Determination of the Labelling Pattern of Dihydroxyacetone and Hexose Phosphate Following a Brief Incubation of Methanol-grown *Hansenula polymorpha* with [14C]Methanol

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The labelling patterns of dihydroxyacetone and hexose phosphate produced after a 2 s incubation of methanol-grown *Hansenula polymorpha* with [14C]methanol have been determined. In dihydroxyacetone over 90% of the radioactivity was located in C-1 and C-3 and less than 10% in C-2. In glucose over 99% of the radioactivity was evenly distributed between C-1, C-3, C-4 and C-6 with less than 1% in C-2 and C-5. The results are consistent with the operation of a previously proposed pentose phosphate cycle of formaldehyde fixation in which dihydroxyacetone is formed by glycolyl transfer from xylulose 5-phosphate to formaldehyde.

**INTRODUCTION**

It has been proposed that methanol assimilation by yeasts proceeds via a pentose phosphate cycle of formaldehyde fixation in which dihydroxyacetone (DHA) is a key intermediate (van Dijken *et al.*, 1978; Babel & Loffhagen, 1979). Evidence for the operation of the DHA pathway has come from enzymic, mutant and whole cell studies (van Dijken *et al.*, 1978; Babel & Loffhagen, 1979; Kato *et al.*, 1979; O'Connor & Quayle, 1979; Waites & Quayle, 1980; Lindley *et al.*, 1980). The initial product of carbon assimilation is considered to be DHA which has been identified as a product of the reaction between pentose phosphate and formaldehyde catalysed by cell-free extracts of *Kloeckera* sp. 2201 (Kato *et al.*, 1979) and *Candida boidinii* CBS 5777 (Waites & Quayle, 1980; O'Connor & Quayle, 1980). Whole cell studies have shown that most of the radioactivity initially incorporated from [14C]methanol into *Candida N-16* or *Hansenula polymorpha* CBS 4732 appears in hexose phosphates (Fujii & Tonomura, 1973; Fujii *et al.*, 1974; van Dijken *et al.*, 1978) and labelled DHA has also been identified as an early-labelled intermediary metabolite in *H. polymorpha* (Lindley *et al.*, 1980).

This paper reports the determination of the labelling patterns of [14C]DHA and [14C]hexose phosphate obtained from whole cell experiments with methanol-grown *H. polymorpha* pulse-labelled with [14C]methanol and shows that these patterns are fully consistent with the operation of the proposed pentose phosphate cycle of formaldehyde fixation.

**METHODS**

*Organism and growth. Hansenula polymorpha* CBS 4732 was grown in a carbon-limited chemostat culture (working volume 750 ml) on the vitamin-supplemented mineral salts medium of van Dijken *et al.* (1976) which contained filter-sterilized methanol (0.4%, w/v). Cultivation was at 37 °C and the pH was maintained at 5.0 with 1.0 M-NaOH. Dissolved oxygen was controlled at 70% saturation during steady state growth with a stirrer speed of 1000 rev. min⁻¹ and dilution rate of 0.075 h⁻¹. This relatively low dilution rate was used in order to maximize methanol oxidation by the culture (cf. van Dijken *et al.*, 1976) thus minimizing residual methanol in the culture which would make the pulse labelling technique less efficient.
Table 1. Stages in the purification of $[^{14}C]$glucose from methanol-grown *Hansenula polymorpha*

Experimental details are given in Methods.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Hexose (μmol)</th>
<th>Inorganic phosphate (μmol)</th>
<th>Organic phosphate (μmol)</th>
<th>Radioactivity (μCi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotary-evaporated extract</td>
<td>230</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>25.06</td>
</tr>
<tr>
<td>Eluate from Dowex 1 (Cl(^{-})) columns after elution with 0-01 m-HCl</td>
<td>545</td>
<td>228.0</td>
<td>1089</td>
<td>320.2</td>
<td>4.789</td>
</tr>
<tr>
<td>Sample following isomerization and dephosphorylation</td>
<td>227</td>
<td>213.4</td>
<td>1289</td>
<td>122.5</td>
<td>4.230</td>
</tr>
<tr>
<td>Eluate from Dowex 50 (H(^{+})) column</td>
<td>275</td>
<td>192.5</td>
<td>1325</td>
<td>137.5</td>
<td>4.080</td>
</tr>
<tr>
<td>Eluate from Dowex 1 (OH(^{-})) column</td>
<td>950</td>
<td>95.0</td>
<td>0</td>
<td>0</td>
<td>1.285</td>
</tr>
<tr>
<td>Eluate from paper chromatography</td>
<td>5</td>
<td>86.4</td>
<td>0</td>
<td>0</td>
<td>0.774</td>
</tr>
</tbody>
</table>

NT, Not tested.

