autoclave for fifteen minutes at 15 pounds pressure. It was necessary to sterilize the lead acetate paper separately because it was found that some blackening occurred when the medium was autoclaved, due probably to a slight hydrolysis of the peptone, since steam at the pressure of the autoclave ionizes more than at atmospheric pressure.

One gram of the feces under examination was weighed out. This merely served as a convenient amount from which to make dilutions since of course the moisture, bacterial content and residue varied enormously in the different samples. A 90 cc. portion of each dilution studied was placed in one of the Erlenmeyer flasks and incubated aerobically at 37°C. The approximate number of millimeters of lead acetate paper blackened was recorded at the end of twenty-four hours, forty-eight hours, seventy-two hours, and seven days. The method was of course, only approximate, but all conditions were kept as nearly identical as

possible. The total forty-eight hour 20°C. bacterial count on standard agar, the total forty-eight hour 37°C. bacterial count on standard agar, and the colon count, using the complete test fermentation tube method, were made in each case (A. P. H. A. Committee, 1912, 1917). In the fermentation tube work, ten tubes of lactose broth were inoculated with 1 cc. each of the dilution selected and several dilutions were always used in order to make sure that some one series of ten tubes would show gas in only part of the tubes. All media were prepared according to the standard method of water analysis of the American Public Health Association (1912, 1917).

The colon count was also made in each sample by plating on Endo medium and counting the colon like colonies directly. This was done as a check on the fermentation tube method and to test the possibility of making direct counts of the colon content of sewage and other materials by the use of Endo medium.

Table 1 indicates the types of feces studied and the data obtained.

The counts are expressed in millions and fractions of millions in order to make the table less bulky. Table 1 indicates that there is a fairly definite relationship between the amount of fecal material in a given solution and the amount of hydrogen sulphide formed, although occasional irregularities appear.

There were some difficulties in making a colon count directly on Endo medium, the most important being that deep colonies are at times difficult to differentiate. On the whole it would seem that the method is as accurate as the fermentation tube method and it is perhaps less cumbersome, especially when dealing with material that is heavily loaded with colon bacilli.

Table 2 gives the minimum number of colon bacilli which produced a perceptible blackening of the lead acetate paper after incubation at 37°C. for twenty-four hours, forty-eight hours, and seven days respectively. It also gives the twenty-four hour 37°C. count, and the forty-eight hour 20°C. count for purposes of comparison with the colon count. The customary 90 cc. dilution was used.

There is no constant relationship between the number of colon bacilli from different animals and the amount or rate of hydrogen sulphide production; and there is also no definite relationship between the numbers of other organisms and hydrogen sulphide

TABLE 1

Summary of examination of feces from different animals

SOURCE	37°, 24-hour standard agar count in millions per gram	20°, 48-hour standard agar count in millions per gram	Colon count in millions per gram on Endo medium	Colon count in millions per gram by fermentation tube	Approximate number of millimeters of lead acetate paper blackened: Dilution = 1:100000			Dilution = 1:1000000			Dilution = 1:10000000			Dilution = 1:100000000		
					24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
Human	39.8	20.9	33.0	30.0	2	30	30	1	5	30	0	0	0	0	0	0
	27.5	6.1	8.0	11.0	0.5	7	30	0	7	30	1	2	4	0	0	0
Bovine	1.74	1.25	0.01	0.1	1	3	5	0.5	1	1	0	0	0	0	0	0
	2.5	1.8	7.5	5.0	4	10	30	1	5	30	0	1	6	0	0	0
Horse	0.31	0.25	0.07	0.1	7	30	30	3	10	25	0	1	2	0	0	0
	55.0	1.0	0.06	0.08	1	30	30	0	12	30	0	1	4	0	0	0
Sheep	90.0	11.0	10.0	5.0	10	15	30	2	7	10	2	4	30	0	1	4
	224.0	215.0	210.0	90.0	3	3	20	1	2	6	0	0	1	0	0	0
Pig	288.0	281.0	233.0	90.0	—	—	—	10	30	20	4	10	30	0.5	1	2
	90.0	50.0	10.0	61.0	2	30	30	1	30	30	9	2	2	0	0	0
Chicken	126.0	26,000.0	20.0	10.0	—	—	—	10	30	30	10	15	30	1	2	5
	28.0	32.0	2.9	4.0	10	30	30	3	20	30	0	15	30	0	0	10
Rabbit*	3.2	2.23	0.01	0.003	1	4	30	1	5	5	0	0	0	0	0	0
	17.0	20.9	0.04	0.01	1	20	30	0	1	30	0	15	30	0	10	30
Dog	53.0	48.0	47.0	50.0	10	15	20	10	15	30	5	20	30	0	20	30
	35.0	34.0	11.0	6.0	—	—	—	5	20	30	5	20	30	0	20	30

* The dilution is one-tenth lower than that given in the column heading.

formation. Certain types of animal feces seem to produce hydrogen sulphide in even higher dilutions than human feces. Therefore this test applied to the direct examination of water would have no value since it is too delicate. It is to be expected

TABLE 2

Relation between the number of bacteria, in the 90 cc. portion of the highest dilution of various types of feces, which produced hydrogen sulphide

SOURCE	COLON BACILLI			37°, 24-HOUR COUNT ON STANDARD AGAR			20°, 48-HOUR COUNT ON STANDARD AGAR		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
Human	2,700	2,700	2,700	3,580	3,580	3,580	1,880	1,880	1,880
	99	99	10	22,750	2,275	227	5,490	549	55
Bovine	9	9	9	157	157	157	110	110	110
	450	45	45	225	22	22	162	16	16
Horse	1	0	0	28	3	3	23	2	2
	1	0	0	4,950	4,950	4,950	90	90	90
Sheep	45	5	1	810	81	1	100	10	1
	8,100	8,100	810	20,160	20,160	2,016	19,350	19,350	1,935
Pig	5,490	549	549	8,100	810	810	4,500	450	450
	210	2	2	160	2	2	153	2	2
Chicken	2	0	0	113	1	1	23,000	230	230
	360	36	4	2,520	252	25	280	28	3
Rabbit	30	3	3	28,800	2,880	2,880	20,000	2,000	2,000
	90	90	90	158,000	158,000	158,000	18,800	18,800	18,800
Dog	4	4	4	5	5	5	4	4	4
	54	—	—	315	—	—	306	—	—

that results of this type would be irregular since many other bacteria aside from colon bacilli are capable of producing hydrogen sulphide. It would be interesting to determine whether there is any difference in the hydrogen sulphide forming power of pure cultures of colon bacilli coming from different sources.

Table 3 summarizes the results of a rather complete bacteriological analysis of samples of water from various sources, the purpose being to compare the hydrogen sulphide test with standard methods.

The samples marked "University of Chicago tap" were specimens from the Chicago mains. Those marked "University of Chicago filtered" were taken from the University of Chicago drinking fountains, the water having been passed through a special filter belonging to the University. It will be noted that the condition of these waters was very good according to the usual standards, yet a small amount of hydrogen sulphide appeared in one of the filtered samples.

The samples marked Omaha House 1, 2, 3, 4, 5, and 6, were taken from the taps of houses in a certain district of Omaha, where several cases of typhoid fever had occurred. These analyses were made to determine the condition of the city water supply. Two series of examinations were made about two weeks apart. There was no evidence of contamination according to standard methods yet hydrogen sulphide was formed in two instances in twenty-four hours, and in most other samples there was an appreciable amount of blackening in forty-eight hours.

These results would indicate that this test is too delicate to be of value in water analysis. Hydrogen sulphide was formed by every contaminated water and by some waters in which there was no evidence of contamination by the usual methods of examination. This agrees with the results obtained by examination of feces from various animals.

TABLE 3

Summary of water analyses

SOURCE	37°, 24-HOUR STANDARD AGAR COUNT PER CUBIC CENTIMETER	20°, 48-HOUR STANDARD AGAR COUNT PER CUBIC CENTIMETER	COLON COUNT PER CUBIC CENTIMETER BY FERMENTATION TUBE METHOD	COLON COUNT ON ENDO'S MEDIUM	APPROXIMATE NUMBER OF MILLIMETERS OF LEAD ACETATE PAPER BLACKENED								
					Dilution = 0			Dilution = 1:10			Dilution = 1:100		
					24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
University of Chicago tap	150	200	0	0	0	0	0	—	—	—	—	—	—
University of Chicago tap. filtered	70	170	0	0	0	0	1	—	—	—	—	—	—
Hobart, Ind	100	220	0	0	0	0	2	—	—	—	—	—	—
Gary, Ind., Depot	46	75	0.3	0	0	0	0	—	—	—	—	—	—
Gary, Ind., Hotel, No. 1	41	70	0	0	0	0	0	—	—	—	—	—	—
Gary, Ind., Hotel, No. 2	60	90	0	0	0	0	0	—	—	—	—	—	—
Gary Depot	30	60	0	0.1	2	4	30	—	—	—	—	—	—
Gary Hotel No. 1	60	40	0	0	2	5	30	—	—	—	—	—	—
Gary Hotel No. 2	45	65	0	0	0	0	0	—	—	—	—	—	—
Gary Depot	60	100	0	0	0	1	3	—	—	—	—	—	—
Gary Hotel No. 1	55	85	0.2	0.4	1	2	3	—	—	—	—	—	—
University of Chicago tap	20	150	0.2	0.4	1	2	3	—	—	—	—	—	—
Lake Geneva, Wis	32	2,600	0	0	0	0	0	0	0	0	0	0	—0
University of Chicago tap	32	48	0.1	0	0	0	0	—	—	—	—	—	—
University of Chicago tap, filtered	4	3	0.1	0	1	10	10	—	—	—	—	—	—
University of Chicago tap	65	410	0	0.3	30	30	30	—	—	—	—	—	—
University of Chicago tap, filtered	2	16	0	0	0	0	0	—	—	—	—	—	—
University of Chicago tap	41	140	0	0	0	0	0	—	—	—	—	—	—
University of Chicago tap, filtered	2	15	0	0	0	0	0	—	—	—	—	—	—
University of Chicago tap	25	400	0	0	0	0	0	—	—	—	—	—	—
University of Chicago tap, filtered	2	38	0	0	0	0	0	—	—	—	—	—	—

Omaha house No. 1 tap	12	7	0	0	0	0	6	0	0	0.5	–	–	–
Omaha house No. 2 tap	14	11	0	0	0	30	30	0	0	30	–	–	–
Omaha house No. 3 tap	2	6	0	0	1	6	30	0	0.5	4	–	–	–
Omaha house No. 4 tap	34	10	0	0	0	12	30	0	0	0	–	–	–
Omaha house No. 5 tap	10	4	0	0	0	4	30	0	0	0	–	–	–
Omaha house No. 6 tap	19	10	0	0	0	6	30	0	0	0	–	–	–
University of Nebraska tap	125	50	0	0	1	2	5	0.5	1	2	0	0.5	–
Omaha house No. 1 tap	9	14	0	0	0	0	0	0	0	0	–	–	–
Omaha house No. 2 tap	9	11	0	0	0	0	0	0	0	0	–	–	–
Omaha house No. 3 tap	7	10	0	0	0	6	30	0	4	10	–	–	–
Omaha house No. 4 tap	12	10	0	0	0	0	0	0	0	0	–	–	–
Omaha house No. 5 tap	13	14	0	0	0	4	10	0	0	0	–	–	–
University of Nebraska tap	8	20	0	0	0	5	30	0	0	0	–	–	–
Well water	870	900	0	0	0	0	0	0	0	0	–	–	–
Calumet River	4,400	–	90	110	–	–	–	1	2	30	1	2	30
Men's Natatorium, University of Chicago	76,000	103,000	60	17	1	12	30	0.5	5	5	0	1	1
Women's Natatorium, University of Chicago	420,000	100,000	10	10	30	30	30	2	4	6	3	4	4
Men's Natatorium, University of Chicago	650	20,000	4	2	4	30	30	0.5	2	2	0	0	0

Effect of the variety of commercial peptone, and of the presence of glucose and sucrose on hydrogen sulphide formation

The hydrogen sulphide producing power of a number of common bacteria was studied in several media. Witte's peptone, "Difco" peptone made by the Digestive Ferments Company, and Fairchild peptone were compared. Four different media were prepared from each brand of peptone, a 3 per cent solution of the peptone containing 0.5 per cent of NaCl, a 3 per cent solution of the peptone containing 0.5 per cent NaCl and 0.5 per cent glucose, a 3 per cent solution of the peptone containing 0.5 per cent NaCl and 0.5 per cent sucrose, and standard nutrient broth. Glucose and sucrose were chosen because Seifert (1909) reports that glucose decreases and sucrose increases hydrogen sulphide formation by B. *paratyphosus B.* Table 4 summarizes the results.

Witte's peptone was the most favorable of the three brands in regard to hydrogen sulphide formation, and Fairchild's was more favorable than Difco. Variable amounts of hydrogen sulphide were formed in Witte and not in Difco peptone media by *Sarcina lutea, Staphylococcus albus, Staphylococcus aureus, B. prodigiosus, B. pyocyaneus, Mic. tetragenus,* a laboratory strain of streptococcus, *B. cloacae, B. subtilis, B. anthracis, B. avisepticus, B. bovisepticus, Sp. metchnikovii, Sp. cholerae, B. lactis-aerogenes,* and *B. mucosus-capsulatus.* Larger amounts of hydrogen sulphide were formed in Witte than in Difco peptone by some of the other organisms.

Hydrogen sulphide was produced in Fairchild and not in Difco media by *B. lactis aerogenes* and *B. cloacae.* It seemed to make no difference which of the four media prepared from each peptone was used. Ordinary broth was as good as any. Glucose and sucrose did not influence the rate or amount of hydrogen sulphide formation.

It was a striking fact that many species of bacteria produced hydrogen sulphide from Witte's peptone and not from the American brands. This was of course due to difference in chemical constituents. Witte's peptone contains more amino

TABLE 4

Relative amount of hydrogen sulphide produced by various bacteria in Witte peptone media

ORGANISM	3% PEPTONE 0.5% NaCl			3% PEPTONE 0.5% NaCl 0.5% GLUCOSE			3% PEPTONE 0.5% NaCl 0.5% SUCROSE			BROTH		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
Sarcina lutea	0	0	1	0	0	0	0	0	2	0	1	1
Staph. aureus	0	0	1	0	7	30	0	7	30	1	4	22
Staph. albus	2	5	12	3	5	10	3	5	8	8	10	20
B. prodigiosus	2	3	12	2	4	7	14	22	30	1	2	12
B. pyocyaneus	0	0	1	0	0	0	0	0	1	0	1	2
Mic. tetragenus	0	0	0	0	0	0	0	0	0	0	1	3
Strep. pyogenes	2	2	2	25	27	27	17	25	30	2	2	2
B. coli	5	15	30	19	20	20	19	22	30	15	25	28
B. lactis aerogenes	2	2	10	19	25	27	15	25	30	3	6	20
B. mucosus capsulatus	0	2	5	6	12	15	4	20	30	3	3	5
B. enteritidis	9	15	27	13	15	20	8	17	30	12	30	30
B. para-typhosus B	9	18	22	7	11	15	7	9	15	15	20	25
B. para-typhosus A	0	0	1	0	0	1	0	0	2	0	0	3
B. typhosus	5	9	15	4	12	15	5	15	25	15	25	30
B. dysenteriae Shiga	0	0	0	0	0	0	0	0	1	0	1	2
B. dysenteriae Flexner	0	0	1	1	1	3	0	0	1	0	1	3
B. proteus-vulgarus	22	27	30	22	25	30	18	27	28	30	30	30
B. cloacae	5	20	27	15	25	30	17	30	30	4	11	25
B. subtilis	30	30	30	22	27	30	22	27	30	25	27	27
B. anthracis	30	30	30	25	30	30	25	30	30	30	30	30
B. moelleri	0	0	0	0	0	2	0	1	2	0	1	3
B. fecalis-alkaligenes	0	1	2	0	1	2	0	1	3	4	6	14
B. bovisepticus	3	3	3	16	18	30	19	25	30	3	3	3
B. avisepticus	2	4	6	12	22	25	0	1	3	9	18	23
Sp. metchnikovii	0	12	25	0	0	0	0	1	25	8	22	27
Sp. cholerae	6	25	28	7	8	9	19	22	30	13	27	30
B. hofmanni	0	0	0	0	0	0	0	0	0	0	0	1

Relative amount of hydrogen sulphide produced by various bacteria in "Difco" peptone media

ORGANISM	3% PEPTONE 0.5% NaCl			3% PEPTONE 0.5% NaCl 0.5% GLUCOSE			3% PEPTONE 0.5% NaCl 0.5% SUCROSE			BROTH		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
Sarcina lutea	0	0	0	0	0	0	0	0	0	0	0	0
Staph. aureus	0	0	0	0	0	0	0	0	0	0	0	0
Staph. albus	0	0	0	0	0	0	0	0	0	0	0	1
B. prodigiosus	0	0	0	0	0	0	0	0	2	0	0	0
B. pyocyaneus	0	0	0	0	0	0	0	0	0	0	0	0
Mic. tetragenus	0	0	0	0	0	0	0	0	0	0	0	0
Strep. pyogenes	0	0	0	0	0	0	0	0	0	0	0	0
B. coli	0	1	1	1	1	2	1	1	2	1	1	1
B. lactis aerogenes	0	0	0	0	0	0	0	0	0	0	0	0
B. mucosus capsulatus	0	0	0	0	0	0	0	0	0	1	1	1
B. enteritidis	15	30	30	4	20	20	4	4	4	22	30	30
B. para typhosus B	10	30	20	12	25	25	6	15	15	22	27	27
B. para typhosus A	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 4—*Continued*

ORGANISM	3% PEPTONE 0.5% NaCl			3% PEPTONE 0.5% NaCl 0.5% GLUCOSE			3% PEPTONE 0.5% NaCl 0.5% SUCROSE			BROTH		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
B. typhosus	25	30	30	0	0	0	4	6	7	25	30	30
B. dysenteriae Shiga	0	0	0	0	0	0	0	0	0	0	0	0
B. dysenteriae Flexner	0	0	0	0	0	0	0	0	0	0	0	0
B. proteus vulgarus	5	10	20	7	10	10	5	6	6	25	30	30
B. cloacae	0	0	0	0	0	0	0	0	1	0	0	1
B. subtilis	0	0	0	0	0	0	0	0	0	0	0	0
B. anthracis	0	0	0	0	0	0	0	0	0	1	1	1
B. moelleri	0	0	5	0	0	0	0	0	0	0	0	0
B. fecalis alkaligenes	0	0	0	0	0	0	0	0	1	0	0	1
B. bovisepticus	0	0	0	0	0	0	0	0	1	0	0	0
B. avisepticus	0	0	0	0	0	0	0	0	0	20	25	25
Sp. metchnikovii	0	0	0	0	0	0	0	0	1	0	0	0
Sp. cholerae	0	0	0	0	0	0	0	0	0	0	0	0
B. hofmanni	0	0	0	0	0	0	0	0	0	0	0	0

Relative amount of hydrogen sulphide produced by various bacteria in Fairchild peptone media

ORGANISM	3% PEPTONE 0.5% NaCl			3% PEPTONE 0.5% NaCl 0.5% GLUCOSE			3% PEPTONE 0.5% NaCl 0.5% SUCROSE			BROTH		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
Sarcina lutea	0	1	5	0	0	2	0	0	0	0	0	0
Staph. aureus	0	1	1	0	0	1	0	0	0	0	0	1
Staph. albus	0	0	0	0	0	1	0	0	0	1	1	4
B. prodigiosus	0	0	2	0	0	1	0.	0	1	0	1	2
B. pyocyaneus	0	0	0	0	0	0	0	0	0	0	0	0
Mic. tetragenus	0	0	0	0	0	0	0	0	0	0	0	0
Strep. pyogenes	0	0	0	0	0	0	0	0	0	0	0	0
B. coli	2	2	3	1	1	2	0	0	1	1	1	2
B. lactis-aerogenes	1	2	3	0	1	2	0	0	1	0	1	6
B. mucosus-capsulatus	0	0	0	0	0	0	0	0	1	0	0	0
B. enteritidis	22	30	30	1	2	3	11	20	27	3	20	30
B. para-typhosus B	25	30	30	26	30	30	15	20	27	15	18	27
B. para-typhosus A	0	0	0	0	0	0	0	0	0	0	0	0
B. typhosus	15	22	25	1	1	2	15	20	27	1	1	15
B. dysenteriae Shiga	0	0	1	0	0	0	0	0	0	0	0	0
B. dysenteriae Flexner	0	0	1	0	0	0	0	0	0	0	0	0
B. proteus vulgarus	25	27	30	25	27	27	11	17	27	1	5	15
B. cloacae	1	2	5	0	1	4	0	0	1	1	2	2
B. subtilis	0	0	0	0	0	0	0	0	0	0	0	0
B. anthracis	0	0	0	0	0	0	0	0	0	0	0	0
B. moelleri	0	1	1	1	1	3	0	0	5	0	0	0
B. fecalis-alkaligenes	0	0	0	0	0	1	0	0	0	0	1	1
B. bovisepticus	2	2	2	0	0	0	0	0	0	0	0	0
B. avisepticus	0	5	5	0	0	0	0	0	0	0	0	0
Sp. metchnikovii	0	0	0	0	0	0	0	0	0	0	0	0
Sp. cholerae	0	0	0	0	0	0	0	0	0	0	0	0

acids than the American products. It probably also has a higher percentage of sulphur bearing constituents, possibly cystin. It would be interesting to determine the relative amounts of cystin in each type of peptone, but this has not yet been done.

A series of experiments was carried out to study the effect of various carbohydrates on hydrogen sulphide formation, by *B. paratyphosus* B. A medium made up of 3 per cent. Witte peptone, 0.5 per cent NaCl and 0.5 per cent of the carbohydrate was used. It was sterilized for twenty minutes in the Arnold on three consecutive days. Three monosaccharides, glucose, levulose and galactose; two disaccharides, lactose and sucrose; and a glucoside salicin were tried. No marked constant effect of these different carbohydrates on hydrogen sulphide formation was noted; this is not quite in accord with the observations of Seifert (1909) who found that the presence of glucose and lactose in peptone media decreased the amount of hydrogen sulphide formed.

Hydrogen sulphide production by B. Paratyphosus A and B. Paratyphosus B. and B. typhosus and B. dysenteriae

A number of strains of *B. paratyphosus* A, *B. paratyphosus* B. *B. typhosus*, and *B. dysenteriae*, were procured. Some of the cultures were obtained from the University of Chicago, some from the University of Kansas, some from the American Museum of Natural History, New York City, and some were freshly isolated from blood or stools at the University of Nebraska, College of Medicine. Their power of producing hydrogen sulphide was tested in a 3 per cent solution of Witte's peptone containing 0.5 per cent of NaCl, the same medium made from Difco and from Fairchild's peptones, respectively, and broth prepared from Difco peptone. All media were sterilized in the Arnold. The results are recorded in tables 5 and 6.

The tables need but little comment. So far as the cultures investigated were concerned *B. typhosus* always produced large amounts of hydrogen sulphide in twenty-four hours or less, while

B. dysenteriae never produced appreciable quantities in twenty-four hours and only a few strains produced traces after a week's incubation. The same distinction held for *B. paratyphosus* A and B. *paratyphosus B.* B always forming hydrogen sulphide in twenty-four hours or less and A forming practically none even

TABLE 5

Relative amount of hydrogen sulphide production by various strains of B. paratyphosus A, and B. paratyphosus B

ORGANISM	1% WITTE PEPTONE 0.5% NaCl			1% FAIRCHILD PEPTONE 0.5% NaCl			1% DIFCO PEPTONE 0.5 NaCl			DIFCO BROTH		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
B. paratyphosus B. 8 ...	15	20	30	22	27	30	30	30	30	25	25	30
B. paratyphosus B. 12...	7	10	30	22	25	30	27	30	30	22	30	30
B. paratyphosus B. 179 .	7	7	12	5	8	10	3	5	8	10	12	12
B. paratyphosus B. 101 .	12	20	25	25	27	30	30	30	30	20	22	25
B. paratyphosus B. 180 .				22	27	30	30	30	30	25	25	28
B. paratyphosus B. 323 .	10	20	30	22	30	30	30	30	30	27	30	30
B. paratyphosus B. 22 ..	8	10	15	20	25	25	30	30	30	30	30	30
B. paratyphosus B. 138 .	9	17	25	15	22	30	30	30	30	25	30	30
B. paratyphosus A. 3 ...	0	1	2	0	0	0	0	0	0	0	0	0
B. paratyphosus A. 158 .	0	1	1	0	0	0	0	0	1	0	0	0
B. paratyphosus A. 9 ...	0	1	1	0	0	0	0	0	0	0	0	0
B. paratyphosus A. 294 .	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyphosus A. 16 ..				0	0	0	0	0	0	0	0	1
B. paratyphosus A. 322 .	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyphosus A. 295 .	0	0	1	0	0	0	0	0	0	0	0	0
B. paratyphosus A. 10 ..	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyphosus A. 4 ...	0	0	0	0	0	1	0	0	0	0	0	0

after a week's incubation. This property, combined with the use of Endo and Russell's medium, should be of considerable value in differentiating these organisms, especially when the detection of carriers is the object in view. Ordinary broth is as satisfactory for this purpose as any medium tried. Sterilization in the autoclave instead of in the Arnold is satisfactory.

TABLE 6

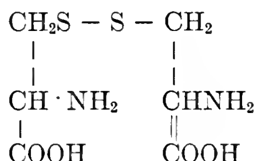
Relative amount of hydrogen sulphide production by different strains of typhoid and dysentery bacilli

ORGANISM	1% WITTS PEPTONE 0.5% NaCl			1% FAIRCHILD PEPTONE 0.5% NaCl			1% DIFCO PEPTONE 0.5% NaCl			DIFCO BROTH		
	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days	24 hours	48 hours	7 days
Typhosus, Hopkins	8	18	25	15	20	30	27	27	30	15	20	20
Typhosus, No. 190	10	20	25	12	22	23	30	30	30	22	22	22
Typhosus, Omaha No. 1.	7	15	20	8	12	15	25	25	25	25	25	25
Typhosus, Omaha No. 2.	12	16	23	10	15	20	20	25	30	20	25	25
Typhosus, Omaha No. 3.	9	13	20	7	15	15	25	27	27	22	25	27
Typhosus, Omaha No. 4.	10	16	22	9	15	20	27	27	27	18	25	30
Typhosus, Omaha No. 5.	12	20	25	10	15	20	25	30	30	15	25	27
Typhosus, Omaha No. 6.	10	18	23	10	15	22	22	25	25	20	25	25
Typhosus, No. 189	9	17	25	12	18	18	27	27	30	20	22	25
Typhosus, No. 197	9	20	22	14	17	22	30	30	30	17	20	27
Typhosus, No. 607				5	15	15	20	25	25	20	25	25
Typhosus, No. 11	10	15	20	9	11	15	30	30	30	15	20	25
Typhosus, No. 608				7	12	12	30	30	30	18	20	20
Typhosus, 5	9	20	25	10	15	15	30	30	30	18	20	22
Typhosus, Cary	12	18	25	15	18	20	20	25	25	20	22	25
B. dysenteriae, Flexner C	0	0	1	0	0	0	0	0	0	0	0	0
B. dysenteriae, Hofmann C	0	0	2	0	0	0	0	0	0	0	0	1
B. dysenteriae Shiga. W.	0	0	0	0	0	0	0	0	0	0	0	0
B. dysenteriae Shiga. C.	0	0	1	0	0	0	0	0	0	0	0	0
B. dysenteriae Strong	0	0	0	0	0	0	0	0	0	0	0	0
B. dysenteriae, Flexner W	0	0	1	0	0	0	0	0	0	0	0	0
B. dysenteriae, 78 W	0	0	1	0	0	0	0	0	0	0	0	0
B. dysenteriae, Omaha No. 1	0	0	1	0	0	0	0	0	0	0	0	0

The relationship between chemical structure and hydrogen sulphide formation

In order to study the relationship between the state of oxidation of sulphur compounds and the ease with which they may be attacked by bacteria with resulting hydrogen sulphide formation, three compounds were selected. Sodium sulphate was chosen as a representative inorganic sulphur compound in which

the sulphur is fully oxidized. Taurin was taken as a representative of the sulphur components of proteins in which the sulphur is fully oxidized. This compound is amino ethyl sulphonic acid $\begin{array}{l} CH_2NH_2 \\ | \\ CH_2SO_3H, \end{array}$ analogous to a sulphate except that one oxygen is replaced by an H. It is also an amino acid. Cystin the disulphide of diamino thiolactic acid was selected because it is an amino acid.



Here the sulphur is in the reduced state. Cystin is the usual sulphur-containing amino acid found in proteins. Cystin and taurin are analogous except as to the state of oxidation of the sulphur.

A medium was prepared as follows: ammonium tartrate 10 grams, anhydrous sodium sulphate, 10 grams, and distilled water to 1000 cc. The mixture was put in culture tubes and autoclaved for ten minutes at 15 pounds pressure. Five organisms were used, *B. coli* and *B. paratyphosus* B, because they are hydrogen sulphide formers which have no proteolytic action when tested in the ordinary media, and *B. fluorescens-liquifaciens*, *B. cloacae*, and *B. proteus-vulgaris*, because they are organisms, with varying degrees of proteolytic activity. Duplicate cultures were made, strips of sterile lead acetate paper were inserted in the mouth of the tubes, and they were placed in a moist 37° incubator. No growth appeared in any of the tubes at the end of seven days incubation under aerobic conditions.

An exactly similar medium to the one given above was made, except that 10 grams of chemically pure glucose was added. After seventy-two hours incubation a vigorous growth appeared in all of the tubes but even after fourteen days incubation there was no perceptible blackening of the lead acetate paper. Evi-

dently carbohydrate was a necessary source of carbon for the metabolism of the bacteria. It was omitted in the first medium in order to learn whether the carbon in the ammonium tartrate could serve this purpose.

Taurin was prepared by the method of Hawk (1918). Its purity was determined by making duplicate analyses for sulphur using the method of Redfield (1912). Theory called for 26.15 per cent and we found 25.90 per cent.

A 0.5 per cent solution of taurin was made in distilled water. It was sterilized with a Berkefeld filter because sterilization by means of heat caused blackening of lead acetate paper, and pointed toward a chemical change in the taurin. Filtration obviated any such possibility.

Duplicate tubes were inoculated with the same series of organisms which were used with the sodium sulphate medium; these were incubated for one week under areobic conditions at 37°C. without growth.

Another medium was prepared, identical with the above except that 1 per cent of chemically pure glucose was added. After one week's incubation a slight growth appeared in the tubes inoculated with *B. proteus-vulgaris*, and *B. fluorescens-liquifaciens*, but not in the others. No blackening of the paper occured in any instance. This would indicate that taurin is not readily attacked by bacteria.

Cystin was prepared by the method of Matthews and Walker (1909).

Its purity was ascertained by determining the percentage of sulphur by the same method used for the taurin. Theory called for 26.72 per cent; we found, 27.04 per cent.

An attempt was made to prepare the ammonium salt of cystin by dissolving the cystin in ammonium hydroxide and evaporating the excess ammonia. The cystin precipitated as soon as the fumes of the ammonia disappeared. Evidently the ammonium salt was easily hydrolyzed.

The citrate of cystin acted in the same way. In order to keep it in solution an excess of citric acid was required which was sufficient to interfere with bacterial growth.

Cystin plus tartaric acid behaved in a similar manner.

Finally a 0.5 per cent. solution of cystin was prepared by adding just enough sodium carbonate to keep the cystin in solution. The medium was sterilized by filtration because heat caused hydrogen sulphide formation. It was placed in sterile tubes and tested for sterility. Tubes were inoculated with the series of organisms used with the sodium sulphate and taurin mediums. After a week's incubation no growth appeared.

Another series of tubes were inoculated with the same bacteria used previously. A bit of sterile litmus paper was added to each tube and sterile 5 per cent. hydrochloric acid added till the litmus was faintly red. The cystin was precipitated as the neutral point was approached but this was ignored. After three days incubation the litmus turned blue and sterile acid was again added till it turned faintly red. Growth appeared in all the tubes and hydrogen sulphide was formed in some of them. Table 7 gives the results.

TABLE 7

Formation of hydrogen sulphide from a solution of cystin, in millimeters of lead acetate paper blackened

ORGANISM	24 HOURS	48 HOURS	72 HOURS	7 DAYS
B. paratyphosus B	0	2	7	7
B. typhosus	0	0	0	0
B. cloacae	0	0	0	0
B. proteus	1	2	4	25
B. fluorescens liquifaciens	0	0	15	25

This experiment was repeated except that the cystin was dissolved in the smallest possible amount of tenth normal hydrochloric acid and tenth normal sodium carbonate was added till a precipitate formed and the medium was only slightly acid, the exact reverse of the preceding method. The results were the same.

Cystin could doubtless have been sterilized by dissolving it in an organic solvent, evaporating this off and adding sterile distilled water but this was not tried.

Probably nearly neutral solutions of cystin in citric acid, in tartaric acid, or in ammonium hydroxide, prepared as in the case of the sodium carbonate solution, would have given similar results but these methods were not tried. The work done seemed to indicate that organic sulphur in the partially reduced condition rather than in the oxidized forms gives rise to hydrogen sulphide when ordinary bacteria act on protein.

SUMMARY

The examination of a number of samples of feces showed no constant difference between the amount of hydrogen sulphide produced by human and animal fecal material. This indicates that a test of samples of water for hydrogen sulphide production would be of no value in distinguishing between human and animal contamination.

The hydrogen sulphide test is too delicate for use in examination of water for the detection of fecal contamination of any type. All contaminated waters examined were positive to this test and many were positive which gave no evidence of contamination by the usual criteria.

A considerable number of common bacteria were able to produce hydrogen sulphide from Witte peptone and not from Difco peptone and some other kinds were able to produce it in larger amounts. Fairchild's peptone yielded more hydrogen sulphide than did Difco peptone. This emphasizes the need for uniform media.

Glucose and lactose had little effect on hydrogen sulphide formation. There was little difference between 3 per cent. peptone solutions and standard beef extract broth.

All strains of *B. paratyphosus* B examined, produced hydrogen sulphide in twenty-four hours or less, while none of the strains of *B. paratyphosus* A were able to do this.

All strains of *B. typhosus* studied produced hydrogen sulphide in twenty-four hours or less and none of the strains of *B. dysenteriae* had this power.

Sodium sulphate was not a source of hydrogen sulphide in the limited number of experiments made.

Taurin was attacked only to a very limited extent by the bacteria used, and none of them were able to split it to the extent of formation of hydrogen sulphide.

Cystin in distilled water was able to support bacterial growth in the case of some of the common bacteria, and even in some instances to permit of hydrogen sulphide formation. This indicates that oxidized sulphur is not readily attacked by bacteria while partially reduced sulphur is completely reduced to hydrogen sulphide.

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A CORRELATION STUDY OF THE COLON-AEROGENES GROUP OF BACTERIA, WITH SPECIAL REFERENCE TO THE ORGANISMS OCCURRING IN THE SOIL

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From the time of the discovery of *Bacterium coli* and *Bacterium aerogenes* by Escherich this group of bacteria has been of profound interest to bacteriologists and sanitarians. As these organisms were first isolated from the human intestine, their presence elsewhere has generally been taken as an index of fecal pollution. They were later found, however, to be normal inhabitants of the alimentary tract of lower animals covering a wide range of species. The problem became still more complicated when it became known that coli-like organisms are widely distributed in nature, particularly in soil and on plants and cereals.

For a time it appeared as if the presence of an organism of the *B. coli* type in water or any other article of human consumption was of no sanitary significance. The subject was so confusing that one German school of sanitarians entirely discarded the colon test. In spite of this situation, the test for *B. coli* has been, and is today, strongly advocated and supported by sanitarians in America, England and France.

Numerous attempts have been made to devise an acceptable system of classification of the colon-aerogenes group, but with very few exceptions these have failed because they did not rest on a sound basis of natural relationships. In the light of recent researches it becomes quite apparent that difficulties in previous classifications were in large measure due to a lack of delicate and exact methods, neglect in the consideration of normal habitat, and faulty interpretation of results. New methods of study have aroused new interest in this group, however, so that within a

brief period of but a few years most important advances in our knowledge have been made. Some of the methods of earlier investigators have given place to newer procedures which rest on broader scientific foundations, as for example the differentiation of types by exact methods of determining the hydrogen ion concentration in media of known composition, and the quantitative relationship of carbon dioxide to the total gas volume. Again, older methods which had been practically discarded have been re-applied and made to serve as very important differentiating tests, as for example the Voges and Proskauer reaction.

In view of the claims held by many investigators that certain types of colon bacilli met with in water have their origin in soil, their occurrence in water being viewed only as the result of soil washings, etc., the question of relationship of types becomes all the more important. Correlation of characters with source, according to such claims, is a problem which requires a most careful and thorough solution, and if true correlation can be established, careful colon bacillus typing will be an essential part of sanitary water analysis.

Both *B. coli* and *B. aerogenes* were long regarded as being of fecal origin. Booker (1891), Hammerl (1897), Hellström (1901), McConkey (1905 and 1909), Clemesha (1912), Rogers and his co-workers (1914–16), Levine (1916), and others showed that the aerogenes type is relatively infrequent in human and animal dejecta, as compared with *B. coli* itself. Winslow and Cohen (1918) estimate the frequency of *B. aerogenes* in animal feces as 0 to 2.6 per cent.

The aerogenes type of colon bacilli appears to be widely distributed in nature, having as a rule a saprophytic existence. Laurent (1899) thought that *B. coli* may lead a parasitic existence on the potato. Klein and Houston a little later (1899–1900) reported the occurrence of both typical and atypical *B. coli* on grains of various kinds. Papasotiriu (1901) obtained results similar to those of Klein and Houston. Prescott (1902 and 1906) showed that coli-like organisms could be found on grains whose contamination with fecal matter seemed very remote. Düggeli

(1904) obtained a large number of similar bacteria from fruits, plants and seeds, and Metcalf (1905) observed the same kind of organisms on flowers, fruits and grains. Bettencourt and Borges (1908) found 12 strains of lactose-fermenting organisms on vegetables and cereals, but only half of these were typical *B. coli*.

McConkey found atypical colon bacilli to constitute 56.2 per cent of the 121 strains isolated from raw water. Houston showed that 13 per cent of his 243 strains from raw water, 5.3 per cent from stored water and 3.2 per cent from stored and filtered water, were atypical *B. coli*. More recently Rogers and his associates (1915–1916) found about 91 per cent of the bacteria on grains, and 33.3 per cent of those in water to belong to the group of non-fecal coli-like bacteria. Their investigations were soon followed by those of Rogers, Clark, etc., Hulton, Greenfield, Levine, Burton and Rettger, Winslow and Kligler, and others.

Houston (1897–1898), studied a large number of soil samples, and came to the important conclusion that true colon bacilli are rarely found in virgin soil, but are present in large numbers in soils that have been grossly polluted with animal matter. Konrich (1910) drew the same conclusions from his examination of 547 samples of soils. Both of these investigators failed, however, to differentiate typical *B. coli* from the non-fecal type (aerogenes-cloacae). Johnson and Levine (1917) found that the aerogenes-cloacae types are the predominant bacteria of this group in the soil. Burton and Rettger (1917) examined 1000 samples of soil, leaves, flowers, etc., and concluded that the cloacae type predominated over its close allies, which is in harmony with the results of Clemesha, who observed that this organism could be isolated readily from soils.

Conflicting reports on the distribution of coli-like bacteria in nature have appeared, however. Clark and Gage (1903) obtained negative tests with grains. Gordon (1904) was unable to isolate lactose fermenters from bran except that of inferior quality. Winslow and Walker (1907) failed to find them in a thorough search in 178 samples of grains and 40 samples of grasses. Neumann (1910) experienced the same difficulty with fresh fruits, but found them on fruits and others foods which had been exposed to human contamination.

The present investigation was planned with the following points in view:

1. To determine the relative frequency of the colon and aerogenes types of bacteria in soils which from all appearances are free from animal pollution.

2. To ascertain whether or not there is a definite correlation between types of bacteria and their origin.

3. To make an extended correlation study of the coli and the aerogenes types of gas-fermenting organisms with reference to some of the most important reactions, particularly the gas ratio, and the methyl red, Voges and Proskauer, and the uric acid tests; and to determine the relative value of these tests in identification work.

4. To determine the relative value of the following media in the present correlation study: (a) The dipotassium phosphate-glucose-peptone (Witte) medium of Clark and Lubs; (b) the same medium with American brands of peptone in place of the Witte; and (c) the synthetic medium of Clark and Lubs.

5. To ascertain whether the coli and aerogenes types of bacteria change their character under prolonged cultivation in a new environment.

METHOD OF COLLECTING SAMPLES

The samples were collected from four main sources in the vicinity of New Haven, Conn., at different seasons and over a period of two years. These sources are East Rock, West Rock, Mt. Carmel and the New Haven Water-shed. In most instances the samples were taken on the summits of the rocks or hills, and in places far remote from human habitations. Chance contamination from birds and wild animals cannot be excluded, however, except perhaps when the samples were taken from underneath precipices and rocks. Thorough sanitary surveys were always made of the surroundings.

Three types of soil were chosen: (1) those from open precipices, (2) from between and under large stones, and (3) from well-aerated open ground. They were selected in such a way as to minimize as far as possible all chances of contamination.

The collecting outfit consisted of a large test tube and a strip of tin one end of which was hammered into a spoon shape. The tin was about an inch longer than the tube, in which it was kept until the time of sampling, both being protected by a cotton plug. This outfit was sterilized in the hot air sterilizer. In taking a sample of soil the dry surface layers were usually removed by a sterile knife and 10 to 15 grams of the soil collected in the tube by means of the tin spatula. The samples were taken to the laboratory immediately.

Four different amounts of each sample were employed in the isolation process, namely 0.01, 0.1, 0.5 and 1.0 gram portions. At first these amounts were weighed out separately in watch glasses, but subsequently they were approximated by comparison with portions of known weight which were used as standards. The different portions of soil were vigorously shaken with definite amounts of water in dilution bottles. The shaking was continued until a uniform emulsion was obtained. After standing long enough for the heavy particles to settle out the supernatant fluid was drawn off and plated on plain agar. The direct plating method for isolation was employed throughout the investigation, in preference to the liquid sugar medium enrichment method. Although the latter method has often been employed, it was feared that its use would disturb the original numerical relationships of the bacterial types.

Litmus lactose agar was at first used in the plating, but was soon found to be very unsatisfactory. Very few red colonies were observed on the plates, and at times not a single red colony was discernable in as many as 20 to 30 plates after forty-eight hours of incubation. This was in harmony with Burton and Rettger's observations on the cloacae-aerogenes type of bacteria, and those of Ayers who showed that alkali-forming bacteria in milk would rarely be noticed on litmus lactose agar.

The plain agar plates were incubated at 30°C. for forty-eight hours. At the end of this period all of the coli-like colonies, noted on examination with the low power objective, were subcultured in lactose fermentation tubes. These tubes were incubated at 30°C. from two to five days, at the end of which time

a sorting out process was conducted. All tubes which failed to show gas production by the end of the fifth day were discarded. The tubes which contained gas were further plated on plain agar, for the purpose of more complete isolation or verification of purity of the cultures. Stock cultures were finally made from well-isolated colonies.

SOURCES OF SAMPLES		NUMBER OF SAMPLES	NUMBER OF SAMPLES YIELDING COLI-LIKE COLONIES	NUMBER OF CULTURES FROM POSITIVE SAMPLES	
				Aerogenes type	Coli type
Soils					
East Rock soil	A	23	7	29	1
	B	25	11	22	1
	C	14	5	20	1
	D	29	13	26	12
West Rock soil	A	23	6	31	0
	B	15	7	13	0
	C	18	11	29	0
	D	8	5	23	5
Water-shed soil	A	19	9	37	0
	B	28	15	40	0
	C	11	4	19	0
Mount Carmel soil	A	12	9	36	0
	B	15	3	37	0
	C	26	6	24	0
	D	6	4	14	0
	E	19	5	27	0
	F	20	7	20	0
Total		317	127	447	20
Feces					
Human (7)		7	7	0	41
Monkeys (2)		2	2	0	30
Horses (4)		4	3	0	31
Cows (3)		3	2	0	23
Sheep (4)		4	4	0	24
Fowls (3)		3	2	0	24
Total		23	20	0	173

For an organism to be admitted into the final collection of strains the following recognized characteristics were required. They must be short rods, staining readily with the ordinary dyes but not by the Gram method. They must fall in the class of non-spore-forming organisms, and possess the ability to attack lactose with the formation of acid and gas.

Altogether 467 strains were obtained from the various soil samples. Of this number 447 were finally identified as belonging to the aerogenes-cloacae subgroup or type, while the remaining 20 were designated as typical *B. coli*.

For comparative study the same types of organisms were sought in human and animal feces, and 173 coli-like organisms were isolated from the feces of 7 men, 2 monkeys, 4 horses, 3 cows, 4 sheep and 3 fowls. No organisms of the cloacae-aerogenes type were found, all of the isolated strains proving to be typical *B. coli*. This should not be regarded as evidence that the aerogenes-cloacae type is not present in the intestines of man and animals. It does indicate, however, that they are of uncommon occurrence there.

The table on page 258 contains a summary of all the samples taken from soil and human and animal feces, showing the number of strains obtained and the types of organisms isolated from each source.

MORPHOLOGY, STAINING PROPERTIES AND MOTILITY

When grown on plain agar the large majority of the strains were alike within narrow limits, and resembled in size and form ordinary *B. coli*, being distinctly rod-shaped, with rounded ends. Some strains were thicker than others, and frequently short forms were observed which were more or less coccus-like. All strains were Gram-negative, although some took the counterstain more deeply than others. All took the ordinary stains readily. Spore formation could at no time be demonstrated.

Motility studies were made on twenty-four hour cultures, grown in Clark and Lubs' medium, and for the greater part of the time by the hanging drop method. The Hesse method of cultivation in semi-solid agar was also employed. While in

many instances motility could be detected without any difficulty, it was at times very difficult to reach a satisfactory conclusion. The following table summarizes the results.

Motility

TYPES OF ORGANISMS	HANGING DROP METHOD		HESSE METHOD		NUMBER EXAMINED
	Motile	Non-motile	Motile	Non-motile	
B. coli	119	54	121	52	173
B. aerogenes	122	325	75	372	447

ACTION ON CASEIN, GELATIN LIQUEFACTION AND INDOL PRODUCTION

The reaction of the different strains in litmus milk was more or less uniform at the end of the observation period, namely seventy-two hours. Some difference was observed, of course, in the rapidity with which visible acid production was brought about; but with few exceptions all of the strains turned the litmus red and coagulated the casein within seventy-two hours. In the exceptional instances application of heat was necessary to bring about the coagulation.

Gelatin liquefaction was determined by the two following methods. In the first, tubes of nutrient gelatin were inoculated (stabbed) with a twenty-four hour agar culture and incubated at 20°C. for six weeks. Observations were made at the end of 3, 7, 14, 21, 39 and 42 days. In the second method, a loopful of a twenty-four-hour broth culture was spread over the surface of standard nutrient gelatin and incubated at 37°C. for thirty days. At the end of five-day intervals the tubes were placed in the refrigerator. Tubes in which no liquefaction was demonstrable were returned to the incubator. The results obtained by both methods are given in the following table.

The superiority of the 37° method is clearly brought out here. According to this, 17 of the aerogenes strains were liquefiers. None of the coli cultures possessed this property.

Gelatin liquefaction

ORGANISMS TESTED	20°C.		37°C.		NUMBER EXAMINED
	+	−	+	−	
B. aerogenes from soil..........	0	447	17	430	447
B. coli from soil...............	0	20	0	20	20
B. coli from feces..............	0	173	Not tested		173

The tests for indol were made by the Salkowski and by the para-dimethyl-amido-benzaldehyde method of Ehrlich (1901). In the former procedure 0.5 cc. of a 10 per cent solution of sulphuric acid and 0.5 cc. of a 0.01 per cent solution of potassium nitrite were added to 5 cc. of a five-day old culture which had been incubated at 30°C. The Ehrlich test was conducted by adding to the same amount of culture material 0.5 cc. of a 2 per cent solution of para-dimethyl-amido-benzaldehyde in 95 per cent alcohol and then concentrated hydrochloric acid drop by drop and in such a way as to keep the two layers of fluid separate. The medium employed in both tests consisted of 10 grams of Witte's peptone, 5 grams of sodium chloride, 0.2 grams of dipotassium phosphate, and 1000 cc. of water. The results of these tests are given in the following table.

Indol production

ORGANISMS	EHRLICH METHOD		SALKOWSKI METHOD		NUMBER OF STRAINS TESTED
	+	−	+	−	
B. aerogenes from soil..........	141	306	80	367	447
B. coli from feces..............	173	0	Not tested		173
B. coli from soil...............	15	5	15	5	20

The marked difference between the results obtained by the two methods with the aerogenes strains from soil is noteworthy, and is due either to the extreme sensitiveness of the Ehrlich reagent, or to the fact that the Salkowski method is in reality not an indicator of indol, but of some other reacting substance, as has been claimed by Kligler (1914) and others. All of the fecal strains of *B. coli* were indol-positive.

CARBOHYDRATE FERMENTATION

A. Acid production

The production of acids and gas by intestinal bacteria was observed by Buchner as early as 1885. He found that the main end products of sugar decomposition by his "Darmbacillus G" were carbon dioxide and fatty acids. Since this time the fermentation reaction in a culture medium has been extensively utilized for the purpose of characterization and classification of bacteria. Its principles have been so firmly established that it is considered one of the very important reactions in the differential studies of organisms of more or less similar as well as widely-divergent types.

At first the acid test was limited to a narrow field, namely, the separation of the colon from the typhoid type of bacteria, as is well illustrated in the early use of the litmus-lactose agar of Wurtz (1892); but at the present time no systematic study of an organism is complete without a resumé of its fermentative properties if it is found to have such. Our chief interest in this paper is, of course, in the colon-aerogenes group of bacteria.

In 1893 Theobald Smith separated the colon bacilli into two groups, according to their action on sucrose. The work of Durham (1900), however, inaugurated the real beginning of the use of the fermentation method in systematic classification. He employed glucose, lactose and sucrose, and characterized *B. communis verus* as glucoso-lactoso-non-sucroso fractor, *B. coli communior* as glucoso-lactoso-sucroso fractor, and *B. lactis aerogenes* as polysaccharid fractor. Ford (1901) chose the same sugars for his classification. He divided the colon group into *B. coli*, *B. lactis aerogenes*, and *B. cloacae*.

McConkey (1905) divided the lactose-fermenting group into the four well-known divisions, based principally upon the action on sucrose and dulcitol, namely: (1) sucrose – dulcitol – (*B. acidi lactici* type), (2) sucrose – dulcitol + (*B. communis* type), (3) sucrose + dulcitol + (*B. communior* type), and sucrose +-dulcitol – (*B. aerogenes* type). This classification for a long time served as a frame-work for investigators in this field. Wins-

low and Walker (1907) found that raffinose is generally attacked by sucrose-positive organisms.

Bergey and Deehan (1908) also adopted McConkey's classification, and by adding other cultural tests extended the grouping in a most bewildering manner. They derived 16 groups and 256 varieties. In 1909 McConkey again subdivided his four main groups by introducing more fermentable substances. Over 100 types were separated out in this way.

Jackson's classification (1911) was a modification of McConkey's four primary divisions. He divided these into 16 distinct types by the additional use of raffinose and mannitol. This was formally adopted in 1912 by the Committee on Standard Methods.

Rogers, Clark and Evans (1914) concluded from their study of the colon-aerogenes organisms in milk and milk products that the acid production from a fermentable substance can not be used to advantage. They claimed that the measurement of the gas ratio is far more reliable and constant than the titratable acidity. In his careful study of the acid production of the colon group in many different sugars and under varying experimental conditions Browne (1914) came to the conclusion that the degree of acidity produced by the various members of the colon group is directly proportional to the complexity of the sugar fermented, and that each type within the group has its own limit of acid toleration. One interesting fact brought out is that strains of colon bacilli isolated from feces produced more acid in fermentable sugars than those which were isolated from oysters.

Kligler (1914) studied 80 laboratory strains of *B. coli* and concluded that salicin fermentation offers a better basis of classification than dulcitol. He thus modified McConkey's scheme as follows: Sucrose − salicin + type (*B. communis*), sucrose + salicin − type (*B. communior*), sucrose + salicin, + (*B. aerogenes*) and sucrose − salicin-(*B. acidi-lactici*). Glycerol was further used, to differentiate *B. aerogenes* from *B. cloacae*, the latter being a non-glycerol fermenter.

Rogers, Clark and Evans (1915) again maintained that "the acid from the fermentation of sugar may be masked by a second-

ary alkali production sufficient in some cases entirely to obscure the acid formation." They, furthermore, discredited the salicin test on the ground that too large a percentage of their own cultures (94.6 per cent) fermented this substance.

Levine (1916) maintained that the origin of a strain correlates better with sucrose than with the sucrose-dulcitol fermentation. In his second paper (1916) he concluded that "quantitative acid production in glucose, galactose, maltose, lactose, sucrose, raffinose, salicin, inulin, mannitol, dulcitol and glycerol is not a reliable index for differentiating colon-bacillus-like bacteria." He believes that gas formation is of more value in classification.

Murray (1916) attempted to differentiate human, bovine and equine types of colon bacilli by means of quantitative acid production. He came to the same conclusions as Levine. "In all cases," he says, "the average acid production for each of the 100 strains of each type resembles that of every other, and also resembles the average acid production of all the strains taken together irrespective of origin."

Hulton (1916) studied 45 strains of colon bacilli from various sources and found that a better correlation was obtained between sucrose fermentation and source than between sucrose-dulcitol fermentation and source, confirming Levine's observations.

It appears very clear, from the above cited observations, that acid production, even when determined by the biometric method, does not furnish a sound basis for the classification of the colon-aerogenes group of bacteria. The relationship between the various cultural and physiological characters and the normal habitat of the organisms studied has been in the past lamentably neglected. This important phase of classification has recently been brought to light, however, by Rogers (1914–1916) and his associates. The grouping of the colon bacilli has by them been greatly simplified and placed upon a more natural and logical foundation. In their papers dealing with the bacteria isolated from milk and milk products, feces and grains, they gave us a sound criterion for the separation of the colon group into two distinct types. This separation was accomplished by the determination of the gas ratio in a glucose medium.

The fallacy of the titrimetric method of determining the amount of acid production by bacteria, and the necessity of the application of more accurate and dependable methods for estimating the H ion concentration in culture media and in bacterial cultures have been clearly demonstrated by Clark and by Clark and Lubs (1915–1917). As a result of their important observations, the colorometric method which has been so advantageously applied by others to biochemical problems in animal physiology has quite generally become a routine part of bacteriological procedure.

B. Hydrogen ion concentration and the methyl red test

The successful attempts of recent years to establish more definite relationships of the important members of the colon group with each other and to place their classification on a more scientific basis were stimulated by the works of Harden (1901 and 1905), Harden and Walpole (1906), and Thompson (1911). They found that the end products of glucose fermentation, such as lactic, succinic, acetic and formic acids, and ethyl alcohol, by *B. coli* differ in quantity from those which are formed by the organisms now known as *B. aerogenes* and *B. cloacae*. Michaelis and Marcora (1912) gave further evidence to show that there is a "physiological constant" in *B. coli* cultures when this type of organism is grown in lactose broth. This constant is evidenced by the cessation of its activity at a definite hydrogen ion concentration of 1×10^{-5}.

The Clark and Lubs (1915) phosphate-glucose-peptone medium serves admirably to apply the above principles to practical use. This, as well as the subsequent synthetic phthalate medium of these investigators, furnishes the necessary conditions to permit an indicator of the right choice to register the hydrogen ion concentration within sharply defined limits, so that the distinguishing character of the indicator is not altered or obscured. Their selection of methyl red as the indicator of merit has also done much to facilitate our study of these organisms.

Clark and Lubs found that *B. coli* produced definite changes in these media, with sufficient acid production to give a brilliant red coloration when a few drops of the methyl red indicator are added, whereas a similar test on cultures of the *B. aerogenes* type resulted in a yellow coloration. More than this, they showed that these reactions correlate in a perfect manner with the source of the strains, and with their gas ratio. These observations were soon followed by fruitful researches of other investigators, and were in a large measure corroborated by Levine, Winslow and Kligler, Greenfield, Fulton, Johnson, Burton and Rettger, and Winslow and Cohen.

There are, however points still undetermined in the methyl red test which require further elucidation. Clark and Lubs emphatically state that the use of peptone other than Witte's would lead to an erroneous reaction. With this brand of peptone there are doubtful reactions which have been termed by Clark and Lubs as the "neutral tints." The typing of bacteria by means of the methyl red test depends upon gradations in color varying from a yellow to a bright red color, and covering a range of 4.2 to 6.0 on the pH scale. Determining the hydrogen ion concentration is comparatively easy when pronounced shades of red are obtained on the addition of the indicator; but when the hydrogen ion concentration is so low that only a yellow color ("neutral tints") is produced by the methyl red, it is imperative that some sort of a scale or limit within the "neutral tints" be established, or the types may be incorrectly placed and the correlations disturbed. Levine (1916) in his first work on the methyl red test encountered this difficulty; and Burton and Rettger (1917) found that the variation in the high ratio cultures was such as to lead them to the conclusion that the biometric method was of little value in the classification. Winslow and Cohen (1918) encountered this difficulty in the Witte peptone-phosphate-glucose medium, as well as in the synthetic medium later devised by Clark and Lubs (1916).

EXPERIMENTAL

In the present investigation the methyl red test has been placed on a more strictly quantitative basis, and exact pH values have been given to the various shades of color that were obtained at different times, both in the "neutral tint" range as well as where the characteristic light rose to deep red colors occurred.

In view of the fact that the inconsistent results obtained by some investigators were attributed by some to the indiscriminate use of different brands of peptone, two well-known products were employed, namely Witte's and Difco. The composition of the medium was the same as that first recommended by Clark and Lubs (0.5 per cent each of di-potassium phosphate, peptone and glucose). The new synthetic medium of Clark and Lubs was also used, for comparison.

No definite standards as to the temperature and incubation time for the methyl red test seems thus far to have been agreed upon by different workers. Clark and Lubs chose 30°C. and an incubation period of three to five days, whereas Levine maintained that no difference could be observed whether the temperature was 30 or 37°C. He held that 3 days' incubation is sufficiently long if the temperature is 37°C. More recently, Johnson and Levine (1916) resorted to forty-eight hours' incubation at 37°; Burton and Rettger (1917), five days at 37°; and Winslow and Cohen (1918), four days at 37°C.

Preliminary studies showed that a large number of the soil organisms refused, at least for a while, to grow at 37°C., especially in the synthetic medium, thus apparently confirming the findings of Rogers and his co-workers, and those of Winslow and Cohen. Throughout the present study the incubation temperature was 30°C. Since there was still a question as to the most desirable incubation period to be employed in order to obtain the most uniform and dependable results, both a three and a five day period were given a lengthy trial with all three of the media.

The synthetic medium had a distinct advantage over the others in being practically if not entirely colorless after sterilization, and hence not obscuring the color reactions. However,

after some preliminary attempts to obtain colorless peptone media, a method was followed by which the peptone-phosphate-glucose medium could be prepared and sterilized so that it remained as free from color as the synthetic. Previously sterilized test tubes are filled with the required amount of medium, and the final sterilization carried out by placing the tubes in containers which have impervious bottoms and sides, as for example canning tins or glass beakers, instead of the usual wire baskets, and sterilizing in the autoclave for ten minutes at an extra pressure of 10 pounds. During the entire course of the investigation very few tubes were found not to be sterile. We made it a common practice not to employ the tubed medium until its sterility had been tested by proper incubation for at least twenty-four hours.

The colorimetric standards were prepared according to the descriptions of Clark and Lubs. Three color indicators were employed, namely methyl red, brom thymol blue and brom cresol purple. Brom thymol blue is particularly well adapted for registering the hydrogen ion concentration of aerogenes cultures. The brom cresol purple was selected because it gives more distinguishable shades of color than the methyl red at the less acid end of the methyl red range where the troublesome neutral tints occur. The following technique was adopted for the test.

Five cubic centimeters of the bacterial culture fluid were transferred to test tubes of uniform diameter, and 0.2 cc. of the indicator added which by the preliminary drop method on a watch glass, or better still on a sheet of glazed paper or cardboard, was found to cover the indicated hydrogen ion range. The tubes were sorted out into three groups, with a fair knowledge of the approximate range of hydrogen ion concentration of each group. The pH value of each culture was recorded by comparison in the comparator with the previously prepared color standards.

A total of 3725 individual hydrogen ion concentration determinations was made in the three media with the two types (coli and aerogenes) isolated from soils and feces. The results are shown in the accompanying charts, in which the frequencies of occurrence are plotted as ordinates, and the corresponding pH values as abscissae. The middle line in the charts is an arbitrary

border or methyl red line to separate the coli from the aerogenes types of organisms. Since the colon type is now accepted as methyl red positive, and since the pH value 6.0 is the end point of the methyl red range, this line which starts at pH 6.0 appears to be the natural line of demarcation between these two types, and to be of value in plotting the charts.

Chart I gives the results of the hydrogen ion concentration determinations of the 447 aerogenes strains grown in synthetic and Witte's peptone media for three days at 30°C. Both curves

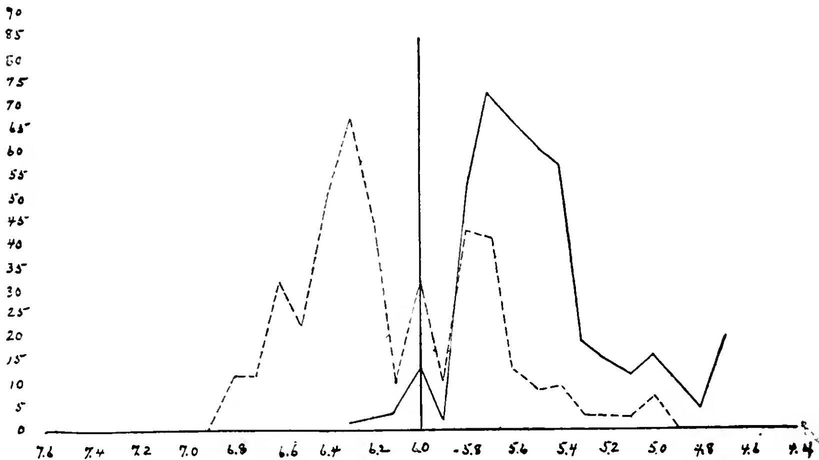

Chart 1. Frequency Curves for B. aerogenes Type (Soil)

Ordinate represents numbers of strains and abscissa the pH values. Three days' incubation at 30°C. Unbroken line, synthetic medium; broken line, peptone-glucose-phosphate medium (both of Clark and Lubs).

show clearly that a three day period of incubation is not sufficient at this temperature for the methyl red test, especially in the synthetic medium, in which almost all aerogenes cultures become methyl red positive. The peak of the curve for the synthetic medium is located at pH 5.7 and even with the clark medium, a large number of aerogenes cultures enter the methyl red range, although not as many, as in the synthetic medium.

Chart II shows the results of five days' incubation in the three media. There is a striking difference between the results of the

three and five-day periods. The additional forty-eight hours' incubation has returned all of the aerogenes strains to their proper indicator ranges in the synthetic as well as in the Witte peptone medium. The peak of the curve in the synthetic medium is now located at pH 6.5 instead of 5.7, the whole curve covering a pH scale of 6.0 to 6.8. In the Witte peptone medium the pH scale extends from about 6.0 to 7.6, whereas the peak lies at 6.8.

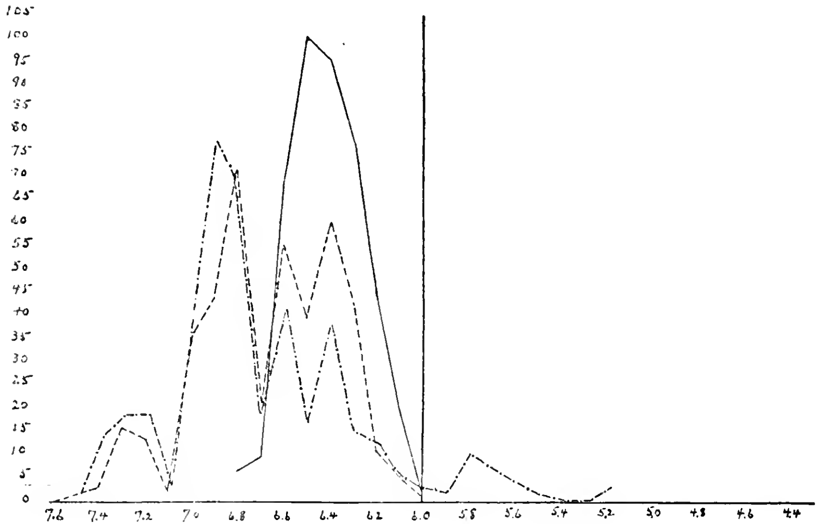

CHART II. AEROGENES TYPE (SOIL)

Five days' incubation at 30°C. Unbroken line, synthetic medium; barred line, Witte peptone-glucose-phosphate medium; barred and dotted line, Difco peptone-glucose-phosphate medium.

When substituting Difco peptone for the Witte in the peptone-phosphate-glucose medium, certain discrepancies occurred, even after prolonged incubation. It is seen that 26 of the aerogenes strains entered the methyl red positive range.

Chart III gives the results of 20 coli cultures isolated from soil, and grown in both the synthetic and the Witte peptone media for three days at 30°C. Chart IV gives the results of the same 20 coli strains in all three of the media after an incubation period of five days.

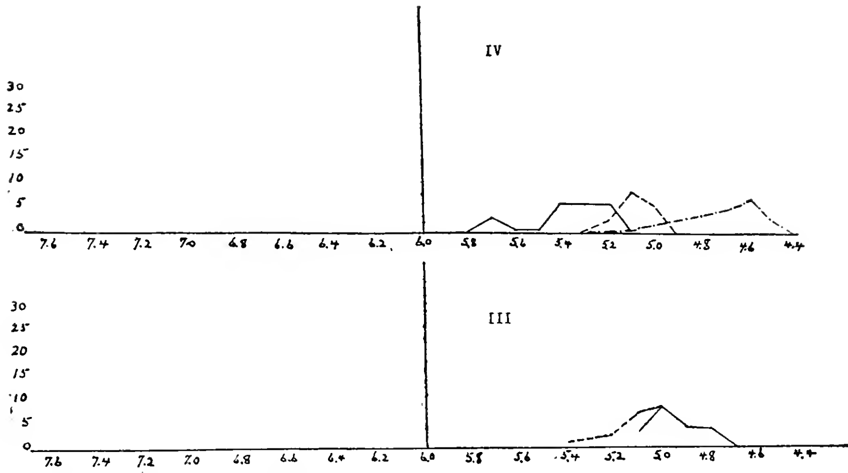

CHART III. (BELOW). B. COLI TYPE (SOIL)

Three days' incubation at 30°C.

CHART IV. (ABOVE). B. COLI TYPE (SOIL)

Five days' incubation at 30°C. Unbroken line, synthetic medium; barred line, Witte peptone-glucose-phosphate medium; barred and dotted line, Difco peptone-glucose-phosphate medium.

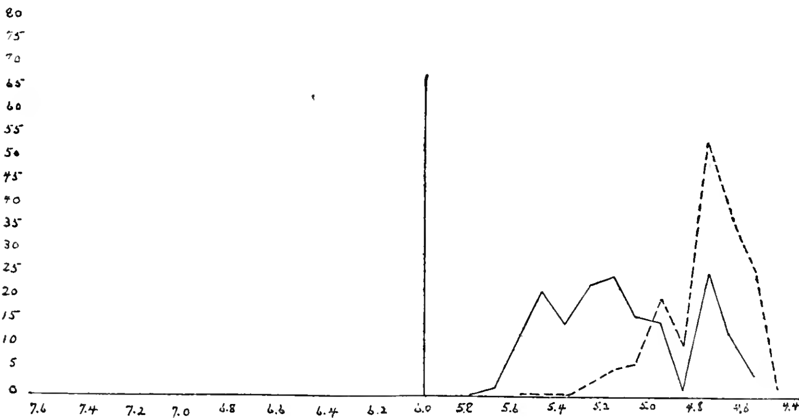

CHART V. B. COLI TYPE (ANIMAL SOURCE)

Five days' incubation at 30°C. Unbroken line, synthetic medium; broken line, Witte peptone-glucose-phosphate medium.

Chart V is plotted with two curves representing 173 coli strains isolated from animal feces. These are the results of the final hydrogen ion concentration determinations in the synthetic and the Witte peptone media after five days at 30°. Again the coli cultures gave uniform results with the methyl red tests. The pH values of these cultures cover a range of from 4.5 to 5.6 in the Witte peptone, and 4.6 to 5.8 in the synthetic medium.

Taking the three coli charts (III, IV and V) together, we find no qualitative differences in the results obtained with the three media or the two incubation periods, in so far as the methyl red test is concerned. This goes to show that incubation for three days at 30°C. is sufficient to give the characteristic methyl red reaction by coli strains from both fecal and non-fecal sources.

A careful analysis of these curves reveals several interesting facts. We find that the hydrogen ion concentration of the aerogenes cultures is generally higher in the synthetic than in the Witte peptone-glucose-phosphate medium either after a three or five days' incubation period. This would indicate that the production of alkalinity by aerogenes strains is more rapid in the Witte peptone medium than in the synthetic. In the former the peak of the H. I. C. curve for the three days' period is in the majority of cultures located at pH 6.3, while in the synthetic medium the peak lies at 5.7. A still more striking difference is shown in the five-day curves. In the synthetic medium the pH peak lies at 6.5, and the curve covers a range of only 6.0 to 6.8, whereas in the Witte peptone phosphate glucose medium the curve extends over a wider pH scale, 6.0 to 7.6, with the peak at 6.8.

In the Difco peptone medium the results are of peculiar interest. Setting aside for the present the few discrepancies (26 out of 447 aerogenes cultures which gave the methyl red positive test), a remarkable similarity between the Witte and the Difco peptone medium curves is observed. They almost parallel each other, from the range of pH 6.0 to 7.5. Their four peaks at pH 6.4, 6.6, 6.8 and 7.3, and the three troughs at 6.5, 6.7, and 7.1 correspond almost exactly.

REVERSION OF REACTION

The principle involved in the differentiation of the coli from the aerogenes type of bacteria by means of the Clark and Lubs media and the methyl red test is as follows. *B. coli* soon reaches a growth-inhibiting or lethal zone of hydrogen ion concentration and remains there; *B. aerogenes,* on the other hand, by virtue of its peculiar metabolic properties, continues its action upon the medium and progressively raises the pH value to a maximum which does not in itself retard further development of the organism. *B. coli* rapidly attacks the glucose, with relatively large acid production, while *B. aerogenes* produces more gas and correspondingly less acid during the early stages of growth than the coli type. *B. aerogenes* brings about a reversion of reaction which after prolonged incubation becomes more and more apparent.

This reversion of reaction in aerogenes cultures has led Kligler and a number of other investigators to assume that it is due to the production of ammonia from a peptone-glucose medium after exhaustion of the sugar. In their further study of the subject, Clark and Lubs disproved this view. They showed that reversion of reaction can take place in a synthetic medium free from peptone, and further proved that an extreme reversion can be obtained in a synthetic medium containing an ammonium salt as a source of nitrogen, although the content of total nitrogen is reduced to a point at which its participation in any form in changes of reaction of the medium would be insignificant. They come to the conclusion, therefore, that "An increase in ammonia may accompany the reversion of reaction, but the amount liberated is inadequate to account for the extent of the reversion." Under such conditions they believed that "It should not be assumed that the reversion is due solely to ammonia production."

