A STUDY OF THE OPTICAL FORMS OF LACTIC ACID PRODUCED BY PURE CULTURES OF BACILLUS BULGARICUS.¹

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(Received for publication, August 13, 1911.)

In 1906 Grigoroff² isolated a bacillus from yoghurt, the Bulgarian fermented milk, which he named B. bulgaricus. This name has since been used to include a general group of bacteria, especially characterized by their ability to produce a higher percentage of acid than other lactic acid forming bacteria. This group of bacteria attracted special attention after Metchnikoff³ suggested that it be established in the alimentary tract to prevent the growth of harmful organisms. The investigations of Freudenreich⁴ and Hastings⁵ have shown it to be of great economic importance in the dairy industry.

Numerous observations have been made on the characteristics of bacteria isolated from a single source, which apparently belong to this group, but few studies have been made of the group as a whole, with a view of correlating the various strains. Such an investigation, however, was made by Heineman and Hefferan.⁶ One of their conclusions was that the lactic acid produced was

¹ Submitted to the faculty of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

² Grigoroff: Deutsche med. Wochenschr., xxi, p. 73, 1895.

³ Metchnikoff: Prolongation of Life, p. 161, 1903.

⁴ Freudenreich: Landwirtschaftliches Jahrbuch der Schweiz, xviii, p. 525, 1904.

⁵ Hastings: Science, xxviii, p. 656, 1908. Hastings and Hammer: Research Bulletin No. 6, University of Wisconsin Agricultural Experiment Station.

⁶ Heineman and Hefferan: Journal of Infectious Diseases, vi, p. 304, 1909.
always inactive. Bertrand and Weisweiller made a chemical study of a culture of B. bulgaricus obtained from Metchnikoff and found that the lactic acid produced was a mixture of the inactive and dextro forms. The lactic acid obtained from Cheddar cheese by Suzuki, Hastings and Hart was in some cases inactive and in others a mixture of inactive and dextro. From these observations we concluded that there were probably different strains of B. bulgaricus which could be differentiated by the optical form of the lactic acid produced. This belief, together with the hope of contributing something to the important problems of the relation of stereochemistry to biological processes, led us to undertake this investigation. This paper is devoted to a discussion of the results obtained and some of the literature bearing directly on this subject.

Certain points in the history of lactic acid are of particular interest in connection with this study. Lactic acid was discovered in sour milk by Scheele about 1780. Liebig isolated an acid from muscle extract in 1847 which he thought was identical with the acid of sour milk. But the following year Englehart showed that the zinc and calcium salts of the two acids differed in their solubility, water of hydration and point of decomposition. He concluded that the acids were not identical but isomeric. The explanation of the isomerism of these two acids was a problem which received the attention of nearly every prominent organic chemist of the time. In a paper published in 1873 Wislicenus, who had previously shown that, of the two acids, only sarco or muscle lactic acid was optically active, summed up the evidence in favor of their structural identity yet dissimilar properties, and concluded that this could only be explained by assuming a different arrangement of the atoms in space. He proposed to call this type of isomerism, geometrical isomerism. The influence of these speculations on the subsequent development of theoretical chemistry is apparent from the statement of van't Hoff that the reflections which led him to his theory of the asymmetric
carbon atom were suggested by the ideas of Wislicenus on the isomerism of sarco and fermentation lactic acids. This problem was finally solved by a study of biochemical processes. In 1889 Nencki and Sieber\(^1\) isolated a micrococcus which in pure cultures produced only dextro lactic acid. The following year Schardinger\(^2\) isolated a bacillus which produced only laevo lactic acid. He mixed equal quantities of zinc lactates prepared from sarcolactic and from the laevo acid produced by his organism, and found that inactive zinc lactate separated out. This established beyond question the relation of the active and inactive lactic acids. The resolution by chemical means of the inactive fermentation acid into its active components has been accomplished by Purdie and Walker.\(^3\)

It is evident from what has preceded that fermentation lactic acid is not, as is frequently stated in text-books, always inactive, but may also be either dextro or laevo rotatory, according to the type of organism employed in the fermentation. Numerous attempts have been made to formulate a definite relation between the ferment and the form of acid produced. Nencki\(^4\) suggested, after isolating his dextro acid forming coccus, that an organism always produced the same optical form of acid, and that this would serve as a certain means of identification. Péré\(^5\), Kayser\(^6\) and Pottevin\(^7\) have obtained results which indicate that the same organism, according to the conditions of growth, can produce either dextro, laevo or inactive acid. The results of these French investigators have been called in question by Kozai\(^8\) in an investigation to explain the paradoxical observation of Gunther and Thierfelder\(^9\) that, while the acid of spontaneously soured milk was usually inactive, \textit{B. lactis acidii}, the chief factor in the souring of milk, when in pure cultures produced only the dextro form.

