Short Communications

Conversion of Oleic Acid into Linoleic Acid by a Subcellular System of Chlorella vulgaris

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Many reports have appeared in recent years on fatty acid synthesis in plant leaves, green algae and chloroplast preparations (Smirnov, 1960; James, 1963a, b; Stumpf & James, 1963; Cheniae, 1963, 1964; Mudd & McManus, 1964). Investigation of the identity of the fatty acids synthesized (James, 1963a, b; Stumpf & James, 1963) showed that entire leaves, or chopped-leaf preparations, synthesize all component fatty acids from acetate. Entire leaves also readily convert oleic acid into linoleic acid and linolenic acid. In contrast with this, leaf-chloroplast preparations appeared to be unable to use oleic acid as a precursor for linoleic acid and linolenic acid, even though these are the major component fatty acids of the particle. However, McMahon & Stumpf (1964) described linoleic acid synthesis from oleyl-CoA in a 'plastid' fraction from safflower seeds.

Chlorella vulgaris cells readily convert oleic acid into linoleic acid and linolenic acid, the conversion being suppressed by incubation in the dark or under nitrogen, as found in the leaf (James, 1963b).

Linoleic acid, as its methyl ester, was isolated by gas–liquid chromatography from an incubation of Chlorella vulgaris with [1-14C]oleate. It was hydrogenated and saponified, and the free stearic acid degraded by the permanganate–acetone α-oxidation method (Murray, 1959). Examination of the reaction products by gas–liquid radiochromatography (James & Piper, 1961, 1963) revealed that only the original stearate contained radioactivity. This indicates that conversion of [1-14C]oleic acid into linoleic acid occurs without randomization of label, confirming a direct precursor relationship.

The choice of Chlorella vulgaris as a source for the preparation of a subcellular enzyme system was suggested by the high content of linoleic acid, the variety of methods available for the disruption of micro-organisms and the ease with which the content of individual fatty acids can be influenced by changing the medium. Synthesis of unsaturated fatty acids is considerably enhanced when a Chlorella culture is transferred from a 'rich' medium (tryptone–glucose) to a 'poor' medium (phosphate buffer). The linoleic acid content increases from 13 to 35% in 6 hr. under these conditions.

The preparation of the active subcellular homogenate takes advantage of this phenomenon and is as follows. A 3-day culture of Chlorella vulgaris, grown in tryptone–glucose, was transferred by centrifugation to 250 ml of 0·2 M-phosphate buffer, pH 7·4, and preincubated in bright light for 3 hr. The cells were subsequently harvested, transferred to a small round-bottomed tube and ultrasonically disrupted (MSE 60 w, 20 kcy/sec., ultrasonic disintegrator) for 1 min. at 15% cell volume in a medium containing sucrose (0·5 M), neutralized ascorbate (0·1 M), sodium chloride (0·1 M) and EDTA (1 mM), adjusted to pH 7·4. The resultant mixture of subcellular components, broken cells and undisrupted cells was then centrifuged at 1000 g for 10 min. to remove undisrupted cells; the supernatant was used for incubation. The absence of whole cells in this supernatant was checked by 'plating out' a small portion on a nutrient–agar plate.

Incubations were carried out with [1-14C]oleic acid for 3 hr. at 25°C in the light and stopped by addition of chloroform–methanol (2:1 v/v). The lipid extract was transmethylated (Stoffel, Chu & Ahrens, 1959) and the fatty acid esters were analysed by gas–liquid radiochromatography (James & Piper, 1961, 1963).

Table 1 illustrates the results so far obtained on cofactor requirements for the desaturation reaction in the Chlorella subcellular system. Undialysed homogenate was used in all cases. No stimulation could be demonstrated on the addition of CoA, ATP and Mg2+, but [1-14C]oleyl-CoA showed a higher conversion than [1-14C]oleic acid itself. The addition of either NADPH or NADH caused stimulation, although that caused by NADPH was generally higher.

