Experiments were then made with several other amides, including some belonging to the aliphatic series. No addition products were obtained with acetamide, salicylamide, oxamide, or succcinamide. Succinimide, on the other hand, gave, with potassium iodide and iodine, the addition product $(C_4H_5O_2N)_4.KI.I_3$. This substance had already been prepared and described by Piutti (*Loc. cit*). Phthalimide also gave an addition product with potassium iodide and iodine which has not yet been analyzed. Benzene sulfonamide gave a fine greenish brown precipitate. Experiments with urea, thiourea and benzoylphenylurea all gave negative results.

Anilides.—Attention was next turned to the anilides, and here it was found that the reaction was fairly general, although substances which gave addition products with potassium iodide and iodine frequently did not do so with hydriodic acid, whereas in certain other cases this relationship was reversed. Among the compounds tested are acetanilide, benzanilide, phenylacetanilide, diphenylbenzanilide, aceto-*o*-toluidide, aceto-*p*-toluidide, benz-*o*-toluidide, and methylacetanilide. In the case of a few of the products formed, analyses have already been made, but it seems best to postpone the discussion of these addition products until some more work has been done. They will, accordingly, be reserved for some subsequent paper.

Summary.

1. Benzamide and some other amides form complex periodides containing iodine and hydriodic acid, or else some metallic iodide. The general composition of most of these compounds is $Am_3.MeI.I_2$ and $Am_2.HI.I_2$, though other combinations are observed.

2. Similar reactions seem to be very general in case of substituted anilides.

3. The compounds described in (1) can exist in combination with iodides of heavy metals, like lead and mercury, forming complex molecular aggregates of exceptionally high molecular weight.

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STUDIES IN BACTERIAL METABOLISM.

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XXXI. The Metabolism of the Typhoid-Dysentery-Alcaligenes Group in Milk.

One of the great problems in modern civilization is that of safeguarding that great river of milk which flows daily into practically every city and town. This problem in the last analysis is a bacterial problem, for those changes which render milk unfit for food are induced in it by the growth of microörganisms. Time, temperature, and the kinds of bacteria which find their way into milk largely determine the changes which it undergoes before it is finally consumed or ultimately unfitted for food. When milk is abnormal to sight and taste, it is no longer salable, but unfortunately these somewhat crude indicators are of little service in detecting the presence of microörganisms which may be more dangerous to health or even life than those which produce the more marked decompositions.

Except in a qualitative way, little is definitely known of the nature or even extent of bacterial action on milk. Souring (coagulation), peptonization, ropiness, and slimy milk are well-known chemical changes which may be regarded as decompositions readily recognizable by the layman as well as by the specialist; but the absence of such obvious changes in physical appearance cannot, in any sense, be a criterion for judging of the fitness of milk or of the products made from it for human consumption. That is to say, the esthetic appearance is not necessarily synonymous with safety —indeed, it may be absolutely the reverse. It should be remembered that milk is much more complex in composition than the ordinary media generally used for bacterial cultivation. This complex fluid, which is a well balanced food for man, contains fats, protein, carbohydrates and salts, the latter probably partly free and partly combined with the protein, all dissolved or suspended in water. With such a variety of constituents to act upon, the decompositions which it undergoes will vary greatly, with the type of organism or organisms which gain access to it and the conditions under which the milk is kept.

Certain types of decomposition might be confidently predicted. Those organisms which attack lactose vigorously would produce greater or lesser amounts of acid by the fermentation of this sugar, with but little coincident action upon the protein, for previous experiments have shown that fermentation takes precedence over putrefaction.[1] With respect to the protein sparing action of fats but little can be stated definitely at the present time. Fats, however, would appear to have but little influence upon the protein sparing action of utilizable carbohydrate, for experiments would suggest that fats are less readily utilizable by the ordinary lactose-fermenting bacteria than are carbohydrates.

Any milk which is available for cultural purposes has undergone a certain amount of decomposition, for bacteria in greater or less numbers are always found in it; indeed, there is strong evidence that even milk drawn from the cow with sterile precautions practically always contains some bacteria.[2] The effect of this early bacterial development upon the

[1] Kendall, *J. Med. Research*, **25**, 155 (1911).

[2] Harding and Wilson, *Tech. Bull.* **27**, N. Y. Agr. Exp. Station, Mar., 1913. For résumé of literature.

composition of the milk prior to sterilization for cultural purposes cannot be predicted. The situation is further complicated by the effect of symbiotic growth of bacteria, for it has been shown, for example,[1] that *B. coli* and *B. mesentericus*, growing symbiotically in milk, produce changes as the result of their mutual development which are greater in intensity and different in kind than the sum of their separate activities. Nevertheless, the best obtainable grades of market milk do not, as a rule, show demonstrable deviations in composition from freshly drawn milk. The observations recorded below were made for the purpose of determining the nature and extent of the changes brought about by the growth of various important types of bacteria in sterile certified milk. These determinations include the changes in reaction, as shown by alizarin, neutral red, and phenolphthalein, which indicate somewhat roughly the differential accumulation of alkaline or acid products, and the action on protein as represented by the accumulation of ammonia, ammonia being the only available index of protein breakdown applicable to this problem. The exact analytical details have been published elsewhere.[2] The cultures have been incubated uniformly at body temperatures. Growth is more rapid under these conditions with the organisms used, and the accumulation of metabolic products should be proportionately greater for this reason, particularly during the early days of incubation. These conditions are not met with in the practical handling of milk but are resorted to here in order to exaggerate the changes which probably would take place at a lower temperature with the same organisms in the same time. Furthermore, it is obvious that pure cultures of bacteria would never be met with in practice, although the net results of associated activity of bacteria in milk are in the last analysis the ones which are of practical importance. An initial investigation, in which the activity of each particular type of organism, *per se*, is studied, should serve as a fundamental introduction to the much more complex subject of bacterial antagonism and symbiosis as it presents itself in the milk problem.

Certain analytical difficulties might be anticipated, particularly in the case of those organisms which cause coagulation, peptonization, or other rather marked physical changes in milk, because of the difficulty in obtaining uniform samples for analysis. While these difficulties have been realized to a certain degree, the determinations which have been made in duplicate have not shown noticeable variations, except in those instances where analyses of the residual milk fats were attempted. The difficulties of obtaining uniform samples for these determinations have been found to be so great that observations along this line have been temporarily discontinued. They will be resumed at a later date, using the modifica-

[1] Kendall, *Boston Med. and Surg. J.*, **163**, 322 (1910).

[2] Kendall and Farmer, *J. Biol. Chem.*, **12**, 13 (1912).

tions of the methods stated above, namely, by introducing the appropriate amounts of milk containing cream into Babcock bottles, sterilizing, inoculating, and making the determinations on the entire sample removed at the appropriate time. The results are given in Table I.

TABLE I.

	Whole milk.					Whole milk.				
Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.
	B. alcaligenes.					*B. dysenteriae*, Flexner.				
1	-0.10	+0.20	-0.10	-0.70	-0.11	+0.30	+0.80	+0.70	0.00	0.00
3	-0.20	-0.30	-0.60	-0.70	-0.11	+0.40	+0.40	+0.20	0.70	0.11
7	-0.90	-1.60	-0.80	+5.60	0.92	+0.30	+0.20	-0.10	0.70	0.11
14	-1.60	-1.90	-2.00	+4.20	0.69	-0.10	0.00	-0.30	0.70	0.11
21	-1.40	-2.00	-2.10	+5.60	0.92	-0.40	-0.10	-0.40	1.40	0.23
28	....	....	....	....	....	-0.40	-0.20	-0.60	1.40	0.23
	B. dysenteriae, Shiga.					*B. typhosus.*				
1	+0.50	+1.00	+1.00	0.00	0.00	+0.30	+0.70	+0.70	-0.70	-0.11
3	+0.10	+0.70	+0.70	0.00	0.00	+0.10	+0.80	+2.90	0.00	0.00
7	+0.40	+0.80	+0.50	0.70	0.11	0.00	+0.70	+2.90	1.40	0.23
14	-0.40	+0.40	+0.10	0.70	0.11	+0.20	+0.30	+2.90	5.60	0.92
21	-0.20	-0.10	-0.20	1.40	0.23	+0.10	+0.50	+1.90	6.30	1.15
28	-0.20	-0.20	-0.40	2.80	0.46	+0.60	+0.50	+1.60	5.60	0.92

B. alcaligenes, an organism which ferments no sugars, produces a slightly alkaline reaction in milk, coincident with a slight increase in the ammonia content, amounting to rather less than 1% of the total nitrogen. These results are in harmony with what is known qualitatively of the changes produced in milk by this organism, and the intensity of the reactions corresponds closely with that obtained under similar conditions in broth cultures.[1]

The Flexner and Shiga types of the dysentery bacillus exhibit an initial acidity followed by a definite return to alkalinity, the degree of alkalinity being greater than that of the inoculated milk. There is very little action upon the protein constituents of the milk. The explanation of this initial acidity followed by an alkaline reaction was first clearly demonstrated by Theobald Smith, who showed by presumptive evidence that market milk normally contains a substance which bacteria utilize like dextrose, the amount being about 0.1%. It will be remembered that the dysentery bacilli and *B. typhosus*[2] utilize dextrose in preference to protein for fuel purposes, consequently this initial acidity observed in milk cultures

[1] Kendall, Day and Walker, THIS JOURNAL, **35**, 1216 (1913).

