XLII. NOTE ON THE VARIATION IN THE END-PRODUCTS OF BACTERIAL FERMENTATION RESULTING FROM INCREASED COMBINED OXYGEN IN THE SUBSTRATE.

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The work of Harden [1901] and of Grey [1919, 1, 2] and others has shown that the end products of the bacterial degradation of glucose and of mannitol are qualitatively the same, but differ considerably in the relative quantity of the substances formed. Thus, under similar conditions of fermentation, *B. coli communis* produces twice as much alcohol from mannitol as it does from glucose, but much smaller amounts of lactic acid from the former than from the latter. With a view to obtaining further information as to the effect of the composition of the substrate on the products of bacterial fermentation it was thought that it would be interesting to investigate the more oxygenated derivatives of glucose. Some information with regard to the intermediate region between complete anaerobiosis and aerobiosis might also be obtained by thus presenting to the organism progressively more oxygen already bound in the substrate molecule.

The result which was anticipated has been demonstrated, namely that increasingly oxygenated end-products are obtained from the anaerobic fermentation of increasingly oxygenated substrates, and some indirect support has been given to the view that certain portions of the substrate molecule provide the molecular framework of particular end-products. These results, however, cannot be accepted as conclusive until the experiments described in this note, the greater number of which have been conducted in peptone solutions, have been repeated with synthetic media of known composition, an opportunity for which has not yet arisen.

The action of *B. coli communis* (Escherich, No. 86 National Type Collection) under anaerobic conditions on a series of progressively more oxidised compounds related to glucose has been investigated qualitatively and quantitatively. A few similar experiments have also been carried out with *B. lactis aerogenes*. Below are the formulae of the substrates used arranged in order of their oxygen content, the most oxygenated compound being on the right.

Mannitol	Glucose	Gluconic acid	Glycuronic acid	Saccharic acid
CH_2OH	CHO	$COOH$	CHO	$COOH$
$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$
CH_2OH	CH_2OH	CH_2OH	$COOH$	$COOH$
$C_6H_{14}O_6$	$C_6H_{12}O_6$	$C_6H_{12}O_7$	$C_6H_{10}O_7$	$C_6H_{10}O_8$

Mannitol, which, strictly speaking, does not come into the series, has not been investigated by the present author, but Grey's figures, obtained under similar conditions, for the action of *B. coli communis* on this substance, are used for purposes of comparison, until figures for sorbitol have been arrived at. The fermentations have all been carried out in the presence of an excess of calcium carbonate. In the case of the acids the neutral calcium salts have been used.

Preparation of gluconic, glycuronic and saccharic acids.

Calcium gluconate was prepared by Kiliani's [1884] method with a few modifications. The method as finally modified is as follows: 400 g. of pure anhydrous glucose are dissolved in 1500 cc. water and shaken mechanically in a strong 2½ litre bottle with 450 g. bromine for 3 hours, during which time the bromine should go completely into solution. The mixture is allowed to stand for 48 hours at room temperature, and then concentrated *in vacuo* at 50° to 550 cc. The hydrobromic acid in a measured portion of this is determined by Volhard's method, and enough lead carbonate added to the reaction mixture to combine with the whole of it. This addition is carried out preferably in a large evaporating basin, the lead carbonate being added in small portions at a time. Finally, the temperature of the whole is raised to boiling point and it is allowed to boil for 3 hours, the volume being maintained by addition of water at 500–600 cc. The excess lead carbonate is then filtered off and washed with a little cold water. Most of the lead bromide crystallises out on standing at 0° for 48 hours. It is filtered off, and washed with cold water, and the washings added to the filtrate. The latter still contains appreciable amounts of bromide.

It is then diluted to 2500 cc. and treated with recently precipitated silver oxide suspended in water until a filtered portion of the reaction mixture gives no precipitate with silver nitrate and nitric acid. The silver bromide is filtered off, and washed, and hydrogen sulphide passed to saturation. The filtrate from the sulphide is evaporated to about 1 litre on the water bath and then boiled with calcium carbonate for 3 or 4 hours to decompose any lactone present. The excess calcium carbonate is filtered off and washed, and the filtrate boiled with two grams of powered respirator charcoal. After filtering and washing, the filtrate is put away for 24 hours, and the crude calcium gluconate filtered off and washed with a little cold water. More crude gluconate may be obtained by concentrating the mother liquors. Yield of dry, crude gluconate, 80 % of theory. On two recrystallisations from hot water the pure salt is obtained.

