Hemin Reversal of Benzene-induced Inhibition of Reticulocyte Protein Synthesis

By Frank J. Forte, Howard S. Cohen, Judith Rosman, and Michael L. Freedman

Benzene (0.056–0.113 M) rapidly and reversibly inhibited protein synthesis in anucleate human sickle cell and rabbit reticulocytes. Hemin (50 μM) both prevented and reversed this effect of benzene. The inhibition in rabbit reticulocytes was accompanied by a conversion of polyribosomes to monoribosomes. The polyribosomal disaggregation required ribosomal movement along mRNA and was also prevented and reversed by 50 μM hemin. Benzene was also shown to inhibit heme synthesis in rabbit reticulocytes while neither ATP nor GSH levels were altered. A translational repressor (HCR) of reticulocyte cell-free protein synthesis was isolated from intact cells incubated with benzene, while no significant amount of HCR was found in cells incubated with both benzene and hemin. These results indicated that benzene inhibits translation at the heme-dependent site of initiation. The clinical implications of these experiments remain to be elucidated.

BENZENE HAS LONG been implicated as having hematologic toxicity. Although many different toxic manifestations (myeloid metaplasia, lymphopenia, acute myeloblastic leukemia, hemolytic anemia) have been described, the most common finding has been pancytopenia.1 In mice, suppression of DNA synthesis of differentiated bone marrow cells has been implicated in the etiology of benzene toxicity.2 On the other hand, it has also been reported that the incorporation of radioactive iron into mouse bone marrow is decreased after a single subcutaneous injection of benzene.3 This experiment raises the question of whether or not benzene could directly affect heme synthesis.

Hemin has been shown to be required by both intact reticulocytes4–10 and their cell-free preparations for maximal globin synthesis.10–16 When intact cells are rendered hemin deficient, the polyribosomes are converted to single ribosomes at the same time protein synthesis is inhibited.5–10 This finding indicates that hemin control is at the site of initiation of protein synthesis. Hemin prevents and reverses this inhibition of initiation in the intact cell.10 When cell-free preparations are incubated without hemin a hemin-controlled translational repressor (HCR) of globin chain initiation has been shown to form in the post-ribosomal supernatant at the same time globin synthesis stops.17,20 Similar HCR may be isolated from intact erythrocytes that have lost protein synthetic capability,21 suggesting that HCR is a physiologic regulator of globin synthesis.

Tryfiates22 has reported that benzene inhibits rat liver protein synthesis...
and disaggregates polyribosomes. Since this effect could be due to hemin deficiency, the present study was undertaken to investigate if benzene inhibits translation in reticulocytes at the hemin-dependent site.

**MATERIALS AND METHODS**

**Incubation of Intact Cells**

Reticulocyte-rich blood was collected in heparin by cardiac puncture of phenylhydrazine-treated rabbits. Human reticulocytes were obtained by venipuncture of two volunteers with sickle cell anemia. The volunteers were fully informed as to the nature of the experiments and gave informed consent for the use of their blood. The cells were centrifuged free of plasma, washed twice with low-magnesium saline, and filtered through nylon to remove neutrophils and monocytes.

Incubations were carried out in a Metabolyte Water Bath Shaker at 37°C with air as the gas phase. The washed reticulocytes were incubated in the low-magnesium saline containing Tris (hydroxymethyl) amino methane hydrochloride buffer, 5 mM, pH 7.4, at 37°C. The concentration of cells in the incubation medium was 1 volume of packed cells per 7 volumes of medium. The concentrations of amino acids, glucose, and iron-transferrin were as previously described. Specific rabbit and human transferrins were used for the rabbit and human cells, respectively. Hemin was prepared as previously described and added to make a final concentration of 50 μM in the incubation medium.

