\( \beta \) Oxidation in Glyoxysomes from Castor Bean Endosperm*

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T. G. COOPER‡ AND HARRY BEEVERS§

From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

SUMMARY

Crude particulate preparations from castor bean endosperm contain most of the \( \beta \) oxidation activity present in initial extracts. Measurements on organelles separated by sucrose density centrifugation of the crude particles show that glyoxysomes (density 1.25) contain more than 80% of the particulate \( \beta \) oxidation activity, with virtually none in the mitochondria (density 1.19). Thiolase shows a similar distribution. Glyoxysomes do not oxidize NADH, but \( O_2 \) is required for \( \beta \) oxidation. Addition of palmitoyl coenzyme A to glyoxysomes results in \( O_2 \) uptake, NADH accumulation, and acetyl-CoA production, in a 0.5:1:1 stoichiometry. Cyanide has no effect on NADH accumulation, but doubles the rate of \( O_2 \) uptake. These data are consistent with an \( O_2 \)-requiring oxidation of the prosthetic group of acyl-CoA dehydrogenase to yield \( H_2O_2 \), which is broken down by catalase in the glyoxysome. Acetyl-CoA produced by \( \beta \) oxidation is consumed in the glyoxylate cycle, which is also located in this organelle.

The reactions of \( \beta \) oxidation are known to occur in the mitochondria in mammalian liver preparations (1). Brdiczka et al. (2) and Green and Allmann (3) have examined the intramitochondrial location of the enzymes of this sequence. Garland (4) and others have demonstrated a tight coupling of \( \beta \) oxidation and \( \alpha \) oxidation in cell-free preparations from these materials has been investigated, particularly by Stumpf and Barber (6), Yamada and Stumpf (7), and Rebeiz and Castelfranco (8). Although the absolute rates are relatively low, it has been established that crude particulate preparations have \( \beta \) oxidation activity. However, most of the activity remains in the supernatant solution under the conditions used.

Recently the glyoxysome, an organelle containing the enzymes of the glyoxylate cycle in amounts consistent with the flux of acetyl units in \( \alpha \) oxidation, has been isolated from fatty seedlings (9, 10). The evidence indicating that most acetyl units are channelled through this organelle in \( \alpha \) oxidation raised the question of how transport of acetyl coenzyme A from the mitochondria, where it was supposedly generated, was brought about. When it was found that enzymes thought to play a role in transport of acetyl groups in other systems (11-13) were present in extremely low amounts, the three organelle fractions (mitochondria, plastids, and glyoxysomes, with respective densities 1.19, 1.23, and 1.26) were examined for \( \beta \) oxidation.

The results show that more than 80% of the particulate \( \beta \) oxidation activity is in fact in the glyoxysomes—virtually none is present in the purified mitochondria. Glyoxysomes do not oxidize NADH, and the provision to palmitoyl-CoA results in accumulation of NADH, uptake of \( O_2 \), and formation of acetyl-CoA.

MATERIALS AND METHODS

Preparation of Cellular Organelles—Mitochondria, plastids, and glyoxysomes were prepared as described earlier (10).

Synthesis of CoA Thioesters—The various thioesters of coenzyme A were prepared by the method of Simon and Shemin (14).

Identification of \( \beta \) Oxidation Reaction Products—Acetyl-CoA produced as a result of \( \beta \) oxidation was trapped by taking advantage of the presence of the malate synthetase which is also present in glyoxysomes. When glyoxylate-\(^{14}C\) is added, acetyl-CoA is converted to malate-\(^{14}C\), which can easily be separated and identified by elution from Dowex with a formate gradient (5). NADH production was measured with a Cary 15 spectrophotometer and \( O_2 \) uptake with a Clark \( O_2 \) electrode connected to a Gilson oxygraph.

RESULTS

Occurrence and Distribution of \( \beta \) Oxidation Activity—The progress of palmitoyl-CoA-dependent reduction of NAD by glyoxysomes is shown in Fig. 1. The observation that 3 to 4 times more NAD is reduced than palmitoyl-CoA provided in the reaction mixture indicates that the substrate has gone through the \( \beta \) oxidation spiral more than three times. After attainment of apparent equilibrium, NADH production was renewed upon further additions of palmitoyl-CoA.