Air dried at room temperature the salt retains one molecule of water of crystallisation, but if the hydrate is left in the hot room at 37° for 3 days the anhydrous salt is produced.

Analysis. Three times recrystallised calcium gluconate left in hot room at 37° for 3 days.

0·5457 g. gave 0·0709 g. CaO = 9·28 % Ca. Calculated for $(C_6H_{11}O_7)_2$ Ca, Ca = 9·30 %.

Glycuronic acid was prepared by Neuberg's [1900] method from euxanthic acid. The glycuronic anhydride was converted into the calcium salt by heating with the calculated quantity of calcium hydroxide.

Saccharic acid was prepared by the method of Tollens and Gaus [1888] as the acid potassium salt. This is twice recrystallised, and then neutralised with ammonia, cooled and treated with the calculated quantity of 10 % calcium chloride, with constant stirring. Calcium saccharate is filtered off and recrystallised from a large volume of hot water. The crystals contain $4H_2O$, which is lost *in vacuo* at room temperature. The calcium salt is not very soluble in water, the saturated solution containing the following quantities of Ca $(C_6H_8O_8).4H_2O$ in 100 cc.: at 0° 0·030 g., at 17° 0·043 g., at 37·5° 0·086 g., at 100° 0·448 g.

Preliminary experiments.

The possibility that these substrates might be of value in characterising organisms of the coli-typhosus group was tested by inoculating tubes containing 1 % of the neutral calcium salts of gluconic, glycuronic and saccharic acids in 1 % peptone with a loopful of an 18-hour culture of 21 organisms of this group, and observing the acid and gas production. Glucose peptone was used as control. All the organisms which produced acid and gas from glucose did so with the three other substrates in 18 hours. Gas and acid production were greatest in the case of glucose, though gluconic acid was only slightly less actively attacked. Morgan's No. 1 bacillus, *B. paratyphosus A* and *B. enteritidis* gave very little acid when grown in calcium saccharate. The extraordinary ability of micro-organisms to obtain the material and energy necessary for growth and reproduction from the most unlikely sources was exemplified by the active way in which all the other organisms of the colon group attacked saccharic acid, a substance more oxidised than acetic acid. The Voges and Proskauer reaction was positive in the case of *B. cloacae* and *B. lactis aerogenes* in tubes containing either glucose or calcium gluconate, but not in the glycuronate and saccharate tubes.

Quantitative experiments.

1. *The action of* B. coli communis *on the mannitol-saccharic acid series of compounds.*

The fermentations were carried out in the apparatus designed by Harden, Thompson and Young [1910]. The hydrogen evolved was not determined in all the experiments, and where this analysis was not done the gas collecting flask was dispensed with and a simplified apparatus employed in which the CO_2 was absorbed directly into potash. The analyses were made on the lines suggested by Harden [1901] and by Grey [1919, 1] with certain modifications rendered necessary by the nature of the substrate.

Excess of precipitated calcium carbonate was always present throughout the fermentation, and the p_H of the medium kept as constant as possible by frequent shaking. The basal medium used was 1 % "bacto" peptone to which was added in various experiments from 1–2 g. % of the substrate. The fermentations were conducted under anaerobic conditions at 37° and were allowed to proceed in each case until the evolution of gas from the reaction flask had practically ceased. The acids obtained were identified by their qualitative reactions, and in many cases by the calcium oxide content of their calcium salts or the silver content of their silver salts. No substance other than those mentioned was found in recognisable quantity in the fermentation mixtures, this qualitative finding being confirmed on the whole by the carbon balance sheets.

The results given below indicate the percentage of the carbon of the substrate destroyed which appeared in the form of the various products of fermentation. Except in the case of the author's glucose figures, the figures are averages of from two to four experiments.