These conclusions of Clark and Lubs do not give us a satisfactory explanation as to the cause of the reversion, nor do they lead us to think that they deny ammonia production in a glucose-peptone medium. It remained for Ayers and Rupp (1918) to furnish a plausible explanation for the phenomenon of reversion in a sugar-peptone medium. They demonstrated that there is

a simultaneous acid and alkaline fermentation in which the salts of organic acids produced from glucose are converted into carbonates or bi-carbonates, which in turn cause the reversion in reaction in a *B. aerogenes* culture.

In our attempts to follow up the work of Ayers and Rupp the Sörensen method of determining primary amino acid was employed, in addition to the ammonia determination method of Folin. It was hoped that some definite relationship between the formol titration and the ammonia figures could be established.

The Clark and Lubs peptone-phosphate-glucose medium was again made use of. Flasks containing 500 cc. of the medium were inoculated with a loopful (standard 4 mm. loop) of a twenty-four-hour culture of *B. aerogenes* grown in the same medium, and incubated at 30°C. At the end of 1, 2, 3, 5, and 10 day intervals the required amount of culture material was removed and subjected to the tests for sugar, amino acids, ammonia and hydrogen ion concentration. Eight representative strains of bacteria were selected for the test. Two were *B. coli* isolated from animal feces, and the remaining six aerogenes types from various apparently uncontaminated soils.

In the following table the Sörensen titration figures are recorded as the numbers of cubic centimeters of $\frac{N}{20}$ NaOH required to neutralize 100 cc. of the test medium. The ammonia figures are given as milligrams of ammonia per 100 cc. of the culture fluid.

The results show several interesting points. A close relationship appears to exist between the sugar utilization and the hydrogen ion concentration. In the aerogenes cultures the complete reversion of reaction took place within five days' incubation, and the sugar supply was practically exhausted in the same period. On the other hand, in the coli cultures the glucose was only partly fermented, and the hydrogen ion concentration remained constant after three days of incubation. No definite relationship between the Sörensen titration and the ammonia figures could be established. Irregularity of ammonia production is noticeable. One point stands out clearly from the rest; the amount of ammonia recorded in the *B. aerogenes* cultures was no greater, after ten days' incubation than in the control.

Cultures			Days	pH	Sörensen	Ammonia	Sugar
Control	Uninoculated			6.9	10.0	3.17	+
Aerogenes type	No. 34 Soil		1	5.2	10.0	2.20	+
			2	4.9	10.0	1.40	+
			3	4.9	20.0	1.70	+
			5	6.1	15.0	1.60	Trace
			10	6.9	10.0	2.10	Trace
	No. 37 Soil		1	5.4	20.0	2.75	+
			2	5.4	10.0	2.10	+
			3	6.1	10.0	1.92	Trace
			5	6.7	20.0	2.55	Trace
			10	6.8	15.0	2.01	Trace
	No. 24 Soil		1	5.9	10.0	1.40	+
			2	5.9	20.0	1.84	+
			3	6.3	15.0	2.50	Trace
			5	6.8	10.0	3.10	0
			10	6.8	10.0	2.54	Trace
	No. 44 Soil		1	5.9	15.0	1.45	+
			2	5.9	20.0	1.75	+
			3	6.3	10.0	2.04	0
			5	6.4	10.0	1.70	0
			10	6.5	35.0	2.80	0
	No. 20 Soil		1	5.9	20.0	1.50	+
			2	5.9	30.0	1.45	+
			3	6.3	20.0	1.78	Trace
			5	6.4	10.0	2.80	Trace
			10	6.8	20.0	3.02	Trace
	No. 21 Soil		1	5.1	20.0	1.64	+
			2	5.0	30.0	2.90	Trace
			3	5.3	10.0	3.10	Trace
			5	6.0	20.0	2.45	Trace
			10	6.5	30.0	2.70	0
Coli type	No. 5 Human feces		1	4.9	15.0	2.40	+
			2	4.6	20.0	3.30	+
			3	4.8	10.0	2.10	+
			5	4.7	10.0	3.10	+
			10	4.7	20.0	3.04	+
	No. 27 Human feces		1	4.8	10.0	1.40	+
			2	4.6	10.0	1.50	+
			3	4.7	15.0	2.90	+
			5	4.7	20.0	2.10	+
			10	4.7	15.0	3.12	+

The interpretation of the results is fraught with some difficulty. Clark and Lubs, and Ayers and Rupp did not deny that ammonia production may accompany the phenomenon of reversion. Kendall's theory of ammonia recession may in part explain a certain phase of reversion, and the claim of Rettger and Berman, and others, that ammonia is a readily available food for bacteria, and that it is not an end product, but an intermediary stage of metabolism, may throw considerable light on the subject.

GAS PRODUCTION

Escherich (1885) clearly demonstrated the production of gas in glucose and lactose media by his two types of intestinal organisms. Arloing confirmed the observation of Escherich. It was not until after the appearance of the fermentation tube (1890–93) of Theobald Smith, however, that the determination of gas volume and ratio formed part of our scheme of differentiation of bacteria. For almost twenty years this method reigned supreme and its accuracy as a quantitative procedure was little questioned.

Although Harden, Walpole and Thompson had improved the Smith method, the analytical error was not eliminated. Keyes (1909) introduced the vacuum method of exact gas analysis, and determined the gas ratio of *B. coli*. His work was followed by that of Rogers and his associates (1914–15), who firmly established the gas ratio of *B. coli* and *B. aerogenes*, namely approximately 1: 1 (CO_2: H) for the former, and 2: 1 for the aerogenes type. Since then there appears to be no doubt that the principle of gas-ratio as a differential test is well founded.

In the present study the gas production was at first observed in the Durham fermentation tube. A gas volume of at least 10 per cent was recorded as a positive test for gas, while the acid reaction in the same tubes was roughly determined with Brom-cresol-purple, a distinct yellow color being recorded as a positive reaction. In the use of the Durham fermentation tube a long inner tube (3 inches) was employed to facilitate the reading of the gas volume, which was done by the Frost gasometer. Al-

though this method is very crude, some interesting data were obtained.

Cultures of the *B. coli* type were seldom observed to attain a gas volume greater than 40 per cent when the medium had 1 per cent of glucose, the amount of gas usually being between 20 and 40 per cent. More specifically, of 173 fecal strains of *B. coli* cultures used only 8 gave a gas volume of 40 per cent, all of the remaining 165 falling below this figure. On the other hand, 322 out of 447 aerogenes strains gave a percentage higher than 40, 74 produced 40, and only 51 gave less than 40 per cent.

On account of the lack of proper facilities gas determination by the exact method was not undertaken until toward the end of the present study. Considerable time was spent in designing a vacuum pump which was a modification of the Sprengel pump, the plan of the Boltwood pump being followed to a large extent. As the Boltwood pump is not adapted for the collection of gases, and as no provision is made for removing the minute air bubbles which collect in the mercury, it had to be further modified. The distinct features of the new design of vacuum pump are: (1) It can be employed for exhaustion of the gases as well as their collection, (2) It possesses a device for the removal of air bubbles from the mercury, and (3) Its operation is automatic and continuous. As it is planned to give a complete description of this apparatus in a separate publication (Chen), further discussion of its design and application is omitted here, except a brief allusion to the following table which gives the representative results of one of the few estimations which were hurriedly made at the close of the present study, and which as far as it goes confirms the gas ratio of Rogers, Clark, etc.

Gas-production in vacuum bulbs

TYPE	SOURCE	TOTAL GAS	RATIO			
			CO_2	H_2	CO_2/H_2	Residue
		cc.				
Aerogenes..........	Soil no. 7	20.80	7.83	3.62	2.16	9.35
Coli...............	Human feces No. 36	11.70	4.26	3.90	1.06	3.54

THE VOGES AND PROSKAUER REACTION

This reaction was first observed by Voges and Proskauer (1898) in their studies on the bacteria of hemorrhagic septicemia. It was soon found that certain members of the colon group gave the reaction, and that on this as a basis the typical and atypical, or the fecal and non-fecal, types coming under this group could be differentiated from each other. Durham (1901), McConkey (1905–09), Archibald (1907), Bergey and Deehan (1908), Ferreira, Horta and Paredes (1908), West (1909), Copeland and Hoover (1911), Clemesha (1912), Kligler (1914), Levine (1916), and more recently Hulton, Greenfield, Johnson, Winslow and Kligler, Burton and Rettger, Winslow and Cohen, and Rogers and his associates have made use of this reaction. As a result the test has been given added significance, and promises to be one of the most important methods of differentiating the coli from the aerogenes type of organisms.

The chemistry of this reaction was not known until the publication of the thorough and painstaking researches of Harden and his co-workers. Harden (1905) in his study of the fermentation of glucose by colon bacilli discovered two important facts concerning the end products of glucose fermentation of the coli and aerogenes types of bacteria. He found that *B. coli* gives a high acidity, with only a partial utilization of the glucose, whereas the aerogenes type produces low acidity and complete exhaustion of the sugar. He concluded that "*B. lactis aerogenes* acts upon glucose in a totally different manner from *B. coli* and is therefore to be regarded as a distinct organism."

Harden and Walpole (1905–06) recognized a new substance, glycol, among the usual products of glucose fermentation, such as lactic, acetic, formic and succinic acids, alcohol and carbon dioxide. According to them, this new substance, crude glycol, contains a large amount of 2: 3 butylene glycol (CH_3-CHOH-CHOH-CH_3) which in the presence of atmospheric oxygen is oxidized to acetyl-methyl-carbinol (CH_2-CO-CHOH-CH_3). This volatile reducing substance is further oxidized in a relatively strong alkaline solution to di-acetyl (CH_3CO-CO-CH_3). The

diacetyl is colorless as such, but when brought into contact with a small amount of peptone or other protein material produces the characteristic eosin-like red coloration.

Walpole (1910) further observed that by passing a current of air through the *B. aerogenes* culture five to six times as much acetyl-methyl-carbinol is produced as when the culture fluid is left undisturbed. Thompson (1911) found that the chemical action of *B. cloacae* of Jordan on glucose is practically the same as that of *B. aerogenes*, in so far as the production of carbinol is concerned.

The actual correlation of the Voges and Proskauer reaction with the source of the organism and with its other characteristics was the work of Levine (1916). Soon after Rogers and his co-workers announced the definite relationship between the gas ratio, gas volume and the hydrogen ion concentration of the coli and aerogenes types of bacteria, Levine showed that an added correlation could be established with the Voges and Proskauer reaction. He found that bacteria of the aerogenes type consistently gave a positive V and P reaction, whereas those which were positive to the methyl red test were regularly V and P negative.

In the present study an attempt was made to determine: first, the relative value of the three media previously described in this paper, namely the Clark and Lubs' Witte peptone-glucose-phosphate, the synthetic phthalate-glucose-phosphate, and the Difco peptone-glucose-phosphate medium, for the study of the Voges and Proskauer reaction; second, the most favorable period of incubation; and third, a rapid and practical method for carrying out the test.

In the original method of applying the Voges and Proskauer test the alkaline fluid was exposed to the atmosphere for twenty-four hours or longer at room temperature. Walpole tried to hasten the oxidation process by passing a current of air through the medium, while West, following "Test No. 1" of Revas, boiled his cultures. More recently Levine introduced various oxidizing agents, and Bunker, Tucker and Green (1918) advocated the use of Syracuse watch glasses. These methods, and particularly the last two, appear to us too uncertain and too laborious.

The technique finally adopted in this work was as follows: At the end of one, three and five days' incubation periods 5 to 6 cc. of the culture fluid were well shaken in the test tube with an equal amount of 10 per cent potassium hydroxide solution. The tubes were placed in an incubator having a constant temperature of 30°C. At the end of one to three hours the tubes were again vigorously shaken until the liquid became foamy. As a rule, the eosin-like coloration made its appearance quickly, and the color deepened throughout the tube, instead of becoming visible as a ring or a surface layer. Without an exception, a decidedly positive Voges and Proskauer reaction (maximum color production) could be observed within a few hours (two to three) by this method. West used a more or less similar shake method ten years ago. He said: "After heating the tube, shake well, or blow through it to bring the red color out."

By the use of the method just described a total of 3725 individual Voges and Proskauer tests were made in the three different glucose-phosphate media, following three different incubation periods. The results are shown in the following chart (VI).

The chart shows clearly that there was no difference in the color reaction as the result of differences in the length of the incubation periods, one, three and five days, or of the different brands of peptone employed. Although the coloration in the synthetic medium was not so uniformly strong as in the peptone media, the reaction was always sufficiently pronounced to be called distinctly positive

Different strains manifested a striking difference in the degree of coloration which they gave. Not a single aerogenes culture, however, failed to give the reaction. On the other hand, not one of the colon strains, either from soil or feces, responded to the test.

An attempt was made further to determine the minimum incubation period in which color production can be obtained. A small collection of typical and representative strains of *B. aerogenes* was tested in the same three media after four, ten and fourteen hours of incubation of the cultures at 30°C. The results showed that a positive Voges and Proskauer reaction may be ob-

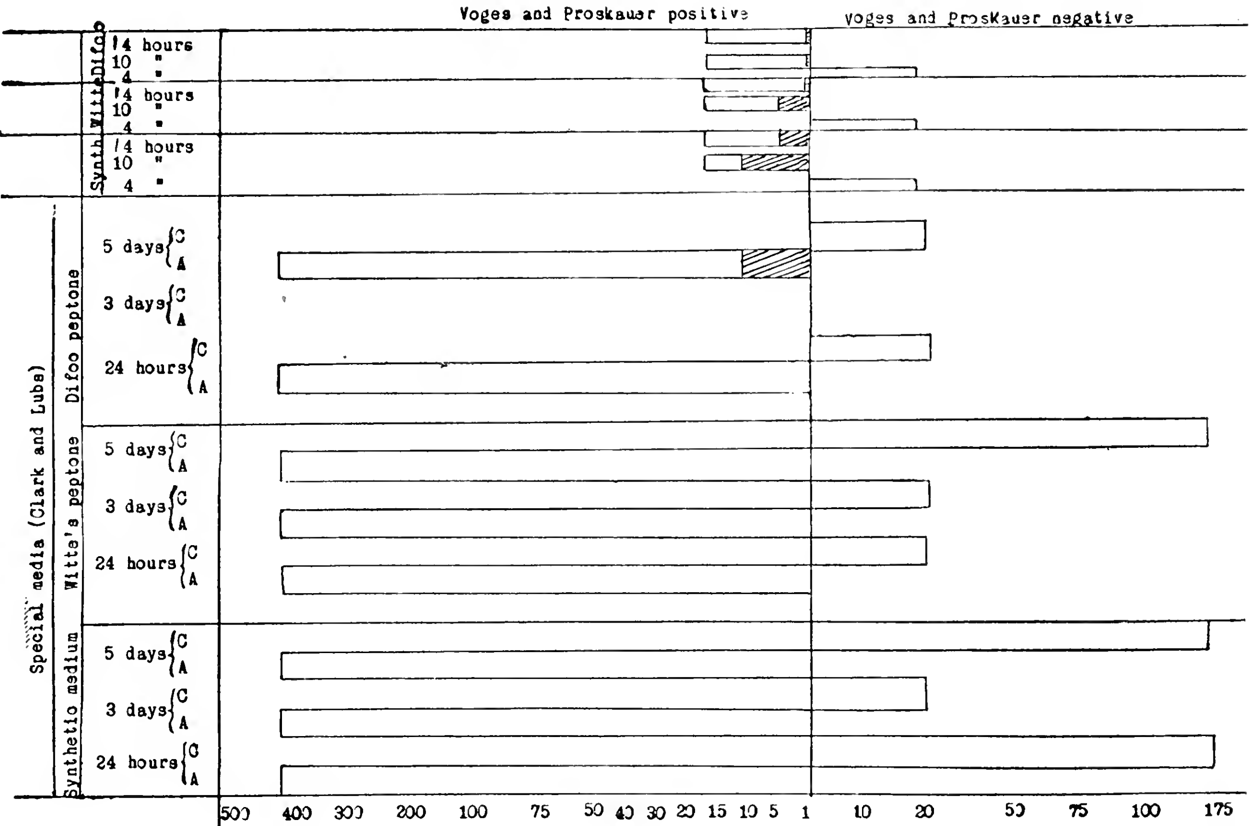

CHART VI. Upper section, showing Voges and Proskauer reaction in very young cultures of B. aerogenes. Lower, main section, showing V. & P. reactions in the different media after one, three and five days' incubation at 30°C.

C = Coli type; A = aerogenes type. The figures below indicate number of strains. Shaded areas show weak reactions.

tained in cultures which are only ten to fourteen hours old (chart VI, upper part).

THE DEPORTMENT OF MIXED CULTURES (COLI AND AEROGENES TYPES) TOWARD THE METHYL RED TEST AND THE VOGES AND PROSKAUER REACTION

One strain of typical *B. coli* of fecal origin was grown in symbiosis with 56 different strains of *B. aerogenes* obtained from soil. Each of the strains had been cultured separately in plain broth for twenty-four hours, and a loopful of each tube was inoculated into the Witte peptone- and the synthetic medium of Clark and Lubs. The mixed cultures were incubated for five days, one set at 24° and a second at 37°C. The hydrogen ion concentration was then determined with methyl red as indicator, and the Voges and Proskauer test was made as described earlier in this paper.

The results of the study are given in the accompanying chart (VII). They show that in the peptone-phosphate-glucose medium, both at 24° and at 37°, the hydrogen ion concentration of the aerogenes type of organism was partly disturbed, and that in the synthetic medium the acidity of the colon type almost entirely obscured that of *B. aerogenes*. The Voges and Proskauer reaction as ordinarily given by the aerogenes type was not at all affected in the synthetic medium at 24°, and only one culture in the peptone medium failed to give the characteristic reaction when grown at 24°C. Of the 56 tubes incubated at 37°, 14 were negative by the V and P test, 4 in the synthetic and 10 in the peptone medium.

Briefly stated, when both types of organisms are present in any given culture the methyl red test reveals the presence of the colon type, while the aerogenes type responds to the Voges and Proskauer test when the temperature of incubation has been as low as 24°. At the higher temperature only a small number of the cultures are negative to the V & P test. The permanency of the Voges and Proskauer reaction is here clearly demonstrated.

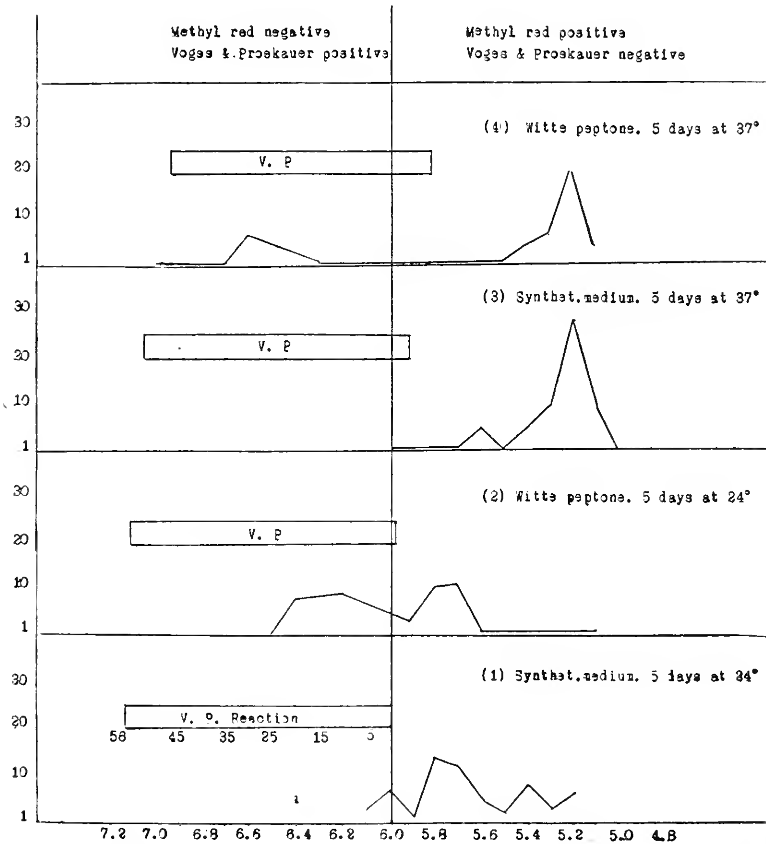

Chart VII. Showing Symbiotic Reactions of One Strain of B. coli With 56 Strains of B. Aerogenes

DIFFERENCES IN NITROGEN UTILIZATION AS A BASIS FOR DISTINGUISHING B. COLI FROM B. AEROGENES. THE URIC ACID TEST OF KOSER

The selective action of bacteria on carbohydrates has long been recognized, and on this principle many tests have been devised, and different types of bacteria have been successfully separated. The differentiation rests, however, on the character of the end products of carbohydrate fermentation, as for example

the formation of acid and gas, and the production of carbinol and diacetyl.

Much attention has also been given in recent years to the differentiation of bacteria on the basis of nitrogen utilization, in the form of ammonia, amino acids, di-amines, nitrates, etc. For example, it was shown by Uschinsky that *B. coli* can attack amino acids like asparagine in a purely synthetic medium, whereas *B. typhosus* is as a rule unable to utilize it as a source of nitrogen. Quite recently Koser (1918 and 1919) succeeded in a somewhat similar manner in differentiating between the *B. coli* and *B. aerogenes* types of organisms.

In his extensive study of nitrogen utilization by bacteria in media of definite chemical composition, Koser observed that *B. aerogenes* readily attacks uric acid or hypoxanthine as the only source of nitrogen, while *B. coli* is void of this property. In other words, he found that the aerogenes type possesses the unique power of seizing upon the nitrogen of the purin ring, whereas the coli type leaves this portion of the uric acid molecule intact, and fails in its development unless some other nitrogenous substance which furnishes available nitrogen is present.

In the present investigation all of the strains isolated from feces and soils were employed in a further study of this phase of bacterial nutrition. They were inoculated into the uric acid synthetic medium of Koser. In a large series of tests other purin bases were employed in the place of the uric acid, the basic composition of the synthetic medium being the same as when the uric acid was used. These substances were xanthine, caffein and theobromin. The uric acid synthetic medium as prepared by Koser has the following composition:

Distilled ammonia-free water	1000.0 cc.
NaCl	5.0 grams
$MgSO_4$	0.2 gram
$CaCl_2$	0.1 gram
K_2HPO_4	0.1 gram
Glycerol	30.0 grams
Uric acid	0.5 gram

The medium was tubed in 5 cc. portions and sterilized for fifteen minutes at 12 pounds extra pressure. The test tubes

which are used must be scrupulously clean; small amounts of nitrogenous matter left on the walls may furnish sufficient nitrogen for the organisms to multiply so as to obscure the sharp distinction between the appearance of the *B. coli* and *B. aerogenes* tubes after the required time of incubation, four to five days at 30°C.

A twenty-four hours growth of the organism under observation was used as a rule as inoculum, and this was transferred with precaution, so as to limit as far as possible the amount of cell material and products of bacterial metabolism which are carried over. The results of the tests with the uric acid medium are summarized in the following table.

Table showing results of uric acid tests

	POSITIVE	NEGATIVE	TOTAL
Aerogenes strains from soils.............	447	0	447
Coli strains from feces....................	0	173	173
Coli strains from soils....................	10	10	20

All of the aerogenes strains (soil) gave a very pronounced growth in the uric acid medium, while none of the fecal strains of the coli type clouded the medium or in any way showed indications of development. The differentiation was in every instance sharp though the degree of turbidity varied much. However, considerable discrepancy was observed among the coli strains isolated from soils. Of the 20 cultures employed 10 were uric acid positive and 10 negative. In the tubes in which the uric acid was attacked the growth was as luxuriant as in typical cultures of the aerogenes type. Lack of correlation here of the 10 uric acid positive strains of soil coli with the methyl red test and Voges and Proskauer reaction may be of some significance as pointing to the possibility that these ten strains are of a type intermediate between the coli and the aerogenes.

The same results were obtained in the xanthine synthetic medium as in the uric acid, except with the soil strains of *B. coli*, which goes to show further that the failure of *B. coli* to utilize the nitrogen is due to its inability to disrupt the purin ring,

which contains the nitrogen. All of the soil coli strains failed to attack the xanthine, and hence reacted like typical *B. coli*. Further investigation may show that xanthine may be superior even to uric acid for differentiating these two types of bacteria, unless types intermediary between coli and aerogenes exist which in uric acid medium are sharply distinguished. With two exceptions, both types of bacteria failed to develop in the caffeine and the theobromine medium. The results with the xanthine, caffeine and theobromine media are shown in the following table.

Table giving results with xanthine, caffeine and theobromine media

ORGANISMS	XANTHINE		THEOBROMINE		CAFFEINE		TOTAL
	+	−	+	−	+	−	
Aerogenes (soils)	55	0	0	55	0	55	55
Coli (soils)	0	20	0	20	0	20	20
Coli (feces)	0	55	2	53	0	55	55
Aerogenes (Am. Mus. Nat. Hist.)	10	0	0	10	0	10	10
B. communior (Am. Mus. Nat. Hist.)	0	5	0	5	0	5	5
B. communis (Am. Mus. Nat. Hist.)	0	5	0	5	0	5	5
Aerogenes (Rogers)	6	0	0	6	0	6	6
Aerogenes (Winslow and Cohen)	5	0	0	5	0	5	5
Total							161

FERMENTATION OF GLUCOSE, LACTOSE, SUCROSE, ADONITOL AND DULCITOL

It was at first intended to classify the present collection of strains according to McConkey's primary divisions, on the basis of fermentation of glucose, lactose, sucrose and dulcitol. Adonitol was also employed because of the claims of Rogers and others that the so-called fecal and non-fecal types of *B. aerogenes* can be differentiated in this way. Owing to our inability to obtain sufficient dulcitol the classification study with McConkey's four sugars was incomplete. The results are presented in the following table.

BACTERIAL STRAINS	GLUCOSE		LACTOSE		SUCROSE		ADONITOL		DULCITOL		TOTAL
	+	−	+	−	+	−	+	−	+	−	
Aerogenes type (from soils)	447	0	447	0	338	109	152	295			447
Coli type (from soils)......	20	0	20	0	15	5	5	15			20
Coli type (from feces).....	173	0	173	0	91	82	30	143			173

Results with dulcitol too incomplete to include in table. The figures indicate both acid and gas production.

All of the strains fermented glucose and lactose. Sucrose was attacked by both the colon and aerogenes types, although the high gas ratio group was as a group more active than the other, excluding the soil coli.

The present results do not bear out the contention that the fecal and non-fecal types of aerogenes may be differentiated by fermentation in adonitol, as 152 soil aerogenes strains out of the 447 attacked the adonitol, whereas the remaining 295 did not. These findings agree in principle with those of Winslow and Cohen, who observed that "a greater proportion of *B. aerogenes* from the unpolluted sources attacked adonite than did those from the polluted waters."

ATYPICAL STRAINS

In the present collection of organisms which resemble the aerogenes type, 18 at first appeared to occupy an intermediate position where complete correlation could not be established. They persisted in giving methyl red positive and Voges and Proskauer positive reactions in all three of the media employed, after one, three and five days' incubation. Impurity of the cultures was suspected and repeated platings were resorted to. In this way the number of non-correlating organisms was reduced from 18 to 4, although contaminating organisms could not be demonstrated.

The methyl red positive strains whose hydrogen ion concentration was on the border line of the methyl red range (5.7 to 5.9) were made to return to their typical methyl red negative reactions by repeated plating, their pH values being raised to

6.2–6.5. This would indicate that variation within certain limits in the hydrogen ion concentration is to be expected. After 39 replatings the above-mentioned 4 strains still failed to correlate, however, in so far as the methyl red test and the Voges and Proskauer reactions are concerned, both being positive. It is unfortunate that we were unable at the time to make exact gas determinations on these 4 strains, since Clark and Lubs have claimed that "when a perfectly clear-cut correlation fails, there is generally found some abnormality in gas-production. . . ."

All of the 4 peculiar strains gave a pronounced Voges and Proskauer test and were able to attack the purin ring in uric acid; hence, in so far as these two reactions are concerned these organisms resembled typical *B. aerogenes.*

Rogers and his associates reported that the majority of their atypical strains gave a pH value ranging from 5.6 to 6.0, and that most of them (11 out of 16) were Voges and Proskauer positive. Repeated replating might have materially reduced their number of atypical strains, as the pH values given by them were so near the border line of the methyl red indicator range, and as a small variation in the hydrogen ion concentration must be expected of all bacteria.

CORRELATION OF CHARACTERS

Chart VIII presents graphically the results obtained in the study of the various characters of the 447 aerogenes and 173 fecal strains of coliform bacteria. The tests embraced the methyl red, uric acid and Voges and Proskauer reactions, deportment towards glucose, lactose, sucrose and adonitol, indol formation and gelatin liquefaction. As a matter of brevity, the results obtained with the 20 coli strains from soil and the 4 atypical strains are not presented here in chart form. They differ little from the above, aside from a difference in indol production and adonitol fermentation, and the points already mentioned with reference to the methyl red, uric acid and Voges and Proskauer.

It will be observed in the accompanying chart that perfect correlation, exists for both the aerogenes (447) and the fecal coli

B. coli Type (Feces)
173 strains

Tests negative 100 90 80 70 60 50 40 30 20 10 0
Tests positive 10 20 30 40 50 60 70 80 90 100

Methyl red test
Uric acid test
V.P. reaction
Glucose
Lactose
Sucrose
Adonite
Indol
Gelatin liquefaction

B. aerogenes Type (Soil)
447 strains

Tests negative 90 80 70 60 50 40 30 20 10 0
Tests positive 10 20 30 40 50 60 70 80 90 100

Methyl red test
Uric acid test
V.P. reaction
Glucose
Lactose
Sucrose
Adonite
Indol
Gelatin liquefaction

CHART VIII. CORRELATION OF CHARACTERS OF B. COLI AND B. AEROGENES TYPES

(173) types, aside from the reactions in sucrose and adonitol, and indol production of the aerogenes type.

A complete study of gas production in the Durham fermentation tube was made of all of these organisms, both as to volume and gas ratio, in so far as the method would permit, 40 per cent being the arbitrary limit placed for the coli type, and above 40 per cent being taken as an indication of the aerogenes type. Though very crude, this method permitted us to assume from the results obtained a complete correlation between gas volume, gas ratio, the methyl red, uric acid and the V and P reactions. However, on account of the very faulty method employed in the quantitative study of gas production these results are not included in the charts or in any of the tables.

GENERAL DISCUSSION

A survey of the present investigation leads to the conclusion that there exist in nature two distinct types within the colon group of bacteria. These two types, now generally designated as *B. coli* and *B. aerogenes*, can conveniently be set apart from each other by the newer tests.

The direct plating method is better adapted for the isolation of the soil organisms than the combined preliminary enrichment and subsequent plating procedure, because it permits of the study of numerical relationships between bacterial types and their habitat and of the determination of the relative numbers of each type.

The failure to isolate *B. aerogenes* from the feces of man and animals in the present study should not be taken as evidence that this organism is absent from the intestine, but rather as a result of circumstances which permitted its being overlooked. This apparent absence or scarcity is not at all surprising, as Rogers and his associates (1914) found only one high ratio strain in 150 coli-like organisms isolated from bovine feces.

Nothing of importance could be gained through the morphological study of this group of bacteria, since both types present practically the same microscopic picture. The indol test is of

little value, also, and should be used with caution. While all of the *B. coli* strains produce indol, the percentage of positive tests with the aerogenes type is too large to make the test a practical one. The Ehrlich method of determining indol formation is decidedly more delicate than the Salkowski sulphuric acid test, and must be regarded as the more reliable. In the present investigation gelatin liquefaction has been of no value as a test.

The constancy of the hydrogen ion concentration in cultures of the *B. coli* type, as determined by the colorimetric method, is proved beyond doubt by the present study. The hydrogen ion concentration can be adequately measured for practical purposes by the methyl red indicator, as Clark and Lubs have claimed. The synthetic medium of Clark and Lubs appears to be best suited for the colorimetric determination of acidity. Witte's peptone-phosphate-glucose medium of Clark and Lubs answers the purpose well for which it is intended provided that it is kept colorless or practically free from color during the process of sterilization.

Owing to the interference of simultaneous alkali production the limiting hydrogen ion concentration of the aerogenes strains could not be so easily determined. The results of the present investigation show that the hydrogen ion concentration of these strains is completely masked after five days' incubation both in the synthetic and in the Witte peptone-phosphate-glucose medium.

A new set of experiments was conducted to determine daily the hydrogen ion concentration of a collection of 60 aerogenes strains in the synthetic medium for a period of three weeks, in order to ascertain the degree of OH-ion concentration reached by the cultures. The H-ion concentration began to decrease at the end of three days of incubation, and the pH value of the OH-ion concentration proceeded to increase steadily and progressively until the end of the experiment. The greatest H-ion concentration was 4.7, and the highest OH 7.4. It must be apparent, therefore, that length of incubation and temperature are two very important factors in the interpretation of results. If the colorimetric determination is made too soon, or the incu-

bation temperature too low, colorimetric determinations with the methyl red indicator may be positive and correlation with the other tests will be lacking.

Difco peptone-phosphate-glucose medium is less well adapted for the study of hydrogen ion concentration of the aerogenes type than the synthetic and the Witte peptone media of Clark and Lubs, and a greater proportion of failures to correlate must be expected, expecially if the exacting conditions as to length of incubation and incubation temperature are not supplied. The difference appears, however, to be chiefly one of degree rather than kind.

The Voges and Proskauer reaction has proven itself in the present investigation to be of very great value in differentiating between the coli and aerogenes types of bacteria. Neither the character of the medium nor the period of incubation seems to interfere with the carbinol formation. A colorless medium is as important, however, for this test as for the colorimetric determination of the hydrogen ion concentration, owing to the fact that, while the characteristic coloration of the tubes cannot be mistaken, it may be at times obscured by coloring matter in the medium itself, especially if the reaction is relatively weak. Furthermore, intelligent execution of the test is absolutely necessary.

The Voges and Proskauer reaction should be observed within shorter incubation periods than have been customary. A weak reaction persists only for a short time, while a strong reaction may last for several days, with a gradual fading away of the color. A period of from two to eight hours may be regarded as sufficient for a positive V and P reaction, if correctly carried out. Abundant oxygenation of the medium after the addition of the alkali, and proper incubation are important factors.

The uric acid test of Koser constitutes another reliable correlation test. Great care must be exercised, however, in the employment of test tubes, etc. which are free from adhering nitrogenous matter. Koser obtained excellent results with uric acid and hypoxanthine. In the present work xanthine, theobromine and caffeine were employed besides uric acid, the xanthine and uric acid giving very gratifying results. No growth could

be obtained, however, in the theobromine and caffeine synthetic media, either of the coli or of the aerogenes type. This was due, in all probability, to an inhibitive or antiseptic action of these substances, even in the small amount in which they were used. *B. aerogenes* is able to attack the purin ring of xanthine, hypoxanthine and uric acid, and thus appropriate nitrogen in a sufficient degree to develop in the synthetic medium more or less luxuriantly, whereas *B. coli* lacks this property. The differentiation is sharp.

SUMMARY

The present study of coli-like organism in soils of known sanitary quality has shown the great predominance of the aerogenes-cloacae type. Of 467 strains of bacteria isolated from various soils 430 were identified as *B. aerogenes*, 17 as *B. cloacae*, and only 20 as *B. coli*. Furthermore, the sources of the coli strains were shown by the sanitary survey to be not entirely free from animal pollution. All of the 173 organisms found in the feces of 7 men, 2 monkeys and 14 domestic animals were typical *B. coli*. It is apparent from these observations that there is a definite correlation between these types of bacteria and their origin.

An almost perfect correlation could be established by the methyl red, Voges and Proskauer and the uric acid tests.

The limiting hydrogen ion concentration of the coli cultures, as determined by the colorimetric method, varied from pH 4.5 to 5.6 in the synthetic medium, and from 4.6 to 5.8 in the Witte peptone-phosphate-glucose medium. The final hydrogen ion concentration of the cloacae-aerogenes type could not be accurately determined on account of the simultaneous acid and alkali production. The pH value obtained under similar conditions ranged from 6.0 to 7.4 in the Witte peptone medium, and from 6.0 to 6.8 in the synthetic medium.

The respective hydrogen ion concentrations of the colon and aerogenes types of bacteria may be adequately determined for practical purposes by methyl red as an indicator, provided the neutral tint reactions are compared with the reactions obtained by brom cresol purple or some other sharp indicator as a check.

The Voges and Proskauer method of distinguishing between *B. coli* and *B. aerogenes* has proven even more satisfactory than the methyl red test, in that it is simple in operation, and when correctly carried out thoroughly constant in its results. When used with precaution, the uric acid test also is of fundamental importance in differentiating fecal coli from the soil aerogenes type of bacteria.

No definite correlation could be established by means of the indol test. Neither did motility study prove to be of practical value.

Adonitol fermentation did not prove itself to be a satisfactory method of differentiating fecal from non-fecal strains of *B. aerogenes*.

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NOTE ON THE FLORA OF THE STOMACH

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Sarcinae and other organisms of undescribed form were observed by Goodsir (1842) in the fluid periodically ejected from the stomach of a patient. Hasse and Kolliker (1847) also reported finding sarcinae in gastric juice. Frerichs (1849) confirmed these findings in dogs with a gastric fistula, while the dogs were in a weakened condition. Studying extracts from his own stomach during fasting Abelons (1888–89) isolated *Sarcina*, *B. pyocyaneus*, *B. lactis-arogenes*, *B. amylobacter*, *Vibrio rugula* and other organisms. Hamburger (1890) reported that gastric juice containing free hydrochloric acid was almost always free from living organisms. From material obtained in the early morning from persons suffering from indigestion, Oppler (1894) found sarcinae in abundance. Kaufmann (1895) in a case of chronic dyspepsia isolated yellow sarcinae, *Micrococcus aurantiacus*, *Staphylococcus cereus-albus*, *Bacillus subtilis*, *Bacillus ramosus*, a large thick bacillus and a short bacillus resembling *B. coli*. One yeast was found in this investigation.

Herter (1907) states that gastric juice in normal abundance as found after a meal acts as a check on the growth to many, and is partially destructive to most, varieties of bacteria. He says that the proteolytic action of pepsin plays a part in this destruction. Gregersen (1916) has shown that gastric juice containing free hydrochloric acid is strongly bactericidal and that the presence of pepsin or combined acid is of no importance in this destruction. Furthermore the presence of bread increased the bactericidal power of the gastric juice to three or four times that of a similar strength of the pure acid in water. Kendall states that certain aciduric bacteria and yeasts may be found occasionally in the normal stomach.

Smithies (quoted from C. H. Mayo, 1914) examined microscopically gastric extracts from some 2400 different individuals with stomach complaint (dyspepsia, indigestion, etc.). He found that irrespective of the degree of acidity of such extracts bacteria were present in 87 per cent of the cases. After cultural studies of the saliva from dyspeptic patients he concludes that pus forming organisms have their growth retarded in gastric juice while bacilli as well as leptothrices thrive in the stomach. The degree of acidity of the extracts is not mentioned.

In investigating the flora of the gastric contents from different individuals, Fowler (1916) found besides bacteria many different yeasts.

The literature on the flora of the stomach has been confined principally to findings that have been made during pathological conditions in the stomach. The degree of acidity and the amount of free HCl present have not always been given due consideration in the determinations.

The individual (Fred V.) from whom the specimens were taken for this investigation has a complete closure of the oesophagus and a gastric fistula. The fistula is of several years standing and does not interfere with his general health which was good at the time this work was carried out. The usual source of contamination from swallowed saliva was eliminated.

Two or three hundred cubic centimeters of sterile water were injected into the stomach three or four hours after a light meal to carry off the residuum. About an hour later a specimen of psychic secretion was taken by aspirating into a sterile bottle. The free and total acidity were determined and plates immediately made. Those specimens only were considered which were clear and of normal acidity, the free acid varying between 0.15 and 0.25 per cent and the total acid between 0.20 and 0.37 per cent. All specimens of normal acidity showed comparatively the same group of organisms present. If the acidity were below normal quite a variety of organisms were contained. On the other hand it seems that relatively few bacteria are able to resist the HCl of gastric juice when in normal concentration.

The count per cubic centimeter of juice from some 20 specimens plated on glucose litmus agar plates shortly after collection varied

between 25,000 and 100,000. With the exception of no. 7, a yeast, these organisms were in relatively small numbers. However this count was reduced more than one half if the specimens were allowed to stand 24 hours at room temperature. The destruction was very marked in all organisms except the one yeast. This yeast not only resisted the acid of the gastric juice but

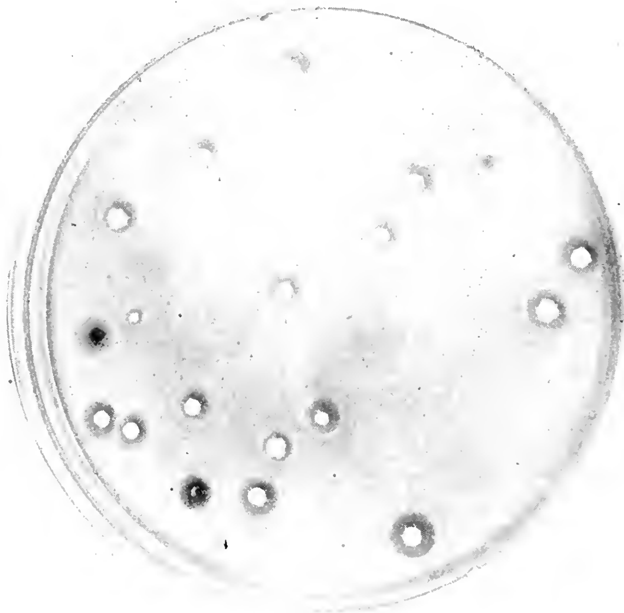

Fig 1. Photograph of a Glucose Agar Plate. This plate was made from a specimen of gastric juice diluted 1–2000 showing predominance of no. 7 by characteristic nail head appearing colonies. Specimen stood for twenty-four hours at room temperature before plating.

thrived in it. Three chromogenic organisms were among those usually found but these seemed to have no greater resistance to the gastric juice than the non-chromogenic forms present.

These findings indicate that the flora of the "empty" stomach of Mr. V. is fairly constant. In clear specimens of normal acidity

the number of different organisms is few and they are unrelated making it impossible to classify them into a definite group or groups. Extracts with acidity below normal have a much higher bacterial content and a greater variety of organisms.

Not being able at the time this work was being carried on to spend further time in the study, a somewhat incomplete description of the following organisms is made necessary:

No. 7. A yeast. Colony on glucose agar, round with rough edges made of projecting filaments; white to gray with raised center giving nail head appearance (see photograph).

Milk. No acid.

Lactose broth: flocculent precipitate after two weeks, no fermentation, no gas, no acid.

Glucose agar stab: no gas.

Glucose litmus agar: Acid formed.

Gelatin: Not liquefied.

No. 4. A yeast. Colony heavy gray on glucose litmus agar, small, acid forming.

Glucose agar slant: Heavy gray waxy growth.

Milk: Acid formed; proteolysis.

Gelatin: Liquifaction.

Lactose broth: Filaments formed; no gas.

Resembles bacterium, has granules and vacuoles; Gram positive.

No. 5. Bacillus.

Colony: Thin, gray and rather large. A short bacillus forming chains.

Glucose litmus agar: No acid.

Glucose stab: No gas.

Litmus milk: Decolorized, no coagulation.

Lactose broth: No gas, no acid, gray precipitate.

Gelatin: Not liquified, (growth at 20 degrees C.).

Non motile, spore forming, Gram positive.

No. 3. Coccus form.

Heavy colony slightly yellow on Russell.

Glucose stab: No gas, no acid.

Lactose broth: Cloudy but not much precipitate, no acid, no gas.

Milk: No acid.

Large cocci with tendency to pairs.

Gram positive.

No. 9. Coccus form.

Colony: White, rough edge made of rounded projections; large.

Grows poorly on glucose agar; grows better on plain agar slightly alkaline.

Milk: No acid until several days, when small amount is noticed.

Large cocci with tendency to bunches.

Gram positive.

No. 10. Sarcina flava.

Colony: Small, yellow, translucent.

Litmus milk: Decolorized, no coagulation.

Gelatin: Not liquified.

In cubes and bunches, not regular even when examined from broth. Lehman and Newman, p. 159; Chester, p. III.

No. 14. Boas Oppler bacillus.

No. 13. A short bacillus with some coccus forms.

Colony: Small, pink on Russell's.

Litmus milk: No acid, no coagulation.

Have not been able to identify.

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THE RELATIVE EFFECT OF PHOSPHATE-ACETATE AND OF PHOSPHATE-PHTHALATE BUFFER MIXTURES UPON THE GROWTH OF ENDOTHIA PARASITICA ON MALT EXTRACT AND CORN MEAL MEDIA

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As described in another report,[1] a mixture[2] of acetic acid and dipotassium hydrogen phosphate (K_2HPO_4) has been used in increasing the buffer effect of several media. As the writers had observed that some kinds of organisms grow upon rather weak solutions of potassium acid phthalate, it was thought well to try the latter salt instead of acetic acid as a buffer material and to test further its effect upon the growth of *E. parasitica* (Murr.) Anders. for which it was desired to use the buffered media. Although acid potassium phthalate and dipotassium hydrogen phosphate do not give as straight a buffer curve as do the acetic acid and dipotassium hydrogen phosphate, and hence have not been used in general culture work, the following results may be of value.

The media used were 2.5 per cent malt extract agar or corn meal extract agar buffered with M/50 molar K_2HPO_4 plus either M/50 acetic acid or M/50 acid potassium phthalate. The pH values of these media are about 5.7 and do not vary from each other in pH by more than 0.2. This pH is at or near the optimum for the organism, as reported in another paper,[3] but is also

[1] Paper read before the Meeting of the American Chemical Society at Philadelphia, September, 1919, and appearing soon in the Journal of Bacteriology.

[2] We also make the same mixture by using weighed quantities of pure anhydrous sodium or potassium acetate and potassium dihydrogen phosphate in proper proportions. Each salt can be sterilized separately in weighed quantities and the mixture can be made afterwards under sterile conditions.

[3] See an article appearing in the Journal of Bacteriology in March, 1920. An exhaustive report on the work on *Endothia parasitica* will appear in the near future. See Science **48**: 449–450, November 15, 1918.

in the pH range which changes very greatly between the straight parts of the neutralization curves for the first and second hydrogen ions of phosphoric acid. Our central idea in using these *buffer mixtures*[4] is to straighten out these H ion inflection curves and hence to keep the pH near the optimum by permitting only small changes in acidity by the organic acids generated by the fungi or bacteria. Acetic, phthalic, formic, malic, asparaginic, aminoacetic and other acids with ionization constants between the three constants for phosphoric acid, i.e., between 1.2×10^{-2} and 1.1×10^{-7} and 1.1×10^{-7} and 10^{-12}, accomplish the desired end excellently.

Since the co-workers of one of us[5] have shown that the activity of both ions and molecules of acids, bases and salts must be measured in all cases, and that in many reactions the nonionized molecules are even more active than the hydrogen or other ions, we shall in all cases consider that the growth curves of every organism are influenced by every ionized and nonionized substance present. In the present case, then, the better growth in the phosphate-phthalate mixture shows that the phthalate or acid phthalate anion, or the nonionized phthalic acid compounds present, must be considered better for *E. parasitica* than the acetate ion or molecular acetic acid compounds. Since, in general, the molecular forms of stronger acids have greater activities than the molecular forms of weaker acids, it will be interesting to see how stronger acids like phthalic increase (or depress) the rate of growth, or other biological factors, more than does the weaker acetic acid, for example, as in the present study. In such comparative studies the factors known to be of great im-

[4] General formulae for calculating the form of the titration curves and data for mixtures of a large number of acids and bases have been developed. These and data on the growth of organisms on a number of such regulated media will be published soon.

[5] Researches by Nirdlinger, Rogers, Shadinger, Loy, Desha, Chandler, Marshall, Johnson, Harrison, Robertson, Myers, Gruse, Shrader, Taylor, and Brown in cooperation with one of us. See Am. Chem. Jour. **39**:, 275 (1908); **49**: 116 (1913); **43**: 519; **49**: 474; **49**: 177, 369; **49**: 122, 132, 485 (1913); **48**: 374; **49**: 350, 378, 396, 403; **41**: 466. Jour. Am. Chem. Soc. **37**: 1902. Jour. Phys. Chem. **19**: 589 (1915); **20**: 365 (1916).

portance, such as the hydrogen ion concentration, must be kept constant while the unknown factors are made the variables.

EXPERIMENTAL

In the first set of comparisons made by the writers[6] on malt extract regulated with these two buffer mixtures, both media were excellent, but the phosphate-potassium acid phthalate mixture seemed to give the better growth. Mr. Hopfield then very carefully repeated the tests and found the better growth with acid potassium phthalate. In order further to verify the results, Mr. Hopfield ran two other sets of tests, side by side, using the same media given above and also media made up in the same way excepting that corn meal gruel was substituted for the 2.5 per cent of malt extract. The corn meal gruel was prepared by cooking 7.5 grams of yellow corn meal and about 200 cc. of water in a water bath at 60°C. for one hour. After allowing the meal to settle the liquid was decanted and fresh water mixed with the meal. After allowing the meal to settle again the water was decanted and added to the liquid first decanted. This liquid was used in making up 250 cc. of each of the two media after adding the proper amounts of K_2HPO_4 and acetic acid or acid potassium phthalate, and agar.

The average diametric growth, in millimeters, of four Petri dishes of each medium is given below:

Medium	*Diametric growth in mm. in 10 days at 25°C.*
2.5 per cent of malt extract, M/50 K_2HPO_4 and M/50 acetic acid..	64
2.5 per cent of malt extract, M/50 K_2HPO_4 and M/50 acid potassium phthalate	80
Corn meal with M/50 K_2HPO_4 and M/50 acetic acid	55
Corn meal with M/50 K_2HPO_4 and M/50 potassium acid phthalate	71

Thus it is very evident that the potassium acid phthalate allows more rapid growth of *E. parasitica,* although both media are excellent.

[6] The data given here were obtained during the summer of 1918.

CONCLUSIONS

1. Malt extract and corn meal extract have been buffered with phosphate-acetate mixtures and with phosphate-phthalate mixtures to straighten out the first phosphate inflection curve and regulate the hydrogen ion concentration accurately between pH = 1 and pH = 8. By using asparaginic acid and other amino-acids the acidity can be regulated accurately between pH = 1 and pH = 13.

2. It is shown that *Endothia parasitica* (Murr.) Anders. grows excellently on all of these media when regulated at optimum values close to pH = 5.7, but that the phosphate-phthalate buffer is somewhat (25 per cent) better. Furthermore the malt extract seems to give slightly (10 per cent) better growth than a corresponding corn meal medium containing the same buffers.

3. It is suggested that biologists should study the influence of all chemical species present, such as the activity of the nonionized acids, bases and salts along with the various simple and complex ions.

A METHOD OF DETERMINING THE RELATIVE TOXICITY OF SODIUM, POTASSIUM, LITHIUM, AND OTHER IONS TOWARD ENDOTHIA PARASITICA: DATA ON SODIUM CHLORIDE

M. R. MEACHAM, J. H. HOPFIELD AND S. F. ACREE

Received for publication February 27, 1920

In making a detailed chemical program in coöperation with Dr. Caroline Rumbold and in connection with her investigations[1] on the injection of trees with chemicals to bring about immunity against attack by fungi and bacteria, it was considered important to conduct a laboratory study with culture media on the relative toxicity or effect of the molecules, anions and cations of various sodium, potassium, lithium, calcium and other salts. As the lithium salts which Dr. Rumbold found most effective were not readily available during the War, the present paper deals with the toxicity of sodium chloride.[2] If a constant hydrogen ion concentration near the optimum for *Endothia parasitica* (Murr.) Anders. were used in the media and if the salts were compared on the basis of chemically equivalent quantities with a common anion, it was thought that the tests would show the relative toxicity of both the cations and nonionized molecules of each salt. Knowing the relative concentrations of the cations and nonionized form of a given salt present in decreasing quantity in a series of samples of the medium, it is easy to calculate the activity of the different ionic or molecular species from a series of simultaneous equations having the form (1) which was

[1] The injection of chemicals into chestnut trees. American Journal of Botany **6**: December, 1919.

[2] Ferdinand Wolesky stated in Papier Zeitung, **21**: 563 (1896) that 0.05 per cent solutions of sodium chloride prevent the growth of organisms on wood pulp. Such a high toxicity for sodium chloride, corresponding to the best creosotes, is not substantiated by the present work in which 75 times as much sodium chloride decreased the growth only to 25 per cent of its normal value.

(1) total activity = cation activity × cation concentration + anion activity × anion concentration + molecular activity × molecular concentration + ...

developed[3] for studying the activities of various ions and molecules in pure chemical reactions. When a given series of salts with a common anion in the same concentration is used, this equation is naturally simplified and gives the values for the specific effects of the cations and molecules.

There are two good ways to vary the cation and molecular species. The first is to use buffer mixtures having common anions but different cations. For example, the mixture of M/50 phosphoric acid and M/50 acetic acid employed as buffer material in the present study can be titrated or neutralized with sodium, potassium, lithium, etc. hydroxides to keep the hydrogen ion concentration at desired values. In such a case the ionization values of these bases and their salts are already nearly identical and the chemical aspects of the problem are much simplified. The second method is simpler in technique though slightly more complicated electrochemically, and involves adding sodium, potassium, lithium, etc., salts with common anion to the medium buffered with the same substances throughout. If the pH of the medium is to be kept absolutely constant while the concentration of one of the salts is to be varied, it is obvious that the salts used must be neutral compounds from strong acids and bases. The chlorides of the metals to be used fulfill this condition as well as any salts. Besides, the chlorides of all these metals except calcium are about equally ionized—another condition to be considered. Even this procedure involves changes in the pH or acidity, because of changing ionization of the buffer salts, but such deviations in pH are known to be small and will be corrected in our future more accurate investigations.

[3] Researches by Nirdlinger, Rogers, Shadinger, Loy, Desha, Chandler, Marshall, Johnson, Harrison, Robertson, Myers, Gruse, Shrader, Taylor, and Brown in cooperation with one of us. See Am. Chem. Jour. **39:** 275 (1908); **49:** 116 (1913); **43:** 519; **49:** 474; **49:** 177, 369; **49:** 122, 132, 485 (1913); **48:** 374; **49:** 350, 378, 396, 403; **41:** 466. Jour. Am. Chem. Soc. **37:** 1902. Jour. Phys. Chem. **19:** 589 (1915) **20:** 365 (1916).

Tests already reported[4] showed that the optimum initial pH for *E. parasitica* is about 5.0. Besides possessing the proper initial pH the medium should have a fairly strong buffer effect in the region between pH = 3 and pH = 7 in order that it may be stable and resist any marked changes in pH which might be brought about through the organic acids generated by the fungus, accidental impurities, carbon dioxide of the air, solubility of glass, etc. Accordingly 2.5 per cent malt extract medium buf-

TABLE 1*

To test the toxicity of NaCl *toward E. parasitica; 2.5 per cent malt extract* + M/50 K_2HPO_4 + M/50 *acetic acid + 1.5 per cent agar; Petri dish cultures*

CONCENTRATION OF NaCl IN MEDIUM	RADIAL GROWTH IN MILLIMETERS IN FIFTEEN DAYS AT 25°C.	CONCENTRATION OF NaCl IN MEDIUM	RADIAL GROWTH IN MILLIMETERS IN FIFTEEN DAYS AT 25°C.
0.0 Normal	†	0.257	26
0.05N Normal	†	0.322	23
0.073N	†	0.363	22
0.0975	†	0.35	24
0.121	39	0.40	20
0.145	38	0.45	20
0.167	35	0.495	19
0.19	35	0.54	12
0.212	28	0.585	13
0.235	25	0.630	15

* Data taken from notes made by Mr. Hopfield.

† Indicates dish completely covered.

fered with M/50 K_2HPO_4 and M/50 acetic acid was chosen. The acetic acid acts as a buffer to smooth out the marked change in pH found in passing from the neutralization of the first hydrogen ion to the second of the phosphoric acid, as discussed in other articles.[5] This medium was brought to a pH of about 5.0 by the addition of 0.5 cc. of normal hydrochloric acid to each 100 cc. To this medium, containing 1.5 per cent agar, was added pure sodium chloride in varying concentrations ranging from 0 to 0.63 normal. Three dishes of each salt concentration were

[4] This Journal, March, 1920. See reference 5, this article.

[5] Address before Amer. Chem. Soc., Philadelphia, September, 1919. Science **48**: 449–450, November 15, 1918. This Journal, March 1920, and unpublished articles.

poured and inoculated. Table 1 shows the average radial growth of the culture in fifteen days at 25°C. The curve sheet contains a graph prepared upon the basis of these data, and shows the decrease in growth with increase of salt concentration.

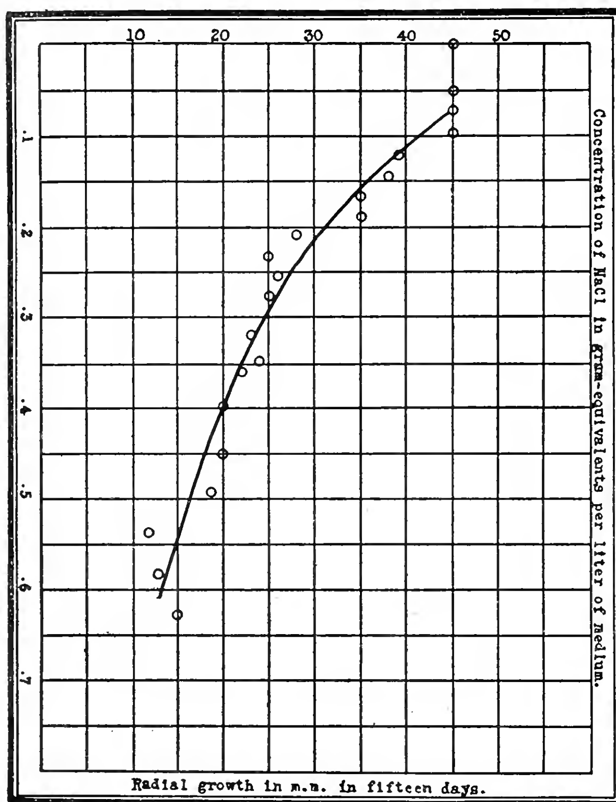

FIG. 1

It is clear from the above table that sodium chloride is only mildly toxic to *E. parasitica* and that solutions as concentrated as 0.63 N, or 3.65 per cent, decrease the growth to possibly 25 per cent of the normal value under the conditions used. Al-

though the points representing radial growth in the graph do not lie exactly on the smooth curve they are sufficiently close to it to show fairly accurately the relation between decreasing growth and increasing sodium chloride concentration. It is clear that this relation is not a straight line but one showing a decreasing ratio (decrease in growth/salt concentration) with increase in sodium chloride. As the ratio (salt ion/salt concentration) also decreases with increasing concentration of sodium chloride, the data may have some weight on the hypothesis that the ions are more active than the salt molecules. This idea should be held with reservations, however, until we can secure data on the other salts by the alternative methods discussed above and learn the specific effects of the various cations, anions and molecules.

CONCLUSION

1. *Endothia parasitica* (Murr.) Anders grows excellently on malt extract, corn meal extract and bean decoction media regulated between pH = 5 and pH = 7, and buffered with M/50 K_2HPO_4 and M/50 acetic acid to prevent the marked changes in pH produced in passing from the neutralization of the first hydrogen ion to the second of phosphoric acid.

2. In such a buffered malt extract medium regulated at about pH = 5, the addition of sodium chloride up to 0.63 N or 3.65 per cent causes a gradual decrease in the rate of growth to about 25 per cent of the normal value. This salt is therefore only mildly toxic, contrary to the statements of Wolesky.

3. The form of the growth curve suggests the possibility that the salt ions are more toxic than the salt molecules, but this conclusion should be considered tentative until extensive work on this and other salts can give the values for the toxicities of the cations, anicns and nonionized molecules of such electrolytes.

REPORT OF COMMITTEE ON DESCRIPTIVE CHART

PART II. REPORT OF PROGRESS DURING 1919

H. J. CONN, *Chairman*, H. A. HARDING, I. J. KLIGLER, W. D. FROST, M. J. PRUCHA, AND K. N. ATKINS

Received for publication January 24, 1920

Two years ago this committee recommended to the Society a new descriptive chart in the form of a folder. The folder was intended primarily for use in instruction, although it was felt that it might be satisfactory in some forms of research work. When first brought to the attention of the Society, this chart provoked much discussion, and it was finally decided that the only way to determine its value would be to print it and test it by use in a practical way. Hence the chart was not officially adopted by the Society, but the committee was directed to have it printed and to distribute it. At the present time the committee does not think it necessary to ask for official adoption of this chart. In the first place, the orders that are constantly being received for it, many of them repeat orders, have endorsed it sufficiently; and in the second place, we hope to draw up a new chart during 1920 embodying some of the suggestions that have been made while the folder has been in use. During the half of 1918 in which the folders were on sale, 10,000 were sold, and during the last year 8000—a total of 18,000. Meanwhile during these same eighteen months, only 4400 of the old charts have been called for.

The extent to which the new chart has been purchased shows that it is generally preferred to the old single page chart. This is natural, for the greatest use of the chart is made in teaching laboratories, and the folder was designed primarily for instruction. It is still in need of revision, however. Besides various matters of detail that need changing, three fundamental ques-

tions have been raised: (1) Should the chart be a folder or a single sheet (i.e., two pages or four)? (2) Shall the use of the group number be continued? (3) Shall a special space be devoted to pathogenesis? These three questions require some discussion.

TWO PAGES VERSUS FOUR PAGES

The committee has never been unanimously in favor of a four page folder. In fact some of us have always been of two minds on the subject. But enquiries made a few years ago seemed to show that nearly all the institutions then printing charts of their own for instruction purposes were using a folder. Accordingly the committee decided in favor of a folder for the instruction chart, thinking that the old card still remained to be used by those who preferred the single sheet. The old card, however, is out-of-date and calls for much unneeded information, and hence does not fill the need for a single sheet chart.

It seems, therefore, as if it would be well to replace the old card of 1914 with a new chart which would be a compromise between the two forms now in use. The experience of the last two years has shown that even research men prefer a chart considerably simpler than the old card and are using the folder to some extent, although they do not like its bulk. Hence it is not impossible that a compromise form might prove more satisfactory both to teachers and to research men than either of our present charts. It should be a single sheet of quarto size, printed on both sides, with the most important information on its face, calling for more information than the folder, but less than the old card, more concise than the folder but with more blank space for sketches and notes than allowed by the old card. If both the new chart and the folder were then put on sale, a couple of years experience would show whether there would be call for both forms or if one could supplant the other.

During the coming year the committee plans to take this matter up with all the institutions that have recently ordered our charts, and to ask the instructors whether they prefer a folder

or a one-page chart. All bacteriological instructors, therefore, who read this report, if using the charts or interested in them, are urged to communicate with the committee chairman[1] and indicate their preferences in this matter. With such information at its disposal, the committee will be able to inform the Society as to what kind of a chart is generally desired, that the Society can act intelligently in deciding whether to approve a revised chart.

THE QUESTION OF THE GROUP NUMBER

The present group number is the weakest part of the chart. It is still there in its present form simply because of the unwisdom of changing it too often and because the committee has never been able to decide what it preferred in its place. As to its value, every opinion is held among the members of the committee: from the conviction that the group number is the most important part of the chart to the feeling that it should be omitted entirely. A compromise judgment is about as follows: that the present group number may be unhesitatingly condemned; that *a* group number, calling for the right information and properly used, might be very valuable; that revision of the present form must be delayed no longer and that the new group number must be in such a form as to prevent confusion with the old one.

One reason for the disrepute into which the group number has fallen is that too much has been expected of it. By some it has been assumed that it could replace the species name, an idea not apparently held by the men who first proposed the group number. It should be regarded merely as the simplest form of concise description of an organism—a sort of short-hand notation for recording the salient characteristics. Its chief value is as an index

[1] At the meeting of the Society it was decided not to continue the committee on the chart, as such, but to appoint a new committee on bacteriological technic. This new committee is to have a somewhat changeable membership, in order that it may be composed of men doing actual work on problems of technic. Its present members are: H. J. Conn (Geneva, N. Y.), chairman, I. J. Kligler, K. N. Atkins, J. F. Norton, and G. E. Harmon. This committee is to continue the work on the chart as a part of its program.

number to be used when the charts are filed according to the characteristics of the organisms.

To avoid further misconception and to emphasize this particular use of the number, the present plan is to call the new form an index number instead of a group number; to drop the generic symbol before it and to put it in such a form that confusion with the old group number will be impossible; and to see that all the information called for in the index number is similarly recorded elsewhere on the chart so that the description will be complete if the student decides not to use the index number. By doing this it is hoped to meet all the criticism that has been directed against the group number.

SPACE FOR RECORDING PATHOGENICITY

Various cards have been devised with space for recording the pathogenicity of an organism. Of these the best have merely the heading pathogenicity followed by a blank space. This feature can very easily be included on the new chart, although this space would be of no use in the case of saprophytic organisms. On the other hand, non-pathogenic organisms generally must be investigated by special tests for which blank space would be desired; and it seems a waste to leave separate blank spaces for both sets of tests. For this reason, the folder has an entire page blank for notes, the idea being that it might be used to record pathogenicity or the special tests made for non-pathogenic organisms. As this use of the space does not seem to be understood by all users of the chart, it is proposed on the revised chart to have a space headed something like this: "Special Reactions and Environmental Relationships (e.g., Pathogenesis)."

METHODS

When the 1914 card was prepared, the glossary and notes were omitted from the back of it on the understanding that a pamphlet was to be drawn up by the committee giving a glossary of terms used on the chart and methods to be employed in making the tests. After some unavoidable delay this pamphlet was finally

prepared and was printed in the Journal of Bacteriology as the report of this committee for 1917. One copy has been furnished with every order of 100 or more charts, and additional copies have been on sale at about cost price. More detailed information as to the acid and nitrate tests was given in the 1918 report, of which, also, copies are on sale. The edition of the 1917 report is not so nearly exhausted that it has been reprinted in slightly revised form as Part I of this report in the last number of this Journal.

Among the special investigations on methods which the committee has undertaken is a study of the Gram stain, and the following paper is the result. This modification of the Gram stain looks very promising because of the stability of the solutions. The committee is recommending it for provisional use, but is not endorsing it until it has been more thoroughly tested. Of the more commonly used methods, the Stirling technic[2] seems to give the most satisfactory results, and can be safely recommended for general use until the value of this more recent technic has been determined by practical use.

[2] Method 1 described in Committee Report for 1918, J. Bact. **4**, 113.

REPORT OF COMMITTEE ON DESCRIPTIVE CHART

PART III. A MODIFICATION OF THE GRAM STAIN

KENNETH N. ATKINS

Dartmouth Medical School

The aniline gentian violet staining solution of the Gram stain is not stable, usually becoming obviously decomposed in a few weeks. One is occasionally apt to use a stain which is too old to give accurate results. The diversity of procedure in using this stain, as noted in textbooks of bacteriology, is doubtless an outgrowth of attempts to make the stain more useful and consistent in its action. The use of a stable staining solution would tend towards uniformity of procedure and accuracy of results. The writer does not wish to introduce a new stain, to be used in place of the Gram stain (Gram, 1884), but rather, to modify that well known stain so that it will be stable while its chemical properties remain unchanged.

The stain is as follows:

Staining solution

Saturated 95 per cent alcohol solution gentian violet........... 1 part
Aniline sulphate, 1 per cent aqueous solution.................. 3 parts

Iodine solution

Iodine.. 2 grams
Normal solution sodium hydroxide........................... 10 cc.
Water.. 90 cc.
(Dissolve the iodine in the sodium hydroxide solution and add the water.)

Decolorizing solution

Alcohol.. 95 per cent

Technique

The slide is prepared as usual and stained for one minute. Wash briefly to remove the excess of stain and apply iodine solution for one minute.

Wash thoroughly before decolorizing in alcohol for two minutes or until the alcohol is no longer colored. In thick smears, as pus, the decolorization may be continued for ten minutes.

Wash. Counterstain. Wash, dry and mount.

In this proposed modification aniline sulphate is used in place of aniline and sodium hydroxide is added to the iodine solution. Otherwise there is no change in the solutions. Aniline sulphate is a stable salt, both in its dry form and in solution. The aniline-sulphate-gentian-violet stain should keep indefinitely. The instant that the modified iodine solution is placed on the slide preparation, stained with aniline-sulphate-gentian-violet, the aniline sulphate is decomposed and free aniline liberated. The effect then, is the staining of the bacteria according to Gram's classical method with a solution that is prepared as used.

The addition of a weak solution of sodium hydroxide to iodine causes the formation of sodium-hypoiodite. In the solution above, the amount of sodium hydroxide is not sufficient to convert all of the iodine to sodium-hypoiodite and leaves an excess of iodine in solution. The amount of free iodine present is about the same as that present in the iodine solution of the Gram stain. This solution contains sufficient hydroxyl-ion concentration to set free aniline from aniline sulphate.

Other aniline salts were tried, such as aniline acetate, oxalate, nitrate and hydrochloride. The aniline acetate was discarded because of its instability. Difficulty was experienced in the use of aniline nitrate and aniline hydrochloride. The bacteria did not take the color of the gentian violet properly and the finished preparation was decidedly lacking in brilliancy. Apparently there was a decided difference in the penetrating power (if the term may be properly used), of the aniline salts. Aniline-oxalate-gentian-violet seemed to penetrate, i.e., stain the bacterial cells in the primary step of the Gram stain, fully as well as aniline-gentian-violet. Aniline-sulphate-gentian-violet also penetrated well but specimens stained with aniline hydrochloride and aniline-nitrate-gentian-violet and washed, dried and examined before the addition of the iodine solution, appeared a light purple color instead of a dark purple. This suggests the testing

of the more uncommon aniline salts with the hope that one may be found which will be entirely stable and have great penetrating power.

As an alternative method, aniline oxalate may be used in place of aniline sulphate in this modified stain. This salt seems to have slightly better penetrating power than the aniline sulphate and has the advantage that staining may be accomplished in thirty seconds instead of one minute. This stain was kept for six weeks in the sunlight out doors and in the incubator for two months and no change could be detected in its staining qualities. It is thought, however, that the aniline oxalate stain would not be as stable as the aniline sulphate stain because aniline oxalate very slowly decomposes in solution, liberating free aniline, while aniline sulphate solution is stable.

The aniline-sulphate-gentian-violet stain as here outlined was tried on various kinds of bacteria and in all cases the results were similar to those obtained with the Gram stain. Control slides of Gram positive organisms made according to the usual Gram method, showed the bacteria slightly less densely stained by this modified method. This difference was no greater than the difference detected in a comparison of slides made with American and Gruebler dye-stuff in the customary manner. Gram negative bacteria with this modified stain seem to be more nearly colorless than the controls made by the Gram method. The degree of difference between a Gram negative and a Gram positive stain seems to be entirely comparable in the two methods. Different strengths of solutions, imported and domestic dye-stuff and various time elements of staining, washing and decolorizing were tried and there seemed to be a fairly wide range of limits, with the production of apparently the same result in the finished specimen. Two points should be noted; first, the primary washing of the slide after staining with aniline-sulphate-gentian-violet should be brief (a long washing of more than half a minute tending, on account of the solubility of the aniline salt, to reduce the brilliancy of the preparation); second, the modified iodine solution should be left in contact with the slide for one min-

ute. With these two exceptions the times of staining and washing may be considerably increased or decreased with little or no apparent change of result.

The writer has received from Prof. C. E. Bolser, of the Chemistry Department of Dartmouth College, important suggestions in this work and desires to express his indebtedness to him.

REFERENCE

Gram, C. 1884 Fortschr. d. Med., **2**, 185.

