Deficient Activity of DPNH-dependent Methemoglobin Diaphorase in Cord Blood Erythrocytes

By Jean D. Ross

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Young infants are unusually susceptible to the development of methemoglobinemia upon exposure to certain medications and toxic agents. Ingestion of well water containing large quantities of nitrate will cause methemoglobinemia in small babies while older members of the family remain unaffected.14 Bismuth subnitrate, benzocaine, resorcin or aniline dye, taken by mouth or absorbed rectally or percutaneously, will produce severe methemoglobinemia in infants in amounts which cause no abnormality in older individuals.5-13

Previous work indicated that umbilical cord blood erythrocytes exhibit a diminished ability to reduce methemoglobin in vitro in the presence of substrate capable of generating reduced diphosphopyridine nucleotide (DPNH). From this it was postulated that the defect was due either to a transient neonatal deficiency of DPHN-dependent methemoglobin reductase, or diaphorase, or to inadequate generation of the DPNH necessary for the action of this enzyme.14 The present study was undertaken to determine whether there is a deficiency of methemoglobin diaphorase† in cord blood erythrocytes. Persistence of such a defect through the first weeks or months of life could explain the increased tendency to acquired methemoglobinemia in this age group.

Materials and Methods

Five to 10 ml.s of blood were used for each determination. Cord blood, obtained from 54 full-term infants, was mixed with 2 ml. of ACD solution† and stored in a refrigerator at 6 C. until processed. Most cord bloods were prepared for assay within 12 hours of collection and none were stored for more than 3 days before use. Fifty-four control bloods from normal adults and patients without red blood cell abnormality were collected either in heparin or in 3 per cent sodium citrate and were used within 6 hours of venipuncture. Previous experiments demonstrated that there was no deterioration of enzyme activity in either cord or control bloods when stored refrigerated in ACD solution for as long

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This work was supported by U. S. P. H. S., National Heart Institute Grant #H-6556.

Submitted June 6, 1962; accepted for publication July 21, 1962.

*The following abbreviations will be used: DPNH = diphosphopyridine nucleotide, reduced form; TPNH = triphosphopyridine nucleotide, reduced form; GAPD = glyceraldehyde-3-phosphate dehydrogenase.

†"Methemoglobin diaphorase," rather than "methemoglobin reductase," will refer throughout this paper to the DPNH-dependent enzyme in order to distinguish it from the TPNH-dependent reductase.

†ACD = acid-citrate-dextrose solution, Baxter, containing in each 100 ml.: 0.44 Gm. citric acid (anhydrous), 1.32 Gm. sodium citrate, 1.47 Gm. dextrose.
as 2 weeks. Also, the enzyme assay was unaffected by the use of different anticoagulant solutions if the blood specimens were fresh.

Erythrocyte diaphorase activity was measured by a modification of the method of Scott. Certain changes in technic were our own, while others, including the more prolonged incubation with sodium nitrite, the method for determining the methemoglobin concentration of the hemolysate and the amount of methemoglobin added to the final test solution, were suggested by Scott.

The plasma and buffy coat were removed from the blood specimen after centrifugation. The erythrocytes were washed three times with cold phosphate buffered saline (nine volumes of 0.9 per cent sodium chloride and one volume of phosphate buffer, 0.1 M, pH 7.4) and the final supernatant solution discarded. To the erythrocytes was added an equal volume of a 1:1 mixture of one per cent sodium nitrite and phosphate saline. The mixture was shaken gently and allowed to stand at room temperature for 60 minutes. The cells were then washed five times with large volumes of cold phosphate saline to remove the sodium nitrite completely. 0.5 ml. of methemoglobinemic cells were added to 9.5 ml. of distilled water. After shaking, the mixture was centrifuged for 10 minutes at 3,000 rpm and the hemolysate separated from the insoluble matter. The methemoglobin concentration was measured as cyanomethemoglobin* by reading a 1:16 dilution of the clear hemolysate in a ferricyanide-cyanide solution (Aculute, Ortho Pharmaceuticals, in a 1:301 dilution) at 540 m\(_\lambda\) in a Beckman model D.U. spectrophotometer. Using this technic in our laboratory, the methemoglobin concentration was derived from the constant:

\[
E \text{ per cent} = 6.7.
\]