_Pulse-labelling procedure_. The pulse-labelling apparatus and the actual experimental conditions used have been described previously (Lindley et al., 1980). An aqueous solution (7.5 ml) containing 350 μCi $[^{14}C]$methanol (1706 μCi mg\(^{-1}\)) (1 μCi = 37 kBq) was injected at a rate of 0.6 ml min\(^{-1}\) into the stream of yeast cells pumped from the chemostat, at a point such that the contact time between injection and the killing solution of methanol was 2 s at 35 °C.

_Isolation of $[^{14}C]$hexose phosphates_. Yeast cells were incubated in the killing solution for 1 h at 0 °C. Glucose 6-phosphate (100 μmol) and fructose 6-phosphate (100 μmol) were added as carriers. The extract was centrifuged at 5000 g for 45 min to remove debris, and the supernatant was reduced to a volume of 230 ml by rotary evaporation in vacuo at 40 °C (Table 1). The pH of the solution was adjusted to 8.0 with 1.0 m-NaOH and then the solution was filtered through Whatman no. 1 filter paper and loaded on to a column (1.5 × 16 cm) of Dowex 1 (X8; Cl\(^{-}\) form; 200 to 400 mesh). The column was washed with distilled water and it was then found that the working capacity of the column had been exceeded as phosphates appeared in the washings. Therefore that portion of the eluate in which phosphate ions had appeared was applied to a second column of Dowex 1 (1.5 × 18 cm). Phosphate-free eluates from both columns were combined and conserved as they contained $[^{14}C]$DHA (Lindley et al., 1980). Sugar phosphates which remained bound on the columns were eluted with 0-01 m-HCl. Fractions containing hexose phosphates (as determined by the methods of Allen (1940) and Bartlett (1959)) from both columns were pooled and the pH was adjusted to 8-0 with 2.5 m-NaOH. To the resulting solution (545 ml), 1-0 M-Tris/HCl buffer pH 8-0 was added to give a final buffer concentration of 0.01 M. It was found that the solution contained more fructose phosphate than glucose phosphate; since it was glucose that was ultimately to be degraded, phosphoglucose isomerase (EC 5.3.1.9; 8 units) was added and the mixture was incubated at 30 °C for 4.5 h, in order to maximize the amount of glucose phosphate present. The reaction was terminated by heating on a boiling water bath for 10 min. Dephosphorylation was carried out by adjusting the pH of the preparation to 4-8 with 2 M-HCl and adding 6-0 ml 0.2 M-sodium acetate buffer pH 4.8 containing 0-01 M-MgCl\(_2\) and 425 units acid phosphatase from potato (EC 3.1.3.2). The mixture was incubated for 16 h at 30 °C, with a few drops of toluene to prevent bacterial growth. The reaction was halted by heating on a boiling water bath for 10 min. The mixture was then cooled and filtered through Whatman no. 1 filter paper, and the volume was reduced to 227 ml by rotary evaporation in vacuo at 40 °C.