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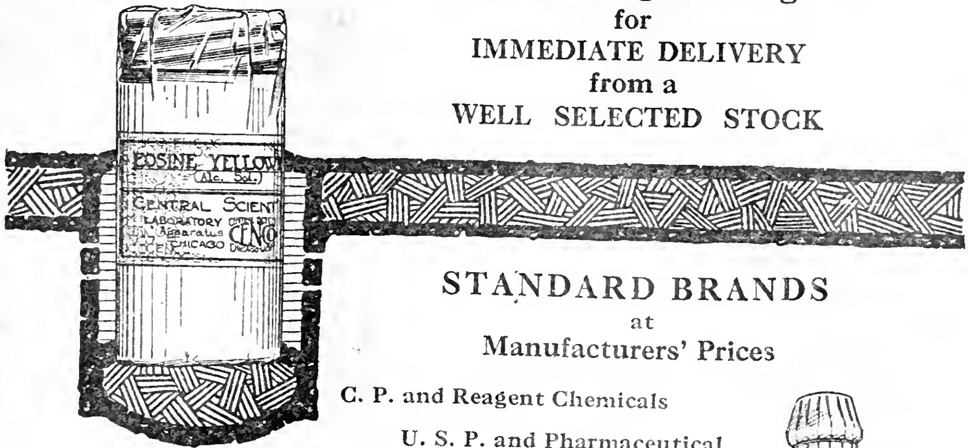

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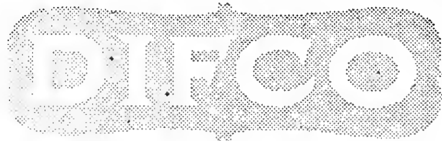

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JULY, 1920

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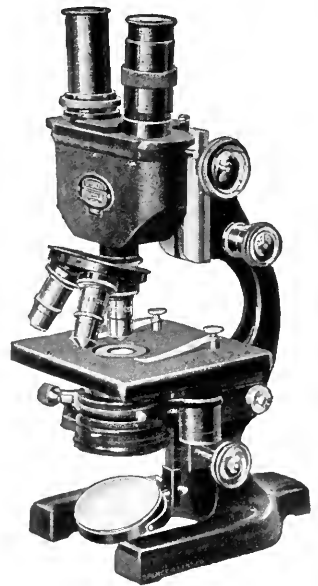

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FURTHER STUDIES ON THE GROWTH CYCLE OF AZOTOBACTER[1]

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At the 1912–1913 meetings of the Society of American Bacteriologists the writer read a paper entitled "A Morphological and Cultural Study of Some Azotobacter," using a series of lantern slides of photomicrographs for illustration. The paper was subsequently published in the Centralblatt für Bakteriologie, 1913, and in the "Transactions of the Royal Society of Canada" of the same year.

Among the observations made in this paper was one regarding the formation in azotobacter cells of two types of granules, one type being stainable, and the other non-stainable, with various bacterial stains. The type that was stainable appeared to represent reproductive bodies or gonidia which on disintegration of the mother cell were liberated, after which they grew into normal azotobacter cells and reproduced by fission for a time until later the cells became granular and again disintegrated with the liberation of reproductive granules. The formation of these reproductive granules with their liberation, on the disintegration of the mother cell, appeared to the writer to constitute a new type of multiplication for azotobacter distinct from the usual method of fission characteristic of bacteria in general. As no reference to such a method of multiplication could be found in the literature, the observations were repeated a considerable number of times to verify the above conclusions.

In the Centralblatt for 1914 the writer published another short article entitled "Further Studies with Some Azotobacter."

[1] Presented before the Society of American Bacteriologists, December 29, 1919.

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This dealt with the viability, thermal death point and formation of involution forms in four varieties of azotobacter under observation.

The studies with these strains of azotobacter were continued for a short time longer, attention being directed more particularly to the disintegration of mother cells with the liberation of the gonidia-like granules and their subsequent development, and numerous photomicrographs were taken of these phenomena. Unfortunately, these studies were interrupted in the latter part of 1914 and were not resumed until the summer of 1919.

In the meantime Löhnis and Smith published in the Journal of Agricultural Research, July, 1916, a remarkable paper entitled "Life Cycles of the Bacteria." In this article the authors state that the life cycle of many, if not all, species of bacteria is much more complex than has been generally considered to be the case. The theory which they advance is that

> All bacteria studied live in an organized and in an amorphous stage. The latter has been called the "symplastic stage," because at this time the living matter previously enclosed in the separate cells undergoes a thorough mixing either by a complete disintegration of the cell wall, as well as cell content, or by a melting together of the content of many cells which leave their empty cell walls behind them. In the first case a stainable, in the latter case an unstainable symplasm is produced.
>
> According to the different formation and quality of the symplasm the development of new individual cells from this stage follows various lines. In all cases at first "regenerative units" become visible. These increase in size, turning into regenerative bodies, which later, either by germinating or stretching, become cells of normal shape. In some cases the regenerative bodies also return temporarily into the symplastic stage.
>
> Beside the formation of the symplasm another mode of interaction between the plasmatic substances in bacterial cells has been observed, consisting of the direct union of two or more individual cells. This conjunction seems to be of no less general occurrence than the process first mentioned.

Whilst Löhnis and Smith base their conclusions on many observations which they made on cultures of various species of bacteria including such diverse forms as *B. subtilis* (*Pseud.*)

fluorescens, Bact. pneumoniae, Sar. flava, Strep. lactis, Lactobacillus bulgaricus and others, they use the azotobacter life cycle more than any other to demonstrate the truth of their conclusions. They give a diagrammatic representation of the life cycle of azotobacter with drawings of the many types of cell structures observed and suggest how these forms may be related to one another. A number of photomicrographs are also presented to the same end in the article.

Though the writer has not so far observed the complexity of the life cycle in any other species of bacteria than the azotobacter, he has observed it in this species, to a greater or less extent, as pointed out in the articles previously referred to, published in 1913 and 1914. He finds, however, that while he agrees with some, he cannot agree with all the conclusions arrived at by Löhnis and Smith with regard to azotobacter.

SPORE FORMATION BY AZOTOBACTER?

In the first place Löhnis and Smith refer to azotobacter as a heat-resistant endospore-forming bacillus, and point out that another investigator, Mulvania, reports the presence of heat-resisting spores in azotobacter. Further, they state that while other investigators failed to report the finding of resistant spores in azotobacter, "They undoubtedly would have found them by a more thorough search." Löhnis and Smith observed two types of spore-forming rods in the complex cycle of the azotobacter, one a small rod and the other a large rod. They also observed non-spore-forming rods both small and large, but neither of these ever developed directly into spore-formers, although the small spore-forming rod sometimes developed into the large spore-forming rod.

They imply that the faculty of heat-resistant endospore production is not constant in the azotobacter as only about 50 per cent of their cultures possessed it and most of these had developed it only after being kept in the laboratory for a number of years.

When the writer was isolating from the soil the varieties of azotobacter which he studied, he frequently found spore-forming rods, both large and small, cropping up in his cultures

and subcultures. But after a prolonged series of alternate replatings and flask cultivations, these spore-forming rods appeared to be eliminated. It was a comparatively simple matter to isolate the spore-forming rods from the azotobacter, but very difficult to isolate the azotobacter from the spore-forming rods. The spore-forming rods on subsequent cultivation proved to be other species than azotobacter.

As stated in the article "Further Studies of Some Azotobacter" previously mentioned, the writer tested the thermal death point of four varieties of this organism from a series of cultures ranging in age from sixteen days to two years and two months, and in every case the cultures heated to 65°C. or over, failed to give any subsequent growth.

In July, 1919, the stock cultures of azotobacter on Ashby's agar that had remained untouched since 1914, or earlier, were again tested for thermal death point in the recognized manner, except that 10 cc. of Ashby's solution was used instead of water or bouillon in the test tubes that were heated. In every case a generous loopful of the culture was transferred to the test tubes, which, on being stirred in, was not thoroughly broken up, as a considerable number of macroscopic particles of culture remained unbroken, thus favoring resistance to heat on the part of the organisms constituting these particles. Again all cultures heated to 65°C. and over failed to give any subsequent azotobacter growth, while all controls gave good azotobacter development. Thus it was concluded that these four varieties of azotobacter even in cultures that had been kept for four years at room temperature, did not produce heat-resistant endospores. However, on plating out on beef peptone agar from these stock cultures, some of them produced colonies of encapsulated spore-forming rods both large and small. These on isolation and subculture did not develop azotobacter characteristics. On Ashby's agar or in Ashby's solution some would not grow at all and others only to a very limited extent, while on beef media they made good development. It was therefore concluded that the resistant spore-formers, which were found present only in very limited numbers and not in all the cultures tested, were contaminations.

SYMPLASTIC STAGE

With regard to the theory of the symplastic stage in the life cycle of azotobacter described by Löhnis and Smith, our observations of the four varieties of azotobacter studied lead us to conclude that there are good grounds for accepting the theory. Such clusters of cells referred to by Löhnis and Smith as "symplasm" have been observed by us in various stages from clusters in which the individual cells were well defined to clusters in which it was difficult to distinguish individual cells but in which minute granules were present in considerable numbers, evidently corresponding to the reproductive granules of Löhnis and Smith. These granules were readily responsive to some stains, particularly Heidenhain's iron haematoxylin, which stains them black, and Neisser's blue, which stains them dark blue, and makes them quite distinct from the surrounding substance. In other masses of "symplasm" these granules were larger in size and were assuming the appearance of individual cells, and in still others they had become small individual azotobacter cells, multiplying by fission.

Previous to the publication of Löhnis' and Smith's symplastic theory, we had considered these clusters to be mechanical agglomerations of cells, their association being not vital but accidental, and the formation of the reproductive granules with their subsequent liberation being identical with that which we described in 1914 as occurring in individual azotobacter cells.

However, from the observations that we have made this year in connection with our cultures of azotobacter, we have come to the conclusion that there are good grounds for accepting Löhnis' and Smith's theory regarding the fusion or mixing together of the protoplasm of those cells which constitute these symplastic clusters. We have observed these symplastic masses in stained preparations from cultures ranging in age from a few days to several months, on beef peptone agar, Ashby's agar and in Ashby's solution, and, notwithstanding the fact that at first we were antagonistic to the theory, we were finally led to accept it by our repeated observations.

CONJUNCTION OF AZOTOBACTER CELLS?

With regard to the claim put forward by Löhnis and Smith that "In addition to the formation of symplasm another mode of interaction between the plasmatic substances in bacterial cells occurs, consisting of the direct union of two or more cells, which conjunction seems to be of no less general occurrence than the process first mentioned," we cannot altogether agree.

In support of their contention Löhnis and Smith present a number of photomicrographs of stained azotobacter preparations. In studying these photomicrographs and comparing them with our own preparations we come to the conclusion that what is here referred to as conjunction of two individual cells is rather the incomplete fission of individual cells in process of division. This process has been observed by us repeatedly, not only in stained preparations, but also in living cultures on agar hanging block, and in hanging drop cultures in moist chambers inoculated from cultures varying in age from one day to several months. In none of these did we observe two cells unite in conjunction, but many times have we observed fission take place in which the cells presented an appearance similar to that shown as "conjunction" in the pictures of Löhnis and Smith.

INVOLUTION FORMS

Löhnis and Smith object to the use of the generally accepted term "involution forms" as applied to those aberrant or abnormal, usually enlarged swollen forms of bacteria that are liable to occur in cultures of most species of bacteria when grown under varying conditions. They contend that "The development of the bacteria is characterized not by the *irregular* occurrence of more or less *abnormal* forms but by the *regular* occurrence of many different forms and stages of growth connected with each other by *constant relations*."

The term "involution forms," the writer understands, is applied to bacterial cells that have assumed swollen, bladder-like, elongated or irregular forms as a result of variations in

temperature or changes in the culture media in which they are growing, and that cells of this type are weakened or degenerated and sometimes devitalized. This being the case, the writer finds that with azotobacter, in addition to there being a "regular occurrence of many different forms and stages of growth connected with each other by constant relations," there is also an irregular occurrence of more or less abnormal forms which merit the term "involution forms." These forms, in great variety of size and shape, are fairly common in old cultures (one to two months) of azotobacter grown in Ashby's solution or on Ashby's agar at room temperature, but when cultures are incubated at 37°C., they become numerous in a few days. Numerous Ashby's agar hanging block cultures in moist chambers were made by the writer in which various involution forms were included in the inoculum. The development of these cultures was observed under the oil immersion lens and in only a very small percentage of cases did the involution forms show any tendency to develop or reproduce. In most cases the involution forms remained dormant either until they were overgrown by colonies of azotobacter developing near them from normal cells included in the inoculum, or until the cultures gradually dried out. Some of these moist chamber cultures were held under observation for fourteen days, sterile water being added to the chamber from time to time to prevent, as long as possible, the drying out of the culture.

In those cases where the abnormal forms reproduced, the process of reproduction at first more closely resembled budding than ordinary simple fission. Irregular rounded projections would develop, apparently from any part of the cell, and in course of a few hours abstriction of these projections would take place, the abstricted portions then proceeding to develop and reproduce by fission.

SUMMARY AND CONCLUSIONS

1. The four varieties of Azotobacter studied by us, which were isolated from garden soil at Guelph, Ontario, in 1910, and were reported on in 1913 and 1914, have a complex life cycle.

2. Individual cells following a period of reproduction by fission, may develop reproductive granules or gonidia, within their cell plasma, which, on disintegration of the mother cell are dispersed, increase in size, become typical azotobacter short rods, ovals or spheres, and reproduce by fission. The young cells are motile.

3. The reproductive granules vary in size, some being very minute. Attempts to pass them through a Berkfeld filter were not successful. They are positive to Neisser's blue and to Heidenhain's iron haemotoxylin stains.

4. Another type of granule, not reproductive and not stainable, possibly composed of reserve food substance, is found associated with the reproductive granules in the mother cell.

5. A "symplastic stage" as described by Löhnis and Smith, has been observed in cultures varying in age from a few days to several weeks. In this symplastic stage aggregations of cells coalesce, the cell walls appear to break down and the plasma of the various cells intermingles, with the resultant production of regenerative granules varying in size from very minute bodies, scarcely discernible with the oil immersion lens, to larger forms that are readily visible when stained. Neisser's blue is a good stain for showing up these granules. On emergence from the "symplasm" these granules grow into young azotobacter cells and reproduce by fission.

6. Varieties 1 and 2 in cultures up to fourteen days old on Ashby's agar produce large capsules; variety 3 produces only small capsules and variety 4 produces no capsules.

7. All four varieties are motile in young cultures on Ashby's agar or in Ashby's solution.

8. In cultures more than fourteen days old, large, spherical, thick-walled cells are common. In varieties 1, 2 and 4 these occur in irregular groups; in variety 3 they occur in tetrads and sarcina packets. These appear to be resting cells or arthrospores, as at this stage multiplication by fission appears to have ceased for the time being. On transference to fresh media these thick-walled cells germinate, the cell plasma emerging from the thick wall as a large rod which at once proceeds to multiply by fission; the young cells are motile.

9. Heat-resistant endospores are not produced.

10. Involution forms varying much in size and shape occur commonly in cultures more than fourteen days old, in Ashby's solution or on Ashby's agar at 25°C. They are particularly numerous when cultures are grown at 37°C.

11. Some involution forms appear to multiply to a very limited extent by a budding process.

PLATE 1

Fig. 1. Azo. 1. Smear from a twenty-four hour culture on Ashby's agar at 25°C., inoculated from a seven-days old culture in Ashby's solution in which rapid growth was occurring and many cells were disintegrating. Smear shows very young cells presumably developed from the regenerative granules dispersed by disintegrated mother cells. Central corpuscles, possibly nuclear bodies, are noticeable in most of the young cells and fission in all stages is apparent; cells were actively motile. Stained with saturated alcoholic gentian violet. × 1000.

Fig. 2. Azo. 1. Cells showing flagella from same culture as figure 1, stained with Moore's modification of Loeffler's flagella stain. × 1000.

Fig. 3. Azo. 4. Resting cells or arthrospores germinating. Preparation in Lugol's solution from a twenty-four hour culture on Ashby's agar at 25°C., inoculated from a one-month old culture in which many resting cells were present. The dark, granular irregular spherical bodies are the resting cells and the light-colored rods are from the germinated resting cells. × 1000.

Fig. 4. Azo. 3. Smear from a four-day old culture on Ashby's agar at 25°C., showing fission first in one direction, then at right angles, giving tetrad forms common with this variety. Stained Neisser's blue. × 1000.

Fig. 5. Azo. 1. Smear from culture on Ashby's agar, six days old at 25°C., showing capsule formation and development of cells in many stages from granules dispersed from disintegrated mother cells. Granular mother cells also in evidence. Stained with saturated alcoholic gentian violet. × 1000.

Fig. 6. Azo. 2. Smear from culture fourteen days old on Ashby's agar at 25°C. showing resting cells or arthrospores. Stained with saturated alcoholic gentian violet. × 1000.

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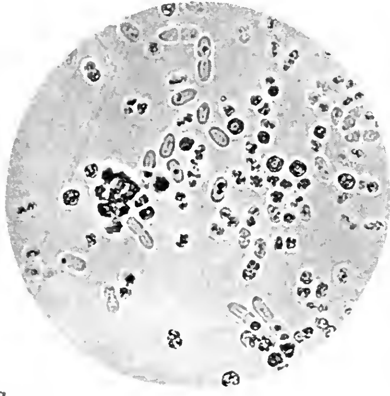

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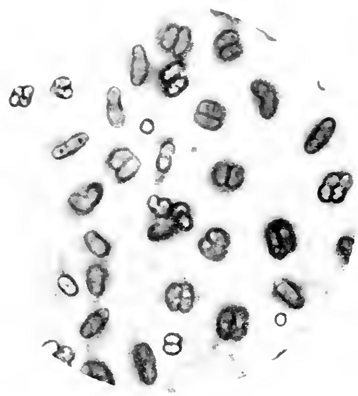

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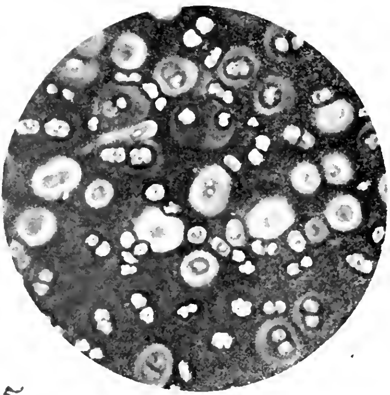

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(Jones: Studies on Growth Cycle of Azotobacter)

PLATE 2

FIG. 1. Azo. 1. Smear from culture one month old in Ashby's solution, showing to the left two mother cells disintegrating, the liberated reproductive granules developing into short rods before dispersal. Stained with Heidenhain's iron hematoxylin. × 1000.

FIG. 2. Azo. 1. Smear from culture fourteen days old on Ashby's agar at 25°C., showing granular mother cells disintegrating. Stained with saturated alcoholic gentian violet which stains the capsule material, forming the background, leaving the organisms mostly negative to the stain. × 1000.

FIG. 3. Azo. 2. Similar preparation to figure 1 from culture of Azo 2 of same age. × 1000.

FIG. 4. Azo. 4. Smear preparation from a culture in Ashby's solution one month old at 25°C., showing various cell forms, particularly dispersed reproductive granules in various stages of development into young cells multiplying by fission. Stained with Heidenhain's iron hematoxylin. × 1000.

FIG. 5. Azo. 4. Similar preparation to figure 4 from culture one month old in Ashby's solution showing young rods and spheres developing from liberated reproductive granules, also small clusters of resting cells.

FIG. 6. Azo. 3. Smear from a twenty-hour old streak culture on Ashby's agar. Most of the cells shown are very young ones developing from dispersed reproductive granules and multiplying by fission. Stained with Neisser's blue. × 1000.

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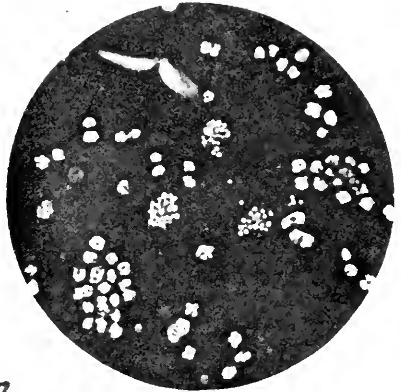

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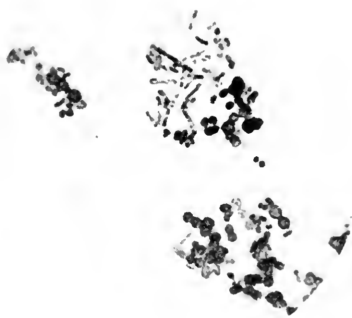

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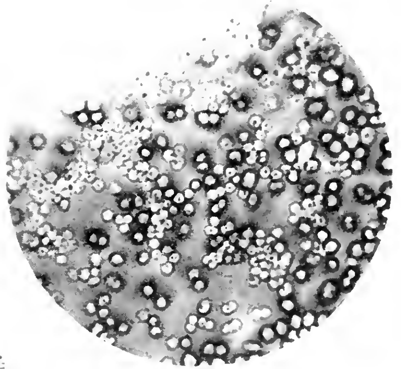

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(Jones: Studies on Growth Cycle of Azotobacter)

PLATE 3

Fig. 1. Azo. 4. Smear from growth five days old on Ashby's agar at 25°C., showing various sized rods positive to the stain and granular spheres mostly negative to the stain, also a cluster of cells probably entering the symplastic stage described by Löhnis and Smith. Stained Heidenhain's iron hematoxylin. × 1000.

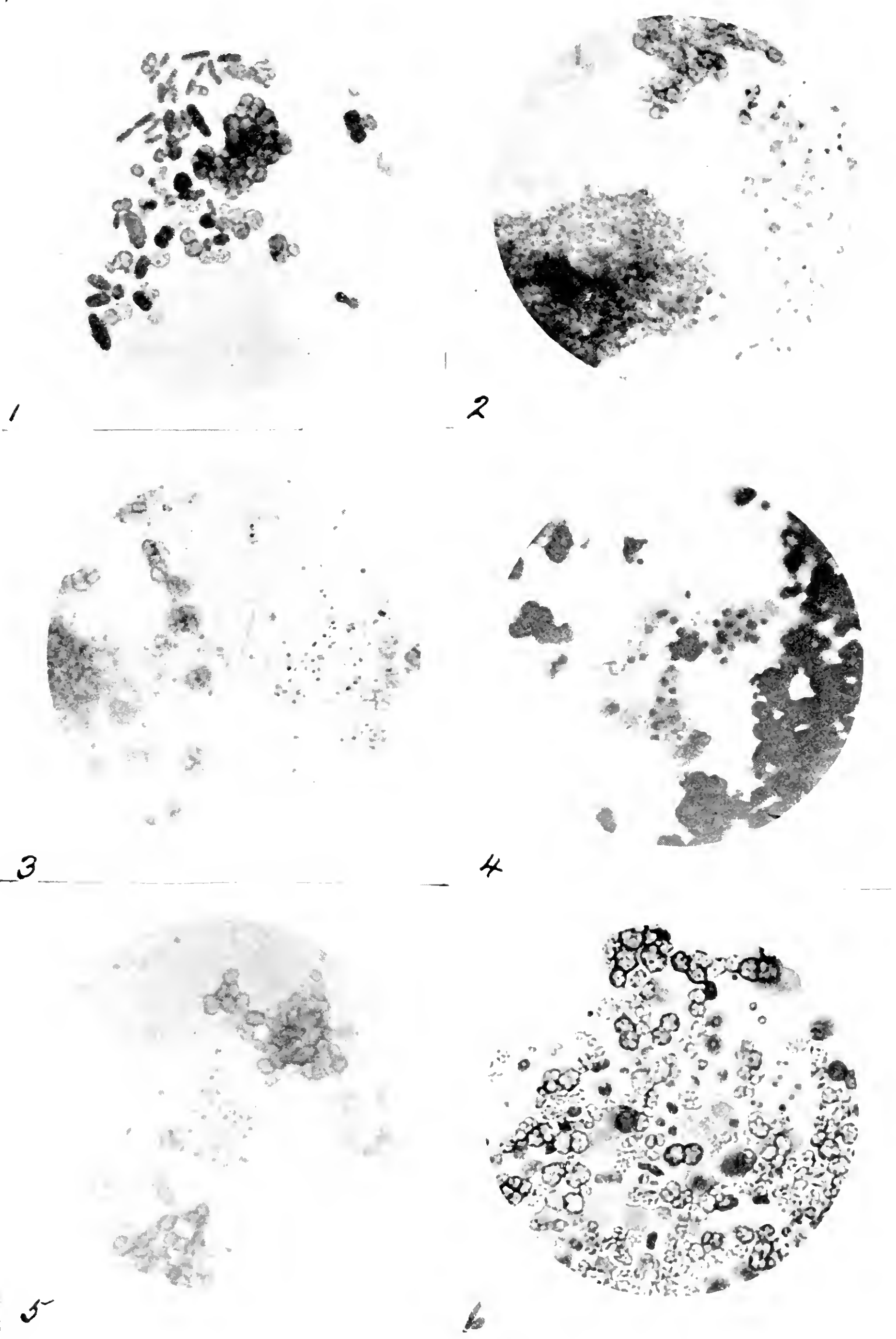
Fig. 2. Azo. 4. Smear from culture sixteen days old in Ashby's solution at 25°C., showing at the top a portion of a mass of cells entering the "symplastic" stage; at the bottom a portion of a mass of cells in an advanced "symplastic" stage where apparently the cells have fused and the reproductive granules are coming into evidence; at the right hand side a number of scattered young cells developed from reproductive granules liberated from another mass of "symplasm." Stained Neisser's blue. × 1000.

Fig. 3. Azo. 3. Smear from a streak culture three weeks old on a beef extract agar plate inoculated from an Ashby solution culture seven days old, showing a few tetrad forms of resting cells and to the left a portion of a "symplasm" in an advanced stage; to the right numerous minute reproductive granules escaped from the symplasm. Around each individual strongly stained granule is a zone of plasma negative or only slightly positive to the stain, clearly discernible in the smear but not showing up well in the photograph. Stained Neisser's blue. × 1000.

Fig. 4. Azo. 3. Smear from colony one month old on beef extract agar showing darkly stained clusters of resting cells also clusters of young cells developed from reproductive granules from disintegrated mother cells. Stained Kutscher's gentian violet; × 1000.

Fig. 5. Azo. 4. Smear from sixteen days old culture in Ashby's solution 25°C., showing two aggregations of cells probably entering "symplastic" stage and in the center a mass of young cells developed from regenerative granules. Stained Neisser's blue. × 1000.

Fig. 6. Azo. 3. Smear from a colony six days old on Ashby's agar at 25°C., showing granular mother cells disintegrating, dispersal of granules and various stages in development of reproductive granules into young cells multiplying by fission. Stained with saturated alcoholic gentian violet. × 1000.



(Jones: Studies on Growth Cycle of Azotobacter)

PLATE 4

Fig. 1. Azo. 2. Smear from culture seven days old on Ashby's agar 25°C., showing large irregular amoeboid cells, varying in density of protoplasm, also some granular forms; stained with Heidenhain's iron hematoxylin. × 1000.

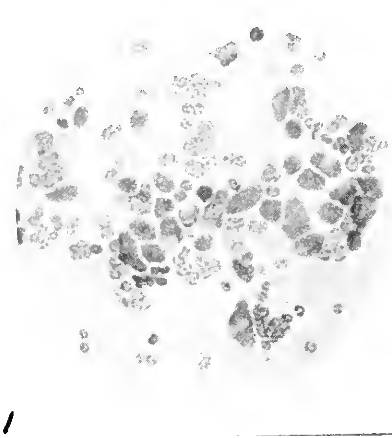
Fig. 2. Azo. 2. Smear from culture on Ashby's agar two days old at 37°C., showing darkly stained granules within the cells; stained Neisser's blue. × 1000.

Fig. 3. Azo. 3. Smear from culture on Ashby's agar two days old at 37°C., showing large granular cells apparently budding. Stained Neisser's blue. × 1000.

Fig. 4. Azo. 1. Large motile cells that were observed to break away from edge of colony four days old growing on agar hanging block in warm stage moist chamber, and swim around for twenty minutes or so in water of condensation accumulated at edge of colony. Photographed in living condition when quiescent. × 1000.

Fig. 5. Azo. 3. Smear from culture seven days old on Ashby's agar at 25° C., showing large granular cells and some involution forms with many small granules, positive to stain. Stained Neisser's blue. × 1000.

Fig. 6. Azo. 1. Smear from culture seven days old at 37°C. on Ashby's agar showing various elongated involution forms, some granular and some with homogeneous protoplasm. Stained with Neisser's blue. × 1000.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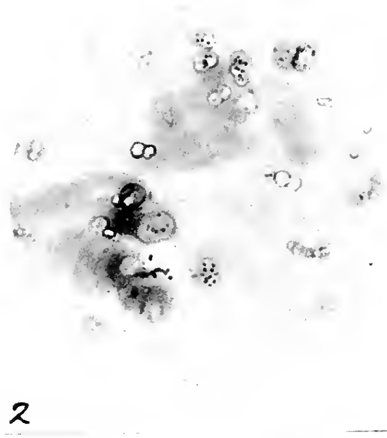
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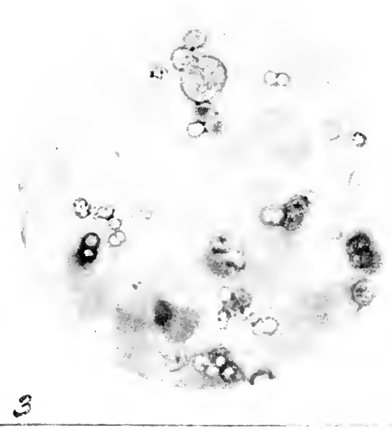
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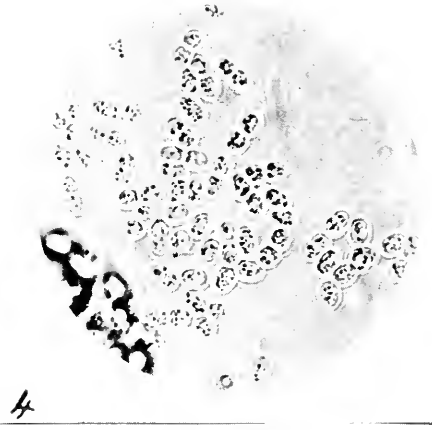
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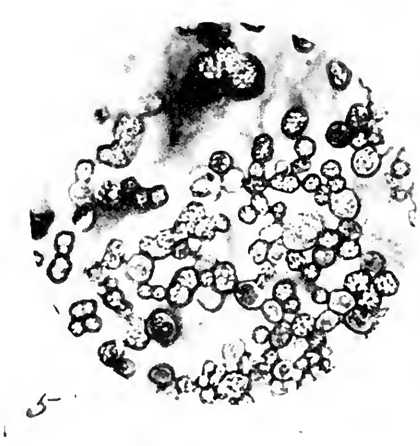
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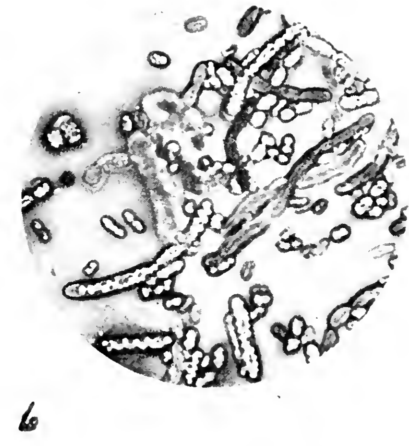
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(Jones: Studies on Growth Cycle of Azotobacter)

THE DIAGNOSIS OF ANTHRAX FROM PUTREFYING ANIMAL TISSUES[1]

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Received for publication December 30, 1919

Of all infectious diseases of animals there is none easier to diagnose bacteriologically, when the material is fresh, than anthrax. In infected tissue, *B. anthracis* is always numerous and present in practically pure culture. This fact, together with the vigor of its growth on the simplest kinds of culture media, make its detection very simple. When the suspected material has undergone a certain amount of putrefaction, however, conditions are changed and difficulties arise. These difficulties are due to two things, viz., first, to the multiplication of a number of species of organisms which resemble the anthrax organism so closely as to cause difficulty in their differentiation; second, to the diminution in the numbers of the anthrax organisms present, this diminution being carried on progressively, as putrefaction proceeds, to the point of complete extinction.

The first difficulty has been satisfactorily overcome but the second cannot be, so long as putrescible tissues are shipped for considerable distances before examination. In such tissues the anthrax organism is usually prevented from sporulating because of lack of sufficient oxygen. In this stage it is quite rapidly destroyed by putrefaction, and, undoubtedly, in a considerable number of positive cases, the evidence is destroyed before the tissue is examined. Methods of sending material which would provide favorable conditions for the anthrax organism to sporulate as well as to reduce the moisture to a minimum, thus preventing the growth of saprophytic organisms, are perfectly feasible and

[1] Presented before Society of American Bacteriologists, December 30, 1919.

practicable but for some reason the practice of submitting putrescible animal tissues for examination has become a habit. A small piece of cotton string soaked in blood or spleen juice, placed in a clean dry bottle and shipped to a laboratory would furnish excellent material for the bacteriologist. The string would slowly dry out allowing any anthrax bacteria which might be present to sporulate. The dry string would then remain in satisfactory condition for bacteriological examination for months. This method is not used, however, and even if used, in a small number of cases putrefaction would have to be dealt with because of the material having been taken from the body of the animal after putrefaction had begun.

My experience with the problem has been in a diagnostic laboratory where animal tissues are received from various parts of the state of New York for examination. Many of the regions are somewhat isolated from the laboratory so far as rapid carrier service is concerned and in a considerable number of cases the material is not properly packed. As a consequence, we have considerable tissue to handle which has partially putrefied.

Tissues received for examination for anthrax consist mostly of blood, pieces of spleen, and ears. Of these, ears are received most often and are, on the whole, the most satisfactory tissue received. To procure an ear it is not necessary to open the carcass, thus spreading infection on the premises by liberating anthrax organisms if they are present, and since the ear contains so little soft tissue it will resist putrefaction much longer than other parts. If the weather is not too hot and the journey is not over twenty-four to forty-eight hours long, ears usually arrive in a satisfactory condition without ice. Many are received, however, which have been in transit for several days up to a week and these are always more or less putrefied. Blood samples, pieces of spleen and other organs almost invariably arrive in bad condition, if sent without ice (and this is frequent) and in not a few cases, even when well iced, because of having been delayed in transit.

Accompanying the specimen or frequently in advance of the arrival of the specimen, a telegram or letter is received requesting

a prompt reply. In many cases the health of other animals is at stake and sometimes it is the health of some of the people who have had to do with the care of the animal while sick or after its death. The element of time required in making a diagnosis is therefore of considerable importance.

LABORATORY PROCEDURE

A smear is first made from the sample of blood, spleen, pulp or blood from a subcutaneous ear vein as the case may be. This is stained with any of the common stains and examined immediately. In case the material is fresh and the case is positive the anthrax organisms will be found in abundance. In these cases the McFadyean reaction works beautifully. If the material has putrefied to any extent, organisms which closely resemble the anthrax bacterium are usually present. There may be several kinds of these organisms in a single sample. In addition to these there are nearly always present some larger organisms of a rod shape and having a slight resemblance to *B. anthracis,* and various organisms of other kinds. I have not had success with the McFadyean reaction in picking out anthrax organisms from such a mixture and the morphology can be taken as of no greater significance than as a suggestion of anthrax.

Cultures are made on plain agar plates. A small bit of tissue or a drop of blood is placed in a tube of bouillon and shaken. From this dilution, a loopful is smeared over the surface of one or more agar plates, using a bent glass rod as a spreader. If the material is relatively fresh and the number of bacteria does not appear to be great, the dilution is done away with, the plates being smeared directly. The cultures are incubated at 37°C. and examined after eighteen to twenty-four hours.

Many of the large organisms having some resemblance to *B. anthracis,* found in putrefying blood and tissue are anaerobes so that samples showing large numbers of these will usually show only a few *Bact. coli* or miscellaneous organisms on the plates. In these cases, a diagnosis of "Unable to find anthrax" is made. Injection of guinea-pigs does not yield any result as far as a diag-

nosis is concerned but the pig usually succumbs in from one to four days with a malignant oedema type of infection. The anthrax organism, if it was originally present, has been destroyed by the putrefaction.

In other cases, microscopic examination of direct smears having shown but very few anthrax-like organisms or none at all, when the culture shows only a few miscellaneous colonies, none of which resemble anthrax, a negative diagnosis is made without subjecting the material to a guinea-pig test.

A glance at the plates after incubation will serve to show a worker with any experience with the subject whether either anthrax or what I refer to as "anthrax-like" colonies are present. The most striking character of these colonies is their "ground glass" appearance, especially at the margins. To the unaided eye, the colony appears like frosted glass. The reason for this ground glass appearance may be readily appreciated when the colonies are moderately magnified. Just as ground glass has its well known opaque, whitish, velvety appearance because of numerous small facets the surfaces of which are facing in many directions and which reflect light from just as many directions as there are faces, so do these colonies have this appearance because they are made up of bacterial filaments arranged in parallel to form bundles which run in many directions and which reflect light as do the facets of the glass. The less the tendency of the organism to arrange itself in parallel chains the finer does the texture of the ground glass become. In colonies of *B. anthracis* the bundles are larger than in most anthrax-like colonies so the reflecting facets are larger and the texture of the ground glass is coarser.

The diameter of the colonies varies, depending on the number on the plate. When not crowded anthrax colonies will reach a maximum diameter of about 5 mm. If given plenty of room some of the anthrax-like colonies will identify themselves by spreading and becoming of much greater size than a true anthrax colony ever reaches. This is not always true, however, especially in a primary culture and the plates as made direct from the suspected material, do not always allow sufficient room for this growth to occur.

Stained preparations will serve to eliminate many of these colonies by showing organisms of a morphology differing sufficiently from *B. anthracis* to admit of a negative diagnosis. These differences are principally in greater length and the possession of ends markedly rounding. Stained preparations will by no means eliminate all of these organisms however for some show bacteria indistinguishable, as far as I am able to determine, from true anthrax. Hanging drops will also eliminate many of these organisms by demonstrating rapid motility and I have no doubt but that other tests would eliminate still others not detected by the methods given. These are all time consuming, however, and, as I have said, this is of importance.

An easy and rapid method has been devised which, during a period of over a year, has picked out readily all true anthrax cases (about 15 in number) from among a considerable number of negatives (about 70), the inoculation of guinea pigs being the criterion. This method consists simply in examining directly with a high power objective the minute structure of the suspected colony. When examined grossly or with low powers, these colonies are so similar as to make differentiation difficult in many cases; but when submitted to a greater magnification differentiation has been made with ease in all cases yet submitted to the test. Some of these differences are difficult to explain and can be appreciated only by actual trial. The method gives at once the morphology of the organism, whether or not it is motile, whether or not spores are present, their location and shape if present, and the relation of the organisms to each other in the colony.

The suspected colony is prepared for examination by laying aside the lid of the Petri dish and dropping a flamed and cooled cover glass directly upon its surface. If one edge of the cover glass is first touched to the surface of the medium at one side of the colony to be examined and then gently lowered with a pair of thumb forceps air bubbles will be avoided as there is sufficient water of condensation on the surface of the agar to fill the space beneath the cover-glass. A drop of immersion oil is now placed on the cover glass, the open plate placed on the

microscope stage and the edge of the colony carefully focussed upon with the $\frac{1}{12}$ mm. objective (fig. 1).

A true anthrax colony under these conditions shows beautiful festoons of parallel, closely packed filaments. The arrangement has been aptly compared to a carefully combed coiffure. Very seldom is the end of any filament seen at the colony margin; they appear to begin and end in the depths of the colony. A rather peculiar character, at first thought, is the fact that the filaments, unless examined very diligently, appear to be homogeneous and do not show the divisions between the individual organisms. I have not seen this character nearly so well marked in any of my anthrax-like colonies. It is undoubtedly due to the almost absolute squareness of the ends of the bacteria. In the case of other organisms which have ends slightly rounded and which form filaments, the individual elements are plainly shown because of the reflection and refraction of light from the curved surfaces of the ends of the organisms. My photomicrograph does not illustrate this character well for the camera seems to have detected the individual organisms much better than the eye is able to do.

The true anthrax colonies show few or no spores until after twenty-four hours when grown on 1.5 per cent beef infusion agar. This point aids in differentiating certain species which sporulate abundantly before this time.

The anthrax-like colonies show various characters which it would not be profitable to discuss here. At least half of these types prove to be motile organisms and are easily eliminated by this character. The motility is observed only at the extreme margin where a comparatively few organisms swim around actively. Many other organisms lie entirely free but with no signs of movement so it is probable that the motile stage is of very short duration. Certain observers have claimed that some organisms of this type were not motile in the primary culture but developed motility later in subcultures. This is probably explained by the observation above.

The arrangement of the filaments in none of the anthrax-like organisms has been exactly like that of the true anthrax. Fre-

quently the free ends of many of the filaments are seen projecting out from the margin of the colony, differing radically in this respect from the anthrax colony. The filaments are never so even and regular, or so homogeneous, or arranged in such smooth sweeping curves in the anthrax-like colonies as in true anthrax. The filaments are usually broken by sharp angular bends here and there, and in some cases they are very short and interlace.

Details of the many anthrax-like organisms encountered in putrefying material are too numerous to give here. To anyone who has a good picture of the true anthrax colony in his mind, it would be an easy matter to eliminate all of the "pseudo" forms which I have encountered and examined as I have described.

I do not advocate the method just described as a method to supersede entirely the use of guinea-pigs in the diagnosis of anthrax from more or less decomposed animal material, but I do believe it is a valuable aid in making a rapid diagnosis and will eliminate the guinea-pig in the majority of cases. In badly decomposed material, I believe cultural methods have certain advantages over the use of animals inasmuch as the anaerobic bacilli present in such material will frequently bring about death of the inoculated animal in less time than is ordinarily required for anthrax material to do this while these organisms offer no difficulties in the cultural method. Furthermore, it is known, in these cases, that the number of viable anthrax organisms are greatly reduced and it is conceivable that those which remain may be so attenuated in some cases that they may fail to infect the animals inoculated. The cultural method offers a small chance of detecting such cases should they occur.

SUMMARY

1. Many anthrax like organisms are encountered in the examination of partially putrefied animal tissues for *B. anthracis*.

2. The McFadyean reaction has not proven of great service in distinguishing the true anthrax from these contaminating forms.

3. Guinea-pig inoculation frequently fails because of the death of the animals from a malignant oedema-like infection before any anthrax organisms which may be present have time to develop.

4. The chief difficulty in the way of a cultural diagnosis of anthrax in these cases, the difficulty in distinguishing, rapidly, between the true anthrax and the anthrax-like organisms has been obviated by a method here given.

EXPLANATION OF PLATE 1

Fig. 1. Microscope, with Open Petri Dish in Place for Direct Examination of Colony

Fig. 2. Anthrax Colony × 20

The black line at one margin is extraneous matter which fell on the open plate while it was being photographed.

Fig. 3. Margin of Anthrax Colony × 100

Fig. 4. Margin of Anthrax Colony × 600

Fig. 5 Margin of an Anthrax-Like Colony × 600

Fig. 6. Margin of an Anthrax-Like Colony × 600

Fig. 1

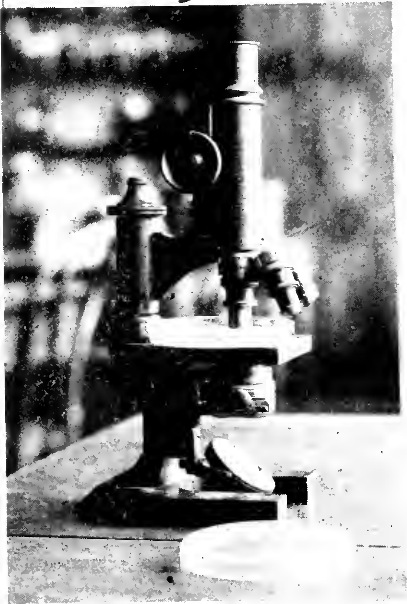

Fig. 4

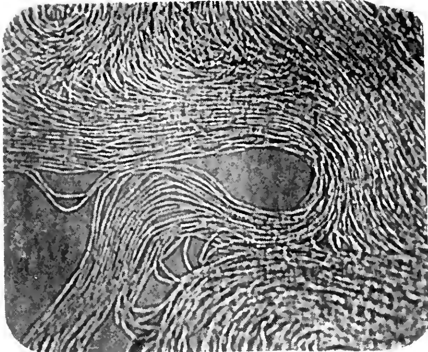

Fig. 2

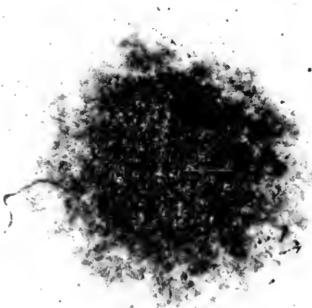

Fig. 5

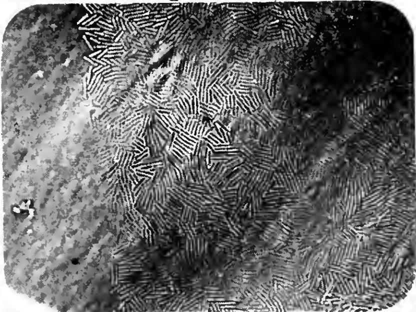

Fig. 3

Fig. 6

(Hagan: Diagnosis of Anthrax)

BACTERIAL DECOMPOSITION OF SALMON[1]

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A study of the decomposition of the food fishes presents an interesting field for both the chemist and the bacteriologist. While the chemistry of fish decomposition has been investigated to some extent, comparatively little has been reported regarding the bacteriology of the problem. Browne (1917) reported the results obtained from his work on the decomposition of various fish during storage in ice stating that autolysis, rather than bacterial action, seems to play the most important part in the initial stages of decomposition. The part played by bacteria in the decomposition of sardines has been studied and reported by Obst (1919). The bacteriology of canned or preserved fish has received the attention of several workers. The question of whether or not the *Bacterium coli* is an inhabitant of the intestines of fish has been investigated by Browne, (1917), Eyre (1904), Houston (1903–04), Amyot (1901), and others but the papers of Browne and of Obst already mentioned seem to be the only studies made of the part played by bacteria in the actual decomposition of the flesh of fish before preserving.

The examination of a large amount of canned salmon at the Bureau of Chemistry during the past year has led to an extensive study of the raw fish from the time it is caught until it is put into the cans. The object of the investigation, from the bacteriological viewpoint, was to determine whether or not bacteria

[1] Presented before American Society of Bacteriologists, December 30, 1919.

[2] Published by permission of the Secretary of Agriculture. In carrying on this investigation valuable criticism and suggestions have been given by Dr. Charles Thom of this laboratory.

were responsible for the decomposition of the salmon and, if such was found to be the case, to establish a correlation between the physical appearance of the fish and the presence of the bacteria after holding the salmon for known lengths of time under known conditions. This necessitated the determination of total counts, and later of the kinds of bacteria, present in the muscular tissue of the salmon and also an investigation as to how these bacteria gain access to the flesh.

Four species of salmon were used in the investigation, namely, the sockeye or red salmon (*Oncorhynchus nerka*), the humpback or pink salmon (*O. gorbuscha*), the silver or coho salmon (*O. kisutch*) and the chum or dog salmon (*O. keta*). All the fish used were caught in fish traps near the San Juan Islands in Puget Sound. The fish were handled as they are handled commercially by the fishermen in that locality and accurate data were kept as to the methods of handling, the length of time out of water, the temperature at which they were held, etc.

The method of procedure was usually as follows: A trip was made to the trap late in the afternoon and the night was spent on the fishing boat alongside the trap. Early the next morning the trap was lifted and emptied of fish. One or two fish were selected for immediate examination and the others were placed in the hold of the boat to be transported to the fish house. On arrival at the fish house the desired number of salmon were placed in a large box in a corner of the fish house. A thermograph was kept with the fish throughout the whole period. Each morning one fish was removed from the box and examined. The general appearance of the fish was noted and recorded. When the odor and appearance of the salmon indicated that the fish were in an advanced stage of decomposition the experiment was terminated and a new experiment begun. Whenever it was found impossible to visit the trap on the fishing boat the fish were received at the fish house and the data in regard to the time and place of the catch were obtained from the fisherman. The fish usually arrived about eight hours after they were taken from the water. In the case of sockeye and humpback salmon three separate catches were allowed to decompose and were studied. The first

lot in each case was a preliminary experiment and the bacteriological examination was not usually conducted on more than the first one or two days. Only one lot of silver and chum salmon was examined. The temperature on the days on which the fish were held never fell below 50° F. nor rose above 70° F.

In the bacteriological examination of the fish total counts of bacteria were made from the muscle tissue of the back and belly of the fish and agar slant cultures were made from various parts and organs of the fish including the mouth, gills, stomach, ceca, intestines, heart, liver and kidney. Cultures in lactose broth fermentation tubes were made from the stomach, ceca, and intestines.

Since the work was necessarily often done in the field at considerable distances from the laboratory, some difficulty was experienced in the plating of the muscular tissue for total count but the technic used, in general, worked very well. The body of the fish was thoroughly washed with alcohol and the alcohol burned off. With instruments sterilized by flaming in alcohol a small flap of skin just posterior to the dorsal fin was carefully lifted and pinned back. A piece of muscle weighing approximately one gram was transferred to a sterile flask of known weight. The flasks used were of thick-walled, heavy glass in order that they might not break under the vigorous shaking necessary to break up the tissue. Known amounts of sterile, broken glass and sterile NaCl solution were added and the whole vigorously shaken until the tissue was thoroughly broken up. This suspension of tissue was diluted and plated according to the usual methods. The flask containing the remaining suspension was tightly stoppered and saved until the laboratory could be reached when it was weighed and the exact amount of original tissue computed. The sample of flesh from the belly was taken in the same manner just posterior to the ventral fin. Glucose agar was used and all incubations were made at room temperature.

The results of the experiments in determining total count are given in tables 1, 2, 3, and 4.

TABLE 1

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNT OF BACTERIA FROM FLESH OF SOCKEYE SALMON		
		First series	Second series	Third series
hours		*per gram*	*per gram*	*per gram*
Within 2	Back	Sterile	Sterile	Sterile
	Belly	Sterile	Sterile	Sterile
24	Back	665	4,000	1,100
	Belly	5,750	8,000	8,000
48	Back		27,000	2,500
	Belly		36,000	15,000
72	Back		8,000,000	410,000
	Belly		12,750,000	920,000
96	Back		50,000,000	900,000
	Belly		155,000,000	6,400,000

TABLE 2

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNT OF BACTERIA FROM FLESH OF HUMPBACK SALMON	
		First* series	Second† series
hours		*per gram*	*per gram*
Within 2	Back	Sterile	Sterile
	Belly	Sterile	Sterile
24	Back	Sterile	120
	Belly	Sterile	1,420
48	Back	2,000	1,750
	Belly	37,000	1,700
72	Back	7,000	660,000
	Belly	50,000	1,600,000
96	Back	15,000	3,100,000
	Belly	60,000	3,500,000

* This lot of fish was thoroughly washed on arrival at the fish house. All blood and slime was removed.

† This lot of fish was left unwashed.

TABLE 3

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNTS OF BACTERIA FROM FLESH OF SILVER SALMON	
		Washed fish	Unwashed fish
hours		*per gram*	*per gram*
Within 2	Back	Sterile	Sterile
	Belly	Sterile	Sterile
24	Back	Sterile	4,700
	Belly	200	71,000
48	Back	5,000	250,000
	Belly	6,500	480,000
72	Back	220,000	470,000
	Belly	2,500,000	2,200,000
96	Back	510,000	4,400,000
	Belly	2,800,000	11,600,000

TABLE 4

LENGTH OF TIME OUT OF WATER	PART OF FISH EXAMINED	TOTAL COUNTS OF BACTERIA FROM FLESH OF CHUM SALMON	
		Washed fish	Unwashed fish
hours		*per gram*	*per gram*
Within 2	Back	Sterile	Sterile
	Belly	Sterile	Sterile
24	Back	860	750
	Belly	2,100	18,000
48	Back	2,600	2,200
	Belly	130,000	28,000
72	Back	480,000	340,000
	Belly	500,000	2,300,000
96	Back	620,000	1,250,000
	Belly	1,180,000	3,410,000

Cultures from the mouth and gills of the fish were taken by simply inserting a sterile loop into these parts and then smearing the adhering mucus over the agar slant. After the material from the back and belly of the fish had been collected and the cultures from the mouth taken, the body cavity was carefully opened and the body wall cut in such a way that it might be pinned back, exposing the viscera. Organs from which cultures were to be taken were seared with a hot instrument and then cut slightly with sterile scissors. The sterile loop was inserted through the small opening and some of the blood and mucus transferred to the agar slant.

Since all the salmon examined were caught during their spawning migration, there was never any food found in the stomach. On rare occasions a small amount of partly digested food would be found in the intestine but for the most part the whole digestive tract appeared to contain nothing but mucus.

In handling one lot of humpback salmon the fish were washed thoroughly with running water, cleaning the bodies entirely of blood and slime. It was noticed that these salmon did not decompose as rapidly as had previous lots and in the subsequent experiments particular attention was given to the effect of washing fish as soon as they were brought ashore.

Examination of the mouths and gills of 41 salmon of various species has shown that microorganisms are always present even when the fish are examined immediately on being taken from the trap. Yeasts, bacilli of various kinds and cocci were usually found in large numbers.

Examination of the stomachs of 36 salmon has shown that no microorganisms are present in this organ during the first twenty-four hours, provided there is no feed in the stomach. In 3 out of 6 lots of fish examined, bacteria were found in the stomach after the fish had been held forty-eight hours and in one other lot the bacteria were recovered from the stomach after holding seventy-two hours.

In examining the ceca of salmon from 7 different lots, bacteria were found in but one lot when the fish were examined immediately on being caught. One lot showed bacteria in the ceca after

twenty-four hours, while in four other lots bacteria were not recovered from this part of the digestive tract until the salmon had been held seventy-two hours.

Living bacteria were found in the intestine of a salmon from one lot immediately on being caught. The intestine in this case contained some partly digested food. In 3 other lots bacteria were not found in the intestine until the fish had been out of water forty-eight, seventy-two and ninety-six hours respectively. In one lot no bacteria were found after the fish had been held for ninety-six hours.

In examining the heart's blood of 4 lots of salmon no bacteria were obtained when the fish was first caught. In 2 lots living bacteria were found in the heart's blood after twenty-four hours, in one lot after forty-eight hours and in the other lot after the fish had been held seventy-two hours.

Blood in the large vessels among the viscera was examined and no bacteria were found in the blood stream when the fish were fresh. In one lot bacteria were obtained from the blood stream after twenty-four hours and in the other lots they were found after forty-eight hours. It was noted that even at the end of ninety-six hours the blood in these vessels had not coagulated.

The kidneys of 6 lots of salmon were examined and in 3 lots no bacteria were found after ninety-six hours. In the other 3 lots living microorganisms were found after the fish had been held forty-eight hours.

The livers of 4 lots of salmon were examined and were always found to be sterile.

Direct smears from the parts examined and smears from the cultures obtained have shown that the organisms obtained from the viscera and the blood stream are at least morphologically similar to those present in the mouth and gills during the first twenty-four hours. These organisms have been isolated and are now being made the subject of a further study, the results of which will be presented in a later paper.

In regard to the physical appearance of the fish it was noted that during the first forty-eight hours, at the temperatures on Puget Sound, no marked decomposition takes place. The eyes of the fish remain bright; the gills are red with no foul odors; the

flesh is firm and sweet and the viscera remain normal in appearance. The fish at the end of the seventy-two hour period were in such a state of decomposition that they were designated as "stale." The eyes became slightly sunken, the gills dark in color and either sour or foul in odor; the digestive tract was darkened and usually rather foul-smelling. The flesh of the salmon at the end of the seventy-two hour period was usually soft with some sour or putrid odor. At the end of the ninety-six hour period the salmon were markedly decomposed and beyond the state where they should be considered as fit for food. The eyes were deeply sunken, the gills blackened and exceedingly foul smelling, the skin dry and cracked and the flesh very soft and putrid. The viscera of such fish were always very much darkened in color and very foul. As will be seen in tables 1, 2, 3 and 4, the total count of bacteria in the flesh from such fish in one case was as high as 155,000,000 per gram.

A study of the data in the tables has demonstrated several interesting facts. The muscular tissue of freshly caught salmon is sterile. In studying the intestinal contents of fishes, Eyre used pieces of flesh of the fish as controls and found them sterile. The counts obtained on the flesh from the belly are always higher than those from the flesh of the back. Since some of the bacteria in the flesh undoubtedly get there through the skin, the fact that the skin of the belly is thinner and more easily broken may help to explain this higher count. The very high counts in the flesh from both the back and belly are sufficient to explain the softening and decomposition of the tissue. In most cases the fish which were washed upon arrival at the fish house had lower total counts than those held unwashed. The washed fish did not decompose as rapidly as did the unwashed fish.

Results of the examination of the various organs of the body would seem to indicate that the sources of infection as regards these organs, and to a considerable extent as regards the muscular tissue also, are the gills and mouth. It would appear that a great many organisms make their way through the blood channels to the viscera and the muscular tissue within forty-eight to seventy-two hours after the fish has been removed from the water. Rough handling of the fish will, however, break the

skin and allow many organisms present on the surface to penetrate the flesh.

In regard to the presence of *Bacterium coli* in the digestive tract it may be briefly stated that in no case was this organism isolated from the ceca or intestines of the salmon examined.

SUMMARY

1. The muscular tissue of freshly caught salmon is sterile.

2. After ninety-six hours at temperatures between 50°F. and 70°F. the total count of bacteria in the muscular tissue has been found to be as high as 155,000,000 per gram. The high counts obtained are sufficient to explain the decomposition of the tissue.

3. Thoroughly washing the fish on arrival at the dock results in lower total counts. The washed fish decompose less rapidly than the unwashed fish.

4. The mouths and gills of salmon contain living microorganisms of various kinds even when fresh from the water.

5. The digestive tract of salmon is sterile when there is no food present. This is in agreement with the findings of Obst in studying sardines.

6. The various organs of the body become infected through the blood vessels usually within ninety-six hours after the fish are caught.

7. Salmon out of water more than forty-eight hours at temperatures between 50°F. and 70°F. are decomposed to such an extent that they are not desirable as food.

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THE FILTRATION OF COLLOIDAL SUBSTANCES THROUGH BACTERIA-RETAINING FILTERS[1]

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It has been noted by the writers that in the preparation of germ-free filtrates, notably in working with the anaerobic organisms, those media which contained raw meat pieces added aseptically were superior to media to which meat cubes were added and which were subsequently sterilized by heat. It was believed—a belief later confirmed—that the presence of the unaltered proteins was chiefly responsible for the superiority of those media of which they were an integral part. However, the addition of meat cubes to previously sterilized media without contamination thereof is difficult and unpractical on a large scale. Therefore, in order to supply proteins unaltered by heat the juice obtained by subjecting finely ground and previously frozen and thawed raw meat was suggested as a substitute for meat cubes in the expectation that this juice could be sterilized by passing through a bacteria-retaining filter.

Filtering of such a colloidal substance was found to be exceedingly difficult. Inasmuch as no literature seemed to be available which offered any assistance it is believed that a report of the technique now used by the writers will prove helpful to others. Preparation of the colloidal substances for the bacteria-retaining filter by preliminary passage through cotton, asbestos wool and filter paper does not appreciably enhance their filterability. However, by adding a sufficient amount of pulverized kieselguhr to the meat juice to make a very thin paste and pouring this mixture over a coarse filter paper, the filtrate so obtained will

[1] Presented before Society of American Bacteriologists, December 29, 1919.

possess a temporary filterability through the bacteria-retaining filter candle, but the lapse of any appreciable time between the preliminary and final filtration permits a reversion of the material into the original state of non-filterability due probably to coagulation of the proteins. Unfortunately, the time required in the accomplishment of this preliminary kieselguhr filtration is sufficiently long to defeat its own purpose and for this reason the kieselguhr mixture is poured directly over the filter candle omitting the paper filtration entirely. The meat juice as it comes from the press is cleared of the coarser particles by centrifugalization. A small amount of kieselguhr is then added to the clarified meat juice and this mixture again centrifuged. After the supernatant fluid is drawn off it is again mixed with a sufficient amount of kieselguhr to make a rather thick mixture, approximately the consistency of butter-milk or a thin gruel. It is this mixture that is poured in direct contact with the filter candle. The best results will be obtained by using a minimum amount of vacuum. By placing the filter candle upright in a mantle, gravitation will materially assist in minimizing the required amount of vacuum necessary to draw the material through the filter candle into the vacuum flask.

In the event that the filtration process cannot be carried out immediately after the expression of the meat juice, some coagulation of the proteins is apt to take place, which should be corrected by centrifugalization just before filtering. It has been noted that the finished product after passage through the bacteria-retaining filter will, on standing or by application of heat, coagulate just as completely as the meat juice that has not been subjected to the filtration process.

By this process, meat juice, milk, hemolized blood corpuscles, etc., may be filtered at a rate which will compare favorably with the filtration of crystalloidal solutions and bouillons.

NOTES ON THE FUSIFORM BACILLI OF VINCENT'S ANGINA

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There is still some difference of opinion expressed in standard texts as to the Gram-staining qualities of the fusiform bacilli found in Vincent's angina and allied conditions. In Hiss and Zinsser it is stated: "Stained by Gram, they are usually decolorized, though in this respect the writers have found them to vary." Stitt calls this bacillus Gram-negative but states that it "is not markedly Gram-negative." No attempt will be made to review completely the literature on this point, but a few statements may be quoted to show that there is such disagreement.

A good summing up of the literature is that of Beitzke (1904) and many of the opinions quoted here are from this article, as well as from those of Weaver and Tunnicliff (1905, 1907) and of Babes (1906).

Weaver and Tunnicliff, from a large variety of material, found the bacilli to be Gram-negative, though they state that, unless thoroughly decolorized by alcohol, the cells retain a very faint bluish color. They mention (1905) that others have found longer decolorization to be necessary; that Vincent, Abel, and Niclotte and Marotte found the bacilli Gram-positive, but that all other authors contradict this. Vincent in his original article on Hospital Gangrene (1896) describes the bacilli as Gram-negative. In a later article (1899) on the anginas, he also describes them as Gram-negative (p. 613) and I can find no reference to them as Gram-positive, such as Weaver and Tunnicliff (1905) (p. 449) cite. Babes (1906) found fusiform bacilli from the gums in scurvy Gram-negative and states that Bernheim and Pospichill, in 30 cases of angina and other conditions found them Gram-negative.

He mentions Veullar and Zuber's having found such bacilli Gram-positive in material from the appendix. An article by Veillon (1898) describes *Fusiformis* from the appendix as Gram-negative (p. 540) and I could find no description of it as Gram-positive in his articles.

In summing up some of the literature on noma, Weaver and Tunnicliff (1907) mention that Foote, Elder, Blumer and MacFarlane, Hofman, Buday and Nicolaysen all found fusiform bacilli Gram-positive, while in their own case of noma they were Gram-negative, "except in the dark spots," though they held the stain longer than the spirilla also present. In some of the last quoted articles, the description does not sound typical of Vincent's organisms and in most no spirilla were present with them. Dick (1913) in 7 post-mortems of meningitis, cerebellar abscess, gangrene of the lung, pneumonia, empyema, and peritonitis, found the fusiform bacilli always Gram-negative. The same was true of the 3 strains which were cultivated. Krumwiede and Pratt, (1913, a, b) of 15 strains from the throat and teeth, found all Gram-negative, in smear and culture. Tunnicliff (1906) who cultivated them, found the fusiforms Gram-negative in cultures.

Altogether of 17 authors or groups of authors who mention the Gram-stain, 6 found the fusiform bacilli Gram-positive, 8 found them Gram-negative, while of the 3 remaining, Foote found them Gram-positive but that care was necessary to avoid decolorizing too much, Hofman, Gram-positive but "care was necessary in differentiation" and Bernheim and Pospichill found them Gram-negative after longer decolorization.

This disagreement might be due to the fact that entirely different organisms of fusiform appearance were present in different cases. In view of the large number of cases of Trench Mouth that we were examining it seemed worth while to test a series of smears against standard Gram staining technique. Unfortunately this was not undertaken while the press of work was on, so records are available for a relatively small number of cases. Only cases which showed a profusion of both bacilli and spirilla were included. Twenty-four examinations on 18 cases could thus

be included, examinations being repeated on 4 of the cases, and three examinations being done on the other case. In 17 examinations, 2 standard methods of staining were used on each, the old long method (one minute carbol gentian violet, two minutes Gram's iodine, five minutes alcohol, one minute safranin) and the newer shorter method (fifteen seconds' gentian violet, washing off twice with Gram's iodine, fifteen seconds' washing with alcohol, one minute safranin). Of the other 7 examinations, the short method was used on 6, the long method on the other. Of the 18 cases, smears were taken in 14 from lesions of the throat or tonsils, in 4 from lesions of the gums.

Of the 24 examinations, the bacilli were found to be markedly more numerous than the spirilla in 17, while the spirilla were equal in number or more numerous in 7. In the cases where examinations were repeated, these relations remained constant in 4, while in the other case the bacilli were first more numerous than the spirilla, and five days later the spirilla were found to be more numerous.

The staining qualities were found to vary markedly with both methods. In the main, the two methods checked, Though in some examinations a larger number of bacilli were Gram-positive by one than by the other method, a majority usually stained in the same way by both methods, in any smear. There was sufficiently marked disagreement to make it questionable which were most numerous in only three examinations. As a check on the methods, it was noted that the streptococci nearly always present in the same smears were always markedly Gram-positive, while the spirillae were invariably Gram-negative by both methods.

Of the 24 examinations, in 12 over half the bacilli were Gram-positive by one or both methods. In 9 over half were Gram-negative. In 3 others, there was disagreement, 2 showing a majority Gram-positive by the short and Gram-negative by the long methods, while 1 showed the reverse relations. Of the 17 in which both methods were used, 7 showed a majority Gram-positive by both methods, 7 a majority Gram-negative by both, while in 3 there was the disagreement noted above. In the 5 cases where repeated examinations were done, the staining

varied as follows: In 4 a majority were Gram-negative at first but examination after two to five days showed a majority Gram-positive. In 1, the majority remained Gram-positive at first and second examinations. The organisms varied greatly in length, and it was thought some difference in staining coincident with the difference in size might be observed. While the longer thread-like organisms which were considered as involution forms, were almost always Gram-negative, the typical fusiforms, of whatever length, showed about an equal number of cells Gram-positive and Gram-negative.

A few attempts were made to cultivate the organisms, to observe whether the Gram-staining properties changed with age. Cultures were made from gingival ulcers, or from the tonsils, on Loeffler's serum made anaerobic by the Büchner-Wright method, also in anaerobic meat-broth and in Veillons. It was found that in the first and second cultures a fair number of slender bacilli could be found, some of which had a distinctly fusiform appearance. The majority of these were Gram-negative, a few Gram-positive. No spirilla were ever found.

One strain from an ulcerated throat showed a good many distinctly fusiform oganisms in the third generation after subculture in aerobic and anaerobic meat-broth for fifteen days. It is interesting that this strain, which in direct smears and first cultures showed a majority of Gram-positive bacilli, showed a much larger number Gram-negative in the second and third generation. In the older growth of the original culture this tendency was even more plainly shown. The aerobic meat-broth after ninety-six hours showed the bacilli all Gram-negative, while in the anaerobic meat-broth about half were Gram-negative. This was the only strain in which such a tendency to Gram-negativeness in well preserved organisms from cultures could be definitely made out. (In several old cultures of the strains showing a few bacilli most were Gram-negative, but a large number of these cells had lost their definite outlines, stained feebly and were considered dead or moribund.) These cultures all had a foul odor.

In one post-mortem an organism was found which from its source and morphology must be considered as at least very closely allied to the bacillus of Vincent. The case was one of death from broncho-pneumonia following a large abscess of the jaw directly continuous with the root of an ulcerated tooth. Smears from the ulcerated area, the abscess, the larynx, the large and small bronchi, all showed a profusion of fusiform bacilli, morphologically indistinguishable from those found in Vincent's Angina. A few ordinary pyogenic cocci were found in the smears but the bacillus was the predominant organism. No spirilla were found in any smears and none of the spore-bearing bacilli to be described. In the direct smears, about half the fusiforms were Gram-positive and half Gram-negative.

In all the aerobic broth cultures from these regions a few fusiform bacilli were found; in some a fairly large number. All showed also many cocci, which proved to be *Streptococcus viridans* and a hemolytic staphylococcus. In a culture from the larynx made on Loeffler's blood serum, the largest number of fusiform bacilli were found, and the fusiforms had greatly increased, relatively to the cocci, after five to six days. Aerobic plates made from this culture showed in twenty-four hours large numbers of peculiar small dew-drop colonies with moist, spreading edges. Second plates from these colonies gave nothing but such colonies appearing like pure cultures. Smears from well isolated colonies, however, while they showed many fusiform bacilli, showed also a club shaped, Gram-positive spore-bearer. On referring back to the original Loeffler's tube a few of these spore-bearers were also found. Cultures from these colonies in aerobic and anaerobic meat-broth and plain broth showed at first nothing but fusiform bacilli, but later in all these cultures, spore-bearers developed. One culture after sixteen days showed only a very few spore-bearers and many perfectly typical fusiform bacilli. Transfers back on plates gave the same colonies with the same mixture of fusiforms and spore-bearers. These transfers from liquid media to plates and back again were repeated as far as the seventh generation with the same results. Orders to the states interrupted the work before the fusiform bacillus could

be isolated from the spore-bearer. It seemed possible that the spore-bearers were a form of the fusiform bacilli appearing when it was grown aerobically and especially on solid media. But remembering that Weaver and Tunnicliff and also Abel found the same intimate relation of the bacilli to the aerobic cocci in isolated colonies, it seemed more likely that the spore-bearer was another organism present in the larynx or as a contaminant in the culture, whose aerobic growth furnished the conditions necessary for the growth of the more anaerobic fusiform bacillus. With regard to the staining characters, the fusiforms in the cultures varied markedly and with no ascribable relation to the conditions present. The question of the pathogenicity of this strain was not determined by animal experiment. Though found in such profusion in the lesions, it may have been only a secondary invader from the teeth following streptococcic infection.

In summing up, it may be said:

1. Bacilli were found to be more numerous than spirilla in 17 out of 24 examinations.

2. The fusiform bacilli of Vincent's angina show much greater variation in their Gram-staining properties than ordinary organisms. Where there were any number of bacilli present, smears were never found which did not show both Gram-positive and Gram-negative bacilli in the same smear. Many were found in nearly all smears which were Gram-negative with Gram-positive granules, most frequently 4 granules in a pair of bacilli, but this number varied. These Gram-positive granules seem to be one of the most constant mophological characteristics of the organisms, almost as constant and distinctive as the polar bodies in *Mycobact. diphtheriae.*

3. A slight predominance of Gram-positive organisms was noted, but not great enough to justify any conclusions.

4. Method of staining is not responsible for the variation noted.

5. The fusiform bacilli may be cultivated anaerobically or aerobically when other aerobic organisms are present. They could not be isolated by our methods, though others have succeeded in doing so.

6. No spirilla were found in cultures.
7. An organism greatly resembling the fusiform bacilli of Vincent's angina was found as the predominant organism in the post-mortem on a case of broncho-pneumonia. It was grown to the seventh generation, but not isolated.

My thanks are due to Captain F. S. Perrings, M. C., in charge of the Laboratory, Evacuation Hospital 19, for obtaining the post-mortem material, and for his coöperation at all times.

Since this paper was finished, I have found fusiform bacilli very like those of Vincent's angina occurring in two unusual locations. One was in smears from a peculiar ulcer surrounding the lower canaliculus; the other in cultures from the pus of a unilateral chalaziosis in which both lids were very extensively involved. In the second case it appeared in pure culture and grew well aerobically on the ordinary media. In both cases it may have been a secondary invader but it is interesting to know that such an organism may inhabit the conjunctival sac and Meibomian glands, a fact not previously recorded, so far as I know. The author hopes to report further on these cases.