\(^{1}\)Nencki and Sieber: \textit{Monatshefte für Chemie}, x, p. 532, 1889.
\(^{2}\)Schardinger: \textit{Monatshefte für Chemie}, xi, p. 545, 1890.
\(^{3}\)Purdie and Walker: \textit{Transactions of the Chemical Society}, 1892, lxi, p. 754, 1892.
\(^{4}\)Nencki: \textit{Centralblatt für Bakteriologie}, ix, p. 304, 1891.
\(^{5}\)Péré: \textit{Annales de l'Institut Pasteur}, vii, p. 737, 1893; xii, p. 63, 1898.
\(^{6}\)Kayser: \textit{Ibid.}, ix, p. 737, 1895.
\(^{7}\)Pottevin: \textit{Ibid.}, xii, p. 49, 1898.
\(^{9}\)Gunther and Thierfelder: \textit{Archiv für Hygiene}, xxv, p. 164, 1895.
Kozai succeeded in isolating three distinct types of lactic acid forming bacteria from milk. Two of these, *B. lactis acidi* and a coccus, produced pure dextro acid and one, a bacillus, produced pure laevo acid. He cultivated these three organisms under various conditions and found that the acid produced by each one was always of the same optical form.

Certain theoretical considerations are of interest in this connection. Bichner and Meisenheimer, and also Herzog, have shown that certain bacteria produce enzymes which, apart from the living cell, are capable of fermenting sugar solutions with the formation of lactic acid. Numerous investigations have shown that the action of most enzymes is specific even to the extent of attacking only one of two optical enantiomorphs. This would possibly indicate the presence of two enzymes in an organism capable of producing inactive or a mixture of active and inactive acids. This appears to offer the simplest explanation of the various acid producing faculties of different organisms.

**THE DISTRIBUTION AND CHARACTERISTICS OF *B. BULGARICUS*.**

Bacilli apparently belonging to this group have been isolated from the oriental fermented milks, yoghurt, kefir, leben, mazun, and gioddu. Freudenreich found several strains of a bacillus in Swiss cheese capable of producing large amounts of acid. Recently Hastings has succeeded in isolating such an organism from nearly every sample of mixed dairy milk examined. In 1895 Boas and Oppler observed a large lactic acid producing bacillus in the gastric fluid of patients suffering from carcinoma of the stomach. Many investigations have since shown that a bacillus of the bul-

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garicus type is common throughout the alimentary tract of man and of some animals. Similar bacteria have also been isolated from malt, kraut, bran and other carbohydrate materials.

An all-sufficient characterization of *B. bulgaricus* cannot be made. The ability to produce large amounts of acid is the most distinctive property. In some cases the acidity may even reach 4 per cent. The bacilli vary in length, occur generally in chains, are Gram-positive and show granules and irregular staining with methylene blue. They grow sparingly or not at all on the ordinary media, but are easily cultivated on media prepared from milk. The optimum temperature is high, generally above 40° C. Milk is usually but not always coagulated. The colonies on whey agar are generally small, almost microscopical, but their size varies greatly with their proximity and the reaction of the medium.

**METHOD OF PROCEDURE.**

To obtain pure cultures sterile milk tubes were inoculated with the material to be examined and incubated at 38° C. Sub-inoculations were made at intervals of about one week until a stained smear showed numerous large bacilli and few if any other forms. The culture was then plated out on whey agar to which a small drop of acetic acid was added to restrain the growth of other bacteria. If growth occurred, a typical isolated colony was transferred from a thinly seeded plate to a sterile milk tube. After several days incubation the culture thus obtained was plated out a second time on the acid whey agar, to make sure that only one type of bacteria was present.

The culture solution used throughout, unless otherwise stated, was 250 cc. of sterile milk to which 4 grams of calcium carbonate were added. The milk for this purpose was procured at the dairy barn of the Wisconsin Agricultural Experiment Station immediately after separation, so that no lactic acid could have been formed before sterilization. This culture solution was inoculated with the pure culture to be studied and incubated 3 to 6 weeks at 38° C. Sufficient dilute sulphuric acid was then added to the

