In medium saturated with oxygen, the cyclopropane synthetase (unsaturated-phospholipid methyltransferase; EC 2.1.1.16) of Proteus vulgaris was generally synthesized after the mid-exponential phase of growth. The enzyme could also be induced by rapidly limiting the oxygen supply, or by initiating respiration on nitrate or thiosulphate following an initial period of growth in a highly aerobic environment. In each of these 'step-down' situations the specific activity of cyclopropane synthetase rose to a maximum prior to the stationary phase of growth and subsequently decreased. The cyclopropane fatty acids, methylene hexadecanoic acid and methylene octadecanoic acid accumulated throughout exponential growth following the induction of the enzyme. During a 12 h period in the stationary phase there was little synthesis of either of the fatty acids, despite detectable cyclopropane synthetase activity in the cells, indicating that essentially all the fatty acid synthesis was complete prior to entering the stationary phase. When nitrate was used as a respiratory electron acceptor, a twofold increase in octadecenoic acid was observed, giving rise to an increase in methylene octadecanoic acid. This increase in octadecenoic acid was not apparent in mutants unable to respire on nitrate.

INTRODUCTION

It is known that the lipid composition of bacteria can vary with the age of the culture. For example, cyclopropane fatty acids generally accumulate during the late-exponential and stationary phases of growth (Cronan, 1968; Halper & Norton, 1975; Jacques & Hunt, 1980). However, the significance of the synthesis of cyclopropane fatty acids (Finnerty & Makula, 1975; Taylor & Cronan, 1976) and the factors controlling the onset of their accumulation are still unclear. For instance, the onset of accumulation of the cyclopropane fatty acid, methylene hexadecanoic acid in Pseudomonas denitrificans depends on de novo synthesis of cyclopropane synthetase (unsaturated-phospholipid methyltransferase; EC 2.1.1.16) (Jacques & Hunt, 1980). Although the enzyme is induced in cells grown on succinate by rapidly limiting the oxygen supply, methylene hexadecanoic acid only accumulates slowly, even after the maximum enzyme activity is obtained, requiring 15 to 20 h to reach a maximum. In contrast, the activity of cyclopropane synthetase does not vary in Escherichia coli (Cronan, 1968). Thus, cultures of E. coli with the same enzyme activity may or may not synthesize cyclopropane fatty acids. A third phenomenon has been reported in cultures of Lactobacillus plantarum, in which 70% of the cyclopropane fatty acids are produced rapidly as the cells enter the stationary phase. In cultures of this organism, the activity of cyclopropane synthetase increases as the stationary phase is approached and then rapidly decreases. The maximum rate of accumulation of cyclopropane fatty acid parallels this peak of enzyme activity (Halper & Norton, 1975).

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In view of the apparent differences observed in the control of cyclopropane fatty acid synthesis in different organisms, the present study was undertaken to determine what factors affect the synthesis of the fatty acid(s) in a facultative anaerobe capable of utilizing a variety of respiratory acceptors. *Proteus vulgaris* was particularly well suited for these studies as it can withstand rapid changes to alternative forms of respiration without detriment to its growth.

**METHODS**

Organism. The H antigenic strain of *Proteus vulgaris* NCIB 4635 isolated on nutrient agar plates was used in this study. Optimum growth of *P. vulgaris* at 30 °C was obtained by supplementing a minimal phosphate-based medium (Hunt, 1959) with Difco Bactopeptone (0-3%, w/v).

Growth conditions for the measurement of cyclopropane synthetase activity. Cultures were grown under one of the following two oxygenation conditions: 'state 1', O₂ (100%) sparged at a rate of 500 ml min⁻¹ (1 medium)⁻¹; or 'state 3', O₂/N₂ (3:97, v/v) sparged at a rate of 200 ml min⁻¹ (1 medium)⁻¹ (Jacques & Hunt, 1980). Additional conditions were defined as 'nitrate respiration' – addition of a sterile solution of potassium nitrate (0-5%, w/v, final concentration) plus N₂ (100%) sparged at a rate of 400 ml min⁻¹ (1 medium)⁻¹ – and 'thiosulphate respiration' – addition of a sterile solution of potassium thiosulphate (0-5%, w/v, final concentration) plus N₂ (100%) sparged at a rate of 400 ml min⁻¹ (1 medium)⁻¹.