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A HIGHLY RESISTANT THERMOPHILIC ORGANISM

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An examination of spoilage samples of "Standard Maine Style" corn which had been packed in the usual manner and processed at 118°C. for 75 minutes showed the presence of a thermophile. The same organism was later found in spoiled cans of string beans and corn on the cob. It appears to be an unknown species and the name *Bacillus stearothermophilus* is proposed for it.

Its cultural characteristics are as follows:

Name proposed: B. stearothermophilus. (N. S.)

Source: Canned corn.

Date of isolation: October 3, 1917.

Vegetative cells: Medium used, agar; temperature, 60 to 65°C; age, twenty-four hours; form, large rods; arrangement, majority single, some in pairs end to end and few in chains of three or four. Size of majority 0.8 by 3.5 micra; ends rounded.

Relation to oxygen: Aerobic, facultative anaerobic.

Endospores: Present; location, polar. Size of majority 1 by 1.5 micra.

Motility: None.

Flagella: None. Muir's modification of Pittfield's method, and Loeffler's method.

Gram stain: Negative.

Nutrient broth: Surface growth, none; no pellicle or ring; clouding of medium uniform, odor, absent; sediment, compact, abundant.

Agar stroke: Growth, moderate; form of growth, generally filiform, sometimes slightly beaded, never spreading; elevation of growth, quite regular; optical character, translucent; chromogenesis, none; color, dirty white; odor, absent; consistency, butyrous.

Agar colonies: Growth moderate; temperature 60° to 65°C. form, circular; surface, smooth; elevation, flat; edge, regular; maximum diameter observed 2 mm., white opaque spot in center surrounded by several concentric rings.

Gelatin: Temperature 20°C., no growth; 60° to 65°C. growth but no liquefaction. (Inoculated tube showed uniform clouding after incubation for three days, solidified when placed in ice-box.)

Glucose agar stroke and stab (litmus): Growth, acid throughout.

Lactose agar stroke and stab (litmus): Growth, acid throughout.

Sucrose agar stroke and stab (litmus): Growth, acid throughout.

Potato: Growth, none.

Potato starch agar: Growth copious.

Corn infusion (litmus): Strong acid after twenty-four hours. Starch digested.

Temperature relations: Optimum temperature for growth 50°C. Maximum 76°C. Minimum 45°C.

Litmus milk: Growth begins after twenty-four hours, acidified, litmus partly reduced. No coagulation after forty-eight hours. After four days litmus completely reduced and casein digested, leaving heavy sediment in bottom of tube. (Control tube showed loss of color only after six days, no coagulation, no digestion.)

Indol production: Absent.

Nitrate broth: Not reduced; ammonia, none.

Glucose broth: Gas production, none.

Lactose broth: Gas production, none.

Sucrose broth: Gas production, none.

The thermal death relations at two temperatures and in media containing different numbers of spores are as follows:

MEDIUM	pH VALUE	TEMPERATURE	SPORES PER CUBIC CENTIMETER	TIME TO KILL
		°C.		
Corn broth	6.1	100	12,500	17 hours
Corn broth	6.0	120	50,000	11 minutes

THE BIOLOGY OF CLOSTRIDIUM WELCHII

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I. INTRODUCTION

The organism causing gaseous phlegmon or emphysematous gangrene was discovered by Welch and Nuttall at an autopsy in 1892 and was named by them *Bacillus aerogenes capsulatus*. In the revision of the nomenclature of bacteriology it was given its present name, *Clostridium Welchii*.

Many investigators have studied this organism and have proposed different theories to explain the symptoms following infection. During the late War, cases of wound infection by *C. Welchii* were reported among the French and British soldiers on the western front. Such infections have frequently led to disastrous results, terminating in death and needless amputation owing to lack of knowledge regarding the causes of the local destruction of tissue.

In 1917 Bull and Pritchett discovered that *Clostridium Welchii* produced a soluble toxin which was capable of causing the lesions characteristic of infections by this organism and which was neutralized by a specific immune serum. These facts have been confirmed by this investigation which was carried on in 1916, 1917, and 1918, to study the biology, pathogenicity, and distribution of the gas bacillus.

II. ISOLATION

The media selected for the isolation of *Clostridium Welchii* were glucose broth, glucose-liver broth, glucose-liver-agar and milk. The liver broth and agar were prepared by the method given in Standard Methods for Water Analysis 1912. Ordinary market milk adjusted to + 1 to phenolphthalein was used. Before sterilization, the surfaces of the media were covered with a layer of paraffin oil (albolin).

Pure cultures were obtained by inoculating tubes of sterile milk, previously covered with oil, with the material supposed to contain *Clostridium Welchii* and heating to 82° to 85° for fifteen minutes. The tubes were then cooled to 37° and incubated at that temperature. The presence of the bacillus in market milk was determined by transferring small portions of the milk to other sterile tubes; or the tubes of market milk were heated directly to 82° to 85° for fifteen minutes, cooled to 37°, and after covering the surface with sterile paraffin wax, incubated.

A positive reaction showed "stormy fermentation," i.e., coagulation of the casein, copious formation of gas, fragmentation of the curd, derangement of the cream layer and a detectable odor of butyric acid. However, milk cultures sometimes contained gas bacilli and yet failed to show this typical reaction due to various reasons. Cultures which showed a positive reaction were inoculated into tubes of glucose-liver broth and incubated from four to six hours, at which time the culture presented a "steaming" appearance. At this time plates were made on glucose-liver-agar, employing anaerobic technique, and incubated at 37°. After sufficient incubation (twelve to twenty hours)

typical *Clostridium Welchii* colonies were fished from around the gas zones and reinoculated into glucose-liver broth and later into milk. The purity of the culture was determined by microscopic examination. Although glucose agar and glucose broth with meat extract gave satisfactory results, liver medium was used in the greater part of this work, since *Clostridium Welchii* grew luxuriantly in the presence of glycogen.

The Welch-Nuttall incubation test was tried in order to prove that the "stormy fermentation" is characteristic of *Clostridium Welchii* and gave positive results throughout, with the recovery of the organism from the organs and body fluids of the animal.

III. DISTRIBUTION

Clostridium Welchii is extensively distributed in nature in the spore form. It has never been found in the vegetative form except in the tissues of the animal body. Members of this group have been isolated from the soil, dust in barns, dirt on laboratory floor, street dirt, from grains, such as gluten, meal, beet pulp and oats, from sewage and oysters, oyster liquor and mud surrounding the oysters.

Clostridium Welchii was present in most of the pasteurized milk examined in Providence, R. I., regardless of its bacterial content. Raw milk samples were consistently contaminated with spores of *Clostridium Welchii* in certain cases, while in other cases negative results were obtained. Clean milk contained *Clostridium Welchii*, although in less numbers and in fewer cases than milk of high bacterial content. Few dairies were continually free from *Clostridium Welchii* infection.

Clostridium Welchii was found on all parts of the cow, on the ceiling and floor of the barn, walls, cobwebs, milk utensils, milker's hands, barn dust and stable air. Milk direct from the cow was free from *Clostridium Welchii* and, therefore, became contaminated through some external source of uncleanliness and carelessness after the milk had been drawn.

Feces from human beings, guinea pigs (rarely), the cow, horse, rabbit, calf, hen, and dog contained spores of *Clostridium Welchii*

in considerable numbers. Two cans of preserved tomatoes packed by the cold pack method showed evidence of gas fermentation and when cultures were isolated anaerobically, *Clostridium Welchii* was found to be the cause of the fermentation. This organism therefore appears to have as broad a distribution as *Bacterium coli.*

TABLE 1

The number of cultures used in the subsequent study by sources

NUMBER OF CULTURES	SOURCE
56	Milk (pasteurized)
469	Milk (raw)
55	Human feces
18	Cow feces
18	Horse feces
10	Guinea-pig feces
626 total	

IV. MORPHOLOGY

1. Size and shape of organisms. *Clostridium Welchii* usually appears in cultures as a straight, plump rod, varying in size from 3 to 6 micra in length and from one to one and a half micra in breadth, according to the nature of the medium. The ends of the rods are slightly rounded or square cut. In old cultures the rods may be slightly curved or may form threads, filaments or long chains. In freshly isolated cultures they occur singly, paired, in clumps and sometimes in short chains of three to six bacilli, with the rods in a straight line or at an angle. Cultures grown for a long time on bloodserum and in liquid media containing coagulated egg-white, show long chains, rather slender threads and filaments, varying in length from 5 to 6 micra to threads extending across the microscopic field. Variation is more pronounced in the presence of a fermentable substance. An acid reaction is more favorable to variation than an alkaline reaction. The rods are more slender in gelatin and nutrient broth than in other media. In milk cultures the organisms are comparatively short, presenting a coccoid appearance. Greater

variation in length and thickness appears in old cultures than in those freshly isolated from the body tissues, although stained preparations from cultures made from body tissues, heart's blood and body fluid occasionally show very long chains and threads.

2. Capsules. Capsules were demonstrated in milk or glucose-liver-broth cultures made directly from body fluids or body tissues, from milk or feces, but cultures grown for any length of time on artificial media could not be made to form capsules unless reinoculated into the animal body or grown in milk. The capsule surrounds the bacterial cell and varies from one to one and a half micra in thickness. Welch's special stain was employed.

3. Staining. Clostridium Welchii stains readily with the ordinary dyes such as gentian-violet, safranin, methylene blue, carbol-fuchsin and eosin, either uniformly or with small unstained areas. In every case the bacilli from young cultures when stained by the regular Gram's method retain their color completely.

4. Motility. Specimens prepared from glucose-liver broth after incubation at 37° for periods varying from one to twenty-four hours do not show motility by the use of the usual hanging-drop method. No attempt was made to stain flagella.

V. SPORE FORMATION

1. Conditions governing sporulation. Sporulation takes place in the following media: Plain peptone water, Dunham's solution, peptone water plus a small amount of coagulated-egg-albumen made neutral or slightly acid, a solution of 5 per cent and 10 per cent coagulated-neutral-egg-albumen, a physiological salt solution plus coagulated-egg-albumen, nutrient gelatin and nutrient agar plus a small amount of coagulated-egg-albumen, plain liver broth, plain nutrient broth, plain nutrient broth plus coagulated-egg-albumen, blood serum, mannitol broth, mannitol-liver broth and a neutral suspension of feces. Strains of *Clostridium Welchii* vary in their ability to form spores in glycerol and inulin broth, with or without liver as a base instead of meat.

In market milk spores of *Clostridium Welchii* may enter as a contamination, but do not germinate except under very special conditions. Spores germinate rapidly in sterilized milk and remain in the vegetative stage without again forming spores. *Clostridium Welchii* has never been found to occur in nature in the vegetative form. This is undoubtedly due to its strictly anaerobic requirement and its inability to withstand an unfavorable environment. *Clostridium Welchii* never sporulates in the tissues, organs and body fluids of the living animals but does sporulate in the intestine.

Spores of *Clostridium Welchii* do not develop below 10°, and die out slowly at this temperature. Market milk, when heated to 80° to 85° for fifteen minutes and then kept on ice, shows no evidence of decomposition or fermentation due to *Clostridium Welchii*, but when allowed to stand above 20°, the characteristic "stormy fermentation" occurs in from twenty-four to seventy four hours. If the milk has not been heated to 80° to 85° for fifteen minutes, *Bacterium coli* and the other facultative aerobes multiply in excess of *Clostridium Welchii* and thus obscure its action, producing a peculiar non-characteristic digestion and decomposition of the milk.

2. Morphology of spores. Great irregularity is observed in the size, shape and location of the spores. They are usually oval, from 1.5 micra to 3 micra long and slightly thicker than the diameter of the bacillus. The spores form in the middle of the rod or slightly toward one end. They present, in preparations stained by Hauser's method, an unstained glistening appearance of a highly refractive character, while the ends of the organism stain deeply and completely.

3. Germination of spores. On germination, the spores show first a change in their refractive property; this is followed by elongation, with a final bursting through of the spore membrane at one pole and the outgrowth of the bacillus. Germination takes place in media containing glucose, galactose, lactose, maltose, sucrose, dextrin and starch. If there is a sufficient amount of the fermentable substance present, all the spores germinate. After all fermentable material is exhausted any

remaining spores fail to germinate and are observed microscopically. On the other hand, it has never been possible to cause sporulation of a vegetative strain in the presence of a fermentable carbohydrate.

4. Relation of spores to acidity. Spores of *Clostridium Welchii* withstand a wide range of reaction, surviving + 12 to − 2 per cent. Ordinarily spores are kept in neutral or slightly acid media but in some cases where all the spores failed to germinate in sugar media they could be recovered for a long time even if the acidity had reached a maximum of + 10 or + 12 per cent. Spores will develop in slightly acid or alkaline media but are formed best in neutral media. An environment of 5 per cent acid or 3 per cent alkali will inhibit the formation of spores but if already formed they will survive such an environment for a long time and can be preserved in such a condition. Media of − 2 reaction did not inhibit the formation of spores from the vegetative forms.

VI. CULTURAL REQUIREMENTS

1. Aerobiosis. *Clostridium Welchii* is an obligate anaerobe which requires strict anaerobiosis for its growth. All anaerobes, especially obligate anaerobes, have an optimum oxygen tension above which growth ceases. Although this tension for *Clostridium Welchii* is greater than for some others of this class, anaerobiosis must be fully maintained to obtain consistent and satisfactory results. Milk tubes which are not rendered strictly anaerobic before inoculation with this organism show either no growth or simple coagulation without a trace of gas formation, presenting that atypical reaction so often reported by investigators. This same phenomenon is observed with stock media which has been sterilized under a film of oil (albolin).

At the end of three months, the oil does not admit oxygen in a sufficient quantity to inhibit the growth of *Clostridium Welchii* when inoculated. At the end of five months, however, oxygen has been absorbed by the medium to such an extent that the optimum aerobic supply is exceeded and consequently growth

does not occur on inoculation. By using media containing different percentages of oxygen, certain strains of *Clostridium Welchii* develop under less oxygen tension than others, but above a certain amount of free oxygen in the medium, growth never occurs. Repeated artificial cultivation for several generations tends to increase the optimum free oxygen allowed, as shown by the fact that the growth approaches nearer and nearer the surface in agar stab cultures after each additional transfer.

Anaerobiosis is effected by simply sterilizing all media under a thin film of oil (albolin). If the medium has remained in the laboratory more than one month after sterilization, it is boiled for one half hour previous to inoculation or resterilized in the auto-clave at ten pounds pressure for fifteen minutes. Anerobic conditions are obtained with plate cultures by covering the surface of the solidified medium with a thin layer of paraffin at the lowest temperature at which this flows. Media which have been sterilized without albolin permit the growth of *Clostridium Welchii* if inoculated directly after sterilization since this organism develops so rapidly at 37° that free oxygen cannot be absorbed sufficiently in a short time to inhibit development. When grown in this manner its life is extremely short as oxygen is soon absorbed in sufficient amount to destroy it. Although a film of oil renders media suitable for anaerobic growth for a longer time than if sterilized without it, simply boiling the media, or resterilization before using, is the only requisite for the growth and isolation of *Clostridium Welchii.*

2. Food requirements. *Clostridium Welchii* grows upon all of the ordinary culture media, although 1 per cent glucose or maltose stimulates its growth when added to plain meat-extract-peptone agar or broth. To maintain the activity of vegetative forms, transplantations are necessary at least every forty-eight hours. In sugar-free broth, sporulation occurs within two to four days. In broth containing a fermentable substance, all vegetative forms of the organism die within three to five days provided it is present in the vegetative form.

Growth in peptone-free media does not occur. A 1 per cent solution of glucose, maltose or lactose without peptone, a physiological salt solution with 1 per cent glucose, maltose or lactose, a 1 per cent glucose, maltose or lactose, a physiological salt and a 1 per cent gelatin solution fail to show any perceptible growth after two weeks incubation.

The spores of *Clostridium Welchii* can be preserved indefinitely in nearly all media in the absence of glucose, galactose, maltose, lactose, sucrose, dextrin and starch. Media which are vigorously fermented by *Clostridium Welchii* do not permit of its sporulation.

3. Temperature requirements. The optimum temperature for this organism is 37°, growth occuring from 10° to 42°. Below 10° vegetative forms die quickly while spores may remain alive for months if a large quantity are present in the medium.

When vegetative and spore forms of *Clostridium Welchii* are exposed to temperatures below the freezing point, an enormous reduction in numbers is observed. In determining this fact for vegetative forms, pure cultures which had been transferred at least twice into freshly sterilized glucose-liver broth were used. A known number of the organisms from these pure cultures were inoculated into freshly sterilized glucose-liver broth and exposed to temperatures ranging from 13° to 36°F. After each twenty-four hours, the actual bacterial content was determined by the dilution method until the organisms failed to develop in a dilution of one per cubic centimeter. In eight days the bacterial content dropped from 50,000,000 per cubic centimeter to less than one per cubic centimeter with a comparatively uniform rate daily. Of the eight cultures tested, all behaved similarly and produced like results.

With spores grown in peptone-egg media for seven days, a similar reduction occurred, although it took a longer period to cause complete destruction.

Freezing vegetative and spore forms of *Clostridium Welchii* effects a steady death rate which leads to the complete destruction of millions of vegetative forms in the course of seven or eight days, while spores fail to develop after ten to twelve days exposure to this environment.

4. Longevity. The longevity of the vegetative forms has already been discussed. If sugar media are used with meat extract as the base, the life of the vegetative organism is about three days; whereas if liver is used instead of meat extract the organism survives from three to five days. In milk, the organism dies out as a rule in from seventy-two to ninety-six hours. In lactose-liver broth the cultures are in the vegetative stage after nine days incubation. Starch and dextrin-liver-broth preserve the life of the organism for four to six days in some cases, but does not cause sporulation, whereas starch and dextrin broth made from meat extract causes the death of the organism in three to four days. Cultures in sugar media in the incubator die out more rapidly than at room temperature.

Cultures which contain spores may be preserved for many weeks, and in some cases for over a year, provided anaerobic conditions are maintained. The numbers of *Clostridium Welchii* present in any culture and the longevity of the organism is inversely proportional to the length of time necessary for the appearance of "stormy fermentation." A covering of oil prolongs the life of this organism appreciably.

VII. CULTURAL CHARACTERS

1. Agar. a. Gas formation. Gas bubbles appear to some extent in plain agar stab cultures, yet much more abundantly and rapidly in agar containing sugar. In plain agar the presence of gas is delayed from two to ten days, while in sugar agar, fermentation takes place very quickly. Bubbles appear at first along the line of growth, but ultimately permeate the entire medium. At 35° to 37° gas production is most abundant.

On plates of glucose agar, numerous gas bubbles are present after 15 hours growth if the plates are heavily seeded with the organism. From the spaces occupied by the bubbles, as well as upon the surface of the agar, a turbid fluid is pressed out, due to the bacteria present. Deep stab cultures in tubes of glucose agar are accompanied by abundant gas production which results in the fragmentation of the medium and the extrusion of turbid fluid on the surface.

On thickly seeded glucose-liver agar plates, abundant gas production occurs, while on plates containing only a few colonies no gas usually occurs; but occasionally two or three colonies on glucose-liver-agar plates will develop sufficient gas to cause a bubble to appear covering the entire plate under the paraffin. This bubble, if punctured, burns with the blue flame characteristic of hydrogen. Gas formation frequently fails to occur in deep glucose-liver agar stab cultures, when only two or three colonies develop in the lower part of the medium.

Nutrient agar of + 1 reaction with 1 per cent glucose or lactose causes a rapid and luxuriant growth with more abundant and speedy gas formation than occurs on plain nutrient agar without sugar. The odor of plain agar cultures is not putrescent or characteristic. The odor of sugar agar cultures resembles that of sour, stale glue, a condition more pronounced with some strains than others.

b. Colonies. Colonies of *Clostridium Welchii* do not develop in plain meat extract agar unless all the oxygen is first removed and strict anaerobic methods employed in the plating. The more strictly anaerobic the conditions employed the larger the colonies as shown by the fact that the colonies in stab nutrient or sugar agar, are very much larger at the lower part of the needle track. In fact when agar stab cultures are inoculated (after sufficient steaming to dispel the air) growth appears most abundant at the bottom of the stab, ceasing to develop some distance below the surface, according to the degree of the anaerobiosis.

Colonies of *Clostridium Welchii* in plain and sugar agar are opaque white, grayish white, or even of a brownish white color by transmitted light, sometimes with a central darker dot, the opacity of the color depending upon the thickness of the colony. The dark center appears in all media in which the organism sporulates.

Young cultures vary in size from 0.5 to 1 mm. in diameter, but frequently increase to diameters of 2 to 3 mms. or even larger. The colonies in plain agar are very minute pin points with fuzzy edges and with a zone around them resembling a halo. A dark center appears in the colony after ten to twelve

days' incubation. The colonies are much larger in media containing sugar than on plain agar. The size of colonies on glucose-liver-agar varies greatly from minute nearly microscopic colonies on thickly seeded plates to those measuring from 3 to 5 mm. on plates with few colonies.

The colonies in stab cultures, and those deep in the agar on plates, appear as spheres or ovals, generally more or less flattened with irregular contours, the irregularity being due to little feathery projections or prongs from the surface of the colonies. The colonies are very firm, and retain their shape and consistency when touched with a needle.

2. Gelatin. Gas is formed in ordinary nutrient gelatin (reaction + 1) neutral glucose-gelatin and neutral glucose-liver-gelatin. The gas production in these media varies, nutrient gelatin showing the least and glucose-liver gelatin the most abundant production. Growth occurs in all three at 20° and 37°, the glucose-liver cultures developing more rapidly and abundantly than nutrient gelatin cultures.

A slight initial softening is observed in the 20° cultures, due to the peptonization of the gelatin at the top of the stab; later the growth settles downward along the line of puncture, until in some cases the entire medium liquefies.

3. Broth. Growth occurs under strictly anaerobic conditions in sugar-free broth, and in broth containing glucose, sucrose, maltose, lactose, dextrin, starch, liver and inulin. The gas is so abundant in the sugar broth that small bubbles of gas rise to the surface and accumulate to form a "foamy" layer, presenting a "steaming" appearance. At 37° growth takes place in two or three hours, the clear broth first becoming cloudy with abundant sediment at the bottom of the tube. When gas formation starts it goes on with great rapidity for about twenty-four hours in plain broth and still longer in sugar broth. After the fermentation ceases the diffuse cloudiness disappears and the sediment settles in the course of a short time, rendering the cultures clear and transparent. The sediment is white, uniform and flaky and more abundant in sugar broth than in plain broth. If the sediment is disturbed, it floats in a viscid thread or cloud produc-

ing again diffuse cloudiness, which quickly settles upon standing. In a few cases the broth is stringy and very viscuous, yet the sediment settles out leaving the supernatant fluid as in the other cases. After the development is complete, the reaction is decidedly acid. The odor of the cultures is not putrescent but resembles sour, stale glue as in agar and gelatin cultures. Glucose, galactose, plain liver, sucrose, maltose, lactose, starch, dextrin and in a few cultures inulin, lead to a most violent fermentation when added to plain broth. Different strains vary widely in their fermentative ability with glycerol and inulin. An extreme acid production is obtained with all the sugars, the maximum reaction being reached in about forty-eight hours. In peptone water containing coagulated egg-white, physiological salt solution and coagulated egg-white, and an aqueous solution of coagulated egg-white, *Clostridium Welchii* causes the so-called "stormy fermentation" which is characteristic in milk. Mannitol broth, 1 per cent casein solution, plain peptone water and Dunham's solution are not fermented appreciably, less than 10 per cent of gas appearing after three days. In media containing coagulated egg-white a black deposit occurred in the white sediment.

4. Milk. After twenty-four to forty-eight hours' incubation at 37°, milk tubes inoculated with pure cultures of *Clostridium Welchii* showed the so-called "stormy fermentation," coagulation and derangement of the curd with gas bubbles rising to the top of the tube. After an incubation of forty-eight hours the digestion is complete, leaving a yellowish whey. Market milk which has been heated for fifteen minutes at 82° and incubated at 37° shows after twenty-four to seventy-two hours variations of the typical reaction from the typical "stormy fermentation" to a slight digestion of the curd, with little or no gas formation. Where gas formation failed to appear the reaction was due, presumably, to insufficient anaerobiosis of the medium. Frequently a culture from a milk tube showing no gas, when inoculated in other milk tubes freshly sterilized, would cause "stormy fermentation" after incubation. Much acid is produced from this fermentation, its production ceasing only upon the exhaustion of the fermentable materials present. Butyric acid is produced

in large amounts, as is detected by its characteristic odor. Litmus milk turns from blue to red very rapidly after inoculation upon incubation showing the increase of acidity.

5. Potato. *Clostridium Welchii* was grown in broth tubes containing pieces of potato and sterilized under oil. Gas production was rapid and growth was abundant in twenty-four hours. The starch was so vigorously attacked in some cases that the potato was penetrated by the organism. The colonies, which appeared on the potato, were thin, moist and grayish-white on the surface and through the pieces. No spores were demonstrated in this medium, either microscopically or experimentally.

6. Blood serum. Growth is abundant on the smeared surface of blood serum after twenty-four hours incubation at 37°. Digestion of the medium soon takes place, changing the consistency of the coagulated serum and liquefying it completely in from four to six days. Cultures live in this medium for at least six months, spores appearing first on the sixth day of incubation.

The colonies on blood serum appear round, about 7 mm. in diameter, opaque grayish-white with finely granular edges somewhat frayed. A foul odor is given off after several days growth. These facts are based on experiments with 20 strains of *Clostridium Welchii* isolated from market milk and inoculated on Loeffler's blood serum.

VIII. CHEMICAL CHARACTERS

1. Action on carbohydrates. a. Gas production. With all the monosaccharides, disaccharides and polysaccharides used, 100 per cent of gas is produced, the time required for such production being from eighteen to thirty-six hours. In nearly every case little or no gas is formed after forty-eight hours. Inulin is fermented in some cases but mannitol and glycerol are never fermented by any strain of this organism.

b. Acid production. The amount of acid produced by the growth of *Clostridium Welchii* in sugar media ranges from less than 1 to 12 per cent as determined by titrating against $\frac{N}{10}$ alkali,

using phenolphthalein as indicator. The most abundant acids are lactic, acetic and butyric. The acidity in all cases attains its maximum between thirty-six and forty-eight hours incubation at 37°, after which there is a slight drop to a value which remains constant indefinitely, regardless of the longevity of the organism.

2. Action on proteins. *Clostridium Welchii* is characteristically a fermentative organism and attacks proteins very slightly, and then only in the absence of fermentable carbohydrates. If sugar is present in a medium containing protein, the organism acts upon it readily, leaving the protein unattacked. Egg-meat mixture is not attacked appreciably, but produces a disagreeable odor upon standing. The egg-meat mixture used is prepared as follows:

A. One-half pound of lean chopped beef is placed in 250 cc. of water, neutralized with sodium carbonate and heated for thirty minutes in the Arnold sterilizer with occasional stirrings. This is then set away in a cold place for several hours after which the fatty scum is removed.

B. The whites of three eggs are placed in 250 cc. of water, neutralized and heated with occasional stirring in the Arnold sterilizer for thirty minutes to coagulate the albumen.

A and B are mixed and 2.5 grams of 0.5 per cent calcium carbonate (powdered) are added. The flask is plugged and sterilized for thirty minutes at 112°.

When this mixture is inoculated with *Clostridium Welchii* there appears just the slightest fermentation due to the small amount of sugar present in the meat and egg.

Growth occurs in a 1 per cent casein solution, but the casein is unattacked by the different strains isolated from milk and feces. Peptone water alone and peptone in physiological salt solution remain unattacked. In liver broth, the glycogen is acted upon very readily with vigorous gas fermentation, whereas the protein is slightly, if at all affected. On albuminous matter alone, however, there appears some proteolytic activity which, when a little sugar is present, is increased enormously.

No evidence of fermentation occurred in cultures of *Clostridium Welchii* grown in peptone water, egg-white and egg-yolk solutions,

a 1 per cent casein solution, and egg-meat mixture incubated for one week at 37.5. In the case of peptone egg-white 100 per cent of gas forms after a week's incubation. After two weeks' growth spores are observed in all the media except casein.

While there appeared to be a slight decrease in the bulk of the proteins in some instances, as well as disagreeable odors, the transformation never assumed the character of real putrefaction. The unpleasant odor is presumably due to the presence of butyric and closely allied acids and not to any product of decomposition of proteins.

By putrefaction is meant the ability to decompose native proteins with the formation or liberation of foul-smelling products, mercaptan, aromatic oxy-acids, etc. Rettger, 1908, in his "Studies on Putrefaction" claims that not all obligate anaerobes have the property of producing putrefaction and has divided the strict anaerobes into four classes, in so far as their bio-chemical characters are concerned, as follows.

First, those that produce very little or no putrefactive changes or fermentation with evolution of gas; second, those that have a strong putrefactive action on native proteins but fail in fermentative properties; third, those which are primarily fermentative organisms and whose putrefactive functions are very slight or perhaps absent; and fourth, those which have very marked putrefactive and fermentative properties. Herter, 1907, ascribes marked putrefactive properties to *Clostridium Welchii* and regards it as being largely responsible for certain intestinal disturbances although he uses the term putrefaction in a much broader sense. Passini, 1905, also asserts that the bacillus of gaseous phlegmon produces an abundance of indol when grown in pure culture on blood serum. This is contrary to my observations on indol production. Tissier and Martelly, 1902, claimed that *C. perfringens* isolated from putrefying meat and resembling *Clostridium Welchii* had a decided putrefying action on blood fibrin although the action was slow. Notwithstanding these views, using the foregoing classification of Rettger's (1908), *Clostridium Welchii* from my observations on the action of sugar-free native proteins would fall in his third class of anaerobes, namely "primary

fermentative and whose putrefactive functions are very slight or absent."

3. Enzymes. Enzyme action is shown in the liquefaction of gelatin and blood serum and the peptonization of milk casein. Diastatic enzymes may be detected by growing cultures of *Clostridium Welchii* in starch solution when the starch is converted into sugars.

IX. CLASSIFICATION

1. Historical. The success that has attended previous investigators in the use of certain carbohydrates and other fermentable substances as a means of differentiating between the different members of a group suggested the use of these substances as a means of distinguishing members of the *Clostridium Welchii* group.

Little has been accomplished in the separation of the members of this group due presumably to the fact that the differences are so small and that it has been impossible to obtain uniform conditions.

Fraenkel, 1902, and Klein, 1900–1902, recognized only one variety of the organisms isolated by them. Schattenfroh and Grassberger, 1900, and Passini, 1905, differentiated two species, virulent and non-virulent. Hitschmann and Lindenthal, 1899, were unable, either from the behavior in the animal body or in cultures, to differentiate varieties. They found tests of pathogenicity especially unreliable as a means of differentiation. Passini, 1904, Werner, 1905, and Rocchi, 1911, by means of serological reactions attempted to distinguish subvarieties but failed. Herter, 1907, was also unsuccessful in his attempt to apply such factors as sporulation, pathogenicity, hemolytic properties, indol production and rapidity of gas formation in man and animals.

Jackson, 1912, described two types, one motile, neutral reaction with no gas formation in lactose bile and 56 per cent gas production in mannitol broth; the other non-motile, 92 per cent gas production in lactose bile and 10 per cent gas production in mannitol broth.

Simonds, 1915, based his classification upon a study of 30 strains according to their fermentative ability in inulin and glycerol broths and their ability to sporulate in neutral media containing these substances. He was able to divide the group into four sub-groups, but he concludes that the source of the culture is no indication of the sub-group to which it belongs although all strains isolated from cow feces and milk fall into the same sub-group.

2. Experimental. The results obtained by this study of over 100 strains isolated from different sources show two possibilities for the classification of *Clostridium Welchii*, namely, (1) the fermentative reactions in glycerol and inulin broth with sporulation in these media and (2) the frequency curves derived from the fermentation of the carbohydrates, glucose, galactose, lactose, maltose, sucrose, starch, dextrin and liver broth. Classification of the *Clostridium Welchii* group could not be effected by the use of such factors as, longevity in different sugars and nutrient broth, sporulation in media of different reactions ranging from 10 per cent acid to 3 per cent alkaline media, thermal death points of vegetative and spore forms, fermentation with acid production in liver media containing the above named carbohydrates, pathogenicity and its relation to virulent and avirulent types, fermentative reactions in protein-free media, liquefaction of gelatin and blood serum, rapidity of gas formation in liver media and animals, motility and the retention of the gram stain. Where variation occurred in these respects there was no characteristic sufficiently stable to serve as a basis for classification. In most of the tests applied, the results were constant throughout and showed the same characteristic with little or no variation.

The pathological study of the organism, as has been discussed in a previous section, reveals interesting and enlightening facts yet they fail to show any characteristic classification. In all cultures isolated from human feces very rapid pathogenic results follow, while from the cow, varying results are obtained. For the most part, however, the bacillus isolated from cow feces fails to set up a characteristic fatal necrosis of the subcutaneous tissue.

a. Classification of *Clostridium Welchii* by fermentative reactions in glycerol and inulin broths. The fermentative reactions of glycerol and inulin broth with sporulation in these neutral media, showed that the members of this group were divided into four subgroups. Fifty-six strains of *Clostridium Welchii* isolated from the following sources: Milk (pasteurized and raw) 42; human stools (normal) 5; cow feces 9; were subjected to this test. All conditions were standardized as far as possible. Pure cultures were obtained through sufficient plating of these organisms and were grown in glucose-liver broth tubes and incubated for another twenty-four hours, after which two drops of these cultures were inoculated into glycerol and inulin. The broths were prepared by rendering neutral to phenolphthalein a 1 per cent solution of peptone and inulin and a 1 per cent solution of peptone and 6 per cent of glycerol. After a forty-eight hour incubation at 37°, the tubes were removed from the incubator and titrated against $\frac{N}{10}$ NaOH with phenolphthalein as an indicator and the acidity recorded. Gas fermentation and sporulation was also determined for all the strains.

Tables 2 and 3 give the results of this study of 56 strains, the four principal subgroups being characterized as follows:

Subgroup 1. Cultures not producing acid or gas from either inulin or glycerol and forming spores in both inulin and glycerol broths. Pathogenicity variable.

Subgroup II. Producing acid with or without gas in inulin but not from glycerol and forming spores in glycerol broth. Pathogenic for guinea-pigs in most cases.

Subgroup III. Producing acid without gas from glycerol but not from inulin and forming spores in inulin broth. Pathogenicity variable.

Subgroup IV. Producing acid from both glycerol and inulin with or without gas production with no formation of spores. Pathogenic in most cases.

The source of the culture gives no indication as to the subgroup to which it belongs as shown by the grouping of the above strains.

TABLE 1

Study of 56 strains from milk, cow feces, and human feces, with regard to acid production and gas, divided into 4 sub-groups according to fermentation of inulin and glycerol

SOURCE	NUMBER	GLUCOSE	GALACTOSE	MALTOSE	LACTOSE	SUCROSE	DEXTRIN	STARCH	INULIN		GLYCEROL		PEPTONE H_2O
		Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid	G	Acid	G	Acid G
Subgroup I													
Milk	7	2.0+	1.2+	1.2+	1.9+	2.6+	1.9+	2.55+	0	0	0	0	0.4−
	18	2.45+	1.7+	1.25+	1.9+	2.7+	2.25+	2.4+	0	0	0	0	0.4−
	20	2.0+	1.2+	1.9+	2.0+	2.6+	3.05+	3.3+	0	0	0	0	0.4−
	21	2.35+	1.15+	0.85+	1.35+	2.65+	2.75+	2.45+	0	0	0	0	0.35−
	24	2.3+	1.15+	1.2+	1.4+	2.7+	1.9+	2.3+	0	0	0	0	0.4−
	26	1.95+	1.15+	1.3+	1.4+	2.05+	2.4+	2.5+	0	0	0	0	0.4−
	1	1.8+	1.3+	1.35+	1.6+	2.55+	3.1+	2.5+	0	0	0	0	0.4−
	6	2.35+	1.25+	1.65+	0.55+	2.95+	2.1+	1.85+	0	0	0	0	0.35−
	C	2.3+	1.2+	1.55+	1.55+	2.6+	3.00+	2.5+	0	0	0	0	0.35−
	36	2.2+	1.0+	1.6+	1.6+	2.6+	2.6+	2.6+	0	0	0	0	0.5−
	T	2.8+	1.85+	1.65+	1.85+	2.65+	2.4+	2.4+	0	0	0	0	0.35−
	R	2.3+	1.5+	1.1+	1.4+	1.0+	2.7+	2.7+	0	0	0	0	0.4−
Cow feces	5	1.9+	0+	1.1+	2.05+	2.6+	3.00+	2.5+	0	0	0	0	0.45−
	7	2.1+	0+	1.8+	0.75+	2.6+	0.6+	3.00+	0	0	0	0	0.4−
	11	2.15+	0+	1.1+	2.0+	2.6+	0.55+	3.00+	0	0	0	0	0.5−
	13	2.3+	0.65+	1.7+	2.1+	2.6+	0.5+	1.6+	0	0	0	0	0.4−
	12	1.85+	0.5+	2.0+	2.0+	2.5+	0.25+	2.6+	0	0	0	0	0.5−

Subgroup II														
Milk	2	2.1+	0.9+	1.9+	1.3+	2.9+	3.1+	2.45+	1.2	30	0	0	0.4−	
	P	2.55+	1.25+	1.85+	1.7+	2.6+	1.65+	1.35+	0.8	40	0	0	0.35−	
	25	2.45+	1.2+	1.7+	1.7+	2.6+	1.7+	1.5+	1.4	30	0	0	0.2−	
	45	2.05+	1.2+	1.2+	1.4+	2.7+	2.35+	1.9+	0.6	40	0	0	0.3−	
	Y	2.25+	1.35+	0.55+	0.65+	2.7+	2.4+	2.1+	1.2	30	0	0	0.4−	
	K	2.55+	1.55+	0.8+	1.65+	3.1+	3.15+	2.95+	0.85	30	0	0	0.35−	
Cow feces	4	2.0+	1.2+	1.1+	2.05+	2.6+	2.5+	1.8+	0.8	40	0	0	0.5−	
	8	1.9+	0+	1.0+	2.0+	2.6+	2.5+	+	1.05	0	0	0	0.6−	
	17	2.4+	0+	1.0+	2.0+	2.45+	+	+	1.05	0	0	0	0.5−	
Human feces	6	2.55+	0.3+	1.7+	1.2+	2.8+	2.9+	3.15+	1.5	0	0	0	0.4−	
	5	2.4+	1.6+	1.8+	1.5+	2.8+	2.9+	3.15+	1.1	0	0	0	0.3−	
Subgroup III														
Milk	3	2.5+	1.6+	2.05+	1.7+	3.0+	2.65+	2.2+	0	0	0.50	0	0.3	0
	4	2.3+	1.55+	1.6+	1.7+	3.1+	2.85+	2.75+	0	0	0.30	0	0.4	0
	9	2.35+	1.2+	1.4+	1.8+	2.5+	1.9+	2.35+	0	0	0.20	0	0.4	0
	11	1.9+	0.9+	1.4+	1.4+	2.5+	1.6+	2.35+	0	0	0.30	0	0.4	0
	12	2.5+	1.3+	1.1+	1.55+	2.75+	2.6+	2.3+	0	0	0.55	0	0.45	0
	13	2.2+	1.5+	1.35+	1.95+	3.05+	2.75+	2.45+	0	0	0.45	0	0.35	0
	17	2.6+	0.85+	1.7+	1.50+	3.00+	1.75+	1.7+	0	0	0.30	0	0.3	0
	22	2.55+	1.9+	2.1+	2.0+	1.4+	2.25+	2.55+	0	0	0.80	0	0.3	0
	J	1.95+	1.3+	0.75+	1.4+	2.55+	2.65+	2.2+	0	0	0.35	0	0.4	0
	N	2.95+	1.8+	1.35+	2.0+	1.1+	2.8+	2.45+	0	0	1.15	0	0.3	0
	U	2.25+	1.25+	1.45+	1.4+	2.25+	2.3+	2.2+	0	0	0.35	0	0.3	0
	40	2.0+	0.8+	1.4+	1.4+	2.15+	2.4+	1.9+	0	0	0.20	0	0.5	0
	42	1.95+	1.25+	1.75+	0.7+	2.7+	2.2+	1.85+	0	0	0.30	0	0.5	0
	47	2.45+	1.45+	1.95+	1.95+	1.15+	2.05+	2.5+	0	0	1.15	0	0.35	0
	27	2.8+	1.5+	0.95+	1.45+	1.5+	2.05+	3.1+	0	0	1.15	0	0.35	0
Cow feces	10	1.85+	0+	0.8+	2.0+	2.5+	2.5+	2.6+	0	0	1.15	0	0.50	0

TABLE 2—*Continued*

SOURCE	NUMBER	GLUCOSE	GALACTOSE	MALTOSE	LACTOSE	SUCROSE	DEXTRIN	STARCH	INULIN	GLYCEROL	PEPTONE H_2O
		Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G	Acid G
Subgroup IV											
Milk	7	2.55+	1.6+	1.05+	1.4+	3.3+	2.55+	2.95+	0.8 30	0.40 0	0.4 0
	8	2.45+	1.55+	1.7+	1.65+	2.8+	2.1+	2.55+	0.8 30	0.40 0	0.4 0
	E	1.85+	1.65+	0.95+	0.65+	2.5+	2.55+	2.25+	0.5 0	0.55 0	0.35 0
	Q	3.05+	1.25+	0.55+	+	1.1+	2.35+	2.35+	1.45 25	1.15 0	0.35 0
	S	2.05+	1.5+	1.65+	1.95+	2.6+	1.7+	1.6+	0.65 50	0.80 0	0.35 0
	V	2.00+	1.25+	1.6+	1.1+	2.3+	2.5+	1.8+	0.6 0	0.35 0	0.4 0
	22	2.6+	1.45+	1.1+	1.05+	3.0+	2.5+	2.5+	1.05 30	1.65 0	0.3 0
	23	2.1+	1.3+	1.15+	0.7+	2.8+	2.7+	1.8+	0.6 25	0.65 0	0.4 0
	31	2.7+	0.5+	1.45+	1.45+	3.25+	2.5+	2.85+	1.4 20	0.6 0	0.45 0
Human feces	4	2.3+	1.65+	1.2+	1.5+	2.75+	2.5+	2.3+	0.7 0	0.8 25	0.4 0
	7	2.0+	1.25+	0.55+	0.55+	2.55+	2.55+	3.0+	0.7 0	0.3 0	0.45 0
	10	1.95+	0.7+	1.75+	0.55+	2.5+	0.75+	3.0+	1.05 0	0.4 0	0.25 0

TABLE 3

Summary of source of principal subgroups

SUBGROUP	MILK	COW	HUMAN	TOTAL
I	12	5	0	17
II	6	3	2	11
III	15	1	0	16
IV	9	0	3	12
	42	9	5	56

b. Frequency curves. Using the results obtained from a study of the 56 strains used in this classification, frequency curves were plotted for the carbohydrates, glucose, galactose, maltose, lactose, sucrose, starch, inulin, glycerol and dextrin and liver.

The curves show an entirely irregular variability without any particular significance.

X. THERMAL DEATH POINT

1. Method. The thermal death point of strains of *Clostridium Welchii* was determined both in the vegetative and spore stage according to the procedure in Standard Methods for Water Analysis, 1912, except that five loopfuls of the culture were used instead of three in order to overcome the influence of the film of oil and insure inoculation of the organism. Cultures were plated at least twice to insure their purity and if on microscopical examination any contamination was present, plating was repeated until a pure culture was obtained. The pure cultures were grown in glucose-liver broth for twenty-four hours and then transferred to fresh glucose-liver broth and grown for another twenty-four hours.

The sources from which *Clostridium Welchii* was isolated to study thermal death point were market milk, human feces, cow feces, and horse feces. In the case of the cultures of market milk, some had been subjected to artificial cultivation for four months, others for three months, and still others for two weeks before testing their thermal death point while the cultures from

the fecal sources were tested as soon as possible after they were isolated in pure culture. For the determination of the thermal death point, tests were made on 75 cultures from market milk, 54 cultures from human feces, 18 from cow feces and 18 from horse feces. The thermal death point of the spores in cow and horse feces was not determined.

2. Thermal death point of vegetative forms. Tubes containing 10 cc. of glucose-liver broth were inoculated with two-tenths cc. of the pure cultures and grown for eight, twenty-four and forty-eight hours at 37.5°. Cultures were also grown in sterile milk. The best and most consistent results were obtained by growing at 37.5° for eight hours. A double water-bath was used to keep the temperature uniform. This bath was heated to the desired temperature and a sufficient supply of tubes each containing 10 cc. of sterile glucose-liver broth was placed in the inner bath. The temperature of the bath was recorded in a control tube by a thermometer placed in the broth which was not allowed to vary more than half a degree in either direction. The water in the bath was agitated by means of a stirring rod throughout the heating. After fifteen minutes exposure at the desired temperature, the broth was inoculated with five loopfuls of the eight-hour broth culture to be tested by simply removing the cotton plug, without removing the tube from the bath. No attempt was made to determine the numbers per cubic centimeter before and after the heat exposure, either of the vegetative or the spore forms. The amount was kept as uniform as possible using the same size loop and uniform 1 cc. pipettes. The inoculated tubes were exposed for fifteen minutes at this temperature and were then transferred at once to a vessel of cold water in order to cool them quickly and prevent further action of the heat upon the bacteria. When cold they were placed in the incubator at 37.5° and kept under observation long enough to ascertain whether growth occurred. *Clostridium Welchii* in the vegetative stage showed evidence of growth in twelve to forty-eight hours if the heat had not killed the organism. Duplicate tubes were inoculated to check the determination and in practically all cases the same thermal death point was obtained.

In no case was there more than a degree difference in duplicate samples. Control tubes were always run at 37° and 80° for fifteen minutes to determine whether the organism was alive before heating and to ascertain the presence of spores. All cultures used showed the characteristic "stormy fermentation" of milk previous to the test. The temperatures used to determine the thermal death point of vegetative forms were 56°, 57°, 58°, 59°, 60°, 61°, 62°, 63° and 80°.

3. Thermal death point of spores. The determination of the thermal death point of *Clostridium Welchii* in the spore stage was determined in a manner similar to that described above using the same pure cultures grown for eight hours by inoculating 0.5 cc. of the "steaming" culture into a neutral medium of peptone-egg and growing at 37.5° for one week. At the end of the seven days' incubation, the resistance of the spores to high temperatures was determined. The cultures to be tested were subjected to temperatures from 85° to 101°, raising the temperature one degree at a time. All the spore cultures survived 86° and some survived 100° for fifteen minutes and thirty minutes. The tubes containing the inoculated material were cooled immediately after the fifteen minute exposure at the desired temperature and incubated at 37° until definite results were obtained. In the majority of cases twenty-four hours' incubation was sufficient for the organism to germinate, but in some cases germination did not occur until after four or five days in the incubator. When doubtful results were obtained, 0.5 cc. of the culture containing spores was inoculated instead of the five loopfuls to make sure that there were sufficient spores present. The use of 0.5 cc. was however resorted to only in exceptional cases, for where negative results were obtained with five loopfuls, the same result was found with the larger quantity.

4. Discussion of results. a. Vegetative forms. The thermal death point of vegetative forms of *Clostridium Welchii* varies according to the source from which the cultures are isolated and the duration and conditions of growth previous to the test. Cultures isolated from market milk survive 56° and die at 61°, although cultures immediately after isolation die at 59°. The

thermal death point of the vegetative forms in human feces is from 59° to 61°. In no case does a vegetative culture survive 63°, provided no spores are present. This is the temperature of pasteurization. Using the data obtained from this work, frequency curves showed no indication of more than one type in this group.

b. Spores. In the case of spores of *Clostridium Welchii*, there seem to be two thermal death points, namely, either the cultures resist 100° for fifteen minutes and longer or they die below 90°. Some of the cultures survive 100° for thirty and forty minutes. Two exceptions arise in the cases of number 11 and number 13 which fail to survive above 95°. Repeated tests confirmed the failure of these strains to survive boiling. Spores from human feces do not survive boiling in any case. Frequency curves show conclusively that the thermal death points of spores fall into two distinct groups.

c. Relationship between vegetative forms and spores. An inspection of the charts shows there is no correlation between the thermal death points of the spores and the vegetative forms for the thermal death point of vegetative forms may range from 56° to 60°, while spore forms of corresponding cultures resist 100°. Moreover, spores dying at 86° to 88° may resist 58° and 59° in the vegetative stage. It is impossible to predict the thermal death point of the spore culture provided the thermal death point of the vegetative culture for the same strain is known or vice versa.

d. Classification of sugar fermentation as compared to thermal death points. The classification of the members of the *Clostridium Welchii* group into four subgroups on a basis of the fermentative reactions in glycerol and inulin, as shown in a preceding section, bears no relationship to the two distinct groups based on the thermal death points of spores. Each of the four subgroups in the classification according to fermentation contains members of both groups of thermal death points. The same is true of the vegetative forms, each group containing cultures whose resistance to heat varies widely.

e. Relation of the time since isolation to the thermal death point. Table 4 shows the effect of prolonged artificial culti-

TABLE 4

Thermal death points

NUMBER ORIGINAL	TEMPERATURE BEFORE TESTING																								
	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	55	56	57	58	59	60	61	62
	Spores																	Vegetative							
Cultures isolated from milk and kept four months before testing																									
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
3	+	+	+	+	+	−	−	−	−	−	−					−	−	+	+	+	+	−	−	−	−
4	+	+	+	−	−	−	−	−	−	−	−					−	−	+	+	+	+	−	−	−	−
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
7	+	+	+	+	−	−	−	−	−							−	−	+	+	+	+	−	−	−	−
8	+	+	+	−	−	−	−	−	−							−	−	+	+	+	+	−	−	−	−
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
11	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−
13	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
14	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	
Cultures isolated from milk and kept three months before testing																									
A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
B	+	+	−	−	−	−	−	−								−	−	+	+	+	+	−	−	−	−
C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
E	+	+	+	−	−	−	−	−								−	−	+	+	+	−	−	−	−	
F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
J	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−		
K	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−		
L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
M	+	+	+	+	+	+	−	−	−	−						−	−	+	+	+	−	−	−	−	
N	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−	−	
O	+	+	+	+	−	−	−	−	−							−	−	+	+	+	+	−	−	−	
P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	
Q	+	+	+	+	−	−	−	−	−							−	−	+	+	+	+	−	−	−	
R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	
U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	

TABLE 4—*Continued*

	Number original	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	55	56	57	58	59	60	61	62
		Temperature before testing — Spores																	Vegetative							
	V	+	+	+	−	−	−	−									−	−	+	+	+	+	−	−	−	
	W	+	+	+	−	−	−	−									−	−	+	+	+	+	−	−	−	
	X	+	+	−	−	−	−	−									−	−	+	+	+	+	−	−	−	
	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	
Cultures isolated from milk and kept one and a half months before testing																										
	45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
	46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
	47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
	48	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	
	49	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	
	50	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	
Cultures isolated from milk and kept two weeks before testing																										
1	20	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	21	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
3	22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−		
	23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−		
	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
	25	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		
6	26	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−		
	28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
	29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−		
	30	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		
	31	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−		
10	32	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		
	33	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	34	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	35	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
11	36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
	37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−		
14	38	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
20	40	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−		
	41	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−		
	42	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	43	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−		
	44	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−		

vation on the thermal death point of vegetative forms of *Clostridium Welchii*. Of 20 strains isolated from market milk and tested after four months' cultivation on artificial media, all were negative at 61°, two survived 60°, nine survived 59° and all survived 58°. Of 24 strains from market milk grown for three months all failed to survive 60°, two survived 59°, 14 survived 58° and all survived 57°. These cultures were kept in the spore form in peptone egg for a month and a half before testing, whereas the first 20 strains were transferred daily or every forty-eight hours to fresh sugar media during the four months. Twenty-five freshly isolated strains grown for two weeks gave the following results:—all survived 56°, 18 survived 57° and eight survived 58°. All cultures after four months survived 58°, after three months 57°, while the corresponding value for freshly isolated strains was only 56°.

Of the spore forms seven out of 20 (35 per cent) failed to survive boiling after four months' growth, eight out of 24 ($33\frac{1}{3}$ per cent) failed to survive boiling after three months' growth and 15 out of 25 (60 per cent) after two weeks' growth.

f. Relation of age of cultures and kind of medium to thermal death point. Tables 5 and 6 show the greater resistance to heat of cultures from milk and feces when grown in glucose-liver broth for more than eight hours before testing. In 13 out of 20 cultures from market milk, growth for twenty-four hours increases the thermal death point 1°. In the other cases no difference was observed under identical conditions. In 19 cultures from human feces, growth for forty-eight hours increased the thermal death point 1° in every case.

Table 5 gives also the results of 19 cultures grown in sterile milk for sixty-six hours in comparison to the growth in glucose-liver broth and shows variations in resistance to heat. Seven of the cultures had the same thermal death point as in the eight-hour glucose-liver broth cultures, six cultures resisted 1°C. higher while the remaining six survived a rise of 2°C. above the eight-hour cultures Probably cultures isolated from feces and grown in sterile milk for eight hours approximate the same resistance to heat as the milk cultures themselves.

g. Thermal death point to cultures isolated from feces and milk. Tables 7 and 8 show the variation in thermal death point of cultures freshly isolated from milk and feces when subjected to the same conditions. Of 25 cultures from market milk all survived 56°, 18 survived 57°, and eight survived 58°;

TABLE 5

Thermal death points. Cultures freshly isolated from human feces and grown for eight hours and twenty-four hours in glucose-liver broth and sixty-six hours in sterile milk

NUMBER	EIGHT HOURS—GLUCOSE-LIVER BROTH								TWENTY-FOUR HOURS—GLUCOSE-LIVER BROTH								SIXTY-SIX HOURS—STERILE MILK							
	Temperature								Temperature								Temperature							
	57	58	59	60	61	62	63	80	57	58	59	60	61	62	63	80	57	58	59	60	61	62	63	80
	Vegetative								Vegetative								Vegetative							
1	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	–	–	–	–	–
2	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–
6	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–
8	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
10	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–
13	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–
16	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–
19	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–
4	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–
5	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–
12	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–	–
14	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–
15	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–	–
17	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–
3	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	–
7	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	–
9	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–
11	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–
18	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–

Spores did not survive 100°C. in any strain.

of 55 cultures from human feces, all survived 59°, 21 survived 60°, and five survived 61°; of 18 cultures from cow feces, all survived 59°, 16 survived 60°, 13 survived 61°, and five survived 62°; and of 18 cultures from horse feces, all survived 57°, 11 survived 58°, ten survived 59° and three survived 60°.

TABLE 6

Thermal death points. Showing greater resistance to heat after longer periods of growth and uniformity of subcultures from same colony. Full line indicates twenty-fourth hour culture. Dotted line indicates eight hour culture. I_B and I_A = subcultures from original sample. 1, 2, 3, 4 = single colonies from subcultures I_B and I_A

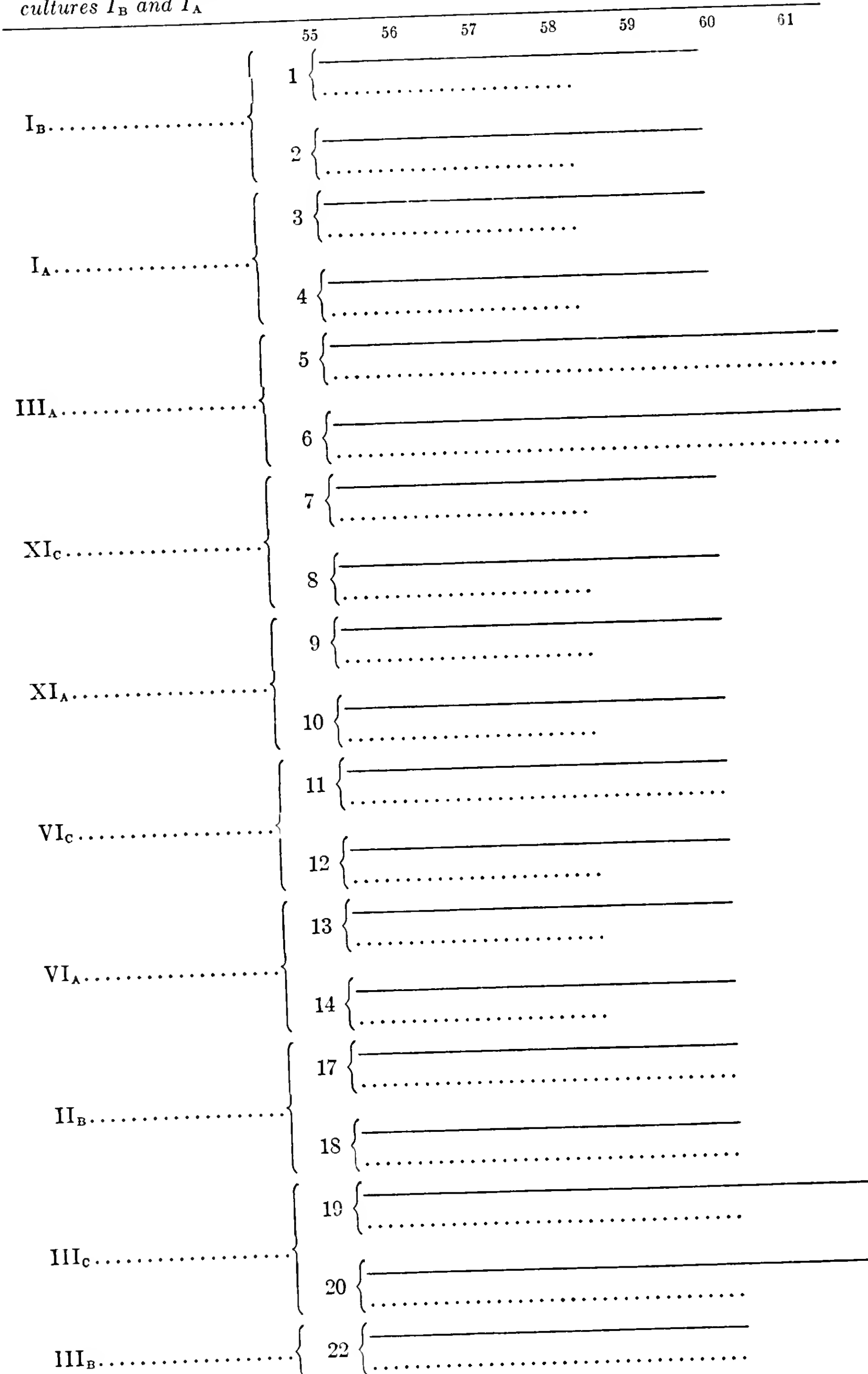

TABLE 7

Thermal death points. Cultures freshly isolated from feces

NUMBER ORIGINAL		TEMPERATURE								
		55	56	57	58	59	60	61	62	63
		Vegetative								
		Human								
N_6	1	+	+	+	+	+	–	–	–	–
	2	+	+	+	+	+	–	–	–	–
	3	+	+	+	+	+	–	–	–	–
N_8	4	+	+	+	+	+	–	–	–	–
	5	+	+	+	+	+	–	–	–	–
	6	+	+	+	+	+	–	–	–	–
E_{11}	7	+	+	+	+	+	–	–	–	–
	8	+	+	+	+	+	+	–	–	–
G_8	9	+	+	+	+	+	–	–	–	–
	10	+	+	+	+	+	–	–	–	–
	11	+	+	+	+	+	–	–	–	–
	12	+	+	+	+	+	–	–	–	–
I	13	+	+	+	+	+	–	–	–	–
	14	+	+	+	+	+	–	–	–	–
	15	+	+	+	+	+	–	–	–	–
	16	+	+	+	+	+	–	–	–	–
III	17	+	+	+	+	+	+	–	–	–
	18	+	+	+	+	+	–	–	–	–
	19	+	+	+	+	+	+	–	–	–
	20	+	+	+	+	+	–	–	–	–
	21	+	+	+	+	+	–	–	–	–
IV	22	+	+	+	+	+	–	–	–	–
	23	+	+	+	+	+	+	–	–	–
	24	+	+	+	+	+	+	–	–	–
	25	+	+	+	+	+	–	–	–	–
	26	+	+	+	+	+	+	–	–	–
V	27	+	+	+	+	+	–	–	–	–
	28	+	+	+	+	+	+	–	–	–
	29	+	+	+	+	+	–	–	–	–
	30	+	+	+	+	+	–	–	–	–
	31	+	+	+	+	+	+	–	–	–
	32	+	+	+	+	+	+	–	–	–
	33	+	+	+	+	+	–	–	–	–
	34	+	+	+	+	+	–	–	–	–
	35	+	+	+	+	+	–	–	–	–
	36	+	+	+	+	+	+	–	–	–

TABLE 7—*Continued*

NUMBER	ORIGINAL	TEMPERATURE								
		55	56	57	58	59	60	61	62	63
		Vegetative								
		Horse								
1	1	+	+	+	−	−	−	−	−	−
	2	+	+	+	−	−	−	−	−	−
2	3	+	+	+	−	−	−	−	−	−
	4	+	+	+	+	+	−	−	−	−
3	5	+	+	+	+	+	+	−	−	−
	6	+	+	+	+	−	−	−	−	−
5	7	+	+	+	+	+	−	−	−	−
	8	+	+	+	+	+	−	−	−	−
	9	+	+	+	−	−	−	−	−	−
10	10	+	+	+	−	−	−	−	−	−
	11	+	+	+	−	−	−	−	−	−
	12	+	+	+	−	−	−	−	−	−
12	13	+	+	+	+	+	−	−	−	−
	14	+	+	+	+	+	−	−	−	−
13	15	+	+	+	+	+	+	−	−	−
	16	+	+	+	+	+	+	−	−	−
14	17	+	+	+	+	+	−	−	−	−
	18	+	+	+	+	+	−	−	−	−
		Cow								
	1	+	+	+	+	+	+	+	−	−
	2	+	+	+	+	+	+	+	+	−
	3	+	+	+	+	+	+	+	−	−
	4	+	+	+	+	+	+	−	−	−
	5	+	+	+	+	+	+	+	−	−
	6	+	+	+	+	+	+	+	−	−
	7	+	+	+	+	+	+	+	+	−
	8	+	+	+	+	+	+	+	−	−
	9	+	+	+	+	+	+	+	−	−
	10	+	+	+	+	+	−	−	−	−
	11	+	+	+	+	+	+	+	+	−
	12	+	+	+	+	+	+	+	+	−
	13	+	+	+	+	+	+	−	−	−
	14	+	+	+	+	+	+	+	−	−
	15	+	+	+	+	+	+	+	+	−
	16	+	+	+	+	+	+	+	−	−
	17	+	+	+	+	+	−	−	−	−
	18	+	+	+	+	+	+	−	−	−

Spores from human feces did not survive 100°C.
Thermal death points of spores not determined for these cultures.

Vegetative cultures of *Clostridium Welchii* isolated from the feces of man, cow and horse were more resistant than those isolated from milk. In all cases, cultures from feces survived 59° for fifteen minutes while cultures from milk survived only 56°. Cultures from cow feces were the most resistant, 28 per cent surviving 62°C. Pasteurization at 63°, however, killed all the vegetative forms of *Clostridium Welchii.*

TABLE 8

Thermal death points. Table showing numbers of cultures from different sources surviving temperatures vs. 56° to 63°C.; numbers surviving boiling in spore stage

SOURCE	TIME SINCE ISOLATION	TOTAL NUMBER CULTURES	TEMPERATURE °C.								SPORE
			56	57	58	59	60	61	62	63	
			Vegetative eight hours growth								
Milk	4 months	20	20	20	20	9	2	0	0	0	13
	3 months	24	24	24	14	2	0	0	0	0	16
	1½ months	6	6	6	5	0	0	0	0	0	3
	2 weeks	25	25	18	8	0	0	0	0	0	10
Human	2 weeks	19	19	19	19	19	11	5	0	0	0
	Freshly isolated	36	36	36	36	36	10	0	0	0	0
Cow	Freshly isolated	18	18	18	18	18	16	13	5	0	
Horse	Freshly isolated	18	18	18	11	10	3	0	0	0	
Combined		166	166	159	131	94	42	18	5	0	

Conclusions. The thermal death point of vegetative forms of *Clostridium Welchii* varies from 56° to 63° for fifteen minutes and is not significant in the differentiation of the members of the *Clostridium Welchii* group.

The thermal death point of spores shows two distinct groups; one whose thermal death point is 87° to 90°, and the other above 100°. Some strains from market milk survive 100° for thirty and forty minutes, while spores from human feces do not survive 100° for fifteen minutes.

There is no correlation between spores and vegetative forms in corresponding cultures, nor is there any relationship between the fermentative reactions and the thermal death points.

Artificial cultivation and cultivation for long periods of time render the organism more resistant to heat. The thermal death points of cultures grown in sterile milk are different from those grown in glucose-liver broth. Sub-cultures in all cases give uniform results under identical conditions.

Cultures from fecal sources are more resistant to heat than those from milk. Pasteurization kills all vegetative forms of *Clostridium Welchii.*

XI. PATHOGENICITY

1. Historical. Since the spores of *Clostridium Welchii* have such a wide distribution in nature, in the air, soil and dust, on the bodies of human beings and animals, on food-stuffs and in the intestinal tract and feces of animals, the question of the possible pathological significance of these spores is an important one. A review of the literature since Welch's discovery of the gas bacillus shows not only a wide diversity of opinion in regard to the pathogenicity of this organism but also varied conceptions regarding its toxicity.

In preantiseptic times, especially in military surgery, wound infection by this organism was much more common than today yet cases are being reported at the present time. In early days the infection was thought by some writers to be due to the penetration of air into the tissues but by most investigators to the decomposition of adipose and bone marrow tissues brought about by contact with the atmosphere. Since the observations of Welch and his associates in the '90's, the investigations have demonstrated that the most common and important cause of gaseous phlegmons or emphysematous gangrene is the "gas bacillus" or *Clostridium Welchii.* Fraenkel, 1902, attributed the general symptoms in this infection to an intoxication due to the decomposition products of the infected tissues while Metchnikoff, 1908, Korentchewsky, 1909, Kamen, 1904, Herter, 1907, Passini, 1905 and others ascribe them to ordinary endotoxin absorption. Other views expressed ascribed the locally destructive effects of the gas bacillus to mechanical action or to the production of fatty acids causing acidosis which brought about fatal effects.

Weinberg, who discovered a strain of gas-forming bacillus isolated from cases of gas gangrene among French soldiers in 1916, held the view that the organisms did not cause the gangrene, but that the condition preceded the infection and he has obtained toxic and antioxic products for various anaerobic bacteria isolated from gangrenous wounds. He has also prepared an antibacterial serum with one of the *Clostridium Welchii* group, but failed to detect either the exotoxin or its corresponding antiserum. Kenneth Taylor in 1916 considered that gaseous gangrene is the result of the mechanical action of the gas in a local focus of developing saprophytic bacteria. His conception is that *Clostridium Welchii* attacks the carbohydrates of the muscular tissue and produces a large volume of gas, which, being unable to escape from the tissues, exerts pressure upon the blood vessels, impeding the circulation so that necrosis results. The necrotic tissue is invaded by putrefactive bacteria which disorganize it.

Wright concluded as a result of his researches that *Clostridium Welchii* operates through the production of an acid condition of the blood and tissues through which the antitrypsin is diminished which permits a tryptic digestion of the proteins so that the bacilli are provided with a highly favorable medium of growth. The intoxication which follows is an acidemia according to his views.

Conradi and Bieling have recently distinguished two phases of action of the bacilli, namely, the fermentative and the saprophytic stages. In the fermentative phase, the carbohydrates are attacked, forming lactic, butyric, proprionic and succinic acids which are the immediate causes of the edema and necrosis of the tissues. In the other phase, the spore-bearing organisms give rise to putrefaction of the dead tissues and consequent intoxication.

Stewart West, following a study of an anaerobic bacillus isolated from an infection in a French soldier, concluded that the bacillus produced no true exotoxin nor was the bacterial protein toxic and believed that acid formation in animal tissue was a powerful factor in its injury.

Simonds was unable to produce toxin in artificial media. Cultures from a normal stool were studied and proved very pathogenic for guinea-pigs but no evidence of toxin production was shown. He believed that the products of the bacteria, and those of other bacteria present, damaged or killed more tissue and thus spread the infection. Living tissue was not damaged, but in order that growth might occur a sufficiently anaerobic condition must be supplied by the dead tissue.

Westenhoffer claims that *Clostridium Welchii* is a pure saprophyte and produces its effects in dead tissue only. For its occurrence unfavorable conditions in the tissues are essential.

Researches by Bull and Pritchett in April and November 1917 have completely overthrown all the conceptions previously held of the manner of the pathogenic action of this organism. Experiments recently performed by them seem to render all the above theories untenable and to establish the fact that the local destruction of tissues and the lethal effects of *Clostridium Welchii* are due to a specific bacterial toxin and not to a blood invasion of the micro-organisms nor to acid intoxication. The work shows definitely that under suitable cultural conditions (broth plus sterile non-denatured muscle and + 0.1 per cent glucose incubated less than twenty-four hours) *Clostridium Welchii* produces a soluble toxin which after separation from the bacilli, is capable of causing the characteristic lesions and possesses the physical and biological properties of an exotoxin. Furthermore, infections can be successfully prevented and controlled by neutralizing the toxin with a specific immune serum as proved by these writers.

2. *Experimental.* The present investigation on pathogenicity is an attempt to differentiate between the human and bovine types and also to present evidence which will substantiate the claims of Bull and Pritchett that the local infection and lethal effects of *Clostridium Welchii* are due to a specific bacterial toxin which can be neutralized with a specific antitoxin.

The modes of inoculation by which the pathogenic properties were demonstrated for *Clostridium Welchii* were subcutaneous, intraperitoneal and intravenous injections of fresh cultures. The

injection of 2 cc. of a virulent glucose broth culture, grown at 37.5° for eight to ten hours, into the subcutaneous tissue of the abdomen of a guinea-pig gave rise to typical gas gangrene and caused the death of the pig in from twelve to thirty hours, depending on the virulence of the culture. When pure cultures of virulent organisms are injected subcutaneously the injection leads to necrosis of all the tissues surrounding the point of inoculation with the presence of rapidly spreading gas phlegmons, giving rise to a local swelling and liquefying necrosis of the involved muscle and skin. The hair of the pig around the inoculated area pulls out very easily, exposing the skin which is free from the body wall. If the animal survives, a large abscess is formed which later bursts, and recovery occurs with eventual sloughing and cicatrization. At autopsy after death an abundant sero-sanguinous exudate is formed extending along the entire abdominal cavity containing *Clostridium Welchii* in pure culture with very few pus cells. There is an extensive muscle necrosis and disintegration of the fat extending from the injection along the greater part of the abdomen and in some cases extending along the legs and up to the neck. The abscess may extend along the entire abdominal and thoracic regions. Muscles and connective tissues are so badly necrosed that they assume a semi-liquid condition filled with gas bubbles, giving off a characteristic strong butyric acid odor. The amount of gas varies very much, in some cases there may be only a few bubbles, and in other cases tissues everywhere are blown up with gas. After death there may be a rapid swelling of the subcutaneous tissues and gas bubbles may be found in the heart, blood vessels and other organs. Certain organs, especially the liver, offer more favorable food supply for growth and development with gas formation than do others, but even in the liver, cases occur where bacilli are present without gas. In the spleen and kidneys gas bacilli are present in clumps, at times, surrounded by necrotic areas with gas formation. The bacilli are most numerous in the disorganized and necrotic tissue, fewer in the bloody exudate and very few or absent in the heart's blood. Cultures were also recovered from the intestines and sometimes from the stomach

and pancreas. Smears showed the presence of capsules and Gram-positive bacilli from the serous fluid and disorganized tissues upon microscopical examination. The gas bacillus seems to select certain tissues and organs of the body, namely, loose connective tissue, voluntary muscles and liver, perhaps on account of the glycogen which they contain. Gas may appear in the tissues as early as eight to ten hours after inoculation.

