Some Factors Affecting Cyclopropane Acid Formation in *Escherichia coli*

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1. The fatty acid composition of the extractable lipids of *Escherichia coli* varied with growth conditions. 2. The principal fatty acids were palmitic acid, hexadecenoic acid, octadecenoic acid and the cyclopropane acids, methylenehexadecanoic acid and methyleneoctadecanoic acid. 3. Cyclopropane acid formation from monoenoic acids was increased by acid media, poor oxygen supply, or high growth temperature. 4. Cyclopropane acid formation was decreased by alkaline media, well oxygenated conditions, the presence of citrate, or lack of Mg^{2+}.

Erwin & Bloch (1964) have pointed out that two chemically distinct pathways are known for the synthesis of mono-unsaturated fatty acids in bacteria. One pathway is anaerobic and produces monoenoic acids by $\beta\gamma$-dehydration of medium-chain β-hydroxy acids and subsequent elongation of the resulting 3-enoates. The other pathway involves the participation of oxygen in the oxidative desaturation of long-chain fatty acyl esters of CoA to the corresponding *cis*-9-enoates. Bacteria that have the anaerobic pathway may be subdivided as follows: (*a*) bacteria with no cyclopropane acids, e.g. *Azotobacter agilis* (Kaneshiro & Marr, 1962) and *Leuconostoc mesenteroides* (Kemp & Rose, 1964); (*b*) bacteria with 19:0▽* acid and little or no 17:0▽ acid, e.g. *Agrobacterium tumefaciens* (Kaneshiro & Marr, 1962), *Lactobacillus casei* (Chalk & Kodicek, 1961) and *Streptococcus lactis* (MacLeod, Jensen, Gander & Sampugna, 1962); (*c*) bacteria with more 17:0▽ acid than 19:0▽ acid, e.g. *Escherichia coli* (Kaneshiro & Marr, 1961), *Serratia marcescens* (Bishop & Still, 1963*a*,*b*), *Salmonella typhimurium* (Gray, 1962), *Aerobacter aerogenes* (O'Leary, 1962) and *Clostridium butyricum* (Goldfine & Bloch, 1961). Bacteria that have the anaerobic pathway may convert monoenoic acids into saturated cyclopropane acids by the addition of the methyl group from *S*-adenosylmethionine across the double bond (Chalk & Kodicek, 1961; O'Leary, 1962; Zalkin, Law & Goldfine, 1963).

The proportion of cyclopropane acid to monoenoic acid was greater at the end of growth than at earlier stages in growth in *E. coli* (Marr & Ingraham, 1962; Law, Zalkin & Kaneshiro, 1963; Knivett & Cullen, 1965), *Agrobact. tumefaciens* (Law *et al.* 1963), *L. arabinosus* (Croom & McNeill, 1961) and *Serratia marcescens* (Law *et al.* 1963; Kates, Adams & Martin, 1964).

The present paper describes factors affecting the fatty acid composition of a strain of *E. coli* harvested during and immediately after the exponential phase of growth. Factors varied were temperature, pH, oxygenation and certain constituents of the medium.

* Abbreviations: the figure before the colon is the number of carbon atoms in the fatty acid, and the figure after the colon is the number of double bonds; ▽ indicates a saturated cyclopropane ring.

MATERIALS AND METHODS

Growth of organisms. E. coli (Pfizer no. FD11396) was a gift from Chas. Pfizer Inc., Groton, Conn., U.S.A., and was maintained on slopes containing (per l.) 28g. of nutrient agar (Oxoid code no. CM3; Oxo Ltd., London). The medium usually employed contained (per 10l.): 50g. of glycerol; 100g. of $(NH_4)_2HPO_4$; 20g. of KH_2PO_4; 10g. of trisodium citrate; 1g. of $Na_2SO_4,10H_2O$; 0·5g. of $MgSO_4,7H_2O$. The pH was adjusted to 7·2 with conc. HCl. Glycerol was the limiting nutrient except in one experiment where an excess of glycerol (150g./10l.) was employed (Fig. 2). Citrate was omitted where stated and in one experiment the Mg^{2+} concentration was halved (Table 2). The medium was usually made up in 10l. batches, boiled and then autoclaved for 1hr. at 15lb./in.^{2}.

