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The Effect of Low Temperatures on Fatty Acid Biosynthesis in Plants

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1. Of three systems, bulb tissue, plant leaf tissue and intact green algal (*Chlorella vulgaris*) cells, only the former shows an increase in rate of formation of unsaturated fatty acids with decrease in temperature. 2. In bulb tissue the oxygen concentration is rate-limiting for synthesis of unsaturated fatty acids at temperatures down to 10°. 3. At elevated oxygen concentrations the formation of unsaturated fatty acids in bulb tissue increases with temperature. 4. The failure of photosynthetic tissues to respond to either lower temperatures or increased oxygen concentrations in the presence of light is attributed to photosynthetic production of excess of oxygen. This is supported by the fact that in the dark a potentiating oxygen effect on the formation of unsaturated fatty acids can be demonstrated. 5. The HCO_3^- ion concentration has a small effect on the formation of unsaturated fatty acids. 6. Elevated content of unsaturated acids at lower temperatures in plants is attributed to increases in oxygen concentration in solution.

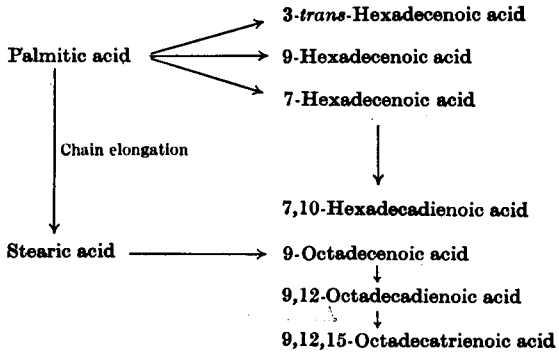
It has long been known that some plants produce a more highly unsaturated seed fat when grown at a lower temperature (Hilditch & Williams, 1964). This effect seems to be limited to certain species, since Canvin (1965) showed that it occurs in the seeds of rape, sunflower and flax, but not of safflower or castor.

No reasonable explanation for this effect has yet been produced. The experimental conditions under which it occurs are such that it could be due either to a direct modification of the enzymes synthesizing the unsaturated fatty acids or to an effect of prolonged growth at low temperatures on the activities of appropriate enzymes.

The chemical pathway of the biosynthesis of unsaturated fatty acids in plants is well defined (Harris & James, 1965; Harris, Harris & James, 1965; Nichols, Harris & James, 1965; Nagai & Bloch, 1965, 1966; Howling, Morris & James, 1967). The sequential series of dehydrogenations is shown in Scheme 1.

The major cofactors so far identified are oxygen, CoA, NADPH, acyl-carrier protein and, in isolated leaf chloroplasts, ferredoxin (Mudd & Stumpf, 1961; James, 1963; Stumpf & James, 1963; Nagai & Bloch, 1966).

In this paper we report a study of the effect of low temperature on fatty acid biosynthesis in leaves, bulb tissue and the green alga *Chlorella vulgaris*.



Scheme 1.

MATERIALS AND METHODS

Chemicals. All solvents were redistilled before use and all chemicals were of A.R. grade. [2-^{14}C]Acetate with a specific radioactivity of approx. 40mc/m-mole (obtained from The Radiochemical Centre, Amersham, Bucks.) was dissolved in water to give a concentration of 10μc/ml.

Chlorella vulgaris. *Chlorella vulgaris* (strain 211/11h) was grown in the 'rich' medium by the method of Harris *et al.* (1965). The cells were resuspended in 0·2M-potassium phosphate buffer, pH 7·4, to give samples of volume 2ml., and approx. 0·25g. wet wt. of cells/sample. Each sample was incubated with 2μc of [2-^{14}C]acetate.