The dephosphorylated sample was then passed through a column (2.5 × 11.5 cm) of Dowex 50 (X8; H\(^{+}\) form; 200 to 400 mesh). The eluate (275 ml) recovered was applied to a column (2.5 × 11.5 cm) of Dowex 1 (X8; OH\(^{-}\) form; 200 to 400 mesh) and eluted with distilled water. Free hexose appeared when 750 ml eluate had been collected; the succeeding 950 ml eluate then contained the required hexose (95.0 μmol). The volume of the hexose solution was reduced to 36 ml by rotary evaporation in vacuo at 40 °C. A sample (200 μl) was chromatographed in two dimensions on Whatman no. 4 chromatography paper using the solvent systems described by Large et al. (1961) and then exposed to X-ray film (Kodak-Kodirex KD5T) for 1 week. Glucose was located on the chromatogram by spraying with aniline phosphate (Waites & Quayle, 1980). The bulk of the radioactivity (87%) co-chromatographed with glucose. The remaining 13% of the radioactivity was located in the Rf regions of fructose and mannose. The volume of the main solution of $[^{14}C]$glucose was further reduced from 36 ml to 5 ml using the method previously described. The entire 5 ml was applied as a stripe on the base line of two Whatman no. 3 chromatography papers and chromatographed in one dimension in phenol/formic acid/water (500 g:13 ml:167 ml). Autoradiography was carried out as described earlier and the glucose band was detected by spraying a small test strip with aniline phosphate. The band containing $[^{14}C]$glucose was cut from the
chromatogram and eluted with 5 ml distilled water. Carrier glucose (2.4 mmol) was added to bring the final amount of glucose to 2.5 mmol. The sample was stored at −15 °C until required.

Isolation of [14C]dihydroxyacetone. The combined, conserved eluates from the initial Dowex 1 (Cl− form) columns, free of phosphates, were pooled (340 ml; 14-16 μCi) and evaporated to dryness in vacuo at 40 °C. To the resulting residue, acetone (10 ml) was added; the acetone-soluble material was concentrated to 1.5 ml in vacuo at 40 °C using an Evapomix (Buchler Instruments, Fort Lee, N.J., U.S.A.) and carrier DHA (1-5 mg) was added. Samples (15 × 100 μl) were applied as single spots to 15 cellulose thin-layer chromatography (t.l.c.) plates which were then chromatographed in two dimensions and autoradiographed as described by Waites & Quayle (1980). One t.l.c. plate was sprayed with aniline phosphate in order to identify the [14C]DHA spot on the X-ray plates. The remaining 14 [14C]DHA spots were carefully removed from the individual t.l.c. plates; the resulting cellulose powder was packed into a column (0-5 × 5 cm) and eluted with 100 ml acetone. The eluate was evaporated to dryness in vacuo at 30 °C and the residue was taken up in 5-0 ml distilled water. To the solution, 148-5 mg carrier DHA was added and attempts were made to lyophilize the DHA. The [14C]DHA failed to lyophilize and remained as a gum, which was redissolved in 4-0 ml distilled water (150 mg; 0-06 μCi). Samples of the solution (two-thirds of the total) were combusted and degraded. To the remaining one-third of the [14C]DHA (50 mg; 0-02 μCi), a further 50 mg carrier DHA was added and recrystallization from acetone (50 ml) was carried out, yielding 40 mg crystals. The crystals were dissolved in 1-0 ml distilled water together with a further 10 mg carrier DHA. Samples of the solution of recrystallized [14C]DHA were combusted and degraded.

Combustion. The specific activity of labelled compounds was determined by total oxidation to CO2 with van Slyke–Folch reagent as described by Sakami (1955).

Degradation of [14C]glucose. Samples of aqueous solution each containing 1 mmol of authentic [U-14C]glucose (0-552 μCi mmol−1) or [14C]glucose from H. polymorpha (0-31 μCi mmol−1) were degraded by the methods of Sakami (1955) as described by Kemp & Quayle (1967). The initial step was a bacterial fermentation with Leuconostoc mesenteroides NCIB 8699.

Degradation of [14C]dihydroxyacetone (Fig. 1). A sample (1-0 ml) of [14C]DHA solution (0-4 to 0-5 mmol; 0-0033 to 0-0123 μCi) was placed in a round-bottomed flask together with 0-25 ml 50% (w/v) periodic acid. The flask was tightly stoppered and incubated in the dark at 20 °C for 1 h. Distilled water (10 ml) was added and the pH was adjusted to 9-5 with 1-0 M-NaOH. The flask was transferred to a steam-distillation unit and the [14C]formaldehyde was steam-distilled off. The 200 ml distillate collected was cooled to 4 °C and the sodium [14C]glycollate remaining in the distillation flask was stored at −15 °C until required. To the distillate, 20 ml 0-1 m NaOH and 20 ml 0-05 m iodine, both at 4 °C, were added. The mixture was incubated at 4 °C for 10 min and then the pH was adjusted to between 3 and 4 with 2 m H2SO4. The resulting formic acid was steam-distilled off as previously described. The distillate was filtered through Whatman no. 1 filter paper and the pH was adjusted to 9-0 with 1-0 m NaOH. The volume was reduced to 10 to 20 ml by rotary evaporation in vacuo at 40 °C, and then this solution was acidified to pH 2 to 3 with 2 m H2SO4. Oxidation of the [14C]formic acid to 14CO2 was carried out using mercuric sulphate as described by Lewis & Weinhouse (1957).