Cells were grown in a fermenter similar to that described by Holme & Edebo (1961). The vessel was a Quickfit (visible flow) pipe section (24in. × 3in.) mounted vertically and lagged. Sterile air (2½l./min.) was forced through three cylindrical Porsilex ceramic sintered spargers (depth 1in., diam. 1⅛in., pore size 10–15μ) (Aerox Ltd., Glasgow) in the base of the vessel. The temperature of the liquid was registered by a mercury thermometer, the bulb of which protruded up through the base. Electrodes were fixed to the thermometer scale at the desired temperature so that any rise and fall of mercury altered the capacitance of the circuit and operated a Tektor proximity switch (Fielden

Electronics Ltd., Manchester), which in turn controlled the voltage supply to a silica immersion heater (Red Rod; Electrothermal Engineering Ltd., London) projecting up through the base of the vessel into the liquid. For the experiment at 23°, the temperature was maintained by circulating water from a constant-temperature water bath through a tube in place of the heater. Sterile medium, stored in 10l. bottles, was blown into the fermenter by a delivery tube through an inlet in the base of the vessel. The inlet was fitted with a clamp so that cells from the fermenter did not contaminate the reservoir. The volume of growth medium in the vessel was 1½–2l. In experiments where the pH was kept constant during growth, a titrator (model TTT1c; Radiometer, Copenhagen, Denmark) controlled the addition of sterile N-NaOH via a delivery tube to the middle of the vessel near a glass electrode. Samples of the growth medium were drawn off via an outlet in the base of the vessel. After appropriate dilution (usually tenfold) the turbidity was measured in a colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a blue filter (OB10). The colorimeter was calibrated with bacterial suspensions of known dry weight.

The inoculum was prepared by transferring cells from a slope to 10ml. of the medium under test in a 50ml. Erlenmeyer flask that was incubated at 37° on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). While still in the exponential phase of growth, inoculum was introduced into the growth medium, the volume being calculated so that after 8hr. turbidity was just visible in the fermenter; regular observations were then commenced. When the cell density reached the desired level, or when growth ceased, the vessel was drained, the sample quickly chilled and the medium then replaced by the same volume from the reservoir. Boost heating was applied to bring the medium to the required temperature; this was done so that those cells remaining in the fermenter provided an inoculum for the next sample and growth could resume without an unduly long lag phase.

In some experiments, cells were grown in 2l. Erlenmeyer flasks containing 100ml. of medium. They were inoculated with 1 drop of inoculum and incubated overnight at 37° on a rotary shaker.

Extraction of lipid from the cells. The samples were cooled rapidly, centrifuged and washed once with water. The packed cells were frozen, freeze-dried and weighed. The dry sample was ground with methanol in a Potter homogenizer and extracted at 45° for 15min. The insoluble material was separated by centrifuging and twice re-extracted with methanol. The residue was then extracted with ethanol–diethyl ether (3:1, v/v) at room temperature. The combined extracts were evaporated to dryness, dissolved in chloroform and filtered. The chloroform-soluble material was dried *in vacuo* to constant weight.

Methylation of the fatty acids. Boron trifluoride in methanol (65g./l.) was prepared by the method of Metcalfe & Schmitz (1961). The methyl esters of the fatty acids were prepared by inter-esterification of the lipid with boron trifluoride–methanol reagent (Coppock, Daniels, Gresham & Howard, 1962), except that a temperature of 60° was used to avoid decomposition of the 17:0▽ acid, which occurred at 100°. Dry lipid (4mg.) was dissolved in boron trifluoride–methanol reagent (0·2ml.) and heated at 60° in a water bath for 30min., after which water (1ml.) was added immediately. The methyl esters were extracted with three portions (1ml.) of light petroleum (b.p. 40–60°). The light-petroleum extract was dried with anhydrous Na_2SO_4 and concentrated under nitrogen in the small flasks described by Vandenhauvel (1963).

Gas–liquid chromatography. Analyses of the methyl esters were carried out on a Pye Argon Chromatograph (W. G. Pye Ltd., Cambridge). Polyethylene glycol adipate on 80–120 mesh Celite (10%, w/w) at 180° and Apiezon at 196° were used as stationary phases. The peaks were identified by comparison with retention data of standards or with published values. The percentage composition of the fatty acids was estimated by the method of Carroll (1961).

