A STUDY OF CERTAIN SALMONELLA AND SIMILAR BACTERIA FROM THE INTESTINE OF THE CHICKEN

by

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INTRODUCTION

The *Salmonella* bacteria are a group of Gram-negative bacilli, morphologically indistinguishable from members of the typhoid and of the colon groups. Their cultural behavior and pathogenic properties indicate that they are intermediate between the colon and typhoid groups. The genus *Salmonella* as defined by Bergey (1934) includes bacteria whose characteristics conform to those of the tribe *Bacterieae*, which ferment glucose with production of acid or acid and gas, which fail to ferment lactose, and which do not liquefy gelatin.

Because of the fact that some species of *Salmonella* are capable of causing a febrile disease in man simulating typhoid fever, the group has been commonly referred to as the "paratyphoid" group. The species which cause this condition are *Salmonella paratyphi*, *Salmonella schottmuelleri*, and rarely *Salmonella hirschfeldii*. A more common pathological condition caused by species of *Salmonella* is "food poisoning", a disease manifested as a gastro-enteritis following the consumption of food contaminated with bacilli of the *Salmonella* group. The species most commonly isolated

from food poisoning outbreaks are Salmonella sertrycke, Salmonella enteritidis, and closely related species.

It is the purpose of this investigation to study the bacteria from the chicken intestine which belong to the genus Salmonella, or which resemble the members of this genus.

Several species of Salmonella have been found in the intestines of chickens. Some of these species are known to be pathogenic for fowls, others have been found to be associated with cases in which there was intestinal parasitism. Of particular importance is the occurrence of S. aertrycke, which is known to be pathogenic for birds and which also has been incriminated as the etiological factor in numerous cases of food poisoning in man. The presence of S. aertrycke in the chicken intestine constitutes a potential source of infection both for birds and for man.

Organisms which superficially resemble the Salmonella types sometimes occur in the chicken intestine. The presence of these bacteria is important because of the fact that on ordinary culture media they cannot easily be differentiated from the true Salmonella. In order to distinguish these organisms from the true Salmonella, it is necessary to carry out an examination which requires the expenditure of both time and energy.

The primary objectives of this study were:

1. To identify the types of Salmonella and similar bacteria from the chicken intestine.
2. To study the distribution of each type.
3. To attempt to enrich the Salmonella bacteria by use of selective media in order to facilitate isolation from mixtures dominated by the common intestinal types.
4. To attempt to correlate bacteriological findings with pathological conditions as indicated by the diagnoses in the various cases.

Following the preliminary study, a number of representative cultures of each major group of organisms was investigated more completely by cultural, biochemical, and serological methods. The purpose of this study was to determine the taxonomic relationship between each of these groups and the recognized species of intestinal bacteria, and to discover characteristics by which rapid identification could be made of members of each group.

The source of material for this study consisted of intestinal contents from diseased chickens upon which post-mortem examinations were made in the poultry disease laboratory of the Department of Bacteriology at Kansas State College.

OCCURRENCE OF THE SALMONELLA AND SIMILAR BACTERIA

Review of Literature

A review of the major studies of the intestinal flora of the normal fowl has been given by Emmel (1930).

In general, it has been found that Escherichia coli and Escherichia communior are the predominating bacteria of the intestine of the normal fowl. In addition to these, large numbers of spore-forming organisms have been found, as well as Gram-positive cocci, molds, lactic acid bacteria, and Aerobacter aerogenes. Emmel (1930) lists two cases in which Salmonella icteroides was isolated.

The members of the genus Salmonella which are pathogenic for birds are S. aertrycke, S. gallinarum, S. pullorum, and S. anatum. Other members of this genus have not often been found in birds. White (1929) stated that only one instance of Salmonella saprophytism in birds has been recorded, a case in which it was claimed that a "paratyphus B bacillus" was isolated from the intestine of a goose.

Emmel (1936) gave the results of analysis of the intestinal contents of chicks suffering from enteritis associated with various types of intestinal parasitism. Examining 232 cases, he isolated S. aertrycke from 77, S. enter-

itidis from 20, S. schottmuelleri from five, S. typhimurium from eight, S. suipestifer from three, S. paratyphi from two, S. anatum from three, and S. pullorum from four. He listed seven cultures as unidentified Salmonella and four as unidentified Eberthella. From 93 cases no isolations were made.

Although many cases have been recorded in which "slow lactose-fermenting" bacteria were isolated from the human intestinal tract, we have found no record of isolation of these organisms from the intestine of the chicken. Lewis and Hitchner (1936) report the isolation of slow lactose-fermenting bacteria which were pathogenic for chicks from the viscera of these birds.

Experimental Methods and Results

Methods of Examination. The samples from which our cultures were isolated were short portions of the ceca of the birds examined. These portions were removed soon after the birds were killed, and were kept in sterile Petri dishes until examined.

With a sterile wire loop, a small amount of intestinal material was streaked over the surface of an eosin-methylene blue agar plate. After 24 hours incubation at 37°C. any small colorless colonies resembling the colonies of members

of the paratyphoid group were inoculated into lactose fermentation tubes. If an organism failed to produce acid or acid and gas in lactose broth in 24 hours, a loopful of the broth was streaked on a second eosin-methylene blue agar plate. After 24 hours incubation, a well isolated colony was fished onto an agar slant for further study. Gram stains were made of the slant cultures and only those which were Gram-negative were saved for further study.

After 25 or 30 cultures had been isolated, their fermentation reactions were studied by inoculation of each culture into fermentation tubes containing one per cent of the various carbohydrates(1). In most cases lactose, glucose, xylose, maltose, and sucrose were used. Ability to produce H_2S in lead acetate medium was also tested.

On the basis of their ability to ferment the common carbohydrates, the cultures were tentatively classified as members of the genus Salmonella. Those cultures which produced acid and gas from glucose, xylose, and maltose and failed to attack lactose and sucrose were called S. aertrycke. Those cultures which fermented glucose and maltose but failed to attack lactose, sucrose, or xylose were tentative-

(1) At the beginning of the investigation fermentation tests were observed for 48 hours only, after which they were discarded. Later they were examined after 14 days.

ly classified as S. paratyphi. All of the cultures designated as S. paratyphi failed to produce H_2S; some of those classified as S. aertrycke produced H_2S while others did not.

A few representative cultures of both groups were saved for complete study; the others were discarded after the tentative classification had been made.

Several cultures were isolated which failed to produce gas from any of the common carbohydrates. These organisms were not studied further and were included in a group which we called "non-gas producers".

The investigation had proceeded for some time before we began to realize the significance of latent fermentation of lactose. After this, all fermentation tests were observed for 14 days before they were discarded. In addition, all cultures which had been saved for further study as described above were reinoculated into lactose fermentation tubes and observed for 14 days.

On the first 330 birds, examinations were made of material from the duodenum and from the middle intestine as well as from the cecum. Study was also made by use of Endo-agar plates streaked in parallel with the eosin-methylene blue plates from the first 95 birds examined.

In most instances these organisms were present in small numbers and a number of enrichment media were used in order

to determine their effectiveness in causing an increase in the number of isolations of paratyphoid bacteria. Most of these media consisted of a base medium to which was added brilliant green and bile. Numerous modifications were made which consisted chiefly in changes in composition of the base medium, changes in concentration of brilliant green, changes in concentration of bile, and changes in the pH of the medium. After the optimum concentration of ingredients was found, the medium of that composition was used unchanged in the routine examination of 205 birds. This composition of this medium and the method of its use will be described on page 19.

For routine analyses, small loopfuls of material from the cecum were inoculated into tubes of the liquid enrichment medium and streaked over the surface of the solid selective medium. After 24 hours incubation at 37°C., colorless colonies were fished from the plates and examined as described above. At the same time loopfuls of the liquid from the enrichment tubes were streaked over the surface of plates of a differential medium. These plates were examined in the same manner after 24 hours incubation.

Results. When the lactose fermentation tests were observed for a period of 14 days, it was found that the entire group of Salmonella-like bacteria was divided into two

distinct groups, one which was unable to ferment lactose and the other which caused latent acidification of the lactose fermentation tube.

Those cultures which failed to ferment lactose in 14 days could be subdivided further on the basis of their ability to produce gas from glucose. Those cultures which produced acid but no gas from the common carbohydrates we called non-gas producers, those which produced both acid and gas we classified as Salmonella.

The cultures which exhibited slow fermentation of lactose could be subdivided into two groups on the basis of their ability to produce secondary colonies on agar plates containing one per cent lactose. We classified those cultures which produced secondary colonies as Escherichia coli mutabile, following the criteria of Neisser (1906) and Massini (1907) for such classification. Those cultures which cause latent fermentation of lactose, but do not produce secondary colonies we called slow lactose-fermenters. It must be borne in mind that the group labeled slow lactose-fermenter does not include E. coli mutabile.

When these subgroups were studied, it was found that the Salmonella, E. coli mutabile, and slow lactose-fermenters could be distinguished from each other by their ability to produce H_2S and their ability to ferment xylose. All of our

cultures of Salmonella produced H_2S and fermented xylose; all cultures of E. coli mutabile failed to produce H_2S and fermented xylose; and all cultures of slow lactose-fermenters failed to produce H_2S and failed to ferment xylose. Using these characteristics as a basis, an empirical classification was made of all those cultures which were discarded without having determined their ability to ferment lactose in 14 days.

The biochemical reactions and classification of all cultures studied is given in Table I. The culture numbers are the serial numbers of the birds examined.

The comparative distribution of each of the major groups is summarized in Table II.

The occurrence of each group in connection with the various pathological conditions existing in the birds is shown in Table III.

In Table IV we have compared the distribution of each group in different levels of the intestine.

Table I. Cultural characteristics and classification of all cultures studied.

Culture Number	Lactose		Secondary colonies	Glucose	Xylose	Maltose	Sucrose	H_2S	Classification
	24 hrs.	14 das.							
3	-			AG	AG			-	E. coli mutabile
5	-			AG	AG			+	Salmonella
8	-	AG	+	AG	AG			-	E. coli mutabile
10	-			A	-			+	Non-gas producer
11	-			A	-			+	Non-gas producer
13	-			A	A			+	Non-gas producer
27	-			A	-			-	Non-gas producer
31	-	A	-	AG	-			-	Slow lactose-fermenter
32	-			AG	-			-	Slow lactose-fermenter
34	-			AG	-			-	Slow lactose-fermenter
39	-			AG	AG			+	Salmonella
40	-			AG	AG			+	Salmonella
41	-			AG	-			+	Slow lactose-fermenter
51	-	-	-	AG	AG			+	Salmonella
60	-			AG	AG			-	E. coli mutabile
64	-			A	±			-	Non-gas producer
65	-	-	-	A	A			+	Non-gas producer
66	-	-	-	A	±			+	Non-gas producer
91	-			A	A			-	Non-gas producer
91	-			AG	AG			-	E. coli mutabile
102	-			AG	AG			-	E. coli mutabile
106	-			AG	AG			+	Salmonella
108	-			AG	AG	AG	-	+	Salmonella
131	-			AG	AG	AG	-	+	Salmonella
151	-			A	-	-	-	-	Non-gas producer
152	-			A	-	-	-	-	Non-gas producer
154	-			A	-	-	-	-	Non-gas producer
167	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
168	-			AG	-	AG	-	-	Slow lactose-fermenter
174	-			AG	AG	AG	-	+	Salmonella
178	-			A	-	-	-	-	Non-gas producer
187	-			AG	AG	AG	-	+	Salmonella
188	-			AG	AG	AG	-	+	Salmonella
200	-			A	-	-	-	-	Non-gas producer
204	-			AG	-	AG	-	-	Slow lactose-fermenter
210	-			AG	AG	AG	-	+	Salmonella
211	-			AG	AG	AG	-	+	Salmonella
212	-			AG	AG	AG	-	+	Salmonella
216	-	AG	-	AG	AG	AG	-	+	Salmonella
221	-			AG	AG	AG	-	-	E. coli mutabile
222	-			AG	AG	AG	-	+	Salmonella
225	-			AG	AG	AG	-	+	Salmonella
228	-			AG	AG	AG	-	+	Salmonella
232	-			AG	AG	AG	-	+	Salmonella
234	-			AG	AG	AG	-	+	Salmonella
236	-			AG	-	AG	-	-	Slow lactose-fermenter
237	-			AG	-	AG	-	-	Slow lactose-fermenter
249	-	A	-	AG	-	AG			Slow lactose-fermenter
370	-			AG	AG	AG	-	+	Salmonella
371	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
381	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
384	-			A	-	AG	-	-	Slow lactose-fermenter
387	-			AG	AG	AG	-	+	Salmonella
388	-			AG	-	AG	-	-	Slow lactose-fermenter
391	-			AG	AG	AG	-	+	Salmonella
392	-			A	-	A	-	-	Non-gas producer
393	-			AG	A	AG	-	+	Salmonella
395	-			AG	AG	AG	-	+	Salmonella
396	-			AG	AG	AG	-	+	Salmonella
397	-	A	-	A	-	AG	-	-	Slow lactose-fermenter
398	-			AG	A	AG	-	+	Salmonella
401	-			AG	AG	AG	-	+	Salmonella
404	-			A	A	-	-	-	Non-gas producer
409	-			AG	AG	AG	-	+	Salmonella
416	-			AG	AG	AG	-	+	Salmonella
417	-			A	AG	AG	-	+	Salmonella
419	-	A	-	A	-	AG	-	-	Slow lactose-fermenter
424	-			AG	-	AG	-	-	Slow lactose-fermenter
427	-			A	AG	AG	-	+	Salmonella
428	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
429	-			AG	-	AG	-	-	Slow lactose-fermenter
432	-			AG	-	AG	-	-	Slow lactose-fermenter
433	-			AG	AG	AG	-	+	Salmonella
434	-			AG	AG	AG	-	-	E. coli mutabile
435	-			AG	-	AG	-	-	Slow lactose-fermenter
438	-			AG	AG	AG	-	+	Salmonella
439	-			AG	AG	AG	-	-	E. coli mutabile
443	-			AG	AG	AG	AG	-	E. coli mutabile
448	-			AG	AG	AG	-	+	Salmonella
449	-			AG	AG	AG	-	+	Salmonella
450	-			AG	-	AG	-	+	Slow lactose-fermenter
457	-			A	-	-	-	-	Non-gas producer