Batch cultures (1600 ml) were grown in 3 litre, three-necked, round-bottomed flasks fitted with dual ceramic spargers. The medium was magnetically stirred and the culture pH and culture redox potential (Eh) were monitored after inoculation with cells grown under 'state 1' conditions according to a strictly defined procedure (Jacques & Hunt, 1980). The cultures were initially grown at 30 °C without pH control under 'state 1' conditions for a period of 2 h to repress the activity of cyclopropane synthetase. The increase in specific activity of the enzyme was followed after changing to one of the three other conditions described above. Similar cultures maintained under 'state 1' conditions throughout their growth cycle were used as controls.

The H₂S formed by 'thiosulphate respiration' was partially removed by the N₂ sparging and was detected in a water trap containing lead acetate (1 M). The nitrite formed in the medium as a result of the reduction of nitrate was measured according to the method of Nason & Evans (1963).

Preparation and analysis of fatty acids. Cells were grown for fatty acid analysis as described above except for the results reported in Table 1. In these instances, larger cultures (15 l) were grown in a glass fermenter fitted with a variable speed paddle-stirrer and a sintered metal sparger. The controlled medium conditions were imposed immediately upon inoculation of the medium with cells (750 ml) which had been grown in flask culture for 16 h in the presence of the appropriate respiratory acceptor. The stirring rate was 200 rev. min⁻¹ under 'state 1' conditions and 50 rev. min⁻¹ for all other conditions.

Samples (100–250 ml) of culture were harvested by centrifugation (6000 g, 4 °C, 10 min). Each pellet was resuspended in 2-5 ml of distilled water and the membrane-bound lipids were extracted according to the method of Bligh & Dyer (1959). Fatty acid methyl esters of each sample were prepared by the addition of NaOH in methanol (2 ml, 0-5 M) to the crude dried lipid in a 15-2 x 2-5 cm screw-top culture tube which was Teflon-lined (Klopfenstein, 1971). The tube was flushed with N₂ and placed in a water bath at 60 °C for 10 min. After cooling to room temperature, 2-5 ml of a 14% (w/v) solution of BF₃ in methanol (Applied Science Laboratories, Pa., U.S.A.) was added, and the tube was again flushed with N₂ and placed in the water bath at 60 °C for a further 45 min. The temperature was maintained at 60 °C to avoid the breakdown of cyclopropane fatty acids that has been observed at higher temperatures (Hancock & Meadow, 1969; Vulliet et al., 1974). After cooling, hexane (3 ml) and saturated NaCl (20 ml) were added and the tube contents were vigorously mixed. The hexane phase was removed and the aqueous phase was re-extracted twice with hexane (3 ml). The combined hexane extracts were dried over anhydrous Na₂SO₄ and the methyl esters of the fatty acids were further purified by applying them in hexane to a 2 x 1 cm column of alumina packed in hexane. Methyl esters were eluted from this column with 5% (v/v) diethyl ether in hexane and evaporated to dryness on a rotary evaporator *in vacuo* below 35 °C. The methyl esters were then diluted with carbon disulphide to a concentration suitable for gas-liquid chromatographic analysis. The fatty acid methyl esters were analysed and characterized on a Bendix chromatograph (Series 2500, The Bendix Corporation, W. Va, U.S.A.) as previously described (Jacques & Hunt, 1980).

Isolation of chlorate-resistant mutants. Chlorate-resistant mutants of *P. vulgaris* were isolated on glucose chlorate agar plates after 24 h growth (Piéchaud *et al.*, 1967). Mutants were selected on the basis of altered colony morphology and transferred into nutrient broth (20 ml). After replating and re-culturing twice to check their homogeneity and stability, selected chlorate-resistant mutants were stored at 4 °C on nutrient agar slopes. Although 12 distinct colony morphologies could be readily discerned on chlorate agar plates, approximately 25% of the mutations were unstable, giving rise to different colony types on re-culturing and re-plating. Only stable colonies were taken for further study.
Cyclopropane fatty acids of *Proteus vulgaris*

Table 1. *Fatty acid composition of P. vulgaris grown under defined conditions*

Cells were grown in 15 l batches in a glass fermenter fitted with a variable speed paddle-stirrer. For 'state 1' and 'state 3' conditions, cultures were sparged with O₂ (100%) at a rate of 500 ml min⁻¹ (1 medium)⁻¹ and with O₂/N₂ (3:97, v/v) at a rate of 200 ml min⁻¹ (1 medium)⁻¹, respectively. The fatty acid composition is given as a percentage of the total fatty acids in cells grown to the late-exponential phase of growth (7-10 h after inoculation). The standard error for any given fatty acid was 0-15 % (four analyses).