Acetyl-CoA was identified as an oxidation product by the
FIG. 1. The dependence of acetylpyridine-NAD reduction upon palmitoyl-CoA (PALM-CoA) in glyoxysomes. The reaction mixture contained, in a final volume of 0.8 ml, 1.3 X 10^{-4} M potassium phosphate buffer, pH 7.5, 5.0 X 10^{-4} M MnCl_{2}, 1.3 X 10^{-4} M CoA, 6.3 μM palmitoyl-CoA, 3.1 mM dithiothreitol, 2.5 mM glyoxylate, 1.4 X 10^{-4} M acetylpyridine nicotinamide adenine dinucleotide, and 180 μg of purified glyoxysomal protein. The reaction was initiated by the addition of palmitoyl-CoA. Subsequent palmitoyl-CoA additions were made as indicated, in volumes of 1 μl. * indicates the use of the acetylpyridine derivative of NAD.

FIG. 2. Column chromatography of the β oxidation reaction product, acetyl-CoA, trapped as malate-^{14}C in the malate synthetase reaction with added glyoxylate-^{14}C. ■, milliequivalents of KOH necessary to titrate the authentic nonradioactive malate results shown in Fig. 2B. Malate-^{14}C, produced from acetyl-CoA and added glyoxylate-^{14}C by malate synthetase in the glyoxysomes, accumulated only when the complete reaction mixture was provided. As shown in Fig. 2A, no acetyl-CoA (malate-^{14}C) was generated when NAD was omitted.

Table I summarizes experiments in which the effects of cofactor omissions on both NAD reduction and acetyl-CoA (malate-^{14}C) formation were investigated. The reaction is clearly dependent on protein (glyoxysomes), palmitoyl-CoA, and NAD. Withholding coenzyme A reduced the reaction rate only partially; this probably indicates that a significant amount of CoA is present in the glyoxysomes or in the palmitoyl-CoA. Omitting MnCl_{2} or dithiothreitol did not strongly affect the rate of the reaction.

In Table II the reactivity of several substrates is shown. The even numbered fatty acyl-CoA derivatives yielded reasonable rates of NAD reduction and acetyl-CoA formation, whereas propionyl- and valeroyl-CoA, the odd numbered derivatives, were poorly oxidized. Caproyl-CoA was used as substrate in some of the later experiments in place of palmitoyl-CoA.

With caproyl-CoA as substrate, NAD was reduced at a constant rate over a 1- to 10-min interval and the rate of reduction was proportional to protein concentration between 8 and 16 μg. The concentration dependence of this series of reactions is shown in Fig. 3. The rate was saturated at roughly 8.0 X 10^{-5} M, and remained essentially the same as the concentration of caproyl-CoA was increased to 38.0 X 10^{-5} M. The response to palmitoyl-CoA concentration is in marked contrast to this, as shown in Fig. 4 (●). Maximum oxidation rates were achieved when the

Table I

<table>
<thead>
<tr>
<th>Deletion</th>
<th>Initial rate of NAD reduction</th>
<th>Malate formed from ^{14}C-glyoxylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.45</td>
<td>996,180</td>
</tr>
<tr>
<td>Protein</td>
<td>0</td>
<td>90,490</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>0.81</td>
<td>17,720</td>
</tr>
<tr>
<td>NAD</td>
<td>64,540</td>
<td></td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>4.17</td>
<td>440,620</td>
</tr>
<tr>
<td>MnCl_{2}</td>
<td>6.15</td>
<td>894,560</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>6.15</td>
<td>705,500</td>
</tr>
</tbody>
</table>