[2] Kendall, *J. Med. Research*, **25**, 155 (1911).

is due to the fermentation of this dextrose-like substance which Theobald Smith has described.[1] The organisms do not ferment lactose, and the return to alkalinity is doubtless due to the formation of basic products from protein which the dysentery bacilli are forced to utilize when this dextrose-like substance is exhausted. It will be noticed that the typhoid bacillus does not exhibit a return to the alkaline reaction. It produces, however, a certain amount of ammonia, indicative of protein breakdown, which appears to be somewhat greater in amount than that produced by the dysentery bacilli and comparable to the amount produced by *B. alcaligenes*. The extent of this protein breakdown, however, is very little, amounting to but 6.3 mg. in 100 cc. of milk in three weeks.

The question might be raised whether this dextrose-like substnace which is fermented by the dysentery and typhoid bacilli is not in reality a mixture of dextrose and galactose formed by the hydrolytic cleavage of lactose during the process of sterilization. A series of experiments conducted in this laboratory, which will be published later, have fully corroborated the observations of Theobald Smith, mentioned above, that such is not the case. The substance which reacts like dextrose is a normal constituent of milk. Further evidence of the correctness of the assumption that fermentation of this dextrose-like substance in milk is responsible for the initial acid reaction produced by the dysentery and typhoid bacilli is furnished by the results obtained in milk to which 1% of dextrose has been added. These organisms produce enough acid in this dextrose milk to cause its coagulation.

This explanation, furthermore, furnishes additional proof of the correctness of the assumption that the terminal alkaline reaction exhibited by the dysentery bacilli is due, in part at least, to the breakdown of protein constituents of milk following the exhaustion of the dextrose. The addition of 1% of dextrose protects this milk protein from attack and the reaction becomes in the latter case progressively acid.

The persistance of an acid reaction in milk cultures of typhoid bacilli is not so readily explained. An explanation of this reaction on the basis of the fermentation of lactose is wholly out of the question, for the typhoid bacillus does not, under any conditions, utilize this sugar. It is conceivable that this organism in breaking down protein after the exhaustion of the dextrose acts upon casein in such a manner as to liberate phosphoric acid, perhaps in the form of acid phosphates. Another possible explanation is the formation of slight amounts of fatty acids from certain constituents of the milk fat.

Conclusions.

1. *B. alcaligenes*, the Flexner and Shiga types of the dysentery bacillus, and *B. typhosus* produce no marked alterations in the appearance of milk,

[1] Smith, Theobald, *J. Boston Soc. Med. Sci.*, **2**, 236 (1898).

nor any noteworthy changes in the composition of milk as indicated by the gross appearance, the changes in reaction, and ammonia production.

2. The presence even of large numbers of these organisms in market milk could not be detected by chemical methods available at the present time during the period when this milk would be salable.

3. The production of an initial acidity followed by an alkaline reaction, which is a well-known cultural characteristic of the dysentery bacilli, is explainable on the basis of the chemical changes which these organisms produce in this medium.

4. The explanation of the permanent acidity exhibited by cultures of typhoid bacilli in milk is not definitely determined.

XXXII. The Metabolism of the Intermediate or Paratyphoid Group in Milk.

The cultural reactions in milk of bacteria belonging to the intermediate or paratyphoid group are, generally speaking, qualitatively like those of dysentery and typhoid bacilli; namely, in the case of *B. paratyphosus alpha* a permanent acid reaction, while other members of this group exhibit an initial acidity followed by a return to alkalinity, resembling the members of the dysentery group in this respect. These observations apply only to the earlier days of growth, however, for it is a matter of common observation that older milk cultures of the paratyphoid group change somewhat in appearance. Cultures of *B. paratyphosus alpha* tend to become somewhat less acid than cultures of the typhoid bacillus, while cultures of *B. paratyphosus beta* and the remaining members of the group become almost opalescent as time goes on, due apparently to a gradual solution of the casein.

The chemical changes which these organisms produce in milk, shown in Table II, resemble those of the dysentery bacilli in a striking manner, both qualitatively and quantitatively, except for the gradual thinning of the medium, mentioned above. This latter phenomenon does not appear to be associated with a noteworthy increase in the amount of ammonia formed or to an unusual degree of alkali production. *B. paratyphosus alpha* produces changes in milk which, measured in terms of changes in reaction and production of ammonia, are very similar to those exhibited by the typhoid bacillus, except that the degree of acidity reached appears to be rather less. This acidity is permanent and is one of the most important cultural characteristics of this organism. This permanent acidity is a noteworthy characteristic of milk cultures of both these organisms. Whether the explanation of this acidity is the same in both cases cannot be stated definitely. *B. paratyphosus beta* differs chemically from *B. paratyphosus alpha* in that the period of initial acidity is followed by a progressively alkaline reaction, the final degree of alkalinity being noticeably greater than that of the uninoculated control. The amount of ammonia

TABLE II.

	Whole milk.					Whole milk.				
Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increses per 100 cc. milk.	NH_3 total N_2 per cent.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.
	B. paratyphosus alpha.					*B. icteroides.*				
1	+0.30	+0.60	+0.70	—1.40	—0.23	+0.40	+0.50	+0.60	2.10	0.34
3	+0.60	+0.80	+0.90	0.70	0.11	—0.70	—0.60	—0.90	1.40	0.23
7	+0.50	+0.70	+1.00	1.40	0.23	—0.40	—1.60	—1.90	2.80	0.46[4]
14	+0.90	+1.10	+1.70	1.40	0.23	—0.20	—3.50	—2.20	2.10	0.34[5]
21	+1.10	+1.00	+0.70	4.20	0.69	—0.40	—3.80	—2.30	2.80	0.46[6]
28	+0.60	+0.60	+0.50	4.20	0.69	—0.40	—3.60	—2.40	3.50	0.57
	B. paratyphosus beta.					*B. Morgan No. 1.*				
1	+0.30	+0.50	+0.60	—0.70	—0.11	—0.10	0.00	+0.50	2.10	0.34
3	—0.70	—0.10	+0.70	0.00	0.00	—0.90	—0.30	+0.40	1.40	0.23
7	—0.60	—1.40	+1.40	1.40	0.23[1]	—1.30	—0.20	+0.70	14.00	2.30
14	—0.80	—2.20	—1.90	2.10	0.34[2]	—0.80	—0.20	+0.80	14.00	2.30
21	—0.50	—1.90	—2.10	2.80	0.46[3]	0.00	+0.20	+1.00	12.60	1.98
28	—0.90	—2.40	—2.00	4.20	0.69	....	....	....	....	....

[1] Milk thin.
[2] Milk thin, somewhat brownish.
[3] Milk very thin; brownish.
[4] Milk somewhat thin.
[5] Milk decidedly thin.
[6] Milk very thin; brownish.

produced is practically the same as that formed by *B. paratyphosus alpha* in milk. By the end of the first week milk cultures of *B. paratyphosus beta* become slightly brown in color and distinctly thinner in consistency than normal milk, and after two or three weeks' incubation the milk becomes almost opalescent.

B. icteroides was at one time regarded as the etiological factor in yellow fever; it is now classified as a member of the hog cholera group. Culturally it resembles *B. paratyphosus beta*, and this cultural resemblance is also manifested chemically, the reactions of the two organisms being very similar. This same brownish decolorization and thinning of milk cultures of *B. paratyphosus beta* is also a prominent feature of milk cultures of *B. icteroides.*

The Morgan bacillus, which it will be remembered is rather frequently met with in the dejecta of babies suffering from diarrhea,[1] is more proteolytic than the other members of the intermediate group. This is in accord with similar observations made in broth cultures.[2]

Conclusions.