Sodium [14C]glycollate remaining from the initial steam distillation was degraded using methods based on those of Lewis & Weinhouse (1957). The sodium [14C]glycollate solution was placed in the degradation flask, acidified with 5 ml 60% (v/v) H2SO4, and 35 ml 0-1 m-ceric sulphate in 10% (v/v) H2SO4 was added. During the degradation 14CO2 was trapped in 2 m NaOH. [14C]Formic acid remaining in the reaction flask was taken to pH 9-0 with 50% (v/v) NaOH, filtered through Whatman no. 1 filter paper and concentrated to 10 to 20 ml by rotary evaporation in vacuo at 40 °C. The solution was acidified to pH 3-0 with 60% (v/v) H2SO4 and any iodine appearing was discharged with 0-1 m-sodium thiosulphate. [14C]Formic acid was steam-distilled off, the distillate was taken to pH 9-0 with 2-0 m NaOH, and the volume was reduced to 15 to 20 ml as previously described. [14C]Formic acid was oxidized to 14CO2 using mercuric sulphate as described earlier.

Assay of 14CO2. On combustion or degradation all carbon atoms were oxidized to 14CO2 which was trapped in 2 m NaOH (CO2-free) and converted to Ba14CO3 by precipitation with 5% (w/v) BaCl2 solution. The Ba14CO3 was collected on filter paper and prepared for scintillation counting as described by Kemp & Quayle (1967). Triplicate samples (25 to 50 mg) were weighed into scintillation vials and 5 ml gel scintillator, consisting of toluene containing 0-4% (w/v) 2,5-diphenyloxazole. 0-01% (w/v) 1,4-di-2-(5-phenyloxazoly)benzene and 4% (w/v)
Cab-o-sil M5, was added with shaking. Samples were assayed for radioactivity in a Nuclear Chicago scintillation analyser (Isocap 300).

Preparation of cell-free extracts. Hansenula polymorpha CBS 4732 and Candida boidinii CBS 5777 were grown in shake flasks as described by van Dijken et al. (1978) and harvested in late-exponential phase by centrifugation at 6000 g for 20 min. Cell-free extracts of C. boidinii were prepared by the method of Waites & Quayle (1980), while those of H. polymorpha were obtained by shaking in a Mickle shaker (Mickle Laboratory Engineering Co., Gomshall, Surrey) at full speed with 4 vol. 20 mM-KH₂PO₄/NaOH buffer pH 7-1 and Ballotini beads no. 12 (1 g ml⁻¹) for 1 h at 4 °C. The preparation was centrifuged at 38000 g for 20 min at 4 °C and the supernatant was used as the cell-free extract.

Enzyme assays. Assays with extracts from C. boidinii and H. polymorpha were carried out at 30 °C and 37 °C, respectively. Triosephosphate isomerase (EC 5.3.1.1) was determined in an assay system (total vol. 1 ml) containing 200 mM-triethanolamine buffer pH 7.6, 0.15 mM-NADH and 0.8 units glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). After 3 min incubation of cell-free extract with the above mixture in order to assess NADH oxidase activity, the reaction was started by adding glyceraldehyde 3-phosphate to a concentration of 6.2 mM. Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) was assayed using the method of van Dijken et al. (1978).

Analytical methods and chemicals. DHA was estimated by the method of Waites & Quayle (1980). Inorganic and organic phosphates were determined using the techniques of Lowry & Lopez (1946) and Allen (1940), respectively. The anthrone method of Bartlett (1959) was used to estimate hexose and hexose phosphate. Protein was measured by the Lowry method with bovine serum albumin as standard. [¹⁴C]Methanol and [U-¹⁴C]glucose were obtained from The Radiochemical Centre (Amersham). DHA and Cab-o-sil M5 (silica, fumed) were purchased from BDH. Glyceraldehyde 3-phosphate was prepared from glyceraldehyde 3-phosphate diethylacetal (monobarium salt) which was obtained from Boehringer, as were the enzymes and sugar phosphates.