With caproyl-CoA as substrate, NAD was reduced at a constant rate over a 1- to 10-min interval and the rate of reduction was proportional to protein concentration between 8 and 16 μg. The concentration dependence of this series of reactions is shown in Fig. 3. The rate was saturated at roughly 8.0 X 10^{-5} M, and remained essentially the same as the concentration of caproyl-CoA was increased to 38.0 X 10^{-5} M. The response to palmitoyl-CoA concentration is in marked contrast to this, as shown in Fig. 4 (●). Maximum oxidation rates were achieved when the
TABLE II
Specificity of β oxidation in castor bean glyoxysomes

For determination of the initial rate of reduction of NAD, cofactor requirements were determined by means of the spectrophotometric assay. The reaction mixture was similar to that in Fig. 1. For determination of malate formation from glyoxylate, reaction requirements were assayed by means of the indirect trapping of the acetyl-CoA. The reaction conditions were similar to those in Fig. 2B. These data were collected from several different experiments.

<table>
<thead>
<tr>
<th>Acyl-CoA addition</th>
<th>Initial rate of NAD reduction (μmol/min)</th>
<th>Malate formed from 14C-glyoxylate (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionyl</td>
<td>0</td>
<td>561,180</td>
</tr>
<tr>
<td>Butyryl</td>
<td>3.22</td>
<td>807,120</td>
</tr>
<tr>
<td>Valeroyl</td>
<td>0</td>
<td>106,980</td>
</tr>
<tr>
<td>Caproyl</td>
<td>4.05</td>
<td>1,831,040</td>
</tr>
<tr>
<td>Palmitoyl</td>
<td>6.45</td>
<td>986,180</td>
</tr>
<tr>
<td>Stearoyl</td>
<td>4.45</td>
<td>1,706,091</td>
</tr>
</tbody>
</table>

Fig. 3. The effect of increasing concentrations of caproyl-CoA upon the rate of reduction of acetylpyridine-NAD. The reaction mixture contained, in a volume of 0.8 ml, 1.3 x 10^-4 M potassium phosphate buffer, pH 7.5, 3.1 mM dithiothreitol, 1.2 x 10^-4 M CoA, 1.7 x 10^-4 M acetylpyridine-NAD, 5.0 x 10^-4 M MnCl₂, 4.7 x 10^-4 M caproyl-CoA (this assumes the synthesis yield of 100%), and 20 μg of purified glyoxysomal protein. The reaction was initiated by the addition of the caproyl-CoA, and was monitored at 363 nm with a Cary 15 spectrophotometer.

substrate concentration was 2.0 μM, and at 1.0 x 10^-3 M the rate was reduced by 88%. As reported by others (15, 16), this detergent effect of long chain fatty acyl derivatives can be modified by the addition of albumin. Fig. 4 (○) shows the response of palmitoyl-CoA concentration when each reaction mixture (0.8 ml) contained 250 μg of albumin (determined in preliminary experiments to be an optimal amount in the presence of 2.5 x 10^-4 M palmitoyl-CoA). A broader optimal substrate range, at concentrations around 3.0 x 10^-4 M palmitoyl-CoA, was observed. The inclusion of albumin thus allows the use of higher substrate concentrations and hence the observation of linear reaction rates for longer periods.

Thiolase Activity in Glyoxysomes—The activity of this enzyme, which splits acetoacetyl-CoA in the presence of CoA to yield 2 moles of acetyl-CoA, was followed by coupling its reaction to that of citrate synthetase. Malate was supplied as the source of the oxalacetate, allowing the net reaction rate to be measured as NAD reduction. The results are shown in Fig. 5. In the absence of glyoxysomal protein, malate, or acetoacetyl-CoA, no

Fig. 5. The effect of various cofactors upon the thiolase reaction. The complete reaction mixture contained, in a volume of 0.8 ml, 1.3 x 10^-4 M potassium phosphate buffer, pH 7.5, 2.5 x 10^-4 M MnCl₂, 3.1 mM dithiothreitol (DTTL), 2.5 x 10^-4 M CoA, 1.4 x 10^-4 M acetylpyridine-NAD, 3.1 mM sodium malate (MAT), 20 μg of citrate synthetase (CIT. SYN.), 4.5 x 10^-4 M acetoacetyl-CoA (AAC), and 20 μg of glyoxysomal protein. The reaction was initiated by the addition of acetoacetyl-CoA. For the various cofactor deletions, the cofactor was replaced by a similar volume of water.
both the \( \beta \) oxidation and thiolase activities were recovered in the 

\[
\text{reaction mixture for 20 min before the glyoxysome preparation was added from the side arm.}
\]