A larger amount of culture is necessary for infection when given intraperitoneally than when given subcutaneously or intravenously yet if lethal doses are used, the same effect takes place in the tissues and body fluids. The lethal dose in intraperitoneal injections was 0.25 cc. for a 607-gram guinea-pig or 0.04 cc. for a 100-gram pig. The minimum lethal dose for subcutaneous and intravenous injections was 0.1 cc. for 400 to 450-gram guinea-pigs. 0.1 cc. was the smallest amount that could be inoculated with the needles available.

The laboratory animal employed for the most part to test the pathogenicity of *Clostridium Welchii* was the guinea-pig, the guinea-pig being more susceptible to infection that the rabbit. As far as possible pigs weighing about 500 grams were used which had not been previously used for experimentation. The subcutaneous injection was adopted chiefly because it proved more successful than the intraperitoneal. The rabbit is more resistant to local infection than is the guinea-pig but nevertheless lesions can be produced by virulent cultures provided larger doses are given.

Subcutaneous inoculation of virulent cultures of *Clostridium Welchii* when injected into a guinea-pig will produce severe local infections, terminating fatally in from ten to thirty-six hours in the majority of cases, the more virulent the organism the quicker being the reaction. In exceptional cases, death has occurred after five to nine days with the recovery of the organism from the tissues at autopsy. In one case a 353-gram pig received 1 cc. injection of number three milk culture and lived for eleven days, having lost 140 grams and developing an abscess over the entire abdominal region which gave off a strong, offensive, putrefactive odor. In cases of sublethal doses or non-pathogenic cultures a

mild local infection occurs from which recovery takes place in a very few hours or the pig remains inactive with no appetite for several days. The injection causes local swelling which after two to three days forms a large abscess and breaks, exposing the abdominal wall. After the abscess opens the pig gains steadily, recovering completely in the course of five to seven days, while the abscess heals and leaves a clean scar.

Cultures of *Clostridium Welchii* vary greatly in virulence. All strains showing any virulence are pathogenic for guinea-pigs, while some may not cause fatal infection in rabbits. The virulence of a strain is increased by passage through the animal body. The source from which an organism was isolated gives no absolute clue as to its pathogenicity. However, of the 9 strains of *Clostridium Welchii* isolated from the feces of man all have proven very highly pathogenic for guinea-pigs and in relatively larger doses, are pathogenic for rabbits. Of the 12 from cow feces, 8 are non-pathogenic and 4 are highly pathogenic. These 4 strains were isolated from 4 cows in the same herd.

The milk cultures used in the test for pathogenicity showed a difference in virulence. Of the 12 cultures tested, 8 were pathogenic in varying degrees; 2 in twelve hours, 2 in forty-eight hours, 1 in eighty hours, 1 in five days, 1 in nine days and 1 in eleven days. Four cultures from milk were grown for twenty-four hours in sterile milk instead of glucose broth before the injection of the animal to see if different culture media would effect the pathogenic result. Apparently growth in sterile milk gives the same result as in glucose broth for the strain was non-pathogenic under both conditions.

Controls were run to check up the results in all cases. The pathogenicity of spores of *Clostridium Welchii* was tested in five cases and gave negative results. To assure the inoculation of spores alone, the cultures containing spores were heated to 80° for fifteen minutes in order to kill all the vegetative forms before the injection into the animal. The spore cultures tested were taken from two strains of *Clostridium Welchii*, one from human feces and the other from market milk, both being virulent for guinea-pigs in the vegetative form. Four of the experiments

were performed on guinea-pigs by injecting the heated spores subcutaneously in 1 and 5 cc. quantities. In each case the animals increased in weight and showed no apparent reaction. The fifth experiment was an intravenous injection of 10 cc. of a spore culture from human feces in a rabbit weighing 2200 grams with negative results.

From these results it is safe to assume that spores heated to 80° for fifteen minutes do not readily germinate in the circulation or in living tissue. Apparently when spores of *Clostridium Welchii* gain entrance to living tissue, they adapt themselves to the amount of oxygen present to such a degree that they will survive for a time but do not produce infection. Thus the healthy human body is protected against infection by the spores of *Clostridium Welchii* which are so widely distributed. In the case of injury to the tissues such as wounds, fractures, etc., there is a sufficient anaerobic condition in the dead tissue to allow the organism carried into the wound to begin to grow and produce its characteristic lesions.

Previous to the investigations of Bull and Pritchett, 1917, no explanation could be given for the fact that spores would not germinate and produce fatal results. When vegetative forms were injected in sufficient quantities in living tissue, inflammation and necrosis of the subcutaneous tissues and muscles developed causing death but no reason could be found for the behavior of spores. Bull and Pritchett, 1917, determined the fact that the toxicity of the fluid is destroyed by heating for 30 minutes at 70° and is greatly diminished at 62° for a similar period. Therefore, it is probable that the toxin was destroyed by heat in these experiments on the effect of the inoculation of spores and hence no necrosis or local lesions were produced. This fact strengthens the view that a toxin is produced during the growth of the bacillus.

3. Effect of acid upon the tissues. The following experiments were conducted to determine whether or not the presence of acid in the culture has any effect upon pathogenicity. Cultures grown for eight to ten hours were injected in most cases, while in a few cases twenty-four hour cultures were used. The acidity

was not calculated in every case before inoculation but those tested showed acidities (Fuller's scale) ranging from 1.2 to 3.5 per cent in terms of N NaOH with phenolphthalein as an indicator. In the case of the twenty-four hour culture where the greatest acidity was produced, mild local infections occurred and in one case death after nine days. In one instance the amount of acid produced by the multiplication of the bacilli in the tissues was determined. Death occurred in twenty hours after the injection of a lethal dose and at autopsy the tissue surrounding the necrotic area was titrated with phenolphthalein as an indicator. It was found that the acidity had reached approximately 12 per cent before the death of the animal, showing again the ability of *Clostridium Welchii* to produce acid by means of utilizing the carbohydrates in the tissues. In two cases, the virulent broth cultures were neutralized with NaOH which when injected in lethal doses in the subcutaneous tissues of guinea-pigs proved fatal, presenting the same symptoms and effects as the unneutralized cultures. The same effects were produced if the sedimented bacilli were injected or the supernatant fluid, provided a sufficient dose was given. Although sufficient experiments have not been performed for definite conclusions regarding the rôle of acidity, yet the results already at hand point conclusively to the exclusion of this factor in bringing about the fatal effects. If the acidity factor aids in the destruction of the tissues, the effect is secondary in the production of fatal results.

4. Discussion of results. Animals are more susceptible, and smaller doses are more liable to kill, when the inoculations are subcutaneous or intramuscular than when other modes of inoculation are employed. The difficulty encountered with the intravenous inoculation for guinea-pigs made it impracticable for routine work. In the one successful attempt, however, it proved very fatal, 0.1 cc. being sufficient to kill a 500-gram guinea-pig. The minimum lethal doses for virulent cultures when injected subcutaneously and intraperitoneally are 0.1 cc. and 0.25 cc. respectively for 600-gram guinea-pigs. The minimum lethal dose was not determined for rabbits.

The guinea-pig is peculiarly susceptible to infection while the rabbit is much more resistant. The rabbit succumbs, however, to the inoculation of a more virulent strain or a larger dose. The duration of the disease in these animals varies from a few hours to several days, complete recovery from a local infection taking one to three weeks. All degrees are observed from a very mild local infection to a severe necrosis, resulting in the liquefaction of the muscle and tissues involved, accompanied by abundant gas production, which results in prostration, complete collapse, and death in a short time. The degree of infection depends on the virulence of the culture.

While no experiments were made to determine the presence of a soluble toxin, yet by eliminating certain factors evidence points strongly to the fact that the local infection and lethal effects of this organism are due to a specific bacterial toxin similar to that of the diphtheria bacillus, as already demonstrated by Bull and Pritchett, 1917. The factor of acidity of the culture appears to be secondary in its effect to its toxic action.

Table 9 gives the results of 55 experiments made on the pathogenicity of cultures of *Clostridium Welchii* isolated from the following sources, human feces 9, cow feces 10 and milk 11. Two cubic centimeters of an eight-hour glucose broth culture were inoculated subcutaneously.

SOURCE	TOTAL TESTED	PATHOGENIC	NON-PATHOGENIC	PER CENT PATHOGENIC
Human	9	9	0	100.0
Cow	10	4	6	40.0
Milk	11	8	3	72.7

Spores from cultures obtained from market milk and human feces, after heating to 80°–82° for fifteen minutes to kill the vegetative forms, did not germinate when injected into the circulation or subcutaneous tissues of guinea-pigs or rabbits and the animals showed no reaction.

TABLE 9

Pathogenicity experiments

WEIGHT	SEX	AMOUNT	STRAIN	SOURCE	ACIDITY	REMARKS
Subcutaneous inoculations						
grams		*cc.*			*per cent*	
186	♀	1.25	6	Human feces	2.5	Fatal in 24 hours. Weight, 186 grams
201	♂	0.75	6	Human feces	2.5	Fatal in 30 hours. Weight, 191 grams
263	♀	0.5	6	Human feces	2.5	Fatal in 12 hours. Weight, 252 grams
367	♂	0.1	6	Human feces	2.5	Fatal in 26 hours. Weight, 317 grams
491½	♂	2.0	4	Human feces	2.3	Fatal in 10 hours. Weight, 468.5 grams
518½	♂	2.0	5	Human feces	2.4	Fatal in 15 hours. Weight, 481 grams
548	♂	2.75	7	Human feces	2.0	Fatal in 15 hours. Weight, 512 grams
550	♂	2.0	13	Cow feces	2.3	Recovered. Reaction slight. Loss in weight
676	♂	0.5	12	Cow feces	1.8	Recovered. Reaction slight. Loss in weight
443	♂	2.0	11	Cow feces	1.2	Recovered. Reaction slight. Loss in weight
443	♂	2.0	8	Cow feces	2.0	Recovered. Reaction slight. Loss in weight
571½	♂	2.0	7	Cow feces	2.0	Recovered. Reaction slight. Less in weight
741	♂	3.0	5	Cow feces	2.0	Recovered. Reaction slight. Loss in weight
499	♂	2.0	5	Cow feces in st. milk		Recovered. Reaction slight. Loss in weight
425	♂	2.0	64	Cow feces		Fatal in 12 hours. Weight, 423 grams
476	♂	2.0	72	Cow feces		Fatal in 27 hours. Weight, 427 grams
548	♂	2.0	65	Cow feces	2.3	Fatal in 12 hours. Weight, 543 grams
616	♂	2.0	71	Cow feces		Fatal in 13 hours. Weight, 590 grams
516	♂	2.0	E 11	Human feces	2.0	Fatal in 13 hours. Weight, 489 grams
540½	♂	2.0	E 8	Human feces	2.1	Fatal in 25 hours. Weight, 502 grams
523	♂	2.0	G 8	Human feces	2.1	Fatal in 38 hours. Weight, 478 grams

$510\frac{1}{2}$	♂	2.0	N 6	Human feces		Fatal in 39 hours. Weight, 469 grams
773	♂	2.0	G 8	Human feces		Fatal in 32 hours. Weight, 770 grams
455	♀	0.5	$22\frac{7}{16}$	Milk	2.5	Fatal in 46 hours. Weight, 439 grams
$448\frac{1}{2}$	♀	1.0	$21\frac{1}{13}$	Milk	2.4	Fatal in 80 hours. Weight, 360 grams
656	♂	2.0	$6\frac{7}{16}$	Milk	2.4	Severe reaction. Abscess. Recovery. Lost 125 grams
666	♂	2.0	$11\frac{7}{16}$	Milk	2.0	Severe reaction. Abscess. Recovery. Lost 140 grams
551	♂	2.0	$8\frac{7}{16}$	Milk	2.5	Severe reaction. Abscess. Recovery. Lost 45 grams
566	♂	2.0	$E\frac{7}{16}$	Milk	1.9	Fatal in 5 days. Weight, 482 grams
322	♀	1.0	$1\frac{7}{16}$	Milk	1.8	Fatal in 12 hours. Weight, 311 grams
306	♀	1.0	$18\frac{7}{16}$	Milk		Fatal in 12 hours. Weight, 300 grams
280	♂	0.5	$8\frac{7}{16}$	24 hour culture	3.0	Recovered. Reaction quite mild. Slight loss
300	♀	0.5	$19\frac{7}{16}$	24 hour culture		Fatal in 48 hours. Weight, 285 grams
353	♂	1.0	$3\frac{7}{16}$	24 hour culture	2.5	Fatal in 11 days. Weight, 219 grams
391	♂	1.0	$12\frac{7}{16}$	24 hour culture	2.5	Fatal in 9 days. Weight, 209 grams
Tests on pathogenicity for rabbits						
2490	♀	5.0	6	Human feces	2.4	Sublethal dose. Mild reaction. Recovery
2300	♀	10.0	6	Human feces	2.4	Sediment pathogenic in 36 hours. 2150 grams
2150	♂	8.0	6	Human feces	2.4	Liquid pathogenic in 28 hours, 1975 grams
Intraperitoneal inoculations						
293	♀	0.5	6	Human feces	2.5	Fatal in 10 hours. Weight, 279 grams
607	♀	0.25	6	Human feces	2.5	Fatal in 10 hours. Weight, 580 grams
$473\frac{1}{2}$	♀	0.1	6	Human feces	2.5	Recovery. Some reaction. Loss in weight
$624\frac{1}{2}$	♂	0.1	6	Human feces	2.5	Recovery. Reaction quite severe. Lost 80 grams
$505\frac{1}{2}$	♂	1.5	4	Human feces	2.3	Fatal in 10 hours. Weight, 484 grams
596	♂	3.0	5	Human feces	2.4	Fatal in 10 hours. Weight, 572 grams
553	♂	0.2	12	Cow feces	1.8	Recovery. Gangrenous. Abscessed. Lost 60 grams
550	♂	2.0	12	Cow feces	1.8	Recovery. Gangrenous. Abscessed. Lost 40 grams
$561\frac{1}{2}$	♂	2.0	13	Cow feces	2.3	Recovery. Gangrenous. Abscessed. Lost 40 grams

TABLE 9—*Concluded*

WEIGHT	SEX	AMOUNT	STRAIN	SOURCE	ACIDITY	REMARKS
				Intravenous inoculations		
grams		*cc.*			*per cent*	
$385\frac{1}{2}$	♂	0.5	6	Human feces	2.5	Recovered quickly. Reaction mild. Small abscess
477	♀	0.1	6	Human feces		Recovered after 10 days reaction. Abscessed
449	♀	0.1	6	Human feces		Fatal in 36 hours. Weight, 402 grams
				Subcutaneous inoculations of spores		
2490	♀	5.0	6	Human	Rabbit	No reaction. Gained weight consistently
2200	♂	10.0	6	Human	Rabbit	Intravenous inoculation. No reaction
$645\frac{1}{2}$	♂	5.0	6	Human	Guinea-pig	No reaction after initial effect of inoculation
522	♂	1.0	6	Human	Guinea-pig	Very slight loss in weight. No ill effects
442	♀	1.0	$1\frac{7}{16}$	Milk	Guinea-pig	No ill effects. Gained weight
				Effect of acidity on toxicity		
540	♂	2.0	E 8	Human	2.1	Fatal in 25 hours. Subcutaneous. Weight, 502 grams
550	♂	2.0	E 8	Human	Neutral	Fatal in 20 hours. Subcutaneous. Weight, 505 grams
491	♂	2.0	4	Human	2.4	Fatal in 10 hours. Subcutaneous. Weight, 468 grams
518	♂	2.0	4	Human	Neutral	Fatal in 15 hours. Subcutaneous. Weight, 481 grams
				Feeding experiments		
425	♀	2.0	6	Human		Fed spores. Gained with no ill effects
401	♀	1.0	6	Human		Fed vegetative. Gained with no ill effects
449	♀	2.0	6	Human		Fed vegetative. Gained. No spores in feces
270	♂	1.0	E 8	Human		Fed vegetative. Gained. No spores in feces
261	♂	2.0	E 8	Human		Fed vegetative. Gained. No spores in feces

XII. IMMUNITY

In the literature dealing with the immunity of animals to inoculation with *Clostridium Welchii* and the production of immune sera, we find views as varied and unreliable as in regard to the other properties of this organism. Very little work has been done on the production of artificial immunity. Previous to 1916, investigators were unable to protect guinea-pigs against infection by injections of bacilli killed either by heat or by chemical substances. A pig which had recovered from one inoculation was believed to be more susceptible to a new injection.

On the other hand Rosenthal and Theroloix, 1909, immunized horses against *Clostridium Welchii* and produced a serum which protected rabbits against intrapleural and intramuscular injections of the living organisms. These results were not in line with other observations made at that time yet later evidence seems to justify their validity.

Robertson, 1916, undertook experiments to determine whether or not immunity could be produced by the prophylactic inoculation of *Clostridium Welchii* and his results showed no immunity in guinea-pigs, and also indicated that the survival of one infection did not confer any immunity at a later period.

As far as is known Bull and Pritchett, 1917, are the only ones who have shown that the toxic products of *Clostridium Welchii* exhibit antigenic activities and give rise to the formation of active antitoxic substances which possess protective and curative properties. Conclusions from their work show that animals may be immunized actively so as to yield an immune serum which neutralizes the toxin perfectly and in multiple proportion.

As stated, different strains vary greatly in virulence and the same strain varies according to cultural conditions. The conditions under which five experiments were performed to produce immunity were not sufficiently controlled to render conclusive evidence; in particular, too much time elapsed between inoculations. With all the conditions controlled and standardized, it is believed however that a lasting immunity can be produced for guinea-pigs. A description of the five experiments follow:

1. A male guinea-pig, weighing 571.5 grams was inoculated subcutaneously with a 2 cc. glucose broth culture 7 of cow feces. Although there was a loss in weight to 547.5 grams at the end of the first day, no discomfort was observed and the pig was lively and in a healthy condition. In forty-eight hours it had gained slightly, weighing 553 grams and its weight remained quite constant for the next few days. This injection was made on January 31, 1917, and proved non-pathogenic. On February 6, the same pig weighing 548 grams was reinoculated with a pathogenic strain to see if any immunity had been produced. Two and three-quarters cubic centimeters of number seven culture from human feces was used subcutaneously. A reaction occurred but did not prove fatal. The pig lost weight consistently, dropping to 457 grams on February 19, thirteen days after the inoculation. A local infection occurred with swelling of the abdomen and gas in the tissues. A large open abscess was formed along the entire right side of the abdomen. On February 19, the abscess began to heal and the animal gained consistently, recovering completely in twenty days. A control pig was inoculated with number seven human feces and died in fifteen hours. It was evident, therefore, that immunity had been produced to a high degree by the previous injection of a non-pathogenic strain.

On March, 20, 1917, this same pig now weighing 528 grams and in excellent condition was inoculated again subcutaneously with 2.5 cc. of a fresh culture of a vegetative organism from human feces. Local swelling was produced and loss in weight was consistent to 467 grams when the abscess broke and recovery seemed probable. Two days after another abscess appeared extending to the neck which further weakened the resistance of the pig and resulted in another loss of weight from 467 to 350 grams. Although the abscess had practically healed the pig was found dead on the fourteenth day. The cause of the death was not primarily due to the infection of *Clostridium Welchii*, but after the resistance had been lowered to such an extent the pig was unable to survive. A smaller dose should have been injected, although some immunity was no doubt produced. The control pig died thirty hours after the injection of a 2 cc. culture subcutaneously.

II. A male guinea-pig weighing 499 grams, was inoculated subcutaneously on February 6, with 2 cc. of culture 5 from cow feces grown in sterile milk. A normal infection occurred resulting in gas in the tissues and a pronounced swelling. Loss in weight continued for two

weeks dropping to 428 grams when the abscess healed and the animal returned to its normal healthy active condition, gaining 50 grams in the next week. On March 20, forty-two days after the first inoculation, 1.5 cc. of a fresh culture from human feces, which was pathogenic for a control pig in thirty-nine hours and produced a violent reaction, was injected into this pig which had completely recovered from the reaction of the non-pathogenic strain. After twelve days the pig succumbed having lost weight consistently going from 527 to 267 grams. The intestine and other organs were exposed due to the open abscess extending the entire length of the abdomen with pus filling the subcutaneous tissues. Immunity must have been produced to have kept the pig alive for so long a time while undergoing such a severe infection.

III. A guinea-pig weighing 656 grams was inoculated subcutaneously on February 6, 1917, with 2 cc. of milk culture 6 isolated on July 16, 1916. The pig lost weight and showed the typical lesions with the presence of gas bubbles under the skin. After the first week the abscess which had formed on the fourth day had practically healed but there was another one forming near the former. The pig weighed 524 grams and was apparently healthy. At the end of the second week the pig had completely recovered from the infection and was in excellent condition. This strain although it produced a moderate infection did not kill the pig.

On March 20, 1917, another injection was given this pig, forty-two days after the first. Two cubic centimeters of a very virulent strain G.8 from human feces was used and proved fatal in twelve hours. If any protection had been produced by the previous inoculation, it had run out before forty-two days. This strain, however, was extremely virulent and needed a powerful immunizing influence to counteract its toxic effect.

IV. A pig weighing 666 grams was inoculated subcutaneously on February 6, with 2 cc. of milk culture 11. A characteristic lesion developed in a few hours resulting in a large abscess and loss in weight to 529 grams in two weeks. After the abscess broke, recovery followed rapidly with the healing of the abscess. In three weeks the animal was as lively as ever. On March 20, the pig weighed 578 grams and was injected with 3 cc. of the same pathogen as used in III, namely, G.8, which was highly pathogenic in fifteen hours in a 600-gram control pig. No immunity was present here for death occurred in twenty hours after severe convulsions. Again the dose was too great and the interval between injections too long.

V. On February 6, 2 cc. of milk culture 8 was injected into a 551-gram pig subcutaneously. Although this strain did not prove fatal in two cases, local swelling occurred accompanied with gas and the formation of an abscess. The reaction was not so violent as in some cases although the animal lost 43 grams. Recovery took place in three weeks.

A second injection was given on March 23, 1917, the pig weighing 617 grams. Two cubic centimeters of a twenty-four hour old culture 19, which had previously proved pathogenic for guinea-pigs, was inoculated subcutaneously and failed to produce a fatal necrosis. Emphysematous crackling due to the formation of gas bubbles occurred, but the reaction was not as severe as in other cases. In two weeks the pig recovered from the second injection and had returned to its active condition. Immunity was thus produced against a virulent culture in guinea-pigs from the same source, namely, milk.

From these five experiments it is evident that immunity can be produced by the injection of avirulent cultures or sublethal doses of virulent organisms.

XIII. EFFECT OF FEEDING CLOSTRIDIUM WELCHII

Previous investigators have met with little or no success by feeding *Clostridium Welchii* to animals. In a few cases diarrhea has been induced in young guinea-pigs and kittens but attempts to cause ill effects in adult animals have met with negative results. Before determining the effect of feeding cultures to guinea-pigs, feces from several animals were collected and tested for the presence of spores and vegetative forms of *Clostridium Welchii*. The feces from guinea-pigs, weighing approximately 400 grams, were collected in the following manner. The sides and belly of the pig were washed with a 5 per cent carbolic acid solution and then the pigs were placed in the sterile container one at a time, and a sample of feces collected. After each sample was taken, the container was thoroughly sterilized. After a sufficient quantity of feces had been excreted the pig was released and the feces analyzed as follows:

The sample of feces was added to 100 cc. of freshly sterilized water and shaken vigorously until the pellets were thoroughly

broken up. One-half cubic centimeter of this emulsion was inoculated into 10 cc. glucose-liver broth tubes (five for each sample) and heated to 80° for fifteen minutes and incubated. Five other tubes were inoculated and incubated directly at 37°. Negative results for the presence of *Clostridium Welchii* in the normal guinea-pig were obtained.

To determine the effect of feeding spore and vegetative cultures to adult and young pigs, six experiments were performed, and in no case was the effect fatal. The cultures were mixed with milk alone, and bread and milk. The pigs refused to eat milk alone with *Clostridium Welchii*, but when mixed with bread, they ate heartily. In the two experiments where the spore cultures were fed there were no apparent disturbance, the pigs seeming as active and healthy as under their normal diet. The feces were normal and hard, and no diarrhea nor intestinal trouble developed. Samples of the feces were analyzed daily for the presence of spores, and vegetative forms. Spores were recovered for three days, after which negative results were obtained. During this time the animals gained weight and seemed to thrive upon their new diet. Two cubic centimeters and 4 cc. of spores were fed from virulent strains to adult guinea-pigs weighing 425 and 445 grams respectively, with negative results.

With vegetative cultures, four experiments were performed using two adult pigs weighing 401 and 449 grams respectively, and two younger pigs, about three months old weighing 270 and 260 grams respectively. Feces before feeding did not contain *Clostridium Welchii*. One cubic centimeter and 2 cc. of a highly virulent strain for guinea-pigs were used in the feeding, soaking bread with milk and adding the culture of *Clostridium Welchii* grown in glucose-liver broth for eight hours. The difference in the age of the pigs did not seem to change the effect on the feeding, for in the fourth pig no discomfort was observed, and a steady gain in weight was recorded. Analysis on the first day showed numerous vegetative forms and the absence of spores in the intestine. The second day after feeding, *Clostridium Welchii* was recovered in all cases as spore. Negative results were again obtained in five days.

The very interesting conclusion from these experiments is that vegetative forms sporulate in the intestines when present either as a result of artificial feeding or inoculation. In all cases of subcutaneous inoculation of vegetative cultures, spores were observed in different parts of the intestine. From the six feeding experiments no ill effects were observed and cultures disappeared quickly from the intestines.

XIV. CONCLUSIONS

1. *Clostridium Welchii* is a non-motile, straight rod, 3 to 6 micra in length and to one and one-half micra in breadth, with the ends slightly rounded or square cut. It stains readily with the ordinary dyes and is Gram positive. Capsules are present in cultures made directly from body fluids or body tissues, from milk or feces, but disappear in succeeding cultures.

2. Sporulation of *Clostridium Welchii* takes place in a great variety of media, provided no fermentable substance is present. Sporulation never occurs in the tissues, organs and body fluids of the living animal but does take place in the intestine. The spores are usually oval, from 1.5 to 3 micra long and form in the middle of the rod or slightly toward one end. The spores germinate when grown in media containing glucose, galactose, lactose, sucrose, maltose, dextrin and starch. They withstand a wide range of reaction, at least from -2 to $+12$. They will germinate in slightly alkaline or acid media but best in neutral media.

3. *Clostridium Welchii* is an obligate anaerobe which requires strict anaerobiosis for its growth. Boiling the media just before using renders it suitable for the growth and isolation of *Clostridium Welchii*. For continued growth on solid media, the surface should be covered with sterile paraffin or other methods for preserving anaerobic conditions should be employed.

4. *Clostridium Welchii* is capable of growth upon all of the ordinary culture media but grows better in the presence of 1 per cent glucose or maltose. Vegetative forms must be transplanted every forty-eight hours. In sugar-free media sporulation occurs in two or three days. The organism does not grow in

peptone-free media. Spores of *Clostridium Welchii* can be preserved indefinitely in all media in the absence of a fermentable substance.

5. *Clostridium Welchii* develops best at 37° to 38°C. Growth does not occur above 42°C. nor below 10°C. From 18° to 35°C. growth is abundant but slow. Freezing vegetative and spore forms effects a steady reduction in numbers which leads to complete destruction of vegetative forms in seven or eight days and of spores in ten to twelve days.

6. Surface colonies of *Clostridium Welchii* in plain and sugar agar are opaque white, grayish white or brownish white in color, by transmitted light, sometimes with a central darker dot. Deep colonies are spheres or ovals. Colonies vary in size from 0.5 to 2 or 3 micra in diameter.

7. Gelatin and blood serum are liquefied. Gas and acid are produced from glucose, galactose, lactose, maltose, sucrose, dextrin and starch. Milk is coagulated with a characteristic "stormy fermentation" in twenty-four to forty-eight hours at 37°. The curd is later digested. *Clostridium Welchii* is chiefly a fermentative organism and attacks proteins only in the absence of a fermentable carbohydrate.

8. The thermal death point of vegetative forms of *Clostridium Welchii* varies from 56° to 63° for a fifteen minute period of exposure. The thermal death point of spores shows two distinct groups; one whose thermal death point is from 87° to 90° and the other which survives 100°.

9. The minimum lethal dose in intraperitoneal injections of vegetative forms is 0.04 cc. of an eight-hour broth culture for a 100-gram guinea-pig, while for subcutaneous and intravenous injections the minimum lethal dose is 0.025 cc. The rabbit is more resistant to infection than is the guinea-pig. Cultures of *Clostridium Welchii* vary greatly in virulence. Cultures containing spores heated to 80° for fifteen minutes are non-pathogenic. Immunity can be produced by the injection of avirulent cultures or sublethal doses of virulent organisms. Feeding vegetative or spore forms has no ill effect.

10. On the basis of acid production and spore formation in glycerol and inulin broths members of the *Clostridium Welchii* group may be divided into four sub-groups.

11. *Clostridium Welchii* is a normal inhabitant of the intestines and is very widely distributed in nature in the spore form. Milk direct from the cow is free from *Clostridium Welchii* but becomes contaminated through uncleanly methods and carelessness in handling so that market milk at the time of delivery consistently contains spores of this organism. The examination of 525 samples of milk indicate that *Clostridium Welchii* is present in most market milk.

The writer wishes to express his most sincere thanks to Prof. F. P. Gorham and Dr. C. A. Fuller for many helpful suggestions in carrying out this study.

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A NOTE ON THE VIABILITY OF MENINGOCOCCI ON YEAST AGAR MEDIUM

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In 1918, the writer showed that a simple yeast medium (extract of baker's or brewer's yeast) could be used for prolonging the viability of the meningococcus. The medium possesses the advantages of requiring no meat infusion or meat extract or peptone. It is prepared with agar as a base and is to be used in the solid or semi-solid form. Observations at the time were extended over a period of six, and in several instances over a period of eleven weeks. More than twenty strains of meningococcus, recent and old, representing normal, irregular and para types isolated from the spinal fluid and the nasal mucosa, were tested on the solid medium and were found fully viable after six weeks on the average and for some strains which were observed beyond that time, for eleven weeks, when stored at 37°C. At room temperature, observations were made for one month only and at the end of this time, cultures were still viable. It was believed that a semi-solid medium was best adapted for unusually prolonged viability of organisms such as the meningococcus and other species.

Some recent observations which I wish to report, point to yeast media as a valuable adjunct in routine bacteriological work, particularly in view of the fact that others, notably Ayers and Rupp recently, have confirmed the adjuvant properties of yeast extracts in culture media.

Representatives of the normal, irregular and para types of meningococcus were seeded in semi-solid yeast agar prepared as described in earlier articles by the writer, and stored at 37°C. and at room temperature (24° to 26°) after a preliminary incu-

bation over night at 37°C. Transplants were made to glucose agar at monthly intervals. Luxuriant growth was obtained with all of the strains studied for a period of five months, after which the tests were curtailed. It is preferable, perhaps, to store cultures at incubator temperature in order to maintain a "body environment," but insofar as viability is concerned, there seems to be no difference between the two methods, for the time observed.

No tests have been made to determine the effect of such storage on virulence. A few experiments, however, have been designed with a view to disclosing any possible changes in agglutinogen or agglutinin content of meningococcus. The results indicate that monovalent sera obtained with strains grown on yeast medium for several months do not differ in agglutinating properties from sera developed with cultures which have been grown on the usual media. Similarly, cultures which have been growing on yeast media for a long time are agglutinated by polyvalent sera to the same extent as are cultures grown on other media.

SUMMARY AND CONCLUSIONS.

Meningococcus cultures in semi-solid yeast agar when stored either at room temperature or in the incubator at 37°C., were still fully viable after five months.

In all likelihood the antigenic nature of the organisms remains unaltered as may be indicated by comparative agglutination tests.

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THE USE OF AGAR SLANTS IN DETECTING FERMENTATION

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In a recent paper (Conn, 1919) one of the writers described the use of agar slants instead of liquid media for detecting acid production by two soil organisms, stating the method to be much more satisfactory for these particular bacteria than the old method. Subsequently this method was given as an alternate procedure in the report of the Committee on the Descriptive Chart (1919). We have recently used the method for a great variety of soil and milk organisms with such great success that it seems worthy of more specific mention. The technic is as follows:

One per cent of the desired sugar is added to an agar medium favorable to the growth of the organisms to be tested. The reaction is adjusted by the use of brom thymol blue to a pH-value between 6.8 and 7.2. Then an indicator is added whose range lies just to the acid side of pH = 7.0, such as china blue or brom cresol purple; or better yet, a mixture of the former with cresol red or sodium rosolate (as recommended by Bronfenbrenner, Schlesinger and Soletsky, 1920), or of the latter with cresol red, in order to detect decreases as well as increases in hydrogen-ion concentration. The following concentrations of indicators are recommended: Brom cresol purple 0.001 per cent; Brom cresol purple purple and cresol red each 0.0005 per cent; China blue 0.0025 per cent; China blue 0.0025 per cent plus sodium salt of rosolic acid 0.005 per cent.

It is convenient to keep the indicators in concentrated alcoholic stock solutions of such a strength that a definite amount can be measured out per litre of medium: i.e., a 1.6 per cent stock

solution of brom cresol purple can be made up and used alone at the rate of 1 cc. to the liter of medium or when combined with cresol red at the rate of 0.5 cc. to the liter.

Inoculate both on the surface and in a stab at the base of the slant. The tubes should be watched day by day until the characteristic changes are complete. Changes in reaction can readily be detected by the color; and gas production can be observed by means of bubbles or cracks in the medium if the agar in the tube is sufficiently deep.

With the organisms studied by the writers, this method has had the advantage of showing acid production after a very brief incubation, sometimes in a few hours only. The acid tends to be localized in one spot at first and can be recognized even though it could not be detected if distributed through the entire tube of medium; hence the method is very delicate. Some organisms are characterized by acid production first at the top of the tube; others show the effect first at the bottom. Sometimes the zone of acid is formed at the top and travels slowly to the bottom, followed by a zone of neutral or alkaline reaction. Such changes are generally constant with a given culture.

One of the greatest sources of error in the ordinary test for acid production is the simultaneous production of acid from the sugar and of alkalinity from the peptone. When both of these activities occur in a tube of agar, however, the acid production generally predominates over the production of alkalinity during the early stages of growth, or if not, the two activities are likely to be localized in separate zones of the agar. For this reason, the agar slant method is a great help in overcoming this source of error.

Bronfenbrenner and Schlesinger (1918) recently recommended a method of using indicator agar for detecting acid production, by placing a series of drops in a single petri dish. Their method has the advantage of reducing the amount of glassware used, but it does not show gas production nor the characteristic localization of acid and alkali brought out by the agar slant.

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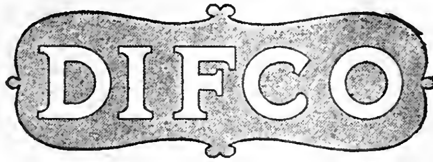

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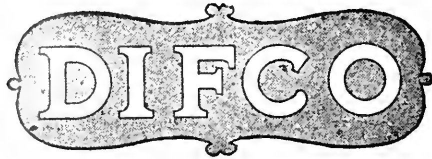

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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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Bromcresol Purple	yellow-purple	5.2-6.8
Litmus (special)	red-blue	5.5-8.9
Bromthymol Blue	yellow-blue	6.0-7.6
Phenol-Red	yellow-red	6.8-8.4
Cresol Red	yellow-red	7.2-8.8
Thymol Blue (alkaline range)	yellow-blue	8.0-9.6
Cresol-phthalein	colorless-red	8.2-9.8
Phenol-phthalein	colorless-red	8.4-9.2

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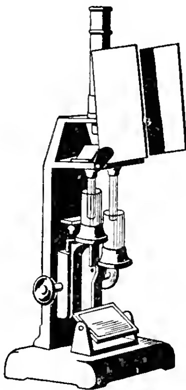

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THE FATE OF BACTERIA OF THE COLON-TYPHOID GROUP ON CARBOHYDRATE MEDIA

O. ISHII

From the Research Laboratory, Western Pennsylvania Hospital, Pittsburgh, Pennsylvania

Received for publication April 7, 1920

In any study of the fermentation reactions of the colon-typhoid group on carbohydrate culture media, it is customary for bacteriologists to make observations as to the quantity of acid, alkali, or gas formation for an unlimited number of days, sometimes for two weeks, or one month, or even for a much longer period. In this study we have found, however, that the organisms of the colon-typhoid group do not live as long as this on certain sugar culture media.

The technique used was as follows. The culture media containing the sugar were made with 1 per cent peptone plus 0.5 per cent sodium chloride, and they were sterilized once in the autoclave under 15 pounds pressure for fifteen minutes; this does not interfere with the differential fermentation reaction of these sugars, as we always have been able to differentiate quite satisfactorily the several strains of the colon-typhoid group with the above method.

Stock cultures were used and fresh subcultures were made in broth culture media. From the latter the sugar culture media were inoculated. The culture tubes were closed with cotton stoppers and kept in an incubator at 37°C. They were examined every day by the streak method on the plain agar plate, also in broth media, one loopful of the bacterial culture being taken from the sugar medium to determine whether or not the organisms were still living.

The results show that the period of life of the colon-typhoid group on certain of the different sugar cultures is short and on

OCT

others long (just the same results were obtained with or without an indicator, either litmus or china blue).

In the glucose-peptone water cultures in most cases, *Bact. typhi* died in three to five days, *Bact. paratyphi A* in three to five days, *Bact. paratyphi B* in three to six days, *Bact. enteritidis* in five to seven days, *Bact. suipestifer* in seven days, *Bact. coli* in six to seven days, *Proteus* in seven days and *Bact. dysenteriae* in three to seven days; but all strains of *Bact. pyocyaneus* still survived after thirty days' growth. The above results with *Ps. pyocyanea* were obtained with strains received from Dr. C.-E A. Winslow, and from Dr. Oscar Teague, Medical School of Columbia University of New York; a few strains, which we have isolated from feces, however, died within ten days in glucose broth, while others survived more than thirty days. It would seem that there are a number of different types of *Ps. pyocyanea* with different biological properties.

In mannitol peptone water cultures, in most cases, *Bact. typhi* died in four to six days, *Bact. paratyphi A* in four to five days, *Bact. paratyphi B* in five to eight days, *Bact. enteritidis* in about eight days, *Bact. suipestifer* in about eight days, *Bact. coli* in five to eight days, *Bact. dysenteriae* in five to ten days (with the exception of one which survived for over thirty days); *Proteus* and *Bact. pyocyaneus* were still alive after thirty days. One special strain of *Bact. dysenteriae* Shiga ※ T (obtained direct from Dr. Shiga, Japan), was still alive after thirty days, though another strain of Shiga no. 1, was dead in ten days. This is very peculiar, since culturally both were otherwise the same and there was no acid fermentation in either case.

In the lactose peptone water cultures of all the strains of *Bact. typhi*, *Bact. paratyphi A*, *Bact. paratyphi B*, *Bact. enteritidis*, *Bact. suipestifer*, *Proteus*, *Bact. pyocyaneus* and of all of the *Bact. dysenteriae* group, none had died within one month, but all strains of the *Bact. coli* group died within five to eighteen days.

In the sucrose peptone water cultures of all the strains used of *Bact. typhi*, *Bact. paratyphi A and B*, *Bact. enteritidis*, *Bact. suipestifer*, *Proteus*, *Bact. pyocyaneus* and of all of the *Bact. dysenteriae* group, none died within a month. Of the seven strains of

Bact. coli used, however, three strains died within five, eight and ten days, while the four other strains remained alive until after one month.

Fate of colon typhoid group on different sugar cultures, inoculated at 37°C. Numerals signify day of death. Mark — still alive thirty days afterward

ORGANISM	SUGAR CULTURES					
	Glucose peptone		Mannitol peptone		Lactose peptone	Sucrose peptone
	Results for all strains	Average number of days	Results for all strains	Average number of days	All strains	All strains
1. *Bact. typhi*	3 to 7	3 to 5	4 to 10	4 to 6	—	—
2. *Bact. paratyphi* A	3 to 7	3 to 5	4 to 8	4 to 5	—	—
3. *Bact. paratyphi* B	3 to 10	5 to 6	4 to 10	5 to 8	—	—
4. *Bact. enteritidis*	5 to 7	5 to 7	7 to 9	8	—	—
5. *Bact. suipestifer*	5 to 8	7	7 to 9	8	—	—
6. *Bact. coli*	5 to 8	6 to 7	5 to 16	5 to 8	5 to 18	3 strains, 5, 8, and 10 days; 4 strains, —
7. Proteus	6 to 9	7	—		—	—
8. *Bact. pyocyaneus*	—		—		—	—
9. Dysentery						
a. Shiga no. 1	3		10		—	—
b. Shiga no. 2	5		—		—	—
c. Flexner no. 1	6		10		—	—
d. Flexner no. 2	7		7		—	—
e. Hiss	7		7		—	—
f. Strong	5		5		—	—

In the above experiments we used 1 or 3 per cent sugar, but the results were almost the same with both concentrations.

From the results of these experiments we conclude that the recording of sugar fermentation reaction after the organisms are dead is unneceessary. It is desired to observe their activity only during their life. However, we still noticed more or less difference in the amount of the acid or alkali, this variation depending upon the absorption of CO_2 from the atmosphere.

For instance, in the glucose culture medium for the *Bact. typhi* and *Bact. paratyphi* group three to four days will be enough to take the record, then the tubes may be discarded; for *Bact.*

enteritidis, *Bact. suipestifer*, or *Bact. dysenteriae* group the period depends upon the strains, but about five to seven days will be enough; for *Bact. coli* six to seven days suffice; in regard to the other bacilli and also the other sugar media reference can be made to the accompanying table; the number of days indicated to study the reaction of the sugar cultures with the colon-typhoid group are sufficient.

"COLOR STANDARDS" FOR THE COLORIMETRIC MEASUREMENT OF H-ION CONCENTRATION pH 1.2 TO pH 9.8

LEON S. MEDALIA

From the Research Laboratories, Department of Biology and Public Health, Massachusetts Institute of Technology

Received for publication April 7, 1920

I. H-ION CONCENTRATION METHOD OF ADJUSTING CULTURE MEDIA BY THE USE OF "COLOR STANDARDS"

Introduction

Sörensen in 1909 demonstrated the value of the "H-ion concentration" as a basis for measuring the reaction in organic fluids as against the "titration" method then in vogue for adjusting culture media. This method has been adopted by practically all research workers in bacteriology ever since the classic work of Sörensen was published, yet no standard text-book in bacteriology however recent, does more than make a passing reference to it, although they all agree upon the value of the proper titration of culture media. It occurred to the writer that the reason why this method of determining reaction was left to the research worker alone, was because of the difficulties encountered in its application. In other words to carry out the standardization of culture media, according to the H-ion concentration properly, as heretofore suggested, one needs to be a fairly well trained chemist as well as a mathematician of no mean degree. To overcome these difficulties and make this very valuable method of adjusting culture media adaptable to the average laboratory, this research was undertaken. Only the successful results and method will be given in this report, all the attempts and experiments that led up to these results being omitted. Expressions that require a knowledge of higher mathematics and chemistry will be

strictly avoided in order not to confuse the average laboratory worker. Those interested in the subject, will find the articles by Sörensen (1912), and Clark and Lubs (1917), of special value.

Principles underlying the H-ion concentration method of titration

This method is based upon the *electrolytic dissociation* theory or *ionic-theory* developed by Arrhenius in 1887, which assumes that acids, bases, or salts, in aqueous solutions are dissociated to a greater or less extent into *ions*, i.e., electrically charged atoms, or groups of atoms. The unit charge is that which is associated with one hydrogen-ion. The strength of an acid in solution, according to this theory, depends upon the free or dissociated hydrogen-*ions* present in that solution. The comparative strength of two equivalent or "normal" solutions will vary according to the ability of the components of each to dissociate and allow free hydrogen-*ions* to accumulate in the solution. The relative amount of free hydrogen-*ions* (percentage dissociation) in "normal" solutions has been found to show wide variations. Thus, the percentage dissociation or the amount of H-ions set free in $\frac{N}{10}$ HCl solution was found (Talbot 1908) to be 90 per cent, while that of $\frac{N}{10}$ acetic acid is 1.4 per cent. Hydrochloric acid according to these findings is therefore 64 times stronger in acidity than acetic acid, although 10 cc. of $\frac{N}{10}$ of either acid will require a similar 10 cc. portion of $\frac{N}{10}$ NaOH to neutralize it. Accordingly the only correct method of measuring the acid strength of a solution is to determine the amount of free H-ions or the *hydrogen-ion concentration* (H.I.C.), of that solution, and *not* to determine the amount of $\frac{N}{10}$ NaOH necessary to neutralize that solution.

Another grave source of error in the titration method in common vogue is the fact that organic substances known as "buffers" when present in solutions (such as culture media) are capable of combining with the $\frac{N}{10}$ NaOH added during the titration, deviating it from and preventing its neutralizing, the acid with which it was meant to react.

From the foregoing it is evident that the NaOH method of titration with phenolphthalein is erroneous and highly misleading.

Method of expressing H-ion concentration—what is pH?

The accumulation of free hydrogen-ions present in a given solution, i.e., the H. I. C. of that solution, can be measured to the minutest amount and has been expressed in terms of "normal solutions." The amounts are so minute that they run up to the billionth or trillionth normal, since the acid strength or the hydrogen-ion content of neutral or even alkaline solutions

TABLE 1

Comparative values of hydrogen ion concentration expressed in "pH" and "normal" solutions

	pH	NORMAL SOLUTION (1 GRAM H TO 1 LITER OF WATER)	MICRONORMAL (μN.) SOLUTION*
$\frac{N}{1}$ HCl	0.0	1.0	1,000,000.0
$\frac{N}{10}$ HCl	1.0	0.1	100,000.0
	2.0	0.01	10,000.0
	3.0	0.001	1,000.0
	4.0	0.000,1	100.0
	5.0	0.000,01	10.0
	6.0	0.000,001	1.0
Neutrality	7.0	0.000,000,1	0.1
Alkaline	8.0	0.000,000,01	0.01
	9.0	0.000,000,001	0.001
	10.0	0.000,000,000,1	0.000,1
	11.0	0.000,000,000,01	0.000,01
	12.0	0.000,000,000,001	0.000,001
$\frac{N}{10}$ NaOH	13.0	0.000,000,000,000,1	0.000,000,1
$\frac{N}{1}$ NaOH	14.0	0.000,000,000,000,01	0.000,000,01

* By Micronormal is meant one-millionth (1/1,000,000) normal. Symbol μN.

is measurable. In order to overcome the unwieldiness of the figures necessary to express the H. I. C. Sörensen suggested the symbol pH to express one-tenth normal beginning on the acid side and going up in negative multiples of one-tenth towards alkalinity. Thus pH 1 equals $\frac{N}{10}$ acid; pH 2 equals $\frac{N}{10} \times \frac{N}{10} = \frac{N}{100}$; pH 3 equals $\frac{N}{100} \times \frac{N}{10} = 1/1000$ normal, etc. The lower the pH of a given solution, therefore, the more acid, or the higher its H. I. C. and the higher the pH the less acid, or the lower is its

TABLE 2

*Comparative values of H. I. C. expressed in pH, with fractions and normal solutions**

pH	NORMAL	MICRONORMAL (μN)
0.0	1.0	1,000,000.0
1.0	0.1	100,000.0
1.1	0.079,43	79,430.0
1.2	0.063,10	63,100.0
1.3	0.050,12	50,120.0
1.4	0.039,81	39,810.0
1.5	0.031,62	31,620.0
1.6	0.025,12	25,120.0
1.7	0.019,95	19,950.0
1.8	0.015,85	15,850.0
1.9	0.012,59	12,590.0
2.0	0.01	10,000.0
2.1	0.007,943	7,943.0
2.2	0.006,310	6,310.0
2.3	0.005,012	5,012.0
2.4	0.003,981	3,981.0
2.5	0.003,162	3,162.0
2.6	0.002,512	2,512.0
2.7	0.001,995	1,995.0
2.8	0.001,585	1,585.0
2.9	0.001,259	1,259.0
3.0	0.001	1,000.0
3.1	0.000,794,3	794.3
3.2	0.000,631,0	631.0
etc.		
4.0	0.000,1	100.0
4.1	0.000,079,43	79.43
4.2	0.000,063,10	63.10
etc.		
5.0	0.000,01	10.0
5.1	0.000,007,943	7.943
5.2	0.000,006,310	6.310
etc.		
6.0	0.000,001	1.0
6.1	0.000,000,794,3	0.794,3
6.2	0.000,000,631,0	0.631,0
etc.		

* This table was prepared from an antilogarithm table by using the antilogarithmic value of 0.9 for pH 1.1, that of 0.8 for pH 1.2, etc.—since pH 1.1 is pH 0.9 stronger than pH 2, and pH 1.2 is pH 0.8 stronger than pH 2, etc.

TABLE 2—*Continued*

pH	NORMAL	MICRONORMAL (μN)
7.0	0.000,000,1	0.1
7.1	0.000,000,079,43	0.079,43
7.2	0.000,000,063,10	0.063,10
etc.		
8.0	0.000,000,01	0.01
8.1	0.000,000,007,943	0.007,943
8.2	0.000,000,006,310	0.006,310
etc.		
9.0	0.000,000,001	0.001
9.1	0.000,000,000,794,3	0.000,794,3
9.2	0.000,000,000,631,0	0.000,631,0
etc.		
10.0	0.000,000,000,1	0.000,1

H. I. C. The relationships of pH to normal are given in tables 1 and 2. Table 1 gives the comparative values of pH and normal solutions, and micronormal solutions. (The term micronormal meaning a millionth normal, symbol (μ.N.) was coined by the writer to facilitate expressing pH values in terms of normal solution.) Table 2 gives the values of pH and its decimal fractions in terms of normality. These tables were thought desirable because we are in a habit of thinking in terms of normality, and not in terms of pH.

Methods used to determine the H-ion concentration

Having decided upon the H-ion concentration as a correct method to determine the acidity of a given solution the question arises how this may be accomplished? Two methods have been described (1) the electrolytic method, and, (2) the colorimetric method.

The electrolytic method, though the more accurate, is highly technical and requires cumbersome apparatus much beyond the possibilities of the average bacteriological laboratory.

The colorimetric method depends on the *color changes* that take place in certain indicators at different acid strength or H-ion concentration. The H-ion concentration has been determined electrolytically for a number of solutions ("buffer solutions") con-

taining various chemicals in different dilutions. Such solutions when accurately duplicated are supposed to be of the same H-ion content, and should therefore show similar variations in color, in the indicators used. These solutions are referred to as "standard solutions" of known H. I. C.

The difficulties met with in preparing the "standard solutions" of known H. I. C. in the average bacteriological laboratory constitute one reason why this method has not come into general use, and furnished the principal stimulus for this research.

Experimental work

In looking over the range of pH of the indicators developed by Clark and Lubs (1917) I found that the sensitive range between the extreme acid color and the extreme alkaline color of each indicator, is pH 1.6. It occurred to me, that by dividing this sensitive range of color, pH 1.6, into *eight equal* parts, one should obtain a range for each indicator of pH 0.2 intervals. This should be accomplished by adding 0.1 cc. of the indicator solution to one test tube containing 10 cc. of an alkaline solution, and 0.7 cc. of the same indicator to another test tube containing 10 cc. of an acid solution—(solutions that will bring out the alkaline and acid colors of the particular indicator)—then looking through the two test tubes placed one behind the other, i.e., superimposing the alkaline and acid colors of the indicator in different strengths[1] the color should be pH 0.2 higher (more alkaline) than the extreme acid color with 0.8 cc. of the same indicator. Increasing by 0.1 cc. in the alkaline solution, and decreasing by 0.1 cc. in the acid solution, in a set of seven pairs of tubes should give us the full range of the indicator at an interval of pH 0.2.

This was tested out with brom thymol blue and it succeeded perfectly, i.e., the green color was found at (pair no. 4) pH 7; or slightly yellowish green at (pair no. 3) pH 6.8, according to this range. (The change of color of this indicator was found by the

[1] Following the procedure made use of by Salm (1904) to determine the half transformation point and that devised by Barnett and Chapman to prepare the color standards for phenol red.

writer to start with pH 6.2 instead of pH 6 as given by Clark and Lubs (1917). Various difficulties had to be overcome in finding the proper acid or alkaline solution that would bring out the extreme colors that would remain fairly permanent. Finally, however, these difficulties were overcome and the proper strength of the indicators was found, that would best serve this purpose.

A method of obtaining the indicators in sterile solutions so that they could be kept indefinitely, and used for testing acid formation by bacteria in cultures without contaminating them was also devised.

Preparation of indicator solutions

a. Stock alcoholic solutions. Two-tenths per cent alcoholic solutions of the indicators were prepared as "stock solutions" by dissolving 0.1 gram of the respective indicators in powder form in 50 cc. of 95 per cent ethyl alcohol (ordinary alcohol), and kept in amber colored bottles, well stoppered with rubber stoppers and paraffined. The following indicators of Clark and Lubs were used, all of which were obtained in powder form from Hynson Westcott and Dunning excepting methyl red which was obtained at the Institute. Range and color changes are as given by Clark and Lubs (1917).

Indicators (Clark and Lubs)

1. Thymol blue acid range	Red-yellow	pH 1.2–2.8
2. Brom phenol blue	Yellow-blue	pH 3.0–4.6
3. Methyl red	Red-yellow	pH 4.4–6.0
4. Brom cresol purple	Yellow-purple	pH 5.2–6.8
*5. Brom thymol blue	Yellow-blue	pH 6.0–7.6*
6. Phenol red	Yellow-red	pH 6.8–8.4
7. Cresol red	Yellow-red	pH 7.2–8.8
8. Thymol blue alkaline range	Yellow-blue	pH 8.0–9.6

* The range of color of this indicator as found by the writer should read 6.2–7.8.

These "stock" alcoholic solutions are best kept in a dark place —closed cupboard, or box.

b. Indicator watery solutions. The strength of the indicator solutions found most useful to prepare the "standard colors" as well as for use in measuring the H. I. C. of fluids were 0.02 per cent of the respective indicators in sterile distilled water, (excepting phenol red in which case 0.04 per cent was found necessary). These solutions were prepared from the "stock alcoholic solutions" by placing 45 cc. (40 cc. for phenol red) of distilled water in each of eight amber colored bottles of proper size stoppered with cotton and sterilized in the autoclave at 10 pounds for one-half hour (rubber stoppers were sterilized alongside). To each of the 45 cc. bottles of sterile distilled water, 5 cc. of the "stock" alcoholic solutions of the indicators were added with sterile pipets in an aseptic way making a 0.02 per cent solution, except in the case of phenol red, in which case 10 cc. of the alcoholic stock solutions were added (to the 40 cc. of water) making a 0.04 per cent solution. These water solutions were kept in the dark, well stoppered with the sterilized rubber stoppers.

The flocculent precipitate of the methyl red, formed when the alcoholic solution was added to the distilled water, was cleared away by the addition of 0.5 cc. of sterile $\frac{N}{20}$ NaOH. This has not interfered with the production of red in the acid, and yellow in the alkaline solutions.

Preparation of "color standards"

For the preparation of "color standards" for standardizing culture media, the regular B. B. H. test tubes were used (thick walled, without lips, 130 by 16 mm. outside diameter) 14 or at most 28 such tubes being necessary. They should be of clear glass and as *nearly alike* in *diameter* as possible. The importance of having the tubes alike in diameter cannot be emphasized too strongly.

Two sets of test tubes, seven pairs in each, are made use of, one set for *brom thymol blue*, and the other for *phenol red*. Seven tubes of each set are filled with approximately $\frac{N}{20}$ NaOH, and the other seven with 0.1 per cent HCl (made from concentrated HCl 0.1 cc. and distilled water 100 cc.). The seven pairs of tubes are

set up in a rack, the tubes containing the acid solution in a row, behind which is another row of seven tubes containing the alkaline solution (fig. 1). Brom thymol blue, 0.02 per cent watery solution, is added to the tubes with NaOH in amounts of 0.1 cc. increasing by 0.1 cc. up to 0.7 cc. from left to right, while in the acid tubes the indicator is added beginning with 0.7 cc. decreasing by 0.1 cc. to 0.1 cc. from left to right, respectively: so that each pair of tubes contains together 0.8 cc. of the indicator, the amount increasing by 0.1 cc. in the tubes with the alkaline solution, while it decreases by 0.1 cc. in the acid solution. The tubes are then labelled in pairs from 1 to 7, the label also bearing the name of the indicator and the pH. The pH of the first pair is always 0.2 higher (more alkaline) than the initial range of the particular indicator. Brom thymol blue, according to my findings, begins to show a change of color at pH 6.2 (no difference in color was found with this indicator between pH 6, and 6.2, but there is a difference between 6.2 and 6.4). The first pair of this indicator will accordingly be pH 6.4, each succeeding pair will increase by pH 0.2 up to the seventh pair which will be pH 7.6. Therefore the seven pairs with brom thymol blue yield a range of "standard colors" of pH 6.4 to pH 7.6, at an interval of pH 0.2, most valuable for titration of culture media since neutrality is at pH 7, which with this indicator is at pair no. 4 yielding a green color—not yellowish green (pair no. 3) nor bluish green (pair no. 5). If a wider range is desired (rarely necessary) a set of "standard colors" with the phenol red can be prepared in the same way as given for brom thymol blue, i.e., the same strength of NaOH and HCl is used, the only difference being the change of the indicator to phenol red. The seven pairs of tubes of this indicator will yield a range from pH 7 to pH 8.2 overlapping at pH 7, 7.2, 7.4, and 7.6. The phenol red does not make as clear cut a range, as easily differentiated at pH 0.2 intervals, as does brom thymol blue and is not really necessary for the titration of culture media which as a rule are neutral, or thereabouts. Figure 1 shows the way the test tubes are set up for the preparation of the "color standards." Tables 3 and 4 give the composition of each pair for the two indicators, the color change of each, as nearly as it can be described, and the pH value of each pair.

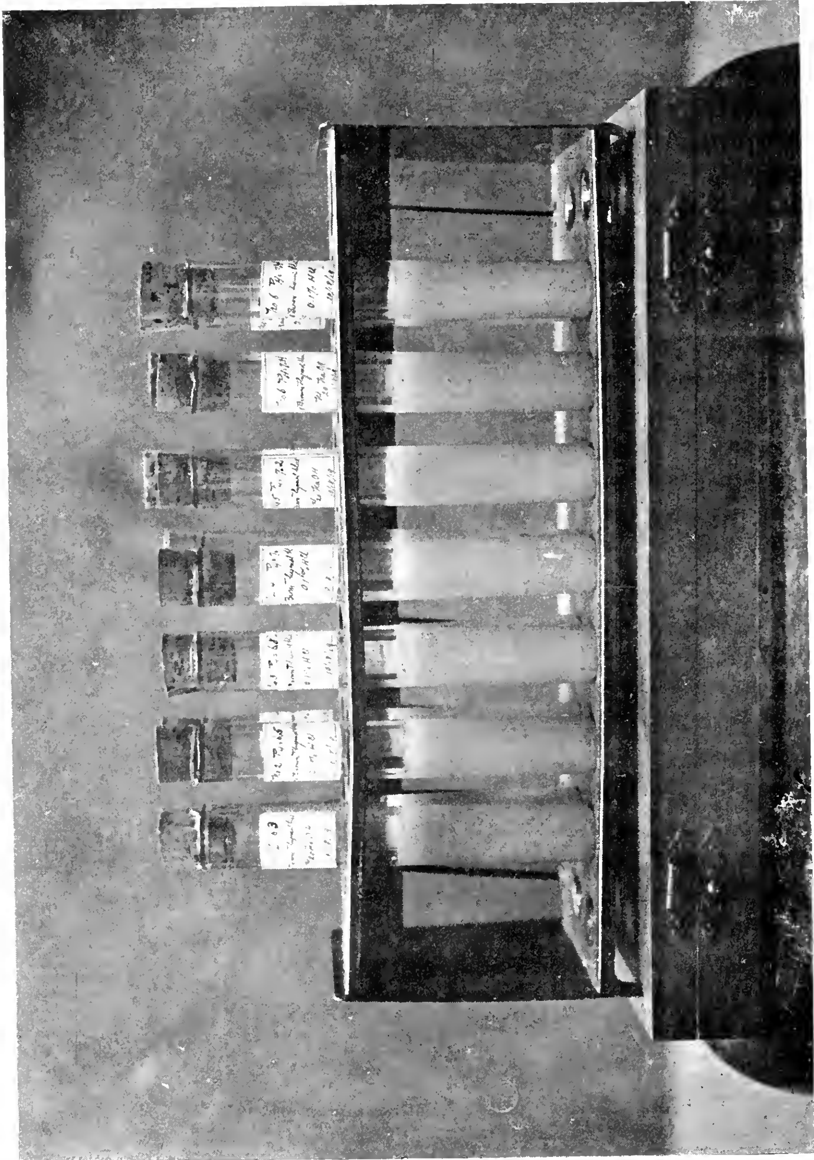

Fig. 1. Black Enamelled Iron Test Tube Support

Single deck with double row of holes containing seven pairs of "color standards" of brom thymol blue. (The second tube of pair no. 6 was placed in front of pair no. 7 by mistake, it should read no. 7 pH 7.6.)

The pH values have been calibrated with solutions of known H. I. C. prepared according to directions given by Clark and Lubs (1917) and cross checked where the indicators overlap.

Keeping qualities of the "standard colors." The standard color solutions have been kept for three months by observing the fol-

TABLE 3

*Composition of "Standard Colors" prepared with brom thymol blue 0.02 per cent watery solution—range pH 6.4 to 7.6**

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 per cent HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	6.4
2	1	10	0.2	2	10	0.6	Lighter yellow	6.6
3	1	10	0.3	2	10	0.5	Yellow-green	6.8
4	1	10	0.4	2	10	0.4	Green	7.0
5	1	10	0.5	2	10	0.3	Bluish green	7.2
6	1	10	0.6	2	10	0.2	Greenish blue	7.4
7	1	10	0.7	2	10	0.1	Blue	7.6

* The useful range of color of this indicator, according to calibration with phosphate NaOH mixture, should begin with pH 6.2, instead of pH 6, as given by Clark and Lubs.

TABLE 4

Composition of "standard colors" prepared with phenol red 0.04 per cent watery solution—range pH 7 to pH 8.2

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 per cent HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*			
1	1	10	0.1	2	10	0.7	Yellow	7.0
2	1	10	0.2	2	10	0.6	Lighter yellow	7.2
3	1	10	0.3	2	10	0.5	Pink yellow	7.4
4	1	10	0.4	2	10	0.4	More pink yellow	7.6
5	1	10	0.5	2	10	0.3	Beginning red	7.8
6	1	10	0.6	2	10	0.2	Slightly more red	8.0
7	1	10	0.7	2	10	0.1	Red	8.2

lowing rules in their preparation. New test tubes were washed in *warm water* (hot water produced a coating on the inside of some of the tubes) dried and filled with the respective $\frac{N}{20}$ NaOH and 0.1 per cent HCl freshly prepared with fresh distilled water. The tubes were then set up and the indicator solutions added, as

described, with pipettes. The tubes were then stoppered with corks previously dipped in hot melted paraffin. This procedure yields tightly sealed paraffined tubes of "standard color" solutions.

Titration of culture media

For the titration of a culture medium[2] three test tubes, similar to those used for the "color standards," are filled, each with 2 cc. of the filtered medium and 8 cc. of distilled water. To one of these 0.8 cc. of the 0.02 per cent of the brom thymol blue indicator is added and compared with the "color standards" tubes. The other two tubes are used to offset the color of the medium in the "comparator" block described below. The fluid to be titrated is placed in the center row of the "comparator" block having two test tubes of distilled water behind it. On either side are placed the two pairs of test tubes nearest to the pH desired (pairs 4 and 5 pH 7 and pH 7.2). In front of each pair is placed the tube containing 2 cc. of the medium and 8 cc. of distilled water to offset the color of the medium. It is absolutely *necessary* to *compensate* for the color of the medium in this way or the result will be misleading. $\frac{N}{20}$ NaOH is carefully run in the tube that is being titrated until it matches pair no. 4 = pH 7. Twenty-five times the amount of $\frac{N}{20}$ NaOH used, will represent the amount of normal NaOH necessary to neutralize 1 liter of the medium.

$$\left(\frac{1000 \text{ cc.}}{2 \text{ cc.}} \times \frac{1}{20} = 25\right)$$

If medium is too alkaline add $\frac{N}{20}$ HCl until the color matches and calculate the amount of $\frac{N}{1}$ HCl to be added to one liter of medium in the same way. The same factor is used whatever the pH value selected for the reaction of the medium. The medium should be retitrated after the addition of the alkali or the acid as the case may be. The use of the "comparator block"

[2] Hot fluid agar or gelatin media, before final filtration, can be titrated as described. The 2 cc. of the agar or gelatin when diluted with 8 cc. of water, remain fluid for a much longer time than is necessary to carry out the titration.

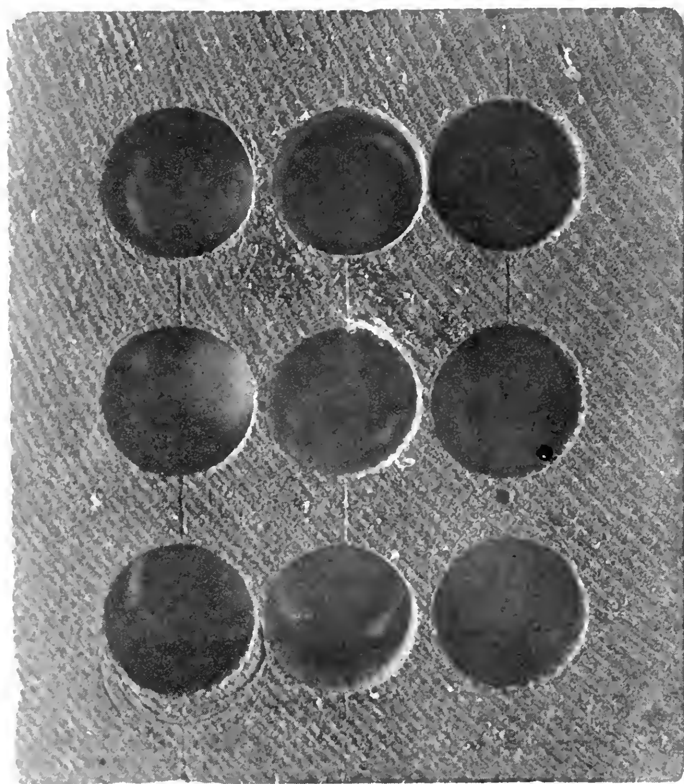

Fig. 2. Top of "Comparator Block," Natural Size—Showing the Three Rows of Holes

In the front, center hole, is placed the tube containing the culture media to be titrated, behind it are placed two tubes of distilled water—on either side are placed the pairs of "color standards" desired with a tube of culture media behind each pair to offset the color of the medium.

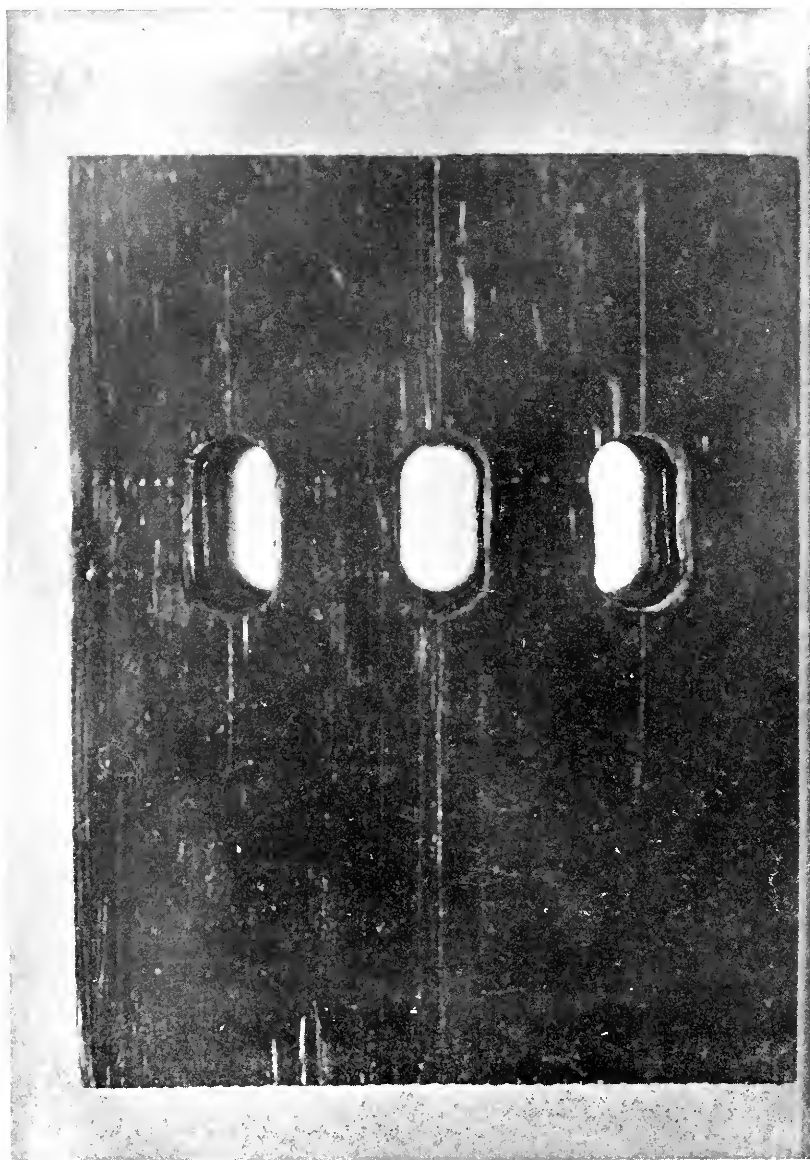

Fig. 3. Front of "Comparator Block," Natural Size

Showing the three slits connecting the three rows of holes holding the test tubes. The slits are used to look through the test tubes as they are placed one behind the other.

such as described by Hurwitz, Myer and Ostenberg (1916) modified to suit our purpose, was found indispensable.

The block is made by boring three rows of three holes in each row. The holes are 2 cm. in diameter and 9 cm. deep. The block measures 13 cm. high by 9 by 10 cm. Each row of holes is connected by a slit that goes all the way through, 2.5 cm. high by 1.25 cm. wide, the upper edge of the slit being 4 cm. from top of block. The block is painted with black enamel paint including the slits. It is best not to paint the holes that hold the test tubes (figs. 2 and 3).

II. pH MEASUREMENT OF ACID OR ALKALI PRODUCTION BY BACTERIA, BY THE USE OF "COLOR STANDARDS"

A wider range is necessary to measure the acid and alkali production by bacteria than that used for the standardization of culture media. "Color standards" prepared from 0.02 per cent solutions of each of the following indicators: Thymol-blue-acid-range; brom-phenol-blue; methyl-red; brom-cresol-purple; cresol-red; and thymol-blue-alkaline-range; in addition to brom-thymol-blue. and phenol-red, used for titration of culture media, already described, will give a range extending from pH 1.4 to pH 9.6 more than sufficient to test the acid as well as the alkali production by bacteria.

The methods for the preparation of "color standards" from these additional indicators are similar to those described in part I. The indicators are used in 0.02 per cent watery solutions prepared from 0.2 per cent stock alcoholic solutions (phenol-red 0.04 per cent).

The solutions necessary to bring out the extreme acid color, and the extreme alkaline color of the various indicators are as follows.

Thymol-blue acid range. The *extreme acid* (red) color of this indicator was brought out by 0.5 per cent HCl; and the *extreme alkaline* (yellow) of this indicator was brought out with 0.001 per cent HCl (1 cc. of 0.1 per cent to 99 cc. of distilled water). Various other strengths of both acid and alkaline solutions were tried, but failed.

