British Journal of Haematology, 1974, **28**, 311.

Ethanol Inhibition of Haemoglobin Synthesis: *In Vitro* Evidence for a Haem Correctable Defect in Normal Subjects and in Alcoholics

M. A. M. Ali and M. C. Brain

Departments of Laboratory and Internal Medicine, McMaster University, Hamilton, Ontario, Canada

(*Received* 8 *February* 1974; *accepted for publication* 2 *May* 1974)

Summary. The effect of ethanol in different concentrations ranging from 0.10 to 0.66 M on globin chain synthesis *in vitro* was studied by measuring ^{3}H-leucine incorporation into human bone marrow suspensions and reticulocyte-enriched blood. With increasing concentration of ethanol there was a decreasing ^{3}H-leucine incorporation. This inhibition was reversed by haem and pyridoxine hydrochloride (PHC) in 1 mM concentration. 5-Aminolaevulinic acid (5-ALA) at similar concentration had no significant effect. The addition of haem to reticulocyte-enriched blood obtained from acute alcoholics increased globin-chain synthesis by 20% whereas PHC and ALA were without effect and none of the additions increased globin synthesis by normal reticulocytes. These data provide direct evidence for the inhibitory action of ethanol on haemoglobin synthesis and suggest that the effect is mediated through inhibition of haem synthesis at a site influenced by pyridoxine.

Transient sideroblastic erythropoiesis is a recognized complication of prolonged ethanol ingestion (Hines, 1969; Hines & Cowan, 1970). Impaired *in vitro* synthesis of globin chains by reticulocytes from patients with congenital and acquired (non-alcoholic) sideroblastic anaemia has been demonstrated by White *et al* (1971). The addition of haem and, in some patients, the addition of 5-ALA significantly increased globin synthesis. In order to elucidate the nature of the ethanol-induced erythropoietic disturbance, the effect of ethanol on globin synthesis by reticulocytes and bone marrow from normal subjects and from patients admitted with acute alcoholism, was studied. Furthermore, the addition of haem, PHC and 5-ALA in the presence of ethanol was investigated. The purpose of this report is to provide evidence for a direct inhibitory effect of ethanol on the haem synthetic pathway.

MATERIALS AND METHODS

Thirty ml of venous blood were collected from laboratory volunteers or from patients with elevated reticulocyte counts into heparin (0.5 mg/ml). After washing three times in isotonic

Correspondence: Dr M. A. M. Ali, Department of Laboratory Medicine, St Joseph's Hospital, 50 Charlton Avenue East, Hamilton, Ontario, Canada L8V 2B2.

saline, 10 ml packed cells were centrifuged at 15 000 *g* for 60 min and the top 2 ml were removed for incubation. The reticulocyte count in these samples ranged between 8 and 24% and was usually three times that of the uncentrifuged blood. Bone marrow samples were obtained from patients with normoblastic erythropoiesis during routine diagnostic procedures. The marrow specimens were collected in heparin and washed three times in isotonic saline.

The reticulocyte-enriched blood and bone marrow samples were suspended in the incubation mixture described by Lingrel & Borsook (1963) omitting leucine, and divided into 1 ml aliquots. Ethanol in a final concentration of between 0.10 and 0.66 M was added in a final volume of 4.00 ml, to all but one (control) aliquot of the incubation mixture, to which was added an equal volume of the patient's plasma. After incubating all the samples for 120 min at 37°C, 100 μCi of ^{3}H-leucine was added and, after mixing, the incubation continued for a further 120 min. Incubation was terminated by washing the cells with isotonic saline at 4°C and the globin was precipitated by the addition of cold acid-acetone as described by White & Ali (1973).

The α- and β-globin chains were separated on carboxymethyl cellulose (CM23) columns with 8 M urea and mercaptoethanol according to the method of Clegg *et al* (1966). The whole of each chain was collected and dialysed against three changes of 2 litres of 0.5% formic acid for 48 hr at 4°C. A 1 ml sample was added to 10 ml of Bray's solution and counted in a Philips liquid scintillation counter (model PW 4570) under a counting efficiency of 25–35%. The total counts incorporated into the globin chains were calculated.

To study the effect of the addition of haem, PHC and 5-ALA on the synthesis of globin in the presence of ethanol the following experiments were performed. The bone marrow samples or the reticulocyte-enriched blood were divided into five equal aliquots. The first aliquot, incubated without ethanol, was the control sample. To the second aliquot ethanol was added in a concentration of 0.33 M in the bone marrow incubations and 0.1 M in the reticulocyte incubations. The third, fourth and fifth aliquots were incubated with haem, PHC and 5-ALA, concentration 1 mM, in addition to the appropriate ethanol concentration. All the samples were then incubated for 120 min at 37°C following which ^{3}H-leucine was added and the incubation continued for a further 120 min. The samples were then washed in isotonic saline and globin was separated as described by White & Ali (1973). The ^{3}H-leucine incorporation in the control specimen was considered to represent 100%, and the results were expressed as % inhibition or stimulation of the first control specimen.

