

Control of Protein Synthesis in Rabbit Reticulocytes

INHIBITION OF POLYPEPTIDE SYNTHESIS BY ETHANOL*

(Received for publication, October 31, 1980, and in revised form, February 12, 1981)

Joseph M. Wu

From the Department of Biochemistry, New York Medical College, Valhalla, New York 10595

Ethanol (0.21–0.84 M) added to rabbit reticulocyte lysates results in a 42–89% decrease of polypeptide synthesis following a 60-min incubation at 30 °C. The onset of inhibition is preceded by a 5–15-min lag. Eukaryotic initiation factor 2 (eIF-2) (85% pure, 0.42–4.2 µg added/30-µl assay) or high concentrations of cAMP, MgGTP, and glucose 6-phosphate (0.55–5.0 mM) partially reverse the inhibition by 0.32 M ethanol. Complete reversal of inhibition was observed with fructose 6-phosphate (0.55 mM). Ethanol was also shown to directly inhibit ternary complex formation between eIF-2, GTP, and initiator Met-tRNA (50% inhibition with 0.5 M ethanol). The inhibitory effect of ethanol on polypeptide synthesis, however, appears to be independent of its effect on ternary complex formation, and may be related to the activation of a translational inhibitor. This tentative conclusion is based on the following results. First, when the postribosomal supernatant or unfractionated lysate is incubated with ethanol, the supernatant or lysate becomes inhibitory to polypeptide synthesis following a 60-min incubation with ethanol at 30 °C. Second, when ethanol-treated postribosomal supernatant is chromatographed on DEAE-cellulose, an inhibitory activity is observed. The peak of inhibition coincides with the elution position of a translational inhibitor termed heme-controlled repressor. Third, pro-heme-controlled repressor becomes more inhibitory to protein synthesis when incubated with ethanol. These findings suggest that ethanol may activate a translational inhibitor by affecting the proper conformational change of a dormant inhibitor.

Excessive and prolonged ethanol ingestion has been amply documented as a causative factor leading eventually to functional and morphological blood abnormalities (1, 2). One of the earliest hematological changes that occur with alcohol ingestion appears to be the vacuolization of primitive bone marrow cells, especially of proerythroblasts (3, 4). For example, infusion of small amounts of alcohol in mothers to delay labor results in vacuolization of red cell precursors in newborn infants (5). White *et al.* have shown that *in vitro* synthesis of globin chains were reduced in reticulocytes of patients with congenital and acquired sideroblastic anemia (6). Subsequently, Ali and Brain demonstrated that the inhibition of

globin synthesis by ethanol in human reticulocytes and bone marrow cells can be reversed by addition of exogenous heme (7). These results suggest that the effect of ethanol on protein synthesis may be coordinated with its effect on heme synthesis.

The interrelationship between ethanol, heme, and protein synthesis has been studied extensively by Freedman *et al.* (8). Using the rabbit reticulocytes and their cell-free extracts as a model system, they have shown that: (a) ethanol inhibits protein synthesis in rabbit reticulocytes subsequent to the inhibition of heme synthesis (8), (b) ethanol inhibits the rate-limiting enzyme, δ -aminolevulinic acid synthetase, of heme biosynthesis (9), and (c) ethanol results in the conversion of polysomes to monosomes in rabbit reticulocytes suggesting that initiation of polypeptides is blocked (8). Because it has been shown that in rabbit reticulocyte lysates, a lack of hemin causes the activation of a preformed translational inhibitor termed heme-controlled repressor (10–12), Freedman and Rosman propose that the primary inhibition of heme synthesis by ethanol results in a secondary activation of HCR,¹ thereby blocking polypeptide initiation (13). Since they also show that hemin addition (50 µM) can only reverse inhibition of polypeptide synthesis by 0.05–0.1 M ethanol, and not inhibition by 0.8 M ethanol, it seems possible that there are at least two different effects of ethanol on polypeptide synthesis.

In this communication, we show that ethanol, at concentrations which are beyond those which are physiologically achievable, directly inhibits the formation of a ternary complex between eIF-2, GTP, and Met-tRNA. Our data also suggest that at these high concentrations, the inhibitory effects of ethanol on protein synthesis may be related to the activation of a translational inhibitor.

