BIOCHEMISTRY OF FILAMENTOUS FUNGI

II. THE QUANTITATIVE SIGNIFICANCE OF AN "OXIDATIVE PATHWAY" DURING THE GROWTH OF Penicillium chrysogenum

E. C. HEATH and HENRY KOFLER

Laboratories of Bacteriology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana

Received for publication June 27, 1955

The direct oxidation of glucose via glucose-6-phosphate, 6-phosphogluconate, ribulose-5-phosphate, ribose-5-phosphate, etc., referred to as the hexosemonophosphate (HMP) or "oxidative pathway", may be an important mechanism of glucose metabolism in various tissues (Dickens, 1952; Horecker, 1953; and Weinhouse, 1954). In fact, Horecker recently postulated that the oxidative pathway, involving the phosphate esters mentioned above and others, may be cyclic in nature and an independent means for the complete oxidation of glucose. In addition to functioning as an oxidative mechanism, the HMP pathway also furnishes such cellular building blocks as ribose (Cohen, 1951), and perhaps others.

This paper presents results of experiments which indicate that a mechanism which preferentially liberates the first carbon atom of glucose plays a major role in the metabolism of glucose during the growth of Penicillium chrysogenum. (Heath et al., 1953; Heath and Koffler, 1953, 1955). The first indication of this was obtained by Blumenthal (1952), who noted that a greater percentage of the radioactivity appeared in the CO₂ from glucose-1-C¹⁴ than from uniformly-labeled glucose (glucose-U-C¹⁴) when resting cells of this organism were allowed to metabolize these substrates. The data of de Fiebre and Knight (1953) obtained in a similar manner with P. chrysogenum strain NRRL 1951B25 corroborate this experience with strain Q176, a "descendant" of the isolate 1951B25. Also members of the genus Aspergillus apparently degrade glucose by first oxidizing C₁ (Arnstein and Bentley, 1953), as is suggested by the following observations: The specific activity of the CO₂ evolved was greatest during the early stages of metabolism but later declined; the reverse was true when glucose-3,4-C¹⁴ was used. A study by Cochrane, Peck, and Harrison (1953) indicates that the oxidative pathway also plays a role in three species of Streptomyces, representative of the mold-like actinomycetes.

METHODS

Culture. Cells of P. chrysogenum strain Q176 were grown and prepared as previously described by Stout and Koffler (1951). Forty-hour-old cells were used in these experiments, except where indicated otherwise.

Cell-free preparation. Approximately 15 g (wet weight) of washed cells were ground with 30 g of alumina A-301 (ALCOA) in a chilled mortar for 4 to 7 min until a smooth, creamy paste was attained (McIlwain, 1948). Ten ml of 0.01 M phosphate buffer, pH 7.4, was then added, and the suspension was centrifuged at 2000 × G for 15 minutes at 0 C. The resulting slightly opaque supernatant liquid was used as the source of enzymes.

Manometric procedures. Oxygen uptake and CO₂ evolution were measured at 30 C according to the procedures described by Umbreit, Burriss, and Stauffer (1949). Corrections were made for endogenous respiration in all calculations. Fluted filter paper was omitted from the center wells of flasks from which C¹⁴O₂ was to be recovered; 0.2 ml of carbonate-free, saturated NaOH was placed into the center well. At the end of the re-
action period, 0.5 ml of 72 per cent perchloric acid was tipped into the flask from the sidearm, and the flask shaken in the bath for at least 1 hour to assure complete collection of CO$_2$.