Plant leaves. Castor-oil plants (*Ricinus communis*) and spinach (*Spinacea oleracea*) were grown in greenhouses. Ten fresh young leaves were laid on top of one another and disks (5mm. diam.; 2·5mg. of tissue) punched from them with a cork borer; 100 leaf disks in 3ml. of 0·2M-potassium phosphate buffer, pH 7·4, and 2μc of [2-^{14}C]acetate were used for each sample.

Narcissus bulbs. The narcissus bulbs used were either ones that had finished flowering and whose leaves were senescing, or ones that had been out of the ground for

about 3 months and were just showing signs of shoot-emergence. The dark outer layers and the stem and roots were removed and the bulb tissue was finely chopped. Chopped bulb tissue (2g.) in 4ml. of 0·2M-potassium phosphate buffer, pH7·4, and 5μc of [2-^{14}C]acetate were used in each incubation.

Incubation studies. Samples were incubated at various temperatures for 5hr., either under air or under various concentrations of O_2, in an illuminated rotary Warburg apparatus (Braun; model V85).

Control samples were incubated under air at 30° for 5hr. in a reciprocating water bath illuminated by four 40w fluorescent tubes giving daylight emission at a distance of 6in.

Gas mixtures of air+O_2 or air+N_2 saturated with water vapour were bubbled through some samples at the rate of about 20ml./min. The concentration of O_2 in the gas mixture was monitored by passing samples of the effluent gas through a Servomex oxygen analyser (Servomex Controls Ltd., Crowborough, Sussex).

Extraction of samples. At the end of incubations the lipids were extracted from all the samples with chloroform–methanol (2:1, v/v) and the water-soluble impurities removed from the lipid extract by the method of Folch, Lees & Sloane-Stanley (1957). The chloroform phase was dried and the lipid residue transmethylated by refluxing it with methanol–benzene–H_2SO_4 (20:10:1, by vol.) for 90min. (Nichols & James, 1964). The resultant fatty acid methyl esters were analysed by gas–liquid radiochromatography on a column of ethylene glycol adipate (James & Piper, 1963; James & Hitchcock, 1965).

Effect of incubation temperature on the formation of fatty acids by Chlorella, *castor leaves and bulb tissue.* Samples of each tissue were incubated at temperatures in the range 10–40°. Control samples of tissue for each temperature change were incubated at 30°.

Effect of a constant concentration of dissolved oxygen at different temperatures on the formation of fatty acids by Chlorella, *spinach leaves and bulb tissue.* Pairs of samples of each tissue were incubated at 10°, 15°, 20°, 25°, 30°, 35° and 40°. One of each pair was incubated under air (i.e. 21% O_2) and the other under one of the following concentrations of O_2 in the gas phase: at 10°, 21%; at 15°, 25%; at 20°, 28%; at 25°, 32%; at 30°, 35%; at 35°, 38%; at 40°, 42%.

Formation of fatty acids by Chlorella *in the dark.* Three samples of *Chlorella* were stored for 24 hr. in blackened flasks in a dark cupboard. One sample was transferred to a normal clear flask and incubated with 3μc of [2-^{14}C]acetate in the light for 5hr. at 20°; 3μc of [2-^{14}C]acetate was added to each of the other blackened flasks and both were incubated in the dark for 5hr. at 20°, one under air and the other under 70% O_2 in the gas phase.

Effect of oxygen concentration on the formation of fatty acids by bulb tissue at different temperatures. Samples of bulb tissue were incubated at 10°, 20° and 30° either under air or under 0, 10, 50, 70, 75, or 100% O_2 in the gas phase.

Effect of temperature on the formation of fatty acids by bulb tissue under 70% *oxygen.* Samples of bulb tissue were incubated either under air or under 70% O_2 in the gas phase at temperatures ranging from 10° to 40°.