<table>
<thead>
<tr>
<th>Fatty acid composition (% of total)</th>
<th>Growth condition:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'State 1'</td>
</tr>
<tr>
<td>Succinate as the major carbon source</td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>3.4</td>
</tr>
<tr>
<td>16:0</td>
<td>49.0</td>
</tr>
<tr>
<td>16:1</td>
<td>30.2</td>
</tr>
<tr>
<td>17:0&lt;sub&gt;ac&lt;/sub&gt;</td>
<td>2.3</td>
</tr>
<tr>
<td>18:1</td>
<td>12.8</td>
</tr>
<tr>
<td>19:0&lt;sub&gt;cyc&lt;/sub&gt;</td>
<td>0.0</td>
</tr>
<tr>
<td>Glucose as the major carbon source</td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>6.0</td>
</tr>
<tr>
<td>16:0</td>
<td>39.1</td>
</tr>
<tr>
<td>16:1</td>
<td>37.8</td>
</tr>
<tr>
<td>17:0&lt;sub&gt;ac&lt;/sub&gt;</td>
<td>2.2</td>
</tr>
<tr>
<td>18:1</td>
<td>12.4</td>
</tr>
<tr>
<td>19:0&lt;sub&gt;cyc&lt;/sub&gt;</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* The figure before the colon indicates the number of carbon atoms in the chain, that after the colon denotes the number of double bonds; cyc indicates the presence of a cyclopropane ring.
† Growth on succinate was poor; the values given are for cells grown for 72 h.

RESULTS

*Fatty acid composition of P. vulgaris grown under defined conditions*

Table 1 shows the fatty acid composition of cells grown in 15 l batch cultures on either succinate or glucose under all four controlled conditions. Only under 'state 1' conditions was there a reduced cell content of the two cyclopropane fatty acids, methylene hexadecanoic acid and methylene octadecanoic acid. Neither carbon source prevented the synthesis of the cyclopropane fatty acids when the other respiratory conditions were imposed. However, cells grown on glucose consistently possessed higher contents of cyclopropane fatty acids especially of the minor C<sub>19</sub> cyclopropane fatty acid, methylene octadecanoic acid.

Assay of cyclopropane synthetase activity

The method described for the assay of cyclopropane synthetase (Jacques & Hunt, 1980) was investigated by examining the enzyme prepared from cells grown for 16 h to stationary phase on succinate or glucose in 1 l flasks containing 250 ml medium and shaken at 50 rev. min⁻¹ (Herbert, 1965). In the presence of 3 mg added lipid and 50 nmol S-adenosyl-L-[methyl-<sup>3</sup>H]methionine (240000 c.p.m.), the activity of cyclopropane synthetase was found to increase linearly with time over 90 min at 30 °C. A linear relationship was also found between the reaction rate and enzyme concentration in the range 0-9 mg total protein (ml assay mixture)⁻¹. The protein concentration used in subsequent studies was between 1.3 and 6.1 mg protein (ml assay mixture)⁻¹ (average 2.8 ± 0.8).

Intracellular localization of cyclopropane synthetase

The sonicated cell-free preparation of *P. vulgaris* which was used for the assay of cyclopropane synthetase (Jacques & Hunt, 1980) was examined to determine whether the enzyme was soluble or particle-bound. Part of a 50000 g supernatant showing a
cyclopropane synthetase specific activity (± s.e.) of 2800 ± 300 c.p.m. h⁻¹ (mg protein)⁻¹  
was centrifuged (100000 g, 4 °C, 1 h) to yield a supernatant with a specific activity of 2750  
± 250 c.p.m. h⁻¹ (mg protein)⁻¹. The original 50000 g pellet had an enzyme specific activity  
of 50 ± 30 c.p.m. h⁻¹ (mg protein)⁻¹, indicating that essentially all of the activity was in the  
supernatant fractions.

