

LXXII.—*Sarcolactic acid obtained by the Fermentation of Inactive Lactic acid.*

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DURING a number of years past, attempts have been made by one of us to induce the fermentation of lactic acid by means of specific micro-organisms which, like the *Bacillus ethaceticus* and the *Bacillus ethacetosuccinicus*, have the power of fermenting a variety of substances, but these endeavours have invariably failed. That lactic acid should thus prove to be a comparatively unfermentable substance is not remarkable when it is borne in mind that it is itself actually produced in larger or smaller quantity in a great number of fermentations.

The fermentation of lactic acid has acquired a particular interest since the fermentative decomposition of the closely allied glyceric acid has led not only to the discovery of one of the two active glyceric acids predicted by theory, but to its preparation on what may be regarded as a large experimental scale (Trans., 1891, 59, 96).

In the case of lactic acid, both the active isomers have been obtained in several different ways: thus the dextrorotary sarcolactic acid (yielding lævorotary salts) occurs, as is well known, in the juices of muscular tissue, and it is also said to have been obtained in numerous impure fermentations, *e.g.*, of dextrin, glucose, cane and milk sugar, &c., whilst more recently Nencki and Sieber (*Monatsh.*, 10, 532—540; *Ber.*, 22, c. 695) have found it in a pure fermentation of dextrose. The lævorotary lactic acid (yielding dextrorotary salts) has been similarly obtained by Schardinger in a pure fermentation of cane sugar (Abstr., 1891, 666).

Both active lactic acids have also been obtained, although not in a state of purity, by Purdie and Walker by crystallising the strychnine salts prepared from the inactive acid (Trans., 1892, 61, 754).

An attempt was made to obtain an active lactic acid by selective decomposition of the inactive acid by Lewkowitsch (*Ber.*, 1883, 2720), who, after growing the mould *Penicillium glaucum* in a nutritive solution of ammonium lactate for some weeks, found that the liquid was dextrorotary, and therefore possibly contained an active lactic acid. Beyond this the investigation does not appear to have been pursued.

This experiment was repeated by Linossier (*Ber.*, 24, c. 660) with the opposite result, namely, that the residual lactic acid yielded lævorotary salts.

In the course of fermentation experiments which we were carrying on in another connection, we obtained a bacterial growth which we found had the power of exciting a vigorous fermentation in suitable solutions of calcium lactate. We do not propose at this stage to enter into a detailed description of the micro-organisms in question or of the various products of the fermentation to which they give rise, but we shall at present confine ourselves to the manner in which this fermentation has lent itself to the preparation of one of the active lactic acids.

Experiment I.—Two flasks containing a sterile solution of the following composition :—

Calcium lactate, 3 grams.	} made up to 300 c.c. with distilled water.
Peptone, 0.3 gram,	
Salt solution, 30 c.c.	
Calcium carbonate, 3 grams.	

were inoculated with a minute quantity of a calcium lactate solution in active fermentation. On the sixth day after inoculation, both flasks were in vigorous fermentation.

The solution in the first flask was allowed to ferment for one week, the liquid was then filtered from the excess of calcium carbonate, the filtrate evaporated down to a volume of about 12 c.c., and on being examined in the 200-mm. tube exhibited a rotation of -1.0° .

The contents of the second flask was allowed to ferment during two weeks, after which it was treated similarly to the above; the filtrate, on being concentrated to 16 c.c., gave a rotation of -0.6° .

In order to ascertain whether this activity was due to the presence of calcium dextrolactate (sarcrolactate), we evaporated the liquid from the first flask nearly to dryness on the water-bath, added a large excess of alcohol, and filtered from some brown insoluble matter, which was again washed with alcohol. The alcoholic liquid was evaporated to dryness, the residue dissolved in hot water, filtered, and the filtrate made up to 12 c.c.; this, on examination in the 200-mm. tube, exhibited a rotation of -0.8° , thus showing that the activity had not been materially altered by the above treatment with alcohol.