Table I.

	Substrate employed					
		Glucose				
Products	Mannitol (Grey, 1914)	(Harden, 1901)	(present author)	Gluconic acid (present author)	Glycuronic acid (present author)	Saccharic acid (present author)
Lactic acid	24·6	39·5	44·6	35·1	17·5	5·8
Succinic acid	6·9	5·6	5·0	9·6	14·4	15·5
Acetic acid	7·0 } 13·9	18·8 } 24·4	16·8 } 21·8	23·4 } 33·0	48·2 } 62·6	49·1 } 64·6
Formic acid	7·4	0·6	0·8	0·5	0·6	2·5
CO_2	27·3	17·2	12·4	13·8	5·0	22·0
Alcohol	27·0	13·1	16·1	10·8	2·6	1·0
Total percentage of substrate carbon appearing as carbon of above compounds	100·2	94·8	95·1	93·2	88·3	95·9

In Fig. 1 the changes in the amounts of the various products in proceeding from mannitol to saccharic acid are shown diagrammatically.

Notes on Table I and Figure 1.

Alcohol. The alcohol obtained from mannitol (Grey) (two —CH(OH).CH_2OH groups) is about twice that obtained from glucose or gluconic acid (one —CH(OH).CH_2OH group) and that obtained from glycuronic and saccharic acids is almost nil (no —CH(OH).CH_2OH groups). The presence of a —CH(OH).CH_2OH group in the substrate appears necessary for the production of alcohol. Thompson [1912] working with organisms of the coli group found that using malic or citric acid as substrate only traces of alcohol were produced. It will be noticed that neither acid contains a —CH(OH).CH_2OH group.

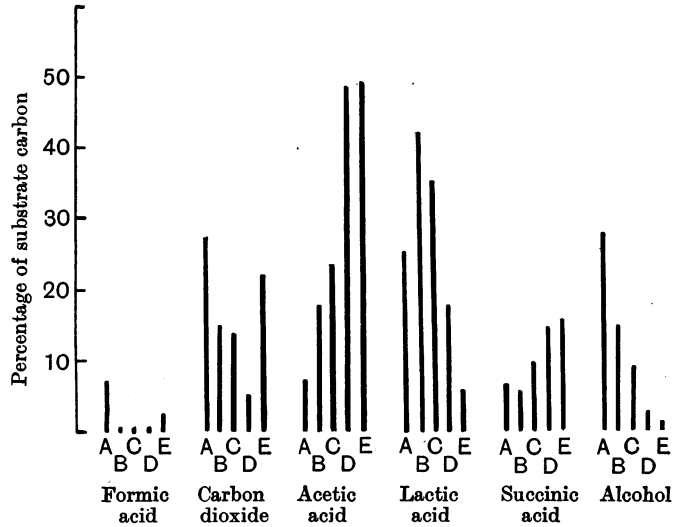

Fig. 1. *A* = amount from mannitol; *B* = amount from glucose; *C* = amount from gluconic acid; *D* = amount from glycuronic acid; *E* = amount from saccharic acid.

Succinic and acetic acids. If succinic acid may be looked upon as two acetic acid molecules joined together [see Grey, 1919, 1] and may be grouped together with it, certain correspondences (which may be fortuitous) appear. Thus the quantity of succinic plus acetic acid produced varies with the number of COOH or potential COOH (*i.e.* CHO) groups in the substance undergoing fermentation. In cases where two COOH (or one COOH and one CHO) groups are present the quantity approaches 66 %, where only one is present it is about 33 %.

Oxalic acid was not found in any of these fermentations.

Methane was not present in recognisable quantity in the gases evolved.

Hydrogen was present in the fermentation gases throughout. Even in the case of saccharic acid, with a relatively large amount of oxygen present in the molecule, a small but quite definite amount of hydrogen was produced.

2. *Action of* B. lactis aerogenes *on mannitol, glucose, gluconic acid and saccharic acid.*

Although *B. lactis aerogenes* is closely related in many of its qualitative reactions to *B. coli communis*, Harden and Walpole [1906] some years ago showed that the end-products of fermentation of glucose and mannitol by this organism, and by *B. coli*, are quantitatively very different. They found that *B. lactis aerogenes* formed, during the fermentation of glucose and mannitol, not only all the end-products of *B. coli* fermentations but also a keto-alcohol, acetylmethylcarbinol, and a glycol, 2 : 3-butylene glycol.