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2Heinemann and Hefferan: *loc. cit.*
solution to release the lactic acid from its calcium and casein combinations, and the lactic acid extracted with ether in a Kutscher-Steudel continuous extractor for at least seventy-two hours. If the solution did not then give Uffelmann's test for lactic acid, the extraction was considered complete. After evaporating off the ether the lactic acid was dissolved in water, a slight excess of zinc carbonate added and the solution boiled for several minutes. The excess of zinc carbonate was then filtered off and the residue washed with hot water. The filtrate and washings were evaporated to crystallization at 60°-70° C. When crystals appeared, the solution was allowed to evaporate at room temperature until only a few cc. of mother liquor remained. This was then set aside in a cool place and after a few hours the mother liquor was drained off and the crystals washed with a small volume of cold water. The mother liquor and washings were again evaporated for a second crop of crystals. The zinc lactate recovered was combined and a portion of the air-dried salt dehydrated at 106°C. Englehart showed that the zinc salt of the inactive acid crystallized with three molecules of water, while the corresponding salt of the active acid carries but two molecules of water. Owing to the small specific rotation (±3.0°) of the active acid a determination of the water of hydration of the zinc salt has been generally regarded as the most satisfactory method for distinguishing these acids. When the zinc lactate is pure it dehydrates readily to a constant weight in from one to two hours; but when impure it loses the last traces of the water slowly and decomposes so readily that the point of complete dehydration is not sharply marked. One part of the zinc salt of the inactive acid is soluble in 58 parts of water at 16°C. and crystallizes out quite pure. At the same temperature one part of the zinc salt of either active acid is soluble in seventeen parts of water and generally carries enough impurities to make it dehydrate with difficulty. In such cases satisfactory results were obtained by dehydrating 0.4 to 0.5 gram samples for fifteen minute periods until the loss in weight did not exceed 0.3 mg. The zinc oxide in the dehydrated salt was determined in nearly every case as a check on the accuracy of the dehydration and the purity of the zinc lactate. A satur-

1Englehart: *loc. cit.*
ated solution of the zinc lactate was always examined in the polariscope before crystallization. In case this preliminary examination and also the water of hydration showed that the salt was probably a pure active zinc lactate, the specific rotation was determined at 22° C. in a 4 dm. tube and at a concentration of 4.122 grams of anhydrous zinc lactate per 100 cc. The specific rotation of zinc lactate varies with the concentration. At a concentration comparable to that used in these determinations Hoppe-Seyler and Araki\(^1\) give 7.552°. The rotation of the zinc lactate is opposite in sign from that of the free acid. In order to get comparative values of the percentage of acid produced, 100 cc. of sterile milk were inoculated with the pure culture and, after thirty days incubation at 38°, the acidity to phenolphthalein was determined by titration against \(\frac{\pi}{10}\) sodium hydrate, and the acidity expressed in per cent of lactic acid.

DISCUSSION OF DATA.

The cultures from human saliva designated I, II and III were obtained by inoculating sterile milk from three separate colonies on the agar plate. The optical form of the acid and also the per cent of acidity reached in thirty days indicate that the cultures were all of the same bacillus. In order to get some data on the form of acid produced under varying cultural conditions, number II was grown in a medium containing 0.5 per cent of peptone and 2 per cent of lactose and also on a medium containing 0.5 per cent of peptone and 2 per cent of glucose. Only a small amount of acid was recovered from these cultures, but in both instances it was of the same optical form as that produced in sterile milk. From these results we must conclude that, so far as this particular organism is concerned, varying both the source of nitrogen and of carbohydrate does not affect the optical form of the acid produced. The ether extract from these cultures gave only a trace of volatile acids.

The morphology and cultural characteristics of the bacillus isolated from human feces were identical with those of the bacillus from saliva. This organism, like the one from saliva, produced

\(^1\)Hoppe-Seyler and Araki: *Zeitschrift für physiologische Chemie*, xx, p. 371, 1895.
### Data.

<table>
<thead>
<tr>
<th>SOURCE OF ORGANISM</th>
<th>HYDRATE WATER IN ZN LACTATE per cent</th>
<th>ZINC OXIDE IN DEHYDRATED SALT per cent</th>
<th>([\alpha]_D^{22}) OF ZN LACTATE 4.122 GRAMS per 100 cc.</th>
<th>OPTICAL FORM OF ACID</th>
<th>ACIDITY IN THIRTY DAYS per cent</th>
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<td>Theory for active acid</td>
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pure dextro lactic acid and also attained very nearly the same acidity in 30 days. These results show the presence of identical, powerful acid-producing strains of *B. bulgaricus* in human saliva and feces.

The bacilli from kraut and malt were very similar to each other. The per cent of acidity attained was less than that of the cultures from saliva and feces, but the form of the lactic acid produced was again in both cases pure dextro. Both cultures contained volatile acids approximating 4 per cent of the total acidity. The considerable volatility of lactic acid with steam does not permit of an accurate determination of small amounts of volatile acids in its presence. The culture from malt had an appreciable odor of butyric acid. The presence of volatile acids was further shown by analyses of the zinc lactate prepared from the ether extract before and after distillation with steam. The latter conformed more nearly to the theoretical values than the former.