RESULTS AND DISCUSSION

The initial step in the assimilation of methanol by yeasts is considered to be the formation of DHA by the following reaction (Kato et al., 1979; Waites & Quayle, 1980; O'Connor & Quayle, 1980):

\[
\text{Xyulose 5-phosphate + HCHO} \rightarrow \text{DHA + Glyceraldehyde 3-phosphate}
\]

If DHA is formed in this way then it may be predicted that the [¹⁴C]DHA arising from pulse-labelling methanol-grown H. polymorpha with [¹⁴C]methanol would initially be labelled predominantly in C-1. Since DHA is a symmetrical molecule this would result in apparently equal labelling of C-1 and C-3, with little or no radioactivity (depending upon the extent of recycling) in C-2 (Fig. 2). Degradation of the first sample of [¹⁴C]DHA (sample A in Table 2) indicated that 82.4 % of the radioactivity was present in C-1 and C-3, but that the label appeared somewhat unequally divided between these two carbon atoms (45.3 and 37.1 %,
Methanol metabolism by H. polymorpha

Table 2. Distribution of radioactive carbon in [14C]dihydr oxyacetone derived from methanol-grown Hansenula polymorpha pulse-labelled for 2 s with [14C]methanol

Chromatographically pure [14C]DHA was mixed with carrier DHA and sample A (0.5 mmol; 0.0123 μCi) was degraded as described in Methods. A further sample, B (0.45 mmol; 0.0033 μCi), was recrystallized from acetone with additional carrier before degradation. The values in parentheses represent the percentage of the total radioactivity in the DHA which is located in each atom.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific radioactivity of Ba14CO3</th>
<th>Sum of specific activities of individual carbon atoms</th>
<th>3 × Specific activity of Ba14CO3 obtained from combustion</th>
<th>Percentage recovery of specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[d.p.m. (mg BaCO3)-1] from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>C-1  120-3 (45-3)</td>
<td>C-2  46-7 (17-6)</td>
<td>C-3  98-4 (37-1)</td>
<td>265-4 277-2</td>
</tr>
<tr>
<td>B</td>
<td>C-1  18-5 (41-3)</td>
<td>C-2  3-8 (8-5)</td>
<td>C-3  22-5 (50-2)</td>
<td>44-8 67-2</td>
</tr>
</tbody>
</table>

respectively). After recrystallization of the [14C]DHA from acetone, 91.5% of the radioactivity was found in C-1 and C-3, but again the radioactivity appeared somewhat unequally divided between the hydroxymethyl groups of DHA (41.3 and 50.2%, respectively). A satisfactory degradation procedure for DHA should, by definition, give a symmetrical distribution of radioactivity in the molecule. The procedure used in the present study has not to our knowledge been reported previously. Specifically labelled DHA is not available commercially and hence we have not been able to test the procedure as with uniformly labelled glucose (see below). Thus, there is an element of uncertainty in the accuracy attainable. It should also be noted that in our hands DHA was not easily crystallized on the semi-micro scale without considerable losses and this prevented us from crystallizing it to proven constant specific activity. Nevertheless, bearing these factors in mind, it is clear that the [14C]DHA was labelled predominantly in the hydroxymethyl groups with only a small amount of the radioactivity (8 to 17%) in C-2. This is in accord with the prediction shown in Fig. 2.

No such experimental uncertainties attend the degradation of the labelled glucose. The production of hexose phosphate from two triose phosphates (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate) via the postulated pathway would lead to hexose phosphate with two hypothetical extremes of labelling pattern, species 1 and species 2 (Fig. 2), the extent to which either would predominate being dependent upon the relative activities of triosephosphate isomerase and fructose-1,6-bisphosphate aldolase. The accuracy of the degradation procedure for [14C]glucose was tested using a sample of authentic [U-14C]glucose (Table 3). The departures from uniformity were relatively small (±3%), with C-1 and C-5 exhibiting the greatest deviations; similar results were obtained by Kemp & Quayle (1967). On degradation of the [14C]glucose from H. polymorpha a very distinct labelling pattern was obtained (Table 3). Over 99% of the radioactivity was found to be fairly equally distributed between C-1, C-3, C-4 and C-6, with less than 1% in C-2 and C-5 combined. This labelling pattern was identical to that of the predicted species 2 (Fig. 2) and was strikingly different from that obtained by Kemp & Quayle (1967) for [14C]glucose from methane-grown Pseudomonas methanica (Table 3), which utilizes the ribulose monophosphate (RuMP) cycle of formaldehyde fixation.