It was decided therefore to examine the chemical action of this organism also on the more oxygenated derivatives of glucose. The methods of fermentation and analysis employed were similar to those used for *B. coli communis*.

The following table shows the results obtained for gluconic acid and saccharic acid, together with those of Harden and Walpole for two other members of the series—glucose and mannitol.

Table II.

	Substrate employed			
	(Harden and Walpole)			
Products	Mannitol	Glucose	Gluconic acid	Saccharic acid
Lactic acid	8·6	5·5	24·1	1·2
Succinic acid	3·2	2·4	2·2	5·4
Acetic acid	2·5	5·1	14·3	50·0
Formic acid	1·5	1·0	2·8	1·5
CO_2	35·5	38·0	24·4	43·1
Alcohol	32·5	17·1	4·8	1·0
Acetylmethylcarbinol	+	+	+	−
Total	83·8	69·1*	72·6	102·2

* The greater proportion of the missing carbon was shown by Harden and Walpole to be present in the form of 2 : 3-butylene glycol.

Notes on Table II.

The experiments reveal the same type of effect as observed with *B. coli*, *i.e.* with increasing oxygen content of the substrate progressive diminution in the alcohol, and increase in the acetic and succinic acids produced. Where no —CH(OH).CH_2OH group is present in the substrate, again no more than traces of alcohol are found. Lactic acid again shows irregularities, reaching a maximum, in the case of *B. lactis aerogenes*, with gluconic acid, whereas with *B. coli* the maximum lactic acid production occurs with glucose.

Acetylmethylcarbinol is not produced from saccharic acid, and is probably, like ethyl alcohol, produced only if there is a terminal CH_2(OH).CH(OH) group present in the fermented molecule. Thompson [1912] found, for example, that *B. cloacae*, which is closely related to *B. lactis aerogenes*, and which produces acetylmethylcarbinol freely from glucose and mannitol, gave no trace of this carbinol when the substrate was malic acid or citric acid, although a straight chain of four carbon atoms is present in both these substances. The present author was unable to detect acetylmethylcarbinol in the products of fermentation of calcium saccharate by *B. cloacae*, but calcium gluconate gave a strong positive Voges and Proskauer reaction.

3. *Stages in the fermentation of calcium gluconate.*

Since the end-products of the fermentation of glucose and of calcium gluconate by *B. coli* were not greatly dissimilar it was decided to investigate the progress of the fermentation of the latter substance to see if the synthetic period of the *B. coli*-glucose fermentation, described by Grey [1919, 2], during which a non-reducing carbohydrate was produced, was also a stage in the fermentation of gluconic acid.

Into each of five flasks, with CO_2 absorbing apparatus attached, were placed 14 g. of calcium gluconate, 7 g. of precipitated chalk and 800 cc. of water. No peptone was used. The flasks were sterilised in the usual way and nitrogen passed through the medium for an hour at 37°. Each was then inoculated with 25 cc. of an emulsion of *B. coli communis* yielding 0·24 g. of dried bacteria per 25 cc. All were placed in the same water-bath at 37°. At the end of 12, 24, 48, 72, 96 hours one flask was removed, and a sample of the medium taken for a bacterial count; the flask was then heated to 70° for half an hour to stop the fermentation, and analyses carried out on the usual lines, using micro-methods wherever possible. The figures obtained are shown in Fig. 2.