The organism isolated from horse feces possessed the morphology and cultural characteristics usually ascribed to *B. bulgaricus* but reached an acidity of only 0.58 per cent in thirty days. This fact might be interpreted to mean that it should not properly be classed as *B. bulgaricus*. Unlike the cultures previously studied it produced pure inactive lactic acid. It possessed a high optimum temperature, did not coagulate milk at all at room temperature and at 38° only after a period of two weeks or longer. The acidity increased uniformly throughout the period of incubation. These are all characteristics of *B. bulgaricus*.

The first culture of the bacillus from cow feces studied gave a zinc lactate containing 17.94 per cent of water and 37.14 per cent of zinc oxide. The figure for zinc oxide indicated the presence of acids other than lactic. A duplicate culture was distilled with steam before neutralizing with zinc carbonate. The volatile acids calculated to lactic amounted to 20 per cent of the total acidity. The zinc lactate recovered was inactive. Although this was a large, irregularly staining bacillus and produced an acidity of 1.20 per cent it is doubtful whether it belonged to the *B. bulgaricus* group because of the large amount of volatile acids produced.

To obtain a culture from milk, a small bottle was completely filled with milk, tightly stoppered and incubated until a stained smear showed the presence of long slender bacilli. The milk wa
Lactic Acid Produced by B. Bulgaricus

then plated out on whey agar. The pure culture contained a mixture of inactive and laevo acids. Analysis of the dehydrated zinc lactate showed that it was not pure. The zinc lactate was decomposed with hydrogen sulphide and the zinc sulphide filtered off. On distilling with steam only a trace of volatile acids was obtained. The solution was then examined for succinic acid. To separate succinic and lactic acids advantage was taken of the insolubility of barium succinate in 60 per cent alcohol. The white precipitate thrown down by the alcohol was filtered off, decomposed by the cautious addition of dilute sulphuric acid, and the filtrate from the barium sulphate evaporated to dryness. When the residue was heated copious white fumes were given off which had the characteristic choking effect of succinic anhydride. The white sublimate obtained after crystallization from water melted at 178°–179° C. The literature gives 180°–182° C. for the melting point of succinic acid. We think this established the presence of small amounts of succinic acid in this culture. Another culture isolated from milk in the same manner gave similar results with the exception that the proportion of laevo to inactive lactic acid present was smaller. These cultures died very quickly and before any results were obtained on the per cent of acidity produced. The biological functions of this bacillus appear quite similar to those of Kozai’s B. acidi levo-lactici halensis, which he concluded produced laevo lactic acid and traces of succinic acid. Bertrand and Weisweiller state that small amounts of succinic acid were produced by the culture of B. bulgaricus obtained from Metchnikoff.

The cultures isolated from Cheddar cheese were kindly furnished by Miss Evans, assistant bacteriologist in the dairy division of the Bureau of Animal Industry, who is working in cooperation with the Department of Agricultural Bacteriology of the University of Wisconsin. No very definite conclusions can be drawn from these results. It appears, however, that at least four different strains of high-acid organisms were present in these cultures, one which produced pure or nearly pure dextro lactic acid, one which produced inactive acid, one which produced a mixture

1 Kozai: Zeitschrift für Hygiene, xxxviii, p. 386, 1901.
2 Bertrand and Weisweiller: loc. cit.
of laevo and inactive acids and one which produced pure laevo acid. It will be remembered that, with the exception of the last named type, organisms of corresponding acid producing functions were isolated from other sources. We were unable to isolate with certainty succinic acid from any of these cultures.

**SUMMARY AND CONCLUSIONS.**

1. Cultures of *B. bulgaricus* were isolated from human saliva, human feces, malt, kraut and Cheddar cheese which produced only dextro lactic acid; from Cheddar cheese which produced dextro lactic acid with a small admixture of inactive acid; from horse feces, cow feces and Cheddar cheese which produced only inactive acid; from milk soured at 38° C. and from Cheddar cheese, which produced a mixture of laevo and inactive acids; and from Cheddar cheese which produced pure laevo lactic acid.

2. Among the bacteria which have been included in the *B. bulgaricus* group, there are strains which may be differentiated by the optical form of the lactic acid produced.

3. Varying both the source of nitrogen and of carbohydrate in the culture media did not alter the optical form of the acid produced by the bacillus isolated from saliva.

4. The dextro lactic acid forming strain predominates and has the power to produce a higher percentage of acid than the other strains.

5. Pure cultures of some bacteria may produce inactive lactic acid, a fact which probably necessitates the presence of both dextro and laevo acid producing enzymes in a single organism.

6. The bacilli of the *B. bulgaricus* type in human feces and human saliva are identical.

7. Some strains of *B. bulgaricus* produce small amounts of succinic acid. This may account for the presence of this acid in Cheddar cheese, although we could not definitely isolate it from any cultures obtained from cheese.

I wish to express my indebtedness to Professors E. B. Hart and E. G. Hastings for their advice throughout this work.
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J. Biol. Chem. 1911, 10:201-211.

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