The Use of Kinetic Isotope Effects in the Study of Metabolic Control

I. DEGRADATION OF GLUCOSE-1-D BY THE HEXOSEMONOPHOSPHATE PATHWAY*

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The observation that molecules substituted with different isotopes may react at different rates has been of great value to the study of chemical reaction mechanisms. The theory and applications of this effect, particularly with isotopes of hydrogen, are well described in the literature (1-3), and frequent use is made of isotope effects in the study of the mechanism of enzyme reactions (4-6). One of the questions that is answered by the comparison of isotopically substituted molecules in a chemical or enzyme-catalyzed reaction is whether the component step concerned with the cleavage of the bond containing the isotope is rate-determining for the over-all reaction. For example, it was demonstrated recently (4) that in the conversion of dihydroxyacetone phosphate to d-glyceraldehyde-3-P by the enzyme triosephosphate isomerase, in which a C-H bond is broken and the proton released into the medium, the reaction proceeds at about half the rate if deuterium is present in the particular position in the substrate instead of hydrogen. This observation provides important information about the meaning of the various kinetic constants measured for the isomerase reaction, and signifies that any mechanism formulated for the reaction must be consistent with the finding that the deprotonation of the substrate is slow relative to the other component steps of the reaction. By extension of this principle, it should be possible to determine whether, in a multi-enzyme sequence of reactions, a particular step is rate-limiting, depending upon whether isotope substitution at the bond broken in that reaction affects the rate of the whole sequence of reactions. For example, it may be asked whether in the conversion of glyceroI to glycogen in the rat, the triosephosphate isomerase reaction is rate-determining.

Since it is known that the isomerase reaction shows a deuterium isotope effect, this question could be approached by the use of glyceroI containing deuterium which would be in the position labilized by isomerase when the glyceroI is converted to dihydroxyacetone phosphate in the rat. The glyceroI should be labeled with C-D so that its rate of incorporation into the glycogen could be conveniently determined.1 However, in preparing this doubly labeled glyceroI, it is necessary that the C-D and deuterium be located in the same molecule, intramolecularly doubly labeled; otherwise, the C-D will not be a tracer for the deuterated species of glyceroI. The complications that may arise from the use of a doubly labeled substrate obtained by mixing the two isotopic isomers are well illustrated by the work of Rachele et al. (7), and by Abeles et al. (6).

In the present work, this approach has been followed to study the so called "hexosemonophosphate pathway," through which the C-1 position of glucose is oxidized to CO₂ (Fig. 1). In this pathway, two reactions, those numbered â and â in Fig. 1, are concerned with the cleavage of C-H bonds of the glucose. This investigation is concerned with a study of the glucose 6-phosphate dehydrogenase reaction (Reaction 1) for a possible isotope effect with substrate containing deuterium in the C-1 position. A large effect was found under conditions in which substrate, coenzyme, and pH were varied over a wide range. The conclusion seemed justified that if the dehydrogenase step were rate-determining for the conversion of glucose-1-C-D to C-D-O₂, this rate should be affected by deuterium substitution for hydrogen in the C-1 position of this glucose. With glucose doubly labeled intramolecularly, glucose-1-D-C-D (actually a mixture of glucose-1-H-C-D and glucose-1-D-C-D), and glucose-1-H-C-D (actually a mixture of glucose-1-H-C-D and glucose-1-H-C-D), the rates of appearance of radioactivity in the CO₂ were compared in whole cell suspensions of human erythrocytes and ascites tumor cells.

In the case of the isomerase reaction, cited above, in which the hydrogen activated is lost to the medium by dilution with the protons of the water (4), the deuterium introduced into the rat in the glyceroI can have no kinetic effect beyond the isomerase reaction. On the other hand, the glucose-6-P dehydrogenase reaction is known to result in the transfer of the hydrogen of the substrate to TPN (8). In the whole cell, the TPN must be regenerated, and if the rate of this process is affected by the presence of deuterium in the TPNH, it may not be possible to interpret unambiguously the appearance of an isotope effect in the over-all process. As will be discussed in detail, the use of intermolecularly doubly labeled glucose, formed by mixing glucose-1-H-C-D with glucose-1-D-C-D, may give results quite different from those obtained with glucose-1-D-C-D. This difference will be referred to as the isotope discrimination effect and will be shown to be of some value in determining the location of the rate-determining step. A preliminary report of this work has been published (9).