**Effect of oxygen tension on the synthesis of cyclopropane synthetase**

Cells grown under ‘state 1’ conditions with succinate as the main carbon source possessed  
little or no measurable cyclopropane synthetase activity until midway through the growth of  
the culture, at an apparent $A_{500}$ of about 1.0 (Fig. 1a). The oxygen tension in the medium  
remained at a saturating level throughout growth whilst the measured $E_h$ value remained high,  
at about +330 mV.

The maximum activity of cyclopropane synthetase was reached in the late-exponential  
phase. However, very little increase in the methylene hexadecanoic acid content of cells  
occurred during the stationary phase. The value only increased from 6.2% on entering the  
stationary phase at 10 h to 9.4% at 24 h, despite the fact that the activity of cyclopropane  
synthetase appeared to be constant over a 12 h period in this phase.

When a culture was ‘stepped-down’ from ‘state 1’ to ‘state 3’ conditions at an apparent $A_{500}$  
of 0.2, the rapid consumption of oxygen from the medium was followed by a drop in the  
culture $E_h$ to a final value of −170 mV (Fig. 1b). Cyclopropane synthetase activity was  
subsequently detected in the cells. The appearance of the enzyme was independent of the cell  
density at which the ‘state 3’ constraint was imposed. The rate of growth and the final cell  
densities, however, were severely impaired by imposing this condition. Also, cyclopropane  
synthetase activity appeared to decrease during the stationary phase.

The maximum activities of cyclopropane synthetase [approximately 3000 c.p.m. h⁻¹ (mg  
protein)⁻¹] attained in cells grown on succinate were comparable for both culture oxygen
Cyclopropane fatty acids of Proteus vulgaris

Fig. 2. Specific activity of cyclopropane synthetase in cells grown on medium containing 0.5% (w/v) glucose: (a) stepped-down from 'state 1' [O₂ (100%); 500 ml min⁻¹ (l medium)⁻¹] to 'state 3' [O₂/N₂ (3:97, v/v); 200 ml min⁻¹ (l medium)⁻¹] conditions at the time indicated by the arrow; (b) stepped-down from 'state 1' to 'nitrate respiration' (KNO₃ (0.5%, w/v); N₂ (100%); 400 ml min⁻¹ (l medium)⁻¹) conditions at the time indicated by the arrow. O, Growth; Aₜₜₜ; △, pH; A, redox potential; Eₜₜₜ; ●, oxygen tension (% maximum saturation with pure oxygen); ■, enzyme specific activity; □, nitrite produced in the culture medium.

conditions. The pH of the medium under both oxygen conditions was constant during culture growth, but rose slightly during the later hours in the stationary phase. Under 'state 3' conditions, the methylene hexadecanoic acid content of cells increased from 4.2%, when step-down conditions were imposed, to 9.3% on entering the stationary phase; a final value of 11.3% was obtained after a further 12 h.

In cultures grown under 'state 1' conditions on glucose as the main carbon source, cyclopropane synthetase was detected initially after the mid-point of growth in a similar manner to cultures grown on succinate. No decline in oxygen tension was observed, and the culture Eₜₜₜ remained high throughout the growth cycle at a value above +300 mV. The cyclopropane synthetase activity reached a maximum of approximately 3000 c.p.m. h⁻¹ (mg protein)⁻¹ and remained at this level for at least 16 h in the stationary phase. The pH remained constant during growth, only falling in the late-stationary phase. The maximum content of methylene hexadecanoic acid measured in such cultures after 24 h was about 9.0%.

The external Eₜₜₜ in a culture grown on glucose remained above +250 mV for 3 h after stepping-down from 'state 1' to 'state 3' conditions, despite undetectable levels of oxygen in the medium (Fig. 2a). The culture Eₜₜₜ then dropped rapidly (within a period of 30 min) and cyclopropane synthetase was subsequently detected in the cells. Culture Eₜₜₜ values as low as −350 mV were then recorded. Subsequently, a drop in pH was observed which was assumed to be due to the secretion of acidic metabolites produced by the cells as a result of the fermentation of glucose under 'state 3' conditions. The nature of these metabolites was not determined.