The predominance of the species 2 labelling pattern over the species 1 pattern would be consistent with the activity of triosephosphate isomerase being several-fold higher than that of fructosebisphosphate aldolase. This was confirmed experimentally: in cell-free extracts of methanol-grown H. polymorpha, triosephosphate isomerase activity was 58-fold greater than fructosebisphosphate aldolase activity [specific activities were, respectively, 51.8 and 0.89 μmol substrate transformed min⁻¹ (mg protein)⁻¹]. In methanol-grown C. boidinii the difference was even more pronounced, triosephosphate isomerase activity being 164-fold
Table 3. Distribution of radioactive carbon in authentic \([U-^{14}C]glucose\) and \([^{14}C]glucose\) obtained from methanol-grown *Hansenula polymorpha* pulse labelled for 2 s with \([^{14}C]methanol\)

The \([^{14}C]glucose\) samples were mixed with carrier glucose and samples (1 mmol) were degraded as described in Methods. The values in parentheses represent the percentage of the total radioactivity in the glucose which is located in each carbon atom.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific radioactivity of Ba(^{14}CO_3) ([\text{d.p.m. (mg BaCO}_3\text{)}^{-1}]) from:</th>
<th>Sum of specific activities of individual carbon atoms</th>
<th>(6 \times \text{Specific activity of Ba}(^{14}CO_3) obtained from combustion</th>
<th>Percentage recovery of specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic ([U-^{14}C]glucose)</td>
<td>C-1: 956.4, C-2: 941.2, C-3: 725.1, C-4: 825.0, C-5: 695.0, C-6: 842.6</td>
<td>4985.3</td>
<td>4894.2</td>
<td>101.8</td>
</tr>
<tr>
<td></td>
<td>(19.2) (18.7) (14.7) (16.6) (13.9) (16.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([^{14}C]glucose) from</td>
<td>C-1: 601.6, C-2: 10.2, C-3: 461.8, C-4: 674.8, C-5: 3.9, C-6: 626.2</td>
<td>2378.5</td>
<td>2597.4</td>
<td>91.6</td>
</tr>
<tr>
<td><em>H. polymorpha</em></td>
<td>(25.3) (0.4) (19.4) (28.4) (0.2) (26.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([^{14}C]glucose) from</td>
<td>C-1: 34.2, C-2: 7.4, C-3: 1.9, C-4: 0.2, C-5: 0.6, C-6: 2.6</td>
<td>46.9</td>
<td>44.4</td>
<td>105.6</td>
</tr>
<tr>
<td><em>Pseudomonas methanica</em></td>
<td>(72.9) (15.8) (4.1) (0.5) (1.2) (5.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Labelling pattern of glucose obtained from methane-grown *Pseudomonas methanica*, following a 3 s incubation at 16 °C with \([^{14}C]methanol\) (Kemp & Quayle, 1967).
greater than fructosebisphosphate aldolase activity [specific activities were, respectively, 37·8 and 0·23 μmol substrate transformed min⁻¹ (mg protein)⁻¹].

In [14C]glucose from H. polymorpha the quantity of label appearing in C-2 and C-5 was even less than expected from the labelling pattern obtained from [14C]DHA. However, in contrast to degradation of DHA, the procedure used for glucose degradation is a well-proven one and hence the results obtained from it are probably more accurate than those for [14C]DHA, especially since the degradation of [14C]DHA gave a higher departure from symmetry than did the degradation of [U-14C]glucose.

It is interesting to recall that the first studies of methanol assimilation by yeasts, encompassing isotopic experimentation with whole cells and crude cell-free extracts, indicated that an assimilation process might operate similar to the bacterial RuMP cycle. Later symmetry than did the degradation of [14C]DHA, especially since the degradation of [14C]DHA gave a higher departure from symmetry than did the degradation of [U-14C]glucose.

In these experiments, the labelling pattern in early-labelled hexose phosphate was not consistent with the operation of the proposed RuMP pathway.

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