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1 Experiments using this approach have been performed. A report is in preparation.
Experimental Procedure

Glucose-1-D used in early experiments was a gift of Dr. Henry Hoberman. That used in all experiments for which data are presented was synthesized from D-glucono-d-lactone by Na-Hg reduction (10) in D$_2$O. For the preparation of glucose-1-D-C$^{14}$, gluconic acid-1-C$^{14}$ from Nuclear-Chicago Corporation was first lactonized (10). The oxalic acid used in the reduction medium was exchanged with D$_2$O by successive evaporations from D$_2$O to assure the highest possible enrichment of the reduction product formed in water of 99.5 atom per cent. Yields of glucose varied from 10 to 50% of the twice recrystallized product which gave a correct melting point (114-146$^\circ$). An isotope dilution experiment was performed to establish the purity of the deuterated glucose. Equal samples of glucose-1-C$^{14}$ were added to solutions containing equal weights of glucose-1-D or of glucose (Mallinckrodt). The two solutions were treated with hexokinase for the preparation of glucose-6-P as outlined below. The radioactivity and the content of glucose-6-P as determined with the dehydrogenase led to values of specific activity that agreed within 3%. The synthesized materials were pure by the reducing sugar test (11); however, the much more specific assay with glucose oxidase coupled to the peroxidative oxidation of the dye, o-dianisidine (Worthington Biochemicals “glucostat” assay) did not suggest high purity even after recrystallization. The oxidase rate was found to be 2.8 times faster with the normal substrate than with the deuterated one at 0.3 mM substrate concentration. This is assumed to be due to a strong isotope effect.$^2$ With the use of large amounts of the “glucostat” reagents and long incubation times, closer agreement for quantitative assay of glucose-1-D and glucose could be obtained, but a difference of 5% remained. This again is attributed to the high isotope effect and the very high Michaelis constant ($K_m$) of the oxidase for glucose, thus making the approach to quantitative oxidation very slow.

In the preparation of the substrates for glucose-6-P dehydrogenase studies, the normal and deuterated species were treated in exactly the same manner throughout. Yeast hexokinase provided by Dr. S. V. Rieder, was incubated with the glucose (10 $\mu$moles) and an ATP-generating system consisting of phosphopyruvate (20 $\mu$moles), Mg$^{2+}$ (5 $\mu$moles), ADP (1 $\mu$moles), and pyruvate kinase in a total volume of 1.0 ml. The glucose-6-P formed was recovered from a Dowex 1 (Cl-) column by eluting with 0.05 N HCl. Similar preparations of glucose-6-P from glucose-1-T did not lose tritium during the preparation or storage in dilute acid in the frozen state. Thus, although direct deuterium analyses were not performed on the product, it may be concluded that no loss of deuterium would have occurred in the enzymatic phosphorylation and subsequent isolation and storage of the deuterated product used in the subsequent experiments.

The yeast glucose-6-P dehydrogenase preparations were products of Sigma Chemical Company and Boehringer and Soh and were essentially free of 6-phosphogluconic acid dehydrogenase. The enzyme preparation from Escherichia coli was made according to the procedure of Scott and Cohen (12) and was 10 times as active with glucose-6-P as with 6-phosphogluconate at pH 7. For the enzyme from human erythrocytes, the supernatant solution from a hemolysate was fractionated and re-fractionated with ammonium sulfate (30 to 50% and 25 to 40% of saturation, respectively). The most active fraction contained about 0.5% 6-phosphogluconic acid dehydrogenase as a contaminant.

The routine assay of the dehydrogenase was conducted in a 1.0-ml volume containing triethanolamine-Cl$^-$ (100 $\mu$moles, pH 7.0), glucose-6-P (1 $\mu$ mole), MgCl$_2$ (5 $\mu$moles), and TPN (0.3 $\mu$ mole). Reduction of TPN was followed at 340 nm at 25$^\circ$. A unit of activity is that which gives rise to 1 $\mu$ mole of TPNH in 1 minute. Glutathione reductase from yeast was a gift of Dr. E. Racker. It was essentially free of glucose-6-P dehydrogenase. The procedure of Grunert and Phillips (13) for the determination of $\bar{SH}$ was followed, with the exception that NaCN was omitted from the test.

Human red blood cells were prepared from blood collected in heparin or citrate by centrifugation and five to six washings with...
cold 0.9% NaCl were carried out. Care was taken to discard the upper surface of the packed cell volume. The packed cells were finally suspended in an equal volume of the phosphate-salt solution used by Nossal (14) and used immediately.

Ehrlich ascites tumor cells and leukemia lymphoblasts (L-5178 Y) were obtained from DBA/2 mice 6 to 7 days after inoculation. The cell suspension was centrifuged at very low speeds (less than 100 X g) at room temperature to separate the tumor cells from the lighter blood cells present in the fluid. Further purification was effected by several washings at room temperature with an incubation medium of the following composition (in /mole per ml): Tris buffer, pH 7.4; 10; NaCl, 88; KCl, 20; MgCl2, 2; and potassium phosphate, 4 (15). The cells, finally suspended in this medium, were essentially colorless. They were preincubated at room temperature for 30 minutes to deplete them of oxidizable substrates, centrifuged, resuspended, and used immediately. The amount of cellular material added to each reaction flask was established in terms of protein determined by the biuret reaction (16).

The whole cell incubations were performed aerobically in conventional 15-ml Warburg flasks which contained 0.2 ml of 10% NaOH in the center well and perchloric acid in the side arm. Incubations were carried out with shaking at 37° for the designated time, after which the acid was tipped into the main chamber of the flask, and shaking was continued for 15 minutes longer. The contents of the center well were immediately removed for the counting of radioactivity. In the leukemic cell experiment, the acid-soluble fraction obtained from several washes of the precipitated material was freed of ClO4− with KOH, and placed on a column of Dowex 1 (formate form). Lactic acid was eluted immediately with 4 Na formic acid. The fraction obtained was twice evaporated to dryness, in a vacuum, and finally analyzed for radioactivity and content of lactate (17).

Tritium and C14 determinations were made by liquid scintillation counting (18) at an absolute counting efficiency of 4%.

RESULTS

Isotope Effect with Glucose-6-P Dehydrogenase—In Fig. 2 are shown typical results obtained with the yeast enzyme at pH 8 in which TPN is held at a concentration much higher than its \( K_{m} \) (0.019 mm) and in which the concentration of glucose-B-P is varied. From the double reciprocal plots are calculated \( K_{m} \) and \( V_{max} \) values derived from \( K_{m} \) and \( V_{max} \) values obtained for the normal and deuterated substrates.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>pH</th>
<th>( K_{H} )</th>
<th>( K_{D} )</th>
<th>( K_{H} ) / ( K_{D} )</th>
<th>( V_{H} ) / ( V_{D} )</th>
<th>( K_{H} ) / ( V_{H} )</th>
<th>( K_{D} ) / ( V_{D} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>5.9*</td>
<td>0.024</td>
<td>0.016</td>
<td>1.5</td>
<td>0.93</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>7.0†</td>
<td>0.033</td>
<td>0.037</td>
<td>1.4</td>
<td>1.0</td>
<td>2.0</td>
<td>1.4</td>
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<td></td>
<td>8.0</td>
<td>0.049</td>
<td>0.094</td>
<td>1.3</td>
<td>1.2</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>0.089</td>
<td>0.089</td>
<td>1.3</td>
<td>1.2</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>9.4‡</td>
<td>0.040</td>
<td>0.024</td>
<td>1.7</td>
<td>1.9</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>6.9†</td>
<td>0.057</td>
<td>0.042</td>
<td>1.7</td>
<td>1.0</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>7.0†</td>
<td>0.073</td>
<td>0.042</td>
<td>1.7</td>
<td>1.0</td>
<td>2.4</td>
<td>1.4</td>
</tr>
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<td>Red cells</td>
<td>7.0†</td>
<td>0.073</td>
<td>0.042</td>
<td>1.7</td>
<td>1.0</td>
<td>2.4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Caeodylate buffer.
† Triethanolamine buffer.
‡ Glycine buffer.

The double reciprocal plots on the right are designated by the vertical lines in the plot on the left. Velocities are given in absorbance changes per minute at 340 nm.