Reticulocyte-enriched blood was obtained from five normal laboratory volunteers and from five patients who were admitted with the diagnosis of acute alcoholism. The ethanolic intake was confirmed by history and physical examination but blood alcohol level was not available.

Tritiated leucine incorporation into globin was determined in the control sample and after the addition of haem, PHC and 5-ALA in 1 mM concentration and results were similarly expressed as % inhibition or stimulation of the first control sample.

RESULTS

Ethanol consistently inhibited the incorporation of ^{3}H-leucine into globin at four different concentrations: 0.10, 0.20, 0.33 and 0.66 M. Table I summarizes the finding in four bone

marrow and two reticulocyte-enriched blood incubations. Inhibition of ^{3}H-leucine incorporation was noticeable at an ethanol concentration of 0.10 M and was marked at 0.66 M. This effect was reproducible and constant both in bone marrow and reticulocyte-enriched blood. The inhibition appeared to be dose related and the higher the concentration of ethanol the more severe was the depression in globin synthesis.

TABLE I. Effect of ethanol on triated leucine incorporation into globin

	Control		*Ethanol* (M)							
			0.10		0.20		0.33		0.66	
Experiment	*dpm/mg globin*	*% Inhibition*	*dpm/mg globin*	*% Inhibition*	*dpm/mg globin*	*% Inhibition*	*dpm/mg globin*	*% Inhibition*	*dpm/mg globin*	*% Inhibition*
Bone marrow	2840	100	2270	74	1815	63	1505	52	225	3
Bone marrow	810	100	585	82	610	75	260	32	120	14
Bone marrow	990	100	900	90	585	89	345	34	60	6
Bone marrow	12675	100	9760	77	8745	68	6085	48	2660	10
Reticulocyte	165	100	140	84	125	75	65	34	10	6
Reticulocyte	215	100	185	86	165	76	95	44	40	18
Mean		100		83		69		41		11
Significance from control			$P<0.01$		$P<0.001$		$P<0.002$		$P<0.001$	

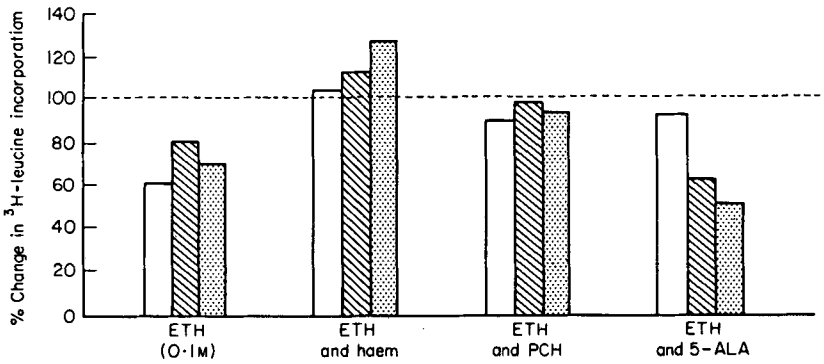

FIG 1. Effect of haem, PHC and 5-ALA on ethanol inhibition of globin synthesis by reticulocyte-enriched blood.

The effect of adding haem, PHC and 5-ALA on the ethanol inhibition of globin synthesis in three reticulocyte-enriched blood samples from non-alcoholic patients is illustrated in Fig 1. ^{3}H-Leucine incorporation in three experiments, when only ethanol was added at a concentration of 0.1 M, was 59, 82 and 71% of the control samples. Haem corrected that inhibition in all the three experiments to 103, 112 and 128% of the control experiment. PHC was similarly effective in abolishing the ethanol inhibition and ^{3}H-leucine incorporation into globin which increased to 91, 98 and 96% of the control experiment. However, 5-ALA

increased the ^{3}H-leucine incorporation from 59 to 93% of the control value in one experiment, but was without effect in the other two experiments which remained at 61 and 52% of the control value.