Techniques involving C$^{14}$. The NaOH solution was removed quantitatively from the center well of the Warburg flasks (or flasks in which cells were grown), and the carbonate collected was precipitated with BaCl$_2$. The BaC$^{14}$O$_3$ was washed twice with 95 per cent ethanol, plated on microporous porcelain discs (Harshaw Scientific Company, Cincinnati, Ohio), 1.5 cm in diameter, and counted with either a thin-window Geiger-Muller tube or a Tracerlab windowless gas-flow counter. Maximum counting error was ±5 per cent. Counts were corrected by a graphical method to the activity at zero self absorption (Schweitzer and Stein, 1950). Specific activities of CO$_2$ were calculated from manometric determinations of the CO$_2$ evolved. Unlabeled NaHCO$_3$ was added as carrier when necessary. Organic materials were combusted by the reagents of Van Slyke and Folch (1940) in the apparatus of Stuts and Burris (1951). Uniformly labeled glucose (glucose-U-C$^{14}$) was added to the National Bureau of Standards through the kindness of Dr. H. S. Isbell; glucose-3,4-C$^{14}$ was generously supplied by Dr. John A. Munts of the School of Medicine, Western Reserve University. All radioactive sugars were shown to be chromatographically pure.

Determination of dehydrogenase activity. The enzymatic reduction of triphosphopyridine nucleotide (TPN) was obtained by measuring increased absorption at 340 m$\mu$ in a model DU Beckman spectrophotometer at room temperature.

Other analytical techniques. Pyruvate was determined by the method of Friedman and Haugen (1943), and glucose by the method of Somogyi (1952). Pentose was determined by the method of Meijbaum as given in Umbreit, Burris, and Stauffer (1949). Pyruvate was degraded to acetate by oxidative decarboxylation with ceric sulfate (Krebs and Johnson, 1937), and the CO$_2$ evolved was analyzed for radioactivity; the resulting acetate was degraded by the method of Phares (1951). Pyruvate and acetate were isolated from reaction mixtures by the celite column procedure of Swim and Krampitz (1954). Unlabeled acids were added as carriers when necessary.

Triphosphopyridine nucleotide (TPN “85”) was obtained from Sigma Chemical Company, St. Louis. The 6-phosphogluconate was obtained as the calcium salt from Biox Chemical Company, New York; glucose-6-phosphate and ribose-5-phosphate as the barium salts from Schwarz Laboratories, Mount Vernon, New York. The salts of the phosphate esters were converted to the acid forms with Dowex 50 (H$^+$), and then neutralized with sodium hydroxide.

RESULTS

Whole cells were permitted to respire on glucose-1-C$^{14}$ for 3 hours in the presence and absence of 6 × 10$^{-4}$ m arsenite. The respiratory CO$_2$ and pyruvate, which accumulated in the presence of arsenite, were collected, and their specific activities were determined. The results of such an experiment are shown in table 1. The specific activity of CO$_2$ evolved in the presence of arsenite is essentially the same as that of the glucose substrate, indicating that there had been a preferential removal of the C$_1$ of glucose, as would occur if the early steps of the HMP pathway were functioning. Even in the absence of arsenite, there was an indication for the selective oxidation of the first glucose carbon, because the specific activity of the CO$_2$ released was greater (900) than would be expected (700) if equal portions of each of the glucose-carbons were converted to CO$_2$. The existence of pyruvate which contains all of its radioactivity in the methyl position indicates that in addition to this oxida-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Micromoles</th>
<th>Total CPM</th>
<th>Specific Activity (CPM/m$\mu$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-C$^{14}$-glucose</td>
<td>5.0</td>
<td>20,400</td>
<td>4,080</td>
</tr>
<tr>
<td>CO$_2$ (+ arsenite)</td>
<td>1.8</td>
<td>7,100</td>
<td>3,940</td>
</tr>
<tr>
<td>CO$_2$ (-- arsenite)</td>
<td>8.4</td>
<td>7,600</td>
<td>900</td>
</tr>
<tr>
<td>Pyruvate (+ arsenite)</td>
<td>3.1</td>
<td>4,500</td>
<td>1,450*</td>
</tr>
</tbody>
</table>

* Specific activity of pyruvate corrected for endogenous accumulation of pyruvate (2.7 m$\mu$) in the presence of arsenite. Stepwise degradation indicates that all of the radioactivity of pyruvate is in the methyl position.
Figure 1. Reduction of triphosphopyridine nucleotide (TPN) by a cell-free extract of *Penicillium chrysogenum* in the presence of glucose-6-phosphate and 6-phosphogluconate.