Glucose-6-P + TPN + = 6-P-gluconolactone + TPNH + H+ (2)

\( {\text{TPNH} + \text{GSSG} + \text{H}^+ \rightarrow \text{TPN}^+ + 2 \text{GSH}} \)
Use of Isotope Effects in Metabolic Control Studies. I

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TABLE II

Isotope effects at varying TPN concentrations

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>TPN</th>
<th>pH</th>
<th>sD</th>
<th>pH/sD</th>
</tr>
</thead>
<tbody>
<tr>
<td>units/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.045</td>
<td>0.0173</td>
<td>0.0083</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>0.014</td>
<td>0.031</td>
<td>0.0167</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>0.014</td>
<td>0.048</td>
<td>0.023</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>0.014</td>
<td>0.054</td>
<td>0.028</td>
<td>1.83</td>
<td></td>
</tr>
</tbody>
</table>

TABLE III

Isotope effects on CO₂ formation from 1-deutero glucose with tumor cells

The main chamber of the Warburg vessel contained: 1.0 ml of cell suspension (3.9 mg of protein for the leukemia cells and 4.5 mg for the Ehrlich cells), plus 10 μmoles of one of the three glucose mixtures noted at approximately 45,000 c.p.m. per μmole. Final volume, 1.2 ml. Incubation at 37°C was for 100 minutes.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Composition of glucose mixture</th>
<th>C³ in center well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C³-H: C³-D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ehrlich cells</td>
</tr>
<tr>
<td></td>
<td>Leukemia cells</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>C³⁴-H: C³₂-H, 1:25,000</td>
<td>12,392 0.275</td>
</tr>
<tr>
<td>2</td>
<td>C³⁴-D: C³₂-D, 1:25,000</td>
<td>10,852 0.248</td>
</tr>
<tr>
<td>3</td>
<td>C³⁴-H: C³₁-D: C³₂-D, 1:250:25,000</td>
<td>20,114 0.433</td>
</tr>
</tbody>
</table>

* Calculated from the c.p.m. in the center well divided by the specific activity of the glucose of the medium.

Use of Isotope Effect in Whole Cell Experiments—Since it has been demonstrated that a considerable isotope effect exists over a wide range of pH values, TPN concentrations, and glucose-6-P concentrations, one may suggest the use of glucose-1-D in comparison with glucose-1-H to determine whether the dehydrogenase is rate-limiting for the formation of CO₂ from the 1-position of glucose in the intact cell. In order to avoid the complications that would arise if much of the CO₂ derived from the glucose has come through the oxidation of lactate, cells were chosen which normally do not oxidize lactate readily. Thus the rate of formation of CO₂ from the C-1 and C-6 positions of glucose was found to be in the ratio of 2.5 for the leukemic cells and 250.25:25,000. Thus the true amount of CO₂ formed from C-1 would be that found in Experiment 2, with the deuterated substrate. Therefore, these data lead to the conclusion that when the normal species and deuterated species of glucose in mixture are presented to these tumor cells, the normal species is utilized at about 1.75 times the rate of the deuterated form. An isotope discrimination effect approaching this magnitude would be predicted from the kinetic data given in Table I and the kinetics of an enzyme acting competitively on two substrates, as seen from the following development. In the case in which
two forms of the substrate, designated \( S \) and \( S' \), are presented together to an enzyme, \( E \), the competitive reactions formally described in Equation 3 occur. The rates of product formation in the forward direction, \( v \) and \( v' \), are \( k_2(ES) \) and \( k_2'(ES') \). The concentrations of the enzyme-substrate complexes are derived from the assumption that they are time-independent:

\[
\frac{d(ES)}{dt} = k_2(E)(S) - (k_1 + k_2)(ES) = 0
\]

\[
\frac{d(ES')}{dt} = k_2'(E)(S') - (k_1' + k_2')(ES') = 0
\]

and so

\[
(ES) = \frac{k_2(E)(S)}{k_1 + k_2} = \frac{(S)(E)}{K_m} = \frac{v}{k_2}
\]

and

\[
(ES') = \frac{k_2'(E)(S')}{k_1' + k_2'} = \frac{(S')(E)}{K'_m} = \frac{v'}{k_2'}
\]