1. Milk containing *B. paratyphosus alpha*, *B. paratyphosus beta*, *B. icteroides*, or the Morgan bacillus, exhibits no noteworthy changes in chem-

[1] Morgan, *Brit. Med. J.*, Apr. 21, 1906, p. 908; July 6, 1907, p. 16; Kendall, Day and Bagg, *Boston Med. and Surg. J.*, **169**, 741 (1913).

[2] Kendall, Day and Walker, THIS JOURNAL, **35**, 1221 (1913).

ical composition, particularly during the early days of incubation. The gradual decrease in opacity, which is a characteristic of old milk cultures of *B. paratyphosus beta* and *B. icteroides* will be of no practical value in detecting the presence of these organisms in market milk.

2. The initial acidity exhibited by all the members of the paratyphosus group is similar in origin to that of the typhoid-dysentery group, due to the fermentation of the dextrose-like substance which appears to be a normal constituent of fresh milk.

3. *B. paratyphosus alpha* reacts like *B. typhosus* in milk; that is, it produces a permanent acidity. The cause of this permanent acidity is not definitely known.

4. The Morgan bacillus is more proteolytic than the other members of the intermediate group.

XXXIII. The Metabolism of the Coli-Proteus-Cloacae Group in Milk.

Among the organisms commonly found in milk are *B. coli*, *B. proteus*, and, perhaps to a lesser extent, *B. cloacae*. *B. coli*, furthermore, is an organism found constantly in the intestinal tract of man and mammals, and its presence in milk is popularly supposed to indicate contamination with fecal matter, hence its presence is frequently regarded as an indication of filth. This conception is undoubtedly attributable in the last analysis to the importance of the colon bacillus as an indicator of pollution in water. The numbers of colon bacilli which may be present in a sample of milk do not necessarily furnish any evidence of the nature or extent of fecal contamination, for there is no way of distinguishing between the initial number of these organisms in a given sample of milk and their descendants. Furthermore, colon bacilli have been shown by many observers to occur in large numbers on dried grains and hay. These organisms are readily detached from these grains, and, together with dust, may readily find their way into the milk pail quite independently of any direct fecal contamination. The subsequent development of *B. coli* and the numbers of it which appear in milk are the results of time and temperature rather than initial seeding, for this organism grows very rapidly in this medium. The analyses recorded in the accompanying chart show that *B. coli* does not attack milk proteins to any appreciable extent, and this might have been confidently predicted, for *B. coli* acts energetically upon lactose, bringing about coagulation in from 24 to 72 hrs. as a rule. The resulting product is potentially buttermilk, that is to say, a pure culture of this organism produces a marked lactic acid fermentation with acid coagulation of the casein. The degree of acidity produced is quite considerable, amounting to more than 5% reckoned in terms of normal acid at the end of the third day. It is a noteworthy fact that *B. coli* does not produce noticeable amounts of gas in milk, although the lactose content of this medium is several times that of lactose broth, in which medium a considerable amount of gas is formed. No satisfactory explanation of this phenomenon is available. It is worthy of note, however, that gas formation proceeds rapidly in milk when *B. coli* is grown symbiotically with certain actively peptonizing bacteria, notably strains of *B. mesentericus*.[1] Here gas formation is actually greater than is the case when *B. coli* is grown in pure culture in lactose broth.

TABLE III.

Days.	Whole milk. Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.
		B. coli.			
1	+0.10	+1.70	+0.20	0.00	0.00
3	+0.50	+4.40	+5.50	0.00	0.00[1]
7	+1.90	+4.30	+5.60	1.40	0.23[1]
14	+2.50	+4.90	+5.30	1.40	0.23[1]
21	+2.90	+5.30	+6.20	2.10	0.34[1]
28	+3.20	+6.40	+6.00	1.40	0.23[1]
		B. cloacae.			
1	−0.10	+0.40	+0.80	2.10	0.34
3	+0.50	+0.70	+0.90	1.40	0.23
7	+0.50	+1.70	+1.20	3.50	0.55[2]
14	+2.50	+2.10	+3.80	4.20	0.69[2]
21	+2.70	+1.80	+3.00	5.60	0.92[2]

Days.	Whole milk. Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.
		B. proteus.			
1	+0.30	+0.60	+0.60	8.40	1.35
3	+1.60	+1.10	+2.80	16.80	2.70[3]
7	+1.90	+2.90	+4.80	27.30	4.38[4]
14	+0.80	+3.00	+5.10	32.20	5.17[5]
21	+1.10	+3.50	+5.30	37.10	5.96[5]
28	+3.20	+2.00	+5.70	36.05	5.85[5]
		B. proteus (20°).			
1	−0.10	+0.30	+0.90	4.90	0.77
3	+1.40	+0.70	+1.60	14.70	2.33[4]
7	+1.30	+1.90	+3.80	22.40	3.53[4]
14	+1.50	+3.60	+4.50	33.60	5.28[5]
21	+1.70	+3.40	+4.60	34.30	5.40[5]

[1] Coagulated. No gas.
[2] Coagulated. Somewhat viscid, no gas.
[3] Coagulated; peptonized.
[4] Coagulated; marked peptonization.
[5] Coagulated: extensive peptonization.

B. cloacae is probably far less commonly met with in milk than *B. coli* or *B. proteus*, although it is frequently found in sewage and sewage polluted water. This organism, like *B. coli*, ferments lactose, but it will be observed that the degree of acidity attained is scarcely one-half that produced by the colon bacillus under the same conditions. The same phenomenon is met with in lactose broth. The degree of proteolysis, as measured by the increased ammonia, is about twice that of the colon bacillus reckoned in terms of milligrams of ammonia per 100 cc. of milk. The amount, however, is not great, being less than 6 mg. in 100 cc., or 60 parts in a million parts of milk. These chemical changes agree closely with those described for this organism in broth cultures[2] so far as the initial stages are concerned. *B. cloacae*, however, does not appear to form alkali in milk even after prolonged cultivation, although it forms alkali in sugar

[1] Kendall, *Boston Med. and Surg. J.*, **163**, 322 (1910).
[2] Kendall, Day and Walker, THIS JOURNAL, **35**, 1227, 1230 (1913).

broth cultures containing 1% of utilizable sugar after an initial acidity. This organism appears to be able to decompose the 1% of sugar of nutrient sugar broths in a comparatively short period of time; the reaction then becomes alkaline, due to the accumulation of products of protein breakdown, for it attacks protein after the sugar is exhausted. The amount of sugar in milk frequently exceeds 6%, which would require a much longer time to decompose than 1%, and it is probable that even after three or four weeks the fermentative process has not entirely ceased. *B. cloacae* does not appear to be able to ferment 6% of sugar completely. Neither *B. coli* nor *B. cloacae* produce any chemical reactions which would indicate that butter fat plays any prominent part in their metabolism when they are grown in whole milk.

B. proteus differs from *B. cloacae* and *B. coli* in that it does not ferment lactose. This has been shown to be the case in nutrient broth which does not contain utilizable sugar.[1] This organism, however, acts vigorously upon milk proteins; even at the end of 24 hrs., 8.5 mg. of ammonia have been formed, and at the end of three weeks it has reached a maximum of about 37 mg., corresponding to the decomposition of 6% of the total nitrogen of the milk. By the end of the third day milk is coagulated and peptonized, and after seven days the peptonization is very extensive. Contrary to what might be expected in the presence of such active proteolysis, the reaction of the milk becomes progressively acid in spite of the rapid peptonization. This might be attributable to the liberation of acid phosphates following the decomposition of the casein or to a partial decomposition of the butter fats with the liberation of fatty acids. Further work, however, will be required to elucidate this point.

A portion of the milk inoculated with *B. proteus* was allowed to remain at room temperature (20°) in order to determine the influence of the temperature of incubation upon the rate of metabolism. The results show that while decomposition proceeded somewhat more slowly, yet it proceeded at a fairly rapid rate, and at the end of the third week it had attained practically the same degree as the corresponding growths incubated at 37°.

Conclusions.

1. *B. coli* and *B. cloacae* ferment lactose vigorously, and their growth in milk is associated with a production of an increased acidity, but there is but little action upon protein constituents of milk as measured by the ammonia content.

