CIII. EFFECT OF ASSOCIATED GROWTH ON FORMS OF LACTIC ACID PRODUCED BY CERTAIN BACTERIA¹.

BY EDWARD LAWRIE TATUM, WILLIAM HAROLD PETERSON
AND EDWIN BROUN FRED.

*From the Departments of Agricultural Chemistry and Agricultural Bacteriology,
University of Wisconsin, Madison, Wisconsin.*

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SPECIFICITY in the production of a given form of lactic acid by cells functioning under normal conditions is usually assumed to be a fixed character. While this may be true, such cells are not lacking in a mechanism for the production of both enantiomorphs. Thus, for example, while the animal cell *in vivo* seems to produce *d*-lactic acid exclusively, Embden and his associates [1912; Oppenheimer, S., 1912; Loeb, 1913; Oppenheimer, M., 1914] found that the enzyme preparations of ground liver, muscle, *etc.* produced both forms, and in some cases produced an excess of *l*-lactic acid. An even more pronounced reversal is reported by Neuberg [1913] in the production of pure *l*-lactic acid from methylglyoxal by the action of muscle and liver extracts.

In the field of microbiology many apparently inconsistent results have been reported. Some investigators [Pottevin, 1898; Péré, 1893, 1898; Kantardjieff and Pappow, 1931; Orla-Jensen, 1919] have asserted that the form of lactic acid varied with the type of carbohydrate, the form of nitrogen, and the temperature of incubation. Others [Kozai, 1899; Nencki, 1891; Currie, 1911-12] have been unable to bring about any such changes in the form of lactic acid by varying the conditions of fermentation. It is impossible to harmonise these conflicting reports but it is probable that some of the contradictions are due to impure cultures and others to faulty analytical procedures such as fractionation of the zinc salts and incorrect interpretation of the data on specific rotation. Most of the papers which report a modification in the form of lactic acid deal with a change from the production of both enantiomorphs to that of but one. Perhaps the most plausible explanation for this change is that of Orla-Jensen [1919]. He concludes that two enzymes, one producing the dextro-acid, the other the laevo-acid, are present in the cell but that under definitely unfavourable conditions the activity of one of the enzymes is inhibited and hence an active acid results. Further support of

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this view is found in the fact that while newly isolated cultures may produce both forms, the same cultures after repeated transfers in the laboratory may lose their ability to produce one of the enantiomorphs.

In the formation of lactic acid from methylglyoxal, using unaltered washed cells, Neuberg and Gorr [1925, 1, 2, 1926], Neuberg and Kobel [1927], Neuberg and Simon [1927, 1931] found an active lactic acid in two cases. However, using enzyme preparations, inactive acid was obtained in all cases. Apart from the possible influence of the substrate, methylglyoxal, on the form of acid produced, the treatment of the cells employed seems to be of primary importance. Where intact resting cells are used there seems to be a possibility of active acid production, but with enzyme preparations inactive acid is formed.

From these facts it appears probable that all cells contain enzymes capable of producing both forms of lactic acid, but that under certain conditions the activity of these enzymes is so controlled that only one enantiomorph appears.

In a former paper [1926] it was reported that a modification of the form of lactic acid could be obtained without disturbing normal growth and reproduction of the cells. This change was accomplished by growing the lactic acid organism in association with a non-lactic acid-producing microorganism. In a mixed culture of *L. leichmanni* and *Cl. acetobutylicum* inactive lactic acid was produced. By itself the former produces only *l*-lactic acid and the latter, in so far as it has been possible to determine, produces no lactic acid at all. Lactic acid-producing bacteria (*L. leichmanni*, and related forms) are frequently found as contaminants in the industrial production of acetone and butanol by *Cl. acetobutylicum*. The association of these two microorganisms is therefore a more or less natural one. The validity of our report has been questioned by Miss Stephenson [1930], who says, "Such a phenomenon would, however, need very rigorous proof before it could be established." We have repeated the work and extended it to various other combinations of microorganisms and find no errors in the former results.