The ratio of the rates of reaction of the two substrates is then given by

\[
\frac{v'}{v} = \frac{k_2'(S')(K_m)}{k_2(S'(K'_m))} = \frac{V_{\text{max}}'(S)(K_m)}{V_{\text{max}}(S')(K'_m)}
\]

in which \( V_{\text{max}} \) is the maximal velocity attained with pure \( S \), \( V_{\text{max}}' = k_2' \) (total), and \( V_{\text{max}}' = k_2' \) (total). A similar equation relating the competitive rates of a deuterated and normal substrate has been given by Abeles et al. (6). To apply this equation to a whole cell experiment in which the substrates for the enzyme are generated from a mixture of deuterated and hydrogen-containing precursors, it is assumed that no selection occurs between the forms of the precursor before presentation to the enzyme; specifically concerning Experiment 3 in Table III, it is assumed that the molar ratio of the two forms of glucose-6-P is the same as that of the glucose from which it is derived in the cell. This is a reasonable assumption, since the intervening reactions are not expected to involve cleavage of the C—H bond in question. In Experiment 3 with deuterated plus normal forms of glucose, the normal species, which is the minor and radioactive component of the mixture, produced \( CO_2 \) from the C-1 position at a rate given by the expression:

\[
\frac{v'}{v} = \frac{p'/t}{T'(S')},
\]

in which \( p \) = counts in the product, \( CO_2 \); \( T \) = total radioactivity in the experiment; \( (S') \) = amount of the normal species; and \( t \) = time of incubation. The rate of conversion of the major, deuterated component of the mixture is obtained directly from Experiment 2 by a comparable expression in which the same amount of radioactivity, \( T \), is present as in Experiment 3 and also the same amount of glucose, since the minor component is only about 1% of the total glucose in Experiment 3. The ratio of the two rates is then given by:

\[
\frac{v'}{v} = \frac{p'(S')}{p(S)}
\]

Combining Equations 4 and 5 results in the final expression:

\[
\frac{p'}{p} = \frac{V_{\text{max}}'(K_m)}{V_{\text{max}}(K'_m)}
\]

This ratio, which is a measure of the preferential utilization of the normal species when in the presence of an excess of the deuterated species, is seen to be independent of the ratio of the two components and determined solely by the kinetic constants of the enzyme for the two substrates.

If the results of Table III indicate that the dehydrogenase reaction and, indeed, Step 3 as a whole is not a major factor in determining the rate of the pathway in these tumor cells, the observation of the isotope discrimination effect in Experiment 3 may permit one to conclude whether the slow step precedes or follows Step 3. Consider the reaction sequence in Equation 6 in which the unprimed letters represent the nonradioactive species

\[
A' \xrightarrow{1} B' \xrightarrow{2} C' \xrightarrow{3} W' \xrightarrow{4} Y' \xrightarrow{5} Z' \xrightarrow{6} p'
\]

which arise from deuterated glucose (AD) and the primed letters represent the C\( ^{\text{H}} \)-tagged species arising from normal glucose (A'H). If AD and A'H are fed into this system as a mixture in which (A'H) \( \ll \) (AD), the ratio A'H/AD may be considered to represent the specific activity of the starting reactant, glucose. This ratio should be maintained in Steps 1 and 2 which do not involve deuterium, B' \( \ll \) BD = A'H/AD = C'H/CD. If Step 1 were slow, and therefore its product completely converted to \( p' \) and \( p \), there would be no discrimination, \( p'/p' = A'H/AD \). Rate limitation in Step 2 would present the same picture. If Step 3 were rate-determining and therefore could be considered irreversible since the following reactions, leading to product, must then be fast, the ratio \( W'/W \) would be greater than C'H/CD and, therefore, \( p'/p' = A'H/AD \). If Step 4 were slow, the products of Step 3 would have ample time to react with XD, XH having negligible by comparison, so that the substrate of Step 3 would tend to become a mixture of C'-D and C-D with continuing interconversions and there would be no discrimination possible. In the event that XD rapidly exchanged with the medium protons, the composition of the substrate would become a mixture of C'H and C—H, and again no discrimination would be found in the eventual product. If Step 5 were slow, the result would be the same unless Step 4 were fast and irreversible, thus assuring the virtual irreversibility of Step 3. In this case, discrimination would be maintained in the ratio \( W'/W = Y'/Y > A'H/AD \), and in the eventual product. Thus, if Step 3 is not rate-determining, isotope discrimination would occur only when succeeding steps are slow, although this is not a sufficient condition for discrimination unless Step 3 itself is irreversible. From this discussion it must follow that in the cells studied,
the rate-controlling steps of the hexose monophosphate pathway occur after the glucose-6-P dehydrogenase step.