Oxidized nicotinamide nucleotides were without stimulating effect, and incubation under nitrogen considerably suppressed the conversion. It is possible therefore that the inhibition found in the
Table 1. Cofactor requirements for the desaturation reaction in the Chlorella subcellular system

Chlorella cell-free homogenate (1 ml) was incubated with 0.5 μCi of [1-14C]oleic acid and other additions as specified. Conditions of incubations are given in the text. All incubations were in light unless otherwise stated.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Substrate</th>
<th>Ratio of specific activities linoleic acid/oleic acid</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[1-14C]Oleate (0.5 μCi) (control)</td>
<td>0.014</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) + CoA (0.5 μmole) + ATP (1 μmole) + Mg2+ (0.25 μmole)</td>
<td>0.014</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleoyl-CoA (0.5 μCi)</td>
<td>0.021</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>[1-14C]Oleate (0.5 μCi) (control)</td>
<td>0.022</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) + NADH (0.5 μmole)</td>
<td>0.048</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) + NADPH (0.5 μmole)</td>
<td>0.055</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>[1-14C]Oleate (0.5 μCi) (control)</td>
<td>0.142</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) + NAD (0.8 μmole)</td>
<td>0.105</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) + NADP (0.8 μmole)</td>
<td>0.140</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>[1-14C]Oleate (0.5 μCi) in air (control)</td>
<td>0.089</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) under nitrogen</td>
<td>0.021</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>[1-14C]Oleate (0.5 μCi) in light (control)</td>
<td>0.023</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Oleate (0.5 μCi) in dark</td>
<td>0.015</td>
<td>65</td>
</tr>
</tbody>
</table>

Dark may result from a decrease in the supply of reduced cofactors and oxygen normally generated by photosynthesis.

These findings suggest a requirement for a reduced nicotinamide nucleotide, oxygen and the CoA ester of the substrate fatty acid. The conversion of oleic acid into linoleic acid in Chlorella vulgaris thus appears to occur by the oxidative desaturation reaction that has been demonstrated by Bloch and his co-workers for the introduction of double bonds into long-chain fatty acids in yeast (Bloomfield & Bloch, 1960; Yuan & Bloch, 1961; Meyer & Bloch, 1963).

Preliminary fractionation has been carried out, separating the homogenate into a 27,000g-sedimented dark-green 'chloroplast fraction' and a light-green supernatant fraction containing small or broken chloroplasts, other subcellular particles and soluble enzymes. The supernatant fraction effects the entire desaturation reaction from oleic acid. The 'chloroplast fraction', however, will not accept [1-14C]oleic acid as a precursor for desaturation, but readily desaturates [1-14C]oleyl-CoA to both linoleic acid and linolenic acid.

The available evidence therefore suggests that the 'desaturase' activity resides in the chloroplast, but requires the CoA ester of oleic acid as substrate, no activating enzyme being present. An oleate-activating enzyme exists in the supernatant, and sufficient chloroplast material remains in this fraction to desaturate the CoA ester once formed.

The skilled technical assistance of Mr R. S. Appleby is gratefully acknowledged.

Smirnov, B. P. (1960). Biochim. biophys. 25, 545.
Concentrations of Acetoacetate and D-(-)-3-Hydroxybutyrate in Rat Liver and Blood

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The measurements and calculations by Bücher and his associates have shown that the oxidized and reduced substrates of certain NAD-linked dehydrogenases and the freely soluble NAD⁺ and NADH are virtually in equilibrium in the cytoplasmic compartment of liver cells (Hohorst, Kreutz & Bücher, 1959). Since the 3-hydroxybutyrate dehydrogenase of rat liver is confined to the mitochondria (Lehninger, or compartment, state of the steady-state to is reflected 1958; acetoacetate hydroxybutyrate: & vivo; however, for methods reported rats in concentrations The vein by Blood pressed between filled with air) acid, after Williamson,* Bucher & Wieland, Matschinsky, Löffler & Müller (1961) reported the ratio 3-hydroxybutyrate:acetoacetate to be 100 in rat liver in vivo; however, these workers used chemical methods for the determination of ketone bodies. The concentrations of acetoacetate and 3-hydroxybutyrate in the liver and blood of fed and starved rats have been measured by an enzymic method (Williamson, Mellanby & Krebs, 1962) and are reported in the present communication.