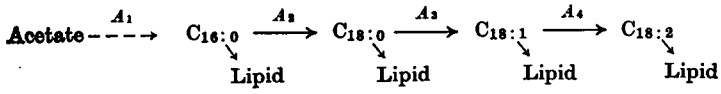
Effect of incubation temperature and HCO_3^- *concentration on the formation of fatty acids by bulb tissue.* Pairs of samples of bulb tissue were incubated at 10°, 15°, 20°, 25°, 30°, 35° and 40°. One of each pair was incubated without $NaHCO_3$ and the other incubated with one of the following amounts of $NaHCO_3$: at 10°, 4mg.; at 15°, 5mg.; at 20°, 6mg.; at 25°, 7mg.; at 30°, 8mg.; at 35°, 9mg.; at 40°, 10mg.

RESULTS

All the published data connecting changes in the degree of unsaturation of plant fatty acids with changes in temperature are based on comparative analyses of fats from plants grown at various temperatures (Canvin, 1965; Hilditch & Williams, 1964). We chose to compare the rates of fatty acid synthesis in plant materials grown at a constant temperature but incubated with labelled acetate at different temperatures. The tissues were chosen to represent a range of plants, being a green alga (*Chlorella vulgaris*), leaf tissue and bulb tissue. To minimize tissue variability samples were randomized between experimental and control flasks and incubated simultaneously.

Palmitic acid is a major component of all the systems studied. The amount of incorporation of labelled acetate into this acid therefore gives a measure of the rate of formation of the saturated acids.

The fatty acid composition of the lipids of these plant systems is a function of the rates of the reactions A_1, A_2, A_3 and A_4 in the sequence:



Any constraint on reactions A_3 and A_4 will cause a change in lipid fatty acid composition such that, provided that there is no feedback inhibition, an increased amount of stearic acid will result.

We have no information about the existence of any feedback inhibition, but it is unlikely that the activity of any of the fatty acid-synthesizing enzymes would change during a 5hr. incubation. The energies of activation of chain elongation and desaturation are obviously different, but there is little reason to expect them to be of opposite sign, which would be necessary to explain a differential temperature effect. With these assumptions, the ratio radioactivity in C_{18} unsaturated acids/radioactivity in stearic acid, which we call 'desaturation', would then give a measure of relative reaction rates controlling the final fatty acid composition. This 'desaturation' for one tissue under one set of conditions relative to that of similar tissue in a

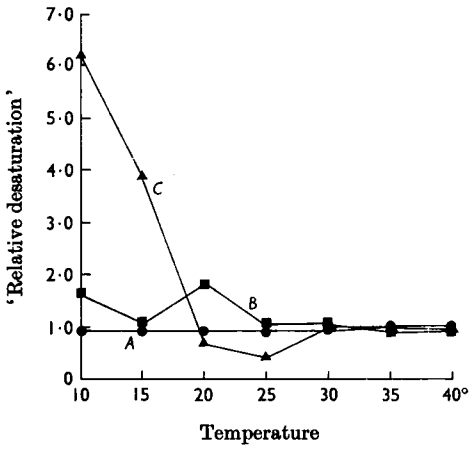


Fig. 1. Effect of incubation temperature on the 'desaturation' in *Chlorella*, bulb and leaf. Samples of *Chlorella*, bulb tissue and leaf tissue were incubated with [2-^{14}C]acetate for 5hr. at 10°, 15°, 20°, 25°, 30°, 35° and 40°. Control samples of tissue were incubated for 5hr. at 30°. The 'relative desaturation' is calculated at each temperature (t) as the radioactivity ratio:

$$\frac{[(C_{18:1}+C_{18:2})/C_{18:0}]_t}{[(C_{18:1}+C_{18:2})/C_{18:0}]_{30°}}$$

▲, Bulb tissue; ■, leaf tissue; ●, *Chlorella*.

control state gives what we call 'relative desaturation'. If the two systems are identical then the 'relative desaturation' will be 1, if the ratio is greater than 1 then the experimental system is synthesizing unsaturated acids more rapidly than the control, and if the ratio is less than 1 the reverse is true. However, since the incubation system (a shaking Warburg bath) differs from the control bath (a simple shaking incubator without a flowing gas stream in the sample flasks), identical incubations yield a 'relative desaturation' usually slightly different from 1. Even so, we are confident that the effects demonstrated are real and not related to the variability of the plant tissues, since the standard state was kept constant throughout any single series of experiments.