Culture Number	Lactose		Secondary colonies	Glucose	Xylose	Maltose	Sucrose	H_2S	Classification
	24 hrs.	14 das.							
458	-			A	-	-	-	-	Non-gas producer
459	-			A	-	-	-	-	Non-gas producer
460	-			A	-	-	-	-	Non-gas producer
464	-			AG	AG	AG		-	E. coli mutabile
465	-			AG	-	AG		-	Slow lactose-fermenter
466	-			AG	-	AG		-	Slow lactose-fermenter
471	-			AG	AG	AG		-	E. coli mutabile
477	-			AG	AG	AG		-	E. coli mutabile
484	-			AG	-	AG		-	Slow lactose-fermenter
485	-			A	-	-	-	-	Non-gas producer
493	-			AG	-	AG		-	Slow lactose-fermenter
494	-			A	-	AG		-	Slow lactose-fermenter
495	-			AG	-	AG		-	Slow lactose-fermenter
498	-			A	-	A		-	Non-gas producer
503	-			A	-	A	-	-	Non-gas producer
507	-			AG	-	AG	-	-	Slow lactose-fermenter
512	-			AG	AG	A	-	+	Salmonella
513	-			AG	-	AG	-	-	Slow lactose-fermenter
523	-			AG	-	AG	-	-	Slow lactose-fermenter
526	-			AG	-	AG	-	-	Slow lactose-fermenter
527	-			AG	-	AG	-	-	Slow lactose-fermenter
531	-			AG	AG	AG	-	+	Salmonella
537	-			AG	AG	AG	-	+	Salmonella
538	-	AG	-	AG	-	AG	-	-	Slow lactose-fermenter
541	-	AG	-	AG	-	AG	-	-	Slow lactose-fermenter
549	-			AG	-	AG	-	-	Slow lactose-fermenter
550	-			AG	-	AG	-	-	Slow lactose-fermenter
551	-			AG	-	AG	-	-	Slow lactose-fermenter
551A	-			AG	AG	A	-	-	E. coli mutabile
552	-			A	-	AG	-	-	Slow lactose-fermenter
553	-			AG	AG	AG	-	+	Salmonella
555	-			AG	-	AG	-	-	Slow lactose-fermenter
556	-			AG	-	AG	-	-	Slow lactose-fermenter
570	-			AG	AG	AG	-	-	E. coli mutabile
571	-			AG	-	AG	-	-	Slow lactose-fermenter
573	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
574	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
575	-	AG	-	AG	-	AG	-	-	Slow lactose-fermenter
579	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
580	-	A	+	AG	AG	AG	-	-	E. coli mutabile
587	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
588	-	A	+	AG	AG	AG	-	-	E. coli mutabile
590	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
593	-	A	+	AG	AG	AG	AG	-	E. coli mutabile
594	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
595	-	-	-	AG	AG	AG	-	+	Salmonella
597	-	-	-	AG	AG	AG	-	+	Salmonella
601	-	A	+	AG	AG	AG	-	-	E. coli mutabile
603	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
606	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
609	-	A	+	AG	AG	AG	-	-	E. coli mutabile
613	-	-	-	AG	AG	AG	-	+	Salmonella
615	-	-	-	AG	AG	AG	-	+	Salmonella
617	-	-	-	AG	AG	AG	-	+	Salmonella
618	-	-	-	AG	AG	AG	-	+	Salmonella
621	-	-	-	AG	AG	AG	-	+	Salmonella
625	-	-	-	AG	AG	AG	-	+	Salmonella
630	-	A	+	AG	AG	AG	AG	-	E. coli mutabile
633	-	-	-	AG	AG	AG	-	+	Salmonella
635	-	-	-	AG	AG	AG	-	+	Salmonella
638	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
639	-	-	-	AG	AG	AG	-	+	Salmonella
642	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
680	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
691	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
692	-	A	+	AG	AG	AG	-	-	E. coli mutabile
701	-	-	-	AG	AG	AG	-	+	Salmonella
702	-	-	-	AG	AG	AG	-	+	Salmonella
704	-	-	-	AG	AG	AG	-	+	Salmonella
710	-	-	-	AG	AG	AG	-	+	Salmonella
717	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
718	-	-	-	AG	AG	AG	-	+	Salmonella
723	-	-	-	AG	AG	AG	-	+	Salmonella
724	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
725	-	A	+	AG	AG	AG	-	-	E. coli mutabile
726	-	-	-	AG	AG	AG	-	+	Salmonella
727	-	-	-	AG	AG	AG	-	+	Salmonella
731	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
732	-	A	-	AG	-	AG	-	-	Slow lactose-fermenter
733	-	AG	+	AG	AG	AG	-	-	E. coli mutabile
736	-	AG	-	AG	-	AG	-	-	Slow lactose-fermenter
744	-	AG	-	AG	-	AG	-	-	Slow lactose-fermenter
748	-	-	-	AG	AG	AG	-	+	Salmonella
755	-	-	-	AG	AG	AG	-	+	Salmonella
760	-	-	-	AG	AG	AG	-	+	Salmonella
761	-	-	-	AG	AG	AG	-	+	Salmonella
763	-	-	-	AG	AG	AG	-	+	Salmonella

A = production of acid
AG = production of acid and gas
\+ = positive reaction
\- = negative reaction
blanks = determination not made

Table II. Number of isolations of each type of organism.

	Number	Per Cent
Total number of birds examined	658	100
Total number of isolations made	169	25.7
Cultures of *Salmonella* isolated	63	9.6
Cultures of *E. coli mutabile* isolated	36	5.5
Cultures of "*slow lactose-fermenters*" isolated	48	7.3
Cultures of "*non-gas producers*" isolated	22	3.3

Table III. A list of the organisms isolated classified according to diagnosis.

Cases	Total cases	Salmonella		E. coli mutabile		Slow Lactose Fermenters		Non-gas Producers		No Isolations	
		Total	Per Cent	Total	Per Cent	Total	Per Cent	Total	Per Cent	Total	Per Cent
Neurolymphomatosis	69	3	4.3	2	2.9	23	33.4	0	0	41	59.4
Taeniasis	129	15	11.6	13	10.1	18	13.9	2	1.5	81	62.9
Ascariasis	76	9	11.8	6	7.9	9	11.8	1	1.3	51	67.2
Coccidiosis	47	7	14.9	1	2.1	9	19.1	1	2.1	29	61.8
Heterakiasis	18	5	27.8	0	0	1	5.6	0	0	12	66.6
Pullorum	53	12	22.7	2	3.8	0	0	0	0	39	73.5
Fowl cholera	60	3	5.0	9	15.0	5	8.3	3	5.0	40	66.7
A Avitaminosis	27	2	7.4	2	7.4	2	7.4	0	0	21	77.8
Fowl paralysis	25	3	12.0	4	16.0	2	8.0	0	0	16	64.0
Tracheitis	22	4	18.2	2	9.1	0	0	0	0	16	72.7
Coryza	26	5	19.2	0	0	4	15.4	0	0	17	65.4
Leukemia	32	0	0	3	9.4	12	37.6	0	0	17	53.0
Ruptured ova	25	0	0	1	4.0	5	20.0	1	4.0	18	72.0
D Avitaminosis	21	3	14.3	0	0	0	0	0	0	18	85.7
Capillariasis	5	0	0	0	0	0	0	0	0	5	100.0
Colibacillosis	11	1	9.1	0	0	0	0	0	0	10	90.9
Leukosis	30	1	3.3	1	3.3	2	6.7	0	0	26	86.7
Fowl typhoid	8	0	0	0	0	0	0	3	37.5	5	62.5
Tuberculosis	4	1	25.0	0	0	0	0	0	0	3	75.0
Peritonitis	5	0	0	1	20.0	0	0	0	0	4	80.0

Table IV. Comparative number of isolations of each group of organisms from different locations in the intestine and their percentage of the 284 birds examined.

Cultures Isolated	Duodenum		Middle Intestine		Cecum	
	Isolations	Per Cent	Isolations	Per Cent	Isolations	Per Cent
Salmonella	4	1.4	3	1.1	10	3.5
E. coli mutabile	1	0.4	3	1.1	3	1.1
Slow lactose-fermenters	9	3.2	13	4.6	12	4.2
Non-gas producers	6	2.1	7	2.5	8	2.8
Total	20	7.1	26	9.3	33	11.6

The enrichment medium which appeared to be most satisfactory was one having a base of the following composition.

Diammonium acid phosphate	0.5 gr.
Ferric citrate	1.0 gr.
Bile (desiccated)	5.0 gr.
Peptone	10.0 gr.
Dextrose	1.0 gr.
Water	1000.0 gr.
pH	6.5

The base medium was tubed in ten cubic centimeter amounts and sterilized in the autoclave at 20 pounds pressure for 30 minutes.

Just before use, enough of an aqueous solution of brilliant green was added to each tube to make a concentration of 1-35,000 of the dye. This medium will be called "brilliant green" enrichment medium.

An "eosin-brilliant green" enrichment medium was made by adding to the base medium the following ingredients: eosin, 1-2,500; brilliant green, 1-35,000; and lactose, 1-100. An "eosin-brilliant green" agar contained the same ingredients with the addition of two per cent agar.

A comparison of the number of isolations made with and without preliminary enrichment is given in Table V.

Table V. The number of isolations made with and without preliminary enrichment.

	Not Enriched				Enriched			
	Eosin-Methylene Blue Agar		Eosin-Brilliant Green Agar		Brilliant Green Medium		Eosin-Brilliant Green Medium	
	Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Per Cent
Number of birds examined	205	100	174	100	195	100	163	100
Total isolations	21	10.2	17	9.8	23	11.8	17	10.4
Salmonella	4	1.9	5	2.9	13	6.7	7	4.3
E. coli mutabile	10	4.9	4	2.3	7	3.6	2	1.2
Slow lactose-fermenters	7	3.4	8	4.6	3	1.5	8	4.9

Recapitulation

1. There occurs in the intestines of diseased chickens a large number of bacteria which resemble the members of the paratyphoid group.

2. These bacteria may be subdivided into four main subgroups: true Salmonella, E. coli mutabile, slow lactose-fermenters, and non-gas producers.

   a. The true Salmonella fail to ferment lactose in 14 days and ferment glucose with production of acid and gas in 24 hours.

   b. E. coli mutabile cause delayed acidification of lactose fermentation tubes. On agar containing one per cent lactose, secondary colonies arise within the initial colonies.

   c. The name slow-lactose-fermenters is used by us to designate those organisms which cause latent fermentation of lactose but which do not form secondary colonies on agar containing lactose.

   d. Non-gas producers is the name we use for the group of organisms which fail to ferment lactose in 14 days and cause fermentation of glucose and other common carbohydrates with the production of acid but no gas.

3. Bacteria of these groups occur in large numbers in cases associated with intestinal parasitism although no one group predominated in these instances.

4. Slow lactose-fermenters occurred in a large number of cases of neurolymphomatosis and leukemia. This is of significance because of the fact that species of Salmonella have been suggested as the etiological factor in these pathological conditions. Since this group produces a delayed fermentation in lactose, it is quite possible that it may lead to considerable confusion in studies of this condition.

5. The different types of bacteria occurred in approximately the same proportions in different levels of the intestine, although all groups occurred in larger numbers in the lower portions of the intestine.

6. Preliminary enrichment increased the number of isolations of Salmonella but did not appreciably change the number of isolations of members of the other groups.

A STUDY OF THE SALMONELLA BACTERIA

Introduction and Review of Literature

The group of paratyphoid bacteria was studied further in order to determine whether or not it conformed to the characteristics of the genus Salmonella in all respects, and

to discover to which species of Salmonella it belongs.

We will not attempt to give an exhaustive review of the literature available on this genus. A comparatively recent and complete summary of the information available concerning this group is that given by White (1929). A more recent review is that of the "Salmonella Subcommittee of the International Society for Microbiology" (1934). This publication is more limited in scope, being concerned primarily with nomenclature. The antigenic complexes of the various Salmonella species are represented in a table which was compiled from the results of the studies made by Kauffman (1930) and White (1927), who studied the antigenic structure of these species independently. Kauffmann's system of designating the different factors was used.

Since we desire to refer to the Kauffmann-White scheme later, we have reproduced it in Table VI.

The O-antigen factors, which are labeled with Roman numerals, are the somatic antigens present at the surface of the cell body. These are entirely distinct from those of the flagella which are spoken of as H-antigens. Many species of Salmonella possess some of the same O-antigen as other species, making possible a division of the genus into five groups on this basis.

Table VI. Antigenic complexes of species of *Salmonella* according to the Kauffmann-White scheme.

Group	Species	O-antigen	H-Antigen: specific	H-Antigen: Non-specific
A	*S. paratyphi* A	I II	a	-
	S. seftenberg	I III	gs	-
	S. seftenberg var. *newcastle*	I III	gs	-
B	*S. paratyphi* B	IV V	b	1,2
	S. typhi murium	IV V	i	1,2,3
	S. typhi murium var. *binns*	IV V	-	1,2,3
	S. stanley	IV V	d	1,2
	S. heidelberg	IV V	r	1,2,3
	S. reading	IV	eh	1,4,5
	S. derby	IV	fg	-
	S. abortus equi	IV	enx	-
	S. abortus ovus	IV	c	1,4,6
	S. brandenburg	IV	enlv	-
C	*S. paratyphi* C	VI VII	c	1,4,5
	S. cholera suis	VI VII	c	1,3,4,5
	S. cholera suis var. *kunzendorf*	VI VII	-	1,3,4,5
	S. typhi suis	VI VII	c	1,3,4,5
	S. typhi suis var. *voldagsen*	VI VII	-	1,3,4,5
	S. thompson	VI VII	k	1,3,4,5
	S. thompson var. *berlin*	VI VII	-	1,3,4,5
	S. virchow	VI VII	r	1,2,3
	S. oranienburg	VI VII	mt	-
	S. potsdam	VI VII	enlv	-
	S. barielly	VI VII	y	1,3,4,5
	S. newport	VI VIII	eh	1,2,3
	S. newport var. *puerto rico*	VI VIII	-	1,2,3
	S. newport var. *kottbus*	VI VIII	eh	1,3,4,5
	S. bovis morbificans	VI VIII	r	1,3,4,5
	S. muenchen	VI VIII	d	1,2

Table VI -- continued

Group	Species	O-antigen	H-Antigen: specific	H-Antigen: Non-specific
D	S. typhi	IX	d	-
	S. enteritidis	IX	gom	-
	S. enteritidis var. danysz	IX	gom	-
	S. enteritidis var. dublin	IX	gp	-
	S. enteritidis var. rostock	IX	gpu	-
	S. enteritidis var. moscow	IX	goq	-
	S. sendai	IX	a	1,4,5
	S. dar-es-salaam	IX	enlw	-
	S. eastbourne	IX	eh	1,3,4,5
	S. panama	IX	lv	1,3,4,5
	S. gallinarum	IX	-	-
	S. pullorum	IX	-	-
E	S. london	X III	lv	1,4,6
	S. anatum	X III	eh	1,4,6
	S. anatum var. muenster	X III	eh	1,4,5

Some species of the group exhibit H specific - H non-specific variation. In the H specific phase, the organism possesses a flagellar antigen which is specific only for certain species of the genus. In the H non-specific phase, the flagellar antigen is not specific for any certain species but reacts with antisera of all diphasic species and with "group race" antisera.

Experimental Methods and Results

In order to determine the antigenic complex of any culture it is necessary to use pure O, H specific, and H non-specific antigens and antisera, since the antigens produced from whole cultures contain an inextricable combination of antigenic factors, making it impossible to obtain distinct agglutination reactions.

Stock Cultures. In order to have standard Salmonella with which to compare our cultures, we collected several recognized species of Salmonella from different sources. A list of our stock cultures and their source is given in Table VII.

Cultural Characteristics. Upon ordinary laboratory culture media the cultures which we classified as Salmonella are indistinguishable from known members of the typhoid-paratyphoid group.

Table VII. Source of stock *Salmonella* cultures.

Culture:	Species	Source
A 1	*S. schottmuelleri* (Rowland)	K.S.C.
A 2	*E. typhi* (Mitchell)	K.S.C.
A 3	*S. paratyphi* (Kessel)	K.S.C.
A 6	*S. schottmuelleri*	K.S.C.
A 7	*S. schottmuelleri*	K.S.C.
A 8	*S. paratyphi*	K.S.C.
A10	*S. aertrycke*	A.T.C.C. No. 854
A11	*S. aertrycke*	A.T.C.C. No. 856
A12	*S. aertrycke* (Newport)	U.S.D.A., B.A.I.
A13	*S. aertrycke* (Mutton)	U.S.D.A., B.A.I.
A14	*S. aertrycke*	Univ. of Ill.
A15	*S. anatum*	Univ. of Ill.
A16	*S. aertrycke*	Univ. of Wis.
A17	*S. aertrycke* (Reading)	Univ. of Wis.
A18	*S. aertrycke* (Tidy)	Univ. of Wis.
A19	*S. aertrycke* (Stanley)	Univ. of Wis.
A20	*S. schottmuelleri*	Univ. of Wis.
A21	*S. anatum* (Aertrycke)	Yale Univ.
A22	*S. aertrycke*	Yale Univ.
A23	*S. aertrycke*	Yale Univ.
A24	*S. aertrycke*	N.J. Exp. Sta.
A25	*Bact. anatum* (Aertrycke)	N.Y. Exp. Sta.
A26	*S. aertrycke*	Univ. of Calif.
A27	*S. aertrycke*	Univ. of Calif.
A28	*S. aertrycke* (Binns)	Univ. of Calif.
A29	*S. aertrycke* (Clitheroe)	Univ. of Calif.
A30	*S. aertrycke* (Glasgow)	Univ. of Calif.
A31	*S. gallinarum*	K.S.C.
A32	*S. gallinarum*	K.S.C.
A33	*S. pullorum*	K.S.C.
A34	*S. pullorum*	K.S.C.
A35	*S. pullorum*	K.S.C.
A36	*S. suipestifer*	K.S.C.
A37	*S. suipestifer*	K.S.C.

K.S.C. = Kansas State College
A.T.C.C. = American Type Culture Collection
U.S.D.A., B.A.I. = United States Department of Agriculture Bureau of Animal Industry

On eosin-methylene blue agar plates they develop into medium sized round colonies, with entire margins, somewhat raised but with a flat surface, homogeneous in structure, butyrous in consistency, translucent to transmitted light, and light grayish-pink in color. Upon aging, the color may become lavender to light purple and the colonies may spread slightly just as do typical members of the Salmonella group.

They grow on nutrient agar slants with a moderately luxuriant amount of growth, filiform, flat on the surface, glistening to reflected light, and translucent to transmitted light.

In nutrient broth they produce a heavy growth, giving a uniform turbidity. No surface film is produced and no appreciable amount of sediment is deposited.

Morphologically these organisms are non-spore forming rods averaging perhaps 0.5 microns in width and 1.5 microns in length, usually single, but occasionally occurring in short filaments. They are negative to Gram's stain.

The fermentation reactions were studied in a medium consisting of two per cent Bacto peptone and 0.5 per cent NaCl in distilled water. Brom thymol blue indicator was added and the medium tubed in test tubes containing inverted glass vials. The tubes were sterilized in the autoclave at 20 pounds pressure for 30 minutes.

The carbohydrates and alcohols to be tested were made up in ten per cent aqueous solutions and sterilized in the autoclave at 20 pounds pressure for 12 minutes.

The sterile solutions of carbohydrates and alcohols were added to the respective tubes in quantities of one cubic centimeter per tube, making a concentration of approximately one per cent. The tubes were incubated for 24 hours at 37°C. and all which were contaminated were discarded. The final pH of the media was very close to 7.0 as indicated by a blue-green color of the indicator.

The tubes were inoculated with a small amount of surface growth from an agar slant using a small wire loop for making the transfer. The results were read as negative, acid production, or acid and gas production at the end of 24 hours incubation at 37°C. All tubes which did not show fermentation were incubated for 14 days before being discarded. In case acid or acid and gas appeared in the tube between one and 14 days, such a change was recorded together with the number of days elapsing before its appearance.

The final pH of the media in the lactose fermentation tubes was determined by means of a quinhydrone electrode potentimeter at the end of 14 days incubation. Some of these values may not be accurate since the quinhydrone de-

termination is not accurate above pH 8.0 and since the medium had evaporated to such a degree that the reaction may have been altered.

Production of hydrogen sulfide was determined by use of Bacto dehydrated lead acetate agar.

Methyl red test and production of acetyl methyl carbinol were determined in Bacto dehydrated M.R.-V.P. medium at four, eight, and 14 day intervals after inoculation.

Production of indol was determined after seven and 14 days incubation in peptone water.

Gelatin liquefaction tests were observed at seven and 14 days after inoculation of tubes of nutrient gelatin.

Motility determinations were made in semi-solid agar as described by Tittsler and Sandholzer (1935).

Action upon milk was observed over a period of 21 days in sterile milk containing brom cresol purple indicator.

Ability to reduce nitrates was determined by following the directions of Levine (1933).

Double sugar agar was used having the same composition as Kligler's lead acetate agar except that the lead acetate was omitted.

Ability to utilize tartrates was determined by use of the medium described by Jordan and Harmon (1928).

The biochemical reactions of our Salmonella cultures are listed in Table VIII together with those of a culture of S. schottmuelleri (A6) and one of S. paratyphi (A8). The biochemical reactions of the known stock Salmonella cultures are given in Table IX.

All of the unidentified organisms studied were very similar culturally. The only characteristic in which there was a distinct difference was in the utilization of inositol. The importance of this difference is minimized by the fact that the same difference existed among our known stock cultures of S. aertrycke. The serological tests, however, indicated no difference between the unidentified cultures which fermented inositol and those which did not. For this reason we considered the fermentation of inositol of minor importance.

These organisms resemble the members of the "paratyphoid B" group but the exact species cannot be determined culturally since there are several which behave alike. For this reason serological studies were made in an attempt to identify the species more exactly.

Table VIII. Biochemical reactions of the Salmonella bacteria studied.

	Lactose	pH in Lactose at 14 days	H_2S	Methyl red	Acetyl methyl carbinol	Indol	Gelatin	Motility	Milk	Reduction of nitrates	Double sugar		Tartrate	Glucose	Xylose	Arabinose	Raffinose	Salicin	Sucrose	Dulcitol	Dextrin	Inositol	Trehalose	Sorbitol	Mannitol	Maltose	Cellobiose
											Slant	Butt															
763	-	8.34	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	-	AG	AG	AG	AG	AG
761	-	8.42	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	-	AG	AG	AG	AG	A
760	-	8.66	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	AG	AG	AG	AG	AG	A
755	-	8.50	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	-	AG	AG	AG	AG	AG
748	-	8.61	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	-	AG	AG	AG	AG	AG
727	-	8.32	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	AG	AG	AG	AG	AG	A
726	-	8.50	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	AG	AG	AG	AG	AG	A
723	-	8.32	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	-	AG	AG	AG	AG	AG
718	-	8.61	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	AG	AG	AG	AG	AG	AG
710	-	8.51	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	AG	AG	AG	AG	AG	A
704	-	8.52	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	+++	-	AG	AG	AG	AG	A
702	-	7.92	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
701	-	8.61	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
639	-	8.36	+	+	-	-	-	+	K	+	K	AG	A	AG	A	AG	-	-	-	AG	++	A	AG	AG	AG	AG	AG
635	-	8.42	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
633	-	8.49	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
625	-	8.39	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
621	-	8.42	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
618	-	8.47	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	-	AG	AG	AG	AG	AG
617	-	8.47	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	-	AG	AG	AG	AG	A
615	-	8.52	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	-	AG	AG	AG	AG	A
613	-	8.59	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	-	AG	AG	AG	AG	AG
597	-	8.46	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
595	-	8.52	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
51	-	8.38	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	A
A6	-	8.19	+	+	-	-	-	+	K	+	K	AG	A	AG	AG	AG	-	-	-	AG	++	AG	AG	AG	AG	AG	-
A8	-	8.22	+	+	-	-	-	+	K	+	K	AG	A	AG	-	AG	-	-	-	AG	+	-	AG	AG	AG	AG	-

A = Production of acid
AG = Production of acid and gas
K = Alkaline reaction

\+ = positive reaction
\- = negative reaction

Table IX. Biochemical reactions of the stock *Salmonella* cultures.

Culture number	Species	Lactose	Glucose	Xylose	Arabinose	Raffinose	Sucrose	Dulcitol	Dextrin	Inositol	Sorbitol	Mannitol	Maltose	Gelatin
A 6	*S. schottmuelleri*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A 7	*S. schottmuelleri*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A8	*S. paratyphi*	-	AG	-	AG	-	-	AG	+	-	AG	AG	AG	-
A10	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	-	AG	AG	AG	-
A11	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	-	AG	AG	AG	-
A14	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	A	AG	AG	AG	-
A16	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A22	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A23	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A24	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A26	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	-	AG	AG	AG	-
A27	*S. aertrycke*	-	AG	AG	AG	-	-	AG	+	-	AG	AG	AG	-
A12	*S. newport*	-	AG	AG	AG	-	-	AG	+	-	AG	AG	AG	-
A13	*S. mutton*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A15	*S. anatum*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A17	*S. reading*	-	AG	AG	AG	-	-	AG	+	AG	AG	AG	AG	-
A18	*S. tidy*	-	AG	AG	AG	-	-	AG	+	A	AG	AG	AG	-
A19	*S. stanley*	-	AG	AG	AG	-	-	AG	-	-	AG	AG	AG	-
A21	*S. anatum*	-	AG	AG	AG	-	-	AG	-	AG	AG	AG	AG	-
A25	*S. anatum*	-	AG	AG	AG	-	-	AG	-	A	AG	AG	AG	-

Table IX -- continued

Culture number	Species	Lactose	Glucose	Xylose	Arabinose	Raffinose	Sucrose	Dulcitol	Dextrin	Inositol	Sorbitol	Mannitol	Maltose	Gelatin
A28	S. binns	-	AG	AG	AG	-	-	AG	±	AG	AG	AG	AG	-
A29	S. clitheroe	-	AG	AG	AG	-	-	AG	±	-	AG	AG	AG	-
A30	S. glasgow	-	AG	AG	AG	-	-	AG	-	AG	AG	AG	AG	-

A = production of acid
AG = production of acid and gas
- = production of neither acid nor gas
± = questionable reaction

Serological Reactions. Since serological studies are frequently made as a basis for classifying this group of organisms, cross agglutination tests were made between representative cultures of our unidentified Salmonella group and known stock cultures of the Salmonella species.

Rather than prepare immune sera against each of our stock strains, a pooled antigen of several of our unidentified cultures was used for immunization of animals. We determined the ability of the serum thus produced to agglutinate suspensions of the stock Salmonella cultures. The numbers used were 727, 710, 633, 625, 621, and 596. A suspension was made of the surface growth from agar slants. The organisms were killed by holding the pooled suspensions in a water bath at 60°C. for one hour.

Two rabbits were immunized with this antigen by giving eight subcutaneous inoculations at four day intervals, increasing the dose from 0.5 cc. of a suspension corresponding to tube No. I of the McFarland nephelometer to 1.0 cc. of tube No. IV. The animals were bled ten days after the last injection.

Two other rabbits were inoculated in the same manner with an S. schottmuelleri antigen. This antigen was a pooled suspension of cultures A1, A5, and A7.

The two immune sera will be designated as "anti-Salmonella" and "anti-Schottmuelleri" sera, respectively. The agglutination titers of stock Salmonellas with these sera are given in Table X. The stock cultures are listed in the groups in which they belong in the Kauffmann-White outline.

From the agglutination tests it is apparent that a group relationship exists between the unidentified cultures and the Salmonella group as a whole.

The only known cultures which were agglutinated completely by the anti-serum of the unidentified cultures were S. reading, S. stanley, and E. typhi. From the Kauffmann-White outline, it is seen that S. reading and S. stanley have the O-antigen factor IV in common. However, it seems improbable that agglutination was due to the O-factor IV, since S. schottmuelleri and S. aertrycke also possess this factor and neither was completely agglutinated. E. typhi and S. stanley have H specific factor d in common, indicating that the unidentified cultures may also possess this factor.

For further serological analysis we immunized rabbits with O-antigens of the unidentified organisms and determined the ability of the immune sera thus produced to agglutinate O-antigens of the known stock Salmonella cultures.

Table X. Agglutination titer of stock *Salmonella* cultures with sera immune to whole cultures of *S. schottmuelleri* and unidentified *Salmonella* species.

Group	Culture	Species	Anti-salmonella serum	Anti-schottmuelleri serum
		"*Salmonella*" antigen	1280	20
A	A 3	*S. paratyphi*	40	0
	A 8	*S. paratyphi*	40	0
B	A 1	*S. schottmuelleri*	40	2560
	A 6	*S. schottmuelleri*	160	2560
	A 7	*S. schottmuelleri*	80	1280
	A20	*S. schottmuelleri*	80	1280
	A10	*S. aertrycke*	80	320
	A11	*S. aertrycke*	160	320
	A14	*S. aertrycke*	40	1280
	A16	*S. aertrycke*	40	160
	A22	*S. aertrycke*	80	320
	A23	*S. aertrycke*	10	320
	A24	*S. aertrycke*	10	160
	A26	*S. aertrycke*	10	80
	A27	*S. aertrycke*	80	80
	A13	*S. mutton*	40	640
	A17	*S. reading*	1280	160
	A18	*S. tidy*	20	2560
	A19	*S. stanley*	2560	20
	A28	*S. binns*	160	160
	A29	*S. aertrycke* (Clitheroe)	80	160
	A30	*S. aertrycke* (Glasgow)	80	160
C	A 12	*S. newport*	320	80
D	A 2	*E. typhi*	2560	40
E	A15	*S. anatum*	20	160
	A21	*S. anatum*	160	160
	A25	*S. anatum*	160	160

The O-antigens of two unidentified Salmonella cultures were used for immunization; culture 723, which was representative of those cultures which fail to ferment inositol; and 718, which is representative of those which ferment inositol.

The O-antigens were prepared from suspensions of whole cultures by destroying the H-antigen according to the method of Gardner (1929). This method consists of treating the heavy suspensions with absolute alcohol for one hour at 55°C. The alcohol was removed by centrifugation and evaporation at room temperature. The cells were resuspended in saline and this suspension was used for immunization of the rabbits.

The titers obtained by tube agglutinations of O-antigens of the known Salmonella cultures by the sera prepared above are given in Table XI.

Complete agglutination was obtained only in cultures of Group C indicating that the O-antigen of the unidentified cultures is the factor VI and perhaps VII or VIII of group C.

The agglutination of several cultures of Group D is rather confusing, especially since a culture of S. gallinarum, A32, was completely agglutinated. The agglutination of either S. gallinarum and S. pullorum of group D must be due to the O-antigen since neither species possesses a flagellar antigen.

Table XI. Titers of agglutination of O-antigens of stock *Salmonella* cultures by O-immune sera of unidentified *Salmonella* cultures.

Group	Culture	Species	Anti-718-O immune serum	Anti-723-O immune serum
	718		5120	5120
	723		5120	5120
A	A 3	*S. paratyphi*	80	80
	A 8	*S. paratyphi*	320	40
B	A10	*S. aertrycke*	40	80
	A11	*S. aertrycke*	0	0
	A14	*S. aertrycke*	10	20
	A16	*S. aertrycke*	0	0
	A22	*S. aertrycke*	0	0
	A23	*S. aertrycke*	0	20
	A24	*S. aertrycke*	40	20
	A26	*S. aertrycke*	0	0
	A27	*S. aertrycke*	0	0
	A17	*S. reading*	0	0
	A18	*S. tidy*	0	0
	A19	*S. stanley*	0	0
	A28	*S. binns*	0	0
	A29	*S. aertrycke* (Clitheroe)	0	0
	A30	*S. aertrycke* (Glasgow)	0	0
	A13	*S. mutton*	20	10
	A 1	*S. schottmuelleri*	160	160
	A 6	*S. schottmuelleri*	20	10
	A 7	*S. schottmuelleri*	320	160
	A20	*S. schottmuelleri*	20	0
C	A12	*S. newport*	1280	2560
	A36	*S. cholera suis*	5120	5120
	A37	*S. cholera suis*	2560	5120

Table XI -- continued

Group	Culture	Species	Anti-718-O immune serum	Anti-723-O immune serum
D	A 2	E. typhi	40	320
	A31	S. gallinarum	80	160
	A32	S. gallinarum	2560	5120
	A33	S. pullorum	40	80
	A34	S. pullorum	160	160
	A35	S. pullorum	320	160
E	A15	S. anatum	10	20
	A21	S. anatum	0	20
	A25	S. anatum	0	0

As far as we know, the structure of none of these particular cultures has been determined by other investigators and it was necessary for us to assume that each of our known stock cultures possessed the antigenic complex as given by Kauffmann and White for cultures of the same name. Time did not permit of a complete analysis of the H specific and H non-specific antigens of the group. Tentatively, we consider these cultures to possess the O-antigen VI, and either VII or VIII, and the H specific antigen d. None of the known cultures possess this combination of antigenic factors. A further study will be necessary to determine the complete antigenic structure, and to determine their exact relationship to the known species of Salmonella.

Recapitulation

1. Culturally and biochemically our unknown cultures of Salmonella bacteria belong to the "paratyphoid B" group. The exact species cannot be determined culturally beyond this point.

2. By use of the Kauffmann-White method of serological receptor analysis, these organisms do not appear to have the same antigenic constitution as any of our known stock cultures.

3. These Salmonella types appear to belong to group C of Kauffmann-White. They possess the O-antigen VI and probably VII or VIII. The H specific antigen seems to be different from that of any of the described members of group C. It appears to be the d factor of E. typhi and S. stanley. For this reason we consider that our unidentified Salmonella cultures are somewhat different from those already described.

A STUDY OF THE SLOW LACTOSE FERMENTING BACTERIA

Review of Literature

The first report of dissociation of bacteria with respect to fermentation of lactose through secondary colony formation was made by Neisser and Massini. They observed this phenomenon in a culture of an organism of the typhoid-colon group which they isolated from the feces of a patient suffering from a feverless gastro-enteritis. Neisser supervised the study of this organism and made a preliminary report in 1906. Massini, who made an intensive study of this organism gave a complete report of his observations in 1907.

Neisser and Massini used the name Bacterium coli mutabile for this organism and defined the species by establishing the criteria by which an organism can be so named.

The culture studied by Neisser and Massini when streaked on Endo agar plates grew as colorless colonies, indicating that it lacked the ability of fermenting lactose. The colonies remained colorless upon aging and never became red. In this respect it appears to be the same as the Salmonella group. However, at the end of about two days there developed in the center of the colorless colony a few minute nodules about the size of pin points. These nodules, or papillae, continued to develop in number and size with increased age of the colony. They increased in number from three or four per colony, at first, to as many as 200 in a few days. They grew to the size of a pin head in many cases. At their appearance, the color of the papillae was yellowish-white, but changed to a dark-red color in a period of usually not less than 24 hours. The papillae appeared first in the central, older portion of the colony but later near the edge of the colony. The actively growing margin of the colony was always free of papillae. If lactose fermentation tubes were inoculated from colonies containing colorless papillae, rapid fermentation of the sugar did not occur. However, if such transfer was made after the papillae became red, rapid fermentation resulted.

Neisser and Massini described this phenomenon as an example of true mutation in the sense of DeVries, and sug-

gested that the name Bacillus coli mutabilis or Bacterium coli mutabile be used to describe it. Their diagnostic characteristic of the group was the formation of papillae within the original colonies on agar plates containing one per cent lactose.

Since the original reports of Neisser and Massini, many studies have been made upon B. coli mutabile, and for the most part their observations have been confirmed.

Massini (1907) described his culture as consisting of short Gram-negative cells with somewhat rounded corners typical of B. coli. Stewart (1926), Kriebel (1934), and Hershey and Bronfenbrenner (1936) describe the organisms studied by them as being coccoid in form. Kennedy, Cummings and Morrow (1932), Fothergill (1929), and Lewis and Hitchner (1936) describe their organisms as being Gram-negative, non-spore forming, short rods.

Neisser (1906) described the colonies of the parent strain as colorless on Endo agar plates. Massini (1907) added that the colonies were round with no especial structural lines or granulations. On plain agar the colony was a light rosette form of a light yellowish color. Lewis (1934) described the colonies as being quite small on agar containing lactose and explained this as being due to the limited capacity of the culture to utilize lactose. Dudgeon

and Pulvertaft (1927) said that the colonies of delayed lactose fermenters were larger than those of the paratyphoid group as a whole. Jones, Orcutt, and Little (1932) described the colonies as smooth, round, and translucent; Fothergill (1929) as well defined, grayish-white, opalescent to opaque colonies. The cultures studied by Lewis (1934) were either colorless on differantial media or lacked the typical metallic sheen of E. coli. Hershey and Bronfenbrenner (1936) found that their cultures might appear in smooth, rough, large mucoid, small translucent, and occasional striated and stellate forms or in more than one of these forms.

Massini (1907) describes the growth on agar slants as a rich colon-type culture, weakly iridescent, with light yellowish-gray coloration.

The cultural reactions of atypical lactose fermenting strains, as described by various authors, are listed in tabular form in Table XII together with the reactions of the cultures studied in this investigation.

In studying the paracolon-mutabile-colon group serologically, Stewart (1926) described the different members as being extremely individual in their antigenic power, as indicated by the fact that an anti-serum against one strain agglutinated only the homologous antigen and its immediate

Table XII. Cultural reactions of atypical lactose fermenters.

	Number of cultures	Lactose	H_2S	Methyl red	Acetyl methyl carbinol	Indol	Gelatin	Motility	Reduction of Nitrates	Glucose	Xylose	Arabinose	Raffinose	Salicin	Sucrose	Dulcitol	Dextrin	Inositol	Trehalose	Sorbitol	Mannite	Maltose	Cellobiose	Galactose	Levulose	Adonitol
Massini (1907)		S				-	-	-		+					-						+	+		+		
Stewart (1926)		S or -					-			+											+	+				
Dudgeon and Pulvertaft (1927)		S or -				+		+		+											+	+				
Fothergill (1929)	32	S or -					-	-		+	+										+	+				
Kennedy, Cummings and Morrow (1932)	22	S		+	-	21+ 1-	-	12+ 10-	+	+	+	+	6+ 16-	15+ 7-	8+ 14-	13+ 9-	21+ 1-	-		18+ 4-	+	+		+	+	5+ 17-
Jones, Orcutt and Little (1932)	38	S	-	+	-		-	+	+	+	+		-								+	+				
Lewis (1934)	9	S		4+ 5-	4+ 5-		4+ 5-	7+ 2-		+				7+ 2-	5+ 4-											
Kriebel (1934)	25	S or -				21+ 4-	-	17- 8+		+		+	2+ 23-					-			+	+				
	11	S				10+ 1-	-	4+ 7-		+		+						-			+	+				
Lewis and Hitchner (1936)		S	+	+	-	-	-	-		+	+	+		-	-	-		-	+	+	+	+	-	+	+	-
Hershey and Bronfenbrenner (1936)	5	S				-	-	+	+						+						+			+		
Kansas State College	48	S	-	47+ 1-	1+ 47-	+	-	29+ 19-	+	+	29+ 19-	+	4+ 44-	25+ 23-	3+ 45-	11+ 37-		-	+	16+ 32-	44+ 4-	+	21+ 27-			

S = slow fermentation
+ = fermentation or positive reaction
- = no fermentation or negative reaction

derivatives. None of these are agglutinated with standard immune sera against members of the typhoid-paratyphoid group. Kriebel (1934), Lewis and Hitchner (1936), Fothergill (1929), and Dudgeon and Pulvertaft (1927) also found no agglutination with members of the typhoid-paratyphoid group. Fothergill (1929) divided 32 cultures which he studied into three general groups on the basis of cross agglutinations. Dudgeon and Pulvertaft (1927) found great type specificity by using precipitin tests.

Gram-negative bacilli which ferment lactose slowly are widely distributed in nature. They have often been found in the human alimentary canal. The culture described by Neisser and Massini was isolated from a case of gastroenteritis. Dudgeon (1926) found them in two per cent of 200 cases including both ill and healthy persons. Kriebel (1934) recovered them during routine carrier examination of a group of normal food handlers. Fothergill (1929) isolated such organisms from the feces of 62.5 per cent of 104 infants suffering from infectious diarrhea. Dudgeon and Pulvertaft (1927) isolated the organisms from cases of chronic infection of the urinary and intestinal tracts. Lewis (1934) isolated latent fermenters of lactose from water, from feces, and from a sample of cheese suspected of having caused a mild food poisoning. Kennedy, Cummings, and Morrow

(1932) obtained them from urine, stools, and water. Jones and Little (1931), and Jones, Orcutt, and Little (1932) reported having isolated organisms which fermented lactose slowly from feces of cows suffering from diarrhea.

The method of isolation used by most workers was that of streaking a sample of feces over the surface of a differential agar plate and isolating from colorless colonies. Such cultures must be studied in lactose fermentation tubes in order to certify their identity as slow fermenters. Eosin-methylene blue agar was used as a differential medium by Lewis (1934), Kriebel (1934), and Fothergill (1929). Hershey and Bronfenbrenner (1936) used agar containing lactose and China blue-rosolic acid indicator while Jones, Orcutt, and Little (1932) used lactose agar containing brom cresol purple. Preliminary enrichment was attempted by Fothergill (1929).

Neisser and Massini observed that after the papillae in the colorless colonies on Endo agar plates had turned dark, one could obtain both "red" and "white" colonies by streaking from the "red" papillae to a fresh Endo plate. The "red" colonies thus obtained were rapid lactose fermenting variants and remained "red" upon further transfer. They did not produce secondary colonies and did not revert to the non-fermenting form. In lactose broth, they caused rapid

fermentation, and on Endo plates they resembled the typical *E. coli*. The "white" colonies obtained from the "red" papillae remain "white" but after a few days they produce papillae which turn dark, and from which both "red" and "white" cultures could be obtained, behaving in all respects like the original "white" culture.

In the remainder of this discussion we shall follow the terminology of Neisser and Massini by using "white" to designate the parent culture and "red" to designate the rapid fermenting variant.

Neisser and Massini's observations upon the isolation of "red" variants have been verified by many who later studied delayed lactose fermenters. Lewis (1934) obtained "red" variants by streaking eosin-methylene blue agar plates from an acid fermentation tube in which latent fermentation of lactose had occurred. Hershey and Bronfenbrenner (1936) obtained a rapid fermenting variant by daily serial transfer in tubes of lactose broth. Jones, Orcutt, and Little (1932) also obtained pure "reds" by passing their cultures through lactose broth and then plating on eosin-methylene blue agar plates. However, they were not able to derive "reds" by culturing from papillae.

The time elapsing before the appearance of secondary colonies was found by Neisser (1906) to be from three days

on; by Massini (1907) to be usually in two days, rarely after the third day, and rarely as early as the first; by Mellon (1925) to be from 72 to 96 hours; by Lewis (1934) to be within five to eight days. Kennedy, Cummings and Morrow (1932) found that eight out of 22 strains gave superimposed pink colonies in five days, the other 14 never showed them.

Massini (1907) described the secondary colonies as small nodules about the size of a pin point, which are yellowish-white on Endo agar. They originate in the central, older portion of the colony on the surface of the agar. Stewart (1926) describes the papillae as minute hemispherical projections varying from 0.05 to 0.1 millimeters in diameter at their earliest appearance. In describing secondary colonies in general, not particularly those of E. coli, Stewart (1928) said that the papillae begin to appear at about the time the colony stops growing, and that they appear in the center of the colony and never in the actively growing margin. There developed what he considered to be a beneficial variation after which the papillae increased greatly in size and overgrew the parent colony. The papillae are bright and shiny when observed by transmitted light and resemble small air bubbles. Kennedy, Cummings, and Morrow (1932) described the papillae as superimposed red colonies appearing as conical elevations on the surface of the

colony and which do not become visible to the naked eye until after 48 hours incubation.

Bronfenbrenner and Davis (1918) cultured slow fermenters in broth containing various concentrations of lactose by transferring serially each day to tubes containing broth of the same lactose concentration. In using one half, one, two, and five per cent lactose they found that more rapid fermentation occurred in broth of higher lactose concentration. Kriebel (1934) grew 12 strains of delayed lactose fermenters in one, two, three, five, and ten per cent lactose broth and found most rapid fermentation in the medium containing five per cent of the sugar. Lewis (1934) also found that more rapid fermentation occurred in five per cent lactose than in higher or lower concentrations.

Kennedy, Cummings, and Morrow (1932) found fermentation to occur more rapidly in Petri dishes and toxin flasks than in test tubes. Hershey and Bronfenbrenner (1936) on the other hand found greater change in a series of sealed fermentation tubes than in a parallel series plugged with cotton. They stated that "it is evident from this experiment that conditions of limited oxygen supply are favorable to the selection of fermenting variants, but only when lactose is accompanied by an additional source of carbon".

Neisser (1906) found that if his culture of B. coli mutabile was streaked serially each day from one Endo plate to another the colorless colonies continued to appear. He found this to occur in a series of 20 daily transfers. Stewart (1928) said that if papillae formation is prevented by daily subculture, no amount of exposure to the sugar will cause variation. Bronfenbrenner and Davis (1918) made serial transfers daily in one per cent lactose broth. At the beginning, acid appeared in five days, but by the fifteenth transfer acid appeared in 24 hours. Jones, Orcutt, and Little (1932) worked with a strain which failed to ferment lactose in 30 days and by repeated transfer caused it to ferment the sugar in four or five days. Nungester and Anderson (1931) stated that variation is speeded up by passage through lactose broth just as "repeated passage of an organism through animals often tends to increase its virulence for these animals". Hershey and Bronfenbrenner (1936) were able to increase the ability of five strains of slow fermenters to attack lactose and this increase in fermentative ability occurred at about the same time that variants appeared on the plates. Kennedy, Cummings, and Morrow (1932) came to the conclusion that serial transfer will cause enhancement of the ability to ferment lactose, but that different strains vary greatly in ease with which this can be

made to occur. Lewis (1934) found that the fermentative ability of some cultures is capable of enhancement while that of others is not. His conclusions were that enhancement would occur if enough cells were carried over in each transfer so as to not exclude all variant cells. Thus he found that if dilutions of one, ten, or 100 million should be carried from tube to tube at 24 hour intervals for 30 days in one cubic centimeter portions, no enhancement of fermentative ability occurred, due to the fact that such dilutions exclude all variant cells. However, if dilutions of 100,000 were used, the fermentative period was shortened to 36 hours, and if lower dilutions were used, rapid fermentation occurred.

The rapid fermenting variant described by Lewis (1934) as having greater size than the colonies of the original white strain on eosin-methylene blue agar, and as having the characteristic color of E. coli, although in most cases the typical metallic sheen of E. coli was lacking. The variant usually had a deep purple center surrounded by a more or less colorless margin. Kriebel (1934) described the variant as producing a typical "coli" colony on eosin methylene blue agar.

The speed of fermentation by a mixture of variant and parent cells appears to depend upon the relative number of variant cells present. Lewis (1934) found that ten per cent "reds" in a mixture caused fermentation in 24 hours. In a culture which ferments the sugar in an average time of 72 hours, there would be approximately one per cent variants in 36 hours, five per cent in 48 hours, and ten per cent in 60 hours. Kriebel (1934) allowed four dissociated cultures to grow in the lactose fermentation tubes for seven days after acid was produced. Upon plating on differential media she found 99 per cent typical coli and one per cent the same as the original strain.

Neisser (1906) observed that the "red" variant colonies bred true upon further transfer, and never showed the original "white" form of colonies. It seems to be agreed by all who have studied E. coli mutabile that under ordinary laboratory conditions, reversion to the "white" form does not occur. Such has been found by Jones, Orcutt, and Little (1932), by Stewart (1928), and by Kennedy, Cummings, and Morrow (1932). Although several reports have been made of reversion from "red" to "white", this can be made to occur only with difficulty. Neisser (1906) caused reversion by growing his culture on phenolized agar. Hershey and Bronfenbrenner (1936) brought about reversion by cultivation in a synthetic

medium containing sodium succinate as the only source of carbon. Nungester and Anderson (1931) were able to produce an unstable non-lactose-fermenting strain from a lactose fermenter. Mellon (1925) reported having transformed a typical strain of B. coli into a mutabile form by aging in glycero-phosphate broth.

Serologically the "red" variant has been found by Stewart (1926), Massini (1907), and by Hall (1935) to be the same as the parent "white" form. Results of agglutination tests were found by Burk (1908) and by Jones, Orcutt, and Little (1932) to be rather unsatisfactory.

A difference in agglutination of "reds" and "whites" was found by Jones, Orcutt and Little (1932) but they decided that this difference was quantitative rather than qualitative.

According to the reports of other investigators the cultural reactions of the variant and the parent strain of these organisms is exactly the same except for rapid fermentation of lactose and the loss of ability to produce secondary colonies on lactose agar.

Neisser (1906) attributed the change in ability to ferment lactose to a true mutation in the sense of DeVries, in which the ability to ferment the sugar was acquired suddenly. Lewis (1934) quoted Burri as believing that the trans-

formation occurred gradually with each cell in the culture capable of being transformed into a rapid fermenter.

Smith (1913) in discussing the production of variants by B. typhosus on dulcite agar suggested that perhaps the organism had exhausted the supply of available nutrients in the medium and was then forced to attack dulcite as a source of energy. Lewis (1934) believed that the "chief factors involved in the formation of secondary colonies are depletion of nutrients and beneficial variation". Both Smith and Lewis expressed the view that lactose did not stimulate variation but rather was a selective agent which preserved the beneficial variation which occurred without environmental stimulus. Bronfenbrenner and Davis (1918) suggest an adaptation of the bacteria to the medium accompanied by concentration of the sugar to the optimum conditions, as being involved in the change.

Stewart (1928) disagrees with the view that a true mutation occurs, because interpolation of characters would not occur with such great regularity, or if they did, the reverse change should occur just as often. He offers the theory that variation occurs as a result of Mendelian variation at a period in which autogamic conjugation occurs, the sugar acting as a selective agent. He considers the primary colony

as developing by somatic division and the papillary division to correspond to reducing or gametic division. His experiments show that the culture need not be aged in lactose, but if the lactose is added at the time at which papillary division is occurring, variation occurs in a few hours. In keeping with the Mendelian theory, Stewart regards the mutabile form as being a heterozygous dominant "white", from which can be derived para-colon as a homozygous dominant which does not ferment lactose, and true E. coli as the homozygous recessive which ferments lactose rapidly. To explain the apparent inability to obtain the homozygous dominant para-colon from mutabile in the laboratory, Stewart suggests that it lacks the ability of growing in competition with mutabile and colon in a mixture.

Mellon (1925) believed that in order to develop the ability to ferment lactose, the organism must pass through the phase in its life cycle in which it is capable of secreting the enzyme lactase. This stage is represented by secondary colony formation and morphologically parallels isogamous conjugation or zygospore formation which has been proved to occur in yeast.

Jones, Orcutt, and Little (1932) determined quantitatively the amount of lactose used by slow-fermenters. They decided that the parent strains of atypical coli were

able to utilize lactose, but due to the production of ammonia, this fermentation did not cause a change of pH in the fermentation tube.

Deere, Dulaney, and Michelson (1936) carried out similar quantitative determinations and found the rate of utilization of lactose by typical coli to be greater than by slow fermenters early in the fermentation period but by the end of two days, at which time Jones, Orcutt, and Little had made their determinations, the amount of lactose used by atypical and typical coli was nearly the same. While these quantitative determinations do not eliminate the possibility of utilization of lactose by the parent culture, Deere, Dulaney, and Michelson (1936) found that acidification in the fermentation tube corresponded so nearly with the appearance of variants on the plates that they believed that changes in fermentative ability must be due to the variants which arise in the culture. Hershey and Bronfenbrenner (1936), on the other hand, could not prove that their variants possessed any metabolic properties which the parent strain lacked, so they concluded that the difference in fermentative ability must be quantitative rather than qualitative.

Neisser (1906) first made use of the name B. coli mutabile to designate a non-lactose fermenting coliform

organism which suddenly acquired the ability to ferment lactose. Their criterion for classification of a coliform organism as B. coli mutabile is the ability to form secondary colonies on lactose agar from which may be derived both rapid and slow fermenting strains. Since Neisser's publication the name B. coli mutabile has sometimes been used to include coli-like organisms which dissociate with respect to other biochemical characteristics such as ability to ferment sucrose.

Many workers have found fault with the use of the name "mutabile" because of the fact that genetically the term mutation is used to designate a change in the chromosomes such as sometimes occurs in the higher forms of life. Since definite chromosomes have not been demonstrated in bacteria, a true mutation could not occur in them. The name Bacterium coli mutabile has been so commonly used that it must be regarded as the correct name for species conforming to the specifications as published by Neisser and Massini, although in order to correspond with the current systems of nomenclature, it is better to call them "Escherichia coli mutabile".

Stewart (1928) regarded E. coli mutabile as forming an intermediate group between para-colon and the typical species of Escherichia. Kennedy, Cummings, and Morrow (1932) re-

garded them as forming a connecting link between the colon-aerogenes group and the paratyphoid group. They were unable to classify these organisms satisfactorily by means of Bergey's system of classification. Lewis and Hitchner (1936) also found them to differ from all recognized genera of Bacteriaceae. Fothergill (1929) discovered differences which eliminated them from all the recognized species of intestinal organisms. Hershey and Bronfenbrenner (1936) found that in some respects the organisms studied by them resembled Aerobacter aerogenes more closely than E. coli. Lewis (1934) suggested the probability that any species of the colon-aerogenes group might occur in the mutabile form.

Many slow fermenters cannot be identified with either mutabile or paracolon since they cannot be shown to form secondary colonies on lactose agar, yet they consistently show latent fermentation of lactose in a liquid medium. Some such organisms were studied by Jones, Orcutt, and Little (1932), which they preferred to call "atypical colon bacilli" rather than either mutabile or paracolon. Kennedy, Cummings, and Morrow (1932) studied 22 slow lactose fermenters, eight of which they readily classified as E. coli mutabile, since secondary colonies were demonstrated in them. They hesitated to regard the other 14 strains as E. coli mutabile since they did not develop secondary colonies.

In the common systems of classification in which dichotomous keys are used in order to identify bacteria, no mention is made of slow fermentation of carbohydrates. Consequently, attempts to use these methods for identification of delayed fermenters leads to error.

Experimental Methods and Results

Cultural Characteristics. E. coli mutabile and slow lactose-fermenters cannot easily be distinguished from members of the typhoid-paratyphoid group by their growth characteristics.

On nutrient agar slants these organisms produce a moderately heavy growth, filiform, flat and transclucent.

In nutrient broth they produce a fairly heavy growth with little surface film or sediment. Total counts indicate that the slow fermenters do not grow as vigorously as do ordinary intestinal organisms.

E. coli mutabile organisms are short thich rods which do not retain the Gram stain. Slow lactose-fermenters are also Gram-negative, but the cells are usually smaller than those of E. coli mutabile and they are almost coccoid in shape.

Slow lactose-fermenters cannot be distinguished from typhoid-paratyphoid organisms by the appearance of their

colonies on eosin-methylene blue agar. The colonies are light pink in color, and medium in size. They are round and usually quite flat.

The colonies of E. coli mutabile also resemble those of the typhoid-paratyphoid group for the first few days of growth. Most of the cultures produce smooth colonies, although it is not unusual to find cultures in the rough form. Occasionally large mucoid colonies are found. No correlation was found between colonial form and ability to ferment lactose. After a few days growth on lactose agar, secondary colonies begin to appear. This usually occurs in from four to seven days. The formation and description of the secondary colonies will be described later.

The biochemical reactions of E. coli mutabile and the slow lactose-fermenters were determined in the same manner as those of the Salmonella group. The results are tabulated in Table XIII. For convenience, the cultures are arranged in groups according to motility and ability to ferment sucrose, salicin, and dulcitol.

All these cultures ferment lactose within 14 days, although the time required for fermentation varies considerably. As a rule, the members of group A require a longer incubation period than do members of the other groups. With several cultures, no gas collected in the inverted vial in

Table XIII. Cultural reactions of delayed lactose fermenting bacteria.

Group	Culture number	Lactose	Days before acidification	pH in lactose at 14 days	Secondary colonies	H_2S	Methyl red	Acetyl methyl carbinol	Indol	Gelatin	Motility	Milk	Double sugar: Slant	Double sugar: Butt	Tartrate	Reduction of nitrates	Glucose	Xylose	Arabinose	Raffinose	Salicin	Sucrose	Dulcitol	Dextrin	Inositol	Trehalose	Sorbitol	Mannitol	Maltose	Cellobiose
A	31	A	7	5.48	-	-	+	-	+	-	-	A	K	A	-	+	AG	-	AG	-	-	-	-	±	-	AG	A	AG	AG	-
	249	A	7	5.65	-	-	+	-	+	-	-	A	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
	397	A	7	5.97	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	274	A	8	5.61	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	575	AG	8	6.02	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	736	AG	8	6.20	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	579	A	9	5.48	-	-	+	-	+	-	-	A	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
	744	AG	9	5.48	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	AG
	642	A	9	5.61	-	-	+	-	+	-	-	A	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	-	AG	-
	541	AG	9	5.75	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	A	-	AG	-
	331	AG	9	5.88	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	A	-	AG	-
	419	A	9	5.92	-	-	+	-	+	-	-	A	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
	574	A	9	6.03	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	AG	-	-	-	±	-	AG	-	AG	AG	-
	538	AG	9	6.10	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	732	A	10	6.03	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	594	A	10	6.16	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	680	A	11	6.29	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	167	A	12	6.56	-	-	+	-	+	-	-	AG	K	AG	-	+	AG	-	AG	-	-	-	-	±	-	AG	-	AG	AG	-
	305	A	9	5.74	-	-	+	-	+	-	-	AC	K	AG	-	+	AG	-	AG	-	AG	-	-	±	-	AG	A	AG	AG	AG
B	725	A	2	4.87	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
	371	AG	3	5.24	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
	731	AG	5	5.65	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
	1233	AG	3	5.70	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	-	-	-	±	-	AG	AG	AG	AG	-
C	587	AG	5	5.64	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	AG	AG	±	-	AG	AG	AG	AG	-
	630	A	5	5.34	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	AG	AG	AG	AG	±	-	AG	-	AG	AG	-
D	717	AG	2	4.94	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	AG	AG	AG	-
	573	AG	4	4.97	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	AG	AG	-
	733	AG	4	5.20	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	A	AG	-	AG	±	-	AG	-	AG	AG	AG
	692	A	4	5.82	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	AG	AG	AG
	381	AG	5	5.97	+	-	+	-	+	-	+	A	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	AG	AG	AG
	216	AG	6	5.41	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	AG	AG	AG
	320	A	7	5.75	+	-	+	-	+	-	+	A	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	AG	AG	AG
	428	AG	7	5.92	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	-	AG	AG
	588	A	8	6.46	+	-	+	-	+	-	+	A	K	AG	-	+	AG	AG	AG	-	AG	-	AG	±	-	AG	-	AG	AG	AG
E	310	AG	2	4.97	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	AG	AG	AG	-
	601	AG	4	4.80	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	AG	AG	AG	-
	691	AG	4	5.17	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	724	AG	5	5.16	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	606	AG	5	5.61	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	A	-	-	±	-	AG	-	AG	AG	AG
	590	AG	5	5.61	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	A	-	-	±	-	AG	-	AG	AG	AG
	8-1	AG	5	6.32	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	609	A	6	5.24	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	580	A	6	5.55	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	8-3	AG	6	5.65	+	-	+	-	+	-	+	A	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	638	AG	7	5.60	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	-	AG	AG	AG
	603	AG	8	5.61	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	-	A	-	-	±	-	AG	-	AG	AG	AG
F	593	A	2	5.03	+	-	+	-	+	-	+	AC	K	AG	-	+	AG	AG	AG	AG	-	AG	-	±	-	AG	-	AG	AG	AG
G	1040	AG	2	5.07	+	-	-	+	-	-	+	AC	K	AG	-	+	AG	AG	AG	-	AG	-	-	±	-	AG	AG	AG	AG	AG

A = production of acid
AG = production of acid and gas
AC = production of acid and coagulation

K = alkaline reaction
+ = positive reaction
- = negative reaction

the lactose fermentation tubes; the others produced only a small amount of gas.

Since these organisms are Gram-negative rods which ferment lactose with production of acid, or acid and gas, and ferment glucose vigorously with production of both acid and gas, they must be regarded as belonging to the genus Escherichia. Groups B, C, D, E, F, and G are considered to be E. coli mutabile since they produce secondary colonies on agar containing one per cent lactose. An exception is observed in culture 1040, group G. This culture produced acetyl methyl carbinol and is methyl red negative in the M.R.-V.P. medium. This culture resembles the Aerobacter genus more nearly than the Escherichia.

In Table XIV we have given a list of the species of Escherichia to which each of these groups of cultures most nearly corresponds according to the classification of Bergey (1934). This should not be considered as an attempt to classify these organisms. Due to the uncertainty regarding the taxonomic position of these bacteria, it seems preferable at present to classify them miscellaneously as E. coli mutabile rather than to attempt to identify them with any species of Escherichia which are now recognized in the common systems of classification.

Table XIV. Species to which the groups of *E. coli mutabile* and *slow lactose-fermenters* most nearly correspond.

Group	Number of Cultures	Motility	Sucrose	Salicin	Dulcitol	Type	Classification by Bergey
A	19	-	-	-	-	*Slow lactose-fermenters*	*E. acidilactici*
B	4	+	-	-	-	*E. coli mutabile*	*E. gruenthali*
C	2	+	AG	AG	AG	*E. coli mutabile*	*E. communior*
D	9	+	-	AG	AG	*E. coli mutabile*	*E. coli*
E	12	+	-	AG	-	*E. coli mutabile*	*E. paragruenthali*
F	1	+	AG	-	-	*E. coli mutabile*	*E. anindolica*
G	1	+	-	AG	-	*E. coli mutabile*	*A. levans*

In order to determine whether or not typical "red" and atypical "white" forms of the same species of Escherichia occurred together in the intestine, we examined several colonies of each type from ten birds.

Plates of eosin-methylene blue agar were streaked just as in the routine analysis. Isolations were made only from those plates which contained both dark and colorless colonies. From such plates, ten colonies of typical dark purple "colon" type colonies and ten colorless colonies were isolated. In case there were not ten well separated colonies on the plate, we isolated as many as possible.

The cultural reactions which are used in Bergey's outline for identification of species of Escherichia were determined for each of these cultures. In order to simplify comparisons of the distribution of both the rapid and slow fermenters, each culture was given the name of the species of Escherichia to which it most nearly corresponds. A list of the species obtained from each bird is given in Table XV.

In only four of the ten birds were there isolated "red" and "white" strains which conformed to the same species. The typical "colon" types belonged to a variety of species while usually the colorless colonies from a given plate were mostly of the same species.

Table XV. The species of typical and atypical *Escherichia* isolated from the intestines of ten chickens.

Bird Number	Number of Isolations	Type of Colony		Classification (Bergey's)
775	13	10 dark colonies	2	*E. neopolitana*
			3	*E. acidilactici*
			1	*E. coli*
			2	*E. communior*
			1	*E. pseudocoscoroba*
			1	*E. enterica*
		3 colorless colonies	3	*E. paragruentbali*
781	10	9 dark colonies	4	*E. neopolitana*
			5	*E. communior*
		1 colorless colony	1	*E. acidilactici*
782	15	10 dark colonies	2	*E. pseudocoloides*
			1	*E. enterica*
			2	*E. anindolica*
			1	*E. paragruentbali*
			3	*E. gruenthali*
			1	*E. coli*
		5 colorless colonies	2	*E. enterica*
			3	*E. coli*
791	12	10 dark colonies	3	*E. anindolica*
			2	*E. enterica*
			3	*E. acidilactici*
			2	*E. coli*
		2 colorless colonies	1	*E. gruenthali*
			1	*E. acidilactici*
793	20	10 dark colonies	2	*E. communior*
			2	*E. acidilactici*
			2	*E. formica*
			3	*E. coli*
			1	*E. neopolitana*
		10 colorless colonies	4	*E. coli*
			5	*E. acidilactici*
			1	*E. formica*

Table XV -- continued

Bird Number	Number of Isolations	Type of Cology	Classification (Bergey's)
795	8	7 dark colonies	1 E. acidilactici
			1 E. paragruenthali
			2 E. enterica
			1 E. coli
			2 E. communior
		1 colorless colony	1 E. formica
807	4	4 colorless colonies	4 E. acidilactici
808	20	10 dark colonies	10 E. communior
		10 colorless colonies	9 E. acidilactici
			1 E. pseudocoscoroba
812	18	10 dark colonies	3 E. pseudocoscoroba
			3 E. neopolitana
			3 E. communior
			1 E. formica
		8 colorless colonies	6 E. neopolitana
			2 E. acidilactici
813	20	10 dark colonies	6 E. pseudocoloides
			2 E. neopolitana
			1 E. pseudocoscoroba
			1 E. formica
		10 colorless colonies	10 E. acidilactici

In Table XVI is given the comparative distribution of each species of slow and rapid fermenting strains of coliform bacteria.

The results of examination of these few birds indicates that E. communior is the predominant species of typical colon bacilli occurring in the intestine. Of the types which produce colorless colonies on eosin-methylene blue agar and which ferment lactose slowly, those corresponding most nearly to E. acidilactici are found in largest numbers.

Serological Reactions. Serological reactions were found to be unsatisfactory as a means of grouping the cultures of E. coli mutabile or slow lactose-fermenters.

Members of these groups were not very antigenic and consequently it was difficult to produce a high titer immune serum.

By use of agglutination tests, there was found to be slight cross agglutination between the cultural groups. Using an immune serum against a pooled antigen of six cultures of group A, agglutination of different cultures was obtained in dilutions varying from 1-10 to 1-5160. This indicates that the cultural group is not uniform serologically.

Dissociation. The cultures which we have designated as slow lactose-fermentersh have never shown papillae on eosin-methylene blue agar plates. The colonies remain small

Table XVI. Distribution of the species of typical and atypical Escherichia organisms.

Species	Typical "colon" types		E. coli mutabile and slow lactose fermenters	
	Total isolations	Per cent of total	Total isolations	Per cent of total
E. coli	8	9.3	7	13.0
E. paragruenthali	2	2.3	3	5.6
E. formica	4	4.7	2	3.7
E. gruenthali	3	3.5	1	1.8
E. enterica	6	7.0	2	3.7
E. acidilactici	9	10.5	32	59.3
E. communior	24	27.8	0	0
E. pseudocoloides	8	9.3	0	0
E. anindolica	5	5.8	0	0
E. neopolitana	12	14.0	6	11.1
E. pseudocoscoroba	5	5.8	1	1.8
Total	86	100.0	54	100.0

and rather flat and the color changes from light pink to a light purple. These colonies can never be distinguished from those of the species of Salmonella by their appearance on differential plates. It was observed that under ordinary conditions in the incubator, the plates begin to dry up and the colonies reach a stage of senescence before the period has elapsed which must pass before they show acidification in the fermentation tube.

The members of all other groups, on the other hand, begin to show papillae in their colonies on eosin-methylene blue agar plates after four or five days, just as described by Neisser and Massini for cultures on Endo agar plates.

The papillae begin to appear first in the central, older portion of the colony as small round projections having the same light pink color as the parent colony. With age, they increase in number and size until the colony is loaded with papillae. In most cases the papillae begin to turn dark in one or two days after their appearance, while in some cases they may remain colorless for a week or more. Occasionally the papillae never become definitely dark.

If a fresh plate is streaked from a papilla, one is supposed to obtain a plate which contains both "red" colonies, which are rapid fermenting variants; and "white" colonies,

which are identical with the original parent strain. If a plate is streaked from a dark papilla one is supposed to obtain a plate predominated by the "red" variety.

Our experience has been that it is quite difficult to obtain "red" variants on plates, due perhaps to the fact that most of the cultures used required five or six days for fermentation of lactose in broth. It seems that the longer the period required for fermentation the more difficult it is to obtain "red" variants on plates. This may be explained by the fact that "reds" are present in greater proportions in the more rapid fermenting cultures.

We have never been able to obtain "reds" by streaking plates from colorless papillae. By streaking plates from the papillae after they have turned dark we can usually find "red" colonies on the plates although in most cases they constitute only a small proportion of the total number.

The "red" variant cultures will be described in a subsequent section.

The cultures of E. coli mutabile cause acidification of lactose fermentation tubes during an incubation period which varied from two to eight days with different cultures. In most cases, the color change from blue to yellow occurred rather suddenly. Most of these cultures produced a small amount of gas.

The slow lactose-fermenters were much slower in causing color change in the lactose fermentation tube, in most cases requiring from seven to 14 days. The color change occurred very gradually and often even after 14 days incubation the broth was not very strongly acid. Only a few of these cultures produced gas and these produced only a very small amount.

None of the cultures which caused delayed fermentation of lactose exhibited latent fermentation of any other carbohydrate.

The method has already been described by which rapid fermenting variants of E. coli mutabile may be isolated from dark secondary colonies. Obviously, it is impossible to obtain "red" variants of slow lactose-fermenters in this way since they produce no secondary colonies under ordinary conditions. For this reason it is necessary to resort to other procedures in order to derive rapid fermenting variants from slow lactose-fermenters.

Rapid fermenting variants of both E. coli mutabile and slow lactose-fermenters may be isolated from lactose fermentation tubes in which delayed fermentation has occurred. The acid fermentation tube contains both "white" and "red" strains. Fermentation is believed to be due to the "reds" which appear in the culture as a result of dissociation. If an eosin-methylene blue agar plate is streaked with a loop-

ful of broth from the acid fermentation tube, there should occur both "red" and "white" colonies on the plate. We have found that even from the old fermentation tube, the "reds" are usually much in the minority as indicated by the number of "red" colonies which appear on the plates. In some cases it was found necessary to streak several plates before a "red" colony could be found for isolation.

If a loopful of broth from an acid fermentation tube is transferred to a fresh tube, fermentation will occur more rapidly than it did in the preceding tube due to more rapid development of the "reds" which are present at the beginning of the incubation period of the second tube. Lewis (1934) suggested that in order to increase the rate of fermentation by serial transfer one must transfer a large enough inoculum so that there will be some "red" cells carried over each time a fresh tube is inoculated. If serial transfers are continued immediately after each tube turns acid, fermentation will occur after a few transfers within 24 hours due to the continued increase in number of "red" cells in the culture. An eosin-methylene blue agar plate streaked from the tube in which rapid fermentation occurred will contain large numbers of "red" colonies. Serial transfers made early in the incubation period will not enhance the fermentative ability of the culture due probably to the fact that

if there are any "red" cells present at that stage, they occur in such small numbers that none or very few of them will be carried over in each successive transfer.

Description of Rapid Fermenting Variants. There is considerable difference in the appearance of red strains from different cultures on eosin-methylene blue agar plates. Some "reds" appear to be typical of the "colon" group, producing dark purple colonies with green metallic sheen. Colonies of this type are most often obtained with "red" strains of cultures which in the "white" form caused fermentation of lactose in three or four days and from which the rapid fermenting variants are easily derived.

Most of the "red" strains produce colonies which are colorless at the end of 24 hours. By the end of 48 hours incubation, these colonies turn dark brown. Usually the colonies are very dark in the center, with a wide margin of a lighter brown. These colonies never exhibit a metallic green color and never resemble typical "colon" colonies. As a rule, the colonies are larger than the "white" colonies of the same strain. All of the "red" strains of the slow lactose-fermenters exhibit this type of colony, as do a large number of the E. coli mutabile.

An important characteristic of the "red" variants is that they do not produce secondary colonies on lactose agar. Upon aging, the colonies become dark brown to black, but papillae never arise within them.

The "red" variants all ferment lactose in the fermentation tube within 48 hours. Those strains which grow as typical Escherichia attack lactose vigorously with production of acid and gas within 12 hours. The strains which are lighter in color on the plates may require from 12 to 48 hours to cause fermentation. In most cases, a small amount of gas is produced although in a few cultures none is produced.

With the exception of their more rapid fermentation of lactose, the "red" variants are identical with the "white" strains from which they were derived in ability to ferment carbohydrates.

In no instance have we observed reversion from "red" to "white" form in the laboratory.

Fermentation Products of Slow Lactose-Fermenters. In order to determine the rate of utilization of lactose by organisms which cause delayed fermentation of this sugar, it was desirable to make quantitative determinations of the amount of lactose present in the medium at intervals during the incubation period.

The possibility existed that the organisms might ferment the sugar rapidly but that products other than acids were formed, or that the acids produced were very weak. In order to discover what products were formed, we analyzed the culture medium at intervals for the common fermentation products.

Jones, Orcutt, and Little (1932) suggested that the apparent latent fermentation of lactose might be due to the production of large amounts of ammonia which neutralized the acid fermentation products, thus causing the reaction of the fermentation tube to remain alkaline. In order to test this possibility, we analyzed the media quantitatively for ammonia nitrogen at each time interval.

A series of tests was conducted in which pH, total lactose, volatile acids, alcohol, lactic acid, total nitrogen, amino nitrogen, ammonia nitrogen, and total counts were determined quantitatively during the incubation period.

The cultures used for the analyses were those numbered 31 and 538, both representative of the slow lactose-fermenters. Cultures from this group were selected because the cultural reactions of all of its members were quite uniform.

Because of the fact that fermentation by these cultures is very slow, analyses were made at the beginning of the incubation period and at the end of five, ten, and 15 days

incubation.

In order to be certain which products were formed from lactose, one series of cultures was grown in broth containing one per cent lactose and another series in the sugar free medium. This made possible a direct comparison of the fermentation products from the two media and the difference could be considered as being due to the fermentation of lactose.

Due to the fact that these cultures did not grow well in synthetic media, we used a medium of the following composition for our analyses:

Proteose peptone	10.0 gr.
K_2HPO_4	1.7 gr.
KH_2PO_4	0.3 gr.
Water .....to	1000 cc.

The above is the sugar free medium. The base for the lactose broth was made of such composition that by adding a sterile aqueous solution of lactose after sterilization of the base, the final composition was the same as that of the sugar free medium with the addition of one per cent lactose.

A titration curve of the buffered one per cent peptone solution of the above composition is given in Figure 1. For comparison we have also given the titration curves of unbuffered one, two, and five per cent peptone solutions. The points of the titration curves were determined by making

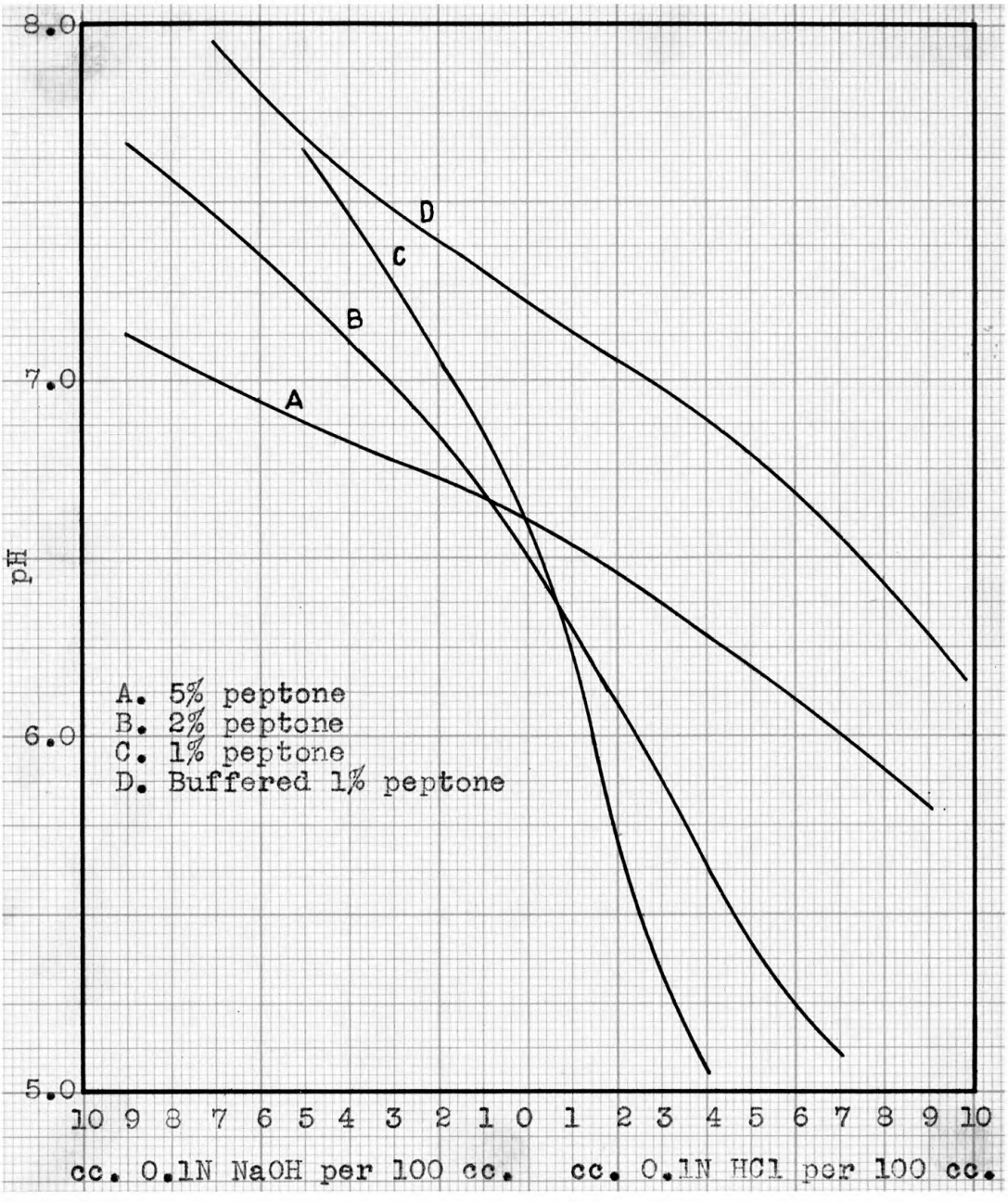

Figure 1. Titration curves of one, two, and five per cent peptone solutions and of buffered one per cent peptone solution.

pH determinations after the addition of standard acidic and basic solutions to the medium. Determinations were first made after each addition of successive one cubic centimeter portions of 0.1N HCl to 100 cc. of the medium. Then similar determinations were made after one cubic centimeter portions of 0.1N NaOH were added to another 100 cc. portion of medium. The pH determinations were plotted against the number of cubic centimeters of standard solutions added and a smooth curve was drawn through the points. All pH determinations were made by use of a quinhydrone electrode potentiometer.

The titration curves show that the base medium used for fermentation analyses is buffered most heavily just above pH 7.0. The medium is buffered slightly less than is five per cent peptone solution.

Both the lactose and sugar free media were used in 800 cc. amounts in liter Florence flasks. Four flasks each of lactose and sugar free broth were used in each series, one flask of each to be used at each five day period.

Each flask was inoculated with 0.0001 cc. of a 24 hour broth culture of the organism used in that series.

After inoculation, each flask was attached to a series of hard glass test tubes in such a way that the evolved gases must bubble through a solution of NaOH. This made

possible a determination of the amount of carbon dioxide evolved.

All cultures were incubated at 37°C.

At the end of each five day incubation period, a flask of sugar free and a flask of lactose medium were removed from the incubator and analyzed.

Before the cultures were opened, a current of CO_2-free air was drawn through the train of tubes in order to remove the evolved gases from the culture. The tube of NaOH was saved for titration of CO_2.

The culture flask was carefully opened and a sample removed to a sterile test tube, the sample to be used for the determination of total count, pH, and total lactose as well as for making Gram stains and streaking eosin-methylene blue agar plates.

From the culture flask about 450 cc. of culture were withdrawn by suction, replacing the volume with CO_2-free air. This sample was preserved at 5°C. in the refrigerator, to be used later for the determination of volatile fatty acids, alcohol, total nitrogen, ammonia nitrogen, and amino nitrogen. The remaining 240 cc. of culture was used for the determination of dissolved and combined carbon dioxide and lactic acid.

For the determination of CO_2, two cubic centimeters of concentrated sulfuric acid were added to the culture and a current of CO_2-free air was drawn through the culture. The current of air was led from the culture through a reservoir containing NaOH. To each reservoir was attached a tower of glass beads so arranged that the current of air issuing from the culture must bubble up through the glass beads which were covered with NaOH solution. Two such reservoirs with absorption towers were connected in series.

Aeration was continued for three hours, after which the NaOH solutions were washed into a volumetric flask and made up to a definite volume. Aliquots of this solution were titrated for carbon dioxide by the method as outlined by Mahin (1932).

The solution of NaOH which was in the fermentation series during incubation was titrated in the same manner. The sum of the average of duplicate determination of each sample was recorded as the amount of carbon dioxide produced, in milligrams per 100 cc.

Determinations of total populations were made by making standard plate counts on the culture at each interval.

The pH of each sample was determined by means of a quinhydrone electrode potentiometer (Leeds and Northrup).

Gram stains were made of each culture.

Eosin-methyelen blue agar plates were streaked from each culture. The plates were examined for 14 days for the appearance of "red" variant colonies or of secondary colonies

For the determination of total lactose, the method of Folin and Wu (1920) was used. The reagents were prepared as described by Mathews (1930). The medium was prepared for analysis by diluting the broth ten times, mixing thoroughly with infusorial earth and filtering through paper. Both the method of preparation of the sample and the method of analysis were found to be satisfactory by Klemme and Poe (1936). A slight blue color which developed in the determinations on the sugar free filtrates was too weak to read against a standard and so was recorded in each case as a "trace".

Volatile fatty acids were determined by the method described by Fred, Peterson, and Davenport (1919). In order to identify the acids, a distillation was made by the method of Duclaux (1895). The distillation curves which were obtained were compared with similar curves for pure volatile acids. In order to identify the unknown acid we consider it to be the same as the pure acid whose curve it most nearly approaches.

The processes involved in the determinations of alcohol were: (1) separation and concentration of the alcohol from the culture, (2) oxidation of the alcohol to the correspond-

ing fatty acid and (3) qualitative and quantitative analysis of the acid thus produced. The first process consisted of distillation of the alcohol from an alkaline sample of the media saturated with common salt. Oxidation was accomplished by refluxing the distillate for one hour after the addition of an oxidizing solution such as was used by Dox and Lamb (1916). The acids produced were analyzed as described above for volatile acids. In no case was there enough acid produced to allow identification by the Duclaux distillation method. The small amounts which were obtained were calculated as ethyl alcohol.

Determination of lactic acid was made by oxidation of the acid to acetaldehyde. The aldehyde was distilled off and titrated in the distillate with standard iodine. The procedure used was that described by Friedemann and Graeser (1933). It was found necessary to use at least five cubic centimeters of media for each determination, a much larger sample than was used by Friedemann and Graeser. This led to considerable interference in the test because of the presence of amino acids.

Total nitrogen was determined by the Kjeldahl-Gunning method, using samples of ten cubic centimeters of culture media. In the first series, this determination was made at each five day intervals. In the following tests, one deter-

mination only was made since the total nitrogen was found to be unchanged during the 15 days incubation.

Amino nitrogen was determined by the Sorenson titration method as described by Koch (1934). Samples of ten cubic centimeters of media were used for each determination and titration was made with 0.01N NaOH.

Ammonia nitrogen was determined by distillation in an apparatus such as was described by Koch (1926) for use in micro-Kjeldahl determination of nitrogen and blood urea. The distillation was carried out as described by Koch (1934) for the analysis of blood urea.

The results of quantitative analysis of the products of fermentation of lactose by "white" strains of cultures 31 and 538 are given in Tables XVII and XVIII, respectively. Each value recorded represents the average of duplicate determinations on the sample. The upper figure in each case represents the analysis on one series of cultures, the lower figure that on a second series.

The Duclaux distillation curves of each sample determined after 15 days incubation are shown in Figures 2 and 3 together with the Duclaux curves for pure acids.

The distillation curves for the acid produced in five and ten day incubation periods were approximately the same as those in the samples incubated 15 days. The curves ob-

Table XVII. Products of metabolism of a slow lactose-fermenter (culture 31) in lactose and sugar free media.

Mg. per 100 cc.	Lactose Media				Sugar Free Media			
	0 days	5 days	10 days	15 days	0 days	5 days	10 days	15 days
pH	7.21	7.08	6.76	6.04	7.23	7.15	7.29	7.18
	7.18	7.10	6.40	5.76	7.24	7.20	7.19	7.21
Lactose	1030	1010	995	931	tr	tr	tr	tr
	1050	1015	990	925	tr	tr	tr	tr
Acetic acid	4.0	22.5	40.2	54.6	3.1	24.1	30.8	28.7
	3.2	23.1	49.2	61.1	3.7	21.6	28.7	29.8
Carbon dioxide	2.7	24.5	34.1	35.9	3.1	23.8	38.0	44.2
	2.1	32.0	28.2	22.8	4.6	28.3	36.8	38.3
Ethyl alcohol	0.7	1.6	1.7	3.9	0.8	1.0	2.5	1.6
	0.3	2.0	4.4	4.9	0.9	1.7	2.3	1.7
Lactic acid	10.7	8.5	9.7	9.4	10.3	7.0	7.0	8.3
	11.9	7.8	8.5	6.4	14.1	6.4	6.4	5.3
Total N	147.5	143.0	146.0	146.0	147.5	146.0	147.0	148.0
	144.2	144.2	144.2	144.2	144.3	144.3	144.3	144.3
Amino N	18.8	23.0	25.0	25.2	19.3	23.1	25.1	27.0
	16.1	19.8	22.9	22.9	15.3	18.3	22.5	25.7

Table XVII -- continued

Mg. per 100 cc.	Lactose Media				Sugar Free Media			
	0 days	5 days	10 days	15 days	0 days	5 days	10 days	15 days
Ammonia N	tr	8.9	9.6	9.6	tr	8.1	9.1	9.8
	tr	10.0	9.7	10.6	tr	8.6	9.0	12.4
Total counts per cubic centimeter	51	112*	73*	85*	52	80*	30*	11*
	39	169	234	98	43	122	16	8

* = million
tr = trace

Table XVIII. Products of metabolism of a slow lactose-fermenter (culture 538) in lactose and sugar free media.

Mg. per 100 cc.	Lactose Media				Sugar Free Media			
	0 days	5 days	10 days	15 days	0 days	5 days	10 days	15 days
pH	7.22 7.28	7.09 7.07	7.02 7.07	6.71 6.55	7.23 7.31	7.20 7.14	7.13 7.16	7.13 7.13
Lactose	1047 1075	1020 1039	1000 1015	965 1003	tr tr	tr tr	tr tr	tr tr
Acetic acid	3.0 2.2	22.6 19.7	28.5 25.9	34.6 39.8	3.3 2.9	19.8 14.0	24.4 25.0	30.8 39.3
Carbon dioxide	3.7 1.2	30.1 17.5	46.6 30.4	34.8 36.0	2.3 2.0	32.9 34.7	69.1 31.5	61.4 29.1
Ethyl alcohol	0.6 0.4	1.9 1.4	1.6 1.7	2.2 4.4	0.6 0.6	1.0 1.6	1.2 1.6	1.1 4.3
Lactic acid	12.7 10.9	12.1 9.5	8.2 7.6	8.6 3.5	13.4 10.2	7.9 8.1	6.7 5.0	9.9 6.0
Total N	143.9 145.0	143.9 145.0	143.9 145.0	143.9 145.0	144.2 144.8	144.2 144.8	144.2 144.8	144.2 144.8
Amino N	14.0 14.7	19.9 19.3	21.0 21.0	22.4 25.9	14.2 14.3	19.8 19.9	22.4 22.4	22.6 27.7

Table XVIII -- continued

Mg. per 100 cc.	Lactose Media				Sugar Free Media			
	0 days	5 days	10 days	15 days	0 days	5 days	10 days	15 days
Ammonia N	tr	8.2	9.3	9.3	tr	8.1	10.8	11.8
	tr	9.3	11.2	11.4	tr	9.6	11.2	11.8
Total counts per cubic centimeter	56	81*	67*	119*	55	122*	69*	49*
	37	216*	186*	186*	44	668*	138*	24*

* = million
tr = trace

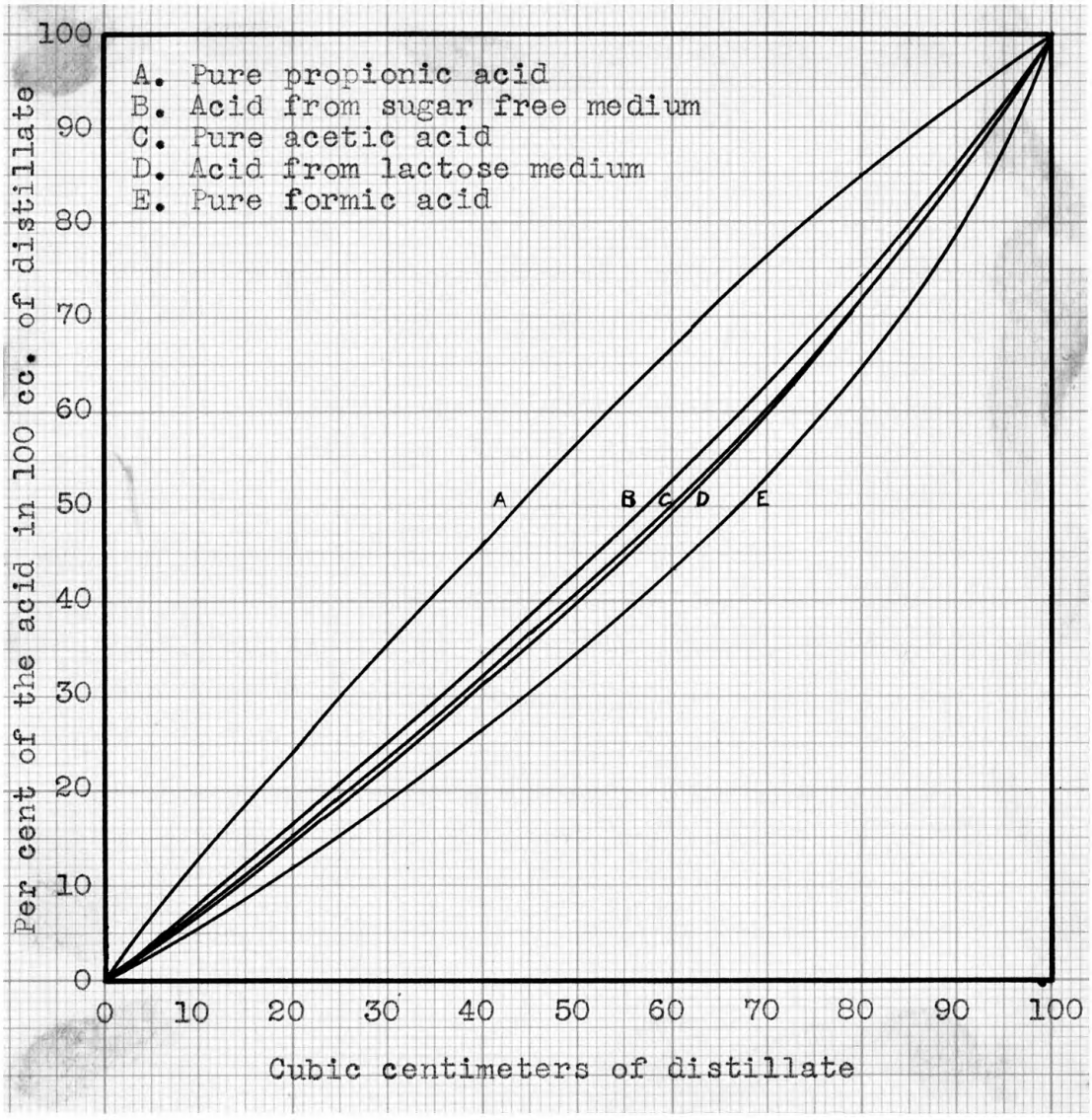

Figure 2. Duclaux distillation curves of pure formic, acetic, and propionic acids, and of the acids produced by culture 31.

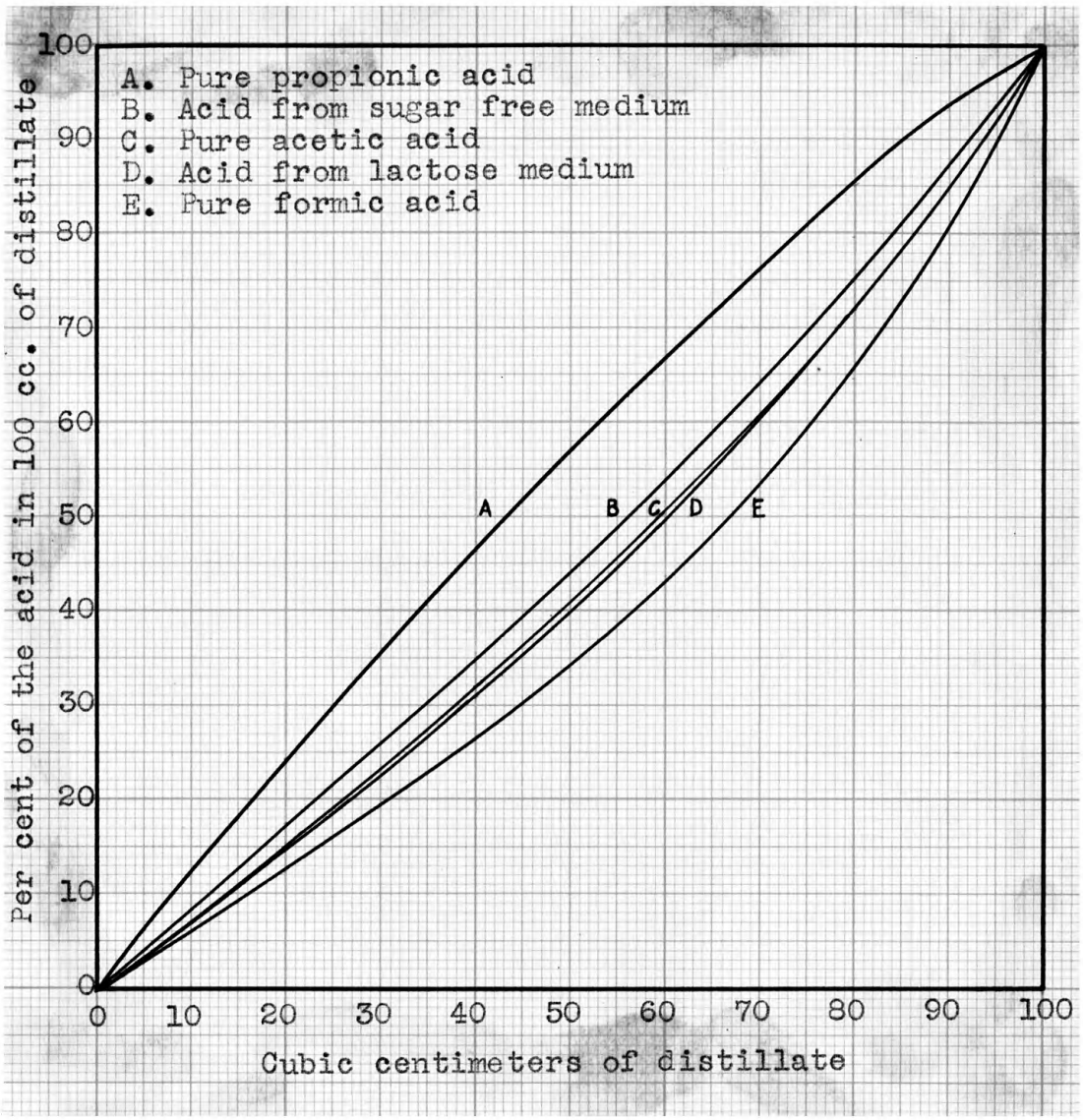

Figure 3. Duclaux distillation curves of pure formic, acetic, and propionic acids, and of the acids produced by culture 538.

tained from duplicate determinations were so nearly alike that it is difficult to plot them on the same graph. For this reason we have plotted only one curve for the acid from lactose medium and one for that from the sugar free. The points on each curve represent the average of four determinations, duplicates on each of two series of tests.

The rate of utilization of lactose by slow lactose-fermenters is very slow and only a small amount of the lactose present is utilized in 15 days. The sugar is used from the beginning of the incubation period, and the rate of utilization does not appear to increase as the incubation progresses. The pH of the lactose broth dropped slowly, corresponding to the decrease in total lactose, while the pH of the sugar free medium remained practically constant.

The volatile acids were identified as acetic acid from the Duclaux distillation curves. The amount of acetic acid increased uniformly both in the lactose and in the sugar free broth. The acetic acid produced in the sugar free medium probably was formed from the amino acids. The excess acetic acid in the lactose broth over that in sugar free broth may be considered as having been produced from lactose. More than that amount of acid may have been produced from lactose due to the "protein sparing" effect of the carbo-

hydrate, although the carbohydrate present is not one which is easily utilized by the organism.

More carbon dioxide was produced in the sugar free than in the lactose broth. This is no doubt produced by decarboxylation of the amino acids.

The amount of alcohol increased very gradually in both media, although the amount is almost negligible in both cases.

The lactic acid determination appears to have been interferred with by the presence of amino acids. In all cases a decrease in the determination was obtained as the incubation progressed.

The amino nitrogen increased as the incubation proceeded, and by about the same amount in both lactose and sugar free broth, indicating approximately equal proteolytic activity in both media.

The production of nearly the same amount of ammonia in both media also indicates that the proteins were attacked to about the same extent in both cases. In neither case was there enough ammonia produced to alter the pH of the medium appreciably.

Determinations of each product in duplicate cultures of the same organism were very nearly the same in most cases. The products of each of the two cultures were also approxi-

mately the same.

The total counts were comparatively low. The lactose medium seemed to favor the slow lactose-fermenters since the total populations did not begin to decrease as early in the incubation period as they did in the sugar free medium.

Gram stains indicated that the cultures were pure in all cases.

No secondary colonies or colonies of "red" variants were found on eosin-methylene blue agar plates. The failure to obtain red variants on the plates was probably due to the fact that the "red" cells were present in a very small minority.

Recapitulation

1. The cultures which cause delayed fermentation of lactose can be subdivided into two groups on the basis of their ability to produce secondary colonies on lactose agar.

2. Those cultures which produce secondary colonies conform to the criteria for classification in the group E. coli mutabile of Neisser and Massini.

3. Those cultures which produce no secondary colonies constitute a group which have not been completely described for which no name has been suggested. We have designated these organisms as slow lactose-fermenters.

4. The slow lactose-fermenters are all similar in cultural reactions and form a uniform group. If classification is attempted by Bergey's system, this group is found to correspond most nearly to the species E. acidilactici.

5. The E. coli mutabile cultures exhibit a variety of combinations of cultural characteristics. Using motility and ability to ferment sucrose, salicin, and dulcitol as bases for subdivision, the E. coli mutabile cultures may be grouped in six subgroups. Except for the latent fermentation of lactose, each of these groups corresponds to a species of the genus Escherichia.

6. Due to the obscurity which exists regarding the taxonomic position of these organisms, our comparison of these groups with the recognized species of Escherichia is for convenience only, rather than an attempt to classify them with described species of this genus.

7. It was found that in some birds, "white" and "red" organisms possessing the same cultural characteristics exist together in the intestines. This suggests the possibility that dissociation from one form to the other may occur in the intestine of the chicken.

8. Rapid fermenting variants of E. coli mutabile may be derived from secondary colonies on lactose agar plates. Rapid fermenting variants of both E. coli mutabile and slow

lactose-fermenters may be isolated from the lactose fermentation tube in which delayed fermentation has occurred.

9. "Red" variants cause fermentation of lactose within 48 hours. Otherwise they are identical in all cultural characteristics as the "whites" from which they were derived.

10. "Red" variants give a characteristic colony on eosin-methylene blue agar plates although in most cases they do not resemble typical Escherichia colonies. No secondary colonies are produced.

11. Slow lactose-fermenters utilize lactose very slowly. The chief product of fermentation of lactose is acetic acid. They are not very active proteolytically.

SUMMARY AND CONCLUSIONS

Bacteria exhibiting the characteristics of members of the genus Salmonella were isolated from the intestines of 9.6 per cent of 658 diseased chickens examined in this study. The species to which these organisms belong could not be determined positively by cultural and biochemical characteristics. Serologically, this group was not identical with any stock cultures of Salmonella with which it was compared. Agglutination tests indicated that these organisms possess the O-antigen VI and the H specific antigen d of Kauffmann and White. It is not known whether or not these

organisms are epidemiologically important.

Organisms which produced no gas from any of the common carbohydrates were isolated from 3.3 per cent of the cases. These organisms were not studied in detail and no attempt was made to identify them. They probably belong to the genera Salmonella, Eberthella, or Shigella.

From 5.5 per cent of the cases we isolated bacteria which belong to the group E. coli mutabile of Neisser and Massini. They ferment lactose slowly in a liquid medium and produce secondary colonies on agar containing one per cent lactose. From secondary colonies or from fermented lactose broth there can be isolated "variant" strains which are capable of fermenting lactose fairly rapidly and which do not produce secondary colonies on lactose agar.

The rapid fermenting variants which we derived from E. coli mutabile cannot be regarded as typical "colon" organisms. In only a few cases did the variant have the typical "colon" appearance on eosin-methylene blue agar plates. Most of the "variants" required from 24 to 48 hours to cause acidification of lactose fermentation tubes and several produced no gas.

In most cases we have found the variant cells to be much in the minority in secondary colonies and in lactose

broth. This makes isolation of pure cultures of the variant much more difficult than one is led to believe from the literature on the subject.

On differential media and in lactose fermentation tubes E. coli mutabile resembles the typhoid-paratyphoid group of organisms until after several days growth. Their presence causes confusion in attempts to isolate members of the typhoid-paratyphoid group and may introduce an error in diagnosis.

Although E. coli mutabile was first described in 1906 and has been studied by numerous workers since that time, no recognition is made of its existence in the common systems of classification now in use. Attempts to classify slow fermenters by use of dichotomous keys always leads to confusion. An error in classification will result if this is attempted before the period required for fermentation of lactose has elapsed.

There are three principal reasons why no classification of E. coli mutabile has been attempted. First, the exact taxonomic position of the group has never been satisfactorily determined and consequently it is not known where the group should be placed in relation to other intestinal organisms. Second, since it is possible that E. coli mutabile organisms are only variants of typical colon species, or vice versa,

it is questionable whether or not E. coli mutabile should be distinguished from the described species of Escherichia. Third, E. coli mutabile includes organisms which exhibit such different biochemical characteristics that it cannot be considered as a single species.

Although the taxonomic position of E. coli mutabile has not been satisfactorily determined, it is agreed that it more nearly corresponds to members of the genus Escherichia than to any other. It is possible that mutabile strains of Aerobacter also exist. Neisser and Massini indicated the relation of E. coli mutabile to the colon types by using "coli" in their name Bacterium coli mutabile. More recently, the generic name Escherichia has been used in place of Bacterium.

If slow fermenting mutabile bacteria are to be classified in the genus Escherichia, it is necessary to observe the fermentation reactions of these organisms after 14 days incubation. Non-lactose-fermenting organisms should be observed in lactose broth for 14 days before making a final classification, in order to eliminate the possibility that they might be mutabile types. The period of 14 days is an arbitrary time limit which was found to be sufficiently long to allow identification of all mutabile cultures examined in this study.

The main difficulty in classifying the mutabile types as Escherichia is in knowing where to place them in relation to the recognized species of Escherichia. If mutabile and colon are variants of each other, it would be no more correct to place them in separate species than it would be to classify smooth and rough strains of the same organism as different species.

Since the characteristic with regard to which dissociation occurs is so important as a means of separating normal intestinal organisms from intestinal pathogens, it seems justifiable to disregard the phylogenetic relationships of the mutabile and colon types until they are better understood, and to regard E. coli mutabile as a species of Escherichia, distinguished from the recognized species by its slow fermentation of lactose. This species would be regarded as consisting of a number of "varieties" which differ from each other biochemically. To conform with the binomial system of nomenclature, the name of the species should be Escherichia mutabile.

This classification of E. mutabile would supply a convenient classification of the mutabile types of coliform bacteria and one which is as nearly correct as can be made at present. Further study of this group will no doubt clarify the uncertain condition which now exists and make pos-

sible an exact classification of the mutabile bacteria. Recognition of the species E. mutabile would eliminate errors in identification which result from attempts to use the systems of classification in their present forms. At present, errors are certain to be made by anyone who is unaware of the existence of the mutabile strains or who is unfamiliar with the characteristics of these organisms.

Slow lactose fermenting bacteria which do not produce secondary colonies on lactose agar were isolated from 7.3 per cent of the birds examined. They dissociate in lactose broth giving origin to rapid fermenting variants which may be isolated in pure culture. The failure of these organisms to produce secondary colonies on agar containing one per cent lactose eliminates them from the E. coli mutabile group as described by Neisser and Massini.

In a few cases we have been able to demonstrate secondary colony formation by these cultures by plating on lactose agar from old lactose broth cultures. This indicates that the ability to produce secondary colonies on lactose agar under ordinary conditions is not a fundamental differential characteristic and should not be used as a basis for separation of other slow lactose fermenters from E. mutabile.

The species E. mutabile should be defined as including those bacteria belonging to the genus Escherichia (lactose

fermented within 14 days) which do not originally ferment lactose but which dissociate in lactose broth, giving origin to variant strains which are able to ferment lactose. The occurrence of dissociation in the lactose fermentation tube causes the phenomenon of "slow fermentation".

No characteristics have been found by which a positive identification of mutabile strains can be made other than by observation of lactose fermentation tests for two weeks, or until fermentation occurs. This precludes the possibility of making a rapid identification of these organisms.

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