Glucocorticoids Directly Affect Spectrophotometry of Bilirubin in Amniotic Fluid

A. Milwidsky,1 S. Yagel,1 M. Chaouat,2 and M. Mayer3,4

Dexamethasone or prednisolone, added in vitro to bilirubin-containing amniotic fluid, produces a time-dependent decrease in the 450-nm absorbance of the pigment. Neither the chemical determination of bilirubin in amniotic fluid nor the lecithin/sphingomyelin ratio as determined by thin-layer chromatography is affected by these glucocorticoids. The effect probably is not a result of displacement of bilirubin from its binding sites on albumin, because the absorbance of a solution of crystalline bilirubin at 450 nm is unaffected by added bovine serum albumin. Light scattering of amniotic fluid increases slightly when dexamethasone is added, whether or not low concentrations of bilirubin (<1.6 \( \mu \)mol/L) are present. Thus the effect on absorbance evidently is not ascribable to supersaturation and formation of a colloidal sol of bilirubin particles. This direct interference of glucocorticoids with the spectrophotometry of bilirubin in amniotic fluid prompts cautious interpretation of such data as an index to the severity of hemolytic disease of the fetus, specifically in cases of Rhesus isoimmunization that are being treated with glucocorticoids.

Additional Keyphrases: fetal status - L/S ratio - drug-induced error - fetal hemolytic disease

It is widely accepted that the absorbance peak of amniotic fluid at 450 nm represents unconjugated bilirubin (1). In uncomplicated pregnancies, as the normal gestation progresses and the fetal liver matures, the peak of bilirubin absorbance at 450 nm becomes smaller, disappearing completely from samples collected at the 36th week (2). Thus, the absorbance of bilirubin gives a good forecast of fetal maturity at any given period of gestation (1, 2).

In pregnancies complicated by Rhesus-factor incompatibility the increase in amniotic fluid bilirubin content was found to correlate with the severity of the hemolytic disease of the newborn (3). Measurement of amniotic fluid bilirubin content by the height of the light-absorption peak at 450 nm is therefore an established procedure for the assessment of the Rhesus isoimmunized pregnancy, and the criteria established by Liley for the accepted range of absorbance values as a function of time of gestation commonly serve as the prime diagnostic index for intraterine transfusion or cessation of pregnancy (3, 4). Consequently, amniocentesis for examination of the absorbance at 450 nm, together with ultrasonic examination of the fetus, offers the current basis on which important clinical decisions in cases of Rhesus incompatibility are made (5, 6).

A common therapeutic approach in Rhesus isoimmunized pregnancies is to administer glucocorticoids, which are expected to reduce the antibody-mediated process of fetal hemolysis and to accelerate fetal lung maturity (7). Several reports have indeed shown that administration of glucocorticoids to Rhesus-sensitized pregnant women produces a decline in the absorbance value of the amniotic fluid at 450 nm (7–9). However, an abrupt increase in absorbance was noted immediately after the steroid injections were stopped (7), and the glucocorticoids produced only minimal, if any, changes in the lecithin/sphingomyelin ratio of amniotic fluid, suggesting that neither the hemolytic disease nor fetal maturity was actually improved by therapy with steroid (8).

In view of the possibility that a steroid-induced decrease in absorbance at 450 nm does not necessarily reflect a true biological effect on bilirubin content, we investigated the direct in vitro effect of glucocorticoids on the spectrophotometric determination of bilirubin in amniotic fluid. Our results show that steroids directly decrease the absorbance peak at 450 nm in vitro, suggesting that direct spectrophotometry of bilirubin is inadequate for estimation of the effect of steroid treatment on the fetal hemolytic disease.

Materials and Methods

Samples of amniotic fluid from normal and Rhesus-sensitized pregnant women were obtained during amniocentesis, performed under sterile conditions for various obstetrical indications. The fluids were centrifuged at 5000 \( \times \) g for 30 min and passed sequentially through Whatman filters no. 2 and 43. The samples were protected from light throughout the procedure and during the different experiments. When indicated, bilirubin standard (Biotrol-bilirubin C et T; Laboratoires Biotrol, Paris, France) was added to the amniotic fluids to yield a final concentration of 3.4 \( \mu \)mol/L. Where applicable, dexamethasone disodium phosphate (Dexacort; Ikapharm, Jerusalem, Israel) or prednisolone sodium tetrahydrophilate (Ultracort H; Ciba-Geigy, Basle, Switzerland) were added to give final concentrations ranging from 1 to 100 \( \mu \)mol/L. Controls received the same volumes of the vehicle alone. Bilirubin was measured in amniotic fluids, either immediately or after incubation with the glucocorticoids at 37 °C in a light-protected incubator for the times indicated below in the footnotes to the appropriate tables.