In the four marrow experiments a higher ethanol conentration was used (0.33 M) and globin synthesis was depressed to 51, 58, 49 and 41% of the control value in the four experiments (Fig 2). As with the reticulocyte incubations, haem was effective in abolishing the ethanol inhibition of globin synthesis which increased to 104, 112, 97 and 92% respectively. PHC,

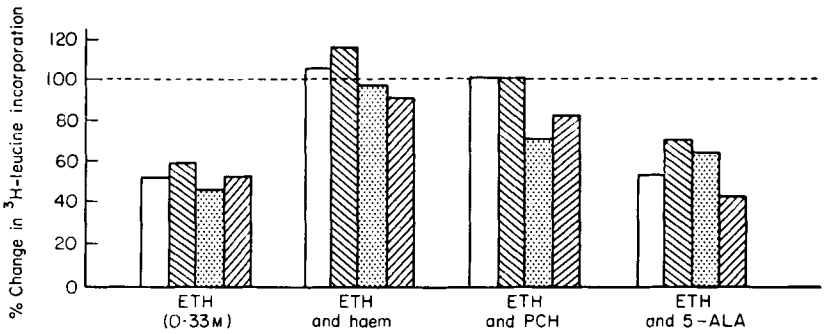

FIG 2. Effect of haem, PHC and 5-ALA on ethanol inhibition of globin synthesis by bone marrow suspensions.

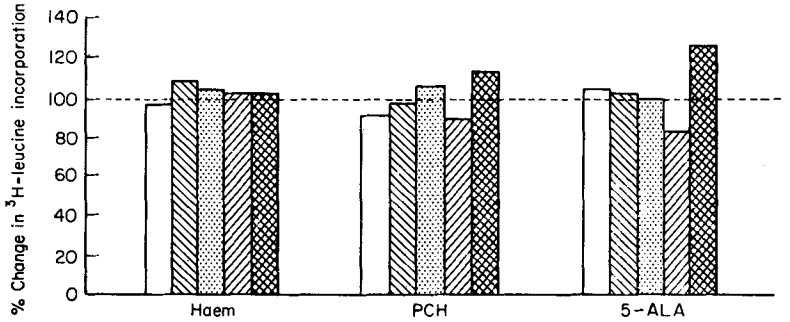

FIG 3. Effect of haem, PHC and 5-ALA or tritiated leucine incorporation into globin chains from normal reticulocytes.

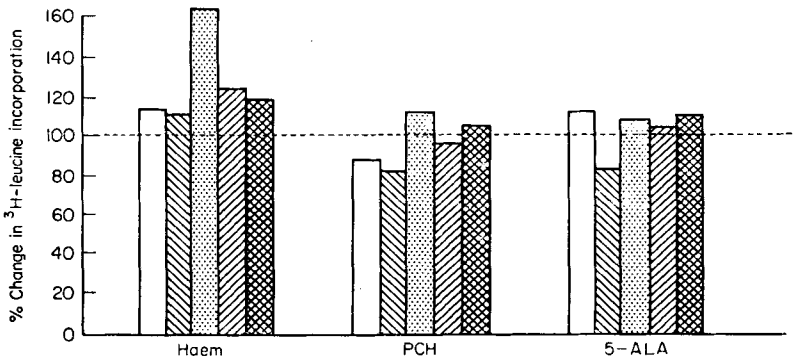

FIG 4. Effect of haem, PHC and 5-ALA on tritiated leucine incorporation into globin chains from reticulocytes obtained from patient with acute alcoholism.

however, counteracted the ethanol in two out of the four incubations which was elevated to 100% in both experiments and was without effect on the other two experiments which remained at 69 and 78% of the control value. 5-ALA failed to overcome the ethanol action in all the four incubations which remained at 51, 68, 61 and 42% of the control experiments.

The incorporation of tritiated leucine into the reticulocytes of the five normal volunteers is shown in Fig 3, where haem, PHC and 5-ALA had no significant effect on tritiated leucine incorporation. Haem, however, increased tritiated leucine incorporation into globin in all the five experiments using reticulocytes from alcoholic patients, which increased to 115, 113, 162, 122, 120% of the control value while PHC and 5-ALA had no significant effect (Fig 4).

DISCUSSION

Ethanol ingestion has been implicated in the development of a number of haematological abnormalities including thrombocytopenia (Sullivan & Herbert, 1964), megaloblastic anaemia (Herbert *et al*, 1958) and transient haemolysis with hyperlipaemia (Zieve, 1958). Of particular interest is the reversible type of sideroblastic erythropoiesis reported by Hines (1969) and postulated to result from abnormal pyridoxine metabolism, possibly at the stage of conversion of pyridoxine phosphate, the coenzyme for 5-ALA synthetase.