The system contained: enzyme extract, 1.0 ml; 0.05 m substrate, 0.1 ml; TPN (1.0 mg/ml), 0.1 ml; phosphate buffer (pH 7.4), 0.01 m, to 3.0 ml total volume. The control cuvette contained all components except TPN. The addition of TPN is indicated by arrows. Glucose or glucuronate ± adenosine triphosphate showed no reaction in the above system.

tive pathway probably also the reactions of the Embden-Meyerhof-Parnas (EMP) scheme occur when arsenite inhibited cells metabolize glucose-1-C\(^{14}\). A split of glucose into two trioses, as postulated in the EMP scheme, and a subsequent equilibration between glyceraldehydephosphate and dihydroxyacetonephosphate should result in methyl-labeled pyruvate from glucose-1-C\(^{14}\). One can infer the existence of glycolysis in *Penicillium* also from the data of Lynen and Hoffmann-Walbeck (1948), who reported aldolase and triosephosphate dehydrogenase activity and the formation of phosphoglyceric acid by cell-free extracts of *Penicillium notatum*, an organism probably related to the one used in this study. Blumenthal, Lewis, and Weinhouse (1954) estimated from the specific activities of compounds derived from glucose-1-C\(^{14}\) and glucose-U-C\(^{14}\) that from 56 to 70 per cent of the trioses formed from glucose by resting cells of *P. chrysogenum* are derived via the EMP process.

The fact that the specific activity of the pyruvate which accumulates from glucose-1-C\(^{14}\) is less (1450 cpm/\(\mu\)m) than would be expected (ca. 2000 cpm/\(\mu\)m) if all of the pyruvate found arose via the EMP reactions indicates that pyruvate was produced also by other means. Since the CO\(_2\) released in the presence of arsenite originated entirely from the first carbon atom of glucose-1-C\(^{14}\) (i.e., its specific activity was undiluted), it appears that under the conditions of the experiment the HMP pathway does not function predominantly as a cyclic mechanism for the oxidation of glucose (Horecker, 1953). However, these data do not constitute critical evidence for such an interpretation, inasmuch as arsenite may be inhibitory at other sites than at the pyruvate stage.

To obtain additional evidence for the potentiality of this organism to perform certain reactions of the HMP pathway, cell-free enzyme preparations were examined as to their ability to catalyze the TPN-linked oxidations of glucose-6-phosphate and 6-phosphogluconate. Figure 1 shows that TPN reduction occurs in the presence of these substrates. The relatively small increase in the optical density on the addition of TPN may have been due to the enzymatic destruction of the coenzyme. Diphosphopyridine nucleotide could not be substituted for TPN, and nonphosphorylated glucose or glucuronate could not serve as substrates even in the presence of added adenosine triphosphate. Apparently also ribose-5-phosphate was metabolized, as was indicated by the rapid disappearance of pentose from a re-

![Figure 2](http://jb.asm.org/) Disappearance of pentose from a reaction mixture containing ribose-5-phosphate and a cell-free extract of *Penicillium chrysogenum*.

The complete system contained 10 ml cell extract and 2000 \(\mu\)g ribose-5-phosphate (or ribose); 1.0 ml aliquots were removed at the times indicated, deproteinized with 5 per cent trichloracetic acid, and analyzed for pentose. The total volume of the reaction mixture was 10.1 ml.
action mixture containing ribose-5-phosphate as the substrate (figure 2); nonphosphorylated ribose was not attacked.