Cl. acetobutylicum affects dextro- and laevo-lactic acid-producing organisms in the same way, *viz.* by causing them to form inactive lactic acid. This property seems to be peculiar to *Cl. acetobutylicum* and is not possessed by closely related butyric acid-forming bacteria. Likewise yeasts, which are usually grown in association with lactic acid-producing organisms, have no influence on the form of acid produced by the latter.

EXPERIMENTAL.

Cultures and media. Pure cultures of the following organisms were used: *Lactobacillus leichmanni*, strains A and D; *Lactobacillus delbrücki*, strains 3 and 4; *Lactobacillus casei*; *Lactobacillus bulgaricus*, strain 18; *Lactobacillus acidophilus*; *Streptococcus lactis*, strain R; *Leuconostoc pleofructi*, strain 3; *Saccharomyces cerevisiae*, strains RS, and 53; *Saccharomyces ellipsoideus*, strain E; *Clostridium acetobutylicum*, strains 105, 300 and 500; *Clostridium* sp., strain 21; and *Aerobacillus polymyxa* (Prazmowski), strain 35.

In testing the yeast cultures alone for the production of lactic acid a 20 % glucose medium was used. A solution containing 3 % glucose and 3 % suspension of malt sprouts was used as a culture medium in all other cases. In experiments dealing with the associated growth of two organisms, both organisms were inoculated simultaneously, except in one case where *Cl. acetobutylicum* was grown a short time before inoculating with *L. casei*. After 24 hours' incubation at 37° an excess of sterile calcium carbonate was added. The degrees of growth of the lactic acid-producer and of *Cl. acetobutylicum* were indicated by the production of non-volatile and volatile acid respectively.

Microscopic examinations of the mixed cultures were made from time to time, the results substantiating the conclusions drawn from the volatile and non-volatile acid-production.

Analytical methods. Sugar, volatile acid and non-volatile acid were determined essentially as described in previous publications. However, instead of ethyl ether, isopropyl ether was used for the extraction of the non-volatile acid. isoPropyl ether gives a solution that is freer from coloured impurities and yields purer zinc salts than does ethyl ether. Zinc lactate was prepared by boiling the extracted lactic acid with an excess of alkali-free zinc carbonate, decolorising and crystallising from 50 % alcohol. In all cases 75 % or more of the acid was obtained as the zinc salt. In all cases an effort was made to obtain as much of the salt as possible. The salt was dried over CaCl₂ and the water of crystallisation determined by heating the salt at 105° for 4 hours. The water of crystallisation of the pure inactive salt is 18.12 % while that of the active salt is 12.89 %. The optical rotation of the salt was determined for a 4 % solution in a 2-dm. tube. The rotation of the salt is opposite to that of the free acid.

Form of lactic acid in pure culture. Table I shows the form of lactic acid produced by nine cultures. Four of these were found to produce *l*-lactic

Table I. *Form of lactic acid produced by pure cultures.*

Organisms	Glucose fermented % of total	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic g. per 100 cc.	Water of crystallisation of zinc lactate %	Specific rotation [α] _D ²⁰	Form of lactic acid
<i>L. leichmanni</i> , A	90	2.7	0.08	2.64	13.23	+8.22	Laevo
<i>L. leichmanni</i> , D	87	2.5	0.04	2.37	13.03	+8.65	Laevo
<i>L. delbrücki</i> , 4	97	2.9	0.09	2.2	13.03	+8.00	Laevo
<i>Leu. pleofructi</i> , 3	97	2.9	0.09	0.64	13.15	+8.10	Laevo
<i>L. delbrücki</i> , 3	87	2.8	0.06	2.32	13.32	-7.36	Dextro
<i>L. casei</i>	83	3.0	0.03	2.8	13.26	-8.65	Dextro
<i>S. lactis</i> , R	90	2.6	0.01	2.41	13.23	-8.65	Dextro
<i>L. bulgaricus</i> , 18	93	2.8	0.06	1.81	15.50	—	Mixture
<i>L. acidophilus</i>	98	2.1	0.08	2.11	18.08	0.0	Inactive

acid, viz. *L. leichmanni*, strains A and D; *Leu. pleofructi*, 3; and *L. delbrücki*, strain 4. Three were found to produce the *d*-acid, viz. *L. casei*; *L. delbrücki*,

strain 3; *S. lactis*. The others, *L. acidophilus*, and *L. bulgaricus* were found to produce mixtures of *d*- and *l*-lactic acid.