Thymol-blue-alkaline-range. The extreme acid (yellow) color was brought out by using 0.001 per cent HCl; the extreme alkaline (blue) color of this indicator was brought out by the use of $\frac{N}{20}$ NaOH.

Brom-phenol-blue. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (blue) color was brought out by $\frac{N}{200}$ NaOH solution, instead of $\frac{N}{20}$ NaOH.

Methyl-red. The extreme acid (red) color of this indicator, was brought out by 0.1 per cent HCl; the extreme alkaline (yellow) color of this indicator was brought out by $\frac{N}{20}$ NaOH.

Brom-cresol-purple. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (purple) color was brought out by $\frac{N}{20}$ NaOH.

Brom-thymol-blue. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (blue) color was brought out by $\frac{N}{20}$ NaOH.

Phenol-red. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (red) color was brought out by $\frac{N}{20}$ NaOH.

Cresol-red. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (red) color was brought out by $\frac{N}{20}$ NaOH.

The procedure is the same as described under part I—seven pairs of tubes are filled, each with 10 cc. amounts of the respective acid and alkaline solutions for each indicator. The seven alkaline (or weak acid) tubes are set up in a row behind the seven acid tubes. The indicator solution is added to the akaline tubes in amount of 0.1 cc. *increasing* by 0.1 cc. up to 0.7 cc. from left to right—while in the acid row of tubes, 0.7 cc. indicator solution is added *decreasing* from left to right by 0.1 cc. down to 0.1 cc. in the last tube. In case it is desired to keep the color standards for any length of time, it is best to stopper the tubes with cork stoppers previously dipped in hot melted paraffin. Solutions treated as just described, have been found to keep well for over three months. It is well to check up these "standard colors" by setting up fresh sets, for the purpose, once a month, or oftener. This can be done very easily and does not take up much time.

TABLE 5

Composition of "standard colors" prepared with thymol-blue-acid—range 0.02 per cent watery solution—range pH 1.4 to pH 2.6

PAIR	TUBE	0.001 PER CENT HCl	INDICATOR	TUBE	0.5 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Red	1.4
2	1	10	0.2	2	10	0.6	Lighter red	1.6
3	1	10	0.3	2	10	0.5	Yellow red	1.8
4	1	10	0.4	2	10	0.4	Red yellow	2.0
5	1	10	0.5	2	10	0.3	Beginning yellow	2.2
6	1	10	0.6	2	10	0.2	More yellow	2.4
7	1	10	0.7	2	10	0.1	Still more yellow	2.6

TABLE 6

Composition of "standard colors" prepared with brom-phenol-blue 0.02 per cent watery solution—range pH 3.4 to pH 4.6*

PAIR	TUBE	$\frac{N}{200}$ NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	3.4
2	1	10	0.2	2	10	0.6	Light yellow	3.6
3	1	10	0.3	2	10	0.5	Beginning pink	3.8
4	1	10	0.4	2	10	0.4	Pink-red	4.0
5	1	10	0.5	2	10	0.3	More red	4.2
6	1	10	0.6	2	10	0.2	Beginning blue	4.4
7	1	10	0.7	2	10	0.1	Blue	4.6

* The blue color of this indicator can be brought out with $\frac{N}{20}$ NaOH, but is not lasting and fades within twenty-four to forty-eight hours, while the $\frac{N}{200}$ NaOH makes a lasting range of "color standards."

TABLE 7

Composition of "standard colors" prepared with methyl red 0.02 per cent watery solution—range pH 4.6 to pH 5.8

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Red	4.6*
2	1	10	0.2	2	10	0.6	Light red	4.8
3	1	10	0.3	2	10	0.5	Yellow red	5.0
4	1	10	0.4	2	10	0.4	Red yellow	5.2
5	1	10	0.5	2	10	0.3	Beginning yellow	5.4
6	1	10	0.6	2	10	0.2	More yellow	5.6
7	1	10	0.7	2	10	0.1	Yellow	5.8

* There was a very slight difference between the phthalate NaOH mixture and the "standard colors" in the first three pairs: Pair No. 1 = pH 4.7; Pair No. 2 = pH 4.9; Pair No. 3 = pH 5.1 according to the phthalate NaOH mixture, the rest were the same in both.

TABLE 8

Composition of "standard colors" prepared with brom-cresol-purple 0.02 per cent watery solution—range pH 5.4 to pH 6.6

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	5.4
2	1	10	0.2	2	10	0.6	Lighter yellow	5.6
3	1	10	0.3	2	10	0.5	Beginning pink	5.8*
4	1	10	0.4	2	10	0.4	Pink	6.0
5	1	10	0.5	2	10	0.3	Beginning purple	6.2
6	1	10	0.6	2	10	0.2	More purple	6.4
7	1	10	0.7	2	10	0.1	Purple	6.6

* According to phthalate and phosphate NaOH mixtures, pairs 3, 4, 5, 6, 7, should read: pH 5.9; 6.1; 6.3; 6.5; 6.7.

TABLE 9*

Composition of "standard colors" prepared with cresol-red 0.02 per cent watery solution—range pH 7.4 to pH 8.6

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	7.4
2	1	10	0.2	2	10	0.6	Lighter yellow	7.6
3	1	10	0.3	2	10	0.5	Pink yellow	7.8
4	1	10	0.4	2	10	0.4	Yellow pink	8.0
5	1	10	0.5	2	10	0.3	Pink	8.2
6	1	10	0.6	2	10	0.2	Beginning red	8.4
7	1	10	0.7	2	10	0.1	Red	8.6

* For the composition of "standard colors" prepared with *brom-thymol-blue* and *phenol-red* see table 3 and table 4.

TABLE 10

Composition of "standard colors" prepared with thymol-blue-alkaline-range 0.02 per cent water solution—range pH 8.2 to 9.4

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.001 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	8.2
2	1	10	0.2	2	10	0.6	Light yellow	8.4
3	1	10	0.3	2	10	0.5	Yellow-green	8.6
4	1	10	0.4	2	10	0.4	Green	8.8
5	1	10	0.5	2	10	0.3	Green-blue	9.0
6	1	10	0.6	2	10	0.2	Faint blue	9.2
7	1	10	0.7	2	10	0.1	Blue	9.4

The detailed composition of the pairs of tubes comprising the "color standards" of each indicator, as well as its equivalent in pH as found by calibration with standard solutions of known H. I. C. prepared according to Clark and Lubs (1917) will be found on tables 5, 6, 7, 8, 9, and 10; and part I, tables 3 and 4. The color of each pair of tubes corresponding to the particular pH is described as nearly as possible.

Procedure for measuring acid production by bacteria

In order to establish the best method to determine the acid production by bacteria quantitatively it was thought desirable to test out the various indicators as to their ability (a) to stand heating while being sterilized; (b) as to the action produced upon such indicators by bacteria. For that purpose the following experiments were undertaken:

Test tubes similar in size to those used for the "color standards" were filled each with 10 cc. amounts of glucose and of lactose bouillon (pH 7) using a 10 cc. automatic filler (fig. 4). These were divided into three sets of sixteen tubes each. To one set of the glucose and one set of the lactose bouillon 0.8 cc. amounts of the respective indicators were added to each tube *before* sterilizing. To another set, the same amount of the respective indicators was added *after* sterilizing, and the third set was used as a control—the indicators being added after inoculation with *Bact. coli* and incubation for twenty-four hours. Sterilization of the three sets was done at the same time by the fractional method in the Arnold sterilizer, on three successive days at 100°C. for three-quarters of an hour at a time.

Thus the *first* set was used to test the ability of the different indicators to stand heat. The *second* set was used to test the ability of the different indicators to resist the action of the bacteria; and the *third* set as a control of the first two sets. Table 11 gives the results of these tests in detail.

Action of heat upon the indicators used

A study of table 11 will show that after sterilization, seven of the indicators: thymol-blue-acid range; brom-phenol-blue; brom-cresol purple; brom-thymol-blue; phenol-red; cresol-red; and thymol-blue-alkaline range; did not change by sterilization in the

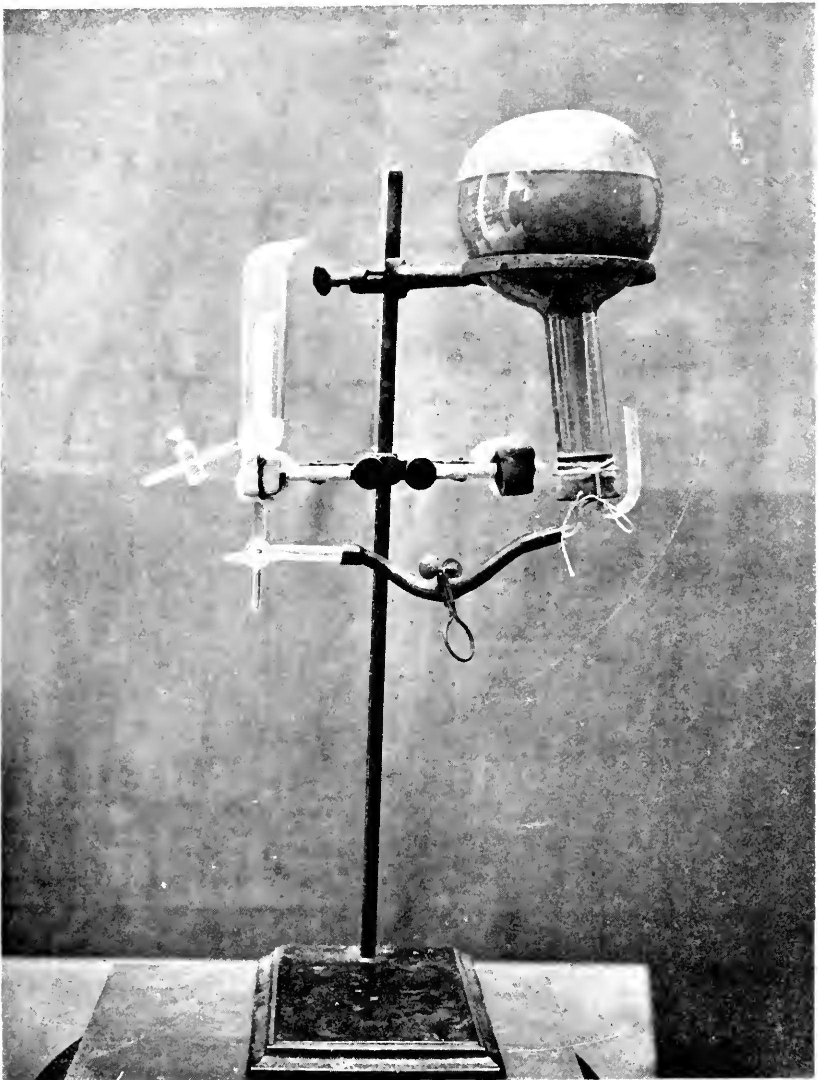

Fig. 4. Automatic 10 cc. Filler

The flask of culture media is inverted and attached by a rubber tube to the 10 cc. automatic pipette (pipette Vanier) consisting of a three-way stopcock which allows the fluid to run into the inside tube which, when filled, measures exactly 10 cc. Any excess fluid flows over into a surrounding tube, which may be emptied by the side stopcock.

Arnold sterilizer. Methyl-red was the only indicator that decolorized on sterilization. This was true of the glucose as well as the lactose bouillon. These findings would justify the conclusion that the indicators enumerated, with the exception of methyl-red can be introduced in the sugar media before sterilization, if sterilization is done by the fractional method. It may be of interest to note here that the reaction of the glucose bouillon with brom-thymol-blue just before sterilization was pH 7, and after sterilization it was found to be pH 6.8; while the lactose bouillon was pH 7 before and the same after sterilization. This would tend to indicate that the glucose bouillon becomes slightly more acid on sterilization.

Action of bacteria upon the indicators

To each of the test tubes of the second set of glucose and lactose bouillon respectively, was added *after* sterilization and with sterile pipettes 0.8 cc. of each of the eight indicators enumerated. The third set was left as a control. All three sets were then placed in an incubator for twenty-four hours to test sterility. When found sterile, the next day they were inoculated with a pure culture of *Bact. coli* (freshly isolated from feces) and placed in an incubator at 37°C. According to the findings as given in table 11 none of the indicators except methyl red were attacked by the bacteria, (*Bact. coli*) used in this test. Methyl red was the only one of the whole group that was decolorized. It would seem fair, therefore, to assume that the seven indicators used may be added before sterilization and inoculation when dealing with *Bact. coli*. When dealing with another organism, it would be wise to test out its effect upon the indicators, or run control cultures to check up findings.[3]

[3] Incidentally it may be noted that the acid production of *Bact. coli* in glucose was found to be the difference between pH 6.8 and 4.8 or pH 2 in twenty-four hours, and that it did not increase in the next twenty-four hours The same organism in lactose produced much less acid in twenty-four hours (a difference of pH 7 to 5.5 or pH 1.5), while in the next twenty-four hours it reached the same acidity as in glucose (the difference of between pH 7 to 5 or pH 2). The third set used as a control, checked up the findings in set no. 1 and no. 2. Methyl-red if it be used, must be added fresh immediately before the reading is made. The ability of sulphonthalein indicators to withstand the action of bacteria, has been recognized by observers and referred to by the committee on descriptive chart. Com. Soc. Am. Bact. (1919).

TABLE 11

Data pertaining to the method of studying acid production by bacteria; also effect of heat and of bacteria (Bact. coli) upon the indicators used in glucose and lactose bouillon

	SET NO. 1. INDICATORS ADDED BEFORE STERILIZATION		SET NO. 2. INDICATORS ADDED AFTER STERILIZATION		SET NO. 3. INDICATORS ADDED AFTER INOCULATION AND INCUBATION	
	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose
			Thymol-blue-acid-range			
After sterilization	No change (more yellow than pair no. 7)	No change in color	No change	No change		
Twenty-four hours incubation, *Bact. coli*	No change	No change	No change	No change	Same as sets 1 and 2	Same as sets 1 and 2
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change
			Brom-phenol-blue			
After sterilization	No change in color	No change	No change	No change		
Twenty-four hours incubation, *Bact. coli*	Almost same as pair no. 7 pH 4.6	More blue than pair no. 7 (more alkaline than pH 4.6)	Same as glucose set no. 1	Same as lactose set no. 1	Same as glucose set no. 1	Same as lactose set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

Methyl red

After sterilization	Decolorized	Decolorized	More yellow than pair no. 7	Same as glucose		
Twenty-four hours incubation, *Bact. coli*	Decolorized	Decolorized	Decolorized	Decolorized	More red than pair no. 1 (more acid than pH 4.7)	Between pair nos. 4 and 5 (pH 5.3–5.4)
Forty-eight hours incubation, *Bact. coli*	Fresh indicator added—pH 4.7 (matches no. 1)	Fresh indicator added—pH 4.9 (matches no. 2)	Fresh indicator added—pH 4.7 (same as set no. 1)	Fresh indicator added—pH 4.9 (same as set no. 4)	Fresh 48 hour culture with fresh indicator—pH 4.7	Fresh 48 hour culture with fresh indicator—pH 4.9

Brom cresol purple

After sterilization	Changed to match pair no. 7 pH 6.7	Slightly more purple than pair no. 7 (more alkaline than pH 6.7)	Same as pair no. 7 pH 6.7	Slightly more purple than pair no. 7		
Twenty-four hours incubation, *Bact. coli*	More yellow than pair no. 1 (more acid than pH 5.4)	Same as dextrose	Same as set no. 1	Same as set no. 1	Same as set no. 1	Same as set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

TABLE 11—*Continued*

	SET NO. 1. INDICATORS ADDED BEFORE STERILIZATION		SET NO. 2. INDICATORS ADDED AFTER STERILIZATION		SET NO. 3. INDICATORS ADDED AFTER INOCULATION AND INCUBATION	
	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose
Brom thymol blue						
After sterilization	Matches pair no. 3 pH 6.8	Matches pair no. 4 pH 7	Same as set no. 1	Same as set no. 1		
Twenty-four hours incubation, *Bact. coli*	More yellow than no. 1 (more acid than pH 6.4)	More yellow than No. 1	Same as set no. 1	Same as set no. 1	Same as set no. 1	Same as set no 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change
Phenol red						
After sterilization	Slightly more yellow than pair no. 1 pH 7	Same as pair no. 1 pH 7	Same as set no. 1	Same as set no. 1		
Twenty-four hours incubation, *Bact. coli*	More yellow than no. 1 (more acid than pH 6.4)	Same as glucose	Same as set no. 1	Same as set no. 1	Same as set no. 1	Same as set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

Cresol red

After sterilization	More yellow than no. 1 pH 7.4 (less alkaline)	More yellow than no. 1	More yellow than no. 1	More yellow than no. 1		
Twenty-four hours incubation, *Bact. coli*	No change in color	No change	No change	No change	No change	No change
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

Thymol blue alkaline range

After sterilization	No change (more yellow than pair no. 1)	No change (more yellow than pair no. 1)	Same as set no. 1	Same as set no. 1		
Twenty-four hours incubation, *Bact. coli*	No change in color	No change	No change	No change	Same color as set no. 1	Same color as set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

The comparator block was used in all the above studies.

Sterile glucose bouillon and lactose bouillon were used in the comparator to offset the color of the bouillon in the studies before inoculation. Twenty-four hours control cultures and forty-eight hours control cultures were used in the comparator to offset the color and turbidity when studying the respective cultures.

Procedure

In order to test the acid production of any given organism it is only necessary to cultivate the organism in 10 cc. amounts of fluid culture media of a known pH, that will best favor its growth. To each of the tubes of culture media is added 0.8 cc. of a 0.02 per cent solution of indicator—either phenol-blue or brom-cresol-purple—before sterilization, or if methyl-red has to be used the 0.8 cc. (of 0.02 per cent watery solution) of this indicator has to be added just before the reading is done, i.e., after sterilization, inoculation, and incubation. The cultures are then inoculated and incubated for as long a time as desired. Control cultures without indicator are also made and incubated alongside of those containing the indicator. The control cultures are used in the comparator block to offset the color and turbidity. The actual reading of the pH is done by placing the culture containing the indicator say brom-cresol-purple in the front of the center row of the comparator block, having two tubes of distilled water behind it while on either side of it are placed the pairs of "standard color" tubes of brom-cresol-purple approaching in color that of the culture with the same indicator; in front of each pair of standard colors in the comparator block one of the control cultures is placed to offset the color and turbidity of the medium. The reading is, then, simply a matter of matching colors, and takes very little time. Thus the actual acid production by any given organism can be easily tested from hour to hour, if desired, without disturbing the culture in any way, except in the case of methyl-red where fresh cultures are necessary for each reading—because of the fact that it is unstable and must be added fresh each time a reading is made.

Testing for alkali production by bacteria

The procedure for testing the alkali production by bacteria could be carried out in a similar way to that just described for the acid production by *Bact. coli.* The only difference is that the proper indicators (phenol-red; brom cresol-red; and thymol-blue-alkaline range) must be substituted for those just described;

otherwise the procedure is exactly the same. It is best to have two different cultures side by side, one for the acid production with its respective indicator (brom-cresol-purple), and another culture for the alkali production with its respective indicator (cresol-red) rather than to use the two indicators in the same culture. The acid and alkali production by the same organism can thus be easily read off without the possibility of the color of either indicator offsetting the other.

III. pH MEASUREMENT OF THE H. I. C. OF OTHER FLUIDS AND SOLUTIONS BY MEANS OF "COLOR STANDARDS"

For measuring the H-ion concentration of other fluids and solutions, acid or alkaline, such as urine, blood serum, etc., the same procedure described under parts I and II may be followed. The color standards and the methods for their preparation will be found described under parts I and II and in the accompanying tables. It may be desirable to describe in detail the testing of urine as a guide to the testing of other fluids.

Procedure

Three test tubes of the same calibre as those used for the "color standards" are each filled with 10 cc. amounts of the urine to be tested, previously filtered. Then 0.8 cc. of the 0.02 per cent solution of the *proper*[4] indicator is added to one of the three tubes with the urine. The test tube is rolled between the palms of the hands until well mixed. This tube of urine containing the indicator is placed in the center hole of the "comparator block" with two test tubes filled with distilled water behind it. The *proper* pairs of color standards are placed, each pair on either side of the urine to be tested, while in front of each pair of color standards, one of the tubes of urine without indicator is placed in the comparator block to offset the color of the urine. The pH represented by the pair of color standard tubes that matches in color that of the urine with the indicator is the pH of the urine.

[4] Some idea as to which is the proper indicator to use may be obtained by using litmus paper. If highly acid to litmus, brom-phenol-blue is used; if slightly acid, methyl-red or brom-cresol-purple is used. If alkaline, brom-thymol-blue or cresol-red should be used. The pH is thus very easily read off.

If highly colored fluids are to be titrated they may be diluted with equal parts or more of distilled water, since the addition of distilled water does not change the H. I. C. materially, if at all. A little experience will greatly facilitate the practical application of this method, with respect to choosing the proper indicator to use, and in estimating readily which pair of color standard tubes to use.

SUMMARY AND CONCLUSIONS

The colorimetric measurement of the H. I. C. by means of the "color standards" described is the simplest method yet published.

These color standards are easily prepared, lasting, and their use is readily applicable in the average bacteriological laboratory.

The practical application of the "color standards" for the titration of culture media, by the H-ion concentration method; also for the pH measurement of the acid or alkali production by bacteria, and for the pH measurement of other fluids as described in this paper, is simple and easily handled, and because of its simplicity, it promises to take a foremost place in the colorimetric measurement of the H. I. C. of any fluid as well as in bacteriology.

In concluding I wish to express my appreciation to Professor W. T. Sedgwick through whose kindness this work was made possible, and to Professor S. C. Prescott, and the other members of the Department of Biology and Public Health of the Institute, for their helpful assistance rendered me in this work.

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A STUDY OF THE ACTION OF EIGHT STRAINS OF *BACT. ABORTIVO-EQUINUS* ON CERTAIN OF THE CARBOHYDRATES[1]

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Abortion in mares is a disease quite widely distributed in the United States. It does not, however, cause as extensive losses, comparatively, as does abortion disease among the bovine species. It has been shown that *Bact. abortivo-equinus* is the causative organism of abortion in mares in the United States and the biology and pathogenesis of the germ have been carefully studied, among others, by Good and Corbett, Meyer and Boerner in this country, and Van Heelsbergen and De Jong, and Dassonville and Reviere in Europe. Abortions by the mares at the Experiment Station at University Farm, Minnesota, have been somewhat frequent. As a preliminary to a detailed study of this infection several strains of *Bact. abortivo-equinus* were obtained and a careful study of the cultural and pathogenic properties of the organisms was undertaken. It was noted that the actions of the different strains varied on the same carbohydrate, notably in the case of lactose. That is, some strains fermented this sugar with the production of gas while others failed to do so. It was thought advisable to make a somewhat detailed study of the action of this germ on the carbohydrates to learn if the fermentive action was constant in the different strains and to determine the cause of possible variations.

Good and Corbett came to the following conclusions as a result of their study of gas production by different strains of *Bact. abortivo-equinus*.

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The bubble, or the small amount of gas, encountered so often in our fermentation tests with *Bacillus abortivo-equinus* in lactose and saccharose broth is not of physical, but of chemical, origin.

Bacillus abortivo-equinus produced approximately 2 per cent gas in lactose in 80 per cent of 116 trials, and in saccharose slightly less than 2 per cent gas in 50 per cent of 56 trials.

Bacillus abortivo-equinus may or may not produce gas in 1 per cent lactose or saccharose broth, even varying in this respect in duplicate and triplicate tests.

Bacillus abortivo-equinus possesses as an original physiologic characteristic the ability, in most cases, to ferment lactose to a small extent, and also, in some cases, to ferment saccharose to a less extent. This characteristic in all probability has not as yet been accentuated by environment.

In these tests the four strains of *B. abortivo-equinus* produced the following average percentages of gas in the carbohydrates which were fermented: xylose 51 per cent; raffinose 39 per cent; arabinose 59 per cent; sorbite 82 per cent; dulcite 95 per cent; glucose 74 per cent; mannite 81 per cent.

De Jong as a result of his original observations states that the germ which he isolated from the uterine exudate of mares which aborted does not form gas in lactose but does in sucrose. In an inaugural dissertation published later Van Heelsbergen working in the same laboratory as De Jong makes the positive statement that *Bact. abortivo-equinus* does not ferment either lactose or sucrose.

Meyer and Boerner state that *B. abortivo-eqinus* does not ferment either lactose or sucrose.

Mudge found that heating in streaming steam seems to hydrolyze lactose and maltose and by sterilizing by filtration this hydrolysis can be avoided.

The study here reported was begun three years ago but owing to the fact that we were unable to obtain certain of the carbohydrates the work was very much delayed. The media used were prepared from lean beef, following the ordinary method. The muscle sugar was removed by the use of *Bact. coli* and then tested to insure that it was sugar free. One per cent of the various carbohydrates was added to this sugar free infusion bouillon.

The inverted vial was used to determine gas production. A portion of the medium, in the case of each carbohydrate, was distributed in test tubes, and sterilized in the Arnold by steaming twenty minutes for each of three successive days. Another portion of the same carbohydrate medium was sterilized by filtration through a Berkfeld filter employing the apparatus shown in figure 1. It will be noted from the cut that the test tubes are

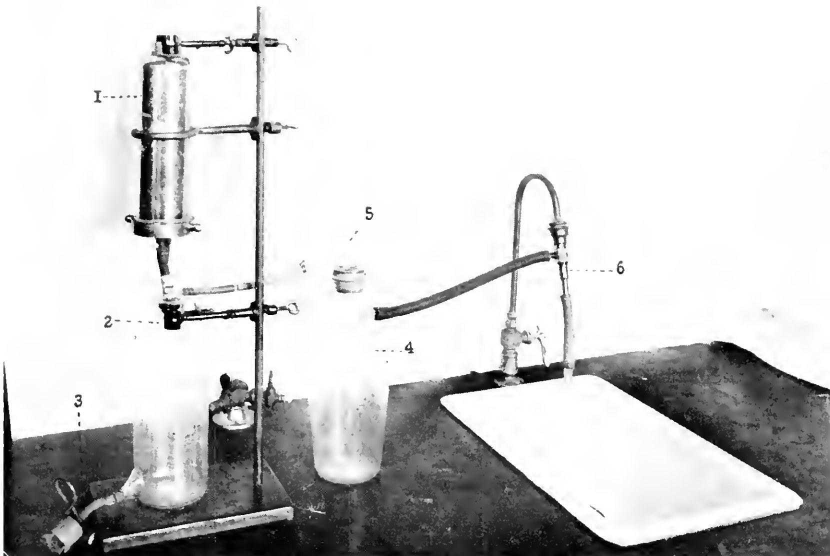

Fig. 1. Apparatus Showing Method of Sterilization of Media by Filtration

1, Berkfeld filter case; *2*, receiver of filtered media; *3*, filling tube; *4*, wash bottle; *5*, air cock; *6*, water suction pump.

filled directly through the tube 3. The glass tube attached to the end prevents bacteria from dropping into the open test tube. The inverted vials were filled by first coating the cotton plugs of the tubes containing the medium with paraffin, allowing it to harden, and then inverting which would permit the medium to fill the vial. This unheated medium was incubated for forty-eight hours and then allowed to stand forty-eight hours at room temperature. The contaminated tubes were then removed.

The strains of *Bact. abortivo-equinus* were obtained as follows: GI, GII, and GIII, were sent us by Professor Good of Kentucky, M.7 and M.8 from the Bureau of Animal Industry, Washington, D. C., H.3 and H.13 from the Laboratory of the Pennsylvania Live Stock Sanitary Board, Philadelphia. Each strain was inoculated in a series of the heated and unheated media. A tube was left uninoculated as a check for each strain which was incubated with the others. In as much as the observations of Good and Meyer were made on the basis of the reaction of the culture,

Table showing the results of gas production by Bact. abortivo-equinus in heated and unheated carbohydrate media. Also a comparison with the results in heated media of other investigators

	HEATED MEDIA	UNHEATED MEDIA	RESULTS OF MEYER AND BOERNER	RESULTS OF GOOD AND CORBETT
Glucose	Gas	Gas	Gas	Gas
Lactose	Gas	No gas	No gas	80 per cent of trials
Sucrose	Gas*	No gas	No gas	50 per cent of trials
Maltose	Gas	Gas	Gas	Gas
Mannitol	Gas	Gas	Gas	Gas
Mannose	Gas	Gas	Gas	Not given
Levulose	Gas	Gas	Gas	Not given
Dulcitol	Gas	Gas	Gas	Gas
Raffinose	No gas	No gas	No gas	Gas
Arabinose	Gas	Gas	Gas	Gas
Rhamnose	Gas	Gas	Gas	No gas
Xylose	Gas	Gas	Gas	Gas
Melezitose	0 (Size of	bubble) 0	Not given	Not given
Inulin	Gas	Gas	No gas	Not given
Salicin	No gas	No gas	Not given	Not given

* None in one trial; very slight in another.

using phenolphthalein as an indicator, it was deemed advisable to continue with this system instead of employing the more accurate pH standard. The tables at the end of the article give the results of these observations, continued in nearly all cases over a period of thirty days. In some instances it was impossible to obtain at the time sufficient of the carbohydrate to complete the series. The figures give the amount of $\frac{N}{20}$ NaOH or $\frac{N}{20}$ HCl (if minus sign precedes) necessary to neutralize five mils of the culture.

The gas production is recorded as accurately as possible from the action in the inverted vials. Observations were made at twenty-four, forty-eight and seventy-two hour intervals. A comparison of the results in regard to gas production in the heated and unheated media is shown in the preceding table. The results of Meyer and Good, both of whom used heated media, are appended.

It will be noted that gas was obtained in lactose only from the heated media. Good and Corbett obtained gas in many trials with this sugar but Meyer and Boerner report "no gas." As a result of our work we believe that the gas production in lactose recorded by Good and Corbett is due to the hydrolysis of the carbohydrate by sterilization. The work of Mudge clearly pointed out that discontinuous Arnold sterilization hydrolizes lactose and the results here recorded show that no gas was produced by any of the strains in the unheated lactose media.

In sucrose bouillon a variation in the results obtained in the heated media is noted. In one trial no gas was produced by any strain, in another only a very small amount. None was found at any time in the unheated sucrose media. Good and Corbett likewise record a smaller number of positive gas productions in this carbohydrate while Meyer and Boerner record "no gas."

In this work all strains have failed to produce gas in raffinose in both the heated and unheated media while all strains have produced gas in rhamnose. The first trial (rhamnose) however, showed much less gas in the unheated media than the second. The fermentation of rhamnose with gas agrees with the work of Meyer and Boerner but is contrary to the results of Good and Corbett. A small amount of gas was formed by all strains in both heated and unheated inulin broth. In as much as *Bact. abortivo-equinus* produces large amounts of gas in maltose, the ease with which this sugar is broken down by heat sterilization is not shown.

The carbohydrates which are fermented with the production of large amounts of gas show a nearly steady increase in acidity during a thirty day period. Dulcitol is an exception to this rule. There seems to be a marked variation in the action of the different strains of *Bact. abortivo-equinus* on this carbohydrate. This

variation is particularly marked in the heated media. In the unheated broth the reactions during the thirty day period are more uniform. The different strains do not show the same variation in the heated and unheated dulcitol media.

Inulin broth, which was fermented with a small amount of gas, shows also a very low initial acidity. Following this the inulin medium gradually becomes alkaline. This is perhaps to be explained in two ways. Inulin is often times impure and according to Hawk often contains a reducing sugar. Also inulin is hydrolyzed readily in the presence of an acid to levulose which carbohydrate is fermented by *Bact. abortivo-equinus* with the production of gas. The bubble of gas formed in melezitose may be due to an impurity in the sugar as the reactions in this media are similar to those in the other non fermented carbohydrates, i.e., a gradually increasing alkalinity.

It would seem that as a result of this work the following conclusions are justified.

1. *Bact. abortivo-equinus* does not form gas in lactose or sucrose. The apparent fermentations of these carbohydrates are often the result of hydrolysis in the sterilizing process.

2. *Bact. abortivo-equinus* does not ferment raffinose. Rhamnose is fermented with gas.

3. A gradually progressive acidity up to thirty days is produced by nearly all strains of *Bact. abortivo-equinus* in carbohydrate media where considerable gas is formed.

4. In carbohydrate broth in which no gas is formed a progressive alkalinity is formed over a thirty-day period.

5. In dulcitol media *Bact. abortivo-equinus* shows considerable variation in acidity and akalinity. This variation is found in unheated as well as heated media.

Note: Because of the necessity of economizing space it was deemed advisable to condense the fifteen tables originally prepared into one which is appended. This was done by computing the average titration figures for the eight strains. The variation in the acidity or alkalinity of the different strains was not large with the exception of their action on dulcitol. If readers desire we can furnish copies of the results obtained for each organism used.

Table showing the average action of eight strains of Bact. abortivo-equinus on the carbohydrates

SUGAR	INITIAL TITRATION	DAYS INCUBATED						
		2	5	10	15	20	30	GAS
								per cent
Heated glucose	0.6	2.9	3.2	3.6	4.1	4.5	6.0	23
Unheated glucose	0.4	3.4	3.7	3.9	4.1	4.5	5.9	20
Heated lactose	1.6	0.5	0.6	−0.5	−0.7	−0.9	−1.5	3
Unheated lactose	1.5	1.5	0.9	−0.6	−0.8	−1.0	−1.1	None
Heated sucrose	0.4	1.0	0.6	0.3	−0.4	−0.6	−1.1	None
Unheated sucrose	0.4	1.1	0.7	0.6	0.0	−0.8	−1.0	None
Heated maltose	2.2	2.2	3.5	4.2	4.5	4.5	5.0	35
Unheated maltose	0.7	2.7	3.2	3.7	4.4	4.5	4.8	29
Heated mannitol	1.1	3.1	3.3	3.4	3.5	3.8	4.5	70
Unheated mannitol	1.0	2.9	3.1	3.0	3.2	3.6	4.1	40
Heated mannose	1.4	2.9	3.2	3.4	3.7	4.1	4.2	50
Unheated mannose	1.4	2.9	3.1	3.3	3.5	3.7	3.9	50
Heated levulose	1.2	3.5	3.9	4.3	4.7	5.3	5.7	30
Unheated levulose	1.2	3.3	3.6	4.1	4.5	4.7	5.7	45
Heated dulcitol	1.5	3.0	3.2	3.3	1.0	0.6	0.8	63
Unheated dulcitol	1.5	2.2	2.5	2.3	1.9	3.0	3.0	40
Heated raffinose	1.1	1.6	−0.5	−0.7	−0.8	−1.1	−1.3	None
Unheated raffinose	1.8	1.4	0.7	−0.8	−1.1	−1.6	−1.7	None
Heated arabinose	1.8	3.1	3.2	3.7	4.4	5.3	5.8	30
Unheated arabinose	1.1	3.3	3.4	3.8	4.1	5.3	5.3	30
Heated rhamnose	1.7	3.4	4.0	4.5	4.8	5.6	6.0	40
Unheated rhamnose	1.5	3.7	4.1	4.6	3.5	4.4	4.0	35
Heated xylose	1.5	2.3	3.1	3.6	4.2	5.1	5.2	30
Unheated xylose	1.1	2.2	2.8	3.1	3.5	4.1	4.2	32
Heated melizitose	1.2	1.2	0.6	0.2	−0.2	−0.5	−0.5	0*
Unheated melizitose	1.0	1.0	0.2	−0.2	−0.6	−0.8	−0.8	0*
Heated inulin	1.7	1.7	0.9	−0.9	−2.2	−1.3	−0.8	3
Unheated inulin	0.6	1.0	0.5	−0.9	−2.2	−1.2	−1.3	5
Heated salicin	1.9	2.0	1.4	0.6	−0.2	−0.8	−0.9	None
Unheated salicin	1.6	1.5	1.0	0.9	−0.8	−0.9	−1.0	None

* Indicates size of the bubble of gas.

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BACTERIOLOGIC PEPTONE IN RELATION TO THE PRODUCTION OF DIPHTHERIA TOXIN AND ANTITOXIN

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I. INTRODUCTION

The necessity of using peptone media in the production of potent diphtheria toxin was recognized by the pioneer investigators in this field of serum therapy. Park and Williams (1896) appear to have been the first to lay any special emphasis on this constituent of the culture medium. After experimenting with varying concentrations of peptone in diphtheria toxin broth, they conclude that the strength of toxin averages greater with higher percentages of peptone (2 and 4 per cent) than with lower percentages (1 per cent). This is corroborated by Theobald Smith (1899), in his publication on the relation of glucose to the production of toxin in bouillon cultures of the diphtheria bacillus. He also proposed the use of a peptone bouillon in which the beef infusion, after preliminary reduction in acidity, was submitted to a fermentation with *Bact. coli*, in order to remove muscle sugar. Previous investigations by Spronck and von Fourenhout (1895) had indicated an inhibitory action of this carbohydrate upon the elaboration of toxin in peptone bouillon cultures of *Corynebact. diphtheriae*.

Martin (1898) attributed unsatisfactory results in toxin production to variations in composition of the peptone then available which appears to have been the Witte product. To overcome this, he proposed what he terms "liquid peptone" obtained from auto-digested swine stomachs which he mixed with an equal volume of veal bouillon. As would be expected, from its com-

position, this medium did not give successful results in the hands of other investigators, and found little application in practice. Spronck (1898) in the same year, recommended the substitution of a boiled and filtered extract of commercial yeast in place of the infusion of veal or beef. However, he specifically required the use of Witte peptone with his yeast product.

Subsequent publications on media for diphtheria toxin production appear to be more concerned with the reaction of the broth, and also, with the kind and condition of the meat used in the infusion. Veal is recommended by some in place of beef, while others insist that the meat before using must be either decomposed or fermented instead of freshly killed. This is in conformity with Theobald Smith's proposal for eliminating muscle sugar, which is assumed to interfere with toxin elaboration. Aside from the mention that the Witte product was employed, no attention appears to have been paid to peptone by later investigators.

The scarcity of Witte peptone during the past few years has again directed attention to bacteriologic peptone and to the methods of producing diphtheria toxin. A number of peptone products have appeared, of differing composition which, while allowing growth of the more common microorganisms and of *Corynebact. diphtheriae*, do not permit of obtaining the strong toxins formerly obtained. The use of trypsinized media as suggested by Cole and Onslow (1916) and the various modifications of Martin's peptone solution which have been recommended have not fulfilled practical requirements.

In the attempt to explain the necessary conditions for the successful application of some of the substitute products, rather unique views have been advanced concerning diphtheria toxin formation. Bunker (1919), in a recent article suggests "that there is a point at which toxin development is at its maximum, before which and after which the potency will be lost." He also states that "if time alone is made the basis of judging when toxin is 'ripe,' it will be only by chance and in spite of technique that any peptone will give satisfactory results." These statements as will be later shown, are contrary to extended practical experience in the routine production of high potency diphtheria toxin.

During the past year, the writer has had an opportunity to study large scale manufacture of diphtheria toxin. This included the preparation of thousands of liters of toxin having an L^+ dose of 0.33 cc., 0.25 cc., or less, and its use in horses for immunization purposes. It is the purpose of this article to consider the essentials for the routine production of high potency diphtheria toxin with special reference to its application in the development of high strength antidiphtheric serum.

II. THE PRODUCTION OF DIPHTHERIA TOXIN

a. Culture to be employed

Practically all of the institutions engaged in the propagation of *Corynebact. diphtheriae* for toxin elaboration employ the strain known as "Park-Williams bacillus no. 8" or "Culture Americana." Recently, six cultures of this strain were obtained from as many different laboratories. All were similarly carried by transplanting from two twenty-four-hour generations on Loeffler slant tubes and then in bouillon, according to technique later described. A decided variation in the strength of the final toxin, from the six cultures, was noted; two gave toxin of which one L^+ dose was greater than 0.5 cc. Only one, from the Hygienic Laboratory of the United States Public Health Service, gave toxin having an L^+ dose of 0.25 cc. or less. The toxins from two of the remaining three cultures gave L^+ doses of 0.50 cc. and the other one had a strength of $L^+ = 0.33$ cc. The advisability of verifying the toxicogenicity, particularly of any new strain of the organism is apparent. The parent culture is best maintained on slant tubes of moist Loeffler blood serum, transplants to bouillon being made as desired.

b. Culture medium

Of the various media recommended for toxin elaboration with *Corynebact. diphtheriae*, we have found plain beef infusion broth containing 2 per cent peptone and 0.5 per cent sodium chloride to be the most satisfactory. Extended trial with veal infusion in

place of the beef has not demonstrated any increase in toxicogenicity or shown any other advantages to warrant its use. Beef from various sources, including both cold storage and fresh products, has been employed in the infusion with equally satisfactory results. The increased acidity at times encountered with cold storage meat has not been found to require any special treatment. Preliminary fermentation of the infusion with a culture of *Bact. coli*, as first recommended by Smith (1899) to remove any fermentable carbohydrates is, at best, an unsatisfactory procedure, and in our experience, entirely superfluous. The formation, by the colon bacillus, of decomposition products which may give trouble on injection of the final diphtheria toxin, must also be taken into consideration.

Allowing the beef to infuse over night is not very practical with large scale preparation. Equally satisfactory results are obtained by adding twice the quantity of water to the minced beef and bringing to a boil in the steam kettle in the course of about an hour and a half. The resultant infusion liquor is then obtained by the use of a suitable press.

Fat must be eliminated from beef infusion, as even traces of fat have a decided inhibitory action on the production of diphtheria toxin. This has been demonstrated on numerous occasions when, out of the same lot of broth, those flasks which showed particles of fat gave final toxin of which the L^+ dose was greater than 0.5 cc., while the strength in the remaining flasks was 0.25 cc. or less. We are probably dealing here with a surface tension phenomenon, similar to that discussed by Larson, Cantwell and Hartzell (1919) in their recent paper on the influence of the surface tension of media on the growth of bacteria. It seems quite likely that the fat depresses the surface tension of the bouillon thus forcing *Corynebact. diphtheriae* to grow beneath the surface with resultant diminution in the formation of pellicle and toxin.

Experimentation with differing concentrations of peptone from 0.5 per cent to 4 per cent has shown that the most potent toxin requires a peptone content around 2 per cent. Amounts up to 4 per cent may be satisfactorily employed, but with no advantage over the smaller concentrations. The peptone used in

preparing the toxin under observation was Bacteriologic Peptone, Parke, Davis and Company, the composition and properties of which have been described in a previous article (Davis, 1917). Twenty grams of peptone and 5 grams of sodium chloride were added to every liter of beef infusion prepared as above, dissolved in the cold and then brought to a boil in the steam kettle to insure thorough solution.

Considerable uncertainty appears to exist as to what is the most satisfactory initial reaction for diphtheria toxin bouillon. Nearly all of the previous investigators, including Roux and Yersin (1888), Spronck (1898), Madsen (1897), Park and Williams (1896), Smith (1899) and Lubenau (1908) have employed either neutralization with litmus, which is crude at best, or "hot titration" with phenolphthalein, a procedure which is admittedly fallacious. The writer (Davis, 1918) in a paper on "Hydrogen ion concentration and toxicogenicity determinations with *Bact. diphtheriae*," has shown that potent toxin is produced in bouillon by *Corynebact. diphtheriae* only when the initial reaction falls within a certain zone of alkalinity, included within the hydrogen ion concentration limits of about 7.0×10^{-8} ($p\dot{H} = 7.2$) to about 5.0×10^{-9} ($p\dot{H} = 8.3$). The maximum degree of potency, however, is obtained when the reaction of the broth comes within the narrow limits of $p\dot{H} = 8.0$ to $p\dot{H} = 8.2$. This may be readily and consistently obtained by following the procedure given below for adjusting the reaction.

1. Transfer 10 cc. of the heated broth to a small Erlenmeyer flask and dilute with about 40 cc. of cold, distilled water. Add 0.5 cc. of a 1 per cent solution (95 per cent alcohol) of phenolphthalein as indicator and titrate to a deep pink color against an $\frac{N}{10}$ NaOH solution. The latter is preferably prepared when required as an exact 1/100 dilution of a $\frac{10}{N}$ stock solution. The amount of the strong ($\frac{10}{N}$) solution required to neutralize per litre of medium is given directly by the burette reading. Bring to a boil again in the steam kettle, and estimate the hydrogen ion concentration.

2. While the more accurate hydrogen electrode method is desirable for comparative and standardization purposes, equally

satisfactory results for routine production may be obtained by the colorimetric method. The simple "comparator" of Hurwitz, Meyer, and Ostenberg (1916) is recommended with standardized boric acid-potassium chloride-sodium hydroxide mixtures of $p\dot{H} = 8.0$ and $p\dot{H} = 8.2$, prepared as directed by Clark and Lubs. Exactly 10 cc. of the neutralized bouillon are transferred to a "comparison" tube, diluted with 10 cc. of distilled water, and mixed well. Ten cubic centimeters of the mixture are now removed to another tube and 0.5 cc. of an 0.02 per cent solution of phenolsulphonphthalein in 50 per cent alcohol next added. Two other tubes are prepared containing 10 cc. respectively of the standardized $p\dot{H} = 8.0$ and $p\dot{H} = 8.2$ mixtures, with 0.5 cc. of the phenolsulphonphthalein solution in each tube. The technique for comparison is as given by Clark and Lubs (1917). Usually, the value will be found to very closely approximate $p\dot{H} = 8.2$. If, as may sometimes happen, the color in the tube containing medium plus indicator is lighter than the compensated $p\dot{H} = 8.0$ standard, $\frac{N}{10}$ NaOH can be added directly to the former tube until the desired tint is reached. The burette reading, multiplied by two (since the equivalent of 5 cc. of the medium is used) gives the amount of $\frac{10}{N}$ NaOH required to correct each liter of broth.

Experience has repeatedly confirmed the observation that toxin of greater potency is obtained from broth contained in large flasks than in small ones. Other conditions being equal, we can expect to find diphtheria toxin of higher strength after growth in a six liter flask containing three liters of broth than in a liter flask containing 500 cc. Three liters of broth, dispensed into 6-liter, Florence type flasks have been employed in producing the toxin under observation.

It has undoubtedly been noted in every laboratory engaged in diphtheria toxin production, that, if for some reason, it becomes necessary to resterilize media, there is a resultant diminution in the strength of the final toxin. From experimentation in progress, to be reported upon in a later publication, it appears that food accessory factors, possibly of a vitamine character, are concerned in the production of diphtheria toxin. This makes it

especially desirable that the sterilization period of the medium should be as short as possible, to reduce destruction of the accessory factors to a minimum and yet be sufficient to ensure thorough heat penetration. Autoclave sterilization at 120°C. (15 pounds steam pressure) for a period not exceeding thirty minutes has given satisfaction.

c. Cultivation

The parent culture of *Corynebact. diphtheriae* on the Loeffler slants is transplanted through several twenty-four hour generations in tubes containing 10 cc. of bouillon to stimulate maximum pellicle formation The tubes are then used to inoculate small "starter" flasks containing 30 cc. of medium which are also incubated for twenty-four hours. The large flasks of broth are now inoculated with the twenty-four hour "starters," allowing one for each large flask. A temperature range of 36° to 38°C. has been found most satisfactory for incubation. Numerous potency tests have demonstrated that at least ten days incubation of the large flasks is necessary to ensure maximum elaboration of toxin and a twelve-day period is desirable. As has been shown in a previous publication (Davis, 1918), toxin of appreciable strength is elaborated by toxicogenic cultures within forty-eight hours. The potency gradually increases to a maximum value about the twelfth day, occasionally sooner. Incubation for an additional period of two weeks, or four weeks altogether, shows no deterioration of the final toxin. That this behavior is not confined specifically to the peptone employed is proved by the fact that, with the procedure as given above, Witte's peptone permits of similar results. In this case, toxin of maximum potency is obtained in the large flasks, only after a two-weeks' incubation.

When cultivated in plain bouillon under the optimal conditions already described, *Corynebact. diphtheriae* causes an initial increase in the hydrogen ion concentration of the medium. This is soon followed by a steady decrease until, apparently, a limiting alkaline reaction is attained. The following table, taken from the

article on hydrogen ion concentration determinations mentioned above (Davis, 1918) shows these changes with a toxicogenic strain.

Changes in H-ion concentration and toxicogenicity during growth of Corynebacterium diphtheriae in bouillon

TIME	C_H	pH	TOXICITY L^+ DOSE
hours			*cc.*
0	7.0×10^{-9}	8.15	
24	2.1×10^{-8}	7.68	1.0
48	3.2×10^{-8}	7.49	0.8
72	3.8×10^{-8}	7.41	
96	3.4×10^{-8}	7.47	0.55
120	2.1×10^{-8}	7.68	
144	2.5×10^{-8}	7.61	0.45
192	2.3×10^{-8}	7.64	
240	1.6×10^{-8}	7.79	0.15
312	8.7×10^{-9}	8.05	0.15
408	7.0×10^{-9}	8.15	0.15
528	5.0×10^{-9}	8.30	0.15

It is further shown in the same publication that the final hydrogen ion concentration of high strength toxin L^+ dose less than 0.25 cc.) after two weeks incubation ranged from $C_H = 1.6 \times 10^{-8}$ (pH = 7.79) to $C_H = 5.2 \times 10^{-9}$ ($pH^+ = 8.28$). At the same time, low strength toxins were obtained, the final H ion concentration of which came within the above limits. It is obvious from the table that in the normal development of *Corynebact. diphtheriae* in bouillon, the organisms may produce the same H ion concentration at two different intervals which represent wide variations in potency. This fact, and what has been stated above, justify the conclusion that there is no direct relationship, during or after growth, between the H ion concentration of the medium and production of toxin.

d. Final operations

The contents of the large toxin flasks, after proper incubation, are checked microscopically to determine purity of culture; 0.4 per cent of purified cresols is then added and allowed to act for, at least, twenty-four hours to ensure thorough germicidal action. As a rule, filtration can be accomplished satisfactorily through paper, otherwise Mandler filters may be employed. During

the above operations and in the finished condition, the toxin should be stored in a cool place.

It has been our experience in evaluating the strength of the diphtheria toxin for injection purposes, that the L^+ dose method (according to the Hygienic Laboratory) furnishes a more reliable index than determination of the minimum fatal dose. Although the theoretical relationship may not exactly hold, the L^+ dose for all practical purposes may be considered as 100 M. F. D.

III. GENERAL OBSERVATIONS

It will be readily conceded that we can best judge the value of what has been presented from the actual results of practical application. Data obtained during the past year in the production of several thousand gallons of diphtheria toxin by the general method indicated, are summarized below.

PRODUCTION RESULTS

Diphtheria toxin

Percentage of total toxin having L^+ dose

ABOVE 0.50 CC. (M. F. D. > 0.005 CC.)	0.50 CC. (M. F. D., 0.005 CC.)	0.33 CC. (M. F. D., 0.0033 CC.)	0.25 CC. OR LESS (M. F. D., 0.0025 CC. OR LESS)
10.2	11.3	35.5	43.0

As may be noted, practically 90 per cent of the toxin produced was of usable strength, $-L^+$ dose = 0.50 cc. or less. Of this, more than 78 per cent was high strength, having an L^+ dose of 0.33 cc. or less, and 43 per cent was so strong that one L^+ dose was 0.25 cc. or less. In view of the fact that only about 10 per cent of the large amount of toxin produced failed to reach a desirable strength, the procedure and medium recommended above can be considered as meeting practical requirements.

It is interesting to note, in connection with the production of diphtheria toxin, that no definite seasonal or weekly variation, as mentioned by MacConkey (1912) was observed. Occasionally, one or two bottles of a large lot would show an inferior or weak toxin, in spite of the fact that, as far as could be determined, the contents of all bottles after filtration should have been identical.

Data have been obtained in this study supporting the view that the troublesome local reactions encountered with horses in diphtheria treatment may be largely attributed to the method of controlling the hydrogen ion concentration of the toxin bouillon. It is a fact that in the use of the toxin under observation, practically no local reactions have been experienced. It is also true that adjusting the toxin bouillon by the colorimetric H ion method discussed above requires considerably less alkali than the use of the inaccurate "hot titration" method formerly employed. Whether it is the decreased amount of alkali used or possibly a diminished content of toxone bodies in the final toxin which is responsible for the favorable results must be left for further study.

Consideration of the foregoing production results would not be complete without data showing the antitoxic response to injection of the diphtheria toxin in horses. In the final analysis, this determines the utility of the toxin and consequently, the value of the methods and culture medium which are recommended. A summary has been prepared in the succeeding table to show the potency of the antidiphtheric serum obtained in the first yield from horses immunized during the past year with the toxin under discussion. The first large scale bleeding has been chosen for the valuation because experience has shown that the potency of this serum represents more closely than that from any of the succeeding bleedings the true value of the toxin injected.

Diphtheria antitoxin

Percentage of new horses yielding serum

UP TO 200 A. U.	200 A. U. TO 500 A. U.	500 A. U. TO 1000 A. U.	1000 A. U. TO ABOVE 1500 A. U.
22.4	27.7	34.3	15.6

Analysis of the table shows that nearly 78 per cent of all the new horses injected during the past year with the toxin under consideration produced a serviceable antidiphtheric serum (i.e., having a potency on first bleeding of 200 antitoxin units or greater). Eighty per cent of the productive horses (or 82 per

cent of the total number) gave serum ranging from 200 to 1000 units, 20 per cent (15.6 per cent of the total number) yielded the high potency products from 1000 to above 1500 antitoxin units, and 44 per cent (34.3 of all of the treated horses) came within the moderately high range from 500 to 1000 a.u. The foregoing and the fact that it has been possible in routine operation to immunize horses to an antitoxin strength exceeding 1500 units per cubic centimeter, can be taken as proof that the toxin used is fully satisfactory to meet all requirements of diphtheria antitoxin-production.

SUMMARY

1. A résumé of the more important literature on the production of diphtheria toxin is given This shows wide divergence of procedure. The recent scarcity of Witte peptone and failure of many substitute products to allow of appreciable toxin formation has further complicated the methods employed.

2. The essentials for the routine production of high potency diphtheria toxin are discussed. It is shown that, other conditions being the same, the toxicogenicity of the culture employed may vary within wide limits, according to the source. The necessity of verifying toxin production with any new strain is made apparent.

3. Plain beef infusion bouillon, containing peptone and salt is recommended for toxin production. Preliminary fermentation of the infusion with a culture of *Bact. coli* is shown to be undesirable. The use of veal infusion in place of beef is unnecessary. Even traces of fat must be avoided in the infusion as it interferes with maximum pellicle formation and thus diminishes toxin elaboration.

4. A content of 2 per cent peptone with 0.5 per cent of salt in the bouillon has been found to be most satisfactory.

5. Maximum strength of the final toxin has been obtained when the reaction of the broth comes within the limits of $pH^+ = 8.0$ to $pH^+ = 8.2$. A procedure is given for adjusting the hydrogen ion concentration to these values.

6. Cultivation for toxin production is best made in large flasks, previously inoculated with twenty-four hour cultures in

small "starter" flasks. Incubation is for at least ten days at 36°–38°C., with a twelve-day period preferred. Data are presented showing that the H ion concentration of the medium during growth cannot be used as an index of diphtheria toxin production.

7. Results are tabulated which have been obtained during the past year in the production of several thousand gallons of diphtheria toxin according to the procedure discussed. Practically 90 per cent of this toxin was of serviceable strength, $-L^{+}$ dose = 0.50 cc. or less. Of this more than 78 per cent was high strength, having an L^{+} dose of 0.33 cc. (M. F. D. = 0.0033 cc.) or less, and 43 per cent had an L^{+} dose of 0.25 cc. (M. F. D. = 0.0025 cc.) or less.

The efficiency of this toxin in the routine immunization of horses for antitoxin is shown by records of antidiphtheric serum production during the past year. Nearly 78 per cent of all new horses on this treatment produced serviceable antidiphtheric serum, i.e., having a potency on the first large scale bleeding of 200 antitoxin units or greater. Of the productive horses, 80 per cent gave serum ranging from 200–1000 units, 44 per cent yielded a product from 500–1000 units, and 20 per cent had serum coming within the very high range from 1000 to above 1600 antitoxic units per cubic centimeter.

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THE NOMENCLATURE OF THE ACTINOMYCETACEAE (ADDENDA)

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Almost simultaneously with the publication of our previous paper with this title (Breed and Conn, 1919) our attention was called to the publication of an article by Merrill and Wade (1919). discussing the identical subject from a somewhat different viewpoint. The facts as given in the two papers supplement ach other, and both papers point out the fact that students of the group face a puzzling problem in regard to the proper name for the group. This arises because the general use of Streptothrix Corda, 1839 renders the term Streptothrix Cohn, 1875 invalid. This would make Actinomyces Harz, 1877 the valid name for the genus in question were it not for the fact that some hold that the use of Actinomyce by Meyen in 1828 invalidates Actinomyces Harz. In this case it appears that Discomyces Rivolta, 1878 becomes the correct name for the genus, although some argue that this name is also invalid.

Merrill and Wade have frankly accepted the principle of priority as final, and contend that Discomyces Rivolta is the only correct term to use, while we have quoted the International Botanical Code (Chap. III, Sect. 2, Art. 20 and Sect. 6, Art. 50) to show that under these rules it is necessary to regard general usage rather than priority in this case, and have suggested the adoption of Actinomyces Harz as a *genus conservandum*. After our paper was written, the Society of American Bacteriologists accepted this suggestion and have included Actinomyces Harz in their list of approved genera.

Since the publication of our paper, our attention has been called to the fact that the difference in spelling and derivation

between Actinomyce and Actinomyces is sufficient to cause them to be regarded as distinct under the International Botanical Code, Chapter III, Article 57.[1] We find that the same thing also holds true under the International Zoological Code, Article 36,[2] a fact that shows general and international acceptance of this view.

Although the stem words from which Actinomyce and Actinomyces are derived have identical meaning in the original Greek, "myce" is derived from the less commonly used feminine word, *μύκη*, while "myces" comes from the masculine noun, *μύκης*. Thus the two generic terms in question ought not to be regarded as homonyms as is done by Merrill and Wade. This view we find has already been expressed by Güssow (1914) in a paper which we had overlooked, and is confirmed by those authorities with whom we have consulted. This being the case legislative action by an International Congress is unnecessary. Actinomyces Harz is valid without such action and should be retained rather than Discomyces Rivolta.

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[1] "The original spelling of the name must be retained, except in the case of a typographic or orthographic error. When the difference between two names, especially two generic names, lies in the termination, these names are to be regarded as distinct even though differing by one letter only. Example: Rubia and Rubus, Monochaete and Monochaetum, Peponia and Peponium, Iria and Iris."

[2] "Recommendations. It is well to avoid the introduction of new generic names which differ from generic names already in use only in termination or in slight variation in spelling which might lead to confusion. But when once introduced, such names are not to be rejected on this account. Examples: Picus, Pica; Polyodus, Polyodon, Polyodonta, Polyodontas, Polyodontus."

PRELIMINARY NOTE ON THE USE OF SOME MIXED BUFFER MATERIALS FOR REGULATING THE HYDROGEN ION CONCENTRATIONS OF CULTURE MEDIA AND OF STANDARD BUFFER SOLUTIONS

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In 1914–1915 Pieper (1917) began, with Humphrey and one of us, the use of a mixture of phosphoric and asparaginic acids in culture media and a number of other buffer mixtures were also employed. Pieper did not have our hydrogen electrode equipment set up to regulate the hydrogen ion concentrations, but this work was already planned. Meacham (1918) then began and has now finished investigations on this very important problem involving the relation between the rate of growth of fungi and the hydrogen ion concentration of culture media containing materials which enable any one now to regulate the hydrogen ion concentration at any desired value with great precision and reproducibility without using the hydrogen electrode or indicator methods. The result of his investigations on the growth of *Endothia parasitica*, *Lenzites sepiaria* and other organisms will appear in other articles.

It is desired to point out here the advantages to be derived by using a *single solution* of possibly two or three acids for giving a *smooth curve* relation between (a) the hydrogen ion concentrations from 10^{-1} to 10^{-14} and (b) the amount of alkali added to such mixture[1] of acids. The desirability of using[2] such a buffer mix-

[1] See also Prideaux (1916), whose excellent work was done after Pieper's but who did not suggest the use of his mixture with culture media. We did not discuss these physio-chemical relations in Pieper's article because he had no opportunity to regulate his media with the hydrogen electrode, this new important phase being taken up by Meacham in 1916–18.

[2] The mathematical side of the theory of buffer mixtures has now been developed in articles already completed, and applied in the measurement of the

ture is clear when we recall that the most important range of acidity in biological work is from a pH of 3 or 4 up to 8 or 9. But it is in just this region that we find the largest change in pH, *and therefore the largest errors,* when acid or alkali is added to the usual unbuffered culture media made from corn meal, beef, etc., which have small buffer properties. This is illustrated clearly in figure 1, where it is shown that, in adjusting a medium, an error of only 0.2 cc. of N/1 alkali in 100 cc. of corn meal extract

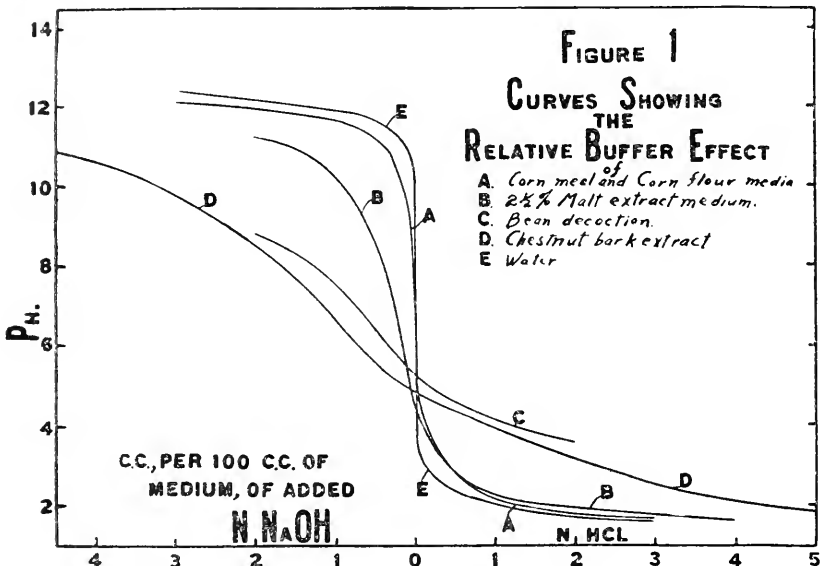

Fig. 1

changes the pH from about 4 to 10 and hence reduces the hydrogen ion concentration from 10^{-4} (or N/10,000) *to one-millionth that value,* or to 10^{-10} (or N/10,000,000,000). If the medium contains a mixture of buffer acids giving a smooth titration curve in this important biological range any desired acidity of the medium

ionization constants of asparaginic, phthalic, tartaric, glycerophosphoric, citric and pyrophosphoric acids. The theory is equally useful for analyzing a total titration curve back into the separate titration curves for each acid (or base) originally present or formed by the bacteria, or for calculating the total titration curve from the separate titration curves for the individual acids (or bases).

can be produced and maintained with small experimental errors, as illustrated in curves C and D in figure 1. In another article we are describing a large number of such useful acid and basic mixtures. For example, the mixture of asparaginic acid ($K_1 = 1.5 \times 10^{-4}$, $K_2 = 10^{-10}$) and of orthophosphoric[3] acid ($K_1 = 1.1 \times 10^{-2}$, $K_2 = 2 \times 10^{-7}$, $K_3 = 3 \times 10^{-12}$), used by Pieper in his synthetic medium gives nearly a straight line for the hydrogen ion concentrations when the equimolecular mixture of acids is treated with successive portions of alkali up to 5 molecules (fig. 2). This practically straight line is formed because a region of sharp inflection in the phosphoric acid titration curve, for example, is straightened out by adding another acid (or base) which, when it is 65 to 85 (especially when it is 75) per cent neutralized, gives a hydrogen ion concentration approximating that of the inflection point (midpoint) of the phosphate inflection curve. The various inflection curves of phosphoric (pyrophosphoric) acid and asparaginic acid are thus mutually nearly annulled. The slight deviation from a straight line will depend also upon the buffer properties of the other materials, if any, present in the medium, such as, for example, Witte's peptone, extracts

[3] Anhydrous sodium glycerophosphate is now sold as a buffer material which we believe will largely replace phosphoric salts for the following reasons. The glycerophosphate is less expensive than the highly purified potassium phosphate. It can be added in sterilized form directly to sterile culture media without producing the precipitate formed by phosphates. It does not precipitate out the calcium, magnesium and other heavy metals which are necessary for bacteria and can therefore be kept in solution with glycerophosphates and studied accurately in different concentrations. The titration curve is very close to that of phosphoric acid and enables workers to duplicate experiments previously buffered with phosphoric acid; the titration curve is furnished by the Grahame Chemical Company, 100 Rockingham Street, Rochester, N. Y., who sell glycerophosphates standardized for pathological and biological work. The glycerophosphates give the organisms a source of carbohydrate food in the glycerol residue which is apparently more readily assimilated than straight glycerol. Avery, Mellon and Acree have grown over 20 organisms, including tubercle bacilli, on solutions buffered with glycerophosphates. The glycerophosphates are stable enough to sterilize in accurately standardized tablets or in solution. The salt can be wieghed out and handled accurately as it is not hygroscopic like anhydrous sodium phosphate, for example. Sucrose-phosphate, mannitol-phosphate and other carbohydrate phosphates have the same desirable qualities and will be reported upon later.

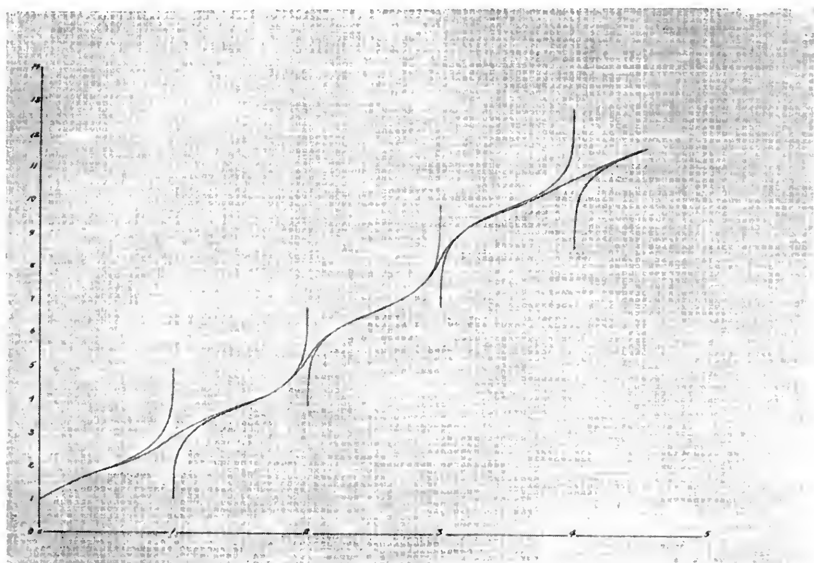

FIG. 2. SEPARATE TITRATION CURVES FOR ASPARAGINIC AND PHOSPHORIC ACIDS AND RESULTANT TITRATION CURVE FOR AN EQUIMOLECULAR MIXTURE OF THE TWO ACIDS

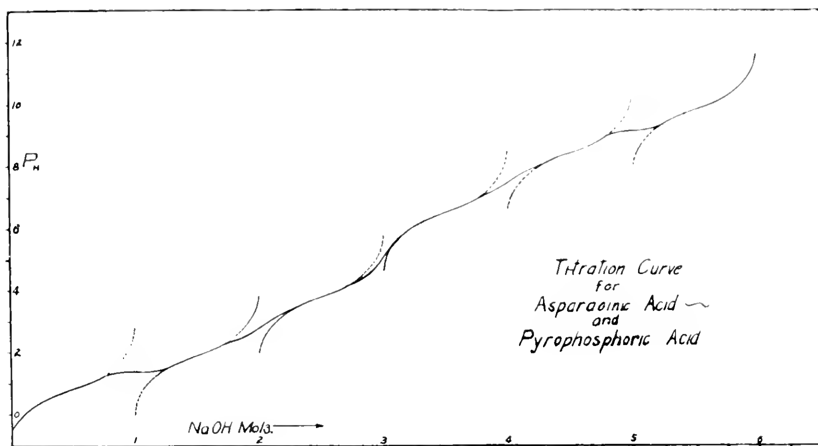

FIG. 3. SEPARATE TITRATION CURVES FOR ASPARAGINIC AND PYROPHOSPHORIC ACIDS AND RESULTANT TITRATION CURVE FOR AN EQUIMOLECULAR MIXTURE OF THE TWO ACIDS

of cornmeal, beans, malt, beef and other nutrient substances, as well as upon the acid or basic products formed by the fungi. An equimolecular mixture of asparaginic (or aminomalonic or acetoacetic) and pyrophosphoric acid ($K_1 = 1.4 \times 10^{-1}$, $K_2 = 1.1 \times 10^{-2}$, $K_3 = 3 \times 10^{-7}$, $K_4 = 3.6 \times 10^{-9}$) also gives nearly a straight line relation between the hydrogen ion concentration and the number of molecules of alkali added (fig. 3). The use of pyrophosphoric acid in this connection is to be recommended for the reason that Bray and Abbott (1909) have found the ionization constants of the third and fourth acid groups to have the above mentioned *unusual* values. It is clear that there would be nearly a straight line relation between the number of molecules of alkali added and the hydrogen ion concentration between about 10^{-6} and 10^{-10}. This is a very important region in the study of seawaters and many natural waters and other solutions which are slightly alkaline and it is doubtless familiar to many that there are practically no organic carboxylic acids having ionization constants between 10^{-6} and 10^{-9} or 10^{-10}. The second acid group of maleic acid has an ionization constant about 3×10^{-7}, and the second acid group of benzylmalonic acid has an ionization constant 4.9×10^{-7}. Outside of phenols and similar substances, there are no organic acids with ionization constants below these values until we come to the aminocarboxylic acids such as asparagin, aminoacetic acid, etc. with ionization constants around 10^{-9} and 10^{-10}. An inorganic substance like pyrophosphoric acid is therefore very welcome to cover this very important range and it has the added advantage that it is not readily decomposed by bacteria or fungi as are the organic acids. Furthermore it does not precipitate out the calcium and magnesium from natural waters or culture media. The only apparently undesirable feature of pyrophosphoric acid is that the work of Abbott (1909) has shown that in acid solutions pyrophosphoric acid is hydrolyzed into the orthophosphoric acid and that the rate of hydration increases with increasing hydrogen ion concentration. It is therefore certain that in very acid culture media containing pyrophosphoric acid there would be a change into orthophosphoric acid. It is clear, however, that there could be

no very great change under average conditions for bacteriological work around 25°C. because the hydrolysis is fairly slow and the first acid group of orthophosphoric acid has an ionization constant about equal to that of the second acid group of pyrophosphoric acid (1.1×10^{-2}). As work with solutions more acid than $C_H = 10^{-2}$ will be rare, the hydrolytic cleavage (of anions and molecules) can be represented by the equation:

$$NaH_3P_2O_7 + H_2O \rightarrow NaH_2PO_4 + H_3PO_4$$

From this equation and the above ionization constants it is seen that there is no appreciable change in the *hydrogen* ion concentration. The preparation of mono-, di-, tri-, and tetra-salts from pure pyrophosphoric acid and the rate of hydrolytic cleavage of each will be studied carefully in this connection. The change of the tetra-salt into the orthophosphate by the action of alkalies will also be studied.

It is highly desirable that the hydrogen electrode be employed more widely in the study of the ionization constants of such acids and in the study of the acidity of such regulated culture media *before and after* the growth of organisms on such media. Professor Abbott and Professor Bray have most generously consented to our work in this field as accessory to their own investigations, which are practically the only authoritative records of the ionization constants of these acids, especially pyrophosphoric acid. We consider their work to be of a very high order of merit and it is hoped that further studies in the application of these materials in culture media and the studies by use of the hydrogen electrode and spectrophotometric methods will be of importance.