The participation of some mechanism (or mechanisms) by which the first carbon atom of glucose is attacked preferentially during the growth of this organism was demonstrated convincingly by the following two experiments: In the first experiment the cells were grown from spores in media containing glucose-1-C\textsuperscript{14}, -2-C\textsuperscript{14}, -3,4-C\textsuperscript{14}, or -6-C\textsuperscript{14}. The spores were inoculated into 25 ml of growth medium contained in 125-ml Erlenmeyer flasks fitted with rubber stoppers supporting NaOH traps. At 10-hr intervals one flask of each series was removed from the shaking machine, and 1 ml of 72 per cent perchloric acid was injected by means of a hypodermic needle and syringe through a diaphragm in the stopper. The flasks then were shaken again for at least 1 hour, a period after which the C\textsuperscript{14}O\textsubscript{2} was analysed in the same manner as that from Warburg flasks. At the same time, fresh NaOH traps were inserted into another series of flasks, so that the release of C\textsuperscript{14}O\textsubscript{2} could be determined for the next 10-hr period. The results of this experiment are shown in figure 3. All values are expressed on the basis of equivalent initial specific activities. These data indicate that the first carbon atom of glucose is oxidized more readily during all phases of growth than any of the other carbon atoms under scrutiny.

![Figure 3](image-url)

**Figure 3.** The relative rates at which the individual carbon atoms of glucose are oxidized during the growth of *Penicillium chrysogenum*.

The details of the experiment are described in the text. Total activity added to each flask = 325,000 cpm.

In the second experiment the cells were grown for 30 hours and harvested as previously described; they were then resuspended in fresh growth medium that contained twice the normal concentration of nutrients but no glucose. One and one-half ml of this suspension was then placed into Warburg vessels. Dilution of this medium by the addition of other components of the reaction mixture resulted in a medium of approximately normal composition. Thirty-hour-old cells continue growing when suspended in fresh medium (i.e., their nitrogen content increases approximately by one third in three hours, the time required for exhaustion of the substrate in these experiments). At zero time 5 \mu M of glucose-1-C\textsuperscript{14}, -2-C\textsuperscript{14}, -3,4-C\textsuperscript{14}, or -6-C\textsuperscript{14} was tipped into the main compartment of the flask. At intervals of 1, 3, 8, 15, 30, 60, 120, and 165 min further metabolism was stopped in one flask of each series by the addition of perchloric acid. The radioactivity in the CO\textsubscript{2} evolved during these experimental periods was analyzed as described before. Figure 4 indicates the initial rate of oxidative attack on the individual carbon atoms of glucose; the inset illustrates the pattern of oxidation for the entire experimental period. The susceptibility of C\textsubscript{1} to oxidative attack is strikingly greater than that of any of the other carbon atoms. During the first 3 minutes for instance, C\textsubscript{1} is oxidized 4 times faster than C\textsubscript{2}, 11 times faster than C\textsubscript{3,4}, and 39 times faster than C\textsubscript{4}.

![Figure 4](image-url)

**Figure 4.** The relative initial rates at which the individual carbon atoms of glucose are oxidized during the growth of *Penicillium chrysogenum*.

The details of the experiment are described in the text. Total activity added to each flask = 44,400 cpm.
Glucose in 25 ml growth medium: 130 mg
Total activity (either from glucose-1-C\textsubscript{14} or glucose-U-C\textsubscript{14}) in 25 ml growth medium: 33,200 cpm
Glucose disappeared during 30- to 40-hr growth period: 61 mg or 15,000 cpm
Total activity in respiratory CO\textsubscript{2} from glucose-1-C\textsubscript{14}: 10,500 cpm
Total activity in respiratory CO\textsubscript{2} from glucose-U-C\textsubscript{14}: 2,600 cpm
Per cent total activity in glucose-1-C\textsubscript{14} utilized appearing in CO\textsubscript{2}: 10,500 cpm/15,000 cpm \times 100 = 70
Activity per carbon atom of glucose-U-C\textsubscript{14} utilized: 15,000 cpm/6 = 2,500 cpm
Activity in CO\textsubscript{2} from glucose-U-C\textsubscript{14} due to glucose-C\textsubscript{2} oxidation: 70 \times 2,500 cpm/100 = 1,750 cpm
Activity in CO\textsubscript{2} from glucose-U-C\textsubscript{14} due to the oxidation of carbon atoms 2 through 6 of glucose: 2,600 cpm - 1,750 cpm = 850 cpm
Total activity in carbon atoms 2 through 6 of glucose utilized: 2,500 cpm \times 6 = 15,000 cpm
Per cent of total activity in carbons 2 through 6 of glucose-U-C\textsubscript{14} utilized appearing in CO\textsubscript{2}: 850 cpm/12,500 cpm \times 100 = 7
Minimum per cent of glucose utilized during the 30- to 40-hr period of growth that was metabolized by a primary attack at C\textsubscript{1}: 70 - 7 = 63