Table II gives the results of experiments with various non-lactic acid-producers in order to make certain that no lactic acid was formed. No test for

Table II. *Test of yeasts and butyric acid-forming organisms for the production of lactic acid.*

Organisms	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic* g. per 100 cc.
<i>S. cerevisiae</i> , RS	19.2	0.08	0.15
<i>S. cerevisiae</i> , 53	19.2	0.08	0.15
<i>S. ellipsoidea</i> , E	19.2	0.08	0.20
<i>Cl. acetobutylicum</i> , 500	2.9	0.87	Trace
<i>Cl. sp.</i> , 21	2.9	0.53	Trace
<i>A. polymyxa</i> , 35	2.8	1.84	Trace

* In all cases the thiophen test for lactic acid was negative.

lactic acid with thiophen was obtained from the non-volatile extracts of any of these fermentations, nor was it possible to obtain any zinc lactate in these cases. The volatile acid production was determined as an index of growth of these organisms.

Table III. *Effect of association on the form of acid produced by l-lactic acid organisms.*

Organisms	Glucose fermented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non-volatile acid, as lactic g. per 100 cc.	Water of crystallisation of zinc lactate %	Form of lactic acid
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 105	2.8	0.39	1.71	18.16	Inactive
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 105	2.9	0.42	1.66	18.16	Inactive
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 105	2.9	0.44	1.58	18.07	Inactive
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 500	2.9	0.11	1.02	18.20	Inactive
<i>L. leichmanni</i> , D and <i>Cl. acetobutylicum</i> , 105	2.6	0.45	0.48	18.03	Inactive
<i>L. leichmanni</i> , D and <i>Cl. acetobutylicum</i> , 500	2.6	0.31	0.31	18.03	Inactive
<i>L. delbrücki</i> , 4 and <i>Cl. acetobutylicum</i> , 500	2.8	0.17	1.38	18.00	Inactive
<i>L. delbrücki</i> , 4 and <i>Cl. acetobutylicum</i> , 500	2.9	0.15	1.42	18.04	Inactive
<i>L. delbrücki</i> , 4 and <i>Cl. acetobutylicum</i> , 500	2.8	0.23	1.70	18.18	Inactive
<i>Leu. pleofructi</i> , 3 and <i>Cl. acetobutylicum</i> , 500	2.9	0.79	0.37	17.50	Inactive
<i>Leu. pleofructi</i> , 3 and <i>Cl. acetobutylicum</i> , 500	2.9	0.57	0.37	18.20	Inactive
<i>Leu. pleofructi</i> , 3 and <i>Cl. acetobutylicum</i> , 500	2.9	0.73	0.34	17.78	Inactive
<i>Leu. pleofructi</i> , 3 and <i>Cl. acetobutylicum</i> , 500	2.9	0.75	0.15	17.65	Inactive
<i>L. leichmanni</i> , A and <i>S. cerevisiae</i> , 53	2.9	0.08	1.89	13.31*	Laevo
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 105†	2.9	—	1.50	12.97*	Laevo
<i>L. leichmanni</i> , A and <i>Cl. sp.</i> , 21	2.34	0.12	2.07	13.14*	Laevo
<i>L. leichmanni</i> , A and <i>A. polymyxa</i> , 35	2.31	0.12	1.75	13.32*	Laevo

* Specific rotation of these samples varied from +7.8° to +8.3°.

† Culture 105 was grown for 24 hours in the medium which was then sterilised and inoculated with culture A. Volatile acid was not determined because the medium was to be sterilised and re-inoculated.