2. The lactose appears to protect the milk proteins from noticeable decomposition.

3. Although *B. coli* and *B. cloacae* ferment lactose, they do not produce noticeable amounts of gas in milk, contrasting in this respect with the

[1] Smith, Theobald, *Am. J. Med. Sci.*, 110, 283 (1895).

extensive gas production in lactose broth, which is a noticeable feature of their growth in this medium.

4. *B. proteus* does not attack lactose; it attacks the milk proteins with the formatiom of a considerable amount of ammonia, amounting to about 6% of the total nitrogen of the milk. At room temperature the proteolytic action is somewhat less rapid, although it proceeds vigorously.

XXXIV. The Metabolism of the Subtilis-Mesentericus Group and B. Pyocyaneus in Milk.

Milk which has been properly pasteurized should contain comparatively few living bacteria. The death rate is greatest among the non-spore-forming organisms; various observers, notably Ayers,[1] have shown, however, that the non-spore-forming lactic acid bacteria very frequently escape destruction in sufficient numbers to bring about souring even of pasteurized milk. This is not always the case, however, and if lactic acid bacteria do survive in small numbers, they may be overgrown or their effects neutralized by the rapid development of spore-forming bacteria, most frequently those of the *subtilis-mesentericus* type. These latter organisms do not ferment lactose and they bring about a rapid, deep-seated change in the protein constituents of milk which are readily recognizable by the extensive peptonization. Lübbert[2] has studied the action of certain peptonizing bacteria belonging to the *subtilis-mesentericus* group, and his experiments indicate that these organisms have no action upon lactose, nor, apparently upon the milk fat; the protein, however, undergoes a deep-seated change. These organisms, furthermore, are very widely distributed in nature, and they form spores which are very resistant to heat and drying. The constant association of these bacteria with hay and grains and the ease with which they are transported with dust makes their abundant presence in milk which has not been handled with the utmost care easily explainable.

It is not certain that these organisms produce disease in man, but there are numerous observations by Flügge, Vincent,[3] and others which would indicate that their entrance into the alimentary tract in large numbers, particularly in young children, would cause severe or even fatal diarrhea and toxemia. The rapidity with which members of the *subtilis-mesentericus* group grow in milk, particularly in the absence of lactic acid bacteria, a condition which is more frequently met with in pasteurized than in non-pasteurized milk, makes this food potentially dangerous. Even at comparatively low temperatures they grow rapidly, so that their increase even in a comparatively short time may be very considerable.

[1] Ayers, Bur. of Animal Ind., *Bull.* **126**, Nov. 14, 1910; *Circular* **184**, Apr. 23, 1912; *Bull.* **161**, Mar. 24, 1913.

[2] Lübbert, *Z. Hyg.*, **22**, 1 (1896).

[3] Flügge, *Ibid.*, **17**, 272 (1896); Vincent, "Acute Intestinal Toxemia in Infants, London, 1911.

TABLE IV.

Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 *total* N_2 per cent.	Remarks.
	Whole milk.					
					B. subtilis.	
1	0.00	0.00	—0.10	0.00	0.00	
3	+0.20	—0.30	—0.40	7.70	1.23	Coagulated; slight peptonization.
7	—0.30	—1.60	—0.30	23.8	3.76	Marked peptonization.
14	—1.50	—2.70	....	73.5	11.80	
21	....	—1.80	—1.40	92.5	14.80	Coagulated; extensive peptonization.
28	....	—3.30	—2.30	109.9	17.62	
					B. mesentericus.	
1	+1.00	+2.80	+3.20	4.20	0.67	
3	+1.90	+4.90	+6.20	11.90	1.91	Coagulated. Some peptonization.
7	+2.30	+5.20	+7.00	32.20	5.17	Coagulated. Extensive peptonization
14	....	....	+6.90	56.70	9.10	
21	+3.00	+2.50	+5.60	95.20	15.30	
28	+3.80	+4.20	+6.60	110.40	17.70	
					B. pyocyaneus.	
1	+0.10	+0.20	+0.10	0.70	0.11	Cream ring slight green-blue.
3	+2.30	+0.70	+1.30	6.30	1.01	Medium thin, yellow-green; slightly viscid
7	+1.20	—1.40	—0.50	51.10	8.20	Medium thin, green-yellow, very viscid
14	+0.30	—2.00	—0.10	123.20	19.80	Very thin, green, very viscid.
21	....	....	—0.20	171.10	27.50	
28	....	....	—1.90	159.90	25.65	

Bacteriologists are by no means in accord in defining precisely the characters which differentiate the members of the hay bacillus and potato bacillus groups. Theobald Smith states that *B. subtilis* ferments no sugars, that is, it is an obligate proteolyte, in other words, and his classification is accepted without question in this work. *B. mesentericus* ferments dextrose, and there is considerable evidence that it forms fatty acids, at least to a moderate extent, from milk fats. Both of these organisms have been found to break down the protein of milk energetically, as is shown in the accompanying tables. This breakdown, furthermore, continues without interruption for at least four weeks at the body temperature, so that there is produced at the end of this time 110 mg. of ammonia above that of the uninoculated controls kept under the same conditions. This breakdown of protein results in the liberation of ammonia which corresponds to nearly 18% of the total nitrogen of the milk. Even these active proteolytic organisms, however, do not produce any considerable amount of protein breakdown during the first 24 hrs. of growth at body temperature, and it is almost certain that if they were kept at room temperature, or at the temperature at which milk is ordinarily shipped, that is, below 50°, these changes would be scarcely detectable in the first few days. This should not be construed, however, to mean that milk containing considerable numbers of these organisms is a fit food for infants.

B. subtilis produces a progressively alkaline reaction in milk, which is readily explainable on the basis of the decomposition of the milk proteins, resulting in the accumulation of ammonia and other basic products. At the third day peptonization is well marked. This peptonization continues until the casein of the milk is almost completely liquefied. *B. mesentericus* produces a progressively acid reaction which manifests itself in spite of the very considerable decomposition of protein and accumulation of ammonia, resembling *B. protens* in this respect.[1] The initial acidity is attributable to the fermentation of the small amount of dextrose or dextrose-like substances normally present in milk. The subsequent development of acid is perhaps due, in part at least, to the breakdown of milk fat and the liberation of fatty acids. Whether a coincident liberation of acid phosphates takes place cannot be stated.

B. pyocyaneus is rarely found in milk which has been properly handled, but it may occasionally find its way into milk through contaminated water, for this organism is sometimes found under these conditions. It is even more active proteolytically than either *B. subtilis* or *B. mesentericus*, and at the end of the third week more than 170 mg. of ammonia, amounting to 27.5% of the total nitrogen in milk, has accumulated. During the first 24 hrs. of incubation the milk becomes blue-green in color, particularly the cream layer, and by the end of the third day the milk is decidedly green throughout. It is then very viscid. By the end of the second week the milk is extremely thin and watery in appearance, and when touched with a platinum needle it can be drawn out into long, slimy threads. The slight acidity which appears during the first three days is perhaps due to the decomposition of certain constituents of the milk fat, for the organism ferments no sugar.

Conclusions.

1. *B. subtilis* and *B. mesentericus* and *B. pyocyaneus* act energetically upon the protein constituents of milk.

2. Their growth in milk is characterized by a noteworthy production of ammonia that is far greater in amount than that produced by the ordinary pathogenic bacteria.

3. The production of ammonia is accompanied by a progressively alkaline reaction in the case of *B. subtilis*, by a transient initial acidity in the case of *B. pyocyaneus*, while *B. mesentericus* produces a progressive acidity.

[1] Kendall and Farmer, *J. Biol. Chem.*, **12**, 215 (1912).

4. *B. pyocyaneus* produces a green coloration in milk even at the end of 24 hrs.

XXXV. The Metabolism of B. Diphtheriae, B. Suipestifer, Vibrio Cholerae, and B. Tuberculosis in Milk.

One of the diseases which is definitely transmissible by milk from man to man is diphtheria. There are numerous instances on record where epidemics varying from a few cases to rather extensive outbreaks have been traced definitely to milk supplies. Almost without exception the evidence is completed by the discovery on the farm or at some station where milk has been handled of a case of diphtheria, or a diphtheria carrier has been recognized.