RESULTS

The extractable lipid of this strain of *E. coli* amounted to 6·5 ± 1% of the dry weight of the cells. No correlation was found between the amount of lipid and growth conditions. Three types of fatty acid were found in the lipid: saturated fatty acids [mainly palmitic (16:0) acid, with small amounts of lauric (12:0) acid, myristic (14:0) acid and stearic (18:0) acid]; monoenoic acids (16:1 acid, 18:1 acid and sometimes traces of 14:1 acid); two cyclopropane acids (17:0▽ acid and 19:0▽ acid, derived from the corresponding monoenoic 16:1 acid and 18:1 acid). Considerable variations in the amounts of the major fatty acids of *E. coli* grown under a variety of conditions were noted (Table 1). The major variations in fatty acid composition were in the unsaturated acids (16:1 acid and 18:1 acid), the cyclopropane acids (17:0▽ acid and 19:0▽ acid) and palmitic (16:0) acid. Changes in the other components were minor and of doubtful significance, and are not discussed.

Effect of temperature on fatty acid composition of growing cells. Cells were grown at 23°, 30° and 37°, and harvested at intervals during the exponential phase of growth and as soon as growth had stopped

Table 1. *Extreme limits of fatty acid composition of the extractable lipid of* E. coli *grown under various conditions*

Experimental details are given in the text.

Fatty acid	Composition (%)
14:0	½–2½
15*	0–2
16:1	1½–29½
16:0	28–46
17:0▽	1½–31
17*	0–4
18:1	5–46
18:0	0–2½
19:0▽	0–15

* Peak with the carbon number (Woodford & van Gent, 1960) shown, but not further identified.

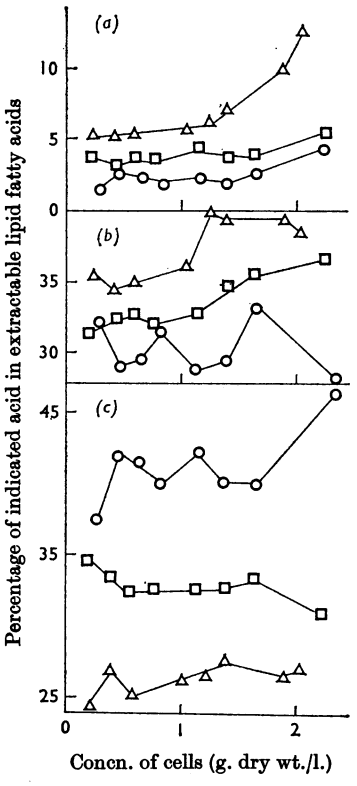

Fig. 1. Percentages of (*a*) 17:▽ acid, (*b*) 16:0 acid and (*c*) 18:1 acid in the fatty acids of extractable lipids from cells grown in a glycerol-limiting medium at 23° (○), 30° (□) and 37° (△) in the fermenter. Cells were harvested during growth at the cell concentrations indicated.

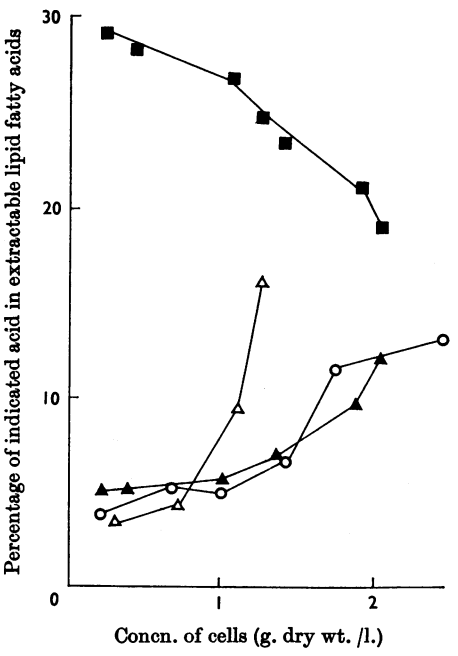

Fig. 2. Effect of medium composition on the amounts of cyclopropane acid in the extractable lipids from cells harvested during growth at 37° in the fermenter: 17:0▽ acid (▲) and 16:1 acid (■) in a glycerol-limiting medium, 17:0▽ acid (○) in a medium containing glycerol in excess (15g./l.); 17:0▽ acid (△) in a glycerol-limiting medium without citrate and maintained at pH7·0.

(preliminary experiments showed that there was no further increase in cyclopropane acid content even after 1½ days). Cells had a higher percentage of 17:0▽ acid (Fig. 1*a*) and of 16:0 acid (Fig. 1*b*) at 37° than at 30° or 23°; the percentage of 18:1 acid (Fig. 1*c*) was lowest at 37°. In these samples 19:0▽ acid was either absent or present in trace amounts, indicating that little or none of the 18:1 acid synthesized was converted into cyclopropane acid.

Effect of constituents of the growth medium on fatty acid composition. Cells were grown at 37° in (1) glycerol-limiting medium, (2) medium containing excess of glycerol and (3) glycerol-limiting medium without citrate. In the medium without citrate, experiments were performed either with the pH maintained constant at 7·0 or without pH control. Cells were harvested at intervals during the exponential phase of growth and as soon as growth had stopped. In the two media containing citrate (Fig. 2), the percentage of 17:0▽ acid was below 6% when the cell density was less than 1g./l. As the cell density increased above 1g./l. the percentage of 17:0▽ acid rose and exceeded 12% in stationary-phase cells. There was also a decrease in the percentage of 16:1 acid. There was little difference in either cyclopropane acids or other fatty acids, not shown here, between cells grown under glycerol-limiting or non-limiting conditions and harvested at comparable cell densities. Cells grown in the absence of citrate had a higher percentage of 17:0▽ acid than did cells grown in its presence, provided that the cell concentration exceeded 1g./l. In this medium the percentage of 17:0▽ acid increased markedly towards the end of growth. Some 19:0▽ acid (1·5%) was present in stationary-phase cells grown in medium without citrate, whereas other cells had none or only traces.

Since stationary-phase cells had the most cyclopropane acid, in subsequent experiments cells were harvested immediately growth had stopped. Cells grown in medium without citrate where the pH was not controlled (Expt. 1 in Table 2) had a slightly higher percentage of 17:0▽ acid (18·8%) than did cells grown in the same medium at pH 7·0 (16·4%). Since Mg^{2+} is required for the synthesis

Table 2. *Effect of* pH *control and* Mg^{2+} *concentration on cyclopropane acid formation in stationary-phase cells grown in glycerol-limiting medium without citrate at* 37° *in the fermenter*

Experimental details are given in the text.

Expt. no.	pH control	Final pH	Concn. of $MgSO_4$ (mg./l.)	Cell density (g./l.)	Fatty acid composition (%) 17:0▽ acid	19:0▽ acid	16:0 acid	16:1 acid	18:1 acid
1	+	7·0	500	1·46	16·4	1·5	41·5	15·0	23·4
1	−	6·6	500	1·51	18·8	1·6	44·3	12·0	21·5
2	+	7·0	500*	1·42	26·9	5·35	43·0	5·85	15·85
2	+	7·0	250*	1·36	13·75	0·9	43·5	12·85	23·85

* Added after sterilization.

of *S*-adenosylmethionine (the active methyl donor in cyclopropane acid formation), the effect of halving the concentration of Mg^{2+} in the medium was tested. In order that all the Mg^{2+} in the medium might be available, citrate was omitted. The culture was maintained at constant pH (7·0). The percentages of 17:0▽ acid and 19:0▽ acid were decreased and those of 16:1 acid and 18:1 acid were raised as a result of halving the Mg^{2+} concentration (Expt. 2 in Table 2). In Expt. 1, magnesium sulphate had been added to the medium before sterilization; after autoclaving there was a slight precipitate, presumably of magnesium ammonium phosphate. In Expt. 2, to minimize any precipitation of Mg^{2+} the requisite amount of sterile magnesium sulphate solution was added directly to the fermenter. The percentages of 17:0▽ acid and 19:0▽ acid were lower in the control cells of Expt. 1 than in the control cells of Expt. 2. This suggested that in Expt. 1 the concentration of Mg^{2+} available to the cells was limiting and thus influenced cyclopropane acid formation.

Effect of pH *on the fatty acid composition of stationary-phase cells.* In a series of growth experiments at 37°, a range of pH (4·5–7·7) that supported growth was chosen (although growth was poor at pH 4·5), the pH was maintained constant and the cells were harvested as soon as growth had stopped. Below pH 6·2 (Fig. 3), the percentage of 17:0▽ acid increased with acidity until at pH 4·5 it was three times that at pH 6·2. Above pH 6·2, there was little change in the percentage of 17:0▽ acid. Below pH 5·5, some 19:0▽ acid appeared. There was a marked decrease in 16:1 acid with increasing acidity below pH 6·2.