The calcium was then precipitated with oxalic acid in the united liquids obtained from both the above flasks, and, after removing the calcium oxalate, the filtrate was evaporated to a thick syrup. The latter was taken up with hot water, and treated with animal charcoal and an excess of zinc carbonate, with which it was boiled and then filtered. The filtrate was evaporated to a small bulk, and then placed in the desiccator to undergo slow evaporation. A number of small wart-like, but not visibly crystalline masses slightly yellow in colour

separated; the quantity, however, was too small for any accurate determinations to be made with it.

Experiment II.—We now proceeded to attempt the preparation of the active lactate on a larger scale, 6 litres of the above 1 per cent. solution of calcium lactate being fermented at once. This liquid was allowed to ferment for 14 days from the time of the first appearance of bubbles of gas, and then filtered from excess of calcium carbonate; the filtrate, after concentration to a volume of 1000 c.c., gave a rotation of about -0.3° in the 200-mm. tube. The calcium was now removed by means of oxalic acid, and the filtrate repeatedly evaporated on the water-bath to remove the whole of the volatile acids. The dark syrup remaining was dissolved in water, and treated with basic lead acetate as long as a precipitate formed, the latter was filtered off, and from the filtrate, which still gave a negative rotation in the polarimeter, the lead was removed by means of hydrogen sulphide. The filtrate from the lead sulphide, which was much less coloured than before, was evaporated to a syrup, and then transferred to a small bottle in which it was freed from water as far as possible by heating on a water-bath, whilst a current of air was aspirated through the bottle. The syrupy residue thus obtained was repeatedly extracted with ether, the ether was removed by distillation, and the residue then remaining, and which should consist of the free active lactic acid, was made up to 10 c.c. with water, but on examination in the 200-mm. tube, practically no rotation was observed. On boiling with zinc carbonate, filtering, and concentrating to about 12 c.c., however, a rotation of -1.0° in the 200-mm. tube was observed. A zinc determination was made in the contents of the tube by precipitation with sodium carbonate in the usual way, 0.1782 gram of ZnO being obtained from 9.59 c.c. of liquid, corresponding to 0.5346 gram anhydrous zinc lactate in this volume, from which follows the concentration—

$$\frac{0.5346 \times 100}{9.59} = 5.575 \text{ grams in 100 c.c.}$$

$$\text{and the sp. rot. } [\alpha]_D = \frac{100 \times -1.0}{1.984 \times 5.575} = -9.04.$$

This figure is in very fair accordance with that given by Wislicenus for zinc sarcolactate, for the concentration 5.36, namely $[\alpha]_D = -8.49^{\circ}$.

The purification of the active substance described above, by extraction with ether in the acid solution, banishes, of course, any suspicion of the activity observed being due to the presence of peptone or other albuminoid substances. It is very possible that a

considerable quantity of the residue insoluble in ether may have been anhydride of sarcolactic acid, for on boiling this residue with zinc carbonate, a laevorotary liquid was also obtained.

We may, incidentally, mention that some of the volatile acids from this fermentation were converted into barium salts, which after drying at 130° were submitted to analysis with the following result:—

0.2845 gave $0.2204\text{BaSO}_4 = 77.47$ per cent. BaSO_4 .

From this it appears that the volatile acids produced in this fermentation consist mainly of butyric acid (barium butyrate yields 74.9 per cent. BaSO_4 , barium propionate 82.33 per cent. BaSO_4), mixed with some of the lower acids of the fatty series. This matter will, however, form the subject of further investigation.

Experiment III.—The previous experiments having yielded only a small quantity of sarcolactic acid, it was our next endeavour to ascertain whether the yield could not be increased by arresting the fermentation at an earlier stage. In this experiment, therefore, the 6 litres of the 1 per cent. solution of calcium lactate were only allowed to ferment for four days after the first appearance of gas-bubbles, or eight days after inoculation. The fermented liquid was treated almost exactly in the same way as in Experiment II (the purification with basic lead acetate was, however, omitted), but only a very small quantity of sarcolactic acid was obtained, whilst a large quantity of inactive acid was found to have remained unchanged. A vigorous fermentation of four days' duration is, therefore, obviously inadequate.

