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Research Article

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A Rabbit Reticulocyte Model for the Role of Hemin-Controlled Repressor in Hypochromic Anemias

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ABSTRACT Hemin allows maximal protein synthesis in intact rabbit reticulocytes and their cell-free lysate preparations by retarding the formation of a translational repressor (HCR) found in the postribosomal supernate. In order to evaluate the role of HCR in the pathogenesis of hypochromic anemias, HCR was isolated and partially purified from intact rabbit reticulocytes incubated in vitro with either 0.1 mM α,α-dipyridyl (an iron-chelating agent) or 0.1 M ethanol. Both of these agents inhibit reticulocyte protein synthesis. Hemin (50 μM) protects against the inhibition by both agents. A ferrous iron-transferrin mixture, however, protects only against α,α-dipyridyl. Both α,α-dipyridyl and ethanol inhibit heme synthesis before the time that protein synthesis is affected, while neither lowers either ATP or GSH levels. These results indicate that while both agents inhibit heme synthesis, α,α-dipyridyl does so by inducing iron deficiency while ethanol works at a non-iron-requiring step. When HCR was isolated from intact cells and assayed in the reticulocyte cell-free systems, plus and minus hemin, premature appearance of HCR was found in cells incubated in vitro with α,α-dipyridyl or ethanol. When hemin was present in the intact cell incubation, the appearance of HCR was retarded. The HCR from α,α-dipyridyl ethanol-treated cells was partially purified and eluted at the same location on a Sephadex G-200 column (molecular weight $\sim 3 \times 10^5$) as that from postribosomal supernates incubated minus hemin. In addition rabbits with phenylhydrazine-induced hemolytic anemia were given intravenous ethanol in vivo at a dose of 0.4 ml/kg. This concentration of alcohol resulted in an inhibition of the rate of heme synthesis and protein synthesis as well as an acceleration of HCR formation in reticulocytes. The HCR from these in vivo treated rabbits was isolated, partially purified, and assayed in an identical fashion as the in vitro experiments. These in vivo experiments further support the physiological and pathophysiological role of HCR in reticulocytes.

On the basis of these results a model for a role of HCR in some of the hypochromic anemias is proposed. In iron deficiency or chronic disease (where iron is not available to the erythroblast for heme synthesis) HCR appears prematurely and inhibits protein synthesis. When heme synthesis is inhibited by ethanol but there is sufficient intracellular iron, HCR appears prematurely and inhibits protein synthesis, iron accumulates in the erythroblast, and the end result is sideroblastic anemia.

INTRODUCTION

Hemin has been shown to be necessary for maximal globin synthesis both in intact reticulocytes (1–7) and in their cell-free preparations (7–13). When intact cells are incubated in the absence of hemin and are rendered iron deficient by an iron-chelating agent, the polyribosomes are converted to single ribosomes (2-7) not attached to mRNA (14), suggesting that when heme synthesis is inhibited, initiation of globin synthesis is also inhibited. Both hemin and a ferrous iron-transferrin mixture prevent and reverse this effect on initiation in intact cells (2-7). Studies utilizing reticulocyte cell-free lysate preparations have shown that in the absence of hemin, a hemin-controlled protein translational repressor of initiation (HCR)[1] forms in the postribosomal supernate at the same time globin synthesis stops (15-19). Hemin markedly retards the formation of the repressor. A similar HCR may be isolated from human

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[1] *Abbreviations used in this paper:* HCR, hemin-controlled repressor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TCA, trichloroacetic acid.

or rabbit mature erythrocytes, but not from reticulocytes (19). This is evidence that HCR plays a physiological role in maturation and cessation of protein synthesis.

Recent work by Ali and Brain (20) as well as in our laboratory (21) has shown that ethanol, in concentrations achievable in humans, is directly toxic to bone marrow and reticulocyte protein synthesis. This inhibition was also accompanied by a conversion of the polyribosomes to monoribosomes. Both the effect on protein synthesis and polyribosomes was prevented and reversed by hemin, but not by iron-transferrin. These data, as well as the finding of raised erythrocyte coproporphyrin and protoporphyrin levels in 11 alcoholic patients with sideroblastic erythropoiesis (22), point towards a defect in heme synthesis in alcohol toxicity, other than iron deficiency.

Both iron-deficient and ethanol-treated reticulocytes, therefore, appear to have decreased heme synthesis and, as a result, an inhibition of protein synthesis. The present study was undertaken in order to investigate if heme synthesis is indeed inhibited in vitro under these conditions and if HCR could be isolated from these cells. Furthermore, we investigated if HCR would form in vivo after injection of ethanol. Our finding that HCR is present in the intact reticulocyte when heme synthesis is inhibited in vitro, either by an iron-chelating agent, α,α-dipyridyl, or ethanol, suggests that HCR is involved in the pathogenesis of some of the hypochromic anemias. The demonstration that HCR is present in vivo in reticulocytes within 5 min after ethanol is injected intravenously is strong evidence for a role of HCR in these anemias.