**Measurement of Protein Synthesis in Intact Cells**

In experiments measuring the incorporation of radioactive amino acid into protein, the total volume of the incubation was 0.2 ml. L-(U)-14C-leucine (3.3 nmoles, 1 μCi) was present throughout the incubation as the only source of leucine. Metabolism was stopped by removing a 25-μl sample into cold 5% TCA. The protein precipitate was then washed three times with cold 5% TCA, once at 90°C with 5% TCA for 20 min, three times with an ethanol-ether (3:1) wash at 62°C for 5 min, and once with ether. The protein precipitate was suspended in ether and plated on 0.45-M Millipore filters. Radioactivity was determined in a Nuclear-Chicago gas flow counter with Micromil window (efficiency ~ 15%).

**Analysis of the Ribosome-Polyribosome Component**

Cells were washed twice with low-magnesium saline, lysed, and cleared of stroma as previously described. One milliliter of the 1:7 stroma-free lysate was layered on 36 ml of a 15%–30% (w/w) linear sucrose gradient in standard buffer (0.01 M Tris, pH 7.4, 0.01 M KCl, and 0.0015 M MgCl2). After centrifugation in a Spinco SW 27 swinging bucket rotor at 4°C for 3 hr at 82,500 g, the gradient was pumped through the flow-through cell of a Beckman Kintrac VII recording spectrophotometer, and absorbance at 260 nm was measured.

**Determination of Reticulocyte-reduced Glutathione (GSH)**

Incubation of reticulocytes with and without benzene was performed as described above. The cells were washed with low-magnesium saline five times at 4°C. Aliquots (0.2 ml) of the packed cells were assayed colorimetrically at 412 nm after reaction with 5,5'-dithio, bis-2-nitrobenzoic acid according to the method of Beutler et al.

**Determination of ATP Concentrations**

ATP concentrations were determined on perchloric acid extracts of reticulocytes by the method of Bucher. Reagents were obtained from the Boehringer Manheim Company.

**Measurement of Heme Synthesis in Intact Cells**

Heme synthesis was determined by the incorporation of L-2-14C-glycine into hemin. Crystalline hemin was prepared by the method of Chu and Chu as modified by Nakao and Takaku. In these experiments the incubations were as described above with a total volume of 4.0 ml. L-2-14C-glycine was added to make a final concentration of 10 μM. Incubations were carried out for 6 hr at 37°C with air as the gas phase. The incubations were stopped by adding the same volume of a 4% TCA solution and heating at 95°C for 10 min. The precipitate was washed three times with 5% TCA, once at 90°C with 5% TCA for 20 min, three times with an ethanol-ether (3:1) wash at 62°C for 5 min, and once with ether. The protein precipitate was suspended in ether and plated on 0.45-M Millipore filters. Radioactivity was determined in a Nuclear-Chicago gas flow counter with Micromil window (efficiency ~ 15%).
glycine (50 nmoles, 2.5 μCi) was present throughout as the only source of glycine. After the 30-min incubation in a 25-ml Erlenmeyer flask, the mixture was transferred to a 12-ml centrifuge tube and the cells washed five times with ice-cold saline. Five milliliters of a mixture of acetone and glacial acetic acid (4:1) were added to the washed packed cells and thoroughly mixed. The tube was centrifuged at 2000 rpm and the supernatant decanted into a 50-ml beaker. These same procedures were repeated three more times, and the supernatant fractions combined in the beaker. About 10 mg of sodium chloride were then added to each beaker. Acetone was removed from the combined supernatants by heating at 70°C for 2-3 hr. During this time crystalline hemin formed. The crystals were washed four times with distilled water and dissolved in 1.5 ml of 5.9 M ammonia water.

Aliquots (0.2-0.4 ml) were plated on planchets and dried. The radioactivity was determined in a Nuclear-Chicago gas flow counter with micromil window. The amount (milligrams) of hemin was determined by weighing the planchets before and after the hemin was plated and dried. The counts were corrected for self-absorption.

Isolation and Assay for Hemin-controlled Repressor (HCR)

Intact rabbit reticulocytes were incubated as described above, but without radioactivity, in a total volume of 8.0 ml either with or without benzene and or hemin. After incubation the cells were washed three times, lysed by addition of an equal volume of ice-cold deionized water, and the stroma removed by centrifugation at 25,000 g for 15 min. The lysates were then centrifuged at 225,000 g for 90 min at 4°C to remove the ribosomes. The resultant postribosomal supernatant was diluted with an equal volume of deionized water and slowly titrated at 0°C to pH 5.0 with 0.1 M acetic acid. The precipitate was centrifuged at 4°C at 1600 g for 15 min, and the pellet was dissolved in a volume equal to the original lysate of 36.7 mM N-2 hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer, pH 7.2. A small amount of undissolved material was removed by centrifugation at 34,000 g for 15 min. This clarified solution has previously been shown to contain the HCR.17821

HCR activity was assayed in the reticulocyte lysate cell-free system plus and minus hemin as previously described.21 The final concentration of hemin in tubes containing hemin was 35 μM. Lysates were obtained from multiple rabbits and tested for incorporating ability at this hemin concentration. Those lysates that showed an incorporation of 13,000-17,000 cpm per 25-μl sample in 1 hr were frozen in liquid nitrogen and stored. These lysates could be used for 1 mo without appreciable change in incorporation capability.

Materials

L-(U)-14C-leucine and L-2-14C-glycine were obtained from the New England Nuclear Corporation; heme from the Sigma Chemical Company; rabbit transferrin from Miles Laboratories, Inc; human transferrin, B grade, from Calbiochem; and GSH for standard curves from the Aldrich Company. Benzene (certified 99 mol %, pure) thiophene free was obtained from Fisher Scientific Company.

RESULTS

Benzene Inhibition of Protein Synthesis

The incubation of either rabbit or human reticulocytes with iron-transferrin and benzene at nonhemolytic concentrations of 0.056-0.113 M resulted in inhibition of protein synthesis. The kinetics of this inhibitory effect in the presence of 0.113 M benzene are shown for rabbit (Fig. 1) and human (Fig. 2) reticulocytes. A decrease in radioactive leucine incorporation was apparent at 2½ min, the earliest time at which samples were taken. When the reticulocytes were incubated simultaneously with benzene and hemin, inhibition of protein synthesis in either rabbit (Fig. 1) or human (Fig. 2) reticulocytes was prevented. In addition, incubation of either rabbit or human reticulocytes with benzene...
for 10 min, followed by the addition of hemin, showed a reversal of the benzene-induced inhibition in both rabbit (Fig. 1) and human (Fig. 2) reticulocytes.

Associated with this inhibition there was a conversion of polyribosomes to smaller aggregates (Fig. 3A and B), predominantly monoribosomes. Table 1 compares the polyribosomal pattern in Fig. 3 with different incubation mixtures. The simultaneous incubation of rabbit reticulocytes with benzene and either hemin or cycloheximide blocked polyribosomal disaggregation. In addition, when reticulocytes were incubated with benzene, washed, and reincubated in the presence of hemin, iron-transferrin, or glucose alone, the polyribosomes reformed.
Effect of Benzene on ATP and GSH Levels

Experiments were performed to determine if benzene had any effect on ATP or GSH levels. There was absolutely no change in either ATP or GSH levels in the presence of 0.113 M benzene (data not shown) in five separate determinations.

Benzene Inhibition of Hemin Synthesis

The 30-min incubation of rabbit reticulocytes with 0.113 M benzene resulted in a marked inhibition of hemin synthesis (Table 2). When earlier times were investigated, similar (~50%) inhibition was noted during the first 5 min of incubation. Incubation with both hemin and benzene resulted in similar inhibition as with benzene alone. Hemin alone in a concentration of 50 μM also inhibited heme synthesis, as has previously been reported.27

Table 1. Effect of Hemin, Cycloheximide, and Removal of Benzene on the Polyribosome Pattern of Intact Rabbit Reticulocytes