Form of lactic acid produced in mixed cultures. As shown by Table III all the *l*-acid-formers, when grown with *Cl. acetobutylicum*, produced *i*-lactic acid, *i.e.* a mixture of *d*- and *l*-acids. When grown with other non-lactic acid-producers, yeasts and butyric acid bacteria, no change of form was detected

although the volatile acid production of the butyric acid bacteria indicated a satisfactory growth. An important point in the association with *Cl. acetobutylicum* is that there is obtained an equimolecular proportion of *d*- and *l*-acids, as indicated both by the water of crystallisation and by the optical inactivity. It should also be observed that among these *l*-acid-producing cultures there are representatives of three distinct classes of organisms.

The effect of associated growth on the *d*-lactic acid-producers is shown by the data in Table IV. The streptococcus, R, easily gave a change of form of

Table IV. *Effect of association on the form of acid produced by d-lactic acid organisms.*

Organisms	Glucose fer- mented % of total	Glucose fer- mented g. per 100 cc.	Volatile acid, as acetic g. per 100 cc.	Non- volatile acid, as lactic g. per 100 cc.	Water of crystalli- sation of zinc lactate %	Form of lactic acid
<i>Strep. lactis</i> , R and <i>Cl. acetobutylicum</i> , 500	96	2.9	—	1.11	16.47	Dextro and inactive
<i>Strep. lactis</i> , R and <i>Cl. acetobutylicum</i> , 500	92	2.8	0.70	0.46	14.71	Dextro and inactive
<i>Strep. lactis</i> , R and <i>Cl. acetobutylicum</i> , 500	92	2.8	0.44	1.16	18.22	Inactive
<i>Strep. lactis</i> , R and <i>Cl. acetobutylicum</i> , 500	92	2.8	0.40	1.11	18.20	Inactive
<i>Strep. lactis</i> , R and <i>Cl. acetobutylicum</i> , 500	92	2.8	0.44	0.94	18.23	Inactive
<i>Strep. lactis</i> , R and <i>S. cerevisiae</i> , 53	97	3.2	0.06	0.39	12.51	Dextro, specific ro- tation of zinc salt — 8.65°
<i>L. casei</i> and <i>Cl. acetobutylicum</i> , 300	91	3.0	0.05	0.81	13.49	Dextro, specific ro- tation of zinc salt — 7.8°
<i>L. delbrücki</i> , 3 and <i>Cl. acetobutylicum</i> , 500	91	3.0	0.11	1.77	13.44	Dextro, specific ro- tation of zinc salt — 8.37°
<i>L. delbrücki</i> , 3 and <i>Cl. sp.</i> , 21	77	2.3	0.04	1.83	12.50	Dextro
<i>L. delbrücki</i> , 3 and <i>Cl. sp.</i> , 21	97	2.9	0.17	1.53	12.06	Dextro
<i>L. casei</i> and <i>Cl. acetobutylicum</i> , 500*	96	2.8	0.40	1.08	16.2	Dextro and inactive
<i>L. casei</i> and <i>Cl. acetobutylicum</i> , 500*	96	2.8	0.40	0.54	15.55	Dextro and inactive

* *Cl. acetobutylicum* grown for 24 hours before inoculation with *L. casei*.

lactic acid, this change being from *d*- to *i*-acid. It was early discovered from microscopic examinations and from volatile acid determinations that the *d*-acid-producing bacteria exerted an inhibitory effect on the acetone-butyl alcohol organism. Where the medium was inoculated simultaneously with both organisms no growth of *Cl. acetobutylicum* took place and the lactic acid produced was consequently active. Therefore the associated organism was grown for 24 hours before inoculation with *L. casei*. Apparently in this case

a sufficient number of cells of the associated organism were present and the acid formed was a mixture of *d*- and *i*-acids. It is probable, since the acetone-butyl alcohol organism did not cause appreciable evolution of gas after the addition of *L. casei*, that the former no longer grew actively, and some of the acid produced by the lactic organisms was active, as is that formed when the two organisms are inoculated simultaneously.