The results reported in this study provide direct evidence for the suppressive action of ethanol on globin synthesis at a concentration of ethanol well above the level encountered in acute alcoholic intoxication, although a concentration of 0.1 M has been reported in severe acute alcoholic intoxication. However, the other concentrations used, namely 0.20, 0.33 and 0.66 M, far exceed the levels attained *in vivo*, and the fatal ethanol concentration is reported to be between 0.15 and 0.20 M (Harger, 1961). That the effect of ethanol on tritiated leucine incorporation is probably secondary to the suppression of haem synthesis is supported by the finding that the addition of exogenous haem (1 mM) to reticulocytes or bone marrow cells from normal and alcoholic patients abolished the inhibitory action of ethanol at two different concentrations, viz 0.10 and 0.33 M. In spite of the pharmacological concentration of ethanol used in the bone marrow incubations, haem prevented the globin synthesis inhibition in all four bone marrow experiments, while PHC was only effective in conteracting the ethanol action in two out of the four experiments and was without effect on the reticulocytes obtained from alcoholic patients.

It is interesting that 5-ALA partially prevented the suppression of ethanol in only one out of the three reticulocyte incubations and was completely ineffective in the bone marrow experiments, for it raises the possibility that pyridoxine may be required for enzymes other than 5-ALA synthetase. Hines & Cowan (1970) have demonstrated in three alcoholic volunteers that excessive ingestion of alcohol affected the conversion *in vivo* of pyridoxine to pyridoxine-5-phosphate (PP). This in turn resulted in depressed haem synthesis and induction of marrow sideroblastic alterations. Our data lend partial support to the hypothesis that alcohol interferes with pyridoxine metabolism as PHC was effective in abolishing the ethanol inhibition in the three reticulocyte incubations and in two out of the four bone marrow experiments. However, the exact role of alcohol in producing these changes needs further clarification.

Further evidence of a disturbance in the haem synthetic pathway due to ethanol was the

finding of raised erythrocytic coproporphyrin and protoporphyrin levels in 11 patients with sideroblastic erythropoiesis, whereas normal values were found in the 15 alcoholic patients with normoblastic erythropoiesis (Ali & Sweeney, 1973).

No definite etiological relationship between the ethanol effect on globin synthesis, the accumulation of iron in the mitochondria and the formation in the marrow of ring sideroblasts could be established in this study. Rubin *et al* (1972) have exposed hepatic mitochondria to ethanol (0.1 M) *in vitro* and reported that the incorporation of ^{14}C-leucine into mitochondrial protein obtained from normal rats was inhibited by 20%. Amino acid incorporation by the isolated inner membrane fraction was also reduced by ethanol. Furthermore, Rubin *et al* (1970) have been able to show that ethanol (0.1 M) *in vitro* decreased the activity of cytochrome P450 reductase and inhibited hepatic microsomal drug metabolizing enzymes.

It is tempting to postulate from the data of Rubin *et al* (1970, 1972) and from our own results that ethanol may interfere with the mitochondrial function of the developing normoblasts in the bone marrow through inhibition of the haem synthetic pathway. This could inhibit cellular respiration and lead to mitochondrial damage, iron accumulation and sideroblastic erythropoiesis.

ACKNOWLEDGMENTS

We are most grateful for the technical assistance of Miss Angela Quinlan. This work was supported by Grant No. MA-4461 from the Medical Research Council of Canada.

REFERENCES

Ali, M.A.M. & Sweeney, G. (1973) Erythrocyte coproporphyrin and protoporphyrin in ethanol-induced sideroblastic erythroporesis. *Blood*, **43**, 291.

Bray, G.A. (1960) A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analytical Biochemistry*, **1**, 279.

Clegg, J.B., Naughton, M.A. & Weatherall, D.J. (1966) Abnormal human hemoglobins. Separation and characterization of the α and β chains by chromatography, and the determination of two new variants, Hb Chesapeak and Hb J (Bangkok). *Journal of Molecular Biology*, **19**, 91.

Harger, R.N. (1961) Ethyl alcohol. *Toxicology* (Ed. by C. P. Stewart and A. Stolman), Vol. 2, pp 85–115. Academic Press, New York.

Herbert, V., Zalusky, R. & Davidson, C.S. (1958) Correlation of folate deficiency with alcoholism and associated macrocytosis, anemia and liver disease. *Annals of Internal Medicine*, **58**, 471.

Hines, J.D. (1969) Reversible megaloblastic and sideroblastic marrow abnormalities in alcoholic patients. *British Journal of Haematology*, **16**, 87.

Hines, J.D. & Cowan, D.H. (1970) Studies on the pathogenesis of alcohol-induced sideroblastic bone-marrow abnormalities. *New England Journal of Medicine*, **283**, 441.

Lingrel, J.B. & Borsook, H. (1963) A comparison of amino acid incorporation into the hemoglobin and ribosomes of marrow erythroid cells and circulating reticulocytes of severely anemic rabbits. *Biochemistry*, **2**, 309.

Rubin, E., Beattie, D.S., Toth, A. & Lieber, C.S. (1972) Structural and functional effects of ethanol on hepatic mitochondria. *Federation Proceedings*, **31**, 131.

Rubin, