Direct Hydrogen Transfer from Reduced Pyridine Nucleotides to Microsomal Cytochrome \(b_5\) Reductase*

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On the basis of several types of experiments (2-7), a mechanism was proposed (7) for the oxidation of reduced pyridine nucleotides by cytochrome \(b_5\) reductase (1.6.2.2, NADH-cytochrome \(b_5\) oxidoreductase). According to this scheme, initially an oxidized flavoprotein-reduced pyridine nucleotide complex is formed involving several nucleotide-protein interactions. This is followed by the formation of an extremely stable complex in which the flavin appears to be reduced and the pyridine nucleotide oxidized. It is characterized by the appearance of a distinct absorption band at 317 mp and may involve an interaction of the two nucleotides. In this mechanism it is assumed that, in the normal catalytic cycle, this complex is oxidized by cytochrome \(b_5\) or any one of several artificial electron acceptors and NADH easily displaces NAD from the oxidized enzyme to begin a second oxidative turn. The enzyme also catalyzes a slow hydrogen transfer between pyridine nucleotides (2). Experiments by Drysdale, Spiegel, and Strittmatter (5) have demonstrated that this hydrogen transfer is direct, stereospecific, and requires the flavin in the enzyme. Furthermore, the hydrogen originally on the NADH at the \(\alpha\) position in the nicotinamide ring does not exchange with protone of the medium at an appreciable rate when the stable enzyme-substrate complex is formed. These results suggested that the direct hydrogen transfer involves the displacement of the oxidized pyridine nucleotide from the reduced flavoprotein complex, followed by a reversal of the steps leading to the formation of the stable complex.

The present work was designed to test this hypothetical mechanism further. First, if the complex of reduced flavoprotein and oxidized pyridine nucleotide is an intermediate in the over-all enzymatic reaction, its rate of formation should be consistent with the kinetics of the over-all reaction. Second, it is important to show that in the formation of the complex there is a direct transfer of the hydrogen atom at the \(\alpha\) position of the reduced pyridine ring to an acceptor group on the enzyme. The experiments below, which utilized several marked deuterium rate effects and a rapid mixing technique, indicate that the cytochrome \(b_5\) reductase system does in fact meet these two additional criteria.

**EXPERIMENTAL PROCEDURE**

The general aerobic procedure for measuring the enzymatic activities of microsomal cytochrome \(b_5\) reductase have been described (3, 9). The kinetic experiments reported below were carried out at 25°, in microcells which contained 0.025 \(\mu\) mole of reduced pyridine nucleotide, 0.05 \(\mu\) mole of potassium ferricyanide (added at zero time), and 10\(^{-5}\) \(M\) enzyme in 0.20 ml of 0.1 \(M\) Tris-acetate-1 \(m\) EDTA buffer, pH 8.1. The oxidation of the reduced pyridine nucleotide was followed for 2 minutes by the decrease in the absorbancy at the wave length for maximal absorption for the particular nucleotide in the 300-400 mp region (10). A correction for the small contribution of potassium ferricyanide to these absorbancy changes was made.

Absorption spectra were measured with a Bausch and Lomb Spectronic-505 recording spectrophotometer which has a 5-A band width from 200 to 700 \(m\)u. The cell compartments were thermostated by circulating fluid from a constant temperature bath through brass blocks surrounding each cell holder. Samples of 0.30 ml could be used by employing the microcell system described previously (6). Time-rate measurements at a single wave length were made with the same instrument with the wave length gear drive disengaged and the braking tube removed from the amplifier circuit.

Temperatures from 0-25° for both the enzymatic assays and the absorption spectra were maintained within 0.2°. In each case the temperature was measured within the experimental cell with a thermocouple probe.

Cytochrome \(b_5\) reductase was prepared as described previously (3, 6, 9). The ratio of the absorbancy at 275 \(m\)p to that at 600 \(m\)p of these enzyme preparations was between 6.3 and 6.7, and the turnover number of the enzyme for NADH oxidation with potassium ferricyanide as electron acceptor was approximately 27,000 to 30,000 moles of substrate per mole of enzyme per minute at 25° in the standard assay system. The reductase was stored at -15° in 0.1 \(M\) Tris-acetate-1 \(m\) EDTA buffer, pH 8.1.