It should be pointed out that the use of the above mixture of asparaginic (aminomalonic, acetoacetic, etc.) acid and orthophosphoric acid (or pyrophosphoric acid) or equivalent mixtures simplifies very greatly the work involved in measuring the useful ranges of indicators, comparisons with culture media, and related fields. This applies to both the buffer solutions[4] alone and those

[4] The Grahame Chemical Company now sells a sterile single buffer solution which remains constant indefinitely and covers all pH values from 1 to 14 very accurately and replaces the 5 or 6 buffer solutions employed heretofore. They also

used in culture media. The buffer solutions are sterilized with thymol, formaldehyde, chloramides, etc. Heretofore investigators interested in these lines have had to use *several different solutions* to get hydrogen ion concentrations between 10^{-2} and 10^{-12} for example. As the preparation of each solution requires considerable care and some of these materials are decomposed by molds, fungi and bacteria, the solutions had to be renewed every few weeks. From our work and that of Prideaux we believe that *one solution* containing asparaginic[5] acid and orthophosphoric (or pyrophosphoric) acid in equimolecular quantities suffices to cover the entire range between 10^{-1} and 10^{-13} in practically a straight line or smooth curve relation between the hydrogen ion concentrations and the number of molecules of alkali added to the mixture of the two acids. We also avoid the use of the boric acid employed by Prideaux which is undesirable in most biological work. The buffer or *culture solutions can be made and kept sterile by making the original solutions decidedly alkaline at ordinary temperatures instead of in autoclaves and afterwards adding a (sterile) strong acid in known amounts to secure solutions having higher hydrogen ion concentrations.* This method is advantageous when the alkali or acid and other materials present interact and change the hydrogen ion concentration when heated in an autoclave.

In making a titration curve on such a strongly akaline solution of the salts of two acids (in equimolecular proportion) we recommend the feature of adding to the alkaline solution in the hydrogen electrode vessel another solution containing the asparaginic and phosphoric acids (in equimolecular proportions and in

furnish sterile buffer tablets, with or without admixed standardized quantities of different indicators, which cover all pH values from 1 to 14 in steps of 0.2 pH. These sterile tablets are added to sterile culture media or to water to give the desired pH values, and have been found very useful in saving time and securing accurate results.

[5] The asparaginic acid can be replaced very advantageously in culture media by $\frac{3}{8}$ equivalent of formic acid, $\frac{5}{8}$ equivalent of acetic acid, and one equivalent of aminoacetic or equivalent acid. For buffer solutions this aminoacetic acid can be replaced by phenolsulphonic acid, thymol, or other suitable and stable substances having ionization constants around 10^{-10}.

the same concentration employed in the first solution) and containing also any desired amount of hydrochloric or other strong acid necessary to liberate any part or all of the asparaginic and phosphoric acids. It is clear that in this way we can keep the concentration of the asparaginic and phosphoric acid (or the salts of these) constant at any desired concentration, say M/10, and at the same time change the hydrogen ion concentration from that of the very alkaline solution up to that of the very acid solution obtained when a sufficient volume of the solution of hydrochloric, asparaginic and phosphoric acid is added to the alkaline solution. It is furthermore clear that a similar procedure would enable the operator to make a choice of the constant concentration at which he could keep the *cations* and *anions* in making such a titration curve. Without further discussion, it is clear that the *one solution* of the mixture of phosphoric and asparaginic acids could be made to give any desired hydrogen ion concentration by simply adding varying known quantities of alkali and water to keep the solutions standard. Likewise, the one solution of disodium (or potassium) asparaginate and tripotassium (or sodium) orthophosphate could be made to give any desired hydrogen ion concentration by simply adding calculated quantities of hydrochloric acid. The above described method of mixing a very alkaline and a very acid solution of equimolecular parts of asparaginic and phosphoric acid (in say M/10 concentrations of each acid in both solutions) will perhaps be a more convenient way of making a titration curve and of getting any desired hydrogen ion concentration by mixing the proper volumes of the two solutions. The hydrogen ion concentration is measured easily by means of the hydrogen electrode, a standard calomel or hydrogen *comparison* electrode and the Loomis-Acree (1911) method of eliminating contact potentials by the use of 4.1N potassium chloride. The errors of the Bjerrum extrapolation method are discussed in another article.

Mr. J. H. Hopfield has completed a titration curve on asparaginic acid from the very acid to the very alkaline regions. The ionization constants of the two acid groups and the basic group have been calculated from his data and will be reported in a

separate article. Miss Margaret Brennan has studied the growth of fungi on such regulated media in continuation of Dr. Meacham's work. It is hoped that other investigators will leave this field to us for a reasonable time so that we shall have an opportunity to complete certain phases of the work which will then be published in detail.

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A MUTATING, MUCOID PARATYPHOIDBACILLUS ISOLATED FROM THE URINE OF A CARRIER

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Mutating strains of paratyphoidbacilli are mentioned several times, especially by Baerthlein who has described different forms of colonies and variants in respect to the size of the bacilli. Recently a mutating form was described by Fletcher who found that the stool of a carrier who was examined at regular intervals suddenly became almost free of the typical colonies of paratyphoidbacilli. Simultaneously he found a new form of bacillus that he had not been aware of before. This microbe formed large wet colonies on the agar and did not agglutinate in a paratyphoidserum. Fletcher considered the microbe a mutating form of paratyphoid and called it mucoid on account of its wet mucuslike consistency.

With the exception of Fletcher the earlier authors on the subject of transmutation in paratyphoidbacilli do not seem to have observed many alterations as to the agglutination in mutating bacilli, and in the case of Fletcher this author does not bring forth any explanation of the lack of agglutinability. We have therefore thought it worth while to present an account of a paratyphoid strain, that we have watched while mutating and made the object of a fairly minute examination.

This strain was isolated from the urine of a man whose record of sickness is as follows:

In December, 1918 he contracted paratyphoid fever of the ordinary type. In January of the following year he had a severe attack of pyelitis of paratyphoid origin. This first attack was followed by numerous others and the patient developed a condition of chronic pyelitis with a continuous discharge of paratyphoidbacilli in his urine. The

strain of bacilli was quite typical until the month of May, when we observed that a new type of colonies became dominant in the cultures. The main characters in which these differed from the ordinary colonies were their large size, their shiny, wet aspect and their lack of agglutinability. The new type was present in great numbers while the ordinary type was markedly reduced. The condition became quite stabile and was the same all the summer and autumn, when the patient passed out of our observation.

The strain studied by us was isolated from the urine in September, 1919, and has now (March, 1920) the same characters as it had immediately after isolation from the patient.

On the surface of litmus agar it forms large, wet, shiny colonies that are much elevated over the surface and have a regularly rounded circumference and top. If growing very close together they flow into each other, forming large irregularly shaped figures, that may be taken to be isolated colonies of a peculiar aspect, but in reality are formed from several colonies melted together. These appearances have led Fletcher to talk about colonies of ameboid shape, an expression that is certainly justified, if it is remembered that these figures are not isolated colonies. Viewed by transmitted light the colonies have a very dense mass that has a distinct reddish hue. Left outside the incubator for some days the colonies, sometimes, dry up, and sink together and the color deepens into a clear blue. In this stage they look more like true paratyphoid than in a fresh condition, when their appearance is distinctly against this diagnosis.

Cultivated in carbohydrates the atypical strain ferments the sugars just as a paratyphoidbacillus should do, and it does not form indol in broth. Examined under the microscope in living condition, the bacilli of the atypical strain are as a rule quite immobile. Here and there is seen a bacillus showing slight movement, in no respect like the common vivid motions of the paratyphoidbacilli. In fixed and dyed films the bacilli are exceedingly small, often being chained together like diplobacilli or even like diplococci.

Testing the agglutinability of these bacilli, we found that they completely lacked the ability to be agglutinated in the prelimi-

nary test directly from the culture test carried out in a drop of serum on a slide. We therefore immunized a rabbit with the atypical strain and another with the normal strain from the same patient. The agglutination was titrated in the serum of the patient.

The result of these tests are as follows:

1. Agglutination test in the patient's serum (kindly recorded by our friend Dr. Kr. Skajaa):

Atypical strain... 1:320
Typical strain 1:320

After absorption with a typical Para. B strain:

Atypical strain... 1:20+
Para. B strain... 1:20+

After absorption with the atypical strain:

Atypical strain... 1:20+
Para. B strain... 1:80+

The serum of the patient thus agglutinates the atypical as well as the typical strain. After absorption the agglutination is abolished or diminished both in case of the atypical and a typical paratyphoid strain.

2. Agglutination in immunesera from rabbits.

TESTED STRAINS	SERUM PRODUCED WITH THE STRAIN								
	Atypical			Normal			Para. B		
	Titer on reading after hours								
	2	24	48	2	24	28	2	24	28
Atypical..........	400	800	3200	800	1600	6400	400	800	3200
Typical............	800	1600	1600	1600	1600	6400	800	1600	3200
Para. B...........	1600	3200	3200	1600	1600	3200	1600	6400	6400

After absorption during 24 hours with the three strains atypical, typical and Para. B 16, the sera showed the following titres:

TESTED STRAINS	ABSORPTION WITH ATYPICAL IN HOMOLOGOUS SERUM	ABSORPTION WITH NORMAL IN HOMOLOGOUS SERUM	ABSORPTION WITH PARA. B IN HOMOLOGOUS SERUM
Atypical	100	200	200
Typical	200	200	800
Para. B 16	800	400	1600

These tests were carried out in cultures from well isolated colonies, but not with one cell cultures. As it, however, was thought advisable to exclude the possibility of our working with cultures containing both atypical and typical bacilli, we prepared one cell cultures after the method of Burri, and made the following serological tests with cultures, that had been obtained from but one atypical bacillus, and that in growth were just as atypical as the cultures had been before.

a. Agglutination in paratyphoidserum:

	2 hours	6 hours
Typical strain	1600	6400
Atypical strain	800	1600

b. Absorption test after Castelliano with atypical strain in 6 hours (antigen added twice):

Agglutination titer after six hours in the incubator

Typical strain	400
Atypical strain	200

c. Complement absorption test:

Antigen. One fiftieth of a twenty-four hours old culture (agar slope), emulsified in saline and killed by heat.

Serum. Paratyphoidserum produced with a standard paratyphoid B strain. Dose: 0.05–0.0001 cc.

Complement. Fresh guinea-pig serum, dose: 0.06 cc.

Time of absorption. Six hours at 8°.

Blood corpuscles of sheep, washed and emulsified as in the test of Wassermann.

Hemolytic amboceptor. As in test of Wassermann.

SERUM	RESULT	
	Atypical strain	Typical strain
cc.		
0.05	Inhibition	Inhibition
0.025	Inhibition	Inhibition
0.0125	Inhibition	Inhibition
0.0063	Inhibition	Inhibition
0.0032	Inhibition	Inhibition
0.0016	Marked inhibition	Marked inhibition
0.0008	Trace of inhibition	Hemolysis
0.0004	Hemolysis	Hemolysis
0.0002	Hemolysis	Hemolysis
0.0001	Hemolysis	Hemolysis
0.0		
Control of antigen	Hemolysis	Hemolysis

The tests show with certainty that the atypical strain is a real paratyphoid strain. Injected into a rabbit it has caused the production of a serum that agglutinates two quite typical paratyphoid strains: and after absorption of two sera with typical strains the titre of agglutination is lowered practically to the same degree in all these three strains. The tests show however that the new strain is much slower in its agglutinating reaction than either of the two typical strains. In all tests the former required forty-eight hours to reach its maximum of agglutination, while this is reached in about twenty-four or even in two hours by the typical ones. Consequently the atypical strain is not less agglutinable than the typical ones, but is slower in responding. This will, however, in practice amount to the same thing, because a strain that does not show an agglutination test in a given time on isolation, especially if this happens in a strain with quite atypical colonies, will be set aside as not being paratyphoid, and thus a carrier will not be discovered.

After having shown that the atypical strain is a real paratyphoid strain, we will have to answer the question, why it shows this peculiar growth. It will already be observed that there are several characters about this strain that point to a special group of microbes, namely the capsule-producing bacteria. The *Bact. pneumoniae* of Friedlaender, for instance, produces such large,

wet colonies, and the bacilli of this group are small bacilli without any motility and also lack agglutinability. These facts rendered it necessary to examine our strain with a capsule dye. It was a fairly easy task to demonstrate great masses of mucus in slides, prepared from our cultures. The bacilli were not surrounded by small distinct capsules as is the case in pneumococci. The capsules were very thick, surrounding one, two or many bacilli in a common cover of mucus. Between the heaps of bacilli there were great masses of mucus, arranged as a homogeneous intercellular substance. This marked formation of mucus might also be demonstrated directly in the cultures, as these were quite sticky and often in agarslope cultures produced mucus that flowed down into the bottom of the tube.

At this point in our investigation we happened to come across a strain of *Bact. pneumoniae* in a case of pneumonia in a guinea-pig. We could now compare the two strains, and we have the greatest difficulty in telling which of the strains was the paratyphoid and which the pneumonia bacillus seen on the plates.

We consequently consider it proved that our atypical strain has obtained the faculty of forming capsules or rather of producing great masses of mucus. This explains very well the peculiar growth, the small bacilli, the slight motility and the slow agglutinability. The strain is of a special interest as it has been watched during its growth in typical manner in the urogenital tract of the patient and has been seen to develop into the capsule producing variety.

It looks as if our strain must be considered a true transmutation of the paratyphoid bacillus. The strain developed the new character quite suddenly and has kept it unaltered for more than $\frac{3}{4}$ year. It thus appears to be very constant, but it would be of interest to show that it kept unaltered even if the strain was cultivated rapidly for several generations from the one directly to the next without being allowed to stand over in the laboratory between the cultures. We consequently secured a well isolated colony and spread it on agar plates. After twenty-four hours of growth a new colony was taken out, spread again and this was done daily for three weeks. The first few cultures showed a

very few typical colonies that in turn were propagated to ascertain whether they kept their typical aspect or reverted to the atypical one. This, however, was not the case. From a typical colony only typical ones grew in the subcultures. On the other hand, when the atypical colonies had been transferred for a few times the fully developed colonies showed only the atypical aspect. At the end of three weeks we had to deal with a culture that never showed any sign of being a mixture of the two kinds of colonies, and since has always kept its character.

Thus it is shown that the atypical strain kept its new character unaltered when propagated rapidly from one artificial medium to the next. We thought it necessary to see if it would also keep this character when living under pathogenic conditions. For this experiment we used white mice, that were very susceptible to inoculations with these microbes. After intraperitoneal injections of small amounts of living bacilli the animals died in about twenty-four hours. From each mouse some blood was taken and injected into a new one without being cultivated on artificial media first. As a control of the bacilli at hand cultures were simultaneously made on litmus agar. After being injected into 8 mice the microbe still gave only atypical colonies in cultures.

As we thought it to be of some interest to find out if the virulence of the atypical strain had altered with the new character we inoculated several white mice with measured volumes of agar slope cultures of the atypical and the typical strain of the bacillus and watched the time when the mice died.

The result of this experiment was as follows:

	AMOUNT OF CULTURE							
	1 loop	1/10	1/20	1/50	1/100	1/200	1/500	1/1000
	Mouse died within hours							
Typical strain	20	20	20	20	30	24	Lives	Lives
Atypical strain	8	24	24	24	Lives	Lives	Lives	Lives

It will be noted that the typical strain kills the mice in smaller doses than the atypical strain. It must, however, be remem-

bered, that the same volume of the atypical strain cannot contain so many bacilli as the typical one, because the mucus produced by the former will take up a space that in the typical strain is filled by bacilli. It is thus obvious that the same amount of culture will contain more bacilli in the typical than in the atypical strain. We cannot therefore expect to find the same titer of virulence in the two strains, unless the virulence of the atypical strain has increased after the transmutation. This is not the case in our strain, the virulence being either the same or slightly diminished.

It has been pointed out that the new character was quite stable as well under saprophytic as under pathogenic circumstances. We are, therefore, justified in calling the alteration of our strain a true transmutation. Why this had developed is a very interesting question which however is, we believe, not solvable. The new character might be looked upon as an effort by the bacillus to obtain a higher resistance against the serumbodies of the probably highly immune patient. In this connection we will point out the fact that increased formation of capsules has been seen in bacteria simultaneously with a strengthened resistance and virulence. Thus Sauerbeck talks about an immunity of the bacilli through a structural adjustment, while Gruber and Futaki have found that weakened anthrax bacilli were not capable of producing such large capsules as the full virulent strains.

In order to try whether our atypical strain might show any difference in its resistance against serum as compared with the typical strain of our patient we carried out bactericidal tests in active human serum with both strains. The result was the following:

SERUM	ATYPICAL STRAIN, COLONIES	TYPICAL STRAIN, COLONIES
cc.		
0.25	0	10
0.125	20	50
0.063	100	Several 1000
0.032	Several 100	∞
0.016	∞	∞
0.008	∞	∞
0.004	∞	∞

The test consequently shows that the capsule producing strain is not the more resistant of the two strains, rather the contrary. This result is in full accordance with our virulence test. If the resistance had been increased in the capsule forming strain it would have been natural that the virulence should have increased also, as the bacilli in the small non-lethal doses in this case would have had the opportunity of growing to a number sufficient to kill before they had been eliminated by the serum of the animal.

The importance of the observation recorded in this paper is mainly in the value it may have for epidemiological practice. It is namely obvious that transmutations of the kind related may hinder the detection of a paratyphoid carrier, as the investigator will pass over colonies of an appearance so unlike paratyphoid-colonies, and especially, if he finds that they do not agglutinate in a specific serum.

Theoretically it is of some interest that we have watched the origin of the transmutated colonies in the urine of the patient as Fletcher already has seen a similar strain arise in the stool of another patient. The two cases considered together give evidence that such transmutations may occur in the alimentary as well as in the urogenital tract of patients.

CONCLUSIONS

The urine of a patient suffering from cystopyelitis quite suddenly showed numerous colonies of an atypical bacterium, while the typical paratyphoid colonies were much reduced in number. The new microbe turned out to be a capsule producing, mucoid paratyphoid strain with the following characters:

1. It grew in large, very wet, slimy colonies with a reddish grey hue.
2. The bacilli were small, the motility slight or lost.
3. The agglutinability was specific, but the reaction was very slow, the maximum reached in a far longer time than was the case with typical strains.
4. The absorption of complement took place exactly in the same doses and in the same time as in tests with the typical strain.

5. The microbe showed the same resistance against normal human serum as the typical strain. Likewise white mice were killed by nearly the same doses of the two strains.

This microbe satisfies all claims that are necessary to establish a real transmutation of bacteria.

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BACTERIOLOGIC STUDIES IN CHRONIC ARTHRITIS AND CHOREA[1]

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I. BACTERIOLOGICAL STUDIES IN ARTHRITIS

This work was part of a study of chronic arthritis undertaken to determine whether any constant cultural or immunologic streptococcus was associated with the disease. At the outset it was found necessary to define clearly the type of the disease studied on account of the ever changing terminology due to etiologic studies, and consequent changes in classification. For instance, one case of gonorrhoeal arthritis may simulate a case of acute rheumatic fever, and another an arthritis deformans. A complete bacteriologic study is necessary for a proper classification, and the bacteriologic studies in arthritis are by no means yet finished. Therefore any classification now given will have to be changed as the result of subsequent studies. However, so that the type of case studied may be somewhat defined, it may be well to state in a broad way a possible general classification.

Arthritis

Non-bacterial in origin

a. Traumatic
b. Metabolic including gout, acute and chronic
c. Following injection of diphtheria antitoxin and other sera
d. Secondary to nervous diseases, e.g., tabes
e. Associated with certain diseases, e.g., scurvy, hemophilia, purpura, etc.

[1] Presented before the Society of American Bacteriologists, December 31, 1919.

Associated with bacteria in origin

a. Acute rheumatic fever

b. Acute suppurative arthritis, e.g., acute pyogenic joint infection.

c. Chronic suppurative arthritis, e.g, tuberculosis of joint.

d. Acute non-suppurative arthritis, e.g., that acute exacerbation which occurs frequently during the course of a chronic non-suppurative arthritis. These attacks are usually mildly febrile, and present a local picture not unlike that of acute rheumatic fever. The joint involved may be one previously diseased, or may be a newly involved one.

e. Chronic non-suppurative arthritis

1. Syphilitic. This condition is much more common than is recognized and is frequently found complicating other types

2. A type of arthritis probably not representing a disease entity, but rather a group of diseases which have been variously termed:

Arthritis deformans, Virchow
Rheumatoid arthritis, Garrod
Osteo arthritis, Garrod
Rheumatic gout, Fuller
Chronic rheumatic arthritis, Adams
Nodosity of the joints, Haygarth, etc.

The dominant features of this type are the tendency to involve many joints especially the smaller ones, the disposition to the destruction of the articular and periarticular tissues resulting in exostoses and ankylosis, and the usual progressive chronicity of the disease.

It is this latter type that was selected for this study. It remains for investigators to determine by etiologic studies whether this type represents a chronic form of acute rheumatic fever, or whether in this type there is included a group of diseases. Also if a constant bacterium is found associated with the disease, it must be determined whether the arthritis is the result of the activity of the bacteria themselves in the articular or periarticular tissues, or the result of toxines elaborated by these bacteria in some remote focus. This work makes no pretense of clearing up these questions, for the streptococcus studied is not

always found, and it has not been determined that the disease is produced by the inoculation of the streptococcus or its toxins. Further work on this subject is now under way.

The literature of this subject is not satisfactory on account of the variations of classification. Very little bacteriologic work has been done on this type. D. J. Davis (1913) found that in this class of case streptococci could rarely be demonstrated excepting at autopsy, but he did occasionally obtain this organism from the regional lymph glands. E. C. Rosenow (1914) found that by making cultures directly from the glands draining the inflamed joints in 54 cases there could be isolated

	Cases
Modified *Streptococcus viridans*	32
Staphylococcus	5
Bact. mucosum	3
Neisseria catarrhalis	1
Gonococcus	1

Moon and Edwards (1917) in 83 cases of chronic arthritis took blood cultures using the technique described by Rosenow and obtained

	Cases
Negative cultures	58
Non-hemolytic streptococcus	18
Bact. mucosum	3
Diphtheroid bacillus	3
Unidentified	1

In acute rheumatic fever much work with not at all uniform results has been done, notably by Poynton and Paine (1913), Beattie and Yates (1912-13), R. I. Cole (1914), H. K. Faber (1915), Swift and Kinsella (1917), E. C. Rosenow (1914), Moon and Edwards (1917) and others. It is difficult to determine what types of cases have been included in these studies. Very frequently cases of typical chronic deforming arthritis at the outset present the picture of acute rheumatic fever; and acute rheumatic fever may extend for months with a low grade fever relapsing into a severe type for a few hours or days at each new focal extension, and terminating either in death, complete recovery, or recovery with damaged tissues and a tendency to relapse. On account of the frequency of these border line cases, the blood

cultures for the present study were taken from patients who were constantly afebrile excepting for the infrequent attacks of fever, lasting for a short time only, during an exacerbation of the arthritis, and the cultures were not taken at the time of the fever. No case is included in this series that did not have at least three months' history of arthritis. It was purposed to include no case that could possibly be considered as acute rheumatic fever. Likewise no case is included with a history of syphilis or a positive Wassermann reaction, or a history of a recent gonorrhoea or a positive gonorrhoeal fixation test.

The technique of blood culture was the same in all cases:

1. North's medium was used. Heating this medium for sterilization caused different batches of the media to increase in acidity to markedly different degrees. It was found necessary for the development of the streptococcus obtained, to titrate the medium so that after the final heating at the time of planting, the reaction was 0.3 to 0.5 $\frac{N}{10}$ acid, using phenolphthalein as an indicator.

2. Five to ten poured plates were used, and planting was done immediately after taking the blood.

3. The concentration of the blood in the plates was varied, so that the first plate had in it only about 0.3 cc. of blood, and the last 5 to 6 cc. This procedure showed that very often a growth would be obtained in the dilute plates and none at all in the more concentrated ones. It may be that the whole blood itself acts to inhibit the development of the growth. It is well known that whole blood makes every attempt to rid itself of bacteria, and does not lend itself to use as a medium as well as other tissues of the body.

4. Incubation of the plates was continued for ten days. To prevent drying, the plates were inverted as soon as they had set, and were then placed over a basin of water in the incubator. The development of the original culture did not appear in two cases until the eighth day of incubation, and the average time was five days. For this reason, some procedure was necessary to prevent drying of the plates, for under the usual conditions the plates dried on the third or fourth day.

5. Transplantation was made as soon as the earliest colony appeared, for in the original culture, there seemed to be only a low degree of viability. More frequently than otherwise no transplant was obtained after waiting for the culture to develop fully. Up to a certain point these cultures seemed to increase in viability by transplantation, but the majority of our cultures did not survive six or eight transplantations.

6. The character of the growth in the original blood culture was not always typically that of *Streptococcus viridans*. Only three produced green in the original culture, but the remaining 11 did so in the transplants. These original colonies appeared like little black spots that could be seen only in a good light. In addition to the fourteen positive blood cultures herein reported we obtained six additional cultures which we were unable to transplant. These colonies were of the type just described; they retained the Gram stain, and presented on the slide the morphology of the other colonies which were transplanted and identified.

7. Identification of the transplants was done by Dr. Thro. All transplants had the same cultural characters:

Did not break up in bile
Did not peptonize milk
Grew in chains in broth
Produced green in blood
Retained the Gram stain
Were of low grade virulency to rabbits

Blood cultures were taken in 104 cases and this organism was found in 14 cases, and no bacteria but this organism were found in any culture. The organism in question can be classed as *Streptococcus viridans*. Control cultures were made from the blood of other patients and no growth of this organism was obtained.

Complement fixation tests with *Streptococcus viridans* antigens were done in all cases. The technique of complement fixation was based on the work of Schwartz and MacNeil (1911) in their study of complement fixation in gonorrhoeal infections, and on the

work of Swift and Thro (1911) in their study of streptococci by the use of complement fixation. The difficulties of the test lie in the preparation of efficient antigens. The one that has given most satisfactory results in our hands is the unkilled, thoroughly washed, salt ground bacterial emulsion. In our comparative experiments this method gave a greater difference between the fixing power and the anticomplementary dose than the Besredka dried antigen, the heated and killed bacterial emulsion, or the extracted filtered antigen. Swift and Thro found the fixing power of an extract made from dried bacteria highest compared to the anticomplementary power. At the beginning of this work, several antigens (5 to 9) prepared from different strains of *Streptococcus viridans* were used. Hastings (1914) found the necessity for the employment of many antigens to be due to the fact that a group reaction could not be obtained. We also found this necessary when working with an antigen of high anticomplementary action. But when an antigen was prepared as just described a polyvalent antigen of pooled strains all from Arthritis deformans gave satisfactory results on account of the higher fixing power—anticomplementary ratio. This is illustrated by the results of case 25 on the table appended. An autogenous antigen was not found more efficient than this polyvalent antigen excepting in case 6. In this case we obtained no inhibition excepting with an antigen prepared from an autogenous tooth culture. For the past two years our bacterial vaccines have been standardized by their nitrogen content. This has been found much more accurate than the Wright method of counting, Of late it has been found convenient to determine a working dilution of our antigens by this method, and usually the fixing power and the anti-complementary dose could be determined in a dilution containing 0.000028 gram bacterial nitrogen per cubic centimeter.

Joint cultures were attempted in 54 cases and *Streptococcus viridans* was obtained in 4 cases. These cultures were taken during an acute exacerbation, and an attempt was made to obtain fluid from the inflamed periarticular tissues as well as from the joint cavity.

TABLE 1

Determination of anticomplementary and fixing power of different antigens with serum from case 25

SERUM CASE 25	ANTIGEN	A	B	C	D	E	F	G	H	I	
cc.	*cc.*										
0	0.05	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.06	0+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.07	0+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.08	0+++	0+++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.09	0 0++	0+++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.10	0 0++	0 0++	0+++	++++	++++	++++	++++	++++	++++	++++
0	0.11	0 0 0 0	0 0 0+	0+++	++++	++++	++++	++++	++++	++++	++++
0	0.12	0 0 0 0	0 0 0 0	0 0++	++++	++++	++++	++++	++++	++++	++++
0	0.13	0 0 0 0	0 0 0 0	0 0 0 0	++++	++++	++++	++++	++++	++++	0+++
0	0.14	0 0 0 0	0 0 0 0	0 0 0 0	++++	++++	++++	++++	++++	++++	0+++
0.02	0.01	0 0 0 0	0+++	0 0++	0+++	0 0 0+	0 0++	0+++	0 0 0 0	0 0 0+	0+++
0.02	0.02	0 0 0 0	0 0++	0+++	0 0 0+	0 0 0 0	0 0 0+	0 0++	0 0 0 0	0 0 0+	0+++
0.02	0.03	0 0 0 0	0 0++	0 0++	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0 0 0+	0+++
0.02	0.04	0 0 0 0	0 0++	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0 0 0+	0 0++
0.02	0.05	0 0 0 0	0 0++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0 0 0 0	0 0 0+
0.02	0.06	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0+
0.02	0.07	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0+
0.02	0.08	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 [illegible]

++++ = complete hemolysis.
0+++ = about 75 per cent hemolysis.
0 0++ = about 50 per cent hemolysis.
0 0 0+ = about 25 per cent hemolysis.
0 0 0 0 = complete inhibition.
A = Washed, unkilled, bacterial emulsion of pooled strains.
B = Washed, killed by 60°C. one hour, bacterial emulsion of pooled strains.
C = Besredka extract of pooled strains.
D = Washed, salt ground bacterial emulsion of pooled strains.
E = Washed, salt ground bacterial emulsion of strain 1.
F = Washed, salt ground bacterial emulsion of strain 2.
G = Washed, salt ground bacterial emulsion of strain 3.
H = Washed, salt ground bacterial emulsion of strain 4.
I = Washed, salt ground bacterial emulsion of strain 5.
J = Washed, salt ground bacterial emulsion of autogenous strain.

The feces of all cases was examined by Gram stain, and those that showed streptococci on the slide were cultured. Streptococci were found by the Gram stain in 42 cases, and were isolated and identified as *Streptococcus viridans* in 5 cases. This is an interesting phase of the work and we are continuing work along this line. The technique of making these cultures was as follows: Select a loopful of clear mucus, wash thoroughly in saline, and plant several plates very thinly. Even by this method we had great difficulty in obtaining the five positive cultures on account of the rapid overgrowth of other bacteria found in the stool.

The technique of culture of the various foci is as follows:

Teeth. When a sinus or pyorrhoea was present a culture was taken directly from the pus. This pus was usually found to contain two or more types of bacteria. When a tooth was withdrawn a culture was taken from the root and the socket, and frequently *Streptococcus viridans* was found in pure culture.

Tonsil. After gargling with sterile saline a loop was inserted as deeply as possible into a crypt of the tonsil using a pillar retractor when necessary for a clear view. If the tonsils were removed a culture was made from the deeper portions through an incision of the tonsillar capsule.

Ethmoid and sphenoid cultures were taken from the discharges from these sinuses after cleansing the nasal cavity with sterile salt solution. The material to be planted was taken as nearly as possible from the opening of these sinuses. In cases 3 and 7 the cultures were taken directly from the sinuses during an operation by E. Ross Faulkner. The other nasal sinuses were cultured in a similar manner.

Prostate. After urinating, the prostate was massaged and the culture made directly from the discharge.

Pyelitis. The sediment from a freshly catheterized urine was immediately planted on North's medium.

Salpingitis. The one case was cultured at the time of operation.

From a study of the table herein appended giving a summary of the 104 cases it is found that

1. In blood cultures *Streptococcus viridans* was found in 14 cases out of 104 cases.

TABLE 2

Review of cases of arthritis

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI
1	M.	38	+	+		0	+	Chronic endocarditis	+	+	
2	M.	41	+	+		+	+		0	+	
3	M.	40	+	+	+	0	+	Acute rheumatic fever	+	+	Ethmoids, sphenoids
4	F.	44	0	+	0	0	+	Chronic endocarditis	+	+	
5	F.	49	+	+		0	+		+	+	
6	F.	44	+	+	0	0	+		+	0	
7	M.	50	+	+		0	+		+	0	Ethmoids, maxillary
8	F.	52	+	+			0		0	+	
9	F.	48	+	+			0		+	0	
10	M.	49	+	+	0	+	+		+	+	
11	F.	46	0	+	+	+	+		0	+	
12	F.	58	+	+	0		0		+	+	
13	M.	39	+	+	0		0	Chronic endocarditis	0	0	Prostate
14	F.	36	+	+	0	0	+		0	+	
15	M.	40	0	0	0		0	Chronic endocarditis, acute rheumatic fever	0	0	
16	M.	39	0	0	–	+	+		+	0	
17	F.	46	0	0		0	+	Acute rheumatic fever	+	0	
18	F.	35	0	0	0	0	+		0	+	
19	M.	47	+	0			0		+	+	
20	M.	39	+	0	0		0	Acute rheumatic fever	0	0	
21	M.	51	0	0		0	+		+	0	
22	F.	52	+	0		0	+		0	0	
23	F.	49	+	0			0		0	+	
24	F.	42	+	0			0	Chronic endocarditis	0	0	
25	F.	44	+	0			0		+	+	

TABLE 2—*Continued*

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI.
26	M.	49	+	0		0	+		0	0	
27	M.	47	0	0	0	0	+		+	0	Ethmoids
28	M.	60	+	0	0	0	+		+	0	
29	M.	62	+	0	0	0	+		0	0	
30	M.	59	0	0	0	0	+		+	+	
31	F.	48	+	0		0	+		0	+	
32	F.	38	+	0		0	+		0	+	
33	M.	47	+	0	0		0		0	0	
34	M.	36	0	0	0	0	+		+	0	
35	F.	29	+	0	0		0		0	0	
36	M.	52	+	0	0		0		0	0	
37	F.	54	0	0	0	0	+		0	0	
38	M.	30	+	0	0	0	+		0	0	
39	M.	38	+	0	0		0		0	0	Ethmoids
40	M.	44	0	0	0		0	Acute rheumatic fever	0	0	
41	M.	36	+	0	0		0	Acute rheumatic fever	+		
42	M.	38	+	0	+		0		+	0	
43	M.	61	0	0			0	Chronic endocarditis	+	+	
44	F.	57	+	0			0		+	0	
45	F.	55	+	0	0		0		+	+	
46	F.	53	0	0	0	0	+		+	0	
47	F.	46	+	0		0	+		+	0	
48	F.	48	+	0		0	+		0	0	
49	F.	57	+	0			0		+	+	
50	M.	46	+	0			0	Acute rheumatic fever	0	0	
51	M.	51	0	0			0		0	0	
52	F.	42	+	0	0	0	+		+	+	
53	F.	51	+	0			0		+	0	
54	M.	52	+	0	0		0		+	+	
55	M.	41	0	0			0	Chronic endocarditis	+	+	
56	M.	43	+	0	0		0		0	0	

TABLE 2—*Continued*

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI
57	M.	45	+	0			0	Acute rheumatic fever	0	+	
58	M.	52	+	0	0		0	Chronic endocarditis	+	0	
59	M.	53	+	0	0	0	+		0	+	
60	M.	45	0	0	0		0		0	+	
61	M.	47	+	0		0	+		0	0	
62	M.	51	+	0		0	+		+	0	
63	F.	48	0	0	0	0	+	Acute rheumatic fever	+	0	
64	F.	39	+	0		0	+		0	0	
65	F.	64	0	0			0		+	0	
66	F.	48	0	0	0		0		0	+	
67	F.	62	0	0			0		0	0	
68	F.	11	+	0			0	Chorea	+	0	Ethmoids, sphenoids
69	M.	19	+	0			0		0	0	
70	F.	28	+	0			0		0	0	
71	M.	42	0	0	0		0		0	+	
72	F.	41	+	0		+	+		+	0	
73	M.	15	+	0			0		+	0	
74	F.	35					0		+	0	
75	F.	36	0	0	0	0	+	Chronic endocarditis	+	+	
76	M.	34	+	0	0		0	Acute rheumatic fever	0	0	
77	M.	48	0	0			0		+	0	
78	M.	51	+	0			0		0	0	
79	M.	39	+	0			0		0	0	
80	F.	33	+	0	0	0	+	Chronic endocarditis	+	+	
81	F.	46	+	0	+		0		+	+	
82	F.	44	+	0	0		0	Acute rheumatic fever	+	+	Maxillary
83	M.	41	0	0			0		+	0	
84	F.	17	0	0			0	Chorea	0	0	

TABLE 2—*Concluded*

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI
85	F.	28	0	0		0	+		0	0	
86	M.	39	0	0	0		0		0	0	
87	M	44	0	0	0		0		0	0	
88	F.	52	+	0		0	+		0	+	
89	F.	52	+	0		0	+		+	0	
90	F.	68	+	0			0		+	0	
91	F.	38	0	0	0		0		0	+	
92	F.	37	0	0	0		0		0	0	Kidney-pelvis
93	F.	39	+	0	0		0	Chronic endocarditis	+	+	
94	M.	44	0	0	0		0		+	+	
95	M.	46	+	0	0		0		+	0	
96	F.	37	+	0	0	0	+		0	0	
97	M.	41	+	0	0	0	+		0	+	
98	F.	36	0	0			0		0	+	
99	F.	42	+	0	0		0		0	0	
101	F.	50	+	0	0		0		0	+	
102	F.	36	+	0	0		0		0	+	
103	F.	48	0	0			0		0	0	
104	F.	39	0	0			0	Acute rheumatic fever	0	0	
100	M.	46	+	0	0		0		+	0	Prostate

In all cases Wassermann and gonococcus fixation tests were negative.

2. Chronic endocarditis was found in 11 cases.
3. A history of acute rheumatic fever was obtained in 12 cases.
4. A history of chorea in 2 cases.
5. Foci of *Streptococcus viridans* infection were found:

	Cases
Teeth	50
Tonsils	40
Ethmoid	5
Frontal	0
Sphenoid	2
Antrum	4

	Cases
Prostate	2
Pyelitis	1
Salpingitis	1

6. Complement fixation with *Streptococcus viridans* antigen was found positive in 68 cases.

7. Joint fluid cultures demonstrated *Streptococcus viridans* in 4 out of 54 cases.

8. No foci of *Streptococcus viridans* infection were found in 20 cases, and of these 20 negative cases 11 gave positive complement fixation tests. In addition to these 20 cases, there were 9 cases in which a streptococcus was seen in the Gram stain of the stool but could not be obtained in culture for identification, and of these, 7 gave positive complement fixation tests with *Streptococcus viridans* antigen.

TABLE 3

Review of cases of chorea

CASE NUMBER	SEX	AGE	WASSERMANN	GONOCOCCUS FIXATION	STREPTOCOCCUS VIRIDANS FIXATION	STREPTOCOCCUS VIRIDANS BLOOD CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	ENDOCARDITIS	HISTORY ACUTE RHEUMATIC FEVER	PRESENT INFLAMMATION JOINTS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	STREPTOCOCCUS VIRIDANS FOCUS NOSE	GONOCOCCUS FOCUS VAGINA	DURATION OF CHOREA
1	F.	13	0	0	+	0	0	+	+	0	0	+	+	0		11 days
2	F.	9	+	0	+	0	0	+	+	0	0	0	+	0		5 weeks
3	F.	14	0	0	+	0	+	+	0	0	+	+	+	0		7 days
4	F.	15	0	0	+	0	0	+	0	+	0	+	+	+		2 weeks
5	F.	11	+	0	0	0	0	0	+	0	+	0	0	0		2 weeks
6	F.	11	0	+	0	0	0	0	0	0	0	0	0	0	+	9 days
7	F.	18	0	0	+	+	+	+	0	0	0	+	+	+		4 weeks
8	M.	9	0	0	+	0	0	0	+	0	0	0	0	0		8 weeks
9	M.	8	0	0	+	+	0	0	+	0	+	0	0	0		10 days
10	M.	14	0	0	+	+	0	+	+	0	0	+	0	0		7 weeks
11	F.	14	0	0	+	0	0	+	+	0	0	+	+	+		4 weeks
12	F.	10	0	0	+	+	+	+	+	0	+	+	0	0		11 days
13	F.	10	0	0	+	+	0	+	+	0	0	0	+	+		15 days
14	F.	13	0	0	+	0	0	0	0	0	0	0	0	+		5 days
15	M.	9	0	0	+	0	0	0	+	0	0	+	+	0		18 days
16	F.	19	0	0	0	0	0	0	0	+	0	0	+	0		11 weeks

II. BACTERIOLOGIC STUDIES IN CHOREA

In January 10, 1913, I reported two cases of chorea in which *Streptococcus viridans* was found in the blood. Since that time I have studied 16 cases in addition. The technique of cultures and complement fixation was the same as was used in the bacteriologic studies in chronic arthritis just reported. In cases 2 and 5 the Wassermann reaction was positive In case 2 there were no other signs of lues, and on account of the absence of both parents, hereditary lues could not be established. Case 5 had an interstitial keratitis, and the mother gave a positive history of lues contracted from the father. Case 6 gave a positive gonococcus fixation test, and was found to have a history of eight months leucorrhoea in which gonococci were found. In this case we could find no focus of *Streptococcus viridans* infection, and the complement fixation was negative with *Streptococcus viridans* antigen. Altogether in 4 cases no focus of *Streptococcus viridans* infection was found. The complement fixation with *Streptococcus viridans* antigen was positive in 13 cases, and negative in 3. The blood cultures showed *Streptococcus viridans* positive in 5, and negative in 11 cases. The feces cultures showed *Streptococcus viridans* in 3 cases, but Gram stain streptococci were found in 9 cases. Other foci of *Streptococcus viridans* infection were found in

	Cases
Tonsils	9
Teeth	8
Nose	5

All cultures were isolated and identified in the manner just described in the bacteriologic studies of chronic arthritis.

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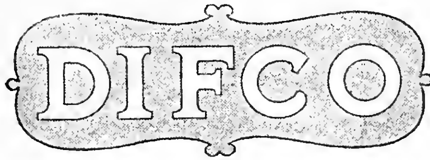

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EDITOR-IN-CHIEF

C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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Bromthymol Blue	yellow-blue	6.0–7.6
Phenol-Red	yellow-red	6.8–8.4
Cresol Red	yellow-red	7.2–8.8
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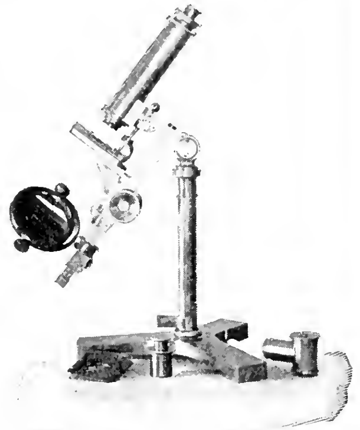
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BACTERIAL INHIBITION

I. GERMICIDAL ACTION IN MILK

WILLIAM H. CHAMBERS[1]

From the Division of Dairy Bacteriology, University of Illinois

Received for publication April 5, 1920

Improvement of our milk supply and the evolution of better methods for its handling will be aided by a more detailed knowledge of the growth of bacteria in milk. The growth of bacteria in bouillon, as shown by Buchanan and others, follows a rather definite curve, a period of delayed multiplication or latency preceding the period of most rapid growth or logarithmic increase. In milk, however, plate counts have, in many cases, demonstrated a decrease of bacteria before the period of logarithmic increase. Whether or not this is a true germicidal action has been the subject of intermittent controversy ever since Nuttall and Buchner first reported a germicidal action in blood in 1888. Later workers, such as Rosenau, have given very complete historical reviews of the early literature, so that only a few points of conflict in ideas are mentioned below.

The earlier workers were sharply divided. Freudenreich, Heim, Hesse, Weigmann, and Kolle found that *Vibrio cholerae* was killed in raw milk, but Honigmann and Basenau observed no germicidal action on this organism. Schottelius reports a better growth of *Corynebact. diphtheriae* in raw milk than in sterilized milk or bouillon. Hunziker in 1901 is apparently the first and only investigator seriously to take account of the possible variation in germicidal power in the milk from different cows. He showed a marked decrease in bacteria in the milk of some cows and none in others, but observed only the action of the milk of individual cows on their own milk flora and used no pure cul-

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tures. Stocking explains the reduction in the number of bacteria in the mixed milk from a herd of thirty cows as due to the death of those organisms which find milk an unfavorable medium while a parallel increase of lactic acid forms is taking place; but he makes no comparison of the behavior of the first group in raw and heated milk to show that the decrease is not due to a property of raw milk only. The relationship between germicidal action and temperature has been demonstrated by Koning, Hunziker, and others. Pure culture work from 1905 to 1909 by Hippius, Coplans, Brudny, Evans and Cope, Heinemann, and Rosenau has shown a definite germicidal action in varying degrees on quite a number of different species of bacteria, but the possibility of variation in individual cows is practically ignored and their conclusions are based on the action of only one or two cows. Heinemann found agglutinins for certain bacteria in milk serum and concluded that they seemed to bear some relation to the germicidal action. On assembling the work of the different investigators, it appears that much of the variation in results is due to a lack of consideration, in each case, of one or more of the essential factors.

It is the endeavor of this experimental work to correlate the important factors, emphasizing the individuality of the cow and the specificity of action of the bacteria, and by direct microscopic examination of the milk which is being tested to follow the growth of the bacteria and observe any evidence of agglutination. The milk was obtained from eleven cows from the University of Illinois herd, selected because in previous experiments they had shown a low germ content in the udder. Three organisms were used for inoculation in pure culture: *Bact. coli*, *Bact. lactis-acidi*, and a brilliant red chromogen isolated from a creamery can and designated Culture R. Near the middle of the milking, about 1 pint of milk, in approximately equal portions from each quarter of the udder, was drawn with aseptic precautions into a small sterile container. One half, to be inoculated raw, was held at 37°C., while the other half was heated to between 85°C. and 90°C. for two minutes and then cooled to 37°C. Ninety-nine cubic centimeter portions of the raw and heated milk in 300 cc.

glass-stoppered bottles were used for inoculation, and equal samples were kept for controls.

The work of Coplans, Rahn, Penfold, Chesney, Salter, and others on the latent period of growth in freshly inoculated bouillon has demonstrated that the most favorable conditions for transferring a culture are during its period of logarithmic increase and between media of the same composition at the same temperature. Therefore a constant temperature of 37°C. was used

TABLE 1

Growth of bacteria in raw milk at 37°C.

TIME	1	2	3	4	5	6	7	8
	OCTOBER 13	OCTOBER 19	NOVEMBER 3	NOVEMBER 14	NOVEMBER 21	DECEMBER 5	DECEMBER 9	DECEMBER 21
	Milk from cow number							
	134 190 212	134 190 212	155 188 134 196	188 134 196	188 134 196	208 180 179	208 201 181	201 208
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	5,100	200	460	1,230	2,290	825	220	390
15		97	500	1,430	2,520			
30		142	550	1,600	2,470	1,210	250	300
60	100	112	440	1,630	3,260	1,610	290	350
90		124	540	1,900	3,450	1,290	250	240
120		210	330	1,970	3,730	1,580	470	470
180	4,900			3,530	4,630		380	480
210						11,400		
240		23	50	9,360	5,750		6,660	4,690
300				14,700	15,700			

throughout the experimental work to give a more intense action over a shorter period of time of observation. The milk inoculations, 1 cc. portions from five-hour milk cultures at 37°C., were high compared to the germ content of the raw milk, thus minimizing the influence of the original udder flora upon the growth in the raw inoculated milk. Numerical determinations were averaged from triplicate plates of 1 per cent lactose agar, made essentially according to the standard methods of the American Public Health Association.

The experimental work was divided into two parts: first, the determination of the individual variation in germicidal action in the milk of the eleven selected cows; and second, the variation in action upon pure cultures in the milk of the cows showing the best germicidal action. Table 1 gives the results of the first part, the study of variation in individual cows. The data comprise eight trials in which the mixed milk from different groups of cows, as indicated by their herd numbers, was plated as one sample. The cows were arranged in groups to introduce organ-

TABLE 2

Growth of bacteria in milk, raw and inoculated with Bact. coli, at 37°C.

TIME	1			2			3		
	JANUARY 26			FEBRUARY 9			APRIL 20		
	Cow 212			Cow 212			Cow 155		
	Uninoculated control	Inoculated		Uninoculated control	Inoculated		Uninoculated control	Inoculated	
	Raw	Raw	Heated	Raw	Raw	Heated	Raw	Raw	Heated
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	970	6,900	8,500	420	31,100	31,800	30	5,600	6,500
30	1,070	13,500	24,800	410	48,400	60,000	30	6,300	15,100
60	890	900	62,000	440	10,100	101,000	50	500	14,800
90	930	2,100	135,800	1,290	9,300	190,000	40	100	50,300
120	1,590	4,800	250,000	5,040	10,400	1,035,000	40	−100	121,800
180	8,360	14,700	5,400,000	21,180	134,000	I*	50	−100	486,000
240	42,300	95,900	12,000,000	69,700	625,000	I*	420	1,600	I*

* I = Innumerable.

isms foreign to each cow's milk into each sample—which proved to be essential in these cases for germicidal action—and to show by elimination which cows possessed the germicidal property. Only three of the eight samples (columns 1, 2, and 3) show a decrease in bacteria distinct enough to be attributed to a germicidal action; the others show only an increase in growth, or a variation within the limits of experimental error. The samples of milk which show a germicidal action (columns 1, 2, and 3) are from six cows, numbers 134, 155, 188, 190, 196, and 212.

Three of these cows, numbers 134, 188, and 196, when tested together twice (columns 4 and 5) show only an increase in growth; hence the decrease in the first three columns is attributed to the three cows, numbers 155, 190, and 212. The milk of the other five cows, numbers 179, 180, 181, 201, and 208, when used in three different combinations, shows no evidence of a germicidal property. Of the eleven cows considered in table 1, only three possess a germicidal power. The individual cow therefore is seen to be an important factor in the study of bactericidal prop-

TABLE 3

Growth of bacteria in milk, raw and inoculated with culture R, at 37°C.

TIME	1			2		
	FEBRUARY 20			APRIL 3		
	Cow 212			Cow 155		
	Uninoculated control	Inoculated		Uninoculated control	Inoculated	
	Raw	Raw	Heated	Raw	Raw	Heated
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	270	162,500	195,000	40	27,300	26,500
30	310	176,000	242,000	60	28,700	38,200
60	420	192,000	360,000	90	24,700	53,400
90	620	342,000	910,000	120	42,400	142,300
120	5,890	1,190,000	1,600,000	190	176,400	491,000
180	27,900	5,000,000	7,150,000	130	930,000	1,260,000
240	72,000	I*	I*	210	3,000,000	7,000,000

* I = Innumerable.

erties in milk, especially if this ratio of 3 to 11 is taken as an indication of the prevalence of these properties among individual cows.

To demonstrate the specificity of the germicidal property for different bacteria, pure cultures were inoculated into the milk of individual cows. From the results reported in table 1, cows 155 and 212 were selected for inoculation tests with pure cultures of *Bact. coli*, *Bact. lactis-acidi*, and Culture R, according to the technique reported above. The growth of the different organisms is recorded in tables 2, 3, and 4, which show the comparable

growth in the uninoculated raw milk control and in the two inoculated samples, raw and heated, which were identical except for the heating. The heated controls are not tabulated, for in every case only a few surviving spore-formers developed during the four-hour period of observation.

The growth of *Bact. coli* in the milk of these two cows corroborates the presence of a bactericidal property in their raw milk which is not evident in the corresponding heated sample. A comparison of the raw and heated milk samples inoculated

TABLE 4

Growth of bacteria in milk, raw and inoculated with Bact. lactis-acidi, at 37°C.

TIME	1			2		
	MARCH 20			APRIL 20		
	Cow 212			Cow 155		
	Uninoculated control	Inoculated		Uninoculated control	Inoculated	
	Raw	Raw	Heated	Raw	Raw	Heated
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	170	17,500	15,700	30	111,700	119,300
30	140	44,500	34,600	30	265,000	251,000
60	110	93,000	80,400	50	295,000	405,000
90	680	166,000	136,000	40	677,000	939,000
120	2,830	416,000	394,000	40	2,200,000	2,550,000
180	11,900	1,800,000	1,250,000	50	I*	I*
240	124,000	I*	I*	420	I*	I*

* I = Innumerable.

with this organism (table 2), shows in every case a marked reduction of numbers of bacteria in raw milk and a rapid increase in numbers in the heated milk. The action is positive, for it is practically the same in two separate tests with cow 212, and is more intense with cow 155, for the count went below the 100 dilution at 120 and 180 minutes. In the milk of cow 212 there is a return of the growth to the normal logarithmic rate of multiplication, after 120 minutes. This germicidal action, as compared to the growth in the heated milk, is illustrated more clearly in chart 1, in which the curves are plotted from the logarithms of the averages of the raw and heated milk samples from table 2.

Culture R (table 3) grows more slowly for the first 90 minutes in the raw milk than in the heated milk. The raw milk growth is distinctly different from that of *Bact. coli*, however, for only a

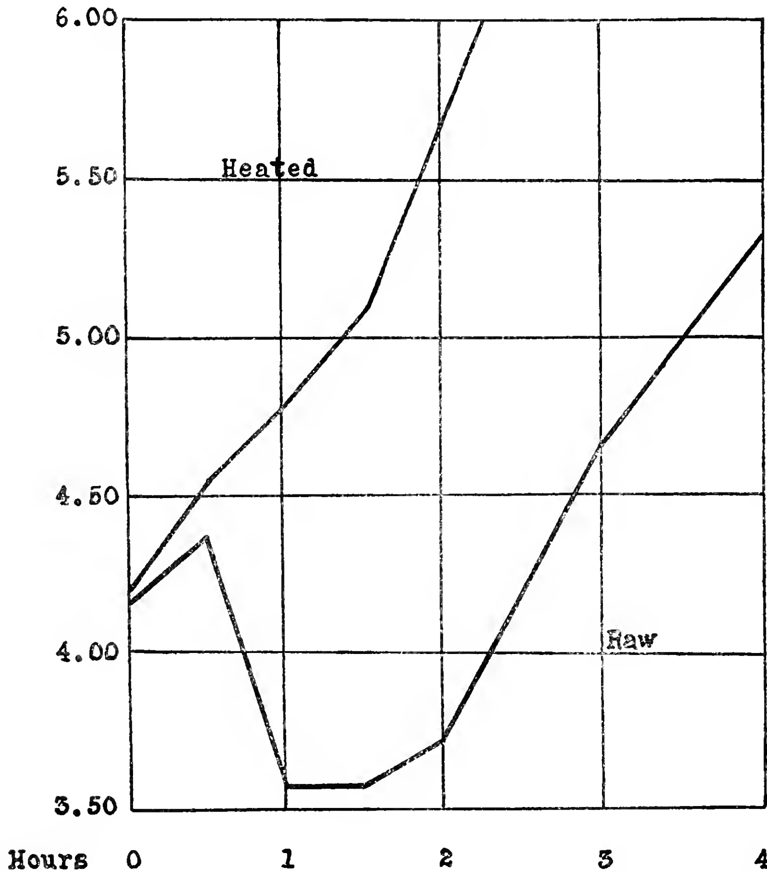

CHART 1. GROWTH OF BACTERIA IN MILK INOCULATED WITH *B. coli* AT 37°C.

lag phase and no germicidal action is evident. This is shown in chart 2, which is based on the logarithms of the averages of table 3. It is noticeable that a type of growth similar to that repre-

sented by the raw milk curve of chart 2, i.e., a growth in which there is no decrease in numbers but a definite lag phase preceding the logarithmic increase, is found in the majority of the uninoculated raw milk samples (table 1, columns 4, 5, 6, 7, and 8, and all the uninoculated controls of tables 2, 3, and 4). Consider-

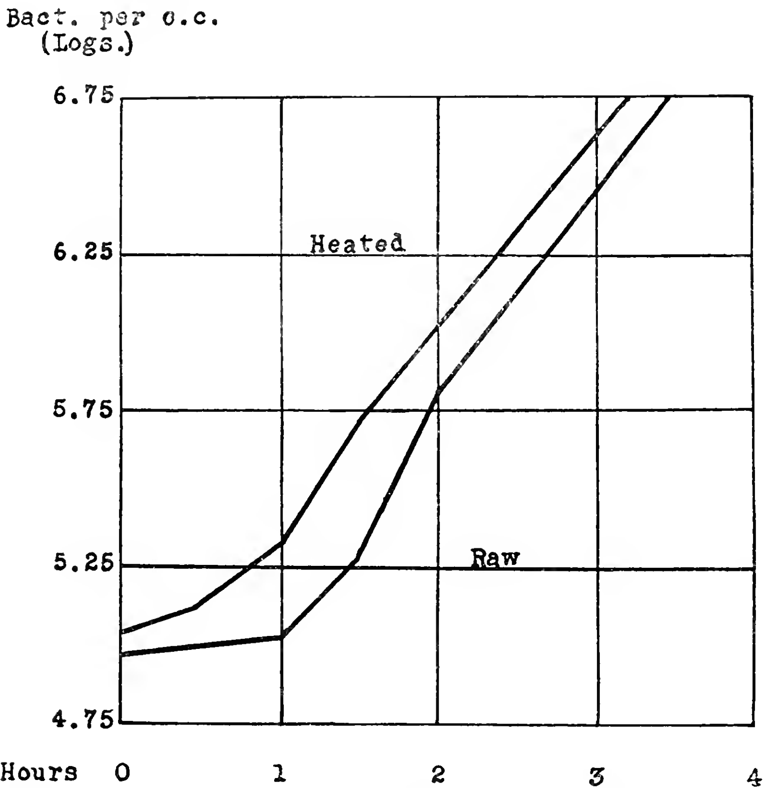

CHART 2. GROWTH OF BACTERIA IN MILK INOCULATED WITH CULTURE R AT 37°C.

ing the number of different species of bacteria represented in the uninoculated raw milk samples, this would seem to be a more common type of growth in raw milk than the germicidal type.

Table 4 shows practically no difference in the rate of multiplication of *Bact. lactis-acidi* in the raw milk and in the heated

milk. Applying the formula of Buchner, Longard, and Riedlin, the average generation time for the period from inoculation to 120 minutes is 26.9 minutes in the raw milk and 26.4 minutes in the heated milk, a difference of only 0.5 minute. In the raw milk of these two germicidal cows there is no bactericidal action on *Bact. lactis-acidi*, and apparently no retarding influence, such as is found for Culture R and the organisms in the uninoculated

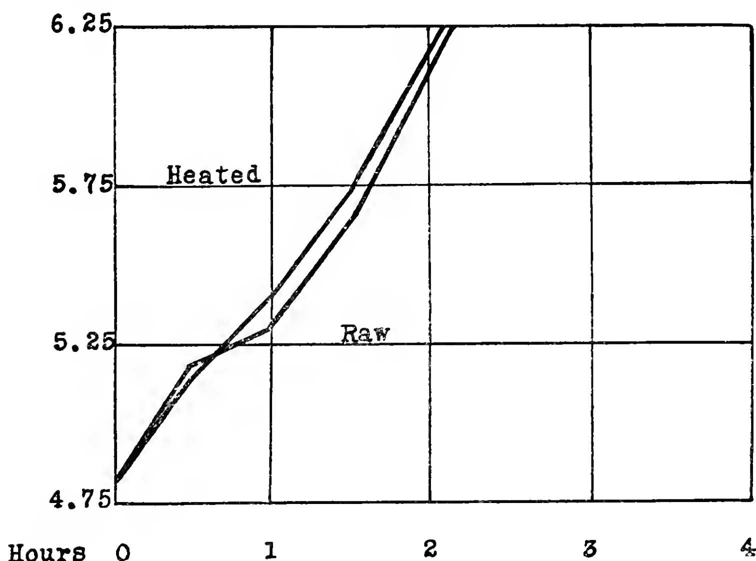

Chart 3. Growth of Bacteria in Milk Inoculated with *Bact. lactis-acidi* at 37°C.

controls. The similarity in the growth curves of the raw and heated milk is shown in chart 3, plotted from the logarithms of the averages from table 4.

This specificity of the germicidal action for the organism is best illustrated by chart 4, in which are assembled the raw milk curves from charts 1, 2, and 3. It is of interest to note that throughout tables 2, 3, and 4 the uninoculated raw milk controls of these two cows, numbers 155 and 212, show no germicidal action

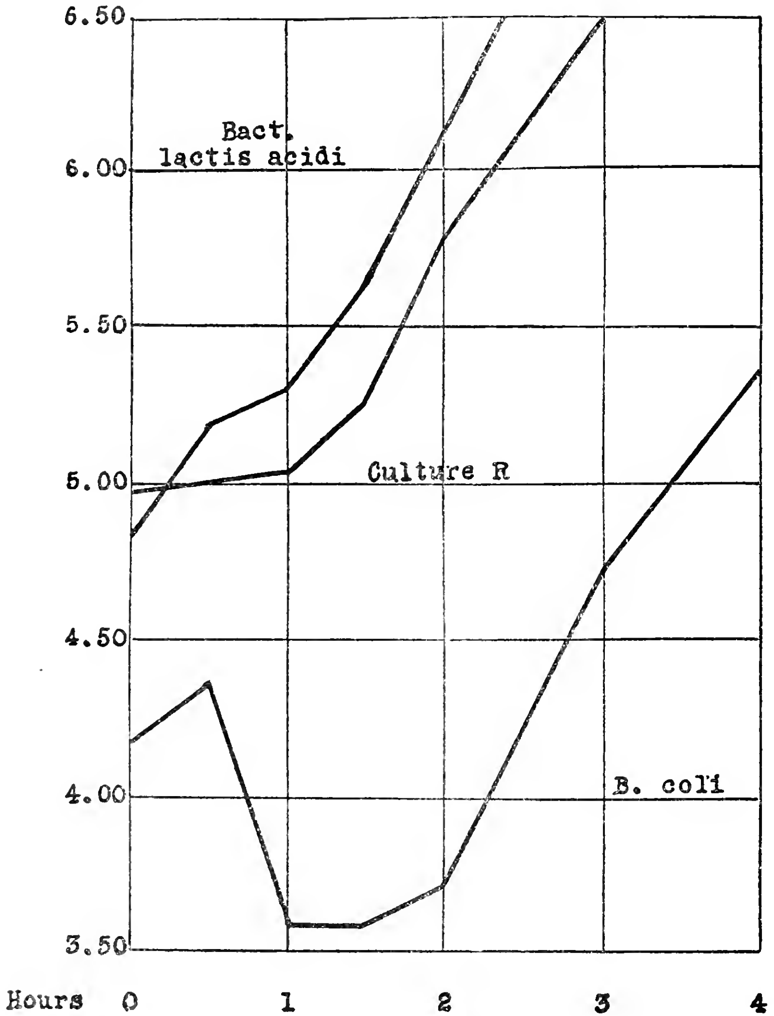

CHART 4. GROWTH OF BACTERIA IN RAW MILK AT 37°C.

toward their own udder organisms, while in table 1 (columns 1, 2, and 3) their milk shows distinct bactericidal properties for the mixed flora of several cows. This also emphasizes the specificity of the germicidal property and suggests an immunity of the organisms constantly present in the cow's own udder.

Observations were made on agglutination in connection with the numerical determination of the bacteria by plate count. The microscopical examinations for agglutination were made according to Breed's method for direct count from the milk cultures just before inoculation, and from the raw and heated milk just after sampling for plating. There was in no case agglutination in the heated milk or in the milk cultures used for the inoculation, which were made from sterilized milk. In the raw milk there was a very well-defined agglutination of Culture R, which appeared in large clumps of from twenty to several hundred, as opposed to an even distribution of individual organisms throughout the preparations made from the comparable heated milk. *Bact. lactis-acidi* showed a similar but weaker action in the raw milk, forming clumps of from four to twenty organisms. The preparations with *Bact. coli* in the raw milk were not so satisfactory, for where the counts were low the scarcity of organisms made microscopical enumeration difficult. A few small clumps were noted in the raw milk smears made at 240 minutes on January 26 and February 9 (table 2, columns 1 and 2).

Several theories have been advanced as explanations of germicidal action. Its specificity and reaction to heat suggest a serologic origin. The presence of agglutinins, antitoxins, hemolysins, opsonins, and other antibodies in raw milk has been frequently reported since Ehrlich's work in 1893. The decrease in bacteria in raw milk has been explained by some investigators as an apparent one, due to agglutination, rather than as a true bactericidal action, but the observations reported above do not show a correlation of agglutination and germicidal action. Summarizing the experimental data, Culture R gives the best agglutination but no germicidal action; *Bact. lactis-acidi* gives a good agglutination and an immediate increase in growth; and *Bact. coli* shows

much weaker agglutination than Culture R and a strong germicidal reaction. If the decrease was only apparent, owing to agglutination, one would expect to find by microscopic count in the comparable raw and heated milk approximately equal numbers of individual bacteria clumped in the raw milk and evenly distributed in the heated milk preparation. Such is not the case. The shaking of the samples and dilutions was uniform and vigorous (thirty times) and a comparison of the plate count and the ratio of clumps in the raw milk to individuals in the heated milk would indicate that the clumps were broken up. For example, a typical case with Culture R showed a plate count ratio of 46 to 100 (raw to heated), and the corresponding stained preparation showed a ratio of 1 to 100 (clumps to individuals). With *Bact. coli* (table 2, column 2) the microscopic count and plate count check very closely The microscopic examination at 120 minutes showed three small clumps in the raw milk in the entire preparation of 0.01 cc., or approximately 7500 bacteria per cubic centimeter, and in the heated milk preparation an average of 1.5 bacteria per microscopic field, or approximately 1,500,000 bacteria per cubic centimeter. This compares very favorably with the plate count of 10,400 per cubic centimeter in the raw milk and 1,035,000 per cubic centimeter in the heated milk (table 2, column 2, 120 minutes). Evidently there is an actual decrease of bacteria with *Bact. coli* as shown by the plate count and the microscope. It is of interest to note the similarity in germicidal action in the growth curves of *Bact. coli* in raw milk (chart 1) and *Bact. coli* in normal rabbit-serum (Chick).

In the freshly drawn mixed milk from a herd of cows the action of bacterial inhibition is variable because of its specificity. The numbers of species of organisms increase as contaminations from the cows, barn, utensils, etc., are added to the original udder flora. Where only the total number of bacteria in the milk is considered, a decrease in numbers may be evident, or one unaffected strain by its rapid increase may completely hide the germicidal action on another less numerous strain, particularly if the action is caused by only a few cows. The predominance of the lactic acid organisms with the increasing age of the milk might be

attributed to bacterial inhibition, for the lactic acid organisms are apparently unaffected by it, increasing immediately from the start, whereas the other organisms seem to be restrained in growth and, in some cases, decreased in numbers.

SUMMARY

1. The combined evidence of microscopic examination and plate count demonstrates a germicidal property, or actual decrease in numbers of bacteria in raw milk under certain conditions.

2. The germicidal property is destroyed by heating to between 80° and 90°C. for two minutes.

3. The germicidal action is specific, depending on both the individual cow and the species of bacteria.

4. No common relation between agglutination and bacterial inhibition is noted, except that both are destroyed by heating the milk.

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BACTERIAL GROUPS IN DECOMPOSING SALMON

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In a previous paper (Hunter, 1920) regarding the decomposition of salmon the total counts of bacteria found in the muscular tissue of the back and belly of the fish were given. The occurrence of these bacteria in the viscera and various organs of the salmon in different stages of decomposition was also discussed. The data presented were all obtained from experiments upon salmon handling as conducted in the Puget Sound region. From the mixed cultures and the plates made in the field 300 cultures were isolated and studied with the idea of grouping or typing the organisms significant in the decomposition of the salmon. Special care was taken to select those colonies which predominated on the plates.

It was evident that many organisms were obtained which played no part in the decomposition of the fish and that there were also many duplicates among the original 300 cultures. In order to eliminate the organisms which did not decompose salmon, and to check off the duplicates, use was made of a specially prepared fish broth.[2] The 300 cultures were grown in this

[1] This work was done under the supervision of Dr. Charles Thom to whom I am indebted for criticism and suggestions.

[2] This medium was prepared in the following manner by Mr. B. A. Linden of this laboratory:

To 1000 grams of finely chopped saltwater trout, or weakfish, from which the skin and bones had been removed, was added 1000 cc. of distilled water and 15 grams of peptone. The infusion was made by heating in the Arnold sterilizer or on a water bath at 95° to 100°C. for one hour with occasional stirrings. The juice was strained through cheese cloth with a meat press, filtered through cotton and the reaction adjusted to neutral. The infusion was then heated in the Arnold for thirty minutes at 100°C. and filtered, using folded filter papers. About 10 cc. was placed in each tube with about 1.5 grams of raw fish. For anaerobic cultures the surface was covered with a layer of liquid petrolatum. The medium was sterilized in the autoclave for fifteen minutes at 15 pounds.

medium, aerobically and anaerobically, for one week at 30°C. They were examined daily for the evolution of foul odors and for any digestion of the fish in the bottom of the tube. At the end of one week each culture was tested for indol production with paradimethylamidobenzaldehyde and hydrochloric acid. Only those cultures which produced indol, foul odors, or both, in this medium were saved for further study. In this manner the original number of cultures was reduced to 65 and these organisms were then studied in more detail in order to check the duplicates. The author, however, is aware that, while the discarded organisms had no putrefactive action in pure culture, they may assist in the decomposition of the salmon when associated with the bacteria which have been studied. In spite of this fact the present study has been limited to those cultures producing indol or foul odors when grown in pure culture.

On the basis of their morphology and their reactions in litmus milk, gelatin, potato, tryptophane broth and glucose, lactose and sucrose broths these 65 cultures were compared and duplicates eliminated so as to reduce the number to 43. Although some of these 43 cultures were very similar in many respects they all varied sufficiently to be considered different organisms and each of the 43 was studied and identified.

Of the 235 cultures rejected as playing no part in the decomposition of the salmon 4 were yeasts, 16 micrococci, 43 streptococci and 172 bacilli of varying morphology. All of the organisms regarded as significant in the decomposition of the fish are bacilli. Six of the cultures are pigment producers while the remaining 37 produce no pigment. The cultural reactions of these 43 organisms are summarized in tables 1, 2, and 3. For convenience the results are given in three separate tables, table 1 being the reactions of those organisms, exclusive of the pigment producers, which liquefy gelatin, table 2, those which do not produce pigment and do not liquefy gelatin and table 3, those which produce pigment.

In studying these bacteria but little attempt has been made to identify any of them, except the pigment producers, as specific organisms. It may be that an extended series of tests in various

carbohydrate media would identify as species or strains those forms which ferment the sugars but for the purposes of this investigation it has been considered sufficient simply to determine the group to which they belong. The studies of Winslow, Kligler and Rothberg (1919) on the classification of the colon-typhoid group and the work of Levine (1918) on the classification of the colon-cloacae group make it possible to identify those bacteria which produce acid, gas, or both, in glucose and lactose. On the other hand the inadequate descriptions in the literature of the non-fermenting bacteria from soil and water made it very difficult to identify unknown organisms of this kind.

In table 1 are tabulated the cultural reactions of 20 organisms which liquefy gelatin. Of these 13 have been identified as resembling *Bact. cloacae* (Jordan, 1890) in that they are small, Gram negative, gelatin-liquefying bacilli which produce acid and gas in glucose, lactose and sucrose, give a positive Voges-Proskauer and a negative methyl red reaction. Eight of these 13 cultures differ from the type in the production of indol.

Of the remaining 7 cultures described in table 1, 3 are very similar to *Bact. formosum* as described by Ravenel (1896) while the other 4 resemble nothing described in the literature and evidently belong in a group of unidentified water and soil bacteria.

The reactions given in table 2 show that of these 17 cultures 3 resemble *Bact. coli*, 2 *Bact. communior* and 3 *Bact. aerogenes*. The reaction in litmus milk in each case is not always characteristic but the reactions in glucose, lactose and sucrose broths and the results of the methyl red and Voges Proskauer tests would place them in the groups indicated. Culture 70 closely resembles *Bact. alcaligenes*. The remaining 8 cultures in this table somewhat resemble a number of organisms described by Chester (1901) in a group of motile, Gram-negative, non-liquefying bacteria from soil and water which form no spores and do not ferment any of the carbohydrates. The original descriptions of these bacteria, however, are inadequate for identification of the organisms studied. Until further work is done on this group, therefore, they cannot be designated other than as belonging to a large group of unidentified bacteria from the water and soil.

