Oxygen Equilibrium of Hemoglobins Containing Unnatural Hemes

EFFECT OF MODIFICATION OF HEME CARBOXYL GROUPS AND SIDE CHAINS AT POSITIONS 2 AND 4

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SUMMARY

Stable ferro-forms of hemoglobins containing various hemes such as dimethyl proto-, dimethyl meso-, etio-, meso-, hemato-, and deuteroheme were prepared by combination of human globin with hemes, followed by reduction with dithionite and purification with carboxymethyl Sephadex column. Sedimentation coefficients of these hemoglobins were all near to 4.0 S and chemical analysis showed that 4 moles of heme were bound per mole of hemoglobin.

Esterification of propionyl carboxyl groups of heme did not change the absorption spectra of hemoglobin; absorption spectra of dimethyl protohemoglobin were essentially identical with those of protohemoglobin, and absorption spectra of dimethyl mesohemoglobin with those of mesohemoglobin. Absorption spectra of etiohemoglobin, in which the propionic acid groups of mesoheme were substituted by ethyl groups, were similar to those of mesohemoglobin. Parameters of oxygen equilibrium, namely oxygen affinity, \( n \) in Hill's equation, and Bohr effect, of hemoglobin containing hemes which lack free carboxyl groups were the same as those for the corresponding hemoglobin with free propionic acid groups.

Oxygen affinities of meso-, deuter-, hemato-, and protohemoglobin, in which heme side chains at positions 2 and 4 are ethyl, hydrogen, hydroxyethyl, and vinyl groups, respectively, were in the ratio of 5:2:1:3:1, corresponding to the reverse order of the negative inductive effects of the groups at positions 2 and 4. These results suggest the effect of \( \pi \) electron of a porphyrin ring on the oxygen binding.

EXPERIMENTAL PROCEDURE

Globin—Globin was prepared from human hemoglobin by the method of Rossi-Fanelli, Antonini, and Caputo (11). The concentration of globin was determined by the Kjeldahl microtitration method with the nitrogen value of 2667 g per mole of monomeric human globin (average of \( \alpha \) and \( \beta \) chain) which was calculated from the amino acid composition.

Protohemin IX and Dimethyl Protohemin IX—Protohemin was crystallized from ox blood by the method of Willstätter (12) and recrystallized three times. Dimethyl protohemin was prepared from protohemin by esterification with methanolic HCl (13). Paper chromatography with 2,4-lutidine-water system (14) gave a single spot with an \( R_p \) value of 0.1. Pyridine hemochrome of dimethyl protoheme gave absorption maxima identical with those of protohemin, namely absorption maxima at 556, 526, and 418 nm, with a millimolar extinction coefficient of 33.4 

Mesohem IX and Dimethyl Mesohemin IX—Dimethyl mesoporphyrin was prepared by the method of Fischer and Orth (15) and iron was inserted by the method of Erdmann and Corwin (16). Mesohemin was obtained from dimethyl mesohemin.
by hydrolysis in methanol containing 1% KOH and a small amount of water at room temperature overnight. Pyridine hemochrome of both mesohemin and dimethyl mesohemin showed absorption maxima at 547 and 518 nm. The amounts of mesohemin and dimethyl mesohemin were measured after conversion to pyridine hemochrome with the extinction coefficient of 33.2 nm\(^{-1}\) cm\(^{-1}\) at 547 nm.

**Hemochrome of Both Mesohemin and Dimethyl Mesohemin** showed absorption maxima at 547 and 518 nm, and showed no shoulder at 556 nm, suggesting that no dehydration of hydroxyl group had occurred during preparation.

**Deuterohemin IX**—Deuterohemoglobin was prepared by the method of Fischer and Hummel (18). Absorption maxima of pyridine hemochrome were at 549 and 519 nm, and showed no shoulder at 556 nm, suggesting that no dehydration of hydroxyl group had occurred during preparation.