METHODS

Collection of rabbit reticulocytes. Reticulocytosis was induced in rabbits by the daily injection of phenylhydrazine for 6 days. The blood was collected in heparin from ear arteries of the animals on the 8th day. The cells were centrifuged free of plasma, the buffy coat was removed, and the cells were washed twice with saline solution (0.14 M NaCl, 5 mM KCl, and 1.5 mM $MgCl_2$) and filtered through nylon to remove remaining neutrophils and monocytes. If the cells were to be used in intact cell incubations, they were suspended in an equal volume of the saline solution.

In vivo ethanol treatment of rabbits. Rabbits with a phenylhydrazine-induced hemolytic anemia were given an intravenous injection of absolute ethanol at a dose of 0.4 ml/kg. The injection was given over a 1-min period and arterial samples were drawn at 5-min intervals. The reticulocytes from these ethanol-treated animals were handled identically to those from non-ethanol-treated animals.

Intact cell incubations. Incubations were performed in a shaking waterbath at 37°C with air as the gas phase. The washed reticulocytes were incubated in the saline solution containing tris(hydroxymethyl)aminomethane hydrochloride buffer, 5 mM, pH 7.4 at 37°C. The concentration of cells in the medium was 1 vol per 7 vol of medium. The concentrations of amino acids and glucose were as previously described (7). Iron transferrin and hemin were prepared as previously described (7). When hemin was added, it was at a final concentration of 50 μM. α,α-Dipyridyl and ethanol, when present, were at concentrations of 0.1 mM and 0.1 M, respectively.

Measurement of protein synthesis in intact cells. The incubation mixture was at a total volume of 0.2 ml. The only source of leucine in these experiments was added as L-[U-^{14}C]leucine (3.3 nmol, 1 μCi). Metabolism was stopped by removing a 25-μl sample into cold 5% trichloroacetic acid (TCA). The protein was washed three times with cold 5% TCA, once at 85°C with 5% TCA for 20 min, once with absolute ethanol, three times with ethanol: ether (3:1) at 62°C for 5 min, and once with ether. The protein was suspended in ether and plated on 0.45-μm Millipore filters (Millipore Corp., Bedford, Mass.). Radioactivity was determined in a Nuclear Chicago gas flow counter (Searle Analytic Inc., Des Plaines, Ill.) with Micromil window (efficiency 15%).

Determination of reticulocyte GSH. Incubation of reticulocytes was performed as described above in a total volume of 2 ml with nonradioactive leucine. The cells were washed with the saline solution five times at 4°C after incubation. Aliquots (0.2 ml) of the packed cells were assayed colorimetrically at 412 nm after reaction with 5,5'-dithiobis-2-nitrobenzoic acid according to the method of Beutler et al. (23).

Determination of reticulocyte ATP concentrations. ATP concentrations were determined on perchloric acid extracts of reticulocytes by the method of Bücher (24). Reagents were obtained from the Boehringer Mannheim Corp., New York. The incubations were performed as described above in a total volume of 8 ml with nonradioactive leucine.

Measurement of heme synthesis in intact cells. Heme synthesis was determined by the incorporation of L-[2-^{14}C]-glycine into hemin. Crystalline hemin was prepared by the method of Chu and Chu (25) as modified by Nakao and Takaku (26). In these experiments the incubations were as described above with nonradioactive leucine in a total volume of 4 ml. L-[2-^{14}C]Glycine (50 nmol, 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. 5 ml of a mixture of acetone and glacial acetic acid (4:1) was added to the washed packed cells and thoroughly mixed. The tube was centrifuged at 2,000 rpm and the supernate decanted into a 50-ml beaker. These same procedures were repeated three more times, and the supernates combined in the beaker. About 10 mg of sodium chloride was then added to each beaker. Acetone was removed from the combined supernates by heating at 70°C for 2–3 h. 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 HCR. Intact rabbit reticulocytes were incubated as described above, with nonradioactive leucine, in a total volume of 8.0 ml either with or without α,α-dipyridyl, ethanol, 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 supernate was diluted with an equal volume of deionized water and slowly titrated at 0° to pH 5.0 with 0.1 M acetic acid. The precipitate was centrifuged at 4°C at 1,600 *g* for 15 min, and the pellet was dissolved in a volume equal to the original lysate of 36.7 mM *N*-2-hydroxyethylpiperazine-*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 (15, 16, 19, 27) when it is present.

In some experiments (as shown in specific figures) further purification was achieved by passing this protein fraction precipitated at pH 5 through a Sephadex G-200 column (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) and collecting the fraction eluted just after the void volume. This procedure separates the HCR from the low molecular weight components and is identical to the method of separation described previously (16, 19). Clemens et al. (27) have also isolated HCR by pH 5 precipitation but achieved further purification by reprecipitation with 30% ammonium sulphate. It would be expected that both methods would give a similar degree of purification.