Effect of incubation temperature on the formation of fatty acids. A comparison of the 'desaturation' of *Chlorella* cells incubated at different temperatures with that of control cells incubated at 30° is shown in Fig. 1 (curve *A*). Clearly the products of fatty acid biosynthesis by *Chlorella* are not appreciably affected by changes in temperature. Under similar conditions, however, leaf tissue shows a 'relative desaturation' slightly higher than 1 at incubation temperatures below 25° (Fig. 1, curve *B*). This effect is small but reproducible.

Table 1. *Effect of incubation temperature on the formation of palmitic acid by bulb tissue*

Samples (2g.) of bulb tissue in 4ml. of 0·2M-potassium phosphate buffer, pH7·4, with 5μc of [2-^{14}C]acetate were incubated for 5hr. at 10°, 15°, 20°, 25°, 30°, 35° and 40°. Control samples of tissue were incubated at 30° for 5hr.

Temperature t	Sp. radioactivity of $C_{16:0}$ at t / Sp. radioactivity of $C_{16:0}$ at 30°
10°	0·5
15	0·6
20	0·5
25	0·4
30	0·6
35	1·0
40	0·5

On the other hand, variation of incubation temperatures in bulb tissue has a very large effect on the 'relative desaturation' (Fig. 1, curve *C*). At 10° the labelling of the C_{18} unsaturated acids is greater by a factor of 2·2 than for the control samples incubated at 30°, giving a 'relative desaturation' of 6·1. The incorporation of [2-^{14}C]-acetate into palmitic acid does not markedly change throughout this range of incubation temperatures (Table 1).

Effect of a constant concentration of dissolved oxygen at different temperatures on fatty acid formation. If it is assumed that at the start of the experiment there are fixed amounts of the fatty acid synthetase and desaturase enzymes, then a decrease in temperature will affect reactions A_3 and A_4 more than reactions A_1 and A_2 only either if there is a marked difference in the energies of activation of the separate reactions or if some cofactor, effective only in some of the reactions, is changed in concentration. The cofactor requirements for the synthetase and desaturase enzymes are markedly different in that the former requires carbon dioxide and the latter requires oxygen; NADPH or NADH are common to all the reactions. Thus the two sets of reactions have requirements for different gases whose dissolved concentrations change relatively with temperature. These changed concentrations will of course affect reaction velocities only if under 'normal' temperature conditions the gas concentrations are rate-limiting.

We therefore attempted to compensate for the effects of temperature on gas solubility by using as a gas phase those oxygen concentrations that would give an identical concentration of oxygen in water at each temperature. It is known that the concentration of oxygen in solution in equilibrium with air at 10° is twice that at 40° (Lange, 1956). The effect of these various concentrations of oxygen in air was studied with *Chlorella*, leaf tissue and bulb tissue incubated at different temperatures.

A comparison of the 'desaturation' of *Chlorella* cells incubated at different temperatures under either air or various concentrations of oxygen in the gas phase (Fig. 2) shows that compensating increases in the concentration of gaseous oxygen exert no effect. A similar study of leaf disks gives similar results (Fig. 2). However, with bulb tissue, the 'relative desaturation' markedly increases with incubation temperature up to 35°, but decreases again at 40° (Fig. 2). If the concentration of oxygen in solution normally limits reactions A_3 and A_4 at high temperatures then the fatty acid composition will change in favour of the saturated acids. Release of this rate-limiting control, by maintaining the concentration of oxygen in solution at the same value as that at 10°, should therefore reverse this effect. The results for bulb tissue show this to be true: the 'relative desaturation' now increases as the activity of the desaturating enzymes increase with rise in incubation temperature. This experiment suggests that the concentration of oxygen is indeed a rate-limiting factor in this tissue. The absence of an oxygen-concentration effect on the 'desaturation' in *Chlorella* and leaf tissue is probably due to the fact that both *Chlorella* and leaves are photosynthetic. Sufficient oxygen for both respiration and fatty acid desaturations would be produced by photosynthesis inside these tissues so that the concentration of oxygen in the gas phase would not be rate-limiting in either of these reactions.

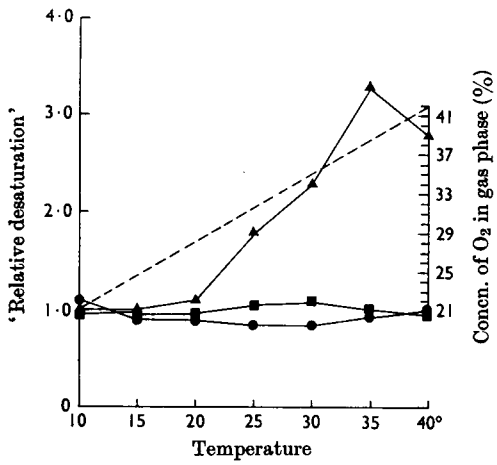


Fig. 2. Effect of a constant concentration of dissolved O_2 at different temperatures on the 'desaturation' in *Chlorella*, bulb and leaf. Samples of *Chlorella*, bulb tissue and leaf tissue were incubated for 5 hr. with [2-^{14}C]acetate either under air or with a constant concentration of dissolved O_2 in solution (see the Materials and Methods section for details) at 10°, 15°, 20°, 25°, 30°, 35° and 40°. The 'relative desaturation' is calculated at each temperature (t) as the radioactivity ratio:

$$\frac{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ O_2^-\ air}}{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ air}}$$

Relative desaturations: ▲, bulb tissue; ■, leaf tissue; ●, *Chlorella*. ----, Concn. of O_2 in gas phase.

Formation of fatty acids by Chlorella *in the dark.* Absence of light diminishes the biosynthesis of all the fatty acids and shows some inhibition of labelling of the unsaturated acids (Table 2). Increase of oxygen concentration in the dark restores the labelling pattern to that found in the light. This supports the concept that in photosynthetic tissue oxygen is not rate-limiting in the light because of its continuous generation inside the tissue.

Effect of oxygen concentration on the formation of fatty acids by bulb tissue at different temperatures. At fixed enzyme activities, assuming that the rate of reaction A_2 is fixed, then the rates of reactions A_3 and A_4 will be proportional to the oxygen concentration if oxygen is rate-limiting. Thus the effect of oxygen concentration at fixed temperatures on the 'desaturation' in bulb tissue was investigated (Fig. 3). The results show that the concentration of oxygen is indeed rate-limiting on desaturations at temperatures down to 10°. At this temperature the 'desaturation' does not rise significantly above the control values, irrespective of increase in oxygen concentration (Fig. 3, curve A). This is probably due to sufficient oxygen being already available in solution for enzyme saturation at this temperature. Increased reaction velocity at

Table 2. *Formation of fatty acids by* Chlorella *in the dark*

Samples of *Chlorella* cells incubated for 5 hr. with 3 μc of [2-^{14}C]acetate either in the dark under air or 70% O_2 or in the light under air.

	Percentage distribution of radioactivity among the fatty acids							
Incubation conditions	$C_{16:0}$	$C_{16:1}$	$C_{16:2}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Radioactivity ratio $(C_{18:1}+C_{18:2}+C_{18:3})/C_{18:0}$
Air, light	21·0	14·1	4·2	2·4	42·0	15·0	1·3	24·30
Air, dark	20·0	13·1	0	3·9	51·0	11·9	0	16·20
70% O_2, dark	23·0	16·9	Trace	0·6	44·2	15·3	0	99·00

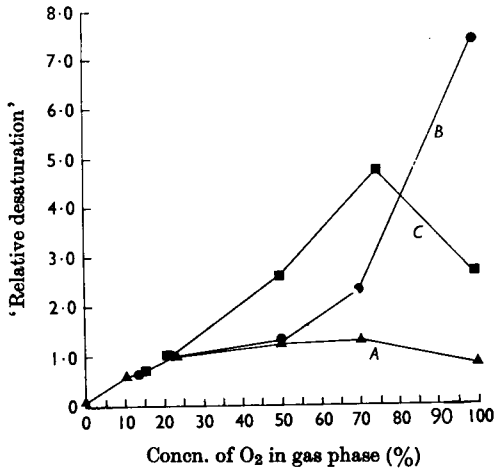


Fig. 3. Effect of O_2 concentration on the formation of fatty acids by bulb tissue at different temperatures. Samples (2g.) of bulb tissue in 4ml. of 0·2M-potassium phosphate buffer, pH7·4, with 5μc of [2-^{14}C]acetate were incubated either under air or under various concentrations of O_2 at 10°, 20° and 30° for 5hr. The 'relative desaturation' is calculated at each percentage concentration of O_2 (x%) in the gas phase and temperature (t) as the radioactivity ratio:

$$\frac{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ x\%\ O_2}}{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ 21\%\ O_2}}$$

Temperature: ■, 30°; ●, 20°; ▲, 10°.

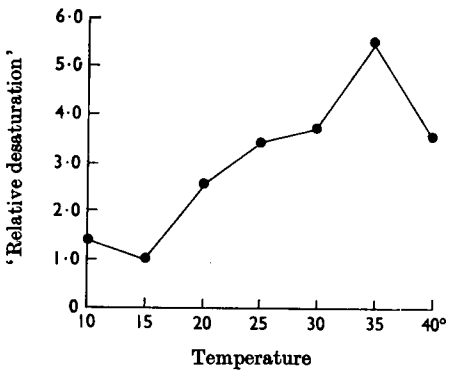


Fig. 4. Effect of incubation temperature on the formation of fatty acids by bulb tissue under 70% O_2. Samples (2g.) of bulb tissue in 4ml. of 0·2M-potassium phosphate buffer, pH7·4, with 5μc of [2-^{14}C]acetate were incubated for 5hr. either under air or under 70% O_2 at 10–40°. The 'relative desaturation' is calculated at each temperature (t) as the radioactivity ratio:

$$\frac{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ 70\%\ O_2}}{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ air}}$$

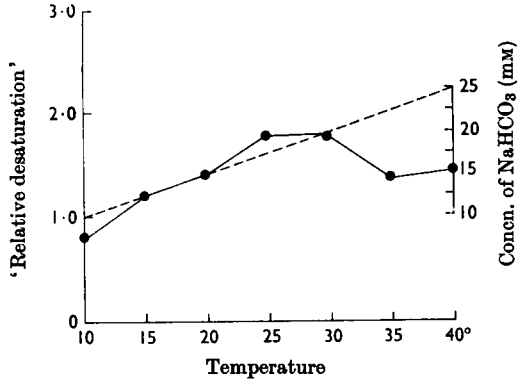


Fig. 5. Effect of incubation temperature and HCO_3^- concentration on the 'desaturation' in bulb. Samples (2g.) of bulb tissue in 4ml. of 0·2M-potassium phosphate buffer, pH7·4, with 5μc of [2-^{14}C]acetate incubated with or without 4mg., 5mg., 6mg., 7mg., 8mg., 9mg. or 10mg. of $NaHCO_3$ at 10°, 15°, 20°, 25°, 30°, 35° and 40° respectively for 5hr. The 'relative desaturation' is calculated at each temperature (t) as the radioactivity ratio:

$$\frac{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ \text{with } HCO_3^-}}{[(C_{18:1}+C_{18:2})/C_{18:0}]_{t,\ \text{without } HCO_3^-}}$$

●, Relative desaturation; ----, concn. of $NaHCO_3$.

20° would render oxygen concentration rate-limiting, hence the increase in the 'relative desaturation' with increase in oxygen concentration (Fig. 3, curve *B*). At 30° there is an increase in the 'relative desaturation' up to 75% oxygen in the gas phase, but at 100% oxygen the rate falls, possibly because of oxidative damage (Fig. 3, curve *C*).

Effect of temperature on the formation of fatty acids by bulb tissue under 70% *oxygen.* When we had established that the oxygen concentration limits the 'desaturation' in bulb tissue (the magnitude of the effect depending on temperature) it was necessary to confirm that the increase in the 'desaturation' with decrease in temperature seen earlier (Fig. 1) is due to increased oxygen availability. Comparisons of the effect on 'desaturation' of a constant high concentration of oxygen in the gas phase (70%) at various temperatures with control samples gives the results shown in Fig. 4. In 70% oxygen, 'desaturation' is markedly increased relative to air at all temperatures down to 15°, showing that above this temperature oxygen was indeed rate-limiting. Below 15° sufficient oxygen is present for the desaturase to operate at maximum velocity, as demonstrated in Fig. 3.

Effect of incubation temperature and HCO_3^- *concentration on the formation of fatty acids by bulb tissue.* As a corollary, it was of interest to investigate

the effects of carbon dioxide concentration on the 'desaturation' in bulb tissue. The results (Fig. 5) show that the 'relative desaturation' increases slightly as the concentration of bicarbonate is increased to a maximum of 20mM-sodium hydrogen carbonate; at concentrations above this the 'relative desaturation' decreases. No pH change occurred in the sample buffer even with 25mM-sodium hydrogen carbonate. This increase in 'relative desaturation' obtained with HCO_3^- is, however, markedly less than that with oxygen (Figs. 2, 3 and 4).

DISCUSSION

These results demonstrate that in non-photosynthetic tissue, with a fixed activity of desaturase enzymes, oxygen is the major rate-limiting factor of desaturation of fatty acids. Since the effect of decreasing the temperature at a fixed gaseous oxygen concentration is to increase the oxygen concentration in solution there is thus an increase in 'desaturation'. In very active photosynthetic tissues such as *Chlorella vulgaris*, oxygen cannot be demonstrated to be rate-limiting for fatty acid desaturations in the light because excess of oxygen is produced within the cell by photosynthesis; only in the dark can an effect of oxygen on the formation of unsaturated fatty acids be demonstrated. This is consistent with earlier experiments with *Chlorella*, when it was impossible to inhibit oleic acid formation by anaerobic conditions in the light (Harris *et al.* 1965). Leaf tissue is not as active photosynthetically as *Chlorella*, since the formation of oleic acid can be supressed by anaerobiosis in the light (Harris, James & Harris, 1967). Thus slight stimulation of the 'desaturation' is seen when the concentration of oxygen in solution is increased by incubation at a low temperature. As bulb tissue is non-photosynthetic, the formation of oleic acid is readily inhibited by anaerobiosis. The cells are entirely dependent on exogenous oxygen and thus all oxygen-requiring reactions in the cell are limited by the availability of oxygen.

The absence of an effect of temperature on the 'desaturation' in bulb tissue between 25° and 35° could be due to competition for oxygen between respiration and fatty acid desaturations. If respiration were to take precedence over fatty acid desaturation an increase in oxygen concentration above that needed for respiration would be expected to increase the 'desaturation'.

The desaturation of fatty acids thus seems to be regulated by the concentration of oxygen in the aqueous phase. As the temperature falls lipids of lower melting point become necessary and these are automatically supplied by the increased oxygen availability. No elaborate controls are thus required and the system is self-regulated. Seed systems would be expected to be similar to other non-photosynthetic tissue such as bulb tissue.

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