**Etiohemoglobin IX**—Crystalline etioporphyrin was prepared from protohemin by the method of Schum (19). Etiohemoglobin was prepared from etioporphyrin by introducing iron by the method of Zaleski (20). Peak wave lengths of absorption maxima in chloroform were (a) 640 nm, (b) 538 nm, (c) 510 nm, and (d) 380 nm; and in pyridine, (a) 630 nm (small), (b) 553 nm (shoulder), (c) 523 nm, and (d) 400 nm. These values were approximately the same as those given by Fischer and Orth (21). Chromatography with 2,4-lutidine-water showed a single spot, \(R_F\) 1.0. As ethiohemin did not dissolve in alkaline pyridine, the concentration was measured by iron determination (22).

**Paper Chromatography of Hemins**—Purity and identity of hemins were examined by a reversed phase paper chromatography, in addition to the measurements of light absorption of pyridine hemochrome described above. Paper chromatography was carried out with a mixture of water-1-propanol-pyridine (5.5:0.1:0.4 by volume) as the solvent system and silicone as the stationary phase, as described by Chu and Chu (23). Hemins were located as dark spots under ultraviolet light. Each of the hemins showed a single spot on the chromatogram. \(R_F\) values were 0.20 for protohemin, 0.33 for mesohemin, 0.60 for deuterohemoglobin, and 0.74 for hematohemoglobin. Ethiohemoglobin, dimethyl protohemin, and dimethyl mesohemin gave an \(R_F\) value of 0. These values compared well with those reported previously (23).

**Oxygen Equilibria**—The method of Asakura et al. (24) was used with minor modifications. Spectra between 500 and 700 nm were obtained at 22°C with a Hitachi EPR-2 recording spectrophotometer (Hitachi Electric Company, Ltd., Tokyo, Japan). Fractional saturation was calculated from extinctions at two wave lengths, \(\alpha\)-band and the minimum between \(\alpha\)- and \(\beta\)-band to cancel small base-line shifts. Good isobestic points evidenced the absence of oxidation or denaturation. Concentrations of hemoglobin were between 4 and \(6 \times 10^{-4}\) M in 0.1 M phosphate buffer.

**Methods and Instruments**—Absorption spectra were obtained at 22°C with a Cary model 14 recording spectrophotometer. Circular dichroism measurements were carried out at 22°C with a Jasco ORD/UV5 spectropolarimeter (Japan Spectroscopic Company, Ltd., Tokyo, Japan) with a circular dichroism attachment. Ultracentrifugal analyses were performed with a Spinco model B analytical ultracentrifuge.

**Results**

**Combination of Hemin Lacking Carboxylic Acid Groups with Globin**—Dimethyl proto-, dimethyl meso-, and ethiohemoglobin were dissolved in warm methanol to a concentration of 2 mg per ml. Acetone, as a solvent for heme, gave poor results. Ten milliliters of the hemin solution was added dropwise and gently stirred to a 20-ml globin solution (the concentration was 1.5 mM heme equivalent) in 0.1 M phosphate buffer, pH 7.0, which was chilled with ice and salt. The mixture was then dialyzed against 0.1 M phosphate buffer, pH 7.0, for 20 hours at 5°C to remove methanol. After dialysis, uncombined heme and denatured globin was removed by centrifugation. The ferrhemoglobin solution was evacuated in a Thunberg tube and reduced by mixing with a small amount of dithionite placed in the side arm. Dithionite showed little deleterious effect in the absence of oxygen. Immediately after the Thunberg tube was open, the hemoglobin solution was applied to a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 6.2, and eluted with the same buffer. The hemoglobin, which was oxygenated during the passage through the column, was absorbed on a carboxymethyl Sephadex C-50 column equilibrated with 0.01 M phosphate buffer, pH 6.2, and eluted by a linear gradient from 0.01 to 0.2 M phosphate buffer, pH 6.2. On these conditions, globin and methemoglobin stayed absorbed on the column. About 30% of the globin was recovered as hemoglobin.