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Polyribosome Pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (IT or hemin)</td>
<td>A</td>
</tr>
<tr>
<td>Benzene, 0.113 M + IT</td>
<td>B</td>
</tr>
<tr>
<td>Benzene, 0.113 M + hemin</td>
<td>A</td>
</tr>
<tr>
<td>Benzene, 0.113 M + IT + cycloheximide, 0.05 M</td>
<td>A</td>
</tr>
<tr>
<td>Benzene, 0.113 M + IT. Washed, reincubated +</td>
<td>A</td>
</tr>
<tr>
<td>IT or hemin or glucose alone, minus benzene.</td>
<td>A</td>
</tr>
</tbody>
</table>

*Polyribosome pattern similar or identical to that shown in Fig. 1. 
†IT, iron transferrin.
Table 2. Benzene Inhibition of Heme Synthesis* in Intact Rabbit Reticulocytes

<table>
<thead>
<tr>
<th>Incubation</th>
<th>cpm/mg t</th>
<th>Per Cent Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6013</td>
<td>100</td>
</tr>
<tr>
<td>Benzene, 0.113 M</td>
<td>2724</td>
<td>45</td>
</tr>
<tr>
<td>Benzene, 0.113 M and hemin 5 x 10^{-5} M</td>
<td>2670</td>
<td>44</td>
</tr>
<tr>
<td>Hemin, 5 x 10^{-5} M</td>
<td>4853</td>
<td>81</td>
</tr>
</tbody>
</table>

*Incorporation of 2-14C-glycine into heme.
†Each determination is the mean of three experiments performed in duplicate.

**HCR in Benzene-treated Cells**

An aliquot of the fraction precipitated at pH 5 was investigated for HCR activity in benzene-treated cells. Approximately 85 μg of protein (determined by E280/E260 method) was added. As shown in Fig. 4, this fraction from benzene-treated intact reticulocytes inhibited the rate and extent of cell-free protein synthesis in both the plus and minus hemin systems. In contrast, a similar fraction from cells treated simultaneously with benzene and 50 μM hemin did not significantly alter the cell-free kinetics (Fig. 4). No inhibition was found in fractions from cells incubated on ice with benzene. The inhibitory fraction was placed on a Sephadex G-200 column and eluted just after the void volume (mol. wt., 3 x 10^3). No inhibitory activity was found in the same fraction from either control or benzene- and hemin-treated reticulocytes.

**DISCUSSION**

Previous studies have shown that hemin is necessary for maximal initiation of protein synthesis in intact reticulocytes. An inhibition of heme synthesis should, therefore, be reflected in an inhibition of protein synthesis which is re-

Fig. 4. Effect of HCR from benzene-treated reticulocytes on cell-free protein synthesis. Approximately 85 μg of protein (determined by E280/E260 method) was added. The intact cells were incubated as shown for 20 min and the HCR-containing fraction isolated as described in Materials and Methods. Assay for HCR activity was in the reticulocyte cell-free lysate system. (A) With 35 μM hemin. (B) Without hemin.
versible with the addition of hemin. In the present study we have shown that benzene inhibits protein synthesis in the presence of iron-transferrin for endogenous heme synthesis in both rabbit and human reticulocytes. The minimum concentration of benzene necessary to inhibit consistently protein synthesis was 0.056 M (438 mg/100 ml). This inhibition was both prevented and reversed by 50 μM hemin, a concentration which has been shown to maintain protein synthesis in the absence of added iron-transferrin for heme synthesis.8-10 While there were differences in the kinetics observed in rabbit and human cells, these might be explained by species or cell age differences.

The effect of benzene was also associated with a reversible conversion of polyribosomes to smaller aggregates, predominantly monoribosomes. These findings are in agreement with those of Tryfiates,22 who found disaggregation of liver polyribosomes after injecting rats with benzene. Since the number of ribosomes on mRNA is directly proportional to the rate of initiation and inversely proportional to the rate of release of ribosomes from mRNA,10 this polyribosomal disaggregation is consistent with an inhibition of initiation with normal elongation and release, as occurs with heme deficiency.8-10 This conclusion is supported by the hemin prevention and reversal of the polyribosomal disaggregation as well as by the failure to disaggregate when elongation is prevented by cycloheximide.