The organism was also grown in swirled Erlenmeyer flasks. The medium in each flask was adjusted to a different initial pH, and at the end of growth the final pH and cell density were measured (Table 3). The percentages of 17:0▽ acid and 19:0▽ acid increased and those of 16:1 acid and 18:1 acid decreased with increasing acidity. The lowest

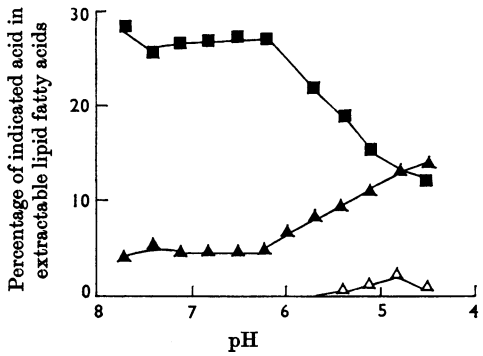

Fig. 3. Effect of pH on cyclopropane acid formation in stationary-phase cells grown in glycerol-limiting medium in the fermenter: percentages of 17:0▽ (▲), 19:0▽ acid (△) and 16:1 acid (■) in the fatty acids of the extractable lipid.

value (14·75%) for 17:0▽ acid in the flask cultures (pH 7·4) was higher than the highest value (13·8%) in the fermenter cultures at the extreme acid pH (Fig. 3). Cells grown in flasks had two to three times as much total cyclopropane acid as cells grown in the fermenter at comparable pH (Table 3). The change in pH in the flask cultures during growth could not account for a difference of this magnitude; since the same medium was used, the significant difference may have been the degree of aeration.

Effect of oxygen supply on the fatty acid composition of stationary-phase cells. The fermenter was normally aerated with 2½ l. of air (500 ml. of oxygen)/min. By mixing nitrogen with air, the oxygen supply could be decreased 100-fold without decreasing the stirring efficiency. Cells were grown under four conditions of oxygen supply, the pH was maintained constant at 7·0 and the cells were harvested when growth had ceased (Expt. 1 in Table 4). In the poorly oxygenated cultures, less growth was achieved and more acid produced. The

Table 3. *Cyclopropane acid formation in stationary-phase cells grown in glycerol-limiting medium at 37° in swirled flasks*

Experimental details are given in the text.

pH		Cell density	Fatty acid composition (%)				
Initial	Final	(g./l.)	17:0▽ acid	19:0▽ acid	16:0 acid	16:1 acid	18:1 acid
8·3	7·4	1·84	14·75	1·55	44·3	14·1	25·4
7·8	7·2	1·78	29·2	9·8	46·0	2·9	7·65
7·5	6·5	1·72	29·4	9·3	44·5	3·7	10·0
6·8	6·3	1·85	30·6	10·65	44·8	1·5	8·0
6·5	5·6	1·53	31·2	14·8	44·5	1·3	4·9
6·2	4·7	1·59	29·2	14·3	44·9	2·1	5·0

Table 4. *Effect of* pH *control, citrate and rate of aeration on cyclopropane acid formation in stationary-phase cells grown in glycerol-limiting medium at 37° in the fermenter*

Experimental details are given in the text.

Expt. no.	Sample no.	Presence of citrate	pH control	Final pH	Aeration (ml. of O_2/min.)	Cell density (g./l.)	Acid produced* (*m*-moles)	Fatty acid composition (%) 17:0▽ acid	19:0▽ acid	16:0 acid	16:1 acid	18:1 acid
1	1	+	+	7·0	500	2·02	27·8	6·1	Trace	41·3	25·2	21·6
1	2	+	+	7·0	100	2·0	30·1	9·7	0·85	39·8	19·15	25·4
1	3	+	+	7·0	20	1·46	59·8	16·65	2·2	41·1	14·1	22·4
1	4	+	+	7·0	5	1·04	68·8	22·9	2·6	42·8	13·0	14·25
2†	5a	−	+	7·0	500	1·31	—	9·6	Trace	39·3	24·7	23·3
2	5b	−	+	7·0	500	1·46	33	16·4	1·5	41·5	15·0	23·4
2	6	−	−	6·6	500	1·51	—	18·8	1·6	44·3	12·0	21·5
2	7	−	−	6·6	50	1·33	—	25·3	7·5	38·95	5·3	20·1

* The titres are not all exactly comparable since the volume of medium in the fermenter may not have been identical each time it was filled and the volume was reduced each time a cell density measurement was made.

† Cells were harvested in the exponential phase of growth.

highest rates of oxygenation produced cells with small amounts of cyclopropane acids. As the oxygen supply was decreased, the proportion of cyclopropane acids increased from 6 to 25%. In Expt. 2 (Table 4), the effect of oxygen depletion was tested in medium without citrate, a condition that resulted in greater cyclopropane acid formation. Samples 5a and 7 were harvested at comparable cell densities, the cells in sample 5a being still in the exponential phase of growth. In sample 5 the pH was controlled at 7·0, and in samples 6 and 7 the pH was not controlled and fell to 6·6; in sample 7 the oxygen supply was decreased to one-tenth of the normal rate. The percentage of cyclopropane acid was slightly greater at pH 6·6 than that of the control (sample 5b), and was even greater when the oxygen supply was decreased (sample 7). However, cells (sample 7) supplied with 50 ml. of oxygen/min. had a higher proportion of cyclopropane acid than did cells (sample 4) supplied with 5 ml. of oxygen/min. in the presence of citrate.