Synthetic hemoglobins showed a single symmetrical peak in the ultracentrifuge. As shown in Table I, sedimentation coefficients for all the hemoglobins were near 4.0 S, suggesting the molecules consisted of tetramers. The ellipticity at 222 nm, which is considered to indicate the \(\alpha\) helix content of the protein, was about the same as that of natural hemoglobin as shown in Table I. The molar ratio of globin (as monomer) to heme to iron in dimethyl protohemoglobin was 1:0.99:1.06; that of globin to heme in dimethyl mesohemoglobin was 1:0.95; that of globin to iron in etiohemoglobin was 1:0.8; indicating that the globin combined equivalent amounts of heme to form hemoglobin.

After measurements of the properties of synthetic hemoglobins, hemins were extracted with acetone-HCl from dimethyl proto- and dimethyl mesohemoglobin, and paper chromatographed with 2,4-lutidine-water system. Each of the hemins gave a single spot with a \(R_F\) value of 1.0, and no hydrolysis of the propionic acid ester in the process of combination was confirmed.

**Table I**

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>(s_{20, w} \times 10^{-11})</th>
<th>(d) at 222 nm (\times 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protohemoglobin</td>
<td>4.27</td>
<td>2.65</td>
</tr>
<tr>
<td>Dimethylprotohemoglobin</td>
<td>4.28</td>
<td>2.57</td>
</tr>
<tr>
<td>Dimethylmesohemoglobin</td>
<td>3.95</td>
<td>2.45</td>
</tr>
<tr>
<td>Etiohemoglobin</td>
<td>4.20</td>
<td>2.79</td>
</tr>
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</table>
**Table II**

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Oxygenated Hb</th>
<th>Deoxygenated Hb</th>
<th>Methemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \lambda )</td>
<td>( \varepsilon )</td>
<td>( \lambda )</td>
</tr>
<tr>
<td>Protohemoglobin</td>
<td>414 135 135</td>
<td>430 140 154</td>
<td>406 151</td>
</tr>
<tr>
<td>Dimethylproto-</td>
<td>541 14.8 15</td>
<td>555 13.4 500</td>
<td>9.5 500</td>
</tr>
<tr>
<td>hemoglobin</td>
<td>577 15.7 630</td>
<td>555 13.6 650</td>
<td>3.8 650</td>
</tr>
<tr>
<td>Dimethylmeso-</td>
<td>414 133 430</td>
<td>140 140 405</td>
<td>151 151</td>
</tr>
<tr>
<td>hemoglobin</td>
<td>540 14.6 135</td>
<td>555 13.6 500</td>
<td>9.4 500</td>
</tr>
<tr>
<td>Mesohemoglobin</td>
<td>577 15.3 630</td>
<td>13.9 620 7.3</td>
<td>3.9 620</td>
</tr>
<tr>
<td>Dimethylmeso-</td>
<td>404 138 421</td>
<td>139 421 420</td>
<td>3.8 420</td>
</tr>
<tr>
<td>hemoglobin</td>
<td>534 13.8 546</td>
<td>13.3 545 140</td>
<td>13.7 620</td>
</tr>
<tr>
<td>Etiohemoglobin</td>
<td>567 12.4 568</td>
<td>13.0 568 181</td>
<td>13.0 620</td>
</tr>
<tr>
<td>Hematohemoglobin</td>
<td>401 138 420</td>
<td>134 420 395</td>
<td>181 395</td>
</tr>
<tr>
<td></td>
<td>537 13.3 540</td>
<td>12.0 540 490</td>
<td>13.2 620</td>
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<tr>
<td></td>
<td>506 11.5 506</td>
<td>405 506 620</td>
<td>6.5 620</td>
</tr>
<tr>
<td>Deuterohemoglobin</td>
<td>402 115 420</td>
<td>119 420 402</td>
<td>119 620</td>
</tr>
<tr>
<td></td>
<td>531 13.5 542</td>
<td>12.9 542 531</td>
<td>119 531</td>
</tr>
</tbody>
</table>

Fig. 1. Absorption spectra of synthesized hemoglobins. Oxygenated and deoxygenated hemoglobins were in 0.1 M phosphate buffer, pH 7.0, and methemoglobins were in 0.1 M phosphate buffer, pH 6.5, at 22°. \( \varepsilon \), absorbance.