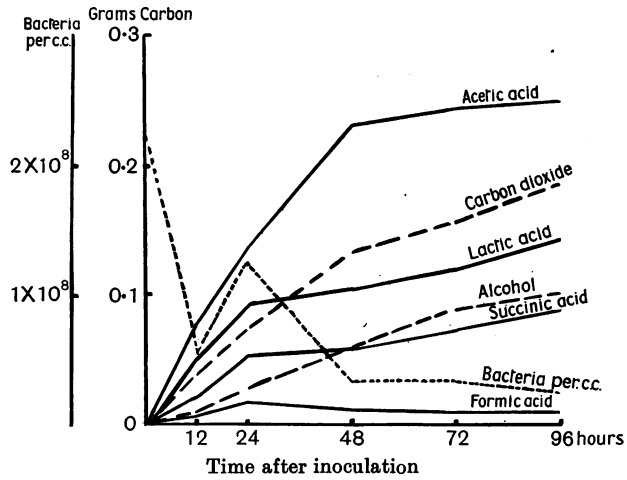

Fig. 2.

The presence of carbohydrate, both reducing and non-reducing, was carefully sought in each flask, but no appreciable amount was produced during the fermentation. Under the conditions of this experiment, with calcium gluconate as substrate, the intermediate production of a non-reducing carbohydrate which occurs in the early stages of the fermentation of glucose by *B. coli*, appears not to take place.

The above experiment also serves to demonstrate the marked difference in the relative proportions of end-products resulting from the fermentation of gluconic acid under varying conditions (peptone present and the culture seeded with a small number of organisms in one case; peptone absent and an emulsion of organisms used in the other) (see Table III). It must be pointed out, however, that, in the 96-hour experiment in absence of peptone, less than one-fifth of the total carbon present in the solution had been broken down, so that the fermentation was still in its early stages.

Table III. *Comparison of products obtained by fermenting calcium gluconate by* B. coli communis *in presence and absence of peptone, shown as percentages of the substrate carbon transformed.*

	Peptone present. Small seeding (fermented till evolution of gas has ceased)	*Peptone absent.* Emulsion of organisms added (after 96 hours' fermentation)
Lactic acid	35·1	17·6
Succinic acid	9·6	11·4
Acetic acid	23·4	33·3
Formic acid	0·5	1·14
CO_2	13·8	25·8
Alcohol	10·8	13·4

The considerable variation in the quantities of fermentation products obtained in the two cases may be due either to the fact that the fermentation was still in progress in the second case, and still far from equilibrium, or that the absolute and relative quantities of these products are dependent on the presence or absence of peptone or the different mode of inoculation. In the case of CO_2 and lactic acid the figures are widely different. It has been assumed that bacteria of the coli-typhosus group grown in a peptone medium containing sugar or other easily fermentable substrate do not attack the peptone to any appreciable extent. The present writer feels that this assumption, particularly in cases where gluconic, glycuronic or saccharic acids are substrates, is open to considerable doubt. Thus there is no question that the organisms increase in quantity during fermentation of glucose in presence of peptone, and the nitrogen required to form the increased quantity of bacterial protein must come from the peptone. When it is remembered that the increased carbon content of the bacteria themselves was not estimated, the amounts of carbon found in the products of fermentation bear in some cases a disturbingly high ratio to the amount of carbon of the substrate consumed. Amino-acids, such as alanine, aspartic and glutamic acids, which are probably liberated in small quantities from protein combination by the enzymes by which the growing organisms provide themselves with nitrogen, are easily capable of slight further change, in presence of the bacteria, to give lactic or succinic acids. It is probable that a proportion of the succinic acid and lactic acid is so derived from the peptone, and until the above experiments are repeated on media which do not contain carbon in addition to that of the substrate, the lactic acid and succinic acid figures, at least, must be regarded with suspicion. It is hoped, later, to repeat this work, using media of known constitution, when it may be found possible to construct more reliable balance sheets, showing the fate of the carbon and hydrogen of the metabolised substrate. With further information as to the heats of combustion of the substances concerned, an energy balance sheet will also become possible. The discussion of the significance of the figures obtained can then be resumed on a far more satisfactory basis.

SUMMARY.

Gluconic, glycuronic and saccharic acids are readily fermented by a large number of organisms of the coli-typhosus group.

Qualitatively, *B. coli communis* and *B. lactis aerogenes* produce from these substances the same group of fermentation products as occur when these organisms act on glucose or mannitol.

Quantitatively, interesting relationships are observed between the chemical composition of the substrate and the quantity of certain of the end-products of fermentation.

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