DISCUSSION

Certain changes in growth conditions (e.g. pH, temperature, oxygenation and medium composition) produced significant changes in the fatty acid composition of cells of *E. coli* (Pfizer no. FD11396). Cyclopropane acid formation during growth varied with the environment, and was increased by acid media, poor oxygen supply, high growth temperature and Mg^{2+}. The highest proportion of cyclopropane acids (nearly 40%) was found in stationary-phase cells grown in flasks (Expt. 1 in Table 5), which was slightly more than in fermenter-grown cells (Expt. 2 in Table 5). As little as 3·9% of 17:0▽ acid was present in some other stationary-phase cells (Expt. 3 in Table 5). Some 17:0▽ acid (1·7%) was always present even in cells grown under conditions unfavourable to cyclopropane acid formation, e.g. exponential-phase cells at 23° (Fig. 1*a*). However, under conditions most favourable to cyclopropane acid formation, exponentially growing cells had a higher cyclopropane acid content

Table 5. *Proportions of cyclopropane acids in the fatty acids of the extractable lipids of stationary-phase cells grown under different conditions in a glycerol-limiting medium at 37°*

Experimental details are given in the text.

Expt. no.	Culture vessel	Cell density (g./l.)	Presence of citrate	pH	Aeration (ml. of O_2/min.)	Fatty acid composition (%) 17:0▽ acid	19:0▽ acid	16:0 acid	16:1 acid	18:1 acid
1	Flask	1·78	+	7·8–7·2	—	29·2	9·8	46·0	2·9	7·65
2	Fermenter	1·42	−	7·0	500	26·9	5·35	43·0	5·85	15·85
3	Fermenter	1·78	+	7·7	500	3·9	0	36·9	28·4	27·6

than did stationary-phase cells grown under conditions unfavourable for such formation.

Of the two cyclopropane acids, the lower homologue was formed preferentially, and usually 9% of 17:0▽ acid was present before any measurable amount of 19:0▽ acid appeared. When the content of 17:0▽ acid amounted to 19% of the fatty acids, that of 19:0▽ acid was about 3%. Once the content of 17:0▽ acid had risen to about 30% of the fatty acids, any further transmethylation appeared to have been at the expense of 18:1 acid to produce 19:0▽ acid.

Cells that had been grown in medium containing citrate had less cyclopropane acids than did cells that had been grown without citrate. A possible effect of citrate may be to limit the availability of Mg^{2+}. Where citrate was omitted (Table 2), and some precipitation of magnesium ammonium phosphate occurred, the limited availability of Mg^{2+} may have restricted cyclopropane acid formation.

Herbert (1965) showed that, during growth of a batch culture, oxygen demand increased and the dissolved oxygen concentration decreased; therefore, unless aeration was very efficient, oxygen was limiting in the later stages of growth. When such conditions of oxygen starvation were reached, the cells produced acid. In a batch culture, as growth continues monoenoic acids are replaced by cyclopropane acids (Law *et al.* 1963; Kates *et al.* 1964), but at the same time cells may be decreasingly deprived of oxygen. In the experiments reported above, cyclopropane acid formation was below 6% in cells harvested at low cell concentrations (0·8 g. dry wt./l.), where oxygen supply was probably adequate, but increased at high cell concentrations, where oxygen supply may have been inadequate. When the supply of oxygen was severely restricted there was definite stimulation of cyclopropane acid formation. It is possible that the greater formation of cyclopropane acids in flask-grown cells compared with cells grown under forced aeration was due to a lack of oxygen, as Schultz (1964) has shown that, in large flasks with cotton-wool plugs, oxygen diffusion through the plug may be slow enough to restrict the growth of cells. The suggestion by Law *et al.* (1963) that cyclopropane rings may prevent peroxidation of unsaturated acids and thus afford the organism some protection against oxygen is not in agreement with our findings that oxygen inhibits cyclopropane acid formation. In its natural habitat, *E. coli* exists under anaerobic conditions and would contain a high proportion of cyclopropane acids.

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