The addition of substrate resulted in a slow rate of \( \text{O}_2 \) uptake, presumably as \( \text{CoA} \) was generated in the system. When only endogenous citrate synthetase was present, the thiolase reaction occurred slowly at first, but increased sharply after 1 min, presumably as \( \text{CoA} \) was generated in the system. When only endogenous citrate synthetase was present, the thiolase reaction was considerably slower. The reaction rate was linearly dependent on protein concentration up to at least 40 \( \mu \text{g} \) per ml, and the normal Michaelis-Menten relationship was observed when substrate concentration was varied. The apparent \( K_m \) for acetoacetyl-\( \text{CoA} \) was \( 8.7 \times 10^{-5} \text{ M} \).

**Distribution of \( \beta \) Oxidation Activity and Thiolase in Subcellular Fractions**—The methods outlined above were applied to the various subcellular fractions obtained from castor bean endosperm by the procedures described in the preceding paper (10). Acetylpyridine-NAD was used in place of NAD as electron acceptor in this survey. As shown in Table III, roughly 80\% of both the \( \beta \) oxidation and thiolase activities were recovered in the crude particulate fraction. When this was separated on a sucrose gradient and the resulting fractions were assayed, the glyoxysomes were found to contain by far the largest fraction of both activities. None was detectable in the mitochondrial fractions.

\( \text{O}_2 \) Uptake during \( \beta \) Oxidation—With the use of larger reaction mixtures, \( \beta \) oxidation was followed on the oxygraph. The cofactor requirements for \( \text{O}_2 \) uptake were found to be the same as those for \( \text{NAD} \) reduction in Table I. Palmitoyl-\( \text{CoA} \) oxidation is shown in Fig. 6, where the reaction mixture contained only an excess of mitochondria (500 \( \mu \text{g} \)) and cofactors initially. The addition of substrate resulted in a slow rate of \( \text{O}_2 \) uptake, which was strikingly increased when 80 \( \mu \text{g} \) of glyoxyosomal protein were added.

It thus appeared that, during \( \beta \) oxidation, \( \text{O}_2 \) uptake occurred concomitantly with \( \text{NADH} \) accumulation and, as shown in Fig. 7, \( \text{O}_2 \) was required for \( \text{NAD} \) reduction. The reaction was performed in an anaerobic cuvette, in which nitrogen of high purity was flushed through the complete reaction mixture for 20 min before the glyoxysome preparation was added from the side arm. As shown there was a very limited reduction of \( \text{NAD} \), which was presumably due to \( \text{O}_2 \) dissolved in the enzyme solution. Opening of the cuvette to the atmosphere (AIR in Fig. 7) resulted in a much more rapid \( \text{NAD} \) reduction. Similar results were obtained when anaerobic conditions were achieved by adding glucose oxidase and glucose.

\( \text{NAD} \) reduction and \( \text{O}_2 \) uptake during \( \beta \) oxidation were measured on parallel samples to yield the data shown in Fig. 8. The ratio of \( \text{NAD} \) reduced to \( \text{O}_2 \) absorbed was roughly 2:1. It therefore seemed clear that for each pair of electrons trapped as \( \text{NADH} \) an additional pair was passed to \( \text{O}_2 \) during \( \beta \) oxidation. Since no electron transport system for \( \text{NADH} \) oxidation exists in the glyoxysome, the \( \text{O}_2 \) uptake observed must be ascribed to oxidation of a component reduced at a stage prior to \( \text{NAD} \) reduction, and thus, by analogy with the mammalian system, presumably a flavoprotein (Fig. 9). Evidence that a direct oxidation occurs.