Experiment IV.—As four days had proved insufficient in the previous experiment, the solution of calcium lactate (1 per cent.) was permitted to ferment for a whole week from the first appearance of gas-bubbles. The subsequent treatment of the fermented liquid was similar to that previously described. The zinc salt, after repeated crystallisation, consisted of small, colourless, wart-like masses, exhibiting no distinct crystalline form to the naked eye, but seen to be composed of minute, prismatic needles when examined with the microscope.

Of this purified zinc salt (rendered anhydrous at 104° C.), 1.1343 gram was obtained, and its solution was made up to 16.6 c.c. (corresponding to a concentration of 6.833 per cent.); on examination in the 200-mm. tube, this gave a rotation of -1.05° , from which follows the specific rotation

$$[\alpha]_D = \frac{100 \times -1.05}{1.984 \times 6.833} = -7.75^\circ,$$

which corresponds very closely with that given by Wislicenus for zinc sarcolactate and a concentration of 6.51 per cent., namely,

$$[\alpha]_D = -7.83^\circ.$$

Experiment V.—In this experiment, a 3 per cent. solution of calcium lactate was substituted for the 1 per cent. solution employed in the previous experiments. The fermentation was interrupted after it had been proceeding for seven days from the first appearance of gas-bubbles.

The residual lactic acid was converted into zinc-salt as usual, and the latter, although mainly composed of the sarcolactate, also contained some inactive lactate, from which, however, it was easily separated by repeated crystallisation. Thus the following polarimetric result was obtained with some of the purified active zinc salt.

$(\alpha)_D = -0.83^\circ$, $l = 198.4$ mm., $c = 4.706$ per cent. anhydrous zinc lactate, from which follows

$$[\alpha]_D = \frac{100 \times -0.83}{1.984 \times 4.706} = -8.9^\circ.$$

Another specimen gave the following polarimetric results.

$(\alpha)_D = -0.55^\circ$, $l = 198.4$ mm., $c = 3.42$ per cent. anhydrous zinc lactate, from which follows

$$[\alpha]_D = \frac{100 \times -0.55}{1.984 \times 3.42} = -8.2^\circ.$$

The specific rotations given by Wislicenus for zinc sarcolactate, using different concentrations, are the following.

$c = 13.98$	per cent.	anhydrous salt.	$[\alpha]_D = -7.30$
9.60	„	„	7.29
6.51	„	„	7.83
5.36	„	„	8.49
4.75	„	„	8.43
4.58	„	„	8.73

results which agree, therefore, very closely with those obtained with our own preparations.

In the above specimen of active zinc salt, the zinc was determined with the following result.

0.6318 gram of Zn salt, dried at $100-101^\circ$, yielded, on incineration with nitric acid, 0.2110 gram of ZnO , corresponding to 26.84 per cent. Zn, whilst for $Zn(C_3H_5O_3)_2$ the calculated percentage of zinc is 26.90.

We also determined the rotation in a stronger solution of this specimen of the active zinc lactate, with the following result.

$(\alpha)_D = -1.65^\circ$, $l = -198.4$ mm., $c = 11.765$ per cent. anhydrous salt, from which follows

$$[\alpha]_D = \frac{100 \times -1.65}{1.984 \times 11.765} = -7.1^\circ.$$

In order to further establish the identity of our zinc salt with that of sarcosolactic acid, we have also determined the water of crystallisation and zinc in a crystallised specimen with the following results

0.4148 gram of crystallised zinc salt lost 0.0535 gram on being dried until constant at 105° , and yielded, on incineration 0.1208 gram ZnO, from which follows.

	Found.	Calculated for (C ₃ H ₅ O ₃) ₂ Zn + 2H ₂ O.
H ₂ O	12.90 per cent.	12.88 per cent.
Zn.....	23.41 „	23.43 „

These figures demonstrate the absolute purity of our zinc salt, as any admixture of inactive salt would have materially altered the percentages, since the inactive zinc lactate crystallises with 3H₂O, thus

	Calculated for (C ₃ H ₅ O ₃) ₂ Zn + 3H ₂ O.
H ₂ O	18.15 per cent.
Zn.....	22.02 „

Calcium Sarcosylactate.—We have also converted the above active zinc lactate into the calcium salt by precipitating the zinc with sulphuretted hydrogen, filtering off the zinc sulphide, and then boiling the filtrate with calcium carbonate. The solution of the calcium salt was then concentrated to suitable bulk for polarimetric examination, the precise quantity of calcium salt present in a given volume being determined in an aliquot part by evaporation and conversion into calcium sulphate.

The following result was obtained.

$(\alpha)_D = -0.63^\circ$, $l = 198.4$ mm., $c = 5.790$ per cent. anhydrous salt, from which follows the specific rotation

$$[\alpha]_D = \frac{100 \times -0.63}{1.984 \times 5.79} = -5.48^\circ.$$

The only previously recorded rotation of calcium sarcosylactate is that given by Wislicenus for a concentration of 5.35 per cent. anhydrous salt, namely,

$$[\alpha]_D = -5.25^\circ.$$

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The two results are, therefore, very closely coincident, and constitute an important confirmation of the identity of our active acid obtained by fermentation with the previously known sarcolactic acid.

In the fermentation in question, therefore, the bacteria have, in decomposing the inactive calcium lactate, attacked the dextrorotary molecules by preference, leaving the lævorotary molecules, or calcium sarcolactate, in the free state. If the fermentation is not interrupted, the lævorotary molecules are apparently also attacked and destroyed by the fermentative activity of the bacteria.

This fermentation of calcium lactate is, therefore, very similar to that of calcium glycerate by the *Bacillus ethaceticus*, in which also the dextrorotary molecules were attacked by preference, the lævorotary calcium glycerate remaining as a residue. In this fermentation of calcium glycerate we have observed a most striking example of adaptation, for when the study of this fermentation was commenced by one of us some years ago, it was found that the *Bacillus ethaceticus* left practically the whole of the lævorotary calcium glycerate untouched, only decomposing the dextrorotary molecules, but by continuous cultivation of the bacillus in solutions of calcium glycerate over a long period of time, its power of fermenting this substance has become so greatly increased, that if the fermentation is now allowed to run its course without hindrance, the yield of lævorotary calcium glycerate is very much smaller than it used to be. In order, therefore, to obtain a more satisfactory yield of the lævorotary calcium glycerate, we now adopt one of the two following methods; the first consists in interrupting the fermentation, after it has proceeded for such a length of time as experience leads us to believe will have sufficed for the destruction of the dextrorotary calcium glycerate without the decomposition extending to the lævorotary molecules, whilst in the second method we inaugurate the fermentation by inoculating with ethacetic bacilli, which have hitherto been strangers to solutions of calcium glycerate.

The results of our investigation may be summarised as follows.

1. By the interrupted bacterial fermentation of ordinary inactive calcium lactate, we have obtained a liquid exhibiting a negative rotation in the polarimeter.
2. From this liquid we have extracted the residual lactic acid, and on converting the latter into the zinc salt, we have by fractional crystallisation obtained a lævorotary zinc lactate, which from its chemical composition and specific rotation proved to be pure zinc sarcolactate.
3. This zinc salt was converted also into a calcium salt possessing the same specific rotation as calcium sarcolactate.

4. When the fermentation was interrupted too early, the active was found to be mixed with a large quantity of inactive lactate, whilst when the fermentation was too long continued, the active lactate was also destroyed.

We propose continuing the study of this fermentation in the hope of obtaining such quantities of sarcolactic acid as will permit of the preparation of sarcolactates for optical comparison with the numerous active glycerates which we have already described.

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