**Preparation of Hemoglobin Containing Heme Modified at Positions 2 and 6—** Concentrations of globin in 0.1 M phosphate buffer, pH 6.5, were calculated from titration of globin with heme according to the method of Rossi-Fanelli et al. (11). Hemin equivalent to globin was weighed, dissolved in a small amount of 0.1 M KOH, and mixed with the globin solution. The mixture was reduced in a Thunberg tube and subjected to a Sephadex G-25 column and a carboxymethyl Sephadex C-50 column chromatography in the same way as described for heme lacking carboxyl groups. To reduce ferrihematohemoglobin, about three times as much dithionite was required as that to reduce other ferrihemoglobins. The yield of oxygenated hematohemoglobin was about 20% because of the denaturation during reduction with the large amount of dithionite. Oxygenated forms of meso-, deuto-, and hematohemoglobins were stable and no oxidation of heme was observed within a week when stored at 5° at pH between 7 and 9.

Identity and the amount of heme contained in the reconstituted hemoglobins were determined after conversion to their pyridine hemochromes. Peak wave lengths of pyridine hemochrome were identical with those of the heme considered to be present. The amount of heme was determined from the intensity of \( \alpha \)-band of the pyridine hemochrome.

**Absorption Spectra of Synthetic Hemoglobin—** Absorption spectra of dimethyl protohemoglobin from 240 to 680 nm are shown in Fig. 1. No difference between the spectra of natural hemoglobin and those of synthetic dimethyl protohemoglobin was observed in both oxygenated and deoxygenated forms (Table II). The \( \alpha \)- and \( \beta \)-band for methemoglobin form of dimethyl protohemoglobin were a little more evident than those for natural methemoglobin. Absorption spectra of dimethyl mesohemoglobin and mesohemoglobin are shown in Fig. 1. Differences between the absorption spectra of dimethyl mesohemoglobin and mesohemoglobin are shown in Fig. 1.
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FIG. 2. Oxygen dissociation curves of dimethyl protohemoglobin and natural human hemoglobin. Conditions were as described in the text. ▲—▲, ⋄—⋄, ■—■, natural hemoglobin; Δ—Δ, ○—○, □—□, dimethyl protohemoglobin.

Wave lengths and extinction coefficients of absorption maxima for other synthetic hemoglobins are summarized in Table II. Oxygen Equilibrium of Synthesized Hemoglobin—Oxygen dissociation curves for dimethyl protohemoglobin and natural hemoglobin at different pH values are compared in Fig. 2. In the figure, the fractional saturation of hemoglobin with oxygen (Y) and the partial pressure of oxygen (p) were plotted logarithmically according to a modified form of Hill's empirical equation, \( \log Y/(1 - Y) = \log K + n \log p \). From the figure, \( p_5 \), the partial oxygen pressure at \( Y = 0.5 \), and \( n \), the heme-heme interaction constant, were obtained. The effect of pH on oxygen affinity is shown in Fig. 6. Dimethyl protohemoglobin and natural hemoglobin showed same values of \( p_5 \) and same extent of pH dependence of \( p_5 \). Values of \( n \) for natural hemoglobin were between 2.6 and 3.0 and those for dimethyl protohemoglobin were between 2.4 and 2.7. Small differences in \( n \) are within the limits of error of the method, so the effect of methylation of protoheme propionyl carboxyl groups on oxygen equilibrium is small or absent. In Fig. 3, oxygen equilibria of dimethyl mesohemoglobin and mesohemoglobin are compared. Again, the oxygen dissociation curves of dimethyl mesohemoglobin are similar to those of mesohemoglobin. The \( n \) values for dimethyl mesohemoglobin were between 1.6 and 2.0, and those for mesohemoglobin were about 1.6 in the pH range studied. Oxygen dissociation curves for etiohemoglobin are shown in Fig. 4. Although the \( n \) value of 2 between pH 9.0 and 6.7 was a little higher than that of 1.6 for mesohemoglobin, oxygen affinity and pH dependence of oxygen affinity of etiohemoglobin were the same as those of mesohemoglobin and dimethyl mesohemoglobin, as shown in Fig. 6. From these results, the propionyl carboxyl groups of heme in hemoglobin are considered to have very little effect on the oxygen equilibrium of hemoglobin. Oxygen dissociation curves for hematohemoglobin and deuterohemoglobin are shown in Fig. 5. The oxygen affinity of deuterohemoglobin is higher than that of hematohemoglobin.