0.05 ml. of 1 M tris (hydroxynethyl) aminomethane buffer, pH 7.55 (Sigma), 0.1 ml. of 0.01 M sodium ethylenediamine tetraacetate (EDTA), and 0.05 ml. of 0.0012 M 2,6-dichlorobenzenoindophenol (Eastman) was added to each of two 10-mm. silica cuvets. To one, the “test,” was added hemolysate equivalent to 2.43 mg. of methemoglobin and water to a total volume in the cuvet of 2.80 ml. and to the other, the “blank,” was added 2.60 ml. of water. These reaction mixtures were allowed to stand for 30 minutes at room temperature to eliminate the effect of endogenous reducing substances. 0.2 ml. of 0.00088 M DPNH (sodium salt, Sigma) was then added to start the reaction and the optical density measured at 3-minute intervals at 600 m\(_\lambda\) in a Beckman model D.U. spectrophotometer at room temperature. Under these conditions the rate of change of optical density was almost linear in both the blank and the hemolysate. The activity of the diaphorase was expressed as change in optical density per minute (\(\triangle m\ O.D. \times 10^4\) per minute, abbreviated, for convenience, to \(\triangle O.D.\) per minute), after correction for the blank.

**RESULTS**

The results are summarized in table 1 and figure 1. The mean erythrocyte diaphorase level of the 54 control bloods was 45.3 \(\triangle O.D.\) per minute with a standard deviation of \(\pm 10.4\), while that of the 54 cord bloods was 26.4 \(\triangle O.D.\) per minute with a standard deviation of \(\pm 8.0\). The difference between the means was statistically highly significant (\(p < 0.001\)). The lowest value among the control bloods was 23 \(\triangle O.D.\) per minute and all but five were above 30 \(\triangle O.D.\) per minute. These results were very similar to those of Scott. In contrast, 19 cord bloods yielded diaphorase values less than 23 \(\triangle O.D.\) per minute and in 33, or more than half of those assayed, the enzymatic activity was below 30 \(\triangle O.D.\) per minute. The diaphorase values for four cord bloods were 15 \(\triangle O.D.\) per minute or less, representing a severe degree of enzyme deficiency.

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*On preliminary experiments, methemoglobin was found to comprise 100 per cent of the total heme pigment.
Table 1.—Summary of Values Obtained for Erythrocyte Diaphorase, Expressed as Δ O.D. per Minute

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>No. in Sample</th>
<th>Mean</th>
<th>S.D.</th>
<th>Significance of Differences between Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>54</td>
<td>45.3</td>
<td>± 10.4</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Cord bloods (all samples)</td>
<td>54</td>
<td>26.4</td>
<td>± 8.0</td>
<td></td>
</tr>
<tr>
<td>birth weight over 7 pounds</td>
<td>26</td>
<td>30.0</td>
<td>± 8.7</td>
<td>p = 0.025</td>
</tr>
<tr>
<td>birth weight under 7 pounds</td>
<td>10</td>
<td>23.0</td>
<td>± 5.5</td>
<td></td>
</tr>
</tbody>
</table>

Representative assay curves, including that of a severely deficient cord blood, are shown in figure 2.

In table 2, the values for 12 fresh cord bloods, processed within 12 hours of collection, are compared with those of eight 3-day-old cord bloods. The mean diaphorase value for the fresh specimens was 26 Δ O.D. per minute and was almost identical with the mean value for all the cord bloods tested. That of the older specimens was 32 Δ O.D. per minute. This would indicate that the longer average storage period of the cord bloods did not result in significant deterioration of the activity of the enzyme. Also, experiments in which blood specimens were stored in ACD solution in the refrigerator at 6 C. for as long
Fig. 2.—Representative curves of the determination of erythrocyte diaphorase assayed on the same day. The ordinate represents the optical density uncorrected for the blank. The abscissa represents the time in minutes. The curves are as follows: 1) *—* is the "blank" (i.e., no hemolysate present); 2) o—o, severely deficient cord blood; 3) x—x, moderately deficient cord blood; 4) *—*, control blood from normal adult.

as 2 weeks resulted in no decrease in enzyme activity (table 3). This stability of diaphorase in the presence of ACD solution has also been described by Scott.15 Thus, the lower enzyme activity of the cord blood erythrocytes as compared to that of the controls could not be ascribed to deterioration following storage.

Diaphorase activity was compared with birth weight in 36 infants. All were full term by dates, and weights ranged from 5 pounds 4 ounces to 9 pounds 5 ounces. There was no linear correlation between birth weight and diaphorase activity. However, when the infants were divided arbitrarily into two groups, those weighing over 7 pounds and those under 7 pounds, it appeared that larger babies generally had a higher mean diaphorase level than did the smaller infants. The mean erythrocyte diaphorase value of 26 infants with a birth weight of over 7 pounds was 30 A O.D. per minute (standard deviation ± 8.7) while that of the 10 babies under 7 pounds was 23 A O.D. per minute with a standard deviation of ± 5.5 (see table 1 and figure 3). The difference between the means was statistically significant (p = 0.025).

**DISCUSSION**

Methemoglobin is produced by oxidative catabolism of hemoglobin during the process of erythrocyte aging17 and is normally present in a constant amount
Table 2.—Comparison of Diaphorase Activity of Cord Bloods Processed within 12 Hours of Collection with Those Used after Storage for 3 Days. (All Were Collected in ACD Solution and Refrigerated at 6°C until Processed)

<table>
<thead>
<tr>
<th>Stored Less Than 12 Hours</th>
<th>Stored 3 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>31</td>
<td>19</td>
</tr>
<tr>
<td>32</td>
<td>28</td>
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<tr>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>41</td>
<td>18</td>
</tr>
<tr>
<td>47</td>
<td>32</td>
</tr>
<tr>
<td>48</td>
<td>27</td>
</tr>
</tbody>
</table>

Mean value 26 32

in the blood. The stable equilibrium between hemoglobin and methemoglobin is maintained by the action of one or more enzymatic reducing systems within the erythrocyte. Two such systems have been described, each with a different coenzyme specificity (fig. 4).

Beginning with Gibson’s studies of patients with congenital methemoglobinemia, considerable evidence has accumulated that the major portion of methemoglobin reduction is mediated via a DPNH-dependent enzyme. The importance of DPNH in the reduction of methemoglobin by the normal human erythrocyte has also been stressed by Gutman.

Recently Scott and Griffith described an enzyme, diaphorase, which is capable of reducing methemoglobin. Upon fractional salting out with ammonium sulfate and adsorption on Ca$_3$(PO$_4$)$_2$ gel, they were able to purify this enzyme 100-fold and determine that its activity in the presence of DPNH is 10 times that observed when TPNH is used as the coenzyme. Its presence can best be demonstrated by its ability to catalyze the reduction of a dye, 2,6-dichlorobenzenone indophenol. The rate of dye reduction by hemolysates at 23°C is 8000 times the reduction of methemoglobin at 37°C. Scott feels that the primary purpose of this DPNH-dependent enzyme is the reduction of methemoglobin since its absence leads to no side-effects other than methemoglobinemia and a compensatory polycythemia.

Studies of Alaskan Eskimos and Athabaskan Indians, among whom there is a relatively high incidence of recessively transmitted hereditary methemoglobinemia, indicated that erythrocytes of individuals with that disorder lack DPNH-dependent diaphorase and that heterozygous, nonmethemoglobinemic carriers of the condition have an intermediate deficiency of the enzyme. These findings support Gibson’s original postulate that the major portion of methemoglobin reduction depends upon the action of a DPNH-dependent enzyme.

A TPNH-dependent enzyme, capable of reducing methemoglobin, was first
Table 3.—Serial Determinations of Erythrocyte Diaphorase Activity Following Storage in a Refrigerator at 6°C. and Using ACD Solution as the Anticoagulant (Values Are Expressed as Δ O.D. per Minute)

<table>
<thead>
<tr>
<th>Time Stored (days)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cord bloods</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>21</td>
<td>16</td>
<td>20</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>30</td>
<td>24</td>
<td>29</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>50</td>
<td>48</td>
<td>49</td>
<td>55</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Adult bloods</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>44</td>
<td>43</td>
<td>47</td>
<td>54</td>
<td></td>
<td>52</td>
<td>33</td>
</tr>
<tr>
<td>(2)</td>
<td>45</td>
<td>45</td>
<td>59</td>
<td>47</td>
<td></td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>43</td>
<td>36</td>
<td>45</td>
<td>44</td>
<td></td>
<td>37</td>
<td>49</td>
</tr>
<tr>
<td>(4)</td>
<td>37</td>
<td>39</td>
<td>41</td>
<td>34</td>
<td></td>
<td>38</td>
<td>37</td>
</tr>
</tbody>
</table>

isolated in the erythrocytes by Warburg and his coworkers25,26 and later by Kiese.27 Gibson also demonstrated the presence of a methemoglobin reducing system that utilized TPNH but felt that, since it required methylene blue for its functioning, it was incompletely represented in the erythrocyte and of very minor importance in vivo.21 In 1957, Huennekens isolated and purified from erythrocytes a TPNH-dependent heme-containing enzyme which catalyzed the reduction of methemoglobin.28,29 This enzyme, like Gibson’s, required methylene blue as an electron acceptor for its function, although later work indicated that free ferric ion may operate in vivo in this capacity.30 The relative importance of TPNH-dependent methemoglobin reductase is not clear. However, with our present knowledge it seems most likely that it plays, at most, a minor role, while the DPNH-dependent enzyme is probably the more important for the reduction of methemoglobin in vivo.

The erythrocyte of the newborn infant is metabolically very different from that of the adult. The glycolytic rate appears to be increased31 and the activity of enzymes such as glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, aldolase, hexokinase, phosphoglyceric acid kinase, and pyruvate kinase appears to be elevated.32,33 The activity of certain other erythrocyte enzymes is decreased. Included among these are cholinesterase, glyoxalase, carbonic anhydrase and catalase.34 In addition, paper and starch block electrophoretic studies have indicated that TPNH-dependent methemoglobin reductase is decreased in cord bloods.36

Certain abnormalities of the intermediates of glycolysis have also been demonstrated. Levels of adenosine diphosphate and triphosphate are higher in the erythrocytes of both full-term and premature infants than in those of the adult.33 2,3-diphosphoglycerate is present in diminished quantities in the red blood cells of newborn rabbits and human infants, and formation of that substance is decreased, as indicated by the incorporation of P32 by cord blood erythrocytes.37 It was previously believed that this abnormality is due to deficient activity of the enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPD), which is responsible for the generation of DPNH during the dehydrogenation of glyceraldehyde. Recent studies, however, indicate that the
activity of GAPD is increased, rather than diminished, in the erythrocytes of newborn infants.\textsuperscript{33}

We have previously demonstrated that cord blood erythrocytes reduce significantly less methemoglobin in vitro than those of adults when incubated with either glucose or lactate\textsuperscript{14} and other authors have made similar observations.\textsuperscript{41,42} Our present studies indicate that the activity of the DPNH-dependent erythrocyte enzyme, methemoglobin diaphorase, is significantly less in cord bloods than in the blood of older subjects. In more than half of our infants this deficiency was of moderate degree and in the range of values seen in heterozygous carriers of hereditary methemoglobinemia, as described by Scott.\textsuperscript{15} The erythrocyte diaphorase activity of an occasional infant was extremely low and approached values seen in the homozygous, or clinically methemoglobinemic individual with the hereditary disorder. An equivalent degree of severity of the defect was detected in our previous studies.

Since the reduction of methemoglobin depends also upon the presence of DPNH, a diminished concentration of that coenzyme could result in decreased reduction of methemoglobin. DPNH is generated in vitro either by the action of lactic dehydrogenase, as is the case when lactate is present in excess in the incubating medium, or, when glucose is used, via the Emden-Meyerhof pathway and GAPD. The latter pathway is also the source of DPNH in vivo. However, it has been shown that GAPD is present in increased amounts in the erythrocyte of newborn infants,\textsuperscript{38} thus ruling out decreased activity of this enzyme as a cause of deficient reduction of methemoglobin.
Fig. 4.—The two major pathways of methemoglobin reductase in the normal human erythrocyte. A.) represents the TPNH-dependent pathway; B.) represents the DPNH-dependent pathway. Abbreviations are as follows: G6P = glucose-6-phosphate; TPN = triphosphopyridine nucleotide; TPNH = triphosphopyridine nucleotide, reduced form; MHb = methemoglobin; Hb = hemoglobin (ferrous form); DPN = diphosphopyridine nucleotide; DPNH = diphosphopyridine nucleotide, reduced form; G6PD = glucose-6-phosphate dehydrogenase.

Deficient amounts of intraerythrocytic DPNH have also been observed in another circumstance. Recently, Löhr and Waller demonstrated that the red blood cells of individuals with primaquine sensitivity, or hereditary deficiency of glucose-6-phosphate dehydrogenase, exhibit a decrease not only of TPNH but also of DPNH.43 One result of this defect is a decrease in the production of lactate, an abnormality which is accentuated in the presence of primaquine43 and acetylphenylhydrazine44 and which is reversible upon the addition of DPNH.44 It is thus possible that a deficiency of glucose-6-phosphate dehydrogenase may result in deficient reduction of methemoglobin not only via the TPNH-dependent pathway45 but also via the DPNH-dependent enzyme system.

We did not assay our cord bloods for activity of glucose-6-phosphate dehydrogenase either in our previous paper or in our present study. However, in the procedure for measuring the activity of methemoglobin diaphorase, DPNH is added to the assay system, thus compensating for any deficiency of that coenzyme in the erythrocyte. We feel it is unlikely, therefore, that decreased production of DPNH is an important cause of the diminished capacity of cord blood erythrocytes to reduce methemoglobin in the presence of lactate or glucose. On the other hand, we believe that our present studies indicate that the latter deficiency is most probably related to diminished activity of methemoglobin diaphorase.

Metabolic abnormalities of the erythrocytes of newborn infants sometimes
Persist for weeks or months after birth. Elevations of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and aldolase are found as late as the sixth month of life. TPNH-dependent methemoglobin reductase does not occur in normal amounts until the infant is 3 months old. Studies of an infant with acquired methemoglobinemia due to acetophenetidin revealed that the activity of erythrocyte diaphorase was still only low normal at the age of 10 weeks, perhaps indicating persistence of the neonatal enzyme deficiency. Six weeks later, diaphorase levels had risen to entirely normal levels in this patient. Moreover, experiments by Künzer revealed that deficient capacity to reduce methemoglobin may continue to be present for the first 3 months of life.

Transient deficiency of the activity of diaphorase in the neonatal period, although not sufficient under normal circumstances to result in clinically apparent methemoglobinemia, may be the cause for the slightly higher methemoglobin values found in normal full-term and premature newborn infants. Moreover, the suboptimal activity of diaphorase is probably inadequate for the reduction of any excess methemoglobin that may be formed and persistence of this enzymatic defect over the first few weeks of life would explain, at least in part, the increased susceptibility of small infants to the development of methemoglobinemia upon exposure to oxidizing agents.

It is also possible that rates of hemoglobin degradation and resultant methemoglobin formation are more rapid in the erythrocytes of newborn infants. Any such increase in the methemoglobin load that is presented to a reducing system that functions suboptimally would rapidly become apparent as clinical methemoglobinemia. Catalase, which protects hemoglobin from oxidative breakdown, is known to be diminished in activity in the neonatal period, and other protective enzymes, such as glutathione reductase or glutathione peroxidase, could perhaps also have reduced levels.

It has been suggested that fetal hemoglobin is oxidized more rapidly to methemoglobin than the hemoglobin of normal adults, although the evidence for this is contradictory. Studies by Künzer et al. indicated that the tendency of hemoglobin to be oxidized spontaneously to methemoglobin is twice as marked in fetal hemoglobin as in the adult form. On the other hand, experiments by Betke et al., using purified hemolysates, revealed no difference in the susceptibility to oxidation of these two forms of hemoglobin.

The metabolic peculiarities of the neonatal period are accentuated in the premature infant. Hyperbilirubinemia of the newborn, which is the result of deficient conjugation of bilirubin with glucuronide, occurs more frequently and is more extreme in premature infants. The elevation of erythrocyte glucose-6-phosphate dehydrogenase of young infants is greater in the premature baby. These findings suggest that more marked derangements of neonatal metabolism may reflect a greater degree of metabolic immaturity.

Abnormalities of methemoglobin metabolism are also more extreme in premature infants. The blood of premature infants generally has a higher methemoglobin concentration than does that of infants born at term, and they are more susceptible to the development of methemoglobinemia upon exposure.
aniline dye. Our studies were confined to infants who were full term by dates, although one infant weighed only 5 pounds 4 ounces and may be considered premature by weight. The lower mean diaphorase level and decreased incidence of normal values in smaller infants, as compared to those found in larger newborns, suggest that there is a greater tendency to metabolic immaturity in small infants than there is in large babies, even when there is no evidence of prematurity by gestational age.

**Summary**

Activity of DPNH-dependent methemoglobin diaphorase was measured in the erythrocytes obtained from 54 cord bloods and 54 adult controls. There was a significantly lower mean level for this enzyme in the cord bloods and the defect was occasionally severe. Mean diaphorase values of smaller infants was less than that of larger infants, suggesting a greater degree of metabolic immaturity in the former group, even in the absence of prematurity. This transient deficiency of methemoglobin diaphorase is probably a major cause for the increased susceptibility of young infants to the development of acquired methemoglobinemia.

**Summario in Interlingua**

Le activitate de DPNH-dependente diaphorase de methemoglobin esseva mesurate in le erythrocytos obtenite ab 54 specimens de sanguine de cordon e 54 specimens de controlo de sanguine adulte. Esseva constatate un significativemente plus basse nivello medie del mentionate enzyma in le sanguines de cordon. In certe casos le defecto esseva sever. Le valores medie pro le diaphorase in infants plus micre esseva plus basse que illos in infants plus grande. Isto suggere, pro le prime del duo gruppos, un plus alte grado de immaturitate metabolic, mesmo in le absentia de prematuritate. Iste transiente carentia de diaphorase de methemoglobin es probabilemente un causa major del intensificare susceptibilitate de juvène infants a contraher methemoglobinemia acquirite.

**Acknowledgments**

We wish to acknowledge the kind cooperation of Dr. John Garry of the obstetrical staff of the Beverly Hospital, Beverly, Mass., and the members of the obstetrical staff of St. Margaret's Hospital, Boston, who supplied the cord blood specimens used in this study.

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16. —: Personal communication.

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