The maximum activity of cyclopropane synthetase observed under imposed 'state 3' conditions was approximately twice that in cells grown under 'state 1' conditions on glucose,
or under either oxygen condition on succinate. A maximum activity approaching 7000 c.p.m. h⁻¹ (mg protein)⁻¹ was observed during the later stages of growth when the culture $E_h$ reached a minimum value. However, the enzyme activity began to decline as cells entered the stationary phase and was low after 12 h in this phase. The culture $E_h$ rose markedly during this period (Fig. 2a). Although the methylene hexadecanoic acid content increased from 4.2% upon inoculation, to 13.2% on entering the stationary phase, a final value of only 16.8% was recorded after 12 h in this phase.

**Effect of ‘thiosulphate respiration’ and ‘nitrate respiration’ on cyclopropane synthetase activity**

Cultures grown on glucose could be readily stepped-down to either ‘thiosulphate respiration’ or ‘nitrate respiration’ without severely impairing the growth rate.

When thiosulphate was used as the terminal respiratory acceptor, the culture $E_h$ reached the lowest measured value of $-480 \text{ mV}$. Cyclopropane synthetase activity was detected following the decline in culture $E_h$, but not until $\text{H}_2\text{S}$ was detected in the medium some 3–4 h after imposing the condition. The activity of the enzyme approached 9000 c.p.m. h⁻¹ (mg protein)⁻¹; this was the highest value recorded and was some threefold higher than that of a similar culture grown under ‘state 1’ conditions. However, the activity of the enzyme declined during the stationary phase in a similar manner to that observed in cultures grown on glucose under ‘state 3’ conditions. Low $E_h$ values persisted during this period.

When cells grown on glucose were stepped-down to ‘nitrate respiration’, the culture $E_h$ dropped from its initial value of $+320 \text{ mV}$ to $-82 \text{ mV}$ over a period of 4 h. During the later stages of growth, the culture $E_h$ began to rise, due to the production of nitrite (Fig. 2b). This increase in culture $E_h$ prior to the stationary phase was followed by a substantial decrease in the specific activity of cyclopropane synthetase. The decrease in activity was far more rapid than that measured during the stationary phase under other culture conditions. The low final cell density indicated the possible inhibitory nature of the nitrite produced by the cells, and this in itself may have adversely affected enzyme activity. Nitrite (50 mM) partially inhibited (23 %) the enzyme in the *in vitro* assay system. However, the addition of both nitrate (0.5 %, w/v) and nitrite (0.1 %, w/v) to a culture grown on glucose under ‘state 3’ conditions was found to produce an immediate increase in culture $E_h$ from $-350 \text{ mV}$ to $-80 \text{ mV}$ over a period of 2 h, without any corresponding decrease in the activity of cyclopropane synthetase. Thus, the presence of excess nitrite in the medium did not appear to affect the activity of the enzyme *in vivo*. The question as to whether nitrite entered the cells under these conditions was not investigated.

The methylene hexadecanoic acid content of the cells increased from 3.8%, on stepping-down to ‘nitrate respiration’, to 10.2% on entering the stationary phase; a final value of 12.3% was recorded after 14 h in this phase. However, a twofold increase in octadecenoic acid and its cyclopropane derivative, methylene octadecanoic acid was observed in cells respiring on nitrate (cf. Table 1). This change in octadecenoic acid content was not as apparent under any of the other imposed conditions.

**Control of the synthesis of cyclopropane synthetase**

No increase in cyclopropane synthetase activity was observed after dialysis of extracts apparently devoid of enzyme activity or possessing low activity. Total recovery of enzyme activity was obtained on addition of extracts devoid of enzyme activity to samples possessing maximum activity, indicating that a reversible inhibition of the enzyme by a low molecular weight molecule was unlikely. The addition of chloramphenicol (1 μg ml⁻¹) to cultures during any one of the step-down conditions described above, however, was found to prevent the appearance of cyclopropane synthetase (data not shown). Enzyme specific activity remained at the basal level (usually undetectable) for upwards of 8 h after imposing any of the three step-down conditions. At least four measurements of enzyme activity were made during this period. Chloramphenicol itself did not affect the activity of cyclopropane synthetase *in vitro*. 
Fatty acid analyses of the lipids of wild-type and chlorate-resistant mutants of *P. vulgaris*