A determination of lactate in the incubations of the leukemic cells with the three kinds of glucose indicated that the same amount was formed in each case, 2.62, 2.56, and 2.52 μmoles. This was to be expected if the isotopically different glucose solutions were otherwise identical, since there should be no cleavage of the 1-C-H bond of glucose by glycolysis. However, the radioactivity in the lactate of Experiment 3 was significantly less than in the others, 1.08 compared to 1.21 and 1.21 μmoles. The lower specific activity of the lactate is a reflection of the preferential oxidation of the C4-H species of glucose-6-P, thus resulting in a pool of glucose-6-P of lower specific activity. This must mean that the glucose-6-P which is in the hexose monophosphate pathway is either the same as that in the glycolytic pathway, or that if the pools are separate, they must be kinetically indistinguishable under these conditions (Step 2b, Fig. 1; rapid).

In Table IV are shown data for a similar experiment with human erythrocytes. In the absence of methylene blue, CO2 production is very low. It is evident that there is a strong isotope effect such that the oxidation of normal glucose occurs at 1.6 times the rate found with glucose-1-D. Thus the rate-determining step must be concerned with the cleavage of a C-H bond in which the H is derived either directly from the C-1 of glucose, Step 3, or from the reduced TPN formed in that step; that is, Step 5 could be rate-limiting if the TPNH in that step mixed with the TPN-D, and hence, its reoxidation rate could be affected. The result of Experiment 3 in comparison with Experiment 2 indicates isotope discrimination to the extent of 1.3. This would be consistent with rate limitation at Step 3, and actually would be required. It is consistent also with rate limitation at Step 5 in the particular case that Step 5b shows an isotope effect with TPN-D, and either Step 3 is irreversible or Step 4 is fast and irreversible. A test of whether Step 5 is rate-determining can be made through the use of glucose-3-D in experiments similar to these.

In the presence of methylene blue, the isotope effect becomes very small, indicating that at this higher rate, the dehydrogenase step does not limit the breakdown of glucose. The fact that the isotope discrimination effect does not occur, even after a longer incubation period is allowed, is consistent with this conclusion. As pointed out earlier, the absence of a discrimination effect does not allow one to conclude that rate limitation precedes or follows the C-H bond-breaking step.

Similar experiments with the leukemic cells in the presence of methylene blue gave similar results. Thus no isotope effect was observed and no enrichment of C14 in the CO2 was obtained with the intermolecularly labeled glucose, contrary to the result without methylene blue. In the presence of dye, the rate of appearance of radioactivity in CO2 was increased 2.16-fold to 3-fold.

**DISCUSSION**

Beyond its relation to the present study, the isotope effect observed in the kinetics of glucose-6-P dehydrogenase clearly points to the step in which hydrogen is transferred as the rate-limiting one under conditions of excess reactants. Thus, product dissociation and whatever conformational changes the protein must undergo subsequent to hydrogen transfer must be relatively rapid reactions.

In order to be assured that an isotope effect found in a particular enzyme reaction would be a promising tool for the study of metabolic control, it is clearly necessary to examine the kinetics of the reaction carefully and to be able to specify the conditions under which the effect can be expected. In the very complete study of Mahler and Douglas (5) on isotope effects in the alcohol dehydrogenase reaction, it was observed that the Km of normal ethanol is 2.3 times greater than that of ethanol-1-D, whereas the Vmax is only 1.8 times greater. This must mean that under conditions of very low ethanol concentration, the deuterated species would actually produce acetaldehyde more rapidly than a comparable amount of normal substrate, and would therefore impose a severe handicap in the use of this isotope effect in studies in vivo. The present study on glucose-6-P dehydrogenase covers the range of conditions in which either substrate or TPN is at high concentration and the other at very low concentration, but it does not give conclusive evidence that the isotope effect would occur when both components were present in exceedingly small amounts. The fact that the isotope effect appears to be completely independent of TPN concentration would strongly suggest that this would be the case.