The diphtheria bacillus, however, as appears to be the case for the majority of those organisms progressively pathogenic for man, does not produce deep-seated changes in the medium in which it is growing. A study of the metabolism of the diphtheria bacillus in milk which appears below shows that this organism produces but very little change in the protein constituents of milk, the amount of ammonia formed being but 3.5 mg. per 100 cc. in 14 days. These figures are in close agreement with those obtained by the same organism incubated under parallel conditions in broth.[1] This organism produces, as the tables show, a progressive acidity in milk, the amount of acid produced, however, being comparatively little. This organism ferments dextrose, and it is certain that at least a portion of this acidity is attributable to the development of acid from the small amount of dextrose-like substance which, as has been stated above,[2] is normally found in fresh milk. Whether any of the acidity produced by this organism is attributable to the liberation of acid phosphates, incidental to the breakdown of casein, or whether it is due to a slight decomposition of milk fats cannot be stated. This organism does not ferment lactose, consequently the acidity observed cannot be attributable to the breakdown of this sugar. The very slight changes in the reaction and composition of milk, as indicated by the tables, show conclusively that this organism could not be detected in milk directly by ordinary chemical procedures.

The bacillus, Swine Plague No. 8, is a member of the hemorrhagic septicemia group pathogenic for swine. It is not an organism which causes noteworthy decomposition of protein. Even in broth containing no sugar, where the conditions for proteolysis are most favorable, this organism has been shown to produce but very little decomposition.[3] It also produces but very little protein breakdown in milk, the maximum amount,

[1] Kendall, Day and Walker, THIS JOURNAL, 35, 1209 (1913).

[2] THIS JOURNAL, Study xxxi. Smith, Theobald, *J. Bact. Soc. Med. Sci.*, 2, 236 (1898).

[3] Kendall, Day and Walker, THIS JOURNAL, 35, 1218 (1913).

TABLE V.

Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.
	Whole milk.				
	B. diphtheriae.				
1	−0.10	+0.10	0.00	1.40	0.23
3	+0.80	+0.20	+0.40	1.40	0.23
7	+1.70	+0.60	+0.30	1.40	0.23
14	+1.00	+0.60	+0.70	3.50	0.56
21	+1.10	+0.50	+1.10	1.40	0.23
..	....	....	....	....	..
	B. suipestifer.				
1	0.00	+0.20	+0.50	2.10	0.32
3	+0.40	+0.20	+0.70	2.80	0.46
7	−0.80	+0.50	+0.70	2.80	0.46
14	−1.50	−0.10	+0.50	2.80	0.46
21	−1.30	−0.90	+1.10	3.50	0.56
..	....	....	....	...	..

Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. increase per 100 cc. milk.	NH_3 total N_2 per cent.
	Whole milk.				
	Vibrio cholerae.				
1	+0.40	+0.50	+1.30	−0.70	0.11
3	+0.80	+0.50	+0.70	0.70	0.11[1]
7	+0.10	+0.70	+1.00	0.00	0.00[2]
14	+0.30	+0.30	+0.90	2.10	0.33[2]
21	+1.80	+1.00	+0.90	2.80	0.46[2]
28	+0.90	+0.30	+0.70	1.40	0.23
	B. tuberculosis "W."				
1	+0.10	+0.20	+0.20	0.00	0.00
3	−0.10	−0.40	−0.50	7.70	1.23
7	−0.60	−1.80	−1.60	7.70	1.23[3]
14	−0.20	−2.70	−1.70	9.10	1.45[4]
21	−0.80	−1.70	−1.80	11.20	1.80[5]
28	−1.00	−2.20	−1.80	12.60	2.02[6]

[1] Slightly coagulated.
[2] Firmly coagulated.
[3] Milk brown; somewhat thin.
[4] Milk brown; thinner.
[5] Milk brown; very thin.

3.5 mg., being found on the 21st day. This slight amount of ammonia, which accounts for but 0.56% of the total nitrogen of the milk, is so slight that it would escape detection. There is a slight production of acid which is persistent when phenolphthalein is used as an indicator, but which is replaced by an alkaline reaction when the milk is titrated with alizarin or neutral red as an indicator, the alkalinity disappearing before the 7th day with the former, and between the 7th and 14th days with the latter. The possible sources of this acid are so numerous that no definite statement can be made about it other than that the initial acidity, at least, can logically be attributable to the fermentation of the small amount of dextrose which is apparently a constituent of normal milk. Organisms of the hemorrhagic septicemia group, it should be stated, have never been found in demonstrable numbers in market milk; consequently, this organism has but little significance in this medium.

The vibrio of Asiatic cholera has at times been reported as transmissible from man to man through milk. The observations of Hesse[1] would indicate that fresh cows' milk exercises a strong bactericidal action upon cholera vibrios and that this bactericidal action is removed when milk is heated for some time. Prolonged heating, according to Hesse, appears to change milk in such a manner that cholera vibrios no longer grow in it. It is

[1] Hesse, *Z. Hyg.*, 17, 270 (1894).

generally assumed that this organism finds it way into milk through contaminated water, for the cholera vibrio, so far as is known, is never found in the intestinal tracts of cattle: it is conceivable that the organisms might be introduced into milk by cholera carriers who have the organisms on their hands. A certain amount of presumptive evidence in favor of this view is furnished by the fact that without exception those cases of cholera which appear to have been acquired from drinking milk have occurred in the Orient where the handling of milk is very much less carefully regulated than in more enlightened communities. The cholera vibrio ferments lactose fairly readily and it is not surprising to find a certain amount of acid development in milk. Kitasato,[1] in 1889, showed that the growth of cholera vibrios in milk was associated with an acid reaction. The amount of acid, however, is not very great, but it is sufficient to cause coagulation on the 7th day. The amount of protein breakdown, as indicated by the accumulation of ammonia, is very small indeed: this is in harmony with what has been observed previously[2] when this organism is grown in ordinary broth containing utilizable sugars, the carbohydrate sparing the protein from breakdown. Here again the changes induced in the milk, even when it is kept at body temperature where conditions are apparently optimum for a rapid development of the organisms, are very slight and would absolutely escape detection by ordinary chemical means.

The distribution of tubercle bacilli, particularly bovine tubercle bacilli, in milk is a problem which has been studied for many years. The consensus of opinion at the present time appears to be that occasionally bovine bacilli which enter the intestinal tract of man with milk—particularly young children—may sometimes result in infection. It is assumed, tacitly at least, that these organisms do not develop appreciably in milk, and there are very few references in the literature referring to the effects of their growth in this medium. Klein,[3] however, has shown that the tubercle bacillus may grown fairly rapidly in this medium. *B. Tuberculosis "W.,"* which has been studied in this connection, is an avirulent human tubercle bacillus which grows fairly rapidly on artificial media. It will be observed from an examination of the accompanying table that this organism produces a fair amount of ammonia in milk, amounting to over 12.5 mg. per 100 cc. in 28 days. Over half of this ammonia is produced by the end of the third day, indicating that at least the rapidly growing varieties of the organism develop in this medium with moderate luxuriance. The reaction produced is progressively alkaline except for the first 24 hours, when there appears to be a very slight acidity. The degree of alkalinity

[1] Kitasato, *Z. Hyg.*, **5**, 494 (1889).

[2] Kendall and Farmer, *J. Biol. Chem.*, **12**, 467 (1912).

[3] Klein, *Cent. Bakt., I. Abt.*, **28**, 111 (1900).

however, never becomes very great and a consideration of the metabolism of this strain would indicate that even rapidly growing tubercle bacilli do not produce noteworthy changes in milk. At the end of the 7th day the milk appears to be somewhat thinner than normal, and by the end of the third week it is very thin, almost opalescent, resembling in this respect milk in which *B. paratyphosus beta* and *B. icteroides* have been growing. It is very probable that this thinning is due to a decomposition of the casein. Monvoisin[1] has called attention to the milk of tuberculous cattle. His results show that milk drawn from cattle with tuberculous udders very frequently exhibits a hypoacidity and the milk itself is very thin, resembling in composition normal blood serum. It would appear that the changes noticed by Monvoisin are qualitatively somewhat analogous to those noted in the experiments described above, particularly with reference to the increased alkalinity of the milk and the gradual thinning. It is by no means to be assumed, however, that the changes observed by this author are parallel in any sense with those observed in milk infected with a rapidly growing, avirulent, human strain of the tubercle bacillus. Moussu and Monvoisin[2] have studied the composition of milk of tuberculous cows somewhat more extensively than the analyses reported above. They find that in cows with tuberculous udders the acidity of the milk decreases as the disease progresses. There is a decrease in the casein, which has also been shown by Storch. The chlorine content of the milk decreases and the normal acidity is reduced 50% or even 75%. The total nitrogen content is also decreased as well as the fat. These changes, taken in connection with the analyses recorded above, indicate that the tubercle bacillus, grown in milk, and in milk drawn from cows suffering from advanced tuberculosis of the udder, shows a noteworthy diminution in its general composition, these changes being a decrease in acidity, or rather, an increase in alkalinity; a thinning of the milk, and, according to Moussu and Monvoisin, a diminution in the amount of butter fat.