For the direct spectrophotometry of bilirubin we used a Gilford 250 scanning spectrophotometer, scanning the wavelength range from 340 to 550 nm. Absorption at 450 nm was determined in a cuvet with a 1-cm lightpath, distilled water serving as the blank. For light-scattering measurements we used a Perkin-Elmer Model 204 fluorescence spectrophotometer equipped with a xenon power supply, and quartz cuvets with a 10-mm lightpath. Excitation was at 294 nm and emission was measured at 297 nm. The measurements were performed at ambient temperature either immediately after the bilirubin and dexamethasone were added (time 0) or 5 min or 24 h thereafter. Chemical determination of bilirubin by the diazo method was according to Malloy and Evelyn (10) as detailed by Kapitulnik et al. (11). The lecithin/sphingomyelin (L/S) ratio was estimated by the Helena (Helena Laboratories, Beaumont, TX).

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Fetal Maturity Test, which is based on thin-layer chromatography of the phospholipids according to Gluck et al. (12).

Results

The absorbance of amniotic fluid at 450 nm is known to represent unconjugated bilirubin. Table 1 shows absorbance values at 450 nm of amniotic fluids from three pregnancies with fetal hemolytic disease of different severity, as is evident from the appreciable absorbance values. Addition of 100 μmol of dexamethasone per liter produced a marked decrease in the absorption values for the three fluids, suggesting a direct effect of the glucocorticoid on the spectrophotometric determination of amniotic fluid bilirubin (Table 1).

Figure 1 shows the results of spectrophotometric scanning of a typical sample of amniotic fluid, obtained at the 38th week of a normal pregnancy, at wavelengths ranging from 350 to 550 nm. While the normal fluid displays a gradual decrease in absorbance throughout this range, addition of exogenous bilirubin produces the characteristic peak at about 450 nm. The subsequent addition of 10 μmol of dexamethasone per liter to the bilirubin-containing amniotic fluid produced a marked decrease in absorbance near 450 nm without a shift in the wavelength of maximal absorbance. In this representative experiment dexamethasone was incubated with the fluid for 24 h at 37 °C. Thus, the characteristic absorbance of bilirubin in amniotic fluid is markedly decreased by the in vitro addition of the steroid. Figure 1 also shows that addition of dexamethasone to a bilirubin-free amniotic fluid had no effect on the absorbance pattern in the wavelength range tested.

Table 2 further shows the effect of two potent glucocorticoids on the height of the bilirubin peak. In this set of experiments the absorbance at 450 nm of 20 amniotic fluid samples was assayed spectrophotometrically 0, 1, 3, and 24 h after glucocorticoids were added. The presence of 10 μmol of dexamethasone or prednisolone per liter caused a decrease in the measured absorbance, an effect that increases with the duration of incubation with the amniotic fluid, being maximal and statistically significant at 24 h of incubation. In other experiments (not shown) the magnitude of the decrease in absorbance was seen to be independent of the concentration of dexamethasone or prednisolone in the range of 1 to 100 μmol/L, while a 0.1 μmol/L concentration of the steroids produced only a small decrease in absorbance. At 0.01 μmol/L, dexamethasone or prednisolone had no effect on the light absorption.

The diminished absorbance at 450 nm in the presence of high dexamethasone or prednisolone concentrations does not reflect a true decrease in bilirubin: incubation of the amniotic fluids with these glucocorticoids for 24 h did not affect the apparent concentration of bilirubin as determined by the chemical (diazo) method (Table 3), nor did it affect the shape of the absorption spectrum of the diazo products.

In other experiments (results not shown) we noted that 10 μmol of dexamethasone per liter had no effect on the electrophoretic determination of the L/S ratio of amniotic bilirubin.

Table 1. Effect of Dexamethasone on Absorbance of Three Amniotic Fluids at 450 nm

<table>
<thead>
<tr>
<th>Control</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.220</td>
<td>0.060</td>
</tr>
<tr>
<td>0.099</td>
<td>0.050</td>
</tr>
<tr>
<td>0.520</td>
<td>0.420</td>
</tr>
</tbody>
</table>

Absorbance was measured at 450 nm in the presence or absence of added dexamethasone, 100 μmol/L. Controls contained the same volume of the vehicle alone. Samples were incubated with the steroid for 4 h before absorbance measured.

![Graph](image)

Fig. 1 Effect of dexamethasone on the light absorption of amniotic fluid in presence or absence of bilirubin

Amniotic fluid samples were incubated for 24 h in the dark in presence (C, III) or absence (C, I) of 3.42 μmol of bilirubin standard per liter, with (dark symbols) or without (open symbols) 10 μmol of dexamethasone per liter. The samples were scanned throughout the wavelength range shown. Samples containing bilirubin were scanned vs the same, bilirubin-free amniotic fluid; samples without bilirubin were scanned vs distilled water as blank.

