PHYSICO-CHEMICAL STUDIES OF *ESCHERICHIA COLI MUTABILE*

by

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B. S., University of Illinois, 1938

A THESIS

submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1939

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INTRODUCTION

Atypical lactose-fermenting organisms have been isolated from the intestines of chickens sacrificed in the Bacteriology Department of the Kansas State College of Agriculture and Applied Science. Because of their similarity to paratyphoid organisms, they are of interest from a taxonomic and biological standpoint.

These organisms were named Bacterium coli-mutabile by Neisser (1906) and Massini (1907). They are gram-negative, short rods and differ from Escherichia coli in that they do not ferment lactose for from four to fourteen days, and produce secondary colonies on solid culture media. On selective media, such as Endo's media or eosin-methylene-blue agar, the organisms give rise to medium-sized, translucent and colorless colonies which are flat and generally smooth. When first isolated from the selective media and placed in a medium containing lactose the organisms will not ferment lactose for a period exceeding four days. If transferred to another tube as soon as acid begins to appear, the period of fermentation becomes shortened and by consecutive transfers the organisms soon ferment lactose in the usual period observed with E. coli.

Correspondingly, if the original colony is not isolated

from the plate, it gives rise to secondary colonies which appear at about the same time that lactose fermentation begins in the fermentation tube. If the secondary papillae-like growths are cultured in a lactose medium it is found that the sugar is fermented in a much shorter period than a tube of lactose medium which has been inoculated with the mother culture.

By conditioning this lactose-fermenting ability with consecutive transplants through a lactose medium until rapid fermentation occurs, one may obtain a rapid lactose-fermenting strain and a latent fermenting strain from the same colony. It is with these corresponding pairs that this paper deals.

Many workers have described this organism and it is possible that many others working with the colon-typhoid-paratyphoid group have encountered E. coli-mutabile and have failed to differentiate between it and the members of the paratyphoid group. Fothergill (1929) isolated the organism from stools of infants suffering from "summer diarrhea" but could not place them in any known genus of gram-negative intestinal bacilli. He described them as being non-motile, unable to liquefy gelatin, latent lactose-fermenters or non-lactose-fermenters, and acid and gas producers in all sugars fermented. Their pathogenicity was unknown. Kennedy,

Cummings and Morrow (1932) isolated similar organisms from routine fecal analyses, typhoid carriers, cases of intestinal toxemia and nephritis. One organism was isolated from water. Lewis and Hitchner (1936) obtained these atypical lactose-fermenting organisms from young chicks suffering from a disorder simulating pullorum disease and described them as gram-negative, actively motile and unencapsulated. They concluded from their studies that the organisms should be considered a distinct type and their taxonomic position should be intermediate between the colon-aerogenes group and the paratyphoids. They also regarded them as pathogenic for chicks and guinea pigs. Bushnell (1938) studied the cultural reactions of 153 cultures and classified them according to Bergey (1934). Of these 13.7 per cent were classed as E. grunthali; 37.9 per cent as E. coli; 44.9 per cent as E. paragrunthali; and 3.47 per cent as A. cloacae. About 3 per cent were classed as "non-gas" producers on lactose.

Hershey and Bronfenbrenner (1936) found the organisms to be quite widespread in samples for routine diagnostic procedures. Lewis (1934) collected his organisms from soil, water, feces and cheese suspected of causing mild cases of food poisoning. Jones, Orcutt and Little (1932) also isolated the organism but obtained it from cows suffering from

infectious diarrhea although they did not conclude that the organism was the causative agent. Penfold (1911), Stewart (1926), Kriebel (1934), Minning (1937) and others have reported the organism. McDaniel (1937), divided the organisms exhibiting delayed lactose fermentation into two groups, namely, "Slow lactose-fermenters" and E. coli-mutabile. The basis for this lies in the observation that while E. coli-mutabile gives rise to secondary colonies, the Slow lactose-fermenters do not have any such papillae-like formations.

STUDY OF CULTURES

The organisms were recovered from the intestines of chickens which had been brought to the Bacteriology Department at Kansas State College. The routine method for isolation was by the removal of portions of the duodenum, the cecum, the upper intestine and the large intestine, streaking upon eosin-methylene-blue agar plates and incubation for 48 hours. Colonies which were typical of the mutabile type were isolated and run through the various laboratory tests. The characteristic diagnostic reactions of E. coli-mutabile are as follows:

E. M. B. plates	translucent, colorless colonies - secondaries (4 days)
Lactose	latent fermentation (4-21 days)
Dextrose	acid and gas (24 hours)

Sucrose	acid and gas (24 hours)
Maltose	acid and gas (24 hours)
Indol	positive
H_2S	negative
Methyl red	positive
Voges-Proskauer	negative
Simmon's citrate agar	no growth and no change
Gram stain	negative, short rod

The organisms used in this study were selected on the basis of these tests and were considered to be typical of the E. coli-mutabile group.

The "Red" form, or rapid lactose fermenting strain, was then obtained from the parent culture by a number of passages through lactose (1 per cent), transfers being made whenever lactose fermentation appeared. When these organisms fermented lactose in 24-48 hours, they were considered as containing a considerable number of the "R" form and they were placed on selective agar plates. From these plates, "red" colonies were isolated and tested for their ability to ferment lactose. Such cultures were "paired up" with the "W", or parent form, from which they were derived. These pairs were grown on inorganic salts agar free of lactose and kept in stock by covering the slant with sterile mineral oil. Cultures which had been recovered within the

year were usually used, as it was found that "S" forms put in stock previous to that had reverted to some extent to the "R" form and did not give the same degree of contrast.

Because of the obvious value of an early identification it seemed advisable to devise a method whereby these latent lactose-fermenting organisms could be differentiated from other colon-typhoid-paratyphoid types. This necessarily demanded a knowledge of the metabolic processes of the organism as compared with those of the other types. With this in mind the writer studied the organism B. coli-mutabile from a standpoint of its physical and biochemical processes.

BIOCHEMICAL STUDIES

Products of Fermentation

In-as-much as the organism differed from the typical colon types only in its fermentation of lactose when following the usual laboratory methods of classification, the logical approach to the problem seemed to be in the quantitative analysis of the end-products of the lactose fermentation.