It has been considered likely for both ascites tumor cells (20) and for mature erythrocytes (19) that the steps controlling glucose oxidation were those involved in TPNH oxidation. The basis for this is that C-1 oxidation to CO2 and oxygen uptake with glucose are greatly stimulated by the addition of methylene blue to the cell suspensions. The present studies do not contradict this conclusion. However, in the two tumors cells studied, the TPNH oxidation coupled with glucose-6-P dehydrogenase cannot be completely rate-controlling, although that coupled with 6-P-gluconate dehydrogenase may be.

In the case of the red cell for which methylene blue addition results in a stimulation of O2 uptake that is often 40-fold, the conclusion that TPNH oxidation is rate-limiting seems strong. The present data are consistent with this hypothesis, since limited TPNH oxidation could cause Step 3 or 5 to be rate-determining. It would seem, however, that effects resulting from the addition to whole cells of compounds such as dinitrophenol and methylene blue, which may give rise to far reaching changes in the state

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**TABLE IV**

Isotope effects on CO2 production in erythrocytes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Composition of glucose mixture</th>
<th>C4 in center well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A: No methylene blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C4H: C4-D</td>
</tr>
<tr>
<td>1</td>
<td>C4-H: C4-D, 1:25,000</td>
<td>1610</td>
</tr>
<tr>
<td>2</td>
<td>C4-D: C4-D, 1:25,000</td>
<td>964</td>
</tr>
<tr>
<td>3</td>
<td>C4-H: C4-H: C4-D, 1:250:25,000</td>
<td>1326</td>
</tr>
</tbody>
</table>

* Calculated from the c.p.m. in the center well divided by the specific activity of the glucose of the medium.
of the cell, may not be associated specifically with one particular metabolic step. It is perhaps the major advantage of the present approach to metabolic rate studies that isotope substitution may be expected to modify the steady state of the cell in minor and very specific ways.

SUMMARY

1. A comparison was made of the kinetics of the glucose 6-phosphate dehydrogenase reaction with the enzyme of yeast acting on normal substrate or substrate substituted with deuterium on C-1. The deuterated substrate was found to react more slowly at all concentrations and over a pH range of 6 to 9.5. At high levels of triphosphopyridine nucleotide (TPN), the Michaelis constant \( K_m \) of the normal substrate was 1.3 to 1.6 larger and the \( V_{max} \) was twice as large as that of the deuterated species. At high levels of glucose 6-phosphate, the \( K_m \) of TPN was independent of the species of substrate used, but the rate with the normal species was twice as rapid at all concentrations of TPN. Similar results were obtained with dehydrogenase preparations from *Escherichia coli* and human erythrocytes.

2. With ascites tumor cells, glucose-1-C\(^{14}\)-D was metabolized to C\(^{14}\)O\(_2\) at about the same rate as the C\(^{14}\)-H species of glucose. Thus, the reaction catalyzed by glucose 6-phosphate dehydrogenase must not be a rate-limiting step in the metabolic sequence. This must also be true for the step or steps responsible for the reoxidation of TPNH to TPN for this dehydrogenase step.

3. Similar studies with human red cells indicated a 60% more rapid rate with the normal species of glucose than with glucose-1-D. Thus, the controlling step must involve cleavage of a C—H bond, the H of which is derived from C-1 of glucose. In the presence of methylene blue, no isotope effect was observed.

4. With intermolecularly doubly labeled glucose, entirely different results were obtained. These were explained by an isotope discrimination effect in the glucose 6-phosphate dehydrogenase reaction. This effect was found to be of value in estimating the location of the rate-controlling step. Thus, in the case of the tumor cells, it could be concluded that the rate limiting step followed the glucose 6-phosphate dehydrogenase step.

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REFERENCES

The Use of Kinetic Isotope Effects in the Study of Metabolic Control: I.
DEGRADATION OF GLUCOSE-1-D BY THE HEXOSEMONOPHOSPHATE PATHWAY
Irwin A. Rose


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