The NAD and NADH were obtained from the Sigma Chemical Company, and the AcPyAD\(^2\) and PyAlAD from Pabst Laboratories. The analogues of NAD are described (3, 9). The kinetic experiments reported below were carried out at 25°, in microcells which contained 0.025 \(\mu\) mole of reduced pyridine nucleotide, 0.05 \(\mu\) mole of potassium ferricyanide (added at zero time), and 10\(^{-5}\) \(M\) enzyme in 0.20 ml of 0.1 \(M\) Tris-acetate-1 \(m\) EDTA buffer, pH 8.1.

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anallogues of NAD with alcohol dehydrogenase systems. The NADH (α-D), AcPyADH (α-D), and PyAlADH (α-D) were prepared with alcohol dehydrogenase systems containing the oxidized nucleotide and C$_2$D$_2$OH, obtained from the New England Nuclear Corporation. A sample of NAD (4-D), kindly supplied by Dr. George R. Drysdale, was used to prepare NADH (β-D) by reduction in an alcohol dehydrogenase system. The AcPyAD (β-D) was prepared by reducing AcPyAD with C$_2$D$_2$OH and alcohol dehydrogenase, oxidizing the reduced nucleotide with a glutamic dehydrogenase system to yield AcPyAD (4-D), heating to denature the glutamic dehydrogenase, and, finally, reducing the nucleotide with a lactic dehydrogenase system to form AcPyADH (β-D).

Rapid Mixing Experiments—For the rapid mixing experiments, the apparatus shown in Fig. 1 was employed. The plunger (a) is Plexiglas and is shaped so that it fits loosely into the microcell. Three holes, approximately 2 mm in diameter, in the bottom of the plunger (b) allow rapid fluid movement into the inner part of the plunger during mixing. The material to be mixed with the cell contents is layered on the base of the plunger over the holes in a small volume (approximately 0.01 to 0.02 ml). The plunger, held firmly in a raised position by the spring in an adapted automatic pipetting syringe (d), is then placed in the top part of the microcell approximately 5 to 8 mm above the solution in the cell (g). Rapid mixing is achieved by depressing this plunger into the solution in the microcell at the proper speed. In the final depressed position (c), the plunger is completely outside the light path of the spectrophotometer, and the light passes through the solution largely contained within the Plexiglas plunger. In all of the experiments described below, the temperature of the solution in the microcell was maintained at 0° as described above. The plunger, along with the layer of solution to be mixed, was always brought to this temperature before mixing by equilibrating the system with the plunger in position directly over the solution in the microcell for 15 to 20 minutes.

To measure the changes in absorbancy that followed rapid mixing, a modified Beckman DU spectrophotometer with a photomultiplier attachment was employed. The microcell attachment made by Pyrocell Manufacturing Company was used in the thermostated cell compartment. The current from the photomultiplier tube was then led directly to a model 350-1800 stabilized Sanborn direct current preamplifier, completely bypassing the normal DU spectrophotometer amplifying circuit, and then to a Sanborn model 332 multiple channel recorder. Since the Sanborn d.c. amplifier has a response time of 1 millisecond, a wide range of zero suppression, and a high degree of stability, this system was ideal for these measurements. The recorder, which gives a linear response, has a 10-millisecond full scale response time so that it became the rate-limiting component of the recording system.

As a test of the response of the apparatus and the rate of mixing by this procedure, a 5- to 10-fold molar excess of hydrochloric acid in 0.01 ml was placed in the plunger and mixed with 0.25 ml of a solution containing phenolphthalein and potassium hydroxide. The reaction, measured by the decrease in transmittancy at 566 mμ, was complete in approximately 30 to 50 milliseconds. In the enzyme experiments described below, 0.01 ml of 7 mM reduced pyridine nucleotide in 0.1 M Tris-acetate-1 mM EDTA buffer, pH 8.1, was mixed with 0.24 ml of 0.01 to 0.06 mM cytochrome b$_1$ reductase in the same buffer. The changes in transmittancy at 460 mμ were recorded. For the results given below, points were taken from these original curves and plotted, in the more easily recognizable manner, as absorbancy changes versus time. A relatively high concentration of reductase was purposely used in these experiments to obtain large absorbancy changes. In this way, optical artifacts, arising from turbulence or small bubbles during mixing, or possibly from a slight degree of incomplete mixing, were minimized. Fortunately, a decrease in absorbancy occurred in the enzyme reaction; thus, the mixing artifacts, which generally yield absorbancy increases, were easily detected and poor experiments could be discarded.

RESULTS

Effects of Deuterium Substitutions on Rate of Pyridine Nucleotide Oxidation—The data of Table I show the effects of deuterium substitution at specific positions in several reduced pyridine nucleotides on their rates of oxidation in the cytochrome b$_1$ reductase system when potassium ferricyanide is the electron acceptor. It is clear that deuterium substitution in the α position of the reduced pyridine nucleotides markedly alters the rate of the over-all reaction. The decrease in the rate of oxidation varies from 3.7-fold for NADH to 8.7-fold for PyAlADH and 10.5-fold for AcPyADH. The same stereospecificity is observed here as in the case of direct hydrogen transfer from one pyridine nucleotide to another (5). Deuterium substitution in the β position yields no rate change. Such a specific rate effect of deuterium substitution on the α side of
is perhaps significant that the data for the three nucleotides shown and temperatures from 0-25° was used to obtain these data.

carbon atom 4 of the pyridine ring indicates that the rate-limiting step in the over-all reaction involves, at some step, the proton originally introduced in this position of the reduced pyridine nucleotide. Since the deuterium rate effects are so large, and the $K_m$ values for the nucleotides are not appreciably changed by the deuterium, the reductase system provides an extremely sensitive and specific method for assaying the deuterium content of the reduced pyridine nucleotide. Since the deuterium rate effects are so large, and the $K_m$ values for the nucleotides are not appreciably changed by the deuterium, the reductase system provides an extremely sensitive and specific method for assaying the deuterium content of the reduced pyridine nucleotide.

**Effect of Temperature on Rate of Oxidation**—To measure the rate of enzyme flavin reduction directly, as a measure of complex formation, the reaction had to be slowed down. The effect that lowering the temperature had on the rate of oxidation of several reduced pyridine nucleotides in the cytochrome $b_5$ reductase system is shown in Fig. 2 in the form of Arrhenius plots. There is approximately a 2.1- to 2.2-fold rate change per 10° for all three nucleotides over the temperature range, 0-25°. It is perhaps significant that the data for the three nucleotides yielded parallel straight lines. Although this method of plotting data is obviously quite insensitive, the results are consistent with the assumptions that, over this temperature range, the reaction and according to the proposed mechanism (7) it is—a plot of the log of flavin reduction versus time was made (Fig. 4). The data for the acetylpyridine analogues of NADH yield straight lines, and from these slopes first order rate constants were calculated. The value for this rate constant falls more than 7-fold when deuterium is substituted for hydrogen in the $\alpha$ position of carbon 4 of the pyridine ring. On the assumption that the actual step of flavin reduction is a first order reaction—and according to the proposed mechanism (7) it is—a plot of the log of flavin reduction versus time was made (Fig. 4). The data for the acetylpyridine analogues of NADH yield straight lines, and from these slopes first order rate constants were calculated. The value for this rate constant falls more than 7-fold when deuterium is substituted for hydrogen in the $\alpha$ position of carbon 4 of the pyridine ring. Clearly, then, in flavin reduction, a specific carbon to hydrogen bond in the pyridine ring is cleaved, and this reaction is the rate-limiting step in enzyme flavin reduction.

In Table II the data for various reduced pyridine nucleotides are summarized. The kinetic data for the enzymatic reactions were compared with the rate constants for flavin reduction, that is, formation of the stable substrate-flavoprotein complex. In the case of the pyridinealdehyde analogue, maximal flavin reduction is 66% in each case. Even at 0°, the NADH reactions with the reductase were too rapid to measure by the present method; they proceeded to completion during the mixing time. For the analogues of NADH, however, the data show, first of all, that the rate of flavin reduction is rapid. The rate of formation of the stable complex in which flavin is reduced is therefore consistent with the over-all kinetics. Second, the specific rate

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Turnover No.*</th>
<th>H rate/D rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>29,600</td>
<td></td>
</tr>
<tr>
<td>NADH (α-D)</td>
<td>8,900</td>
<td>3.00</td>
</tr>
<tr>
<td>NADH (β-D)</td>
<td>29,700</td>
<td>1.00</td>
</tr>
<tr>
<td>AcPyADH (α-D)</td>
<td>3,560</td>
<td></td>
</tr>
<tr>
<td>AcPyADH (β-D)</td>
<td>3,530</td>
<td>0.99</td>
</tr>
<tr>
<td>PyAlADH</td>
<td>414</td>
<td></td>
</tr>
<tr>
<td>PyAlADH (α-D)</td>
<td>48</td>
<td>8.7</td>
</tr>
</tbody>
</table>

* The standard assay system, at 25°, described in “Experimental Procedure,” was used. Results are expressed as moles per minute per mole of reductase.

\[ \text{Fig. 2. The effect of temperature on the rate of oxidation of pyridine nucleotides by cytochrome } b_5 \text{ reductase.} \]

\[ \text{Fig. 3. The rates of flavoprotein reduction by AcPyADH and AcADH (α-D). The rapid mixing system described in “Experimental Procedure” was employed.} \]

\[ \text{Vol. 237, No. 10} \]
systems can be obtained in which the enzyme flavin is partially rate-limiting for both flavin reduction and the over-all enzymatic reaction when potassium ferricyanide is the electron acceptor. This finding is interpreted as evidence that when potassium ferricyanide is the electron acceptor, the enzyme flavin is essentially completely oxidized. This would be expected when flavin reduction is the rate-limiting step in the over-all enzymatic reaction when potassium ferricyanide is the electron acceptor.

**Effect of Electron Acceptors on Level of Flavin Reduction during Enzyme Turnover**—The first two lines of Table III show that, during the enzymatic reaction catalyzed by the reductase, in which either PyAlADH or PyAlADH (α-D) is the electron donor and potassium ferricyanide the electron acceptor, the enzyme flavin is essentially completely oxidized. This would be expected when flavin reduction is the rate-limiting step in the over-all reaction. However, if an electron acceptor such as indigotetrasulfonate, which has a relatively high \( K_m \) value in the reductase system (9), is used at various concentrations, systems can be obtained in which the enzyme flavin is partially reduced (Lines 3 to 6) during oxidation of the NADH analogues. Thus, a step subsequent to flavin reduction, involving interaction of the reduced enzyme complex with the electron acceptor, becomes rate-limiting. Conditions were chosen for the last four experiments in Table III in such a way that for each analogue this steady state level of reduced flavin is sensitive to deuterium substitution in the \( \alpha \) position in the reduced pyridine ring. These data illustrate the fact that in the cytochrome bs reductase system the rate of enzyme flavin reduction may or may not be rate-limiting, depending upon the reactivities and concentrations of the electron acceptor and donor.

**DISCUSSION**

Because the spectral and chemical properties of the stable complexes formed from reduced pyridine nucleotides and cytochrome bs reductase (2-7) were most easily explained by assuming that the flavin was reduced and the pyridine nucleotide oxidized, a mechanism utilizing such a complex as an intermediate was postulated for the reactions catalyzed by this enzyme (4, 5, 7). The studies of Drysdale, Spiegel, and Strittmatter provide important evidence in support of this scheme. The results presented here document the dependence of the rate of flavin reduction on both the concentration of the electron acceptor and the rate of reduction of the flavin. These data illustrate the fact that in the cytochrome bs reductase system the rate of enzyme flavin reduction may or may not be rate-limiting, depending upon the reactivities and concentrations of the electron acceptor and donor.

**TABLE II**

Comparison of rates of enzymatic reaction with rates of enzyme flavin reduction by various reduced pyridine nucleotides

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Turnover No.</th>
<th>( k_1 ) for flavin reduction</th>
<th>Turnover No.</th>
<th>( k_2 ) for flavin reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>86.6</td>
<td>68.6</td>
<td>3.66</td>
<td>8.0</td>
</tr>
<tr>
<td>NADH (α-D)</td>
<td>17.7</td>
<td>8.0</td>
<td>3.06</td>
<td>8.0</td>
</tr>
<tr>
<td>NADH (β-D)</td>
<td>0.0</td>
<td>1.00</td>
<td>0.80</td>
<td>1.0</td>
</tr>
<tr>
<td>AcPyADH</td>
<td>8.33</td>
<td>8.0</td>
<td>10.4</td>
<td>7.34</td>
</tr>
<tr>
<td>AcPyADH (α-D)</td>
<td>8.33</td>
<td>8.0</td>
<td>8.0</td>
<td>0.95</td>
</tr>
<tr>
<td>PyAlADH</td>
<td>0.06</td>
<td>1.02</td>
<td>8.7</td>
<td>8.5</td>
</tr>
<tr>
<td>PyAlADH (α-D)</td>
<td>0.08</td>
<td>1.02</td>
<td>8.7</td>
<td>8.5</td>
</tr>
</tbody>
</table>

* The standard assay system, at 0°, as described in “Experimental Procedure” was used for these determinations. Results are expressed as moles per second per mole of reductase.

† The rapid mixing system described in “Experimental Procedure” was used, and the rate constant was determined in each case as described in Fig. 4.

**TABLE III**

Effect of electron acceptor on level of enzyme flavin reduction during reduced pyridine nucleotide oxidation by cytochrome bs reductase

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Electron acceptor</th>
<th>Concentration of electron acceptor (mM)</th>
<th>Enzyme flavin reduction (μM)</th>
<th>Deuterium rate effect on nucleotide oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyAlADH</td>
<td>Potassium ferricyanide</td>
<td>20</td>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>PyAlADH (α-D)</td>
<td>Potassium ferricyanide</td>
<td>20</td>
<td>0</td>
<td>8.5</td>
</tr>
<tr>
<td>AcPyADH</td>
<td>Indigotetrasulfonate</td>
<td>67</td>
<td>50-60</td>
<td>3-4</td>
</tr>
<tr>
<td>AcPyADH (α-D)</td>
<td>Indigotetrasulfonate</td>
<td>67</td>
<td>5-15</td>
<td>3-4</td>
</tr>
<tr>
<td>PyAlADHII</td>
<td>Indigotetrasulfonate</td>
<td>12</td>
<td>0-60</td>
<td>3-4</td>
</tr>
<tr>
<td>PyAlADH (α-D)</td>
<td>Indigotetrasulfonate</td>
<td>17</td>
<td>10-15</td>
<td>3-4</td>
</tr>
</tbody>
</table>

* The extent of flavin reduction was measured continuously at 460 nm with the Spectronic-505 recording spectrophotometer as described in “Experimental Procedure.” The microcells, at 0°, contained 0.04 μmole of reduced pyridine nucleotide, 0.004 μmole of cytochrome bs, and the electron acceptor as shown, in 0.30 ml of 0.1 M Tris-acetate-1 mM EDTA buffer, pH 8.1.

† The rate of reduced pyridine nucleotide oxidation was followed in a duplicate system as described in “Experimental Procedure.”

**Scheme 1**

The bar represents the protein.
matter (5), demonstrating that the reductase catalyzes a direct and flavin-dependent transfer of hydrogen from one pyridine nucleotide to another, suggested that a direct hydrogen transfer from NADH to the enzyme flavin occurs if this complex is an intermediate in the slow hydrogen transfer reaction as well as in the rapid reactions catalyzed by the reductase. The experiments described here provide evidence that this direct hydrogen transfer does occur.