Table 2. Effect of Glucocorticoids on the Absorbance of Bilirubin-Containing Amniotic Fluids at 450 nm

<table>
<thead>
<tr>
<th>Incubation, h</th>
<th>Control</th>
<th>Dexamethasone</th>
<th>Prednisolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.674 ± 0.041</td>
<td>0.657 ± 0.042</td>
<td>0.578 ± 0.071</td>
</tr>
<tr>
<td>1</td>
<td>0.753 ± 0.012</td>
<td>0.636 ± 0.020</td>
<td>0.544 ± 0.063</td>
</tr>
<tr>
<td>3</td>
<td>0.657 ± 0.053</td>
<td>0.596 ± 0.047</td>
<td>0.400 ± 0.040</td>
</tr>
<tr>
<td>24</td>
<td>0.598 ± 0.043</td>
<td>0.407 ± 0.036</td>
<td>0.544 ± 0.063</td>
</tr>
</tbody>
</table>

Absorbance of normal amniotic fluids, to which 3.42 μmol of bilirubin was added per liter, was measured at 450 nm against the same, bilirubin-free amniotic fluids. Dexamethasone or prednisolone to a final concentration of 10 μmol/L were added and readings were done at the indicated times after addition of the steroids. Results are mean absorbance values ± SEM for 20 controls, 20 dexamethasone-containing samples, and five prednisolone-containing samples. Controls contained the vehicle alone.

Table 3. Effect of Glucocorticoids on the Chemical Determination of Bilirubin in Amniotic Fluid by the Diazo Method

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Apparent bilirubin concn, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.17</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.17</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0.17</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>3.16</td>
</tr>
<tr>
<td>Bilirubin + dexamethasone</td>
<td>2.94</td>
</tr>
<tr>
<td>Bilirubin + prednisolone</td>
<td>2.91</td>
</tr>
</tbody>
</table>

Samples of amniotic fluid from a normal pregnancy were incubated for 24 h at 37 °C in the dark with bilirubin or the vehicle and 10 μmol/L dexamethasone or prednisolone or a combination of bilirubin and a glucocorticoid. Total bilirubin content was assayed by the diazo method. Results are taken from one of three similar experiments.
fluids. Dexamethasone did not affect this ratio in the "imma-
ture" or "borderline" standard marker mixtures that are
commercially available for the electrophoretic determina-
tion of L/S ratios in amniotic fluid.

One possible mechanism of the steroid-induced decrease
in absorption of amniotic fluid bilirubin is steroid-dependent
displacement of the pigment from its binding sites on some
protein(s) in the amniotic fluid. However, as shown in Table
4, the glucocorticoids also decrease the light absorption
of bilirubin in albumin solution, in the absence of amniotic
fluid, indicating that the effect does not depend on the
presence of some specific component of the amniotic fluid.

A second plausible mechanism is formation of a colloid
suspension of the pigment, a condition known to coincide
with a reduction in light absorption (13). The steroids could
favor the formation of a colloidal sol as a result of bilirubin
supersaturation. Because colloid formation increases the
light scattering (14, 15), we followed the light scattering of
different concentrations of bilirubin in amniotic fluid in the
absence and presence of dexamethasone. The results (Table
5) indicate that a small increase in light scattering occurred
at low bilirubin concentrations (0.4 and 0.8 μmol/L), as a
result of addition of 10 μmol of dexamethasone per liter.
However, at higher concentrations of bilirubin (1.6 and 3.2
μmol/L) dexamethasone did not increase the scattering,
suggesting that the steroid does not enhance formation of
bilirubin colloid. Dexamethasone alone, in the absence of
bilirubin, also increased the light scattering. Lack of an
effect of dexamethasone on bilirubin aggregation was simi-
larly noted with bilirubin dissolved in 1 g/L solutions of
albumin instead of amniotic fluid, and with bilirubin solu-
tions incubated for only 5 min or 1 h with the steroid (results
not shown).