HCR activity was assayed in the reticulocyte lysate cell-free system plus and minus added hemin as previously described (19). The final concentration of hemin in those tubes containing hemin was 35 μM. Lysates were obtained from multiple rabbits and tested for optimal incorporating ability at this hemin concentration. Those lysates that showed an incorporation of 13,000–18,000 cpm/25-μl sample in 1 h were frozen in liquid nitrogen and stored. These lysates could be used for 1 mo without appreciable change in incorporating capability. Each incubation tube (0.24 ml final volume) contained the following ingredients added in this order: (*a*) 20 μl of 4.2×10^{-4} M hemin in 50 mM Tris-Cl (pH 7.9) or 20 μl of Tris-Cl without hemin; (*b*) 100 μl of lysate; (*c*) 40 μl of partially purified repressor from incubated intact cells or the same fraction from unincubated cells; (*d*) 80 μl of master mix containing salts, buffer, amino acids, L-[U-^{14}C]leucine (2.64 nmol, 0.8 μCi) and an energy source as previously described (19). Cell-free incubations were performed at 34°C in plastic tubes in a rotating waterbath.

Measurement of cell-free protein synthesis. 25-μl samples were removed from the incubation mixture to 6 ml of ice-cold 0.02 M NaCl containing 20 μmol of L-(^{13}C)leucine. 2 ml of 20% trichloroacetic acid was added to this mixture as it was vigorously agitated. After 15 min at 0°C, 20 min at 85°C, and another 15 min at 0°C, the solution was filtered through a Millipore filter (0.45 μm pore size). The precipitate was washed with 5% cold TCA, dried, and counted in the gas-flow counter.

Materials. L-[U-^{14}C]Leucine and L-[2-^{14}C]glycine were obtained from New England Nuclear, Boston, Mass.; heme from the Sigma Chemical Co., St. Louis, Mo.; rabbit transferrin from Miles Laboratories, Inc., Elkhart, Ind.; and α,α-dipyridyl and ethanol from Fisher Scientific Co., Pittsburgh, Pa.

RESULTS

α,α-Dipyridyl and ethanol inhibition of intact reticulocyte protein synthesis in vitro. As has previously been reported (7), in vitro incubation of intact reticulocytes for 30 min minus iron-transferrin or hemin did not result in significantly decreased protein synthesis from the control value of 100% (Table I, columns A–C). Incubation of cells without either hemin or iron-transferrin and in the presence of 0.1 mM of the iron-chelating agent α,α-dipyridyl (column D) resulted in significant inhibition (41.1%). When the cells were incubated with 0.1 M ethanol and either with or without iron-transferrin, significant inhibition (39.7 and 42.8%, respectively) was found (columns H and G). Incubation of the cells with dipyridyl and iron-transferrin or hemin or with ethanol and hemin did not result in a significant decrease from the control value of 100%

TABLE I

*Effect of α,α-Dipyridyl and Ethanol on Rabbit Reticulocyte Protein Synthesis in the Presence and Absence of Iron-Transferrin or Hemin**

	Addition								
	A	B	C	D	E	F	G	H	I
Exp.	Iron-transferrin	None	Hemin	α,α-Dipyridyl	α,α-Dipyridyl +iron-transferrin	α,α-Dipyridyl +hemin	Ethanol	Ethanol +iron-transferrin	Ethanol +hemin
	cpm/mg				*Percent of control*				
1	12,796	89.2	140.3	57.2	64.9	98.2	39.7	44.0	85.9
2	21,682	78.5	90.9	67.2	102.8	89.2	32.4	54.4	63.0
3	10,756	113.6	149.3	37.8	46.8	127.5	48.9	57.4	104.2
4	20,844	100.7	81.3	64.7	122.9	98.8	34.5	56.5	57.9
5	16,258	87.4	118.4	55.4	79.4	81.5	75.1	61.7	93.5
6	15,695	93.4	138.0	73.8	86.8	125.5	78.7	71.7	138.8
7	12,669	116.5	120.4	56.0	145.1	98.8	91.0	76.2	127.0
Mean	100%	97.0	119.8	58.9	92.7	102.8	57.2	60.3	95.8
SEM		5.30	9.69	4.35	12.77	6.58	9.04	4.11	11.45
95% confidence		84.03–109.97	96.09–143.51	48.26–69.54‡	60.20–123.95	86.7–118.9	35.08–79.32‡	50.24–70.36‡	67.78–123.82

* Incubation for 30 min at 37°C.

‡ Differs from 100% with $P < 0.05$.

TABLE II

*α,α-Dipyridyl and Ethanol Inhibition of Heme Synthesis in Intact Rabbit Reticulocytes**

Exp.	Control	α,α-Dipyridyl	Ethanol
	cpm/mg	*% control*	
1	5,509	53.2	73.1
2	4,331	84.1	72.6
3	7,661	53.8	68.0
4	3,395	50.4	58.7
5	4,945	24.3	40.6
6	8,331	48.0	54.5
7	6,260	72.1	71.6
8	5,987	82.4	86.7
9	6,845	76.9	87.9
10	9,770	82.3	64.2
Percent control	100	62.8	67.8
SEM		6.26	4.52
99.9% confidence interval		32.88–92.72‡	46.19–89.41‡

* Incubation for 30 min at 37°C.
‡ Differs from 100% with $P < 0.001$.

(columns E, F, I). A comparison of the significance of the difference between dipyridyl alone (column D) and dipyridyl and iron-transferrin (column E) or dipyridyl and hemin (column F) was performed with Student's *t* test for paired samples. Both dipyridyl and iron-transferrin and dipyridyl and hemin were significantly higher than dipyridyl alone ($P < 0.05$ and $P < 0.01$, respectively). By similar analysis ethanol and hemin was significantly higher than either ethanol alone or ethanol and iron-transferrin ($P < 0.01$). There was no significant difference between ethanol either with or without iron-transferrin.

α,α-Dipyridyl and ethanol inhibition of heme synthesis in vitro. Reticulocytes were incubated under similar conditions as in the protein synthesis experiments but in a total volume of 4 ml with radioactive L-[2-^{14}C]glycine. During the 30-min incubations, no significant difference in heme synthesis was found if the cells were incubated either with or without iron-transferrin. In order to study α,α-dipyridyl and ethanol inhibition of heme synthesis in the same experiment, therefore, the control incubations were performed without iron-transferrin. As can be seen in Table II, α,α-dipyridyl and ethanol inhibited heme synthesis by 37.2 and 32.2%, respectively. Incubation with hemin and either α,α-dipyridyl or ethanol (Table III) resulted in similar inhibition as with each agent alone. Hemin alone in a concentration of 50 μM also inhibited heme synthesis to similar degree as has been reported previously (28).

Table IV shows the results of a time-course of α,α-dipyridyl and ethanol inhibition of heme and protein synthesis. The effect on heme synthesis was apparent at 2 min while the effect on protein synthesis was significant at 5 min. This demonstrates that the effect on heme synthesis occurs before the inhibition of protein synthesis.

Effect of α,α-dipyridyl and ethanol on ATP and GSH levels in vitro. Reticulocytes were incubated under identical conditions as in the previous experiments. The total volume in the ATP experiments was 8 ml and in the GSH experiments, 2 ml. During the 30-min incubations, no significant difference in either ATP or GSH concentration was found if the cells were incubated either with or without iron-transferrin. In order to study α,α-dipyridyl and ethanol effect on ATP and GSH in the same experiment, the control incubations were performed without iron-transferrin. As is shown in Table V, incubation with α,α-dipyridyl or ethanol did not result in any significant decrease in either ATP or GSH concentrations.

Formation and isolation of the hemin-controlled repressor. A rabbit reticulocyte postribosomal supernate was incubated minus hemin for 60 min. HCR was isolated by precipitation at pH 5 as described above and resuspended in a volume of HEPES buffer equal to the original supernate. Dilutions were made, as shown in Fig. 1 and assayed in the cell-free lysate system plus and minus hemin.

Fig. 1 shows the necessity of having hemin in the cell-free lysate system. Without hemin, both the rate and extent of protein synthesis were markedly reduced after 5 min of incubation. When HCR was added to the plus and minus hemin system, both rate and extent of protein synthesis were inhibited. Dilutions of HCR showed progressive diminishing of this inhibitory effect. At a 100-fold dilution, some stimulation was apparent. When HCR was placed on a Sephadex G-200 column, it eluted just after the void volume, as has previously been reported (19).

TABLE III

*α,α-Dipyridyl and Ethanol Inhibition of Heme Synthesis in Intact Rabbit Reticulocytes in the Presence of Hemin**

	cpm/mg‡	*% control*
Control	5,560	
α,α-Dipyridyl, 0.1 mM	2,891	52.0
Ethanol, 0.1 M	2,535	45.6
Hemin, 5×10^{-5} M	4,448	80.0
Hemin + α,α-dipyridyl	2,836	51.0
Hemin + ethanol	2,391	43.0

* Incubation for 30 min at 37°C.
‡ Each number is the mean of three separate experiments performed in duplicate.

TABLE IV

Time-Course of α,α-Dipyridyl and Ethanol Inhibition of Heme and Protein Synthesis in Rabbit Reticulocytes

	Time in minutes				
	2	5	10	20	30
Heme synthesis*					
Control, *cpm/mg*	823	1,485	4,206	6,681	12,117
α,α-Dipyridyl, % *control*	82.5	72.5	78.2	42.9	47.8
Ethanol, % *control*	67.2	57.1	65.5	66.0	61.6
Protein synthesis‡					
Control, *cpm/mg*	1,794	3,486	6,127	10,073	14,964
α,α-Dipyridyl, % *control*	112.0	71.4	75.0	65.3	53.3
Ethanol, % *control*	94.7	57.2	58.3	65.0	58.2

* Incorporation of L-[2-^{14}C]glycine into hemin.
† Incorporation of L-[U-^{14}C]leucine into protein.

Isolation of HCR from α,α-dipyridyl and ethanol-treated cells in vitro. Intact reticulocytes were incubated in vitro in the presence of 0.1 mM α,α-dipyridyl and 0.1 M ethanol with and without hemin for 20 min. The postribosomal supernates from these cells inhibited the cell-free lysate systems plus and minus hemin (data not shown). HCR fractions were then isolated as described in Methods and added to the cell-free lysate systems. The controls contained the same fraction from intact cells incubated with glucose alone or with glucose and hemin for 20 min.

Fig. 2 shows the results when the HCR fraction was isolated from intact cells incubated in vitro with α,α-dipyridyl and added to the cell-free lysate system plus and minus hemin. There was inhibition of the cell-free system both plus and minus hemin. A similar fraction from intact cells incubated with α,α-dipyridyl and hemin, however, did not inhibit either the plus or minus hemin cell-free lysate.

In Fig. 3, the effect of adding the HCR fraction isolated from intact cells incubated in vitro with ethanol to the cell-free lysate system plus and minus hemin is shown. There was inhibition of the cell-free system, plus and minus hemin. A similar fraction from intact cells incubated with hemin and ethanol, did not inhibit the cell-free lysate test systems.

In all of the experiments, the fraction from hemin-treated cells showed stimulatory activity. That this was not due to additional hemin is excluded as the lysates were already at the hemin optimum. When the HCR fractions from α,α-dipyridyl and ethanol-treated cells were placed on Sephadex G-200 column, the more purified inhibitory fraction eluted just after the void volume, corresponding to an approximate molecular weight of

TABLE V

Effect of α,α-Dipyridyl and Ethanol on ATP and GSH Levels in Intact Rabbit Reticulocytes

Condition*	ATP‡	GSH‡
	mg/0.25 ml cells	
Control	0.67±0.02	64.0±8.8
α,α-Dipyridyl, 0.1 mM	0.69±0.02	63.2±9.2
Ethanol, 0.1 M	0.67±0.02	64.2±8.7

* Incubation was 30 min at 37°C in the absence of iron-transferrin.
‡ The values given are the means of five determinations±SE.

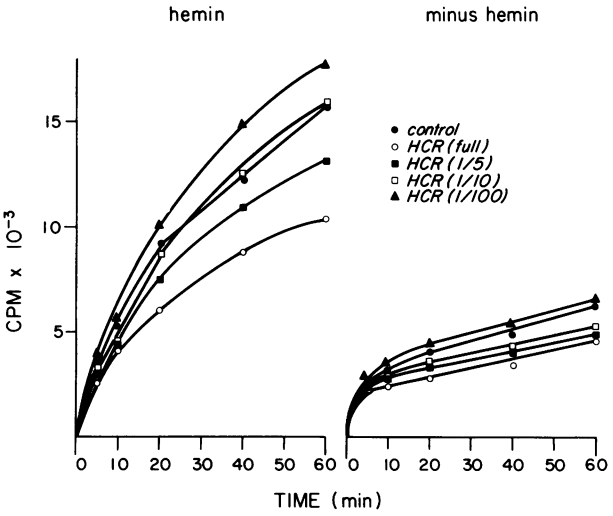

FIGURE 1 The effect of HCR from a reticulocyte postribosomal supernate incubated minus hemin for 60 min on the cell-free lysate system plus and minus hemin. HCR was isolated by precipitation at pH 5 as described in Methods and diluted as shown. The control contained a similar fraction from unincubated reticulocytes. Each point is the mean of three separate experiments. The ordinate refers to amino acid incorporation into protein.

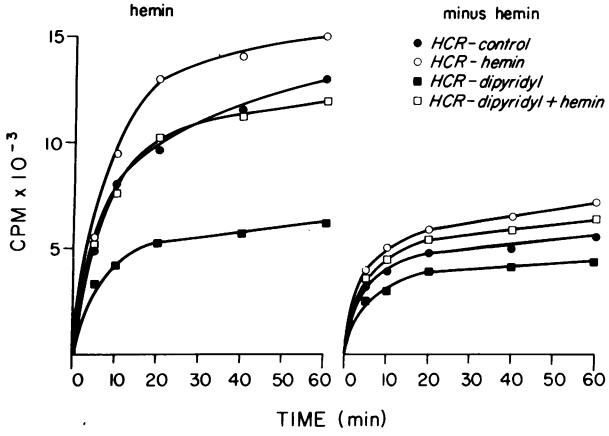

FIGURE 2 The effect of HCR from intact reticulocytes incubated with 0.1 mM α,α-dipyridyl on the cell-free lysate system plus and minus hemin. All intact cell incubations were for 30 min at 37°C. The control intact cell incubation was without either hemin or iron-transferrin. Hemin when included in the intact cell incubation was present at a concentration of 5×10^{5} M. HCR was isolated by precipitation at pH 5 as described in Methods. Each point is the mean of five separate experiments.

3×10^{5} (Fig. 4). These fractions cochromatographed with rabbit reticulocyte HCR and is where human cell HCR has been found (19). The slight stimulatory activity seen in the fraction from hemin-treated cells also eluted at this point in the Sephadex G-200 column.

Inhibition of reticulocyte protein and heme synthesis after in vivo ethanol treatment of rabbits. After rabbits were given an intravenous injection of absolute ethanol (0.4 ml/kg) reticulocytes were obtained from

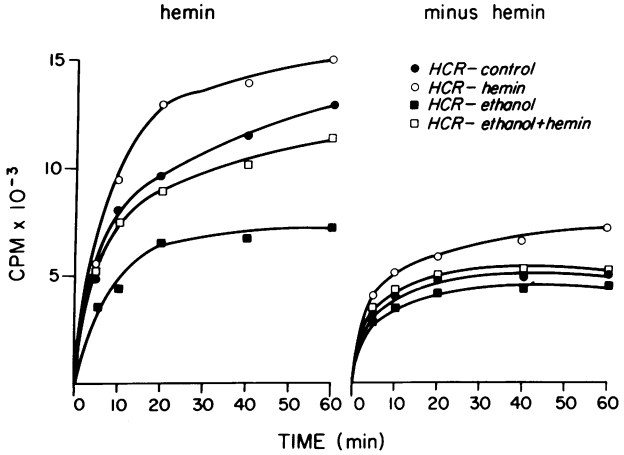

FIGURE 3 The effect of HCR from intact reticulocytes incubated with 0.1 M ethanol on the cell-free lysate system plus and minus hemin. The intact cell incubations and isolation of HCR were as described in Fig. 2. Each point is the mean of five separate experiments.