Some properties of the active agent. That the shift in the form of acid can be brought about only through the agency of the living cell or some heat-labile substance elaborated by it was demonstrated by us [1926] and confirmed by the following experiment. A 24-hour culture of *Cl. acetobutylicum* was sterilised. This treatment would destroy enzymes and other heat-labile substances but would leave intact the other fermentation products. The medium was then inoculated with *L. leichmanni* and incubated for several days. An abundance of lactic acid was produced but it was all laevorotatory.

Next an effort was made to determine if the agent responsible for the change in the form of lactic acid could pass through a collodion or viscose membrane (Table V). Sacs made of these materials were filled with medium

Table V. *Experiments with viscose and collodion sacs.*

Organisms	Type of sac	Test for*		Water of crystallisation of zinc lactate %	Specific rotation $[\alpha]_D^{20}$	Form of lactic acid	Remarks
		<i>Cl. acetobutylicum</i> in lactic culture	Lactic acid organism in <i>Cl. acetobutylicum</i> culture				
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 500	Collodion	+	+	18.10	0.0°	Inactive	Organisms passed through membrane
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 500	Collodion	+	+	18.20	0.0°	Inactive	Organisms passed through membrane
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 500	Collodion	+	+	18.00	0.0°	Inactive	Organisms passed through membrane
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 500	Viscose	—	—	13.3	+7.8°	Laevo	No effect of <i>Cl. acetobutylicum</i>
<i>L. leichmanni</i> , A and <i>Cl. acetobutylicum</i> , 500	Viscose	—	—	13.2	+7.9°	Laevo	No effect of <i>Cl. acetobutylicum</i>
<i>L. casei</i> , and <i>Cl. acetobutylicum</i> , 500	Viscose	—	—	13.5	—8.65°	Dextro	No effect of <i>Cl. acetobutylicum</i>
<i>L. casei</i> , and <i>Cl. acetobutylicum</i> , 500	Viscose	—	—	13.5	—8.22°	Dextro	No effect of <i>Cl. acetobutylicum</i>

* Growth of *Cl. acetobutylicum* was tested for on the lactic side of the membrane by inoculation into corn-mash. Growth of lactic acid organisms was tested for on the *Cl. acetobutylicum* side of the membrane by streaking on a glucose peptone agar plate.

and were suspended in some more of the same medium. The medium inside the sac was inoculated with one organism and that outside the sac with the other organism. Collodion sacs proved unsatisfactory as the two organisms penetrated the membrane and were found both inside and outside the sac. *i*-Lactic acid was formed as a result of the intimate growth of the two organisms. Viscose sacs were found to keep the two organisms separated throughout the fermentation, but an active lactic acid was formed in such

fermentations. It seems probable that most of the products of *Cl. acetobutylicum* passed through the membrane, since similar viscose sacs have been found to be highly permeable to aniline dyes and plant viruses. It would appear, therefore, that the two types of cells must be in intimate contact or that the influential product of *Cl. acetobutylicum* is incapable of passing through the membrane.

SUMMARY.

The effect of associated growth on the optical form of lactic acid produced by certain bacteria has been studied. Four *l*-lactic acid-forming bacteria, and three *d*-acid-producers were grown with four non-lactic organisms, in various combinations.

When the lactic acid bacteria, which by themselves produce only *d*- or *l*-acid, were grown in association with the acetone-butyl alcohol organism, *Cl. acetobutylicum*, *i*-lactic acid was formed.

Intimate contact between the lactic acid bacteria and *Cl. acetobutylicum* seems to be necessary to bring about the change since no effect was obtained when the two organisms were separated by a viscose membrane. The active agent is apparently not diffusible and is destroyed by heat.

The ability to bring about this change in the form of lactic acid seems to be the peculiar property of *Cl. acetobutylicum*. Closely related butyric acid bacteria had no effect. The yeast, *S. cerevisiae*, did not bring about any change in the form of lactic acid.

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