We also considered the possibility that the benzene could reduce the oxygen supply to the cells during the incubations. The lack of decreased ATP and GSH concentrations, however, was against this conclusion. Furthermore, when the cells were incubated in a nitrogen atmosphere for 30 min, similar kinetics with and without benzene were obtained.

Direct demonstration of benzene inhibition of heme synthesis has also been presented in this study. It should be pointed out that the inhibition is more rapid than when hemin or iron-transferrin is omitted from the incubation medium,4-6 or when the cells are made iron deficient with an iron-chelating agent.7-10 Possibilities for this finding could be rapid uptake of benzene into the cells and direct inhibition of an enzyme rather than depletion of endogenous iron. This view that iron was not depleted was supported by the reversal of polyribosomal disaggregation when benzene was removed and the cells incubated in the absence of either iron-transferrin or hemin. The exact site of inhibition of heme synthesis, however, remains to be elucidated.

The effect of hemin in reticulocyte protein-synthesizing systems is still in dispute. Considerable evidence, however, has been collected in different laboratories that in heme deficiency a translational repressor forms in the post-ribosomal supernatant10,21 which reduces the level of met-tRNA f associated with the native subunits.28,29 The rate of polypeptide chain initiation in heme-deficient lysates can be maintained or restored by a ribosomal preparation which has been subfractionated to yield an initiation factor IF-MP which mediates binding of met-tRNA f to the 40S ribosomal subunit.10 Clemens et al.30 have proposed that HCR directly inhibits IF-MP-dependent binding of met-tRNA f to the 40S subunit. Balkow et al.,31 however, have suggested that the impaired binding is a secondary phenomenon, with the primary effect of HCR being deacylation of the met-tRNA f on the 40S subunit.
On the other hand, Raffel et al.\textsuperscript{32} have suggested that hemin mediates the formation of an active initiation factor complex (stimulator) from inactive lower molecular-weight components. This work, however, did not consider the formation of HCR in postribosomal supernatants. While it is possible that the “inactive component” (minus hemin) is an inhibitor and the “active component” (plus hemin) is a stimulator, no evidence has yet been presented to this effect.

Our work supports the view that with decreased amounts of hemin a repressor (HCR) forms to inhibit initiation. HCR did not form in cells incubated with both benzene and added hemin, even though heme synthesis was inhibited to the same degree. This finding indicates that benzene itself does not directly inactivate an initiation factor, but rather inhibits protein synthesis indirectly via the formation of HCR. Furthermore, these results indicate that diminished heme synthesis by itself does not inhibit protein synthesis. Hemin synthesis is necessary only to maintain a concentration of hemin sufficient to prevent HCR formation.

Our studies, therefore, demonstrate a direct translational mechanism of benzene toxicity, namely a heme-dependent block of protein synthesis. The block is presumably not DNA related, since anucleate reticulocytes are thought not to synthesize DNA. Hemin has also been shown to be necessary for maximal nonglobin protein synthesis in reticulocytes,\textsuperscript{33,34} platelets,\textsuperscript{35} Krebs ascites tumor cells,\textsuperscript{34} and brain and liver cells.\textsuperscript{32} It is possible, therefore, that hemin is a universal requirement for maximal initiation of all protein synthesis. If this hypothesis is indeed true, reported effects of benzene on DNA synthesis\textsuperscript{2} could conceivably result from a decreased translation of proteins necessary for DNA synthesis. Of course our studies do not exclude a separate and perhaps an even more important direct effect on DNA synthesis or transcription.

Our in vitro experiments have not established an in vivo toxic dose for benzene. Even with constant shaking, benzene does not remain dispersed throughout the medium. We therefore do not know the concentration of benzene actually responsible for toxicity. Furthermore, we cannot say that clinical benzene toxicity results from a heme-dependent inhibition of initiation of protein synthesis or if indeed benzene itself rather than a metabolite is the actual toxic agent. Further experiments will hopefully provide answers to these important questions.

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FJ Forte, HS Cohen, J Rosman and ML Freedman