Conclusions.

(1) The diphtheria bacillus produces no visible change in milk even after three weeks' growth at body temperature, and chemically the changes are so slight as to escape detection by ordinary chemical methods.

(2) The cholera vibrio produces an acid coagulation in milk by the end of the third day, which is attributed to the fermentation of the lactose. There is but little coincident protein breakdown, the lactose protecting the milk proteins from degradation.

(3) The rapidly growing, avirulent, human tubercle bacillus grows luxuriantly in milk, producing a moderate breakdown of the proteins and a progressive alkaline reaction. After a week's incubation the milk is

[1] Monvoisin, *Rev. d. Med. Veterin.*, **87**, 16 (1910); *Compt. rend.*, **21** (1909).

[2] Moussu et Monvoisin, *Compt. rend.*, **62**, 26 (1907).

distinctly thinner in consistency and slightly brownish in color. At the end of three weeks the milk is decidedly brownish and almost opalescent.

XXXVI. The Metabolism of Certain Members of the Coccal Group in Milk.

There has been a great deal of discussion regarding the significance of the streptococci found in cows' milk. The most common variety of these, *Streptococcus lacticus*, (Kruse),[1] is culturally very similar to the *Streptococcus pyogenes*, according to Kruse,[1] Heinemann,[2] and others. This organism, together with its numerous variants, appears to be an almost constant inhabitant of ordinary milk, in which medium it usually produces an acid coagulation. Streptococci which are but imperfectly differentiated from *Streptococcus lacticus* are found frequently in the milk drawn from cows with inflammed udders, and epidemics of sore throat, frequently of a severe nature, have also been attributed to streptococci which belong to this same group.

Streptococcus pyogenes grows with moderate luxuriance in milk, producing an acid coagulation of the casein on the third to the seventh day or even earlier, due to the fermentation of lactose. It might be confidently predicted, and the table shows, that there is no considerable coincident breakdown of protein, which is indicated by the relatively slight increase in the ammonia content of the milk even after several days' incubation. Aside from the coagulation which this organism, *Streptococcus pyogenes*, brings about, there is no noticeable change in the composition of the milk, and the changes even when measured quantitatively are very little.

Staphylococcus aureus is much more actively proteolytic than the Streptococcus. This organism ferments lactose as well as other sugars, and it is not surprising to find that the reaction becomes progressively acid, the acidity amounting to 7% at the end of the 14th day. Coagulation is already complete at the end of the third day and there appears to be a certain amount of coincident peptonization which is most marked by the end of the third week. Whether this peptonization is in reality a true liquefaction of the casein coagulum or a contraction of it cannot be stated definitely; from the relatively small amount of ammonia produced, amounting to but 12 mg. in 14 days, it would seem that the proteolytic activity exhibited by this organism, although somewhat greater than that of the Streptococcus, is not sufficient to account for what appears to be a considerable degree of peptonization. The proportionate increase in ammonia formed by the Staphylococcus in milk, as compared with that of the Streptococcus, is in about the same proportion as the ammonia formation by these organisms in broth under the same conditions, and it would appear that the utilization of protein by the Staphylococcus is accompanied

[1] Kruse, *Centr. Bakt.*, 34, 737 (1903).

[2] Heinemann, *J. Inf. Dis.*, 3, 173 (1906).

by a somewhat deeper-seated change or changes of the protein than is the case when the Streptococcus utilizes the same protein. Whether *Staphylococcus pyogenes aureus* or the chromogenic variants of this organism are to be regarded as pathogenic bacteria occasionally present in milk cannot be definitely stated. This organism usually appears to gain entrance to the tissues of man through abrasions in the skin, or occasionally through damaged mucous membranes. It is quite unlikely, however, that the mucous membrane of the gastro-intestinal tract is a portal of entry for this organism.

TABLE VI.

	Whole milk.						Whole milk.				
Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. per 100 cc. milk.	NH_3 total N_2 per cent.	Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. per 100 cc. milk.	NH_3 total N_2 per cent.
	Streptococcus pyogenes.						*Mic. zymogenes.*				
1	+0.40	−0.30	+0.10	0.00	0.00	1	0.00	+0.80	+2.20	2.80	0.44
3	+0.30	−0.20	+0.30	2.10	0.32	3	+1.30	+3.10	+5.40	5.60	0.88[4]
7	+0.30	+0.30	+0.30	1.40	0.22[1]	7	+3.40	+2.50	+6.30	5.60	0.88[4]
14	+0.50	+0.40	+0.50	2.80	0.44[1]	14	+3.30	+3.60	+7.50	8.40	1.32[4]
21	+0.30	+0.70	+0.60	3.50	0.55[1]	21	+3.30	+3.80	+7.10	8.40	1.32[4]
	Staphylococcus aureus.						*Mic. melitensis.*				
1	+0.30	+0.80	+1.20	2.10	0.32	1	+0.20	+0.10	+0.70	0.70	0.11
3	+2.30	+1.60	+2.80	11.90	1.87[2]	3	+0.10	+0.40	+0.10	1.40	0.22
7	+3.00	+4.70	+6.60	11.90	1.87[2]	7	−0.40	+0.50	+0.50	2.10	0.32
14	+3.40	+5.70	+7.00	12.60	1.98[3]	14	−0.70	+0.50	+0.60	2.80	0.44
21	+3.90	+8.40	+6.80	9.10	1.43[3]	21	−1.50	+0.60	+0.60	3.50	0.55

[1] Coagulation.

[2] Coagulation; peptonization slight (?).

[3] Coagulation; peptonization marked.

[4] No visible coagulation; peptonization (?).

Micrococcus zymogenes reacts very similarly to *Staphylococcus aureus* in milk, producing in it visible changes which are manifested by a coagulation and separation of serum, by a moderate accumulation of ammonia (amounting to about 9 mg. in 100 cc. of milk), and by a progressively acid reaction which amounts to 7.5% at the end of two weeks. This organism, like *Staphylococcus aureus,* ferments lactose, and the accumulation of acid is, therefore, readily explainable. The comparatively small amount of ammonia formed would speak in favor of the separation of serum as being due to a mechanical contraction of the casein coagulum rather than to an extensive liquefaction of the casein.

Micrococcus melitensis, which is the etiological agent of Malta fever is transmitted to man through the milk of Maltese goats. It is also found in the urine of these animals. Malta fever has recently become endemic in certain parts of the United States, particularly Texas, where it has been introduced by the importation of goats from the Island of Malta. *Micrococcus melitensis* grows slowly in milk, produces but little ammonia and a slight, progressive acid reaction which is indicated by an increase in the titration values both with phenolphthalein and neutral red. With alizarin as an indicator the reaction after the third day becomes slightly alkaline; the explanation for this phenomenon is not known.

Conclusions.

(1) *Streptococcus pyogenes, Staphylococcus aureus,* and *Micrococcus zymogenes* produce an acid coagulation in milk which is visible on the third day; occasionally it may be visible somewhat earlier, and less typically, somewhat later.

(2) The amount of acid formed varies with the organism, *Streptococcus pyogenes* producing relatively little acid, *Staphylococcus pyogenes* and *Micrococcus zymogenes* producing considerable amounts of acid.

(3) *Staphylococcus aureus* and *Micrococcus zymogenes* form somewhat more ammonia than does the *Streptococcus pyogenes* under the same conditions in milk.

(4) Milk cultures of *Staphylococcus aureus* and *Micrococcus zymogenes* exhibit a considerable accumulation of clear serum after coagulation is complete: whether this is to be construed as a true liquefaction of the casein (peptonization), or a mechanical contraction of the casein with the extrusion of whey is not definitely known.

(5) *Micrococcus melitensis* grows slowly in milk, produces no visible change and but very little quantitative change in chemical composition.

XXXVII. The Metabolism of Certain Bacteria in Skimmed Milk, Whole Milk, and Cream.

In a series of previous communications relating to the metabolism of certain bacteria in certified whole milk containing 3.6% butter fat,[1] a number of reactions, chiefly increases in acidity, were encountered, for which no satisfactory explanation could be advanced. The character of these reactions suggested that they might be attributable to the action of these organisms upon some constituent or constituents of the cream, possibly by means of a fat-splitting ferment or lipase. Attempts to study the chemical changes in the cream were temporarily defeated because of the difficulties encountered in obtaining representative samples for analysis.

The observations recorded below were made with a view of determining, grossly at least, the effect of butter fat (cream) upon the metabolism of these bacteria. For this purpose certified skimmed milk (containing 0.15% of butter fat), certified whole milk (containing 3.6% of butter fat), and certified cream (containing 40% of butter fat), prepared in the usual

[1] Kendall, Day, and Walker, "Studies in Bacterial Metabolism, XXXI–XXXVI," inc., THIS JOURNAL, 36, 1920 (1914).

manner discussed above, were inoculated under like conditions with certain of these organisms, and these cultures, respectively in skimmed milk, whole milk, and cream, were incubated under parallel conditions and studied at appropriate intervals. The analyses follow:

Discussion.

If butter fat exerts any true sparing action for protein, it should be manifested by noteworthy differences in the amounts of ammonia produced when the same organism is grown, respectively, in skimmed milk, containing but little butter fat, and in cream: that is to say, less ammonia would be found in the cream cultures than in the corresponding skimmed milk cultures. The acidity of the cream cultures under these conditions might be confidently predicted to be greater than those in skimmed milk, provided carbohydrates played no part in the reaction. On the other hand, if the increase in acidity observed in these cultures be due to a fat-splitting ferment (a lipase) excreted by the bacteria or liberated from them as the organisms are autolyzed, the cultures should show an increase in acidity without a noteworthy diminution in ammonia production. In other words, a true fermentation of the butter fat[1] might be expected to shield the protein of the milk from bacterial breakdown, precisely as utilizable sugars protect the protein under the same conditions, although perhaps not to the same extent; while, on the contrary, the action of a ferment, a lipase in this instance, might be conceived to be more or less independent of proteolysis, in which case the reaction might become progressively acid, although proteolysis progressed normally. It is conceivable that these differences might be masked on the one hand by certain mechanical factors, particularly prominent in cream, for this medium forms a compact layer practically impermeable to air, and it is quite possible that the organisms might, therefore, grow less luxuriantly than would be the case in skimmed milk where access of air is more easily obtained. On the other hand, a resultant acid reaction might be met with even though the proteolysis proceeded normally, because the amount of acid produced by the activity of the lipase might be more than sufficient to neutralize the alkalinity of the basic products resulting from the protein breakdown. The metabolism of such a culture should show a progressive proteolysis of greater or lesser magnitude associated with a progressive acid reaction.

The organisms studied in this connection were *B. typhosus*, *B. paratyphosus alpha* and *beta*, *B. coli*, *B. proteus*, *B. pyocyaneus*, and the avirulent tubercle bacillus "W," all of these organisms being represented in the earlier studies on the subject.

B. typhosus and *B. paratyphosus alpha* and *beta*, as shown in the table, do not exhibit any marked differences in metabolism whether they are

[1] Rubner, *Arch. Hyg.*, **38**, 67 (1900), believes that bacteria ferment fats, but do not split fats, by lipase action.

TABLE VII.

	Skim milk.					Whole milk.					Cream.				
Days.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. per 100 cc. milk.	NH_3 total N_2 per cent.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. per 100 cc. milk.	NH_3 total N_2 per cent.	Alizarin.	Neutral red.	Phenolphthalein.	NH_3 mg. per 100 cc. cream.	NH_3 total N_2 per cent.
B. typhosus.															
1	+0.60	+0.50	+0.60	0.70	0.10	+0.20	+0.30	+0.80	0.70	0.11	—0.50	+0.20	—0.40	1.40	0.32
4	+1.10	+1.10	+1.10	2.80	0.40	0.00	+0.80	+1.40	0.70	0.11	0.00	+0.30	+0.70	1.40	0.32
7	+1.20	+0.90	+1.40	1.40	0.20	+0.20	+0.60	+1.40	2.10	0.32	+0.60	+0.20	+0.90	1.40	0.32
14	+2.50	+1.20	+1.20	2.80	0.40	+1.80	+0.70	+1.30	3.50	0.55	+0.60	+0.70	+0.90	2.80	0.64
21	+2.40	+1.20	+1.30	3.50	0.50	+1.60	+0.50	+1.20	4.20	0.64	+0.10	+0.50	+0.80	2.80	0.64
B. paratyphosus alpha.															
1	+0.60	+0.20	+0.50	0.70	0.10	+0.20	+0.40	+0.80	2.10	0.32	+0.10	+0.30	+0.40	0.70	0.16
4	+0.50	+1.20	+1.10	2.10	0.30	+0.10	+1.10	+1.50	2.80	0.44	—0.40	+0.40	+0.30	1.40	0.32
7	+0.50	+0.90	+1.20	2.10	0.30	+0.30	+0.90	+1.50	4.20	0.64	—0.90	+0.80	+0.40	0.70	0.16
14	+1.10	+1.10	+0.90	3.50	0.50	+0.70	+0.70	+1.00	4.20	0.64	—0.60	+0.60	+0.40	1.40	0.32
21	+0.90	+1.00	+0.60	2.10	0.30	+1.20	+0.70	+1.30	4.20	0.64	—0.80	+0.10	+0.20	1.40	0.32
B. paratyphosus beta.															
1	0.00	+0.50	+0.70	0.70	0.10	—0.30	+0.10	+0.60	0.70	0.11	—0.40	+0.20	+0.30	1.40	0.32
4	0.00	—0.20	—0.20	—0.70	—0.10	—0.50	—0.60	—0.30	0.00	0.00	—0.90	0.00	+0.40	0.70	0.16
7	—0.10	—1.40	—1.90	1.40	0.20	—0.00	—2.00	—1.40	0.70	0.11	—1.30	—1.30	—0.60	0.00	0.00
14	—1.20	—2.00	—1.70	2.80	0.40	—1.40	—3.40	—1.20	1.40	0.22	—1.70	—1.80	—0.80	0.70	0.16
21	—0,80	—3.70	—1.90	5.60	0.80	—0.50	—1.90	—1.40	2.80	0.44	—0.80	—1.30	—0.90	2.10	0.48

B. coli.															
1	+0.40	+3.40	+4.60	—0.70	—0.10	0.00	+3.20	+4.80	0.00	0.00	+0.90	+1.40	+2.40	—0.70	—0.16
4	+1.50	+3.60	+5.10	—1.40	—0.20	+0.80	+3.30	+4.40	0.00	0.00	+0.80	+2.20	+2.10	—0.70	—0.16
7	+3.10	+5.10	+5.60	0.00	0.10	+1.90	+4.10	+5.20	0.00	0.00	+0.60	+2.10	+2.80	0.00	0.00
14	+3.70	+5.50	+5.70	+0.70	0.10	+2.90	+4.50	+5.10	0.70	0.11	+0.70	+3.00	+3.00	+0.70	0.16
21	+3.80	+5.80	+6.00	+1.40	0.20	+3.10	+4.60	+5.30	1.40	0.22	+1.40	+3.30	+3.20	+1.40	0.32
B. proteus.															
1	+0.30	+0.60	+1.00	8.40	1.20	—0.70	+0.30	+1.70	11.90	1.87	—0.10	+0.50	+0.90	4.20	0.95
4	+1.20	+1.30	+3.10	18.90	2.70	+0.60	+1.10	+2.90	21.00	3.30	+0.50	+0.60	+1.60	11.20	2.54
7	+1.80	+5.70	+4.40	24.50	3.50	+0.50	+2.20	+4.30	26.60	4.18	+0.90	+0.80	+2.40	14.00	3.18
14	+1.20	+5.60	+5.60	28.70	4.10	+0.30	+2.10	+5.20	31.50	4.95	+1.00	+0.90	+2.90	39.20	8.90
21	+1.00	+5.20	+5.60	32.20	4.60	+1.40	+1.00	+5.30	33.60	5.28	+0.90	+1.00	+3.70	61.60	14.00
B. pyocyaneus.															
1	+0.80	—0.20	+0.10	—1.40	—0.20	0.00	—0.10	+0.40	0.00	0.00	—1.60	+0.30	+0.30	0.70	0.16
4	....	....	....	11.90	1.70	....	....	....	14.00	2.20	+1.90	—0.10	+0.40	2.10	0.48
7	+1.30	—1.00	—2.70	35.70	5.10	+2.30	—4.50	+3.30	67.90	10.65	+1.40	—2.20	+2.80	18.90	4.29
14	+1.30	—3.70	—2.80	151.90	22.70	+2.30	—5.80	—2.80	137.20	21.50	+2.50	—2.60	+1.30	50.40	11.40
21	+0.60	—3.60	—2.70	154.70	22.10	+2.20	—6.40	—2.70	154.70	24.40	+2.10	—3.40	+0.50	72.10	16.30
B. tuberculosis "W."															
1	+0.60	+0.60	+0.20	1.40	0.20	+0.10	+0.10	+0.10	1.40	0.22	—1.20	+0.10	—0.10	0.70	0.16
4	—0.70	—1.40	—1.30	7.00	1.00	+0.50	—0.50	—0.40	10.50	1.65	—0.80	—0.10	—0.10	2.80	0.64
7	—1.10	—1.70	—1.80	0.10	1.30	—1.20	—1.30	—0.80	11.20	1.76	—0.20	—0.20	—0.20	3.50	0.80
14	—0.50	—1.90	—1.80	11.90	1.70	—0.60	—1.80	—1.10	13.30	2.09	+0.40	0.00	—0.70	7.70	1.74
21	0.00	—1.90	—2.30	12.60	1.80	0.00	—1.70	—1.80	11.20	1.76	+0.50	—0.30	—0.90	6.30	1.43