Discussion

Spectrophotometry of amniotic fluid bilirubin at 450 nm
is quenched by the presence of the glucocorticoids dexam-
ethasone and prednisolone, either of which decreases the height
of the absorbance peak of bilirubin at 450 nm. The main
significance of our findings is the potential for error: if the
spectrophotometric bilirubin measurement is subject to er-
or owing to the direct effect of glucocorticoid in the concen-
tration present at the time the sample is drawn, then it
would be of no value in many Rh-isoinmunization patients
with glucocorticoids. In these cases the severity of the
hemolytic disease may be grossly underestimated. The
glucocorticoid-induced decline in absorbance might also
interfere with accurate determination of fetal maturity in
normal pregnancies when the mother is receiving glucocor-
ticoid hormones. These findings suggest that the previously
reported decrease of bilirubin after administration of gluco-
corticoids is falsely exaggerated, and that extreme caution
should be exercised in the interpretation of the direct
spectrophotometric measurement in cases where glucocorti-
coid is being administered. Interestingly, previous studies
showed that although glucocorticoid treatment altered the
bilirubin content as measured spectrophotometrically in
amniotic fluid, it did not affect the lecithin/sphingomyelin
ratio (8). Furthermore, unexplained differences were report-
ed between results of spectrophotometry, L/S ratio, and
outcome of pregnancies in cases of Rh-isoinmunization
with glucocorticoids (8, 9). Possibly dexamethasone
produces a decrease in absorbance without improving the
hemolytic disease of the fetus. This possibility is supported
by the finding that the rapid decrease in amniotic fluid
bilirubin also occurs in Rh-negative fetuses after therapy
with betamethasone (8). In addition, betamethasone admin-
istration did not produce prenatal evidence of improvement
in fetal pulmonary maturity (8). Concordantly, we find no
direct effect of the glucocorticoid on the chromatographic
determination of the phospholipid ratio.

On the basis of the present observations we support the
suggestion of Caritis et al. (8) that management of the Rh-
sensitized pregnancy be based on absorbance values ob-
tained before steroid therapy and that the effect of steroid on
the L/S ratio for prediction of the fetal condition after
5 glucocorticoid administration needs to be established.
Because our studies demonstrate that glucocorticoids do not
affect the chemical determination of bilirubin, the chemical
evaluation is superior to spectrophotometry for fluids ob-
tained from glucocorticoid-treated patients.

The mechanism of the effect of glucocorticoids on the
direct spectrophotometric determination of bilirubin re-
mains to be elucidated. We find that it is unlikely to be due
to a displacement of bilirubin from binding sites on some
specific components in the amniotic fluid, because the effect
can also be reproduced with bilirubin dissolved in albumin
solution (Table 4). Albumin is the major protein in amniotic
fluid (16) and suspensions of bilirubin and albumin duplica-
tate the absorbance peak seen in the amniotic fluid at 450
nm (5); the steroid could potentially displace the pigment
from albumin. However, we did not observe a difference in
absorbance at 450 nm between bilirubin alone or in the
presence of albumin.

Another possible explanation—that the steroids produce
supersaturation and thereby enhance aggregation of the
pigment with a resulting decrease in absorbance (13–15)—

**Table 4. Effect of Dexamethasone on Absorbance
of Bilirubin Solutions at 450 nm**

<table>
<thead>
<tr>
<th>Bilirubin, μmol/L</th>
<th>Control</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.040</td>
<td>0.040</td>
</tr>
<tr>
<td>0.8</td>
<td>0.110</td>
<td>0.060</td>
</tr>
<tr>
<td>1.6</td>
<td>0.300</td>
<td>0.150</td>
</tr>
<tr>
<td>3.2</td>
<td>0.650</td>
<td>0.450</td>
</tr>
<tr>
<td>PBS</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>0.8</td>
<td>0.080</td>
<td>0.080</td>
</tr>
<tr>
<td>3.2</td>
<td>0.450</td>
<td>0.340</td>
</tr>
</tbody>
</table>

Stock crystalline bilirubin (2.0 mmol/L) was first dissolved in 100 mmol/L
NaOH and subsequently diluted in either 1 g/L bovine serum albumin solution
or phosphate-buffered saline (PBS) to give the indicated final concentrations
of the pigment. Absorbance was measured at 450 nm in bilirubin-free solvent.
Dexamethasone was added to give a final concentration of 10 μmol/L, and
reading was made 2 h afterwards. Results are mean values for three
determinations.

**Table 5. Effect of Dexamethasone on Light
Scattering of Bilirubin in Amniotic Fluid**

<table>
<thead>
<tr>
<th>Bilirubin, μmol/L</th>
<th>Control</th>
<th>Dexamethasone (10 μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>0.4</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>0.8</td>
<td>51</td>
<td>62</td>
</tr>
<tr>
<td>1.6</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>3.2</td>
<td>78</td>
<td>79</td>
</tr>
</tbody>
</table>

Light scattering was measured at a×1 setting of the fluorimeter, with
excitation at 294 nm and emission at 297 nm. Amniotic fluid from a normal
pregnancy, containing the indicated concentrations of exogenous bilirubin,
were incubated for 24 h in absence or presence of 10 μmol dexamethasone
per liter. Values are mean of three determinations on each sample.
was denied by the lack of a significant effect of the steroids on the light scattering of bilirubin solutions.

A third possibility, that the glucocorticoids enhance the rate of degradation of bilirubin in amniotic fluid, is supported by the finding that the decrease in absorbance is proportional to the incubation interval. However, the effect is also noted in albumin solutions, and the pigment concentration as determined by the chemical (diazo) method is not affected by the steroids (Table 3), so this explanation should also be abandoned. Future studies are needed to clarify the mechanism of the effect.

References