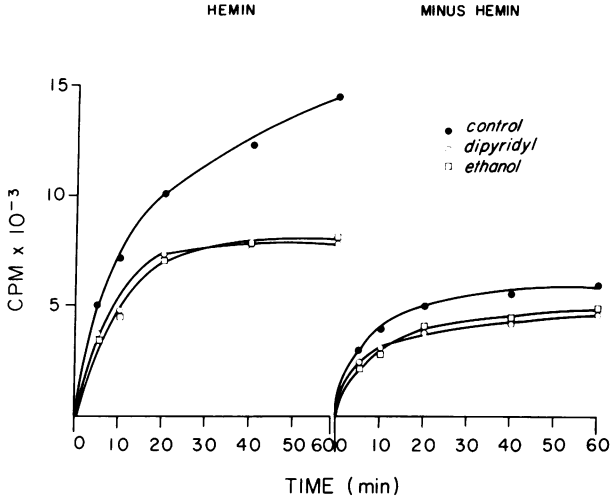

FIGURE 4 The effect of partially purified HCR from α,α-dipyridyl- and ethanol-treated cells on the cell-free lysate system plus and minus hemin. HCR was isolated by precipitation at pH 5 and further purified through a Sephadex G-200 column. The inhibitory fractions corresponded to a molecular weight of 3×10^{5}. The control was a similar fraction isolated from intact cells incubated without hemin or iron-transferrin. Each point is the mean of three separate experiments.

these animals and incubated in vitro to measure rates of protein and heme synthesis. Fig. 5 shows that there was a decreased initial rate of both protein and heme

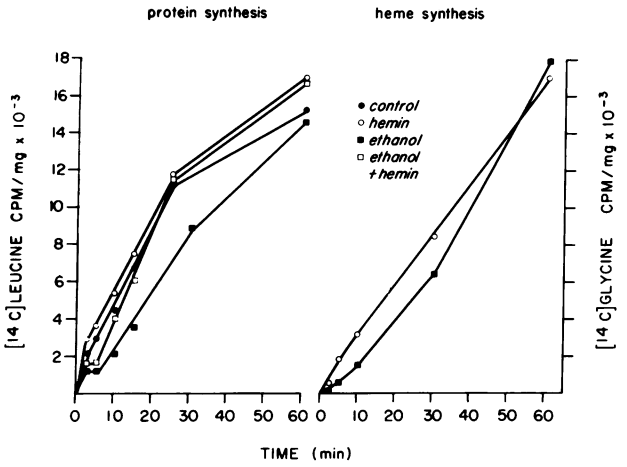

FIGURE 5 The rate of protein and heme synthesis in rabbit reticulocytes after intravenous injection of ethanol (0.4 ml/kg) in vivo. The rabbits received ethanol, 0.4 ml/kg, and intact reticulocytes were incubated in vitro as described in Methods. Each point is the mean of three separate experiments. (●) 0-time reticulocytes (before ethanol) incubated in vitro with iron-transferrin. (○) 0-time reticulocytes incubated in vitro with hemin, 50 μM. (■) Reticulocytes obtained 5 min after ethanol injection in vivo, incubated in vitro with iron transferrin. (□) Reticulocytes obtained 5 min after ethanol injection in vivo, incubated in vitro with hemin, 50 μM.

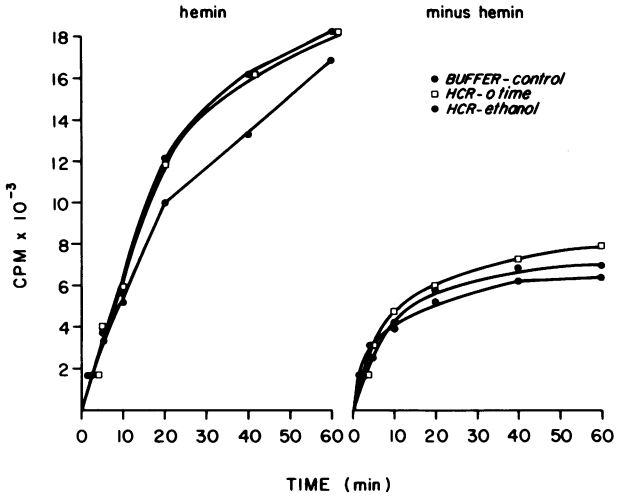

FIGURE 6 The effect of HCR from reticulocytes obtained 5 min after in vivo injection of ethanol (0.4 ml/kg). The control is the reticulocyte lysate cell-free system without any additions except buffer. The 0-time HCR was from the rabbit reticulocytes obtained before alcohol injection, the ethanol HCR from reticulocytes from the same rabbit 5 min after alcohol injection. Each point is the mean of five separate experiments.

synthesis when intact reticulocytes, obtained 5 min after completion of the alcohol injection, were incubated with iron-transferrin and without hemin. This inhibition of early rate could also be demonstrated in reticulocytes obtained up to 60 min after ethanol injection. When these cells from ethanol-treated rabbits were incubated with hemin there was a more rapid return of the rate of protein synthesis to that of the control. When these cells were incubated without iron-transferrin and hemin kinetics identical to that with iron-transferrin were obtained. It was not posible to measure heme synthesis in the presence of hemin due to the above-mentioned hemin inhibition of heme synthesis.

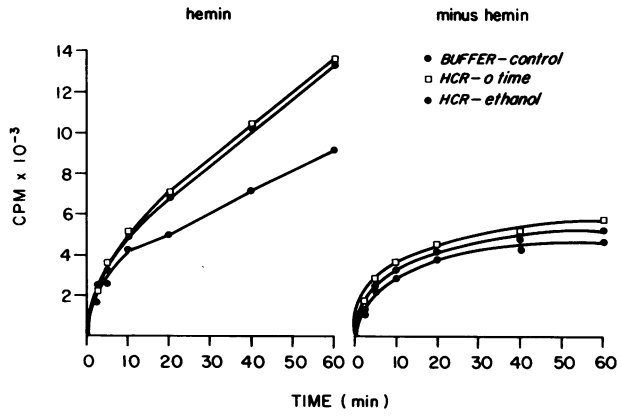

FIGURE 7 The effect of partially purified HCR from reticulocytes obtained 5 min after in vivo injection of ethanol. The collection of reticulocytes was the same as in Fig. 6, but HCR was further purified through a Sephadex G-200 column. Each point is the mean of three separate experiments.

Isolation of HCR from reticulocytes of in vivo ethanol-treated rabbits. Reticulocytes were obtained before and 5 min after in vivo ethanol treatment of rabbits. HCR fractions were isolated from these cells as described in Methods and added to cell-free lysate test systems plus and minus hemin. Fig. 6 shows that addition of an HCR fraction from reticulocytes obtained from the rabbits before the injection of ethanol (0 time) did not inhibit the cell-free system plus and minus hemin. Indeed, there was consistent stimulation of the minus hemin system. In contrast when the same fraction isolated from reticulocytes from the same animal 5 min after ethanol injection was aded to the test systems, inhibition of the rate of protein synthesis was seen in both the plus and minus hemin cell-free systems after approximately a 5-min lag period.

There HCR fractions were then further purified on Sephadex G-200 columns. Consistent inhibition of both the plus and minus hemin test cell-free systems was seen with HCR fractions from reticulocytes obtained 5 min after ethanol injection (Fig. 7). The HCR fractions from reticulocytes before ethanol injection were noninhibitory and were slightly stimulatory, particularly in the minus hemin system.

DISCUSSION

In the present study we have presented direct evidence that both ethanol and the iron-chelating agent α,α-dipyridyl inhibit reticulocyte heme synthesis in vitro before the time that protein synthesis is inhibited. We have confirmed that the inhibition of protein synthesis seen with α,α-dipyridyl is prevented by both hemin and iron-transferrin (7), while that seen with ethanol is prevented only by hemin (20, 21). It has previously been demonstrated that both α,α-dipyridyl (7) and ethanol (21) exposure results in a conversion of polyribosomes to single ribosomes. The effect on polyribosomes is also prevented by hemin in both cases (7, 21), but iron-transferrin only protects against α,α-dipyridyl (7). 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, this disaggregation in heme deficiency has been interpreted as evidence for an inhibition of initiation, with normal elongation and release (7). We have also shown in the present study that this inhibition is not due to decreased ATP or GSH levels. This is particularly important as recent work has shown that when GSH is converted to GSSG there is an inhibition of initiation similar or identical to that of hemin deficiency (29, 30).

We have also confirmed that reticulocyte lysate cell-free systems require hemin for maximal protein synthesis beyond the first 2–5 min of incubation (7–13). Considerable evidence has been presented from different laboratories and confirmed in this study showing that in the absence of hemin in the cell-free incubation, a hemin-controlled translational repressor (HCR) with an approximate molecular weight of 3×10^5 forms at the same time protein synthesis stops (15–19). In the present study we present evidence that intact reticulocytes do not contain HCR when they are incubated with hemin or before they lose their heme-synthesizing capability. On the other hand, when they are made hemin deficient with α,α-dipyridyl or ethanol, HCR forms. When hemin is added together with α,α-dipyridyl or ethanol to the intact cell incubation, the formation of HCR is retarded. These data strongly suggest that HCR is involved in the cessation of intact reticulocyte protein synthesis when heme synthesis is inhibited.