MATERIALS AND METHODS

Rabbit reticulocyte lysates were purchased from Clinical Convenience Products (Madison, WI). L-[³⁵S]Methionine and [U-¹⁴C]leucine were obtained from New England Nuclear. Other materials were from sources described previously (14–16). Met-tRNA_i, crude initiation factors, and partially purified eIF-2 were prepared as described (14, 15). Highly purified eIF-2 (85% pure) was a generous gift from Dr. William Merrick (Department of Biochemistry, Case Western Reserve University). Cell-free protein synthesis were done in 30-µl aliquots and 5-µl aliquots were removed at the indicated times to determine the incorporation of radioactive leucine into polypeptides (14). Met-tRNA_i·eIF-2·GTP ternary complex was measured by the retention of the complex on Millipore filters (15). Pro-HCR was obtained according to the procedure of Gross by chromatography of the postribosomal supernatant on CM-Sephadex (17).

Chromatography of N-Ethylmaleimide or Ethanol-treated Postribosomal Supernatant—Postribosomal supernatant (4.5 ml) was made 5 mM in N-ethylmaleimide and incubated for 15 min, 37 °C. The incubated mixture was made 7.5 mM in dithiothreitol and incubated for an additional 15 min, 4 °C. Alternatively, postribosomal supernatant (supplemented with 50 µM hemin) was incubated with 0.84 M ethanol for 60 min, 30 °C. Treated postribosomal supernatant was applied separately to identical DEAE-cellulose columns (1.5 × 12 cm) previously equilibrated with Buffer A (20 mM Tris-HCl, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol, pH 7.5) and eluted with Buffer A. Fractions (4.5 ml) were collected until no more protein was eluted (as monitored by absorbance at 280 nm). The column was further eluted with Buffer A supplemented with 250 mM KCl and 4.5-ml fractions were collected. Aliquots of each fraction was

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ The abbreviations used are: HCR, heme-controlled repressor; Met-tRNA_i, initiator tRNA; eIF-2, eukaryotic initiation factor 2.

assayed for inhibitory activity using fresh reticulocyte lysates (2 μ l/30- μ l assay).

Treatment of pro-HCR with Ethanol—Pro-HCR (1.8 mg/ml) was incubated with or without 0.84 M ethanol for 60 min, 30 °C (total volume 0.5 ml). Following incubation, the mixture was dialyzed against 2 changes of 500 ml of Buffer A for 90 min, 4 °C. Aliquots of the dialyzed pro-HCR was tested for inhibitory activity in a fresh reticulocyte lysate.

RESULTS AND DISCUSSION

Fig. 1A shows the effect of varying concentrations of ethanol on the kinetics of leucine incorporation into polypeptides. Ethanol (at all concentrations tested) has minimal effect on the initial rate of polypeptide synthesis (0–5 min), but greatly reduces the rate after 5–15 min. A 50% inhibition is observed with 0.21 M ethanol following a 60-min incubation. The two-phase kinetics has been typically observed in rabbit reticulocyte lysates which lack hemin, or in lysates to which GSSG or double-stranded RNA has been added (18–20).

Because it is generally believed that a key step in the regulation of eukaryotic peptide chain initiation is the formation of a ternary complex between eIF-2, GTP, and Met-tRNA_i (21–23), we investigate the possibility that ethanol directly interferes with ternary complex formation. Results in Table I show that ethanol reduces ternary complex formation.

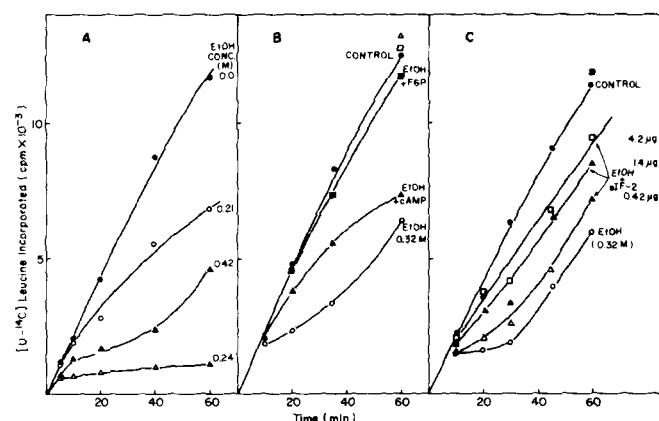


FIG. 1. Effects of ethanol on kinetics of leucine incorporation in reticulocyte lysates. Incubations were at 30 °C and 5- μ l aliquots were removed at times indicated. A: \bullet — \bullet , control; \circ — \circ , plus 0.21 M EtOH; \triangle — \triangle , plus 0.42 M EtOH; \square — \square , plus 0.84 M EtOH. B: \bullet — \bullet , control; Δ — Δ , control plus cAMP (5 mM); \square — \square , control plus fructose-6-P (F6P) (0.5 mM); \circ — \circ , plus 0.32 M EtOH; \blacktriangle — \blacktriangle , EtOH plus cAMP (5 mM); \blacksquare — \blacksquare , EtOH plus fructose-6-P (0.5 mM). C: \bullet — \bullet , control; \blacksquare — \blacksquare , control plus 4.2 μ g of eIF-2; \circ — \circ , plus 0.32 M EtOH; \triangle — \triangle , EtOH plus eIF-2 (0.42 μ g); \blacktriangle — \blacktriangle , EtOH plus eIF-2 (1.4 μ g); \square — \square , EtOH plus eIF-2 (4.2 μ g).

TABLE I

Effect of ethanol and methanol on ternary complex formation by eIF-2 from rabbit reticulocyte lysates

Addition	[³⁵ S]Met-tRNA _i bound ^a
	pmol
Plus eIF-2 (1.2 μ g)	0.23
Plus eIF-2 and ethanol	
0.34 M	0.13
1.36 M	0.07
3.40 M	0.03
Plus eIF-2 and methanol	
0.5 M	0.21
2.0 M	0.13
5.0 M	0.13

^a GTP-dependent binding of [³⁵S]Met-tRNA_i (1.2 pmol, 25,000 cpm, 95% 5% trichloroacetic acid-precipitable) was assayed in a 50- μ l reaction. Background without eIF-2 (0.01 pmol) has been subtracted.

TABLE II

Effects of nucleotides and phosphorylated sugars on inhibition of polypeptide synthesis by ethanol in rabbit reticulocyte lysates

Addition	Polypeptide synthesis ^a	
	–Ethanol	+Ethanol ^b
	cpm	
None	17,480	10,560
+ cAMP/ (5 mM)	18,560	13,700
+ MgGTP (1.67 mM)	19,370	15,810
+ 2-Aminopurine (2.5 mM)	17,510	8,100
+ Glucose 6-phosphate (0.55 mM)	22,170	15,860
+ Fructose 6-phosphate (0.55 mM)	20,060	18,830
+ Fructose 1,6-diphosphate (0.55 mM)	19,720	8,750

^a Incubations were at 30 °C, 60 min. Ten aliquots were removed and processed for leucine incorporation into polypeptides.

^b Ethanol was added to a final concentration of 0.32 M.

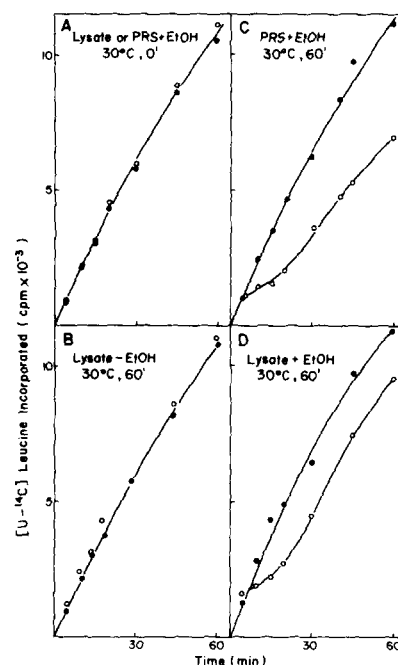


FIG. 2. Effects of ethanol-treated lysate or postribosomal supernatant on polypeptide synthesis in fresh reticulocyte lysates. \bullet — \bullet , fresh lysate plus aliquot of lysate or postribosomal supernatant (PRS) not treated with EtOH; \circ — \circ , fresh lysate plus EtOH-treated lysate or postribosomal supernatant.

A 50% inhibition was observed with the addition of 0.5 M ethanol. At the same concentration, methanol has no apparent effect on ternary complex formation (Table I).

If polypeptide synthesis inhibition by ethanol is totally due to its effect on ternary complex formation, addition of eIF-2 should reverse the inhibition. Consequently, a number of experiments were designed to test this possibility. Results in Fig. 1C show that highly purified eIF-2 (85% pure) gives a significant reversal of inhibition by 0.32 M ethanol. High concentrations of cAMP, MgGTP, and glucose 6-phosphate were equally as effective (Table II). Complete reversal of inhibition was observed with fructose 6-phosphate (Fig. 1B, Table II). In contrast, fructose 1,6-diphosphate or 2-aminopurine were without effect (Table II). Measurement of ethanol at 0 or 60 min shows that its concentration remains constant throughout the incubation period.² Since ternary complex

² The concentrations of ethanol in samples without cAMP or with cAMP (5 mM) or with fructose-6-P (0.55 mM) at 0 or 60 min were 0.316 M, 0.32 M, 0.34 M, 0.33 M, 0.33 M, 0.337 M, respectively.

inhibition by ethanol was not reversed by these compounds (data not shown), this suggests that ethanol must exert another effect in translational inhibition which is independent of ternary complex inhibition.

We next consider the possibility that ethanol activates a translational inhibitor. This was tested by incubating lysates or postribosomal supernatant (both supplemented with 50 μ M hemin to suppress HCR activation) with 0.84 M ethanol for 0 or 60 min at 30 °C. An aliquot of the diluted incubated lysate or postribosomal supernatant (ethanol concentration was 0.09 M after dilution and was not inhibitory to protein synthesis) was then added to fresh reticulocyte lysate to test for inhibitory activity (Fig. 2). No inhibition was evident in the samples incubated with ethanol for 0 min (Fig. 2A) or without ethanol for 60 min (Fig. 2C). However, following a 60-min incubation, lysates or postribosomal supernatant treated with ethanol becomes inhibitory (Fig. 2, B and D). The inhibition was characterized by an interesting pattern of leu-

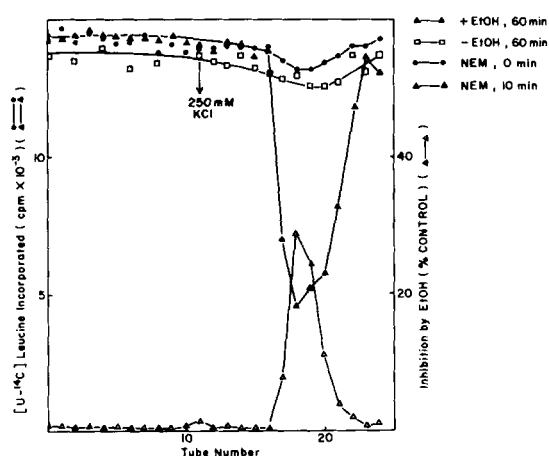


FIG. 3. Chromatography of *N*-ethylmaleimide- or EtOH-treated postribosomal supernatant on DEAE-cellulose. For details, see "Materials and Methods." Inhibition by ethanol was measured following a 20–30-min incubation at 30 °C whereas inhibition by *N*-ethylmaleimide (NEM)-treated samples was measured following a 60-min incubation at 30 °C.

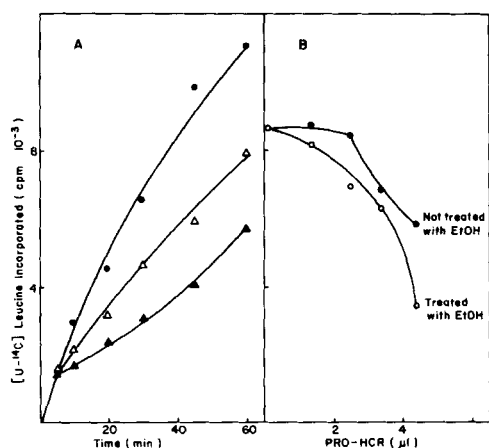


FIG. 4. Effects of ethanol-treated pro-HCR on polypeptide synthesis in fresh reticulocyte lysates. The protein concentration of pro-HCR after dialysis was 1.6 mg/ml. A: ●—●, control; △—△, plus pro-HCR incubated for 60 min at 30 °C without EtOH and then dialyzed (4 μ l/30- μ l assay); ▲—▲, plus ethanol-treated, dialyzed pro-HCR (4 μ l/30- μ l assay). B: varying amounts of non-EtOH-treated, dialyzed pro-HCR were tested for inhibition of protein synthesis in fresh reticulocyte lysates. Incubations were for 60 min, 30 °C.

cine incorporation into polypeptides. There was no inhibition for the first 5–10 min. This was followed by a period of 10–15 min when leucine incorporation was reduced by 80%. Subsequently, at 20–30 min, the rate increased to the control rate. Previously, Balkow *et al.* have reported on the same type of kinetics of protein synthesis (24) and have suggested that the transient inhibition may involve the activation of the "heme-reversible form" HCR (24). Since the conversion of pro-HCR to HCR has been suggested to involve a conformational change (25, 26), it seems possible that a similar conformational change may result from the addition of ethanol. We have further examined whether ethanol can activate a HCR-like translational inhibitor by two different types of experiments. First, when ethanol-treated postribosomal supernatant was chromatographed on DEAE-cellulose, a peak of inhibitory activity was observed which coincided with the elution position of HCR (Fig. 3). Second, to test directly whether ethanol can activate a translational inhibitor, pro-HCR (isolated by CM-Sephadex chromatography) was incubated with ethanol at 30 °C for 60 min. Ethanol was subsequently removed by dialysis (see "Materials and Methods"). Results in Fig. 4 clearly show that without treatment with ethanol, some inhibition was observed in the pro-HCR preparation following dialysis. However, the inhibitory activity was greatly increased by prior treatment of pro-HCR with ethanol. These observations strongly suggest that ethanol, at concentrations which are unlikely to be physiologically achievable, can directly activate a translational inhibitor in the lysate. Although these results may not be related to the physiological effects of ethanol, they are of significance in that ethanol may be used to investigate the various conformational aspects associated with the activation of a translational inhibitor in the lysate. Experiments are presently in progress to further purify this inhibitor and to study the mechanism whereby it inhibits protein synthesis in rabbit reticulocyte lysates.

REFERENCES

- McCurdy, P. R., Pierce, L. E., and Rath, C. E. (1962) *N. Engl. J. Med.* **266**, 505–507
- McCurdy, P. R., and Rath, C. E. (1980) *Semin. Hematol.* **17**, 100–102
- Eichner, E. R., and Hillman, R. S. (1971) *Am. J. Med.* **50**, 218–232
- Sullivan, L. W., Adams, W. H., and Liu, Y. K. (1977) *Blood* **49**, 197–207
- Loz, R., and Montoya, M. (1971) *J. Pediatr.* **79**, 1008–1016
- White, J. M., Brain, M. C., and Ali, M. A. M. (1971) *Br. J. Haematol.* **20**, 263–269
- Ali, M. A. M., and Brain, M. C. (1974) *Br. J. Haematol.* **28**, 311–316
- Freedman, M. L., Cohen, H. S., Rosman, J., and Forte, F. J. (1975) *Br. J. Haematol.* **30**, 351–362
- Ibrahim, N. G., Spieler, P. J., and Freedman, M. L. (1979) *Br. J. Haematol.* **41**, 235–243
- Hunt, T. (1976) *Br. Med. Bull.* **32**, 257–261
- Revel, M., and Groner, Y. (1978) *Annu. Rev. Biochem.* **47**, 1079–1126
- Safer, B., and Anderson, W. F. (1978) *Crit. Rev. Biochem.* **5**, 261–290
- Freedman, M. L., and Rosman, J. (1976) *J. Clin. Invest.* **57**, 594–603
- Wu, J. M., Cheung, C. P., and Suhadolnik, R. J. (1978) *J. Biol. Chem.* **253**, 7295–7300
- Wu, J. M. (1980) *Biochem. Biophys. Res. Commun.* **92**, 452–458
- Wu, J. M., and Ibrahim, N. G. (1980) *FEBS Lett.* **119**, 25–28
- Gross, M. (1977) *Arch. Biochem. Biophys.* **180**, 121–129
- Kosower, N. S., Vanderhoff, G. A., and Kosower, E. M. (1972) *Biochim. Biophys. Acta* **272**, 623–637
- Hunt, T., and Ehrenfeld, E. (1971) *Nature* **230**, 91–94
- Wu, J. M., Cheung, C. P., Bruzel, A. R., and Suhadolnik, R. J. (1979) *Biochem. Biophys. Res. Commun.* **86**, 648–653

21. Levin, D. H., Kyner, D., and Aces, G. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 41-45
22. Schreiber, M. H., and Staehelin, T. (1973) *Nature New Biol.* **242**, 35-38
23. Dasgupta, A., Das, A., Roy, R., Ralston, R., Majumdar, A., and Gupta, N. K. (1978) *J. Biol. Chem.* **253**, 6054-6059
24. Balkow, K., Hunt, T., and Jackson, R. J. (1975) *Biochem. Biophys. Res. Commun.* **67**, 366-375
25. Gross, M., and Rabinovitz, M. (1972) *Biochim. Biophys. Acta* **287**, 340-352
26. Trachsel, H., Ranu, R. S., and London, I. M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3654-3658