TABLE 1

Cultural reactions of gelatin-liquefying bacteria from decomposed salmon

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIO-CHEMICAL REACTIONS							TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer	
110	Gills and intestines	Small bacilli	0	Peptonized	0	+	0^x	0	0	0	0	
137	Mouth	Medium size bacilli	0	Peptonized	0	+	0	0	0	0	0	
139	Mouth	Small bacilli	0	No change	0	0	0	0	0	0	0	
184	Kidney	Small bacilli	0	Decolorized	0	+	0	0	0	0	0	*Bact. formosum* (Ravenel)
192	Gills and flesh of belly	Small bacilli	0	Decolorized	0	+	0	0	0	0	0	*Bact. formosum (Ravenel)*
168a	Gills	Small bacilli	0	Decolorized	0	+	0	0	0	0	0	*Bact. formosum* (Ravenel)
103a	Mouth and intestines	Small bacilli	0	Peptonized	+	+	A^y	0	0	0	0	
70a	Flesh of belly	Short, thick bacilli	0	Coagulated	+	+	AG^z	AG	AG	0	+	*Bact. cloacae*
118	Gills	Short, thick bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
130a	Mouth	Short, thick bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
138	Mouth and gills	Small bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
144	Gills and stomach	Short, thick bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
156	Gills and ceca	Short, thick bacilli	0	Coagulated	+	+	AG	AG	AG	0	+	*Bact. cloacae*
205a	Flesh	Medium size bacilli	0	Acid	+	+	AG	AG	AG	?	+	*Bact. cloacae*
235	Flesh of back	Medium size bacilli	0	Acid	+	+	AG	AG	AG	0	+	*Bact. cloacae*

253a	Flesh of back	Medium size bacilli	0	Acid	+	+	AG	AG	AG	?	+	*Bact. cloacae*
262	Flesh of belly	Medium size bacilli	0	Acid	+	+	AG	AG	AG	0	+	*Bact. cloacae*
273	Flesh of back	Medium size bacilli	0	Acid	0	+	AG	AG	AG	0	+	*Bact. cloacae*
292	Flesh of belly	Medium size bacilli	0	Acid	+	+	AG	AG	AG	0	+	*Bact. cloacae*
1	Ceca	Short, thick bacilli	0	Coagulated	+	+	AG	AG	AG	0	+	*Bact. cloacae*

x—0 indicates neither gas nor acid produced.
y—A indicates acid but no gas produced.
z—AG indicates acid and gas.

TABLE 2

Cultural reactions of non-liquefying bacteria from decomposed salmon.

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIO-CHEMICAL REACTIONS							TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer	
F	Intestine	Small bacilli	0	Decolorized	0	+	0[x]	0	0	0	0	
76	Flesh of belly	Small bacilli	0	Peptonized	0	+	0	0	0	0	0	
92a	Gills	Small bacilli	0	Decolorized	+	+	0	0	0	0	0	
108	Blood	Short, thick bacilli	0	No change	0	+	0	0	0	0	0	
125	Gills	Slender bacilli	0	No change	+	+	0	0	0	0	0	
125a	Gills	Small bacilli	0	No change	+	+	0	0	0	0	0	
190	Mouth	Medium size bacilli	0	Decolorized	0	+	0	0	0	0	0	
291	Flesh of belly	Medium size bacilli	0	Decolorized	0	+	0	0	0	0	0	
70	Flesh of belly	Small bacilli	0	Alkaline	0	+	0	0	0	0	0	*Bact. alcaligenes*
200	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG[z]	AG	0	+	0	*Bact. coli*
203	Flesh of back	Medium size bacilli	0	Acid	0	+	AG	AG	0	+	0	*Bact. coli*
285	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG	AG	0	+	0	*Bact. coli*
261	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG	AG	AG	+	0	*Bact. communior*
268	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG	AG	AG	+	0	*Bact. communior*
265b	Flesh of back	Medium size bacilli	0	Decolorized	+	+	AG	AG	AG	0	+	*Bact. aerogenes*
259	Flesh of back	Medium size bacilli	0	Acid	0	+	AG	AG	AG	0	+	*Bact. aerogenes*
95	Gills	Medium size bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. aerogenes*

[x]—0 indicates neither acid nor gas.
[z]—AG indicates both acid and gas.

TABLE 3

Cultural reactions of pigment producing bacteria from decomposing salmon

CULTURE	SOURCE	SHAPE	PIGMENT	GRAM STAIN	LITMUS MILK	BIO-CHEMICAL REACTIONS							ORGANISM RESEMBLED
						Indol	Gelatin liquefied	Nitrates reduced	Glucose	Lactose	Sucrose	Motility	
C	Intestine	Small bacilli	Green	0	Peptonized	±	+	0	A[y]	0	0	+	?
216	Flesh of back and belly	Small bacilli	Green	0	Peptonized	0	+	0	A	0	0	+	?
14a	Slime and belly	Short, thick bacilli	Orange	±	No change	0	0	0	0[x]	0	0	0	*Bact. breve* (Frankland)
113b	Stomach	Medium size bacilli	Orange	0	Peptonized	+	+	+	A	0	0	0	*Bact. caudatum* (Wright)
180	Stomach	Medium size bacilli	Orange	+	No change	0	0	+	0	0	0	+	*Bact. aurantiacum* (Frankland)
124	Ceca	Slender bacilli	Flesh-colored	0	Peptonized	+	+	0	A	0	0	+	*Erythrob. carnicolor* (Frankland)

[x]—0 indicates neither acid nor gas produced.
[y]—A indicates acid but no gas.

Of the 6 cultures producing pigment on gelatin and agar 3 produce an orange pigment, 1 a flesh-colored pigment and 2 a greenish pigment. Cultures 14a, 113b and 180, producing orange pigment, have been found to resemble closely *Bact. breve* (Frankland, 1894), *Bact. caudatum* (Wright, 1895) and *Bact. aurantiacum* (Frankland, 1894) respectively. The organism producing a flesh-colored pigment, 124, is very similar to *Erythrobacillus carnicolor* (Frankland, 1894). The original descriptions of these bacteria are not adequate for positive identification but the reactions of the organisms studied have been found to check with the limited descriptions given in the literature. The two cultures producing green pigment, C and 216, do not closely resemble any bacteria described. These two organisms are undoubtedly identical and might well have been considered as a single culture. All cultures of C and 216 have an aromatic odor.

No strict anaerobes and no spore-forming bacteria were isolated from the decomposed salmon. The absence of bacteria of the mesentericus group was surprising in view of the fact that during 1919 an examination of 530 cans of salmon packed in Alaska showed 42 per cent of the cans to contain living bacteria of this group (Hunter and Thom, 1919). No such organisms, however, have been isolated from the salmon caught and packed in the Puget Sound region. In considering the source of the organisms found in the uncooked fish it is evident that neither strict anaerobes nor spore-forming bacteria should be expected. As shown in the previous paper on the subject (Hunter, 1920) the digestive tract and viscera of the salmon are sterile when there is no food in the tract and when the fish are examined immediately after they are caught. The presence of bacteria in the gills, mouth and on the skin at all times and the late appearance of these organisms in the digestive tract and in the viscera indicate that the infection after death is from the outside inward rather than from the inside outward as is ordinarily found in the case of warm-blooded animals. We would expect the bacteria in the gills and mouth and on the skin to be those forms, the natural habitat of which is the sea-water in which the salmon live.

Identification of these 43 cultures has shown that this is actually the case. Most of the bacteria isolated from the decomposed salmon were originally described as water or sewage organisms, although a few were originally isolated from the soil. While these facts do not absolutely preclude the possibility of strict anaerobes and spore-formers being present, actual examination of 300 cultures from a large number of salmon in various stages of decomposition has shown that the flora of decomposed salmon is that of the sea-water from which they were taken. Furthermore, there was apparently no contamination with spore-forming organisms in the boats or in the cannery subsequent to the catching of the fish. The bacteria isolated from fish held for four days in the cannery are the same forms which were isolated from the gills and mouth when the salmon were examined immediately after being caught.

The bacteria responsible for the decomposition of the fish are not, in the strict sense, putrefactive and there is no rapid digestion of the flesh of the fish in the cultures. Each culture examined, however, did give some sort of "off" or foul odor in the fish medium and many showed the character of indol production when grown in this medium. It was noted that when grown in a beef infusion broth, with or without a small amount of carbohydrate added, no odors suggestive of decomposition were obtained. This makes it apparent that the flesh of fish is more susceptible to decomposition by ordinary water bacteria than is the flesh of the higher animals.

Although the salmon, when not feeding, has no bacteria in its intestines which may play a part in its subsequent decomposition, the accumulation of ordinary water bacteria in the gills and mouth and on the skin provides a flora which brings about a slow decomposition of the body of the fish after death.

SUMMARY

In a study of the bacteria which decompose raw salmon, 43 cultures were isolated as significant. On identification 21 cultures were found to be members of the colon-cloacae group. One culture resembled *Bact. alcaligenes* and 3 cultures resembled

Bact. formosum. Six cultures produced pigment. Twelve cultures not identified to type, evidently belong to a large group of water and soil bacteria which have not been adequately studied.

The bacteria isolated from decomposing salmon were found to be those which are described in the literature as water, sewage and soil organisms.

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THE INFLUENCE OF VARIOUS CHEMICAL AND PHYSICAL AGENCIES UPON BACILLUS BOTULINUS AND ITS SPORES

I. RESISTANCE TO SALT

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A number of statements are made in various text books concerning the vitality and longevity of *Bacillus botulinus* and its spores, as influenced by various chemical and physical agencies. Having a number of different strains of *B. botulinus* in pure culture, it seemed desirable that these statements should be evaluated in the light of past personal experiences with various strains of this bacillus. A study of the various agencies has been taken up in the order of their importance as nearly as practicable but from the nature of the problems it has been possible to conclude some of the less important phases first. For this reason the studies of the resistance of *B. botulinus* and its spores to salt are presented at this time. The other data will be published in the order of their completion.

The statement is made in Kendall's "General Pathological and Intestinal Bacteriology," that pickling in 10 per cent salt solution is destructive to the spores of *B. botulinus*, killing them within a week, and in Herzog's "Disease-Producing Micro-organisms," 5 or 6 per cent salt is said to prevent the multiplication of the bacillus.

Nineteen cultures of *B. botulinus* were used in the tests. Some of these strains may be identical, as a full history was not sent by some of the laboratories from which they were obtained.

The medium used was glucose pork gelatin-broth having a reaction of −0.5, and the percentages of salt used were from

one to ten inclusive, the solutions being made by adding sufficient broth (specific gravity taken roughly as 1.00) to 1, 2, 3, etc., grams of salt to make 100 cc. Each separate concentration of broth was inoculated heavily with *B. botulinus* so that the resistant minority would survive. A layer of sterile paraffin oil about 1 to 1.5 cm. deep was placed on the broth cultures, which were cultivated at room temperature. Examinations, macroscopic, and microscopic when necessary, were made every twenty-four hours for ten or eleven days with the first five strains and for seven days only with the remainder. Where tubes showed no growth, transfers were made from the tube of next lower concentration showing growth. Growth is shown first by cloudiness, then generally by gas production (more or less vigorous), followed by a precipitate, more or less heavy. Where growth did not show by these signs transfers were made into similar broth containing only 0.5 per cent salt and incubated. Examinations were made of the 10 per cent salt broth cultures only, after considerable periods of time as follows:

	days
Strains GO6, Mul 433, Alb 175, Alb 175B, Chi 595, C91 and GS	27
Strain Columb 31	28
Strains Mul 435, Mul 471, and AM of NH	31
Strains NBS., Columb 1 and N. Y. City B. of H	32
Strains Cal I, Cal II, Cal III, Cal IV and Cal VI	49

Table 1 indicates the growth in salt broth for the nineteen strains used. The occasional strains showing no growth at various times and salt concentrations are indicated.

It will be noted by examining the above table that only two out of the total of nineteen strains failed to show growth at any percentage of salt from 1 to 5 within the seven day period, and further that these two strains were both positive at the end of 27 days.

Table 2 indicates the record of the individual strains in the different salt broths. In this table the + sign signifies positive growth throughout the whole period of time unless otherwise indicated. It will be noted that ten out of the nineteen strains showed growth throughout the whole period.

Strains Cal I, Cal II, Chi 595, Mul 435, and AM of NH, are from the same original source and are of low virulence, and the results are practically the same, i.e., growth occurred throughout in all percentages of salt, and their vitality was not apparently impaired by a sojourn for 49, 27 or 31 days respectively in 10 per cent salt broth. These results are not confined to strains of low virulence, however. Similar results were obtained with strains of high virulence such as Cal IV, NBS, GO6, Mul 433 and Alb 175B, which survived 10 per cent salt for 49, 32, 27, and 27 days respectively. Cal III, Cal VI, and Mul 471, virulent strains, were the only strains virulent or non-virulent showing

TABLE 1

Resistance of B. botulinus

AGE	PER CENT SALT					
	1	2	3	4	5	10
days						
1	+	+°	+°	+	+	
2	+	+	+	+	+	
3	+	+	+	+	+	
4	+	+	+	+	+	
5	+	+	+	+	+	
6	+	+	+	+	+	
7	+°°°	+°°°	+°	+°°	+°°°	
8*	+	+	+	+	+	
9*	+	+	+	+	+	
10**	+	+	+	+	+	
11*	+	+	+	+	+	
27						+
28						+
31						+°°°°
32						+
49						+***

° Alb 175 negative.
°° Alb 175 and Alb 175B both negative.
°°° Alb 175B negative.
°°°° Strain Mul 471 doubtfully positive at this time.
* Strains Cal II, Cal III, Cal IV, and Cal VI only, examined.
** Strain Cal I only, examined.
*** Strain Cal III, only, negative.

TABLE 2

Resistance of individual strains of B. botulinus

STRAIN	PER CENT SALT					REMARKS
	6	7	8	9	10	
Cal. I	+	+	+	+*	+*	* These were negative the eighth and ninth days
Cal II	—*	—**	—**	—***	—****	* Positive after the first day ** Positive after the second day *** Positive after the second day, negative the third day and fourth days, then positive throughout **** Positive after the third day
Cal III	+	+	+	+	+*	* Negative the forty-ninth day
Cal IV	+	+	+	+*	+**	* Negative after the sixth day ** Negative the eleventh but positive the forty-ninth day
Cal VI	+	+	+	+	+*	* Negative the forty-ninth day
NBS	+	+	+	+	+	
Columb. 1	+	+	+	+	+	
GO6	+	+	+	+	+	
Columb. 31	+	+	+	+	+	
New York City Board of Health	+	+	+	+	+	
Mul 435	+	+	+	+	+	
Mul 471	+	+	+	+	+*	* Growth questionable on thirty-first day
Mul 433	+	+	+	+	+	
Alb 175	+	—*	—*	—**	—*	* Positive after the first day ** Negative after sixth and seventh days
Alb 175B	+*	+	+**	+*	+	* Negative after the fifth day ** Negative after the sixth day
Chi 595	+	+	+	+	+	
AM of NH	+	+	+	+	+	
C91	+	+	+	+	+	
GS	+*	+*	+	+*	+*	* Growth negative on the seventh day. In 10 per cent salt growth was positive however at the twenty-seventh day

growth at 11 days yet apparently not surviving 10 per cent salt at the end of 49 or even 31 days. All the other strains with two exceptions gave good growth every day for seven or eleven days after inoculation, and in addition, from 27 to 49 days after inoculation, in 10 per cent salt broth. Quantitative results were not obtained except in that a loop transfer from the 10 per cent salt broth culture gave a definite and decided clouding in the alkaline glucose pork gelatin-broth in 24 hours in every case as stated, with but two or possibly three exceptions. These results were checked microscopically.

These data prove quite conclusively that a large number of strains of *B. botulinus* are not inhibited by percentages of salt ranging from 1 to 10, when growing in a medium of an alkalinity of −0.5. This may not signify that *B. botulinus* will survive 10 per cent or less salt used in pickling, as the additional factor of acidity enters here, and this anaerobe is said to be injured—some strains quite decidedly so—by even a slightly acid medium (+0.5 to +0.8). However, the results obtained above seem to indicate strongly that it is not the salt which is the inhibiting factor in the destruction of *B. botulinus* and its spores by pickling solutions.

The data for the above tables were worked out by Miss Ruth Normington, a post-graduate student.

TIME SAVING BACTERIOLOGICAL APPARATUS

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In bacteriological laboratories the preparation of media and the cleaning of used apparatus and the sterilization of the same are often problems of difficult solution. Especially is this true in laboratories where all the media are furnished the students and all the apparatus such as Petri dishes and tubes, etc., are cleaned and sterilized for them. This practice is being followed in the bacteriological laboratories at the University of Illinois.

This paper gives a brief description of apparatus which we have found to be time saving and consequently expense saving in our work. No claim of originality is made. These descriptions are given with the hope that they may be of some use to others who have similar problems to work out.

APPARATUS FOR COOKING MEDIA

This is a double walled steam jacketed trunnion kettle. Similar kettles are used in hotel kitchens and in ice cream plants. Figure 1 shows its general construction and appearance.[1] It is made of one piece of aluminum and hence its inner surface has no seams or joints. It can be used where a supply of steam is available. About 2 to 5 pounds of steam pressure is sufficient for cooking. The kettle used in our laboratory has a capacity of eight gallons.

The main virtue of this kettle lies in the fact that a large quantity of media can be prepared in a short time. It takes less than five minutes to bring 30 quarts of cold water to a boiling point.

[1] This photograph was kindly supplied by the Aluminum Cooking Utensils Company, New Kensington, Pennsylvania.

Fig. 1. Steam Jacketed Trunnion Kettle Used for Cooking the Media

Fig. 2. Hotel Size Gas Oven Used for Hot Air Sterilizer

APPARATUS FOR STERILIZING WITH HOT AIR

This apparatus is a large gas oven, known as the hotel size oven, because such ovens are used extensively in hotel kitchens. Figure 2 shows its general appearance. The oven is made of non-rustable metal and is constructed on the principle of the fireless cooker, being well insulated and the flame not entering the chambers.

Its main virtues are, first, its size—about 2500 petri dishes can be sterilized at one time; second, ease of sterilization—being well insulated, the oven is heated to a certain temperature and the gas is then turned off; third, the uniformity of sterilization—all parts of the oven are evenly heated. The oven has given great satisfaction.

APPARATUS FOR CLARIFYING MEDIA

Filtration of agar and gelatin has always been a time consuming and unsatisfactory procedure. This is evidenced by the many different methods which have been described in the literature and by the detailed instructions given in the methods for preparing these media. The Laboratory Super-Centrifuge,[2] figure 3, makes it possible to secure clear media without the use of egg albumin and without the trouble of filtering through cotton or paper. With the trunnion kettle and the super-centrifuge, the preparation of a large quantity of media becomes a simple operation. A concrete example may be cited. It took one and one-half hours to prepare, cook, and clarify 25 liters of agar, ready for tubing. With 25 liters of agar there is a loss due to clarifying of not over 500 cc.

These Super-Centrifuge machines may be driven by compressed air or by high pressure steam. Ours is attached to both systems, but we find that the compressed air is more satisfactory for our purpose. A speed of 30,000 revolutions per minute gives a satisfactory filtrate. Equally satisfactory results are secured

[2] This illustration was kindly supplied by the Sharples Company of West Chester, Pennsylvania.

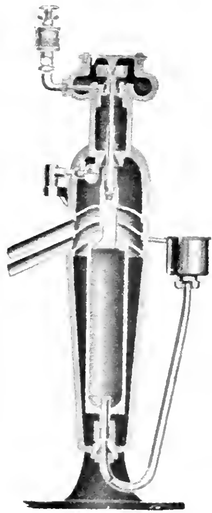

Fig. 3

Fig. 4

Fig. 3. A Cross Section of the Super-Centrifuge
Fig. 4. Test Tube and Flask Washing Machine Driven by Electric Motor

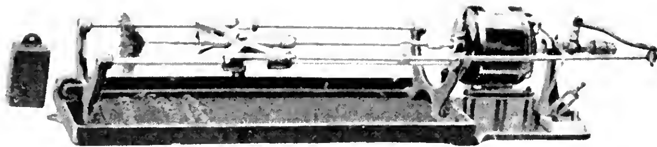

Fig. 5

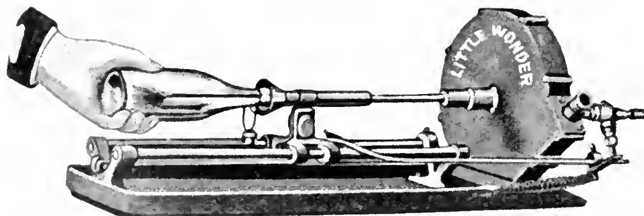

Fig. 6

Fig. 5. Modification of Figure 4
Fig. 6. Test Tube and Flask Washing Machine Driven by Water Motor

with agar as with gelatin. The value of this apparatus is especially noticeable with a stiff agar (2.5 per cent), used in such medium as Endo's agar. With the old methods of clarification the filtration of such stiff agar was a task.

APPARATUS FOR WASHING

The washing of large quantities of test tubes and other apparatus by hand is time consuming and expensive. The high cost of laboratory apparatus makes it imperative that test tubes be cleaned and sterilized as soon as possible in order that they may not be out of use for any extended period of time. We resorted to the use of the contrivance which is used with success in the carbonated drink industry and while it is not directly suited to all bacteriological apparatus, it is serving well its purpose. This apparatus consists of a revolving brush attached to the shaft of an electric motor. Figures 4, 5 and 6 show the general construction of this apparatus. Those in figures 4 and 5 are driven by an electric motor while that in figure 6 is driven by a water motor. Figures 5 and 6 are equipped with a contrivance to admit clean water into the test tube or the bottle while it is being washed by the revolving brush. Any size brush can be attached to the shaft.

In our experience we find that the simple machine shown in figure 4 is as satisfactory as those with the contrivance for admitting water. If these machines are run carefully, breakage of glass ware is a negligible factor with any of them.

MILK-POWDER AGAR FOR THE DETERMINATION OF BACTERIA IN MILK

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Before presenting descriptions of new media for the determination of bacteria in milk, we wish to call attention to the fact that the media described give not only quantitative but, to a certain extent, qualitative results. If the new media achieved merely the same results as those now well known, we should not feel warranted in taking up the reader's time.

It is believed that the time has passed when one can be satisfied with total counts only. Mere quantitative results in bacteriological milk analysis can not be interpreted satisfactorily unless something of the history of the milk is known. Often this "something" is missing; then one must interpret the count as due to this, that, or the other condition or a combination of them. This is not an attempt to discredit the total bacterial count, for it is very valuable as it stands, in milk-control work, but it is hoped that some of the media described in this paper will increase its value.

The media extensively used in milk-control work have been (1) infusion agar, described in the standard methods of the American Public Health Association in 1910, and (2) extract agar, later adopted as a standard medium, its formula having been published by the association in 1916.

Many who use the extract agar realize that it does not give the high counts which are obtained on the infusion agar, but use it because it is the standard medium for milk analysis. Even the members of the Committee on Standard Methods recognized the fact that extract agar is defective, as is shown by the state-

ment on page 5 of the 1916 Standard Methods that "The standard methods, for example, are not such as give a proper count of lactic acid bacteria." In this connection we must say that we see no reason for not taking into account the lactic-acid bacteria. They are of as much significance in tracing the history of a sample of milk as any other type of bacteria, and especially so when their proportion to some other type, such as peptonizers, can be determined. Furthermore, it is believed, since extract agar does not readily support the growth of certain types of lactic-acid bacteria, that this is the most important reason for variations in counts observed when the new standard medium is used. There are certain types of lactic-acid bacteria which will grow slowly on extract agar, and their colonies may be either just visible or not show at all after forty-eight hours' incubation at 37°C.

In our opinion, a medium should be used (1) which will be relatively cheap, (2) which will give the highest count with colonies large enough to be readily counted, and (3) which will give different pictures with different milks; that is, it should be qualitative so far as possible. If bacteria are in milk there is no logical reason for counting only a portion of them. A bacterial count is made to determine the number of bacteria in milk, and if one medium shows higher counts than another, it is evident that the highest count comes closest to the actual number of bacteria in the milk. With either the infusion or extract agar medium nothing but a simple count can be obtained. If a medium can be devised which gives an indication of different groups of bacteria or even only of a single group, in other words, a medium which will give any information relative to the bacteria in milk besides a simple count, it should prove to have distinct advantage over the media now in common use.

GENERAL SCOPE OF THE WORK

In 1911, one of us (Ayers (1911)) described a casein medium which was adapted to the bacterial examination of milk. On this casein medium peptonizers could be readily detected and

counted and the total count obtained as well. It was found that the total counts on the casein medium were decidedly lower than on infusion agar unless the plates were incubated for six days. This fact, of course, made the medium almost valueless for routine control work. Attempts to make a medium by using skimmed milk, with its casein dissolved, and adding peptone and extract were not successful.

Having in mind this casein medium, with its advantages and defects, it seemed probable that skimmed-milk powder could be used in place of purified casein, and that with some additional nutritive substances a cheap and valuable medium could be prepared which, in forty-eight hours, would give counts at least as good as the present standard extract agar and also give a count of the peptonizers present.

In this work about 50 different media were made, containing milk powder alone and in combination with meat extract, yeast extract, peptone, peptone and meat extract, and meat infusion. Ordinary agar was tried, also agar with some of the calcium and magnesium salts removed by precipitation with sodium dibasic phosphate, and agar in which they were partly removed by washing.

The media suggested in this paper are not the result of a "lucky shot." They were developed during a study of various combinations which were in every case compared with the standard extract agar. This work resulted in the examination of about 400 samples of milk of different kinds, that is, raw, pasteurized, and certified; which, figuring the different combinations, is equivalent to the plating of about 1750 samples of milk on a single medium.

This is mentioned only to impress upon the reader that the directions given for making the media are based on considerable experimental data which it is impracticable to include in this paper. We do not feel that the media are beyond improvement, but hope the directions will be followed until they have been given a fair trial.

We will describe a skimmed-milk powder medium containing meat extract and peptone, a modification of this medium, and a milk powder medium containing no meat extract or peptone.

MILK-POWDER AGAR

Medium A. Ingredients for one liter of medium

(a) { 5 grams skimmed-milk powder; 1 gram sodium dibasic phosphate. (Sorensen's phosphate[1]) ($Na_2HPO_4 + 2H_2O$) } In 250 cc. distilled water

(b) { 5 grams peptone; 3 grams extract } In 250 cc. distilled water

Mix (a) and (b) and add 500 cc. of double strength (3 per cent) washed-agar solution.

DETAILED DIRECTIONS FOR PREPARATION OF 1 LITER OF MEDIUM

Milk-powder solution

The detailed directions must be followed accurately if satisfactory and constant results are to be obtained. The medium is very easy to prepare when the various steps are understood and the process completed once. It may appear complicated because of the complete details which are given of each step in the process and which make the preparation of the medium appear somewhat long.

[1] Sorensen's phosphate is sodium dibasic phosphate with 12 molecules of water of crystallization ($Na_2HPO_4 + 12H_2O$) which has been *air dried* so that instead of containing 12 molecules of water it has 2 molecules. Anhydrous sodium dibasic phosphate takes up water easily as it stands in the laboratory and that containing $12H_2O$ loses water easily, so that neither of these can be depended upon to be definite in regard to water content. To overcome this difficulty, Sorensen air-dried $Na_2HPO_4 + 12H_2O$ for about two weeks and found that the water of crystallization was reduced to $2H_2O$ and that it remained practically constant. Sorensen's phosphate can be purchased or for practical purposes prepared in the laboratory by taking the ordinary dibasic phosphate with $12H_2O$ and spreading it out on filter paper and allowing it to remain at room temperature in a dry place for fourteen days. This phosphate ($Na_2HPO_4 + 2H_2O$) is used in the preparation of the medium so that a definite amount can be weighed out at any time and the milk-powder solution will always have practically the same hydrogen-ion concentration. If this phosphate is purchased, care should be taken to be sure it contains two molecules of water and is not anhydrous.

To make the milk-powder solution *"a"* use a good grade of skimmed-milk powder made by the *spray* process, and prepare the following solutions:

(1) 5 grams milk powder
20 cc. distilled water
(2) 1 gram sodium dibasic phosphate (Sorensen's phosphate)
5 cc. distilled water

(When making more than 1 liter, the same proportions of milk powder to water and phosphate to water must be used; therefore, to make 5 liters multiply each amount by 5.)

Weigh out 5 grams of skimmed-milk powder and pour on to 20 cc. of *cold* distilled water in a small beaker. Stir until thoroughly dissolved. In another beaker, dissolve 1 gram of sodium dibasic phosphate (Sorensen's phosphate) in 5 cc. of distilled water. Warm to dissolve phosphate quickly. Sorensen's phosphate, $Na_2HPO_4 + 2H_2O$, must be used.

Add the phosphate solution (2) to the milk powder (1). Place the beaker, containing (1) and (2) mixed, in a water bath with water at about 30°C. and heat the milk powder phosphate solution to about 60°C. This should take about 10 minutes. At temperatures between 50 and 60°C. a flocculent grayish precipitate will appear. Continue the heating until the precipitate appears, then steam in an Arnold or other steamer for five minutes, or until the precipitate appears white. Then dilute the milk-powder solution about one-third with distilled water and steam five minutes longer. Too long steaming will cause the solution to turn dark and should be avoided. The whole heating period should not be more than twenty or twenty-five minutes.

Decant the solution while hot on to a filter paper, taking care to keep the precipitate in the beaker until most of the liquid is through. Then pour the precipitate on the filter and wash with a little distilled water. If the milk-powder solution has been properly heated it will filter readily, provided the filter paper is not too hard. "J. Green" Grade 588 and "Ilmenau" filter paper have given good results.

The filtrate, which is of a yellowish-white color, will appear cloudy and can not be filtered clear in the concentration used. This makes no difference, because it is clear in the dilution of the final medium.

Make up the filtered milk-powder solution to 250 cc. with distilled water. This completes the milk-powder solution "a."

PEPTONE-EXTRACT SOLUTION

To make the peptone-extract solution "b" dissolve 5 grams peptone and 3 grams Liebig extract in 100 cc. distilled water by steaming in the Arnold sterilizer or by boiling over flame for twenty minutes. Filter until clear and make solution up to 250 cc. with distilled water. This completes solution "b." "Difco" peptone has been used in our experiments because of its hydrogen-ion concentration, which is near the neutral point, and because, with the extract in the proportion of 5 grams peptone to 3 grams extract, a precipitate is usually formed which permits filtration with a resulting clear solution. For a standard medium, whatever makes of peptone and extract are selected should be universally used.

The milk-powder solution "a" is now mixed with the peptone extract solution "b" which gives a total volume of 500 cc. To this mixture 500 cc. of double strength (3 per cent) washed-agar solution is added. This completes the medium, which is now ready for sterilization.

THE USE OF DISTILLED WATER

We specifically mention distilled water. Tap water may or may not contain dissolved substances, the effect of which on media making and bacterial growth is unknown. By the use of distilled water this uncertainty is obviated and one is assured of a constant definite solvent, "standard" everywhere.

WASHED-AGAR SOLUTION

A stock solution of double strength (3 per cent) washed agar is prepared and put up in flasks and sterilized. This agar is then

ready at any time for use. The agar should be put up in flasks in amounts suitable for the amount of medium to be made at any one time. It is not desirable to use part of a flask of agar, then resterilize and hold for future use. Repeated heating lowers its jelly strength. The flasks should be stoppered to prevent evaporation. To prepare a liter of 3 per cent washed agar, weigh out 30 grams of agar and place in a flask with 2000 cc. of distilled water. This proportion should always be used. Allow it to stand for twenty-four hours, at room temperature, with occasional shaking. Then pour off as much water as possible, using a piece of cheesecloth over the top of the flask, and add distilled water enough to make up again the original volume.

Allow the agar to stand another twenty-four hours, then pour off the agar on to a cotton-flannel cloth in a funnel and wash once with a liter of distilled water. Let the agar drain and then press out as much water as possible by squeezing the filter cloth with the hands. A container large enough to hold the agar is counterpoised on the laboratory scales, and the agar placed in it. In the opposite pan is placed 30 grams for the agar and 1000 grams for the weight of the water in which the agar is to be dissolved. Then water enough is added to make up this weight. This will make a liter of 3 per cent agar. Dissolve the agar by heating in the Arnold sterilizer, then filter through cotton flannel or absorbent cotton until clear.

REASONS FOR USING WASHED AGAR

There are two reasons for using washed agar; first, because it makes possible the preparation of the previously described medium without the formation of a precipitate upon sterilization; and second, because washed agar gives higher counts than ordinary agar, at least with some samples of milk.

When ordinary or so-called purified agars are used in a medium with milk powder, a heavy precipitate forms upon sterilization, probably due to the precipitation of calcium and magnesium phosphate with some casein. This will not occur if enough sodium bicarbonate is added to the medium to bring the hydro-

gen-ion concentration to pH 7.6. The medium with this pH, however, does not give satisfactory results as to counts.

By the addition of $Na_2HPO_4 + 2H_2O$ to the agar during preparation, the calcium and magnesium salts are partly precipitated and can be removed by filtration. This agar when used in the medium causes no precipitation upon sterilization but cuts down the count. From our experiments it is believed this reduction may be due to the increase in the phosphate content of the medium.

Washing the agar as described gives an agar which works perfectly because the calcium and magnesium content is reduced to a considerable extent and a readjustment of the medium to a suitable pH is not necessary.

We will consider the question of the special value of washed agar in raising counts, in another paper.

NO ADJUSTMENT OF REACTION NECESSARY

It will be noted that no adjustment of reaction has been mentioned. If the medium is made according to directions, it will have a hydrogen-ion concentration of about pH 6.8 when "Difco" or Parke-Davis peptone is used and about pH 6.7 with Fairchild peptone. To be safe, it is desirable to check the reaction of the milk-powder peptone-extract solution for its hydrogen-ion concentration with indicators[2] for each new lot of skimmed-milk powder, peptone, and meat extract used. This can easily be determined in a sufficiently accurate manner by adding 5 drops of phenol red indicator to a tube containing 5 cc. of the double-strength milk-powder peptone-extract solution diluted to 10 cc. with distilled water and 5 drops of brom-cresol purple to another tube of the solution. The reaction is correct when the tube with phenol red is yellow and the one with brom-cresol purple is purple. If the tube with phenol red is red, add $\frac{N}{10}$ HCl, calculating the amount necessary to change it to yellow

[2] See paper by Clark, W. M., and Lubs, H. A. The colorimetric determination of hydrogen ion concentration and its application to bacteriology. Journ. Bact., **2**, p. 1, 1917.

with phenol red, and purple with brom-cresol purple. From this computation it is easy to figure the amount of *N* HCl required to bring the total volume of solution to the proper pH. If the color is greenish or yellow with brom-cresol purple add $\frac{N}{10}$ NaOH and calculate the amount of *N* NaOH necessary to bring the total volume of solution to a point where it is purple with brom-cresol purple and yellow with phenol red. This is a very rough way of obtaining a reaction of about pH 6.8 but is accurate enough for this medium. This process need only be carried out, as previously stated, with each new lot of ingredients used. The addition of washed agar does not appreciably affect the reaction.

To prepare the *stock* solution of phenol red indicator take 0.2 gram of the powdered phenol red and add 11.4 cc. of $\frac{N}{20}$ NaOH. Warm and agitate until dissolved, then make up to 100 cc. with distilled water. Take one volume of this stock solution and 9 volumes of distilled water to make the *test* solution. Use 5 drops of this test solution to 10 cc. of medium at 45°C. in a test tube.

The *stock* solution of brom-cresol purple is made by adding 14.8 cc. $\frac{N}{20}$ NaOH to 0.4 gram of powdered brom-cresol purple. This is warmed and when dissolved is made up to 100 cc. with distilled water. To make the *test* indicator solution mix one volume of stock solution with 9 volumes of distilled water. Use 5 drops to a tube with 10 cc. of medium at 45°C. to indicate the hydrogen-ion concentration.

COUNTS OBTAINED WITH THE MEDIUM

Comparisons were made of the counts on standard extract agar, standard infusion agar (1910 standard methods) and milk-powder medium. The plates were incubated at 37°C. for forty-eight hours and a hand lens was used in counting. Various kinds of milk were examined, such as raw, pasteurized, and certified milk. The results of this work are shown in table 1. It will be seen that with three exceptions the counts on milk-powder agar were higher than on the standard extract agar. With the

TABLE 1

Comparison of bacteria counts on extract agar, infusion agar, and milk-powder agar A

EXTRACT AGAR. STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR. STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK POWDER AGAR A.	INCREASE OVER EXTRACT AGAR
		Raw milk		
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
650,000	1,080,000	66.0	1,250,000	92.0
1,110,000	1,890,000	70.0	1,760,000	59.0
1,410,000	1,930,000	34.0	1,890,000	34.0
1,160,000	6,110,000	425.0	5,830,000	400.0
1,580,000			4,410,000	180.0
246,000	2,110,000	450.0	687,000	188.0
239,000	1,460,000	510.0	1,500,000	520.0
3,370,000			12,160,000	262.0
1,760,000	2,930,000	66.0	3,500,000	99.0
2,300,000	3,530,000	53.0	4,700,000	100.0
5,120,000			14,720,000	186.0
690,000	860,000	29.0	960,000	39.0
480,000	950,000	100.0	900,000	87.0
59,000	173,000	193.0	215,000	264.0
54,000	220,000	300.0	249,000	366.0
1,100,000	780,000	62.0	2,000,000	82.0
112,000	198,000	76.0	204,000	82.0
217,000	900,000	232.0	970,000	258.0
75,000	510,000	580.0	720,000	860.0
6,800,000	46,200,000	517.0	41,200,000	506.0
2,860,000	6,400,000	124.0	9,800,000	243.0
119,000			447,000	275.0
159,000			256,000	61.0
192,000			284,000	48.0
87,000			124,000	42.0
1,360,000			2,790,000	105.0
124,000			149,000	21.0
1,210,000			1,590,000	31.5
380,000			320,000	−18.0
325,000			380,000	17.0
139,000			303,000	118.0
263,000			391,000	48.5
720,000			1,400,000	94.5
990,000			1,230,000	24.0
130,000			297,000	128.0
154,000			308,000	100.0
141,000			257,000	82.0
620,000			870,000	40.0
196,000			450,000	129.0
184,000			348,000	89.0
167,000			355,000	112.0
9,700,000			16,300,000	68.0
1,200,000			1,580,000	31.0

TABLE 1—*Continued*

EXTRACT AGAR. STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR. STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK POWDER AGAR A.	INCREASE OVER EXTRACT AGAR
		Raw milk—*continued*		
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
75,000			105,000	40.0
2,090,000			2,460,000	7.0
60,000			90,000	50.0
4,400,000			6,600,000	50.0
46,000			94,000	104.0
73,000			117,000	60.0
87,000			84,000	−9.0
64,000			85,000	32.0
4,300,000			9,300,000	110.0
43,000			71,000	65.0
127,000			227,000	80.0
227,000			416,000	83.0
163,000			224,000	37.5
145,000			190,000	31.0
2,990,000			3,360,000	20.0
46,000			60,000	30.0
20,000			58,000	190.0
15,700,000			14,500,000	−9.0
4,200,000			5,700,000	35.0
339,000			690,000	104.0
30,300,000			128,000,000	76.0
428,000			740,000	73.0
6,500,000			10,600,000	63.0
48,500,000			153,000,000	202.0
287,000			390,000	36.0
1,420,000			2,200,000	55.0
830,000			1,430,000	72.0
		Pasteurized milk		
1,000	68,000	679.0	117,000	11,600.0
3,500	64,000	1,725.0	125,000	3,470.0
41,900	46,000	9.0	62,000	48.0
13,000	20,000	54.0	46,000	254.0
4,800	36,200	652.0	120,000	2,400.0
4,500	25,000	455.0	128,000	2,745.0
5,700	32,000	465.0	140,000	2,350.0
1,500			95,000	6,230.0
3,800	168,000	384.0	191,000	400.0
200	162,000	80,000.0	159,000	75,000.0
6,800	23,400	244.0	16,900	148.0
18,700	49,000	162.0	32,800	75.0
30,000	52,000	73.0	62,000	107.0
51,000	63,000	23.5	111,000	123.0
100,000	112,000	11.0	103,000	3.0

TABLE 1—*Concluded*

EXTRACT AGAR. STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR. STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK POWDER AGAR A.	INCREASE OVER EXTRACT AGAR
Pasteurized milk—*continued*				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
1,400	36,800	2,500.0	50,000	3,470.0
2,700			7,400	174.0
238,000			288,000	21.0
236,000			313,000	33.0
11,000			55,000	400.0
9,700			56,000	475.0
52,000			154,000	127.0
1,400			61,000	425.0
580,000			2,440,000	320.0
44,000			156,000	255.0
12,500			33,300	166.0
13,200			23,500	78.0
25,000			120,000	41.0
62,000			271,000	330.0
7,700			108,000	1,440.0
980,000			1,240,000	26.0
1,330,000			1,650,000	24.0
66,000			339,000	410.0
220,000			405,000	84.0
300,000			472,000	57.0
47,000			166,000	250.0
36,000			194,000	430.0
24,000			550,000	2,000.0
28,000			370,000	1,220.0
480,000			1,600,000	230.0
600,000			1,040,000	73.0
206,000			470,000	128.0
140,000			760,000	449.0
700			3,700	428.0
300			3,400	1,000.0
6,200			410,000	6,900.0
13,100			560,000	4,200.0
6,400			173,000	2,780.0
16,000			15,500	55.0
9,600,000			17,900,000	65.0
34,800			920,000	2,550.0
Certified milk				
4,900	5,700	16.5	5,200	6.0
8,500	12,400	46.0	14,500	70.0
4,100	7,600	85.0	6,200	51.0
37,800			39,500	4.5
36,000			78,000	110.0
29,100			39,200	25.0

exception of the certified milk the counts were very much higher, the approximate percentage increase over the extract-agar count ranging from 3 to 75,000 per cent. The colonies were very much larger and could be readily counted. Particular care was taken with the count of the colonies on the standard extract agar and we believe our counts are higher than would have been the case if the plates had been counted in the average laboratory, because when there was a question of doubt about a colony it was always included. This was done to avoid favoring the media in which we were interested. The greatest differences in count were usually found in pasteurized milk.

As to the counts on milk-powder agar and infusion agar (1910 standard) it will be noted that of the 36 samples of milk plated, in 22 cases the counts were higher on the milk-powder agar. In some cases they were practically the same or within the limits of error, but in other cases they were distinctly higher.

It seems unnecessary to discuss the results further, for a study of the figures in the table will show clearly the remarkable increase in count obtained on milk-powder agar over that from the standard extract agar.

If the bacteria were not in the milk they would not have been on the milk-powder agar plates; and if one is determining the number of bacteria in a sample of milk one must, if possible, use a medium on which they will grow.

The fact that the medium shows high counts is not its only merit. It is possible with this medium to obtain a direct count of the number of peptonizing bacteria, of strong acid-forming, and of weak acid-forming bacteria in a sample of milk. The medium has been purposely arranged so as to be slightly cloudy after sterilization. After forty-eight hours' incubation, colonies of strong acid-forming bacteria have a white cloud about the colony, while the peptonizers have a clear zone, after the plate has been flowed with acid. Even though the plates are white, due to the precipitation of the dissolved casein through acid formation, it is easy to count the colonies. The colonies of weak acid-forming bacteria can be determined by adding enough brom-cresol purple indicator to cover the plate. This should

remain on the plate for five minutes or until it has penetrated through the agar. All the acid-forming colonies show a yellow color against a purple background. Of course when the plate is crowded the whole plate may be yellow (acid). If desired, brom-cresol purple can be added to the medium at the time of preparation. To get the proper color, add 8.0 cc. of stock solution to each liter of medium; the preparation of the stock solution is described on page 573.

The peptonizers should not be counted until the plate has been flowed with a 5 per cent solution of acetic acid, because the medium is often rendered sufficiently acid to cause a precipitation of the casein except about colonies which produce a strong alkaline reaction. The zone about such colonies under this condition may be clear and resemble peptonization. Flowing with acid will cloud these zones unless there is true peptonization. After flowing the plate with acid allow the acid to stand on the milk-powder medium for ten minutes, then make the count of peptonizers. The acid precipitates the dissolved casein of the milk powder except about colonies where it has been digested. Evidence of this casein peptonization is shown by a clear zone, and colonies should only be counted as peptonizers when there is a definite, clear zone. The activity of the bacteria in the colony can be further estimated by the diameter of the zone. In case no peptonizers show on the plate which is being counted, they should be counted on plates with a lower dilution. They can be easily counted even though the plate is crowded.

When the plate has been flowed with brom-cresol purple, then with acetic acid, the plate is yellow instead of white. This does not interfere in any way with the counting of the peptonizers.

The milk-powder medium, therefore, gives a picture which varies greatly with different samples of milk. Sometimes there are numerous colonies of strong acid-forming bacteria, sometimes none, sometimes many peptonizers and again only a few. While the relation between these various colonies and the total count has not been fully worked out, we believe that there are certain correlations which will be found to have a definite relation to the history of the milk.

To take the fullest advantage of the medium the counting procedure should be as follows:

1. *Make a total count.*
2. *Count strong acid-forming colonies.* Those with a cloudy zone about them or a slight hazy edge.
3. *Count weak acid-forming colonies.* This is done by flowing the plate with brom-cresol purple and counting the total acid colonies and subtracting the number of strong acid-forming colonies.
4. *Count peptonizers.* Flow plate with a 5 per cent solution of acetic acid before counting.
5. *Calculate the number of alkali formers and inert colonies.* To do this add the total number of acid colonies to the number of peptonizers and subtract from the total count.

MILK-POWDER MEDIUM "B"

Feeling that there might be some objection to the use of a medium which was slightly cloudy, it was decided to work out a medium which would be clear. This milk-powder medium differs from the first merely in the amounts of peptone and meat extract used. To make this medium, simply substitute in the formula for medium "A" the following: 2 grams of peptone and 1 gram meat extract instead of 5 grams peptone and 3 grams extract. The medium in other respects is made exactly the same. No adjustment of reaction is necessary, as it will be about pH 7.0 to pH 7.1, brown or slightly red brown with phenol red.

The counts obtained on this medium and other media are shown in table 2. It will be seen that they are just as high on the whole as on milk-powder medium "A" and in many cases higher. The colonies of strong acid-forming bacteria can be seen on this medium with a small white zone about them. They are not so distinct, however, as on the milk-powder medium "A." In order to distinguish colonies of peptonizing bacteria it is necessary to flow the plate with 5 per cent acetic acid. This precipitates the casein and causes the clear medium to have a white, opaque appearance. After flowing with acid, clear zones may be seen about the colonies of the peptonizers.

TABLE 2

Bacterial counts on milk-powder agar A and B compared with extract agar and infusion agar

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER AGAR B (0.2 PER CENT PEPTONE), (0.1 PER CENT MEAT EXTRACT)	INCREASE OVER EXTRACT AGAR	MILK-POWDER AGAR A, (0.5 PER CENT PEPTONE), (0.3 PER CENT MEAT EXTRACT)	INCREASE OVER EXTRACT AGAR
			Raw milk			
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
690,000	860,000	29.0	990,000	42	960,000	39
480,000	950,000	100.0	1,090,000	112	900,000	87
59,000	173,000	193.0	180,000	200	215,000	204
54,000	220,000	300.0	229,000	324	249,000	360
1,100,000	1,780,000	62.0	2,600,000	136	2,000,000	82
112,000	198,000	76.8	213,000	90	204,000	82
271,000	900,000	232.0	840,000	210	970,000	258
75,000	510,000	580.0	570,000	660	720,000	860
6,800,000	46,200,000	517.0	44,200,000	550	41,200,000	500
2,860,000	6,400,000	124.0	10,200,000	257	9,800,000	243
			Pasteurized milk			
3,800	168,000	384.0	196,000	415	191,000	400
200	162,000	80,000.0	192,000	90,000	159,000	75,000
6,800	23,400	244.0	23,900	250	16,900	148
18,700	49,000	162.0	32,300	73	32,800	75
30,000	52,000	73.0	86,000	187	62,000	107
51,000	63,000	23.5	77,000	51	111,000	123
100,000	112,000	11.0	134,000	13	103,000	3
1,400	36,800	2,500.0	70,000	4,900	50,000	3,470
			Certified milk			
4,100	7,600	85.0	6,500	58	6,200	51
4,900	5,700	16.3	6,500	32	5,200	6

This milk-powder agar "B" does not give quite such characteristic pictures with different samples of milk as does the first mentioned milk-powder agar "A." However, it is a clear medium and is cheaper than medium "A."

MILK-POWDER YEAST AGAR

It is sometimes said that it is impossible to expect uniformity in bacteria counts as long as peptone and meat extract are so variable. Of course every one will agree that different makes

of peptone will not be exactly the same and the peptone of one manufacturer may vary from time to time. Even meat extract is not constant in composition. In view of these facts it may be well to eliminate peptone and meat extract from standard media and so discount any possible effect of variations in their composition. With this thought in mind a medium was devised consisting of skimmed-milk powder, yeast extract and washed agar. Yeast extracts (Ayers and Rupp, 1920) have been used extensively in these laboratories and found to be very satisfactory for many purposes.

Ingredients for 1 liter of medium

(a) 5 grams skimmed-milk powder; 1 gram sodium dibasic phosphate. (Sorensen's phosphate) — In 250 cc. distilled water

(b) 10 grams pure dry fresh yeast. In 250 cc. distilled water

Mix (a) and (b) and add 500 cc. of double strength (3 per cent) washed-agar solution.

DIRECTIONS FOR PREPARATION OF MEDIUM

The milk-powder solution should be prepared as previously described.

The yeast extract is made from dry fresh (not autolyzed) yeast which contains no added fillers. To facilitate filtering, the yeast should be dried at a temperature of from 105° to 110°C. for five hours. The extract is made by steaming 10 grams of yeast in 100 cc. distilled water for 45 minutes in the Arnold, then filtering until the filtrate is clear and brilliant. When most of the extract has been filtered, 100 cc. of distilled water should be poured on the filter to wash out the remainder. Make up to 250 cc. with distilled water. The filter papers previously mentioned for filtering the milk-powder solution give good results with yeast extracts. In order to obtain a clear, brilliant filtrate, it is usually necessary to pour the filtrate back into the filter a few times until it clogs slightly.

The milk-powder solution and yeast extract are then mixed and 500 cc. of double strength (3 per cent) *washed* agar is added.

The washed-agar solution is prepared as described. No adjustment of reaction is necessary, as the final hydrogen-ion concentration of the medium will be about pH 6.7.

BACTERIAL COUNTS ON MILK-POWDER YEAST MEDIUM

The counts obtained on the milk-powder yeast agar, as well as on standard extract and standard infusion agar, are shown in table 3. The figures show that the counts on the milk-powder yeast were much higher than on standard extract agar and that they agree closely with the counts on standard infusion agar.

Yeast extract was used extensively in other combinations with good results, and while we have only a few results on the medium just described it is believed it can be used to advantage with skimmed-milk powder as a substitute for peptone and meat extract. Further work is necessary before this point can be definitely settled.

The milk-powder yeast agar does not appear to give quite such high counts with all samples as the powder, peptone, meat-extract agars A and B, but it has the advantage of being more nearly uniform in composition. This is obvious for the reason that the yeast extract can be made in the laboratory in a definite way from a dry preparation consisting of a definite species of pure yeast.

The milk-powder yeast agar is a clear medium on which colonies of the strong acid-forming bacteria can be seen, although not so distinctly as on the other media described in this paper. Peptonizers can be readily observed by flowing the plate with 5 per cent acetic acid.

THE USE OF SKIMMED-MILK POWDER

Dissolved skimmed-milk powder is used as a foundation for the media described in this paper and not as merely something to be added to some other medium. The use of skimmed-milk powder must be considered in this light in order that its value may be fully appreciated.

TABLE 3

Comparison of bacteria counts on extract agar, infusion agar, and milk-powder yeast agar

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER YEAST AGAR	INCREASE OVER EXTRACT AGAR
		Raw milk		
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
690,000	860,000	29.0	810,000	17.0
480,000	950,000	100.0	730,000	52.0
59,000	173,000	193.0	165,000	180.0
54,000	220,000	300.0	209,000	287.0
1,100,000	1,780,000	62.0	1,530,000	39.0
112,000	198,000	76.8	185,000	65.0
271,000	900,000	232.0	660,000	143.0
75,000	510,000	580.0	690,000	820.0
6,800,000	46,200,000	517.0	41,600,000	497.0
2,800,000	6,400,000	124.0	5,800,000	103.0
43,000			80,000	86.0
127,000			198,000	56.0
227,000			342,000	39.0
163,000			200,000	36.0
145,000			158,000	8.0
2,990,000			3,370,000	13.0
46,000			62,000	34.0
20,000			60,000	200.0
		Pasteurized milk		
3,800	168,000	384.0	202,000	432.0
200	162,000	80,000.0	192,000	90,000.0
6,800	23,400	244.0	23,400	244.0
18,700	49,000	162.0	32,200	73.0
30,000	52,000	73.0	76,000	153.0
51,000	63,000	25.5	66,000	29.5
100,000	112,000	11.0	107,000	7.0
85,000			132,000	67.0
62,000			291,000	370.0
7,700			114,000	1,530.0
980,000			1,190,000	22.0
1,330,000			1,400,000	5.0
66,000			317,000	380.0
220,000			265,000	21.0
300,000			302,000	0.6
47,000			289,000	510.0
36,000			163,000	353.0
24,000			330,000	1,260.0

TABLE—3 *Continued*

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER YEAST AGAR	INCREASE OVER EXTRACT AGAR
Pasteurized milk—*continued*				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
28,000			560,000	1,900.0
480,000			2,000,000	325.0
600,000			1,410,000	135.0
206,000			450,000	118.0
140,000			510,000	264.0
700			2,400	243.0
300			2,300	667.0
Certified milk				
4,100	7,600	85.0	6,800	66.0
4,900	5,700	16.3	5,900	20.0
36,000			69,000	92.0
29,100			37,900	32.0

A medium with skimmed-milk powder and washed agar without any other nutritive material gives counts much higher than the standard extract agar. As may be seen in table 4, the counts were much higher except in the case of the last sample. In many samples the counts on the milk-powder agar, with no other nutritive ingredients, checked with those on standard infusion agar, while with a few samples the count was decidedly lower. The colonies, however, on this medium are very small in most cases; therefore, it is necessary to supply some other nitrogenous material to stimulate growth.

Peptone and meat or yeast extract, whichever may be used, are added in order to increase the nutritive value of the medium. Milk powder supplies lactose to the medium in small amounts, which assists materially in stimulating and supporting bacterial growth, as has been shown by the work of Sherman (1916) and others. We do not feel, however, that the lactose is the only reason for the high counts obtained on media containing dissolved milk powder.

The increased count over extract agar and the large size of the colonies obtained on the media described in this paper are

due, we believe, to a combination of the following factors: the nitrogenous portions of the milk powder, the lactose, the buffer content, and the use of *washed* agar.

When 5 grams of skimmed-milk powder per liter of medium is used, figuring on an average analysis of 50 samples of milk powder, there is added about 0.25 per cent lactose and 0.17 per cent protein (mostly casein). From results of the analysis of skimmed-milk powder which were kindly supplied to us by Dr. J. T. Kiester, of the Bureau of Chemistry, United States Department of Agriculture, we have calculated the difference in percentage of lactose and protein if 5 grams of powder containing the highest and lowest percentages found among 20 samples had been used.

TABLE 4

The value of milk powder as a nutrient material

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER AND WASHED AGAR. NO OTHER INGREDIENTS	INCREASE OVER EXTRACT AGAR
		Raw milk		
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
690,000	860,000	29.0	770,000	11.5
480,000	950,000	100.0	840,000	75.0
59,000	173,000	193.0	164,000	178.0
54,000	220,000	300.0	162,000	200.0
1,100,000	1,780,000	62.0	1,670,000	50.0
112,000	198,000	76.8	176,000	57.0
271,000	900,000	232.0	810,000	199.0
6,800,000	46,200,000	517.0	36,600,000	395.0
2,860,000	6,400,000	124.0	4,800,000	68.0
		Pasteurized milk		
3,800	168,000	384.0	101,000	165.0
200	162,000	80,000.0	186,000	85,000.0
6,800	23,400	244.0	24,100	255.0
18,700	49,000	162.0	29,700	62.0
30,000	52,000	73.0	60,000	100.0
51,000	63,000	21.5	68,000	33.4
100,000	112,000	11.0	114,000	11.0
1,400	36,800	2,500.0	36,000	2,510.0
4,100	7,600	85.0	5,500	34.0
4,900	5,700	16.3	2,000	−59.0

The range in the percentage of lactose in the medium would be only 0.03 per cent and in protein also 0.03 per cent. These differences are so slight that they would exert no influence on the medium. It is realized that there may be other differences in skimmed-milk powder, small differences which may not be apparent from the ordinary chemical analysis, and which may be ascribed to the condition of the milk before it was dried, or perhaps to methods of drying.

We have obtained the best results when using a skimmed-milk powder made by the spray process, and suggest that if milk powder is used in a standard medium a spray-process powder be specified, and one prepared by some one company, perhaps even from some special milk.

THE ESTIMATED COST OF DIFFERENT AGAR MEDIA

We are not prepared to say just how important the cost of a medium is, but it will always be a factor and therefore must be considered.

The cost of different agar media has been calculated on a basis of the cost of ingredients shown in the list.

Cost of ingredients

Peptone	$4.80 for 500 grams
Meat extract	3.70 for 1 pound
Dry yeast	3.75 for 500 grams
$Na_2HPO_4 + 2H_2O$	1.50 per pound
Skimmed-milk powder	.50 per pound
Lean beef	.35 per pound
Agar (shred)	.90 per pound

Approximate cost of agar media

	per liter
Standard infusion agar about	$0.46½
Standard extract agar about	10
Milk-powder agar "A"	.10¾
Milk-powder agar "B"	.06¼
Milk-powder yeast agar	.11¼

In making calculations we have not figured the cost of washing agar. This should not add much to the cost. From these figures it may be seen that the milk-powder media cost about

the same as standard extract agar, with the exception of medium B, which costs only about 6 cents a liter. It seems evident that no objections can be raised to milk-powder media on the ground of cost.

SUMMARY

1. Formulae for three agar media are given, two of these media containing skimmed-milk powder with different amounts of peptone and meat extract. The third contains skimmed-milk powder and yeast extract, no peptone or meat extract being used. This may be a distinct advantage if the variation in composition of peptone and meat extract plays an important part in connection with the bacteria count.

2. Any of the skimmed-milk powder media described give counts very much higher than standard extract agar and the colonies are very much larger. The larger size of the colonies makes the counting process much more accurate. Counts obtained on the powder media with peptone and extract are on the average higher than those obtained on the old standard infusion. On milk-powder yeast agar they may be, in general, slightly lower.

3. With these milk-powder media it is possible to obtain not only a total count, but a count of colonies of strong and weak acid-producing, alkali-forming, inert and peptonizing bacteria. The plates therefore, give quantitative and qualitative results, at least so far as these groups of bacteria are concerned.

4. The importance of using washed agar in media for bacterial counts will be discussed further in another paper.

5. It is hoped that these media will be given a thorough trial, for it is felt that any one of the three possesses decided advantages over the present standard extract agar.

No claim is made that the skimmed-milk powder media can not be improved; we hope they can. As they stand at present, however, any one of the three has advantages enough over the extract agar to replace it as a standard medium for the determination of bacteria in milk, because: first, they show higher counts than the standard extract agar and therefore counts

more closely related to the actual number of bacteria in the milk; secondly, they give different "pictures" when different samples of milk are plated; and, thirdly, this is accomplished without any significant increase in expense. In fact, one of these media is much cheaper than extract agar. All the milk-powder media are very much cheaper than the old standard infusion agar.

6. The full value of these milk-powder agar media can be determined only by the results obtained from the analysis of a large number of samples of milk. While our experiments are extensive, we realize that they only indicate the probable value of the media.

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THE USE OF WASHED AGAR IN CULTURE MEDIA

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In connection with the preparation of milk-powder agar it was found that it was necessary to wash the agar in order that it might be used in the medium without causing a precipitate during sterilization. Qualitative tests indicated that the calcium and magnesium salts in the agar probably combined with the phosphate used in the preparation of the milk-powder solution and the phosphates were precipitated during sterilization. To overcome this difficulty, the agar was washed in distilled water in order to reduce its salt content.

Soil bacteriologists have employed washed agar for many years. It has been washed in order to remove as much as possible of the soluble organic matter which is believed to be detrimental to the growth of nitrifying bacteria. Sternberg in his "Textbook of Bacteriology" recommended that agar be soaked in cold water for twenty-four hours; this was advised in order to facilitate dissolving and filtering. It is the practice in some laboratories to wash agar, apparently more for the sake of a cleaner preparation than for any other reason.

Washed agar gave such satisfactory results in the milk-powder medium that its use in the regular standard peptone extract medium was tried. It is the purpose of this paper to present briefly the results of this work and to show the effect of washing on the calcium and magnesium content of agar.

The results in table 1 show in a decided manner the effect on the bacterial count of using washed agar. While there was not an increase in all samples, there was in many cases an increase beyond any experimental error. As a rule, the higher counts were found when pasteurized milk was examined.

TABLE 1

Bacteria counts on standard extract agar using shred and washed agar

STANDARD EXTRACT AGAR	STANDARD INGREDIENTS AND WASHED AGAR	APPROXIMATE INCREASE OVER STANDARD EXTRACT AGAR
Raw milk		
bacteria per cc.	*bacteria per cc.*	*per cent*
650,000	990,000	52.0
1,110,000	1,530,000	37.5
1,410,000	1,940,000	37.5
1,160,000	5,180,000	345.0
1,580,000	3,620,000	129.0
246,000	432,000	75.0
239,000	1,010,000	320.0
3,370,000	11,580,000	246.0
1,760,000	3,040,000	72.6
2,300,000	4,330,000	88.2
5,120,000	15,360,000	199.0
139,000	198,000	42.5
263,000	346,000	31.5
720,000	1,010,000	40.0
990,000	800,000	−23.0
130,000	224,000	72.0
154,000	259,000	68.2
141,000	144,000	2.1
620,000	790,000	27.0
196,000	232,000	18.4
184,000	230,000	25.0
167,000	189,000	13.2
9,700,000	16,800,000	73.0
30,300,000	50,400,000	66.0
15,700,000	3,200,000	−20.0
4,200,000	3,500,000	−8.0
339,000	264,000	−7.8
428,000	189,000	−44.0
6,500,000	6,100,000	−9.5
48,500,000	36,300,000	−7.5
287,000	228,000	−8.0
1,420,000	1,050,000	−7.0
830,000	740,000	−9.0
Pasteurized milk		
1,600	2,100	31.0
1,000	8,000	700.0
3,500	17,000	385.0
41,900	63,000	50.0

TABLE 1—*Continued*

STANDARD EXTRACT AGAR	STANDARD INGREDIENTS AND WASHED AGAR	INCREASE OVER STANDARD EXTRACT AGAR
Pasteurized milk—*continued*		
bacteria per cc.	*bacteria per cc.*	*per cent*
13,000	22,000	69.0
4,800	66,000	1275.0
45,000	76,000	69.0
5,700	68,000	1090.0
1,500	20,100	1240.0
52,000	84,000	61.5
1,400	3,100	121.0
580,000	870,000	50.0
44,000	94,000	135.0
12,500	18,600	48.0
13,200	19,700	48.0
62,000	88,000	42.0
13,100	15,700	19.0
6,400	37,000	480.0
10,000	14,900	49.0
9,600,000	11,520,000	19.0
34,800	71,000	104.0

The washed agar used in these experiments was prepared as follows: For 1 liter of double strength (3 per cent) agar, 30 grams of shred agar was placed in a flask with 2000 cc. of distilled water. This was allowed to stand for 24 hours at room temperature. At the end of this period as much water as possible was poured off, a piece of cheesecloth being placed over the top of the flask. Fresh distilled water was added to make up for the water poured off. The agar was allowed to soak another twenty-four hours, after which it was thrown on to a cotton-flannel cloth in a funnel and washed once with 1 liter of distilled water. The agar was allowed to drain and as much as possible of the remaining water was pressed out by squeezing the filter cloth with the hands. A container large enough to hold the agar was counterpoised on the laboratory scales, and the agar was placed in it. In the opposite pan was placed 30 grams for the weight of the agar and 1000 grams for the weight of the water in which it was to be dissolved. Then water enough was added to the agar to make up this weight, it was then dissolved and filtered.

After the agar is washed, it may be air dried, and then used the same as ordinary agar. It is only necessary to use 1 per cent of the dry material to obtain a medium having the same jelly strength as a 1.5 per cent shred agar medium.

Various methods were tried for washing shred agar, and the effect of these treatments on the calcium and magnesium content will be of interest. As shown in table 2, ordinary shred agar contained about 16 per cent of moisture. On an air-dry basis

TABLE 2

Analyses of various kinds of agar

KIND OF AGAR	MOISTURE	ON DRY BASIS			
		Ash	CaO	MgO	Protein
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Shred agar					
Sample 1	16.37	4.46	1.17	0.79	2.29
Sample 2	15.97	4.48	1.13	0.76	2.23
Commercially purified agar					
Lot No. 1	12.83	4.54	1.41	0.64	1.66
Lot No. 2	8.82	3.77	1.12	0.55	1.40
Washed agar					
24 hours (A)	14.21	3.37	0.74	0.53	
48 hours (B)	14.62	2.99	0.70	0.39	
72 hours (C)	15.45	2.74	0.61	0.34	
24 hour washed 30 grams to 5000 H_2O	14.32	2.74	0.61	0.43	
NaCl-acid treated agar	12.70	3.37	0.39	0.08	1.35

the per cent of ash was about 4.4. The calcium content was about 1.15 per cent, calculated as CaO, and the magnesium about 0.77 per cent, calculated as MgO.

Two lots of commercially purified agar were examined. These were purchased at different times, and probably do not represent one orginal lot of the material. The agar was said to contain a minimum amount of moisture, when packed, and to have been specially treated to reduce the extraneous matter, inorganic

salts, and acidity. As the results show, the moisture content of this agar was variable, but the content of calcium was no lower than that of shred agar, although the magnesium had been slightly reduced. The protein content, however, was about 50 per cent of that of shred agar.

The analysis of washed agar showed a decided decrease in the percentage of calcium and magnesium, and experiments were made to determine the time required to wash the agar, starting with 30 grams to 2000 cc. of water. Agar "A" was held twenty-four hours. Agar "B" was held forty-eight hours, but after the first twenty-four hours the 2 liters of water were replaced with fresh water. Agar "C" was held seventy-two hours, the water being removed and replaced with fresh distilled water at the end of each twenty-four hours. The agar was air dried by spreading it on filter papers after washing.

It will be seen from the table that the moisture content of the three lots of agar was very similar to that of shred agar. The most interesting effect of washing was the reduction of the calcium and magnesium salts. The forty-eight-hour period of washing, which is the one we have used most extensively, reduced the CaO from about 1.1 per cent to 0.7 per cent, and the MgO from about 0.78 per cent to 0.39 per cent. The calcium and magnesium content was reduced sufficiently by this treatment to give satisfactory results in the milk-powder agar medium; that is, enough was removed so that the phosphates did not precipitate upon sterilization. Agar washed twenty-four hours in the proportion of 30 grams to 2000 cc. of water was not entirely satisfactory and, as the results show, there was but little further reduction in the calcium and magnesium by washing for seventy-two hours.

It was found that by increasing the amount of distilled water, good results could be obtained in twenty-four hours. When 30 grams of shred agar was held in 5000 cc. of distilled water for twenty-four hours the calcium and magnesium content was found to be 0.61 per cent and 0.43 per cent respectively, when calculated on a moisture-free basis.

The results obtained by the addition of NaCl and HCl to the wash water are interesting. Various amounts of salt and acid were used, their use having been suggested by Dr. Zoller of these laboratories. The best results were obtained as follows: 20 grams of shred agar was added to 1000 cc. of distilled water containing 10 grams of NaCl and 5 cc. of N/10 HCl. This was allowed to stand for six hours at room temperature, then the salt and acid solution was poured off and replaced by 1800 cc. of fresh distilled water. This was allowed to stand 18 hours, which made the washing period twenty-four hours. The agar was then poured on a cotton-flannel cloth in a funnel and washed with 500 cc. of distilled water, then allowed to drain and as much water pressed out by hand as possible. The agar was finally air dried.

The agar treated in this manner showed a lower calcium and magnesium content than any of the others. Reference to the table shows that it contained 0.39 per cent of CaO, and 0.08 per cent MgO, while the protein content was reduced from about 2.2 per cent to 1.35 per cent.

Media made with this agar did not, however, give quite such satisfactory counts as that made with washed agar, but its use has not been tried out very extensively. This method of washing, however, has possibilities of considerable value. The principal point of interest at present in connection with the use of NaCl and HCl is the fact that they assist in the removal of the calcium and magnesium salts.

We were very much interested in the results obtained with washed agar. It was valuable to us because it made possible a milk-powder agar medium which would stand sterilization without precipitation of the phosphates, but the fact that higher bacterial counts were often obtained when it replaced ordinary shred agar in the standard extract medium led us to wonder just what might be the explanation.

In connection with the milk-powder medium it had been noticed that the presence of too much phosphate often lowered the bacterial counts. Even the increase from 0.1 per cent to 0.2 per cent seemed to have a marked effect. Besides this, in

certain milk-powder media the milk powder was dissolved in such a way as to leave the calcium phosphate in the medium. With such media low counts were noticed.

Having these facts in mind and knowing that at least one of the effects of washing agar was a partial removal of the calcium and magnesium salts it occurred to us that this lowering of the percentage of these salts might be one of the reasons for the increased counts when washed agar was used in the standard medium. To confirm this opinion, counts were made on three media, all of which contained 0.5 per cent peptone and 0.3 per cent extract, and had the same reaction but made up with different kinds of agar. One contained regular shred agar, one washed agar, and the other washed agar with sufficient $CaSO_4$ and $MgSO_4$ added to make up for the calcium and magnesium (calculated as CaO and MgO) removed by washing.

TABLE 3

Effect of addition of calcium and magnesium salts to a washed-agar medium

STANDARD EXTRACT AGAR, A. P. H. A. 1916	STANDARD EXTRACT WASHED-AGAR AND Ca AND Mg SALTS	STANDARD EXTRACT WASHED AGAR
bacteria per cc.	*bacteria per cc.*	*bacteria per cc.*
6,200	5,800	8,800
13,100	8,100	15,700
6,400	6,700	37,000
10,000	12,000	14,900
9,600,000	9,550,000	11,500,000
34,800	30,600	71,000

A few samples of pasteurized milk were plated on these three media. The results in table 3 indicate that the addition of these salts to the washed-agar medium reduced the counts so that they agreed with those on the regular agar medium.

We do not intend to convey the idea that this is an explanation of the higher counts. Perhaps similar results may never be obtained again, but at least they indicate that these salts may play an important part in culture media.

SUMMARY AND CONCLUSIONS

1. The standard extract medium with washed agar showed in many cases, when market milk was examined, a much higher count than the same medium with regular shred agar.

2. Washing agar reduced its content of calcium and magnesium salts.

3. A few experiments indicated that the removal of these salts was a factor in the cause of the higher counts. This point, however, is merely suggested by the results and not definitely proved.

4. Since certain samples of milk show a higher count when plated on a washed-agar standard extract medium than on the regular standard extract medium, it seems evident that washing removes something detrimental to the growth of certain species of bacteria. This naturally suggests that a further study is needed of the value of washed agar in lines of bacteriological work where it has not been used.

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