On the basis of the rapid mixing studies, the formation of the stable reduced cytochrome b₅ reductase-NAD complex would appear to involve the reaction sequence shown in Scheme 1. Cleavage of the carbon-hydrogen bond involving the α-hydrogen atom of the reduced nicotinamide ring occurs during reduction of the flavin. Previous studies (5), demonstrating a direct and stereospecific hydrogen transfer between pyridine nucleotides and the absence of an appreciable exchange between the α-hydrogen of NADH with protons of the medium, indicate that the α-hydrogen is not lost to the medium during the formation of the stable complex. Therefore, the proton is still in the complex, and the most likely site for it is in the reduced isoalloxazine ring of the flavin, as Scheme 1 indicates. The kinetic studies on flavin reduction have demonstrated that the rate of this reaction is sufficiently rapid to allow the reduced flavoprotein-oxidized pyridine nucleotide complex to participate as an intermediate in the normal oxidative cycle or in slow hydrogen transfer involving analogues of NAD. If this postulated mechanism is correct, a further consequence of this reaction sequence should be emphasized (5). In view of the assumed case with which protons of the N–H or O–H groups of free reduced flavins should exchange with those of water, the retention of a specific proton in a reduced flavoprotein complex would seem to point to some interaction of the reduced flavin with the pyridine nucleotide or the protein, perhaps actually involving this specific proton.

This hypothetical mechanism should be tested further. The rapid mixing experiments only represent direct measurements of the cleavage of a carbon-hydrogen bond in reduced pyridine nucleotides. By isolating the stable complex formed from the enzyme and substrate containing deuterium in the α-position, it should be possible to demonstrate in an equally direct fashion that deuterium is retained in this complex, as previous exchange experiments suggest (5). In addition, modification of the reductase with flavin analogues substituted for FAD may yield preparations with characteristics that will provide a method for identifying the location of the deuterium in the reduced flavoprotein-pyridine nucleotide complex.

The data concerning the rate-limiting step during the over-all enzymatic reaction and the steady state levels of flavin reduction during such reactions with different electron acceptors have further implications for the reaction mechanism. Previous work (2–7) has already demonstrated that the reduced flavoprotein-oxidized pyridine nucleotide complexes and the complete enzyme system, during rapid turnover, are both insensitive to excess p-chloromercuribenzoate, a reagent that readily complexes an essential sulphhydril group in the isolated, oxidized reductase (3). It was suggested (7) that these data could be interpreted by assuming that the enzyme, during rapid turnover, was in the form of the stable reduced flavoprotein-oxidized pyridine nucleotide complex, in which the sulphhydryl group is protected from p-chloromercuribenzoate. Even though conditions can be chosen (Table III) to make the reaction of the electron acceptor with reduced enzyme the rate-limiting step, and thereby maintain the flavin largely in the reduced form during the reaction, the rate-limiting step in systems utilizing potassium ferricyanide as electron acceptor and any one of several reduced pyridine nucleotide analogues as electron donor is the rate of flavin reduction, and in this case the flavin is largely in the oxidized state during these reactions. Since these enzyme systems containing potassium ferricyanide are among those previously shown to be insensitive to p-chloromercuribenzoate, it must now be assumed that, in addition to the stable reduced flavoprotein-pyridine nucleotide complex, the mechanism for pyridine nucleotide oxidation may involve an additional intermediate in which the flavin is oxidized and the essential sulphhydril group is also protected from the mercurial. Experiments in progress on the characteristics of the PyAlADH reactions with the enzyme and the effect of p-chloromercuribenzoate on the flavoprotein in systems in which the steady state level of flavin reduction is varied may serve to test this possibility.

**SUMMARY**

1. The rate of oxidation of several reduced pyridine nucleotides in the cytochrome b₅ reductase system was shown to be decreased markedly by the substitution of deuterium in the α position of the reduced pyridine ring. This effect is stereo-specific: β-substitution has no effect on the reactions.

2. In rapid mixing experiments, at low temperatures, it was possible to show that, for two of these nucleotides, the deuterium rate effects on the over-all reaction were reflected almost quantitatively in the rate of flavoprotein reduction.

3. These data indicate that a specific carbon-hydrogen bond in the reduced pyridine ring is cleaved during the formation of the reduced flavoprotein-pyridine nucleotide complex, and that this reaction is the rate-limiting step in the over-all reaction when potassium ferricyanide is the electron acceptor.

4. A mechanism, involving direct hydrogen transfer from the pyridine nucleotide to the flavoprotein, was considered on the basis of these data and earlier work.

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Direct Hydrogen Transfer from Reduced Pyridine Nucleotides to Microsomal Cytochrome b5 Reductase
Philipp Strittmatter


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