To minimize randomization and the resulting

---

For other methods of calculating the quantitative significance of the oxidative pathway, see Bloom et al. (1953); Abraham et al. (1954); and Blumenthal et al. (1954).
### TABLE 2

**Minimal participation of an oxidative pathway during short intervals of growth of Penicillium chrysogenum**

<table>
<thead>
<tr>
<th>Period</th>
<th>Glucose-1-C(^14)</th>
<th>Glucose-U-C(^14)</th>
<th>Minimal Per Cent Oxidative Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM in glucose utilized</td>
<td>CPM in CO(_2) % C(_1) in CO(_2)</td>
<td>CPM in glucose utilized</td>
</tr>
<tr>
<td>hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34-34.25</td>
<td>6270</td>
<td>4380</td>
<td>70</td>
</tr>
<tr>
<td>34.5-34.75</td>
<td>5700</td>
<td>3650</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>(70 – 12)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The organism was grown in nonradioactive medium; at 34 hr., glucose-1-C\(^14\) or glucose-U-C\(^14\) (101,800 cpm) was added to the flask, and the organism was allowed to grow for 15 min., at which time perchloric acid was added to stop metabolism. The respiratory CO\(_2\) was collected, and the radioactivity was determined as described in the text. Aliquots were removed for glucose analysis when radioactive glucose was added, and also immediately before the perchloric acid was added. Calculations were made as outlined in the text.

Inaccuracies in the interpretation of these data, the minimal participation of an oxidative pathway was also studied during relatively shorter intervals within the period of most active growth (30 to 40 hr). The cells were grown on unlabeled glucose until the beginning of the interval to be studied; at that time glucose-1-C\(^14\) or glucose-U-C\(^14\) was added to the flask. The contents of each flask were acidified after \(\frac{1}{4}\) hr, and the radioactivity in the CO\(_2\) was analyzed as before; the same method of calculation was applied to the data as in the preceding experiment. Table 2 shows that the estimate regarding the minimal participation of a pathway by which the C\(_1\) of glucose is attacked preferentially is not affected appreciably by the length of the experimental period during which the measurement is being made (\(\frac{1}{4}\) hr vs. 10 hr, figure 5).