**Table III**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>( \beta ) Oxidation</th>
<th>Thiolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5 K supernatant</td>
<td>6.8</td>
<td>35.8</td>
</tr>
<tr>
<td>Crude particulate</td>
<td>25.8</td>
<td>115</td>
</tr>
<tr>
<td>Gradient supernatant</td>
<td>0</td>
<td>11.5 (28)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proplastids</td>
<td>2.8 (19)</td>
<td>6.3 (15)</td>
</tr>
<tr>
<td>Glyoxysomes</td>
<td>11.4 (81)</td>
<td>22.9 (57)</td>
</tr>
</tbody>
</table>

**Fig. 7.** The requirement of oxygen for \( \beta \) oxidation in glyoxysomes. The complete reaction mixture contained, in a volume of 2.4 ml, 8.5 \( \times 10^{-3} \text{ M potassium phosphate buffer, pH 7.5} \), 1.0 \( \times 10^{-3} \text{ M MnCl}_2, 2.1 \text{ mM dithiothreitol, 2.1} \times 10^{-4} \text{ M CoA, 2.3} \times 10^{-4} \text{ M acetylpyridine-NAD, 4.2} \mu \text{mol palmitoyl-CoA, and 80} \mu \text{mol of glyoxyosomal protein. Prior to initiation of the reaction, by addition of protein and palmitoyl-CoA from two separate side arms, the contents of the cuvette were bubbled for 20 min with nitrogen of high purity (containing less than 1 ppm } \text{O}_2 \). At the point indicated (+ AIR) the cuvette was opened and bubbled for 5 sec with air, after which acetylpyridine-NAD reduction (\( \beta \) oxidation) was again followed at 363 nm.

**Fig. 8.** The effect of mitochondria and glyoxysomes upon palmitoyl-CoA-stimulated oxygen uptake. The reaction mixture contained, in a volume of 4.0 ml, 2.5 \( \times 10^{-3} \text{ M potassium phosphate buffer, pH 7.5, 2.5} \times 10^{-4} \text{ M MnCl}_2, 1.4 \times 10^{-4} \text{ M CoA, 1.4} \times 10^{-4} \text{ M NAD, 2.0 mM glyoxylate (used to remove the generated acetyl-CoA), and 500} \mu \text{g of mitochondrial protein. The oxygen uptake was monitored at } 25^\circ\text{. Additions of palmitoyl-CoA (PALM-CoA) and glyoxysomes were made as indicated.**
yielding H$_2$O$_2$ is given by the data in Fig. 10. The addition of cyanide resulted in a doubling of the rate of O$_2$ uptake, as would be expected if the catalase in the glyoxysome were inhibited. NADH production, however, was unaffected by cyanide.

**Stoichiometry of $\beta$ Oxidation**—Relationships between the amounts of reactants and products are summarized in Table IV. For convenience of presentation, it is assumed that the O$_2$ uptake observed with glyoxysomes alone is in fact due to reoxidation of the prosthetic group (flavin) reduced at the fatty acyl-CoA dehydrogenase step. Although the details of this hydrogen peroxide-producing reaction are not established, it has been shown that neither methylene blue, 1,6-dichloroindophenol, nor ferricyanide will substitute for O$_2$, but that phenazine methosulfate is partially effective.$^1$

Experiment I (Lines 1 and 2 in Table III) is that shown in Fig. 11, in which oxalacetate,$^{14}$C and citrate synthetase were added to a complete reaction mixture with palmitoyl-CoA as substrate. Under these conditions malate,$^{14}$C formation is a measure of NADH formed and citrate,$^{14}$C formation measures acetyl-CoA production. As shown in Fig. 11 and Table III, the ratio of NADH to acetyl-CoA is very close to unity.

Experiment II in Table III is that in Fig. 12. The total O$_2$ uptake observed when mitochondria were added during $\beta$ oxidation in the glyoxysomes measures the oxidation of all reducing equivalents, i.e. NADH and the reduced flavin, whereas O$_2$ uptake by the glyoxysomes alone measures only the O$_2$ concerned in reactions prior to NADH formation. The data indicate that equal amounts of reducing equivalents are generated at the

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$^1$ T. G. Cooper and H. Beevers, unpublished data.
TABLE IV
Stoichiometry of β oxidation in castor bean endosperm

The data for Experiment I were taken from Fig. 11; II, from Fig. 12; and III, from Fig. 8.