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FIG. 3. Oxygen dissociation curves of dimethyl mesohemoglobin and mesohemoglobin. ▲—▲, ⋄—⋄, ■—■, dimethyl mesohemoglobin; X—X, Δ—Δ, ○—○, □—□, mesohemoglobin.

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FIG. 4. Oxygen dissociation curves of etiohemoglobin. Conditions were as described in the text.
The n value for deuterohemoglobin showed a small pH dependence, and n changed from 2.50 at pH 8.3 and to 1.65 at pH 6.6. Hematohemoglobin showed a n value of 2.0 in the pH range studied. The results obtained from hematohemoglobin are different from those reported by Antonini et al. (10) that the hematohemoglobin showed no heme-heme interaction and had higher affinity of oxygen than deuterohemoglobin and protohemoglobin.

Effects of pH on the oxygen affinity of various hemoglobins are compared in Fig. 6, plotting log p\textsubscript{4} against pH. All hemoglobins showed Bohr effect and the magnitudes, (d log p\textsubscript{4})/ (d pH), in Fig. 6 are about 0.45 for proto-, dimethyl protohemato-, and deuterohemoglobin, and are about 0.32 for meso-, dimethyl meso-, and etiohemoglobin. Oxygen affinity of meso-, deutero-, hemato-, and protohemoglobin, in which heme side chains at positions 2 and 4 are ethyl, hydrogen, hydroxyethyl, and vinyl groups, respectively, was in the ratio of 5:2:1:3:1, corresponding to the reverse order of the negative inductive effect of the groups at positions 2 and 4.

**DISCUSSION**

Detailed analysis of the effect of chemical modification of heme on oxygen equilibrium requires the preparation of stable reduced hemoglobin to which oxygen binds. The globin prepared from human hemoglobin by the method of Rossi-Fanelli et al. (11) could be coupled with protoheme to produce a hemo-globin having the same absorption spectrum, sedimentation coefficient, and other properties as natural human methemoglobin. One of the difficulties in obtaining oxygenated and deoxygenated hemoglobin was the reduction of methemoglobin. O'Hagan (2) reported that numerous attempts failed to obtain in oxygenated hemoglobin that lacked free carboxyl groups α- and β-peak at the same relative height as in normal hemoglobin. Antonini et al. (10) reported that they could not obtain stable ferro-form of hematohemoglobin and chlorohemoglobin. Our method of reducing hemoglobin with dithionite or borohydride in the absence of oxygen, followed by quick removal of the reductant by a Sephadex column, gave the stable ferrhemoglobin in good yields. Another difficulty in obtaining the hemoglobin that lacked heme carboxyl groups was the use of organic solvent for the hemin which was insoluble in aqueous solution. The satisfactory recovery of hemoglobin was attained by the use of concentrated globin solution and hemins dissolved in methanol.

Dimethyl protohemoglobin gave the same absorption spectra, oxygen dissociation curves, and sedimentation coefficient as those of natural hemoglobin. A difference was observed only in the chromatography of the hemins extracted from the hemoglobins. Similar relationships were observed between the properties of dimethyl mesohemoglobin, etiohemoglobin, and mesohemoglobin. Previous reports (2, 3) suggested that propionyl groups might be necessary for the stability of hemo-globin linkage and protein structure, although not essential for oxygenation. These suggestions were deduced from the instability and poor spectral peaks of the hemoglobin that lacked carboxyl groups (2) and from the results comparing the affinity of ethyl isocyanide for protohemoglobin and etiohemoglobin, which have different groups at positions 2 and 4 in addition to substitutions of carboxyl groups by ethyl groups (3). Perutz et al. (25) showed by x-ray analysis that propionyl carboxyl groups made polar contacts with globin, and suggested that all polar contacts were in water so that their contribution to the binding energy should be small. Our results with dimethyl protohemoglobin, and etiohemoglobin suggested that propionyl carboxyl groups have small or no effect on oxygen equilibrium of hemoglobin. Kinetic constants were not measured, but considering the fast rates of combination and dissociation of oxygen with hemoglobin, propionyl carboxyl groups may not be essential for the physiological function of hemoglobin. The carboxyl groups of heme may play some role in the bio-

![Fig. 5. Oxygen dissociation curves of deuterohemoglobin and hemato-hemoglobin.](image)

![Fig. 6. Effect of pH on the oxygen affinity of synthetic hemoglobins and natural human hemoglobin. Conditions were as described in the text. X, natural hemoglobin; Δ, dimethyl protohemoglobin; □, hemato(hemoglobin); O, deuterohemoglobin; ●, mesohemoglobin; ■, dimethyl mesohemoglobin; Δ, etiohemoglobin.](image)
synthesis of hemoprotein, for instance leading heme a slight solubility in aqueous media.

The effect of substituents at positions 2 and 4 on ligand binding has been studied by Caughey (26) and the importance of electron withdrawal by the substituents was discussed thoroughly. The rates of formation of hemoglobins from globin and hemes modified at positions 2 and 4 have been reported (27). The ratio of the reaction were in the order of proto-, meso-, and deuterohemins. The order of vinyl, ethyl, and hydrogen, is consistent with "electron-donating" power, and the effectiveness of donor-acceptor interactions between heme and globin is suggested. The slowest binding rate of hemohemate was correlated with the hydrophilic nature of its hydroxyl groups, which were unsuitable to be buried in the hydrophobic interior of globin. Our results on oxygen equilibria of hemoglobins containing unnatural hemes, however, showed that the oxygen affinity increased in the order of proto-, hemato-, deutero-, and mesohemoglobin in correspondence to the decrease in negative inductive effects of the groups at positions 2 and 4 of the hemes. When hemoglobin is formed, the oxygen affinity seems to be determined differently from the rate of the combination of heme and globin. The possibility that the altered oxygen affinity of synthesized hemoglobin is caused by the alterations of globin was denied by Rossi Fanelli et al. (9) from the results that the protohemoglobin reconstituted from apomesohemoglobin showed the same oxygen affinity as that of natural hemoglobin.

Antonini et al. (10) reported that the oxygen affinity of hemoglobins containing hemes modified at positions 2 and 4 were in the increasing order of proto-, deutero-, hemato-, and mesohemoglobin. In their report, hemohemoglobin showed no heme-heme interaction and soon became turbid. The discrepancy about hemohemoglobin from our results might be caused by the unstability of their hemoglobin, which may be denatured during oxygen equilibrium determination and may have shown apparently high oxygen affinity and lack of heme-heme interaction. They ascribed differences in oxygen affinity among the unnatural hemoglobins to the varying hydrophobic character of the side chains, but the order of hydrophobic nature seems not exactly in accord with their results. As the order of oxygen affinity corresponds to the order of electron-withdrawing effect of side chains, the effect of side chains seems to be more direct than through the macromolecular environment. The results are consistent with the explanation of oxygen binding of hemoglobin proposed by Caughey (26) and by Falk, Philips, and Magnusson (28), that the withdrawal of π electron of porphyrin ring increases the strength of the iron to porphyrin nitrogen π-bonds at the expense of the iron to oxygen π-bonds.

The results of kinetic work on reconstituted hemoglobins by Antonini and Gibson (7) showed that the largest differences between proto-, meso-, and deuterohemoglobin were in the rate of dissociation of the first oxygen molecule from saturated hemoglobin. The oxygen affinity calculated from the kinetic constants were in the decreasing order of meso-, deutero-, and protohemoglobin, which is compatible with the results obtained from equilibrium measurements. However, from kinetic work with carbon monoxide, ethyl isocyanide, and various apohemoproteins, Brunori et al. (6) showed that effects arising from interaction of the protein with the heme side chains at positions 2 and 4 were very different in the various cases. For the present, the explanation of the effects of modification of side chains by the negative inductive effect can account for the oxygen equilibrium of hemoglobin, and is put forward as a basis for further investigation.

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REFERENCES

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