Review of Literature. Jones, Orcutt and Little (1932) suggested that there is a growth retardation on the part of the mutabile strains and that there is an alkaline produc-

tion occurring upon this retardation. This alkalinity was then supposed by the authors to be the important factor in "masking" the true fermentation and giving an obvious latent action. Hershey and Bronfenbrenner (1936) found that the multiplication rate and the velocity of lactose fermentation were influenced by the concentration of lactose in the medium and that the effect of the rate of fermentation was direct as well as dependent upon changes in the rate of growth.

In the present studies it will be seen that the concentration of the ingredients of the culture media were quite consistent throughout, thereby eliminating any error that might be due to an inconsistency.

Deere, Dulaney, and Michelson (1936) studied the utilization of lactose by B. coli-mutabile. They found that the slow fermenting forms utilized small quantities of lactose before the lactose fermenting variants could be demonstrated. However, the lactose fermenting variants had to appear before an appreciable amount of lactose was fermented.

Little other information on the biochemical analysis of the lactose fermentation of these strains has been published. However, Stephenson (1930) gave the results found on typical colon organisms and it was decided that quantitative tests

for these same products would be appropriate for the mutabile strains. However, after several analyses on the fermentation products of a lactose-peptone broth, it became evident that the results would not be significant unless a carbon balance could be computed. This was, of course, impossible as long as an ingredient such as peptone was present. This led to the adoption of the synthetic salts-lactose broth from which a carbon balance could be calculated.

Experimental Methods. An inorganic broth was found to be a suitable basic medium for growth, as it was well-buffered and did not contribute carbon or necessary growth elements that could not be easily measured. To this basic medium was added an available source of carbon which was, in this case, enough lactose to make a 1 per cent solution. The medium consisted of the following:

KH_2PO_4	1.0 gm.
$MgSO_4 \cdot 7H_2O$	0.7 gm.
NaCl	1.0 gm.
$(NH_4)HPO_4$	4.0 gm.
Iron	Trace
H_2O	1000 cc.
Lactose	10 gm.

Batches of 4.2 liters were made at one time so that the Florence flasks in which the fermentation occurred could be filled with 600 cc. each. Six flasks were used for actual fermentation studies, while one flask was held for the control determinations, or 0-day determinations. These flasks

were then plugged with cotton, sterilized at 15 pounds for 15 minutes, and allowed to stand over night in the incubator to test their sterility.

With sterile conditions assured, the flasks were then inoculated. Three were inoculated with the R-strain and three with the corresponding W-strain as aseptically as possible. Each flask was used for a complete determination. That is, one for a 2-day analysis, one for a 5-day analysis, and the last one for a 10-day analysis.

After incubation, each flask was attached to a sodium hydroxide absorption tower filled with glass beads and containing 0.1 N NaOH. The entire system was fitted to be gas-tight and the culture then placed in a 37°C. incubator for 10 days. Samples were then removed at the intervals 2, 5, and 10 days, and the following determinations made: carbon dioxide, lactose, lactic acid, succinic acid, volatile acids, alcohols, and ammonia nitrogen.

Carbon dioxide. When the cultures were removed from incubation they were placed on a suction and CO_2-free air drawn through the culture for 30 minutes to remove the CO_2 which was then absorbed by means of the alkali in the absorption tubes. One hundred cc. was then withdrawn from the Florence flask attached to another absorption tower and acidified with H_2SO_4. The same procedure was again followed,

alkali removed and double titrated with 0.1 N HCl, using phenolphthalein and methyl orange as indicators. The non-acidified culture gave up the "free" or uncombined CO_2 whereas the acidified culture lost its CO_2 which was in the "combined" form of carbonates. However, the total "free" CO_2 was divided by 6 to give the CO_2 formed in 100 cc. of culture. The two amounts (free and combined CO_2) were then added to give the total mgms. CO_2/100 cc. of culture.

Calculations: Mgm. CO_2 per 100 cc. = HCl x norm x $\frac{\text{mol. wt. of } CO_2}{2}$

pH: The pH was determined on a Northrup and Leeds quinhydrone pH potentiometer. It is considered to give results that check within 0.04 pH. Five to ten cc. of the cultures were used for this determination and the usual technique was employed.

Lactose. Lactose was determined colorimetrically, using the method of Folin and Wu (1920). Reagents were prepared as suggested by Mathews (1930).

Into each of two test tubes were measured 9.2 cc. distilled water and 0.8 cc. of the culture. Into two other tubes was measured 9.0 cc. water and 1.0 cc. of a standard 0.08 per cent lactose solution. One-half gram of Infusorial earth was then added to each tube and thoroughly mixed. Each sample was then filtered through a No. 2 filter paper

and the filtrate collected in a clean dry test tube.

Duplicate samples of 2.0 cc. of each filtrate were measured into Folin-Wu sugar tubes and 2.0 cc. alkaline copper tartrate were added. These tubes were then placed in a boiling water bath and left for six minutes. After cooling to room temperature, (or for two or three minutes) 2.0 cc. of the phosphomolybdate solution was added. When all bubbling had ceased the tubes were made up to volume (25 cc. mark) and mixed thoroughly. After 5 minutes the unknowns were compared against the standards in a colorimeter. It was often necessary to make up two or three sets of standards as the lactose content in the cultures was continually decreasing as cultures were incubated.

Calculations: mg. lactose per 100 cc. =

$$\frac{\text{Reading of standard} \times \text{mg. in standard}}{\text{Unknown reading} \times \text{fraction of 100 cc.}}$$

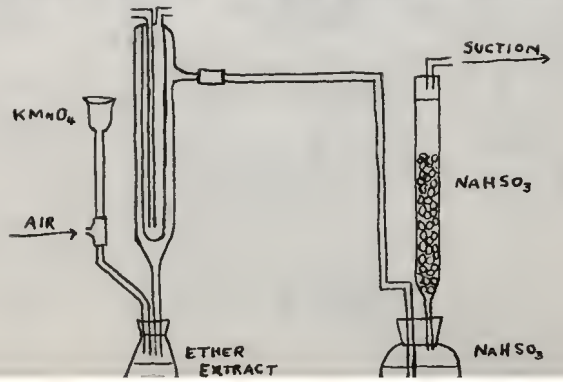
Lactic Acid. The principles involved in the determination of lactic acid were worked out chiefly by Friedemann, Cotonio, and Shaffer (1927). These principles are, namely: (1) removal of interfering substances by ether extraction, (2) further removal of interfering substances by reflux, (3) oxidation of lactic acid to acetaldehyde, and (4) combination of acetaldehyde production by back-titration of bisulphite.

For the lactic acid and succinic acid determinations,

100 cc. of culture were taken from the flask, placed in an evaporation dish and evaporated to approximately 15 cc. on a steam bath. This was then acidified with H_2SO_4 until the end-point of congo-red was reached. The acidified and condensed culture was then taken up with anhydrous sodium sulfate, placed in a Soxhlet extracting thimble and extracted over night. The ether was boiled by means of an 100 watt bulb fitted into a tin, box-shaped unit which gave a good consistent temperature.

After extraction, 100 cc. of distilled water was added to the ether and the ether boiled off, leaving the extracted material in the water. This was then divided in two, setting aside one portion for the succinic acid determination.

The other half was then made slightly alkaline with NaOH, boiled for one or two minutes to break up any lactides that may have formed, and acidified with 10 cc. of 2M. phosphoric acid and 5.0 cc. of 10 per cent manganese sulfate. The volume was made up to about 100-150 cc. and placed in a 300 cc. Erlenmeyer flask which was then placed in the oxidizing apparatus as shown below:



The manganous salts increase the rate of oxidation and the yield of acetaldehyde which is formed by oxidation with potassium permanganate. The manganese salts and permanganate react to give manganite or MnO_2 which is the effective oxidizing agent of lactic acid. If the manganese sulphate were not present, the permanganate would continue to oxidize the acetaldehyde. This procedure, including the extraction was suggested by Nelson and Workman (1937).

A slight suction was applied at the top of the absorption tower so that a slow current of air was circulating through the system. Heat was then applied to the Erlenmeyer containing the ether extract and boiled slowly while the $KMnO_4$ was added drop by drop. When the extract no longer caused the permanganate color to disappear the $KMnO_4$ was shut off and the solution boiled for 5 minutes longer. At this time, the air current was shut off, the bisulphite and glass beads rinsed into a beaker and immediately titrated.

The excess bisulphite was titrated with a 1.0 N iodine solution using starch as an indicator. A small amount of sodium bicarbonate was added to the solution to liberate the combined $NaHSO_3$ which was rapidly titrated with 0.05 or 0.1 N standard iodine solution. The end point was that point at which the color produced failed to disappear after 20-30 seconds.

Calculations: 1 cc. 0.1 N iodine = 4.5 mgm. lactic acid mgm. lactic acid per 100 cc. = titration x 4.5 x 2

Succinic Acid. The remaining one-half of the ether extract was then neutralized with an excess of $CaCO_3$, heated on the steam bath for 30 minutes and filtered. The filtrate contained quantities of calcium succinate if present. The filtrate was then evaporated to a volume of 10 cc. and ethyl alcohol added to give a final concentration of 85 per cent by volume. This was placed in the refrigerator for eight to ten hours, filtered and washed with 85 per cent alcohol. The crucibles were dried at 100°C. and weighed. The weight of calcium succinate times 0.756 equals the weight of succinic acid in the aliquot. This method was also suggested by Nelson and Workman.

Calculations: Mgm. succinic acid per 100 cc. wt. x 0.756 x 2.

Volatile Fatty Acids. Into an 800 cc. Kjeldahl flask, there were measured 50 cc. of culture, 20 gm. of NaH_2PO_4, 15 cc. of concentrated (85 percent) phosphoric acid, a drop or two of mineral oil being added to prevent "bumping", and about 300 cc. of H_2O. This mixture was then placed on a distillation apparatus and distilled until 10.0 cc. of distillate was neutralized with one to five drops of N/100

NaOH. The first 10.0 cc. of distillate were titrated with 0.01 N NaOH and set aside for the Duclaux (1895) method of acid identification. The remaining distillate was also titrated.

The first 10.0 cc. of distillate was made to a 100 cc. volume and placed into a 250 cc. extraction flask. To this was added 10.0 cc. of approximately 0.2 N H_2SO_4 along with a pinch of talcum to insure uniform boiling. The flask was attached to a Hopkins Condenser and distilled slowly by heating with a burner. The distillate was collected in 20.0 cc. portions up to 100 cc. Each portion was titrated with 0.1 N NAOH. The titration of 100 cc. was considered total acid and titrations of 20, 40, 60, 80 and 100 were calculated as per cent of the total. These values were compared with those found by Duclaux for pure acids. The acid was then identified as that whose rate of distillation it most nearly approached.

Calculations: Mgm. acid/100 = Total titration of culture x norm. of NaOH x mol. wt. x 2.

Alcohols. To 100 cc. of culture were added 40 gm. NaCl and a few drops of mineral oil. This was then distilled until 55 cc. of distillate collected from the condensor. To the distillate were added 10 cc. of CO_2-free water and 10 cc. of oxidizing solution. (10 gm. $K_2Cr_2O_7$ in 70 gm. CO_2-free water plus 20 gm. H_2SO_4). This mixture

was put in a 250 cc. flask, attached to a Hopkins condensor and refluxed for one hour over a low flame. This converted the alcohols present to the volatile acid whose carbon atom content was the same.

The same procedure was then followed as for the calculations of volatile acids.

Calculations: Mgm. alcohol per 100 cc. = Total titration x normality x mol. wt. alcohol.

Ammonia N_2. Ammonia nitrogen was determined by the Kjeldahl method. Ten cubic centimeters of culture were taken for the determination and placed in a Kjeldahl flask, enough water being added to make a volume of about 200 cc. This was then placed on a distillation apparatus and 10 cc. of standard N/10 H_2SO_4 were measured into a receiving flask and made up to a volume of approximately 150 cc. The tip of the tube from the condensor was immersed in the acid of the receiving flask, the culture made slightly alkaline with NaOH and the solution boiled for ten or fifteen minutes. Most of the ammonia N_2 is distilled off in the first minute but boiling is continued for some time to complete the process.

The acid in the receiving flask was then titrated with standard alkali. From this titration the amount of acid used was calculated. The ammonia N_2 was the only source of

nitrogen available to the organisms so its utilization was nearly a direct measurement of metabolic activities.

Calculations: Mgm. ammonia N_2 per 100 cc. = cc. H_2SO_4 used to absorb NH_3 x factor x 10.

Factor = Mgm. N_2 taken up by 1.0 cc. H_2SO_4.

Results. Tables 1 to 6 give the results of an analysis of the products formed during the fermentation of lactose. The 0-day column is for the results of the uninoculated broth, which was the same as the other six samples. The 2-day column indicates the results obtained from an analysis of broth in which the organisms had grown for 2 days. The same is true for the 5-day column which is for a 5-day broth culture, and the 10-day is for a 10 day broth culture. The figures for total carbon near the bottom of the table represent the total carbon accounted for in that particular sample. The percentage of carbon which was recovered in the end products is the result of the total carbon of that particular sample divided by the total carbon calculated for the original, or 0-day sample. All results included in these tables are in terms of milligrams percentage or milligrams per 100 cc. of culture.

Table 1. Comparisons of lactose fermentation of "R" and "W" strains of culture 413A.

		"R"			"W"		
Products	0-day	2-day	5-day	10-day	2-day	5-day	10-day
Lactose	1060.00	824.00	760.00	672.00	992.00	932.00	634.00
pH	7.20	5.10	4.96	4.86	6.84	6.10	4.68
Carbon dioxide	5.00	42.00	286.00	363 .00	16.00	72.60	326.00
Lactic acid	9.00	84.00	86.00	74.00	9.00	68.00	86.00
Succinic acid	0.00	Trace	Trace	Trace	0.00	0.00	0.00
Acetic acid	0.00	28.60	26.40	25.00	12.00	16.00	46.00
Alcohol	0.00	38.00	36.00	42.40	3.00	20.00	42.00
Ammonia N_2	52.16	44.26	38.00	37.50	48.30	39.60	36.70
Total carbon	424.00	404.24	445.00	428.70	411.00	416.89	416.70
% carbon recovered		95.2%	104.9%	100.9%	96.9%	98.32%	98.2%

Table 2. Comparisons of lactose fermentation of "R" and "W" strains of culture 942.

		"R"			"W"	
Product	0-day	2 day	5-day	10-day	2-day	5-day	10-day
Lactose	1100.0	796.00	664.00	669.00	1024.00	972.00	682.00
pH	7.2	5.48	4.76	4.64	6.87	6.62	4.80
Carbon dioxide	5.0	126.60	272.00	324.00	22.00	64.80	265.00
Lactic acid	9.0	.86	112.00	104.00	9.00	42.00	106.00
Succinic acid	0.0	Trace	Trace	Trace	0.00	0.00	0.00
Acetic acid	0.0	30.00	40.00	36.20	14.00	26.00	46.00
Alcohol	0.0	34.00	52.20	39.10	8.00	16.00	37.00
Ammonia N_2	56.0	47.40	41.20	38.60	52.00	49.80	42.40
Total carbon	440.0	417.00	430.80	432.20	429.00	442.00	428.00
% carbon		94.7%	97.9%	98%	97.8%	100.4%	97.2%

Table 3. Comparisons of lactose fermentation of "R" and "W" strains of culture 428.

Product	"R" 0-day	"R" 2-day	"R" 5-day	"R" 10-day	"W" 2-day	"W" 5-day	"W" 10-day
Lactose	1120.0	864.00	787.00	704.00	1056.00	964.00	722.00
pH	7.2	5.68	4.85	4.90	6.90	5.42	5.33
Carbon dioxide	5.0	181.00	203.5	237.00	18.04	104.00	250.8 0
Lactic acid	9.0	121.00	167.4	160.65	10.70	66.00	90.00
Succinic acid	0.0	Trace	Trace	Trace	Trace	Trace	Trace
Acetic acid	0.0	22.86	44.0	25.40	20.40	50.20	26.40
Alcohol	0.0	20.3	41.6	52.80	15.90	23.20	67.40
Ammonia N_2	52.0	44.25	36.5	27.60	48.60	39.30	34.70
Total carbon	452.0	473.00	482.7	448.00	448.00	472.30	438.40
% carbon		104%	106%	99%	99%	105%	96.9%

Table 4. Comparisons of lactose fermentation of "R" and "W" strains of culture 911.

Product	"R"				"W"		
	0-day	2-day	5-day	10-day	2-day	5-day	10-day
Lactose	1797.00	1649.00	1276.00	1460.00	1728.00	1425.00	1450.00
pH	6.9	5.51	5.08	4.91	6.65	5.15	4.91
Carbon dioxide	5.0	93.20	261.48	207.30	69.70	195.04	197.68
Lactic acid	9.0	9.60	9.00	9.00	9.20	9.00	9.00
Succinic acid	0	34.4	193.00	105.00	Trace	154.00	194.50
Acetic acid	0	31.24	41.24	33.60	57.36	24.48	24.00
Alcohol	0	5.1	34.27	22.30	Trace	34.50	43.00
Ammonia N_2	71.94	51.78	40.29	42.84	67.98	44.82	42.85
Total carbon	723.7	717.70	698.00	712.00	733.00	706.20	748.80
% carbon		99.1%	96.5%	98%	102%	97.5%	103.4%

Table 5. Comparisons of lactose fermentation of "R" and "W" strains of culture 418A.

Products	"R"				"W"		
	0-day	2-day	5-day	10-day	2-day	5-day	10-day
Lactose	985.00	770.00	714.00	640.00	914.00	892.00	584.00
pH	6.86	5.20	5.21	4.87	6.83	5.24	4.76
Carbon dioxide	5.00	33.45	279.00	391.60	20.00	76.30	347.60
Lactic acid	9.00	54.00	54.40	27.00	9.00	72.00	48.60
Succinic acid	0.00	Trace	Trace	0.00	0.00	0.00	0.00
Acetic acid	0.00	32.04	27.60	24.00	13.00	19.20	44.40
Alcohol	0.00	24.32	22.08	29.20	2.30	17.53	25.53
Ammonia N_2	46.24	38.75	22.10	34.80	40.50	32.81	33.49
Total carbon	386.00	364.00	406.30	397.40	383.15	414.00	379.00
% carbon		94%	105%	102%	99.2%	107%	98%

Table 6. Comparisons of lactose fermentation of "R" and "W" strains of culture 1233.

		"R"			"W"		
Products	0-day	2-day	5 day	10-day	2 day	5 day	10-day
Lactose	1797.0	1500.00	1293.00	1290.00	1714.00	1224.00	1221.00
pH	6.9	5.65	4.96	4.88	6.8	4.93	4.88
Carbon dioxide	50.0	105.60	217.80	196.70	42.68	272.00	229.70
Lactic acid	9.0	9.00	9.00	9.00	9.00	9.00	9.00
Succinic acid	0.0	195.00	376.30	115.00	73.70	349.00	368.00
Acetic acid	0.0	28.80	25.20	120.00	1.68	26.40	29.00
Alcohol	0.0	19.32	31.42	17.70	1.47	17.57	25.30
Ammonia N_2	71.9	48.28	40.29	7.31	54.06	43.69	22.10
Total carbon	736.0	732.00	758.00	677.20	730.00	729.90	728.90
% carbon		99.4%	102%	91.9%	99.1%	99%	99%

Discussion. The biochemical analyses of the end-products of lactose fermentation accounted for what might be considered all of the carbon initiated into the nutrient medium. The "W" strain in each case exhibited latent formation of total acids and carbon dioxide, although small amounts of these were produced by the second day. However, the amount of acid and gas was small and would not be sufficient to produce the visible changes apparent in the acid and gas reaction of the fermentation tube.

Four of the organisms studied did not produce succinic acid either in the "R" form or the "W" form but produced lactic acid, whereas the other two organisms did not form lactic acid but produced great quantities of succinic acid. This production of succinic acid may be due to an increased concentration of carbon dioxide in these two cultures, as suggested by Elsden (1938) although the mechanical set-up was the same in each case and the carbon-dioxide was forced out through the absorption tubes in an identical manner in each culture.

No differences other than a delayed action in the "W" form were found between the "R" and "W" cultures. By the end of ten days incubation, it was found that the "W" strains had utilized the same amount of lactose as the "R" strain.

Reversal of Mutation

Review of Literature. Several workers since Neisser have attempted to reverse the normal dissociation processes of E. coli-mutabile. That is, they hoped to get the typical lactose fermenting strain to give rise to the latent fermenting variant.

Neisser (1906) caused the reversion in one case, by weekly passages through phenol agar. However, he could not repeat the experiment. Hershey and Bronfenbrenner (1936) used a synthetic medium with sodium succinate as the sole source of carbon to revert the mutation and reported it successful. Similar results were obtained by Winning (1937) who cultivated the organisms in peptone water for 21 days. Lewis and Hitchner (1936) failed to change the rapidly fermenting type by growing in a lactose-free medium for four months. Eisenberg (1918) exposed E. coli to petroleum ether for one and one-half hours and produced mutants which lacked the power of lactose fermentation, and remained thus affected for 20 generations. Bungester 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 E. coli into a mutabile form by continued cultivation in glycerin-phosphate

broth.

In a review of enzyme variation, Yudkin (1938) stated that when microorganisms produce enzymes de novo, or in increased amounts in the presence of specific substrates, they may be looked upon as examples of chemical adaptation. He stated further that when enzyme production is associated with the presence of its substrate, it is regarded as possible through (1) natural selection--genetic mutation or, (2) chemical adaptation--an enzyme arising as a response to its chemical environment. Neisser (1906) and Massini (1907) considered B. coli-mutabile as a mutation in the sense of DeVries, whereas Lewis (1934) concluded that the lactose acts as a selective agent but does not exert a specific exciting stimulus to variation. He also found that large inocula speeded up the fermentation of lactose and concluded that it was because of a greater number of the mutant forms. Penfold (1911) believed that enzymes from mutants do not become lost, but go into a minority. That is, they retard until their effects are negligible. These reports did not indicate well-established facts, so it seemed necessary to attempt to simulate conditions which might occur within the intestine of a normal chicken. That is, to establish a medium in which normal colon strains might revert to an unstable mutable form and at the same

time afford some enlightenment upon the stability of the enzyme system.

Experimental Methods. In an attempt to cause the R-forms to revert to the original (W-forms) characters, the organisms were grown in broths in which the only sources of carbon were either sodium succinate, sodium glutarate, or sodium glutamate.

The following synthetic base as given by Stephenson (1930) was found to be satisfactory:

KH_2PO_4	0.1 gm.
$MgSO_4 \cdot 7\ H_2O$	0.07 gm.
NaCl	0.1 gm.
$(NH_4)_2HPO_4$	0.4 gm.
Iron	Trace
Water	100 cc.

To this base were added the sodium salts to give a final concentration of 1.0 per cent. Inoculations of both "R" and "W" forms and a typical colon type were made and the tubes incubated for three days before transfer. This was continued for 45 days while eosin-methylene-blue agar plates were poured at frequent intervals.

Results. Attempts to cause the typical lactose-fermenting colon strain to give rise to the latent fermenting strain ended with six strains showing inhibition of lactose fermenting ability for from 2-4 days after growing them on sodium salts of glutamic, glutaric and succinic acids for a period of six weeks. On eosin-methylene-blue agar plates,

the colonies were colorless and typical of the latent-fermenting strain. However, after 2-4 days they returned to their original appearance on these plates and fermented lactose rapidly when transferred to fermentation tubes.

These results led the writer to believe that the mechanism of lactose fermentation was one of selection and that although the enzyme responsible for the fermentation of lactose is inhibited by the growth of the organism on a lactose-free medium, it is not completely lost and will become a dominant or active characteristic when the organism is again put in the presence of lactose.

RESPIRATORY ENZYME STUDY

Review of Literature

Much work on respiratory enzymes has been done since the beginning of the century. Wieland (1913), Quastel and Whetham (1925) and Stephenson (1928) were pioneers in the field of the study of respiratory enzymes, and their theories and works are very ably discussed by Stephenson (1939). Two distinct types of respiratory enzymes studied were:

1. The dehydrogenases which activate the hydrogen of organic molecules.
2. The oxidases which activate molecular oxygen to accept hydrogen.

Wieland first put forward the theory that biological oxidations consist in the catalytic transfer of hydrogen to some reducible substance. When oxygen enters into the oxidation of the molecule it first forms a hydrate and is subsequently dehydrogenated. For example, acetaldehyde oxidized through its hydrate to acetic acid.

This catalytic transfer refers to the activation of the molecule to liberate its hydrogen or to the activation of oxygen to receive it, or both, if the reaction is under aerobic conditions. The former activity is termed "dehydrogenation" and is catalyzed by "dehydrogenase". The activation of atmospheric oxygen is termed "oxidation" and the enzyme is called "oxidase".

Some organisms such as E. coli have been found to have as many as fifty or more separate dehydrogenations and it would seem that it would be necessary for the organism to produce as many enzymes. Quastel (1926), however, has sought to avoid this multiplicity of enzymes, which act in the capacity of dehydrogenase, by suggesting a general mechanism which accounts for all of them. In brief, the Quastel theory depends upon adsorption of the molecule to the cell surface (internally or externally). This results in an inactivation by which the configuration of the molecule is altered. This alteration causes a gain or loss of hydrogen.

Keilin (1929) gives a discussion on the nature of the oxidases, whereas Hopkins (1926) covers the characteristics of the dehydrases. It would be quite useless to attempt a summary of all the work done in this field. The essence of the work is summarized by Krebs (1938) in his three formulae:

1. Substrate + carrier → reduced carrier + oxidized substrate.
2. Reduced carrier + O_2 → carrier + H_2O.

   Carrier and reduced carrier cancel out leaving:
3. Substrate + O_2 → oxidized substrate + H_2O.

It has been quite well established by Keilin (1938) that the substance acting as carrier in aerobic respiration is cytochrome. That is, hydrogen is not directly transferred to oxygen due to the great span in oxidation-reduction potential, and therefore must be carried by an easily reducible compound. Cytochrome within the organism acts in this capacity. The cytochrome is reduced and is then acted upon by the oxygen which has been activated by oxidase and gives up its hydrogen to it. This reaction may be stopped at various stages by certain poisons, but since they do not apply to this investigation, I shall not discuss them here.

Besides the production of water in the final hydrogen

transfer, quantities of hydrogen peroxide are formed. It appeared that there must be either a great peroxide tolerance on the part of the organism or some mechanism for its disposal. This reasoning led to the investigation of catalase, and a simple method was devised for the determination of its activity in an effort to determine the relationship of its action to the two strains of E. coli-mutabile.

Experimental Methods

Reduction tests were run on the organisms using the method of Thunberg (1920).

A 48 hour culture of organisms (both "R" and "W") was suspended in saline solution and washed three times by centrifugation, made up to a suspension of 20 times the turbidity of McFarland nephelometer tube number I, and aerated by passing CO_2-free and sterile air through the cultures for 20 minutes.

A wire grid was arranged within a large desiccator so that ordinary test tubes could be held under water and upright against the inside. This made it possible to see the reduction tests more easily. The water was held at 37°-38°C. and the tubes were then filled with the following:

1.0 cc. suspension of organisms.

2.0 cc. buffer solution K_2HPO_4 - KH_2PO_4(pH 7.4)

1.0 cc. of 1-5000 methylene-blue (H-acceptor)

1.0 cc. of M/100 (H-donator)

The air was then expelled from the desiccator by suction and time for complete reduction of the methylene-blue to the colorless leuco-base was observed.

The hydrogen donators used were:

Sodium lactate	Sodium glutamate
Sodium citrate	Asparagine
Glycerol	Galactose
Maltose	Sodium succinate
Sodium pyruvate	Sodium fumarate
Cystiene	Glucose
Mannitol	Sodium formate

The oxidase system was studied by the oxygen uptake of the organisms when in the presence of these organic substances:

Sodium lactate	Sodium formate
Glycose	Lactose
Sodium citrate	Sodium pyruvate
Sodium succinate	Sodium acetate
Sodium fumarate	Cystiene

The Warburg apparatus was used for these latter studies. It consists of a constant temperature bath with shaker attachment; flasks to receive organisms, buffer and substratum, and with a cup for filter paper soaked with sodium hydroxide for absorbing the CO_2 as formed. Leading from this flask is a calibrated U-shaped glass tube which contains a fluid, (Brodie solution*). This fluid will

*The composition of Brodie solution is as follows: 500 cc. water, 23 gm. NaCl, 5 gm. sodium tauroglycocholate and a few drops of an alcoholic thymol solution.

rise or fall in the calibrated U-tube with a difference of pressure between the interior of the vessel and the outer air. When the organisms utilize oxygen and produce carbon-dioxide, it is absorbed by the sodium hydroxide in the inner cup and the actual oxygen uptake is measured by a rise of the fluid in the U-tube.

Catalase activities were studied by devising a simple method for the quantitative determination of oxygen freed from a definite amount of hydrogen peroxide added to the suspension of organisms. This was done by the utilization of the Warburg apparatus. One cc. of culture was placed in the flask and 2.2 cc. of buffer solution added. In the side arm was placed 0.5 cc. of hydrogen peroxide, which was poured into the flask as soon as the contents had reached a constant temperature. The catalase produced by the organisms split the hydrogen peroxide, caused a production of free oxygen and subsequent internal pressure which forced the liquid in the U-tube downward. Since the volume of the flask is known, the amount of gas freed could be calculated. A comparison of the "R" and "S" strains was made.

Results

Tables 7, 8 and 9 contain the data obtained in the study of the respiratory enzymes. The anaerobic dehydro-

genation of twelve organic compounds by "R" and "W" forms is shown in table 7, when the results are recorded as time required (in minutes) for the methylene-blue to accept hydrogen and to become completely reduced to a leuco-base.

Aerobic oxidases were not studied from a standpoint of hydrogen transfer, but from a standpoint of oxygen uptake, and table 8 shows the results of these studies. The oxygen taken up by the organisms in the presence of various substrates is tabulated as cubic millimeters of oxygen.

Table 9 shows the relative measurements of catalase activity in six pairs of E. coli-mutabile. Since catalase acts directly upon hydrogen peroxide to liberate oxygen, the measurement of this liberated oxygen should be a determination of the catalase activity, if all other factors are constant. The results given are in terms of cubic millimeters of oxygen liberated under constant temperature and pressure, and with equal amounts and concentrations of bacterial suspension and hydrogen peroxide.

Discussion of the Enzyme System

The results of the studies on the respiratory enzymes brought to light many interesting facts which may serve as a basis for further investigations. When a comparison is made of the results of the dehydrogenase activity and the

Table 7. Reduction time (in minutes) of methylene-blue in the presence of various H-donators.

Chemical	428		911		588		400A		418A		953	
	R	W	R	W	R	W	R	W	R	W	R	W
Sodium formate	7	7	42	31	15	15	7	4	12	11	5	3
Sodium succinate	17	10	12	18	4	5	13	9	8	8	7	3
Sodium lactate	14	8	6	5	8	8	4	9	9	9	5	6
Glucose	5	3	6	4	2	4	5	2	3	2	3	1
Maltose	14	9	7	9	4	5	6	3	3	2	8	4
Mannitol	5	3	9	8	5	5	11	8	4	4	8	5
Sodium fumarate	16	8	-	66	6	6	27	13	11	13	16	6
Sodium glutamate	25	18	-	-	16	18	28	30	20	20	24	12
Sodium pyruvate	9	6	39	42	8	8	12	9	5	6	11	6
Sodium acetate	39	27	-	-	-	-	25	33	28	22	50	21
Sodium citrate	50	50	75	75	75	75	75	75	75	60	70	70
Lactose	-	44	75	75	75	75	35	75	75	70	75	75

Table 8. Cubic millimeters of oxygen taken up at end of sixty minute period.

	418A		40CA		413A		8785A$_1$	
	R	W	R	W	R	W	R	W
Glucose	266	280	127	137	255	226	136	126
Sodium acetate	240	249	122	-	98	107	80	79
Sodium lactate	288	269	179	139	134	154	58	47
Sodium succinate	218	194	120	150	124	87	84	104
Sodium pyruvate	210	243	101	118	108	120	74	68
Sodium fumarate	203	237	130	132	12	62	71	87
Sodium formate	116	109	99	92	-	-	80	80
Sodium citrate	32	52	16	8	15	13	-	-
Cystiene	53	32	31	-	63	70	29	36
Lactose	30	22	10	11	97	90	13	12

Table 9. Catalase activity as measured by oxygen liberation from hydrogen peroxide.

Organism	c.mm. oxygen liberated
M461 R	214.0
M461 W	197.0
413A R	243.0
413A W	241.0
414A R	182.0
414A W	130.0
8785A_1 A R	132.0
8785A_1 A W	125.0
412A R	125.0
412A W	160.0
M6 R	45.0
M6 W	66.0

oxidase reactions when placed in the presence of various organic compounds, it is seen that there is a correlation in all cases except, perhaps, in the cases of sodium acetate and lactose. The former is nearly inactive as a hydrogen donator when under anaerobic conditions (when methylene-blue is the H-acceptor), whereas it ranks with succinate and pyruvate when oxygen accepts the hydrogen. This may possibly be due to the difference in oxidation-reduction potential, or may be due to an inhibition of dehydrogenase activity when oxygen is absent. This latter may be explained by the necessity of oxygen for the formation of the intermediate hydrated forms which would be necessary for the second step leading to dehydrogenation.

The case of lactose is a similar one. It did not contribute to the reduction of methylene-blue in less than 90 minutes and yet it was fairly active under aerobic conditions. In no case was it definitely established that the "R" form or the "W" form differed consistently from each other in these regards, with the possible exception of lactose under aerobic conditions. However, the variation in this case was very slight and not significant.

The results obtained by the peroxide test for catalase are quite self-explanatory. It is evident that there is no difference in the catalase production of the two

organisms. However, it is interesting to note that the organisms are all well able to break down the hydrogen peroxide formed by the oxygen when accepting hydrogen.

SEROLOGICAL STUDIES

Serological studies are often used for classifying members of the colon-typhoid-paratyphoid group and they also give some indication of characteristic protein structure. This would seem to be, then, a logical approach to the study of the protein composition of E. coli-mutabile.

McDaniel (1937) when investigating E. coli-mutabile in chickens found that the organisms were not highly antigenic and that it was difficult to produce a high titer immune serum.

Results

The serological tests are recorded on the following page. The antiserum was obtained from normal rabbits that had been injected eight times, over a period of one month, with doses increasing from 0.5 cc. of a suspension with a turbidity of nephelometer tube number 1 to 2.0 cc. of the suspension paralleling nephelometer tube number 2. The uppermost chart represents the direct agglutination reactions while the lower chart shows the titer of the serum when used in cross agglutination.

AGGLUTINATION REACTIONS

Organism	Serum	Titer
428 R	428 R	1-640
428 W	428 W	1-2560
1300 R	1300 R	1-320
1300 W	1300 W	1-160
1059 R	1059 R	1-640
1059 W	1059 W	1-640
1233 R	1233 R	1-10240
1233 W	1233 W	1-2560

CROSS-AGGLUTINATION

Organism	Serum	Titer
428 W	428 R	1-40
428 R	428 W	1-10240
1300 W	1300 R	1-160
1300 R	1300 W	1-10240
1059 W	1059 R	1-320
1059 R	1059 W	1-320
1233 W	1233 R	1-10240
1233 R	1233 W	1-10240

Discussion

The results obtained in these serological tests confirmed the former work of McDaniel. The titers were quite high in each case, but no significant differences could be found in the two strains by cross-agglutination. These results show without a doubt that the antigenic principles of the organisms are identical in spite of the difference in the rate of lactose fermentation.

PHYSICAL STUDIES

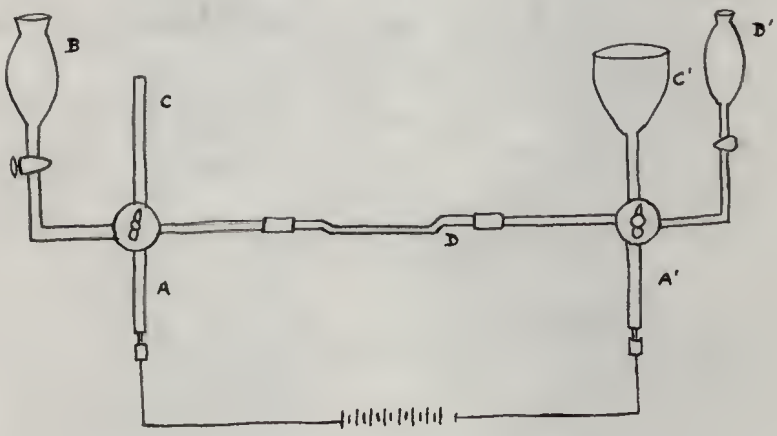
Electrophoresis

Review of Literature. Since the early work of Northrop and DeKruif (1922) in the studies of cataphoretic migration of bacterial cells, a good deal of work has been done on this measurement of organisms' potential drop between the Helmholtz double layer and the medium. Chapman and Lieb (1929) found that three strains of E. coli maintained a constant velocity potential over a period of five years on artificial media and that strains of E. coli isolated from persons whose sera fixed complement with E. coli antigen had a different frequency distribution of electrophoretic migration velocities from those strains isolated from persons whose sera did not fix complement with the same anti-

gen.

With this in mind it was deemed advisable to calculate the migratory velocities of the two strains of E. coli-mutabile in the event that the serological tests proved a difference in protein consistency in the two strains.

Methods. The equipment used was that of Northrop-Kunitz with the Mudd Assembly. The procedure was followed as outlined by the Arthur H. Thomas Company, Philadelphia, Pennsylvania.



A & A' = Zn electrodes
B & B' = $ZnSO_4$ electrode vessel
C' = Funnel for organism
C = Overflow tube
D = Cataphoresis cell

Experimental Results. The following results are recorded as an average time for an organism to traverse 0.0312 mm. within an electrical field which is constant, (135 volts are applied at the zinc electrodes). The results are the average of ten readings taken at the first and second stationary levels of the electrophoretic cell.

Time in seconds/0.0312 mm.

Organism	R-strain	W-strain
8785 A_1A	1.67	2.20
942	2.60	2.88
412 A	3.40	3.42
413 A	2.30	3.80
M 6	2.48	2.30
418 A	3.17	2.80
400 A	2.90	3.30
428 A	2.40	2.66

If the enzyme system of the two strains differed to any extent, it would be expected that the organisms would be antigenically specific. This was not shown to be true for it was found that the organisms gave good cross-agglutination. This fact would lead us to believe that the electrophoretic migration velocities of the two organisms would be the same, if we should accept the work of Chapman and Lieb (1929). The results obtained in this study in-

dicated that five of the "R" strains had a higher electrophoretic velocity than did their corresponding "W" strain, two were lower while one pair had essentially the same velocity.

SUMMARY

B. coli-mutabile is a colon type of organism whose taxonomic position has not been ascertained. The organism is a latent lactose-fermenting type which gives rise to colorless colonies on selective media used in the isolation of members of the colon-typhoid-paratyphoid group. These characteristics cause a great deal of confusion in diagnostic procedures and require several days' incubation before lactose is fermented and a report may be made. Corresponding with the latent fermentation of lactose is the production of papillae-like secondary colonies within the colorless colony on the selective agar plates. These secondary colonies contain rapid fermenting organisms and account for the late fermentation. By culturing the rapid fermenting strain("R") on an inorganic salts-agar and by cultivating the mother strain ("W") on this same medium, one may then have both variants of the same organism.

The term "R", or "red" form, was used from the fact that the rapid fermenting organismsappear as red colonies on eosin-methylene-blue agar. They are developed by al-

lowing secondary colonies to appear in the mother colony and by subsequent passages through lactose broth. The "W" or "white" forms, are the original mother cultures which give the delayed fermentation and are cultured directly from the chicken intestine.

Investigations have been made to determine:

1. Characteristic differences of the slow lactose-fermenting strain "W", and the rapid fermenting "R" strain of E. coli-mutabile which could afford some approach to a new means of identification.
2. The enzyme structure of the two strains.
3. Reasons why the two strains differ.

Chemical analyses of the products of lactose fermentation were made. These products were found to be carbon dioxide, lactic acid, succinic acid, acetic acid, (formic acid, doubtful), and ethyl alcohol. Between 95-105% of the carbon was accounted for in each case. This difference was attributed to the inaccuracy of the methods used and experimental error. It was found that although the "W" strain utilized some lactose by 24-48 hours, it did not utilize an appreciable amount for 5-10 days. At the end of 10 days incubation, both strains were the same.

The rapid fermenting organisms were caused to show inhibition of lactose fermenting ability for 2-4 days by

growing them on sodium salts of glutamic, glutaric and succinic acids. This experiment was quite significant as it indicated a selective action of the substratum on the organism rather than a mutation in the sense of DeVries.

Studies on the electrophoretic migration velocity indicated that there was a difference in the potentials of the two organisms' surface.

The serological reactions verified the results of other workers in that the antibody titers of the organisms were low and that cross agglutination occurred between the "R" and "W" strains.

The Thunberg technique and Warburg apparatus were used in the study of respiratory enzymes on resting bacteria. That is, the organisms were washed and aerated and did not proliferate to any appreciable extent. The results obtained showed a direct correlation between dehydrogenase and oxidase activities in the instances where glucose, sodium lactate, sodium succinate, sodium pyruvate, sodium fumarate, and sodium citrate were used as hydrogen-donators. In the cases of lactose and sodium acetate, the substratum was found to be more active as a hydrogen-donator when oxygen acted as the acceptor than when methylene-blue was used for that purpose.

Catalase tests were run, using an original technique

that required the use of the Warburg apparatus. It was made evident that both "R" and "W" forms of E. coli-mutabile were prolific catalase producers.

These studies suggested that the respiratory mechanisms of B. coli-mutabile caused the following reactions under aerobic conditions:

$$\text{Substrate} + \text{carrier} \xrightarrow{\text{dehydrogenase}} \text{oxidized substrate} + \text{reduced carrier}$$

$$\text{Reduced carrier} + O_2 \longrightarrow \text{carrier} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O$$

Under anaerobic conditions it was concluded that the following reactions occurred:

$$\text{Substrate} \longrightarrow \text{hydrated substrate}$$

$$\text{Hydrated substrate} + \text{H-acceptor} \xrightarrow{\text{dehydrogenase}} \text{oxidized substrate} + \text{reduced H-acceptor} + CO_2$$

CONCLUSIONS

The results of this investigation show that the variants differ only in the velocity of their lactose fermentation. Although it has been shown that lactase is present in the slow lactose fermenting variant in small amounts, it cannot be designated a constitutive enzyme in the same sense as that referring to the enzymes attacking dextrose, maltose, etc. There seems to be a definite relationship between a lactose environment and the in-

creased lactose production. On the other hand, it cannot be strictly called an adaptive enzyme for it is not produced "de novo" when the organism is placed in the presence of lactose.

The origin of the variant form becomes a perplexing problem. If the rapid fermenting variant was considered to be a contaminant of the slow fermenting organism, it would appear that a pure culture of the "W" form could be obtained, and that it would be an organism which would not attack lactose at all. This has never been accomplished, however, and leads the writer to believe that the variant is in reality, a mutant form. The causative agent of this mutation must be a factor arising from the metabolism of the mother culture. Once the newly formed enzyme is established within the culture it becomes enhanced by the lactose present and the lactose containing organism becomes dominant due to the selective action. It appears that more information on the subject of bacterial variation must be secured before definite conclusions may be drawn.

ACKNOWLEDGMENT

The studies made in this thesis were under the direction of Dr. L. D. Bushnell. The writer is deeply indebted to Dr. Bushnell for his interest and many helpful suggestions that made the completion of this thesis possible.

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