<table>
<thead>
<tr>
<th>β oxidation product</th>
<th>Measured product</th>
<th>Amount (mM)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>Palmitoyl-CoA-dependent production of malate</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>Palmitoyl-CoA-dependent production of citrate</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ reduced flavin</td>
<td>Palmitoyl-CoA-dependent O₂ uptake in glyoxysomes + mitochondria</td>
<td>607</td>
<td></td>
</tr>
<tr>
<td>Reduced flavin</td>
<td>Palmitoyl-CoA-dependent O₂ uptake in glyoxysomes alone</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td>Experiment III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>Palmitoyl-CoA-dependent accumulation of NADH in glyoxysomes</td>
<td>247</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Palmitoyl-CoA-dependent O₂ uptake in glyoxysomes</td>
<td>107</td>
<td></td>
</tr>
</tbody>
</table>

* Although flavin was used for convenience of presentation in analogy to the mammalian system, there is no evidence to show either the details of the oxidation reaction or the prosthetic groups involved.

flavin and NAD stages. Experiment III is that shown in Fig. 8, where NADH formation and O₂ uptake were measured on parallel samples and the ratio observed was slightly over 2.

β oxidation by the glyoxysomes alone therefore results in the formation of acetyl-CoA, NADH, and O₂ uptake in a 1:1:0.5 stoichiometry. The addition of cyanide, by inhibiting catalase (Fig. 10), changes this relationship to 1:1:1.

DISCUSSION

The data show, firstly, that in the endosperm of the germinating castor bean the enzymes concerned with β oxidation are localized primarily in the glyoxysome, and not in the mitochondria. Hutton and Stumpf have independently arrived at a similar conclusion. The generation of acetyl-CoA in this organelle from long chain fatty acids in vivo might explain why acetate escapes oxidation through the tricarboxylic acid cycle and is exclusively channeled through the glyoxylate cycle in this tissue (Canvin and Bevers), although other controls are not of course excluded. The information presently available allows us to picture the reaction in the glyoxysome as shown in Fig. 13.

In general, the reactions of the β oxidation sequence in the glyoxysomes appear to be similar to those established through

2 Dr. P. K. Stumpf, personal communication.
the extensive work with mitochondria from mammalian liver (3, 4). However, there are important differences in the way in which the reducing equivalents are transferred to O2. The sequence in the glyoxysome is not tightly coupled to a cytochrome-containing electron transport sequence; all of the evidence shows that this organelle contains no such mechanism. NADH accumulates, and the O2 uptake observed is apparently that ascribable to oxidation of flavin generated in the fatty acyl-CoA dehydrogenase reaction. The details of this reaction are not clear, but apparently H2O2 is produced and broken down by the very active catalase present in the glyoxysome.

The sustained operation of β oxidation by this organelle in vivo would clearly require a mechanism of NADH oxidation, as indeed would the operation of the glyoxylate cycle (9, 10). The mitochondria from this tissue are capable of oxidizing NADH through a conventional electron transport system, but the possibility is not excluded that a shuttle involving the very active glutamate:oxalacetate transaminases and malic dehydrogenases present in both organelles functions in the transfer of reducing equivalents to the mitochondria.

A further question concerning metabolite transfer into organelles is that of introducing the fatty acid into the glyoxysomes. As shown, the isolated glyoxysomes are capable of oxidizing palmitoyl-CoA directly. The free acid is also oxidized if CoA and ATP are added, but at a lower initial rate. The transport system which operates in mammalian mitochondria does not operate in the glyoxysome, since carnitine derivatives were not oxidized.

REFERENCES
β Oxidation in Glyoxysomes from Castor Bean Endosperm
T. G. Cooper and Harry Beevers

J. Biol. Chem. 1969, 244:3514-3520.

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