developed in skimmed milk, whole milk, or cream. The amount of protein breakdown recorded in percentage is about the same for each of these organisms in each of the media.

B. typhosus and *B. paratyphosus alpha* produce a permanent acid reaction in skimmed milk as well as in whole milk. It will be remembered that an acid reaction was produced by these organisms in whole milk in the above-mentioned studies.[1] The growth of these organisms in these media furnishes no definite explanation for this acid reaction.

B. paratyphosus beta, similarly, exhibits an initial acidity, which, however, is followed by an alkaline reaction. It is apparent that the variation in composition of the media has not influenced, in any noteworthy manner, either the nature or the extent of the metabolism of these three organisms.

B. coli does not exhibit any noteworthy differences in metabolism in the three media, although the reaction produced is somewhat more acid than it is in either whole milk or cream. The amount of ammonia, however, formed in skimmed milk does not differ materially from that observed in whole milk or cream, and, aside from the slight difference in reaction noted, the organism appears to thrive equally well in the three media, producing in them changes of about the same magnitude.

B. proteus forms more ammonia both relatively and absolutely in cream than it does in either skimmed milk or whole milk. This organism, it will be remembered, does not ferment lactose; consequently, this sugar cannot exert any sparing action for the protein constituents of milk and cannot have any part in the production of acid in milk. Why the protein content of cream should undergo a greater decomposition than the protein of skimmed or whole milk is not readily explainable. Notwithstanding the increase of proteolysis in cream as contrasted with that observed in skimmed or whole milk, *B. proteus* effects a noteworthy decomposition of protein in either instance, amounting to 33 mg. of ammonia in the milk containing less butter fat, and to about 60 mg. of ammonia in the cream. The reaction is somewhat less acid in cream, probably due, in part at least, to the proportionately greater accumulation of ammonia. It is difficult to explain the acid reaction produced by this organism on the basis of information contained in the tables.

B. pyocyaneus appears to act less energetically upon the proteins in the cream media than the proteins contained in whole or skimmed milk. There is associated with this restriction of proteolysis, amounting to about 50%, a progressively acid reaction to phenolphthalein in cream. Reactions to alizarin and neutral red are less accurate, for the color change induced in the milk as the result of the growth of *B. pyocyaneus* obscures the end point and makes the titrations less accurate. The noteworthy

[1] Kendall, Day and Walker, "Studies in Bacterial Metabolism, XXXI," This Journal, 36, 1920 (1914).

difference in reaction observed in cream as compared with skimmed and whole milk would appear to rule out the possibility of a restricted growth of the organism in cream as a possible explanation for the decrease in proteolysis. This reaction reaches a maximum corresponding to 2.8% acid on the 7th day, and then decreases as ammonia formation proceeds. Remembering that *B. pyocyaneus* ferments no sugars, this acid reaction, which appears during the initial stages of development of the organism in cream and also in skimmed and whole milk before proteolysis has progressed to any considerable degree, would lead to the plausible assumption that this acidity may be due, partially at least, to a fat-splitting ferment. Sommaruga[1] has shown that both *B. typhosus* and *B. pyocyaneus* produce lipases in gelatin and agar media containing oil or fats. The decrease in acidity after the first week may, perhaps, be due to the gradual accumulation of ammonia, ammonia formation progressing rapidly at this time. It is stated by Michaelis[2] that alkalies tend to inhibit the action of lipases of vegetable origin.

The avirulent, rapidly growing, human tubercle bacillus "W" shows no noteworthy differences in metabolism or reaction when it is grown, respectively, in skimmed milk, whole milk, and cream. It should be observed, however, that the reaction in cream is less alkaline than is the case in either skimmed milk or whole milk. Whether lipases play any part in this reaction or not cannot be stated definitely. The organism produces a moderate amount of breakdown of the proteins of the three media respectively, and the results obtained in whole milk correspond closely with those recorded in the previous experiment in the same media.[3]

Conclusions.

(1) *B. typhosus*, *B. paratyphosus alpha* and *beta*, *B. coli*, and the tubercle bacillus "W" do not exhibit any marked differences in their nitrogen metabolism as measured by ammonia formation, or in their reactions to various indicators when they are grown under similar conditions in whole milk, skimmed milk and cream.

(2) The permanent acid reaction, which is a feature of the growth of *B. typhosus* and *B. paratyphosus alpha*, in whole milk, is also produced in skimmed milk and cream. The initial acidity followed by an alkalinity, which is a characteristic of *B. typhosus* and *B. paratyphosus beta* in milk, is also observed in cream.

(3) *B. proteus* is more proteolytic in cream than it is in whole milk or skimmed milk.

[1] Sommaruga, *Z. Hyg.*, **18**, 454 (1894).

[2] Michaelis, Abderhalden's "Handbuch d. biochem. Arbeitsmethoden," Vol. III, p. 23.

[3] Kendall, Day and Walker, "Studies in Bacterial Metabolism, XXXV," THIS JOURNAL, **36**, 1933 (1914).

(4) *B. pyocyaneus* is less proteolytic in cream than it is in whole milk or skimmed milk.

(5) The presence of certain pathological bacteria, *B. typhosus*, and *B. paratyphosus alpha* and *beta*, cannot be detected in milk by the chemical changes which they induce in it.

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STUDIES IN BACTERIAL METABOLISM.

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XXXVIII. Observations on Fat-Splitting in Milk by Bacterial Lipase.

A study of the metabolism of certain bacteria in sterile whole milk disclosed certain reactions, chiefly relating to the production of titratable acid in the presence of the progressive formation of basic products of protein breakdown, which could not be explained by any available information. *B. typhosus* and *B. paratyphosus alpha*, for example, produced a progressively acid reaction in milk, while *B. paratyphosus beta*, an organism very closely related, both qualitatively and quantitatively, produced a terminal alkalinity in the same medium under the same conditions. Similarly, *B. proteus* and *B. mesentericus* produced a progressive acid reaction, while *B. subtilis* produced a progressively alkaline reaction, notwithstanding the fact that all three organisms are strongly proteolytic. Several possibilities present themselves to explain these results:

(1) It might be assumed that there is a liberation of phosphoric acid, probably as acid phosphates, during the course of the bacterial digestion.

(2) The fermentation of fats in the Rubner sense,[1] with the liberation of fatty acids from the cream, might be a possibility.

(3) These organisms may secrete lipases, which break down the fatty constituents of the milk, perhaps independently of the protein metabolism.

(4) Other causes, as, for example, the possible formation of acid-reacting products, the result of protein decomposition.

There is no direct evidence for any of these assumptions, so far as these studies have shown: there is a certain amount of *à priori* objection to possibilities 1, 2, and 4, chiefly theoretical, however, and due to imperfect knowledge of the nature of intermediary metabolism of bacteria. To assume the presence of a fat-splitting ferment would appear to be a most logical hypothesis to consider first, and with this possibility in view the following experiments were made:

Broth cultures of *B. typhosus*, *B. coli*, *B. proteus*, *B. subtilis*, *B. mesen-*

[1] Rubner, *Arch. Hyg.*, **38**, 67 (1900).