**DISCUSSION**

In the interpretation of these data advantage is taken of the fact that this organism assimilates a large portion of the glucose utilized. No hint as to the mechanism of metabolism could be gained, if the substrate were oxidized to completion, because the CO\(_2\) produced would have the same specific activity, regardless of whether it came from uniformly labeled or 1-C\(^14\)-labeled glucose (of the same initial specific activity). If the 3-carbon fragments formed from glucose via the EMP reactions and those arising through the HMP oxidative pathway were in equilibrium with each other, and then oxidized to completion by the same route, CO\(_2\) of the same specific activity would result irrespective of the pathway. On the other hand, if degradation products of glucose are converted to cellular materials rather than CO\(_2\), differences will occur in the amount of glucose activity eventually recoverable as CO\(_2\), depending on whether uniformly labeled glucose or glucose-1-C\(^14\) is used and which part of the glucose molecule becomes assimilated. For example, if CO\(_2\) and a 5-carbon compound were the only two products arising directly from glucose during a given experiment, all of the radioactivity in the CO\(_2\) would have to come by oxidative decarboxylation when glucose-1-C\(^14\) is the substrate. The fate of glucose during growth, of course, is not that clearly defined. Consequently, the differences in the percentages of radioactivity in the CO\(_2\) from glucose-U-C\(^14\) and glucose-1-C\(^14\) allow one to estimate only the minimal but not the actual participation of a mechanism that liberates the C\(_1\) of glucose preferentially; as stated above, all of the radioactivity that appears in the CO\(_2\) from glucose-1-C\(^14\) may have come through such a route. The data presented in this paper, therefore, can be interpreted as evidence that the first carbon atom of glucose is the first to be oxidized to CO\(_2\) when the percentage of the initial substrate activity appearing in CO\(_2\) from glucose-1-C\(^14\) is greater than that from glucose-U-C\(^14\), and that the mechanism or mechanisms involved in this oxidation are of major quantitative significance in the breakdown of glucose when such a difference is large.
The interpretation of these data is based on the assumption that the first carbon atom of glucose is oxidized preferentially to CO₂ when still a constituent atom of a C₅ compound, and not of a breakdown product of glucose. Among the known mechanisms by which P. chrysogenum can accomplish such an oxidation, the HMP oxidative pathway seems to be the most plausible. This is consistent with (1) the ability of cell-free extracts of P. chrysogenum to perform several of the reactions envisaged to occur in the HMP pathway, (2) the relative vulnerability of the various carbon atoms of glucose to oxidation (C₁ > C₂ > C₄,₄ > C₄), and (3) the position of radioactivity in pyruvate derived from glucose-1-C¹⁴ in the presence of arsenite. The fact that arsenite leads to the accumulation of methyl-labeled pyruvate from glucose-1-C¹⁴ minimizes the possibility that the preferential liberation of CO₂ from glucose-C₁ was brought about via the decarboxylation of carboxyl-labeled pyruvate, which may have been formed by a mechanism similar to the one operating in Pseudomonas saccharophila (Entner and Doudoroff, 1952; MacGee and Doudoroff, 1954) and P. fluorescens (Wood and Schwert, 1953; Kovachevich and Wood, 1954). None of the known metabolic conversions that pyruvate is likely to undergo could account for the preferential oxidation of its methyl carbon to CO₂. Moreover, since no other carbon atom of glucose gives rise to CO₂ as readily as does the first carbon atom (figures 3 and 4) it is apparent that, if the radioactivity of C₁ because of skeletal rearrangements during the growth of the organism should have found its way into 6-carbon compounds (or compounds derived from them) at positions other than those predicted, it would be less likely to appear as CO₂ than if it had remained in the C₁ position. This is another reason why these data must be regarded as minimal measurements. Even so, the data show that at least two-thirds of the glucose utilized during the growth of this organism is degraded via a mechanism or mechanisms by which the carbon atom initially released in the CO₂ is the first carbon atom. Blumenthal et al. (1954), using a somewhat different approach, estimated that about one-third to one-half of the glucose was dealt with in such a manner by resting cells of P. chrysogenum. Growing cells might have a greater tendency to use a mechanism like the HMP pathway, because of the steady withdrawal of intermediates such as ribose for synthetic purposes; this may explain the somewhat higher percentage figure obtained in the present experiments. In any case it is clear that an "oxidative pathway" constitutes a quantitatively significant mode of metabolizing glucose in this organism.

ACKNOWLEDGMENTS

The authors owe a great deal to Dr. Harry Bevers for his valuable suggestions during the course of this work, and to several others for comments regarding the writing of this paper. One of us (H. K.) also wishes to express his gratitude to his colleagues at the School of Medicine, Western Reserve University, for making his sojourn there during 1953-1954 a most stimulating experience, and to the John Simon Guggenheim Memorial Foundation for making this stay possible.

SUMMARY

Growing cells of Penicillium chrysogenum metabolize at least two-thirds of the glucose utilized through a mechanism (or mechanisms) involving a primary oxidation of the first carbon atom to CO₂. The data presented are consistent with the belief that this mechanism is the hexosemonophosphate oxidative pathway.

REFERENCES


Arnstein, H. R. V. and Bentley, R. 1953 The biosynthesis of kojic acid. I. Production from (1-C¹⁴) and (3:4-C¹⁴) glucose and (2-C¹⁴)-1:3-dihydroxyacetone. Biochem. J. (London), 54, 493-508.