Experiments with in vivo treatment of rabbits with ethanol showed that HCR forms in reticulocytes within 5 min after injection of the alcohol. The reticulocytes obtained under these conditions exhibited a decreased initial rate of both heme and protein synthesis. These experiments are strong evidence for a physiological and pathophysiological role of HCR in cessation of protein synthesis when heme synthesis is inhibited.

HCR has been reported to reduce the level of Met-tRNAf associated with ribosomal subunits (27, 31-32). This repression may be overcome by a ribosomal preparation which has been subfractionated to yield an initiation factor "IF-MP" which mediates binding of MET-tRNAf to the 40S ribosomal subunit (27). Clemens et al. (27) have proposed that HCR directly inhibits IF-MP-dependent binding of Met-tRNAf to the 40S subunit. Balkow et al. (33), however, suggested that the impaired binding is a secondary phenomenon, with the primary effect of HCR being deacylation of the Met-tRNAf on the 40S subunit. To add to the complexity, Raffel et al. (34) have presented evidence that hemin promotes protein synthesis by mediating the formation of an active initiation factor complex (stimulator) from inactive lower molecular weight components. While it is possible that the "inactive component" (minus hemin) is a repressor, and the "active component" (plus hemin) is a stimulator (perhaps even IF-MP), no evidence has yet been presented to this effect. It is clear that the relationship between hemin, initiation factors, repression, and stimulation still needs much more clarification.

The kinetics of inhibition seen with HCR isolated from dipyridyl- and ethanol-treated cells are identical to that described as "hemin-irreversible-repressor" (15, 16, 19). It has been suggested, on the basis of incubation of reticulocyte postribosomal supernates without hemin, that a "hemin-reversible repressor" (15, 16, 19) forms from a pro-inhibitor state. Only with prolonged incubation does this repressor convert to "hemin-irreversible" state (15, 16, 19). However, in the present study when we diluted samples of "irreversible-repressor" (Fig. 1) the kinetics observed in the diluted samples were identical to that of "reversible repressor." These results suggest that the differences in kinetics observed between so-called reversible and irreversible forms of HCR reflect differences in the amounts of the same protein. Since high concentrations of hemin also inhibit protein synthesis (35), it might be that in our test cell-free lysates we cannot add sufficient hemin to reverse the activity of HCR. An alternative explanation is that dilution itself alters the configuration of the inhibitory protein. The data do not allow a choice between these or still other alternatives.

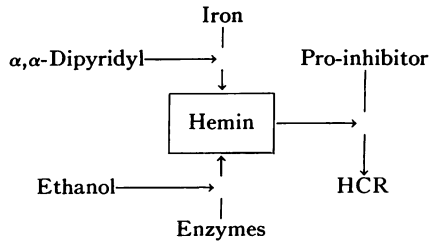

FIGURE 8 Schematic representation of the postulated interaction between α,α-dipyridyl, ethanol, and hemin in reticulocyte protein synthesis. Hemin synthesis requires both iron and enzymes. α,α-Dipyridyl decreases the availability of iron for hemin synthesis, while ethanol presumably is an enzyme inhibitor. When the concentration of hemin is decreased by either agent, HCR formation is not prevented and protein synthesis is inhibited.

Simultaneous addition of hemin with either α,α-dipyridyl or ethanol in vitro did not result in the formation of HCR, even through heme synthesis was inhibited to the same degree. This indicates that these concentrations of α,α-dipyridyl and ethanol do not directly effect initiation, but rather inhibit protein synthesis indirectly via the formation of HCR. Furthermore, these results show that diminished heme synthesis alone does not inhibit protein synthesis if sufficient hemin is present. Heme synthesis is necessary only to maintain a concentration of hemin sufficient to prevent HCR formation.

The effects of α,α-dipyridyl in vitro and in vivo suggest model systems for the study of some of the hypochromic anemias (Fig. 8). In iron deficiency (α,α-dipyridyl experiments), where the cells are depleted of iron for heme synthesis, there would be decreased hemin in the developing red cell, and HCR would form at an earlier time to inhibit protein synthesis. In the anemia of chronic disease, where total body iron is normal, iron availability to marrow cells is diminished (36). Thus, heme synthesis would be decreased, as in iron deficiency

(α,α-dipyridyl experiments), and HCR would form and inhibit protein synthesis. In sideroblastic anemias, as suggested by the ethanol experiments, even though sufficient iron is available to the cells, heme synthesis is also decreased. HCR would form to inhibit protein synthesis, but iron would accumulate to give the picture of sideroblastic erythropoiesis (*20–22*). This hypothesis does not imply that there is a greater quality of HCR found in these conditions. Since HCR is found in mature erythrocytes (19) and the appearance was accelerated in the in vivo treatment of rabbits with ethanol, it seems that in pathological conditions there is a premature appearance of HCR in developing erythroid cells.

Furthermore, the hemin effect is not specific to the synthesis of globin. Recently, it has been shown that hemin is necessary for maximal protein synthesis of the nonglobin proteins of the reticulocyte (*27, 37-39*), as well as for Krebs II ascites tumor cells (40), platelets (41), and brain and liver cells (34). Thus, one may speculate that the suppression of heme synthesis and enhancement of HCR possibly might have many more complex effects rather than solely the inhibition of hemoglobin synthesis and production of hypochromic red cells.

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