Male rats weighing 100–150 g, were used in this work. Blood samples were obtained from the tail vein by the method described by Williamson & Wilson (1965). The rats were killed by dislocation of the neck, and the liver was removed rapidly and pressed between metal clamps previously cooled in liquid air (Wollenberger, Ristau & Schoffa, 1960). The frozen tissue was transferred to a mortar filled with liquid air. The average time elapsing between killing the animal and deep-freezing the tissue was 10 sec. The tissue was then pulverized to a fine powder (with frequent additions of liquid air) and transferred to a weighed plastic centrifuge tube containing 2 ml of frozen 30% (w/v) perchloric acid. After reweighing, the powdered tissue (3–4 g.) was intimately mixed with the perchloric acid, care being taken not to allow the mixture to thaw. Ice-cold distilled water (10 ml) was added and the mixture homogenized in the centrifuge tube with a glass pestle. Denatured protein was removed by centrifugation in the cold at 10 000 g for 20 min. The supernatant fluid was adjusted to pH 5–6 with 20% (w/v) potassium hydroxide and the precipitate of potassium perchlorate centrifuged off. The yellow supernatant fluid was then shaken for 30 sec. with Florisil (0–1 g./ml.). This treatment removed flavines from the solution and decreased the non-enzymic oxidation of NADH that was observed with untreated samples. Experiments showed that the recovery of acetoacetate, 3-hydroxybutyrate, lactate and pyruvate was not affected by this procedure. The Florisil was removed by centrifugation and the supernatant fluid used for the determination of ketone bodies. These were determined essentially by the method of Williamson et al. (1962), except that, for 3-hydroxybutyrate, malate was determined first (Hohorst et al. 1959) with a hydrazine–tris–hydrochloric acid buffer, pH 9·5, and then the pH was adjusted to 8·5 to allow the determination of 3-hydroxybutyrate on the same sample. The 3-hydroxybutyrate dehydrogenase used in this work was prepared by the method of Williamson et al. (1962) and purified further on DEAE-Sephadex. It had a specific activity of 3 i.u./mg. of protein, and contained about 1% of malate dehydrogenase and no detectable lactate dehydrogenase. As a control on the validity of the rapid freezing technique described above pyruvate and lactate were determined in livers from fed animals according to the method of Hohorst et al. (1959). The mean value for the ratio lactate:pyruvate was 13 (eight rats), which is in reasonable agreement with the value of 11 obtained by Hohorst et al. (1959), who removed the livers under anaesthesia. Storage of the deep-frozen liver overnight at −15 ° resulted in a loss of 80% of the acetoacetate but caused no appreciable change in the 3-hydroxybutyrate value.

The results presented in Table 1 show that when rats were starved for 24 hr. there was approximately a 15-fold rise in the concentration of ketone bodies in the liver, but starvation for a further 24 hr. caused no further increase in ketone-body concentration. There was some variation between indi-

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individual rats: for example, the lowest value recorded during the 48 hr. starvation period was a total of 2.13 μmoles/g. fresh wt. and the highest value 3.59 μmoles/g. fresh wt. The ratio 3-hydroxybutyrate:acetoacetate ranged from 1.7 to 4.9 but was not significantly altered by starvation. However, anoxia led to a large increase in the ratio: for example, when a liver sample was removed 1 min. after killing the animal the 3-hydroxybutyrate:acetoacetate ratio obtained was 14, whereas a value of 2.8 was found for the same liver by the standard procedure. Similar changes in ratio were observed by Hohorst, Kreutz & Reim (1961) for the cytoplasmic redox couples, and the increase in the ratio 3-hydroxybutyrate: acetoacetate can be interpreted as an increase in the concentration of mitochondrial NADH due to the anoxia.

The total ketone-body concentrations in blood are similar to the values for liver in the 24 hr. and 48 hr. starved rats, but the ratio 3-hydroxybutyrate: acetoacetate tends always to be lower than the ratio found in liver and this is very marked in the fed animal (Table 1).