When cells were cultured anaerobically in the presence of nitrate, they possessed not only nitrate reductase activity but also elevated contents of octadecenoic acid. The effect on the fatty acid composition of cellular lipids upon initiating conditions of 'nitrate respiration' was studied in wild-type cells and in three chlorate-resistant mutants (designated H2, H6 and H9) grown on glucose. The three mutants were known to lack both formate-linked nitrate reductase activity and formate hydrogenlyase activity when grown under 'state 3' conditions in the presence of nitrate (unpublished observations). Stepping-down to conditions of 'nitrate respiration' completely inhibited growth in mutant H9, but not the parent culture or mutants H2 and H6. While the three mutants showed an immediate rise in their contents of methylene hexadecanoic acid once 'nitrate respiration' was imposed, no changes in their contents of octadecenoic acid were apparent, the values remaining constant at approximately 13 % of the total fatty acids. In contrast, the parent strain showed an increase in both methylene hexadecanoic acid and octadecenoic acid once 'nitrate respiration' was imposed. After a further 8 h, the octadecenoic acid had increased from 14% to 24% of the total fatty acids.

**DISCUSSION**

During the hyperbaric oxygen conditions imposed under 'state 1', the cyclopropane synthetase of *Proteus vulgaris* initially remained repressed and cyclopropane fatty acids did not accumulate during exponential growth. Synthesis of the enzyme could be initiated by stepping-down to one of the three other respiratory conditions where either oxygen was limiting, or an alternative terminal respiratory acceptor was utilized. The main accumulation of the cyclopropane fatty acid(s) occurred after the appearance of the enzyme but before the stationary phase was reached. This contrasts with the results obtained with *Pseudomonas denitrificans* (Jacques & Hunt, 1980), where a direct relationship was observed between cyclopropane synthetase activity and fatty acid production during the stationary phase.

Only under 'state 1' conditions did the activity of cyclopropane synthetase in *P. vulgaris* remain constant for 12 h in the stationary phase. In all other cases the activity of the enzyme decreased during this period in a similar manner to that observed in cultures of *Lactobacillus plantarum* (Halper & Norton, 1975). In *P. vulgaris*, the amount of cyclopropane fatty acid(s) that had accumulated prior to the stationary phase, appeared to depend on the maximum activity of cyclopropane synthetase. In cells grown on succinate and stepped-down to 'state 3' conditions, for instance, the fatty acid accumulated to a level approximately half that in cells similarly grown on glucose, which possessed twice the enzyme activity.

It is possible that substrate availability in the form of either ATP or S-adenosylmethionine limited the accumulation of cyclopropane fatty acids in *P. vulgaris* during the stationary phase. Alternatively, an overriding control of cyclopropane fatty acid production at the level of enzyme activity might occur in these cells in a manner similar to that proposed for *E. coli* (Cronan, 1968; Cronan et al., 1974). Unlike *E. coli*, the enzyme in *P. vulgaris* appeared to be inducible as the addition of chloramphenicol prevented the appearance of the enzyme under all conditions, indicating that de novo protein synthesis was required for expression of enzyme activity.

Cultures of *P. vulgaris* grown under 'nitrate respiration' also had increased contents of octadecenoic acid. Each of the chlorate-resistant mutants H2, H6 and H9 failed to show any increase in this fatty acid when stepped-down to conditions of 'nitrate respiration'. The increase in octadecenoic acid observed under 'nitrate respiration' in the parent strain, resulted in more methylene octadecanoic acid being synthesized regardless of the carbon source in the medium (cf. Table 1).

In summary, cyclopropane synthetase appears to be an inducible enzyme in *P. vulgaris*, its synthesis being promoted by anaerobic or microaerophilic conditions. The activity of the enzyme generally rises to a maximum just before the stationary phase and then decreases.
The two fatty acids, methylene hexadecanoic acid and methylene octadecanoic acid are preferentially synthesized during the period of maximum enzyme synthesis, indicating that other factors influence the activity of the enzyme in the stationary phase.

This work was supported in part by a Postgraduate Fellowship awarded by the C.S.I.R.O. The author wishes to thank Associate Professor A. L. Hunt for helpful discussion during the course of this work.

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