According to Krebs, Mellanby & Williamson (1962) and Shuster & Doudoroff (1962) the equilibrium constant, K, of the 3-hydroxybutyrate-dehydrogenase system is 1.42 x 10⁻⁹. Substituting a value of 3.0 for the ratio 3-hydroxybutyrate: acetoacetate in the equation

$$K = \frac{[\text{acetoacetate}] [\text{NADH}] [\text{H}^+]}{[\text{3-hydroxybutyrate}] [\text{NAD}^+]},$$

a value of 23 is obtained for the ratio NAD⁺:NADH within the liver mitochondria (assuming pH 7.0). This is in striking contrast with the ratio for NAD⁺:NADH in the cytoplasmic compartment of the liver cell, namely 1.8 x 10², estimated by Hohorst et al. (1959).

We thank Professor Sir Hans A. Krebs, F.R.S., for advice and encouragement. M. B. W. acknowledges the support of the Wellcome Trust.


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**Table 1. Concentrations of acetoacetate and d-(-)-3-hydroxybutyrate in rat liver and blood**

<table>
<thead>
<tr>
<th>Nutritional state of animals</th>
<th>Acetoacetate</th>
<th>3-Hydroxybutyrate</th>
<th>Ratio 3-hydroxybutyrate: acetoacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanced diet</td>
<td>0.057±0.02</td>
<td>0.144±0.04</td>
<td>2.6±0.5</td>
</tr>
<tr>
<td>Starved for 24 hr.</td>
<td>0.62±0.12</td>
<td>1.18±0.46</td>
<td>3.1±0.7</td>
</tr>
<tr>
<td>Starved for 48 hr.</td>
<td>0.69±0.18</td>
<td>2.02±0.33</td>
<td>2.71±0.41</td>
</tr>
</tbody>
</table>

The experimental conditions are given in the text. The results are expressed as μmoles/g. fresh wt. of liver (± s.e.).
Biochem. J. (1965) 94, 19c

The Effects of Cyclopropane Derivatives on Ketone-Body Metabolism in vivo

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Although the concentrations of ketone bodies in rat liver in vivo show a 15-fold increase on starvation, no significant change in the ratio 3-hydroxybutyrate:acetoacetate occurs (Berry, Williamson & Wilson, 1965). As this ratio may reflect the oxidation-reduction state of the mitochondrial NAD system it was decided to investigate the effect of inhibition of hepatic oxidation of fat on the ratio. Hypoglycin A (2-methylenecyclopropanylalanine) is a toxic amino acid, isolated from the seeds of Blighia sapida (Hassall, Reyle & Feng, 1954), which inhibits fatty acid oxidation in vivo (Holt & Benedict, 1959; McKerns, Bird, Kaleita, Coulomb & de Renzo, 1960). Hypoglycin and a simpler derivative, cyclopropanecarboxylic acid, decrease the amount of ketone bodies formed by rat-liver slices from endogenous substrates or added butyrate (E. V. Ellington, D. H. Williamson & M. B. Wilson, unpublished work). The present communication reports the effects of injection of these cyclopropane derivatives on the concentrations of acetoacetate and 3-hydroxybutyrate in rat liver and blood.

Male rats that had been starved for 48 hr. were given an intramuscular injection of either hypoglycin or cyclopropanecarboxylic acid (sodium salt; 10 mg./100 g. body wt.); control rats received a saline injection. Blood samples (100–150 mg.) were collected from the tail vein on weighed strips of nylon material. After reweighing, the sample was transferred to a centrifuge tube containing 2 ml. of 3% (w/v) perchloric acid and mixed thoroughly. Denatured protein was removed by centrifugation and the supernatant fluid neutralized with 10% (w/v) potassium hydroxide. Ketone bodies were determined on the supernatant fluid obtained after the removal of the crystalline potassium perchlorate. The animals were killed by dislocation of the neck and the livers treated as described in the preceding communication (Berry et al. 1965), which also contains information on the analytical methods used in this work.

Within 1 hr. of injection of the cyclopropane derivatives there was a decrease in the ratio 3-hydroxybutyrate:acetoacetate in blood, and this was maintained at the lower level for several hours, whereas there was little change in the ratio for the control animals (Table 1). The mean values for the ratio in livers removed 2 hr. after injection of the animals were similar to those for blood: control animals, 3·0; hypoglycin-treated animals, 1·0; cyclopropanecarboxylate-treated animals, 1·5.

This decrease in the ratio can be interpreted as an increase in the ratio NADH:NAD+ at the site of 3-hydroxybutyrate dehydrogenase (i.e. within the mitochondria) and this in turn might result from inhibition of fat oxidation. Further evidence for this suggestion that inhibition of fatty acid oxidation decreases the concentration of mitochondrial NADH can be deduced from liver perfusion experiments reported by Wieland & Matschinsky (1962). Perfusion with oleate resulted in a rapid rate of ketogenesis and a ratio 3-hydroxybutyrate:acetoacetate 2–3 in the perfusion medium. Glycerol was then added to the medium; the production of ketone bodies ceased and the ratio fell below 1.

In the starved animal, inhibition of fat oxidation in the liver should result in a decrease in ketone-body concentration in the liver and blood, assuming the metabolic block to be before the entry of acetyl-CoA.
Table 1. Effects of hypoglycin and cyclopropenebutyrate on the concentrations of acetoacetate and 3-hydroxybutyrate in rat liver and blood.

<table>
<thead>
<tr>
<th></th>
<th>(a) N-Cl</th>
<th>(b) Hypoglycin</th>
<th>(c) Cyclopropenebutyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Acetoacetate</td>
<td>Acetoacetate</td>
</tr>
<tr>
<td></td>
<td>Acetoacetate</td>
<td>bodies</td>
<td>bodies</td>
</tr>
<tr>
<td>Hypoglycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>0.03</td>
<td>0.71</td>
<td>0.23</td>
</tr>
<tr>
<td>Liver</td>
<td>0.02</td>
<td>0.72</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Results that had been received for 48 hr, were given an intramuscular injection of (a) 0.9% NaCl, (b) 2% hypoglycin (10 mg/100 g body wt) or (c) 2% cyclopropenebutyrate (10 mg/100 g body wt). Blood samples were collected at timed intervals and the results (expressed as µmol/kg of blood) are from a typical experiment. The values for liver were obtained from a patient experiment. All the other experimental details are given in the text.

into the tricarboxylic acid cycle, but the results presented in Table 1 show that this is not the case in these experiments. On the contrary, apart from a decrease during the first 2 hr. with hypoglycin, the ketone-body concentration in blood actually rose by at least 100% in 4 hr. (Table 1). A possible explanation for this anomalous finding would be inhibition of the peripheral utilization of ketone bodies, which could lead to an increased ketone-body concentration although the rate of hepatic ketogenesis was inhibited. Experiments with kidney homogenates confirmed this suggestion: the utilization of acetoacetate by kidneys removed from hypoglycin-treated or cyclopropenebutyrate-treated rats 3 hr. after injection (measured in the system described by Kulka, Krebs & Eggleston, 1961) was found to be inhibited by about 90%, although the endogenous respiration was not affected. The initial decrease in ketogenesis observed with hypoglycin can also be explained on this basis. It has been shown that it is oxidized to methylenecyclopropane carboxylic acid in the liver (Holt, Chang, Holt & Böhm, 1964), and there may be a time lag before sufficient of this product (which appears to be the inhibitor of fatty acid utilization in liver) accumulates in the peripheral tissues to inhibit acetoacetate utilization. It might be argued that the decrease in the ratio 3-hydroxybutyrate: acetoacetate observed in these experiments is a direct result of the under-utilization of acetoacetate; however, although this explanation is valid for blood, owing to the absence of 3-hydroxybutyrate dehydrogenase, it does not hold for liver.

These results suggest that the cyclopropene derivatives inhibit two sites of fat metabolism: one concerned with the oxidation of fatty acids and the other with the utilization of ketone bodies. This inhibition of ketone-body utilization may prove a useful tool in the study of acetoacetate and 3-hydroxybutyrate metabolism.

We thank Professor Sir Hans A. Krebs, F.R.S., for helpful discussions and encouragement, Dr E. V. Ellington for the isolation of hypoglycin and Professor S. J. Landor for a gift of synthetic hypoglycin. M. B. W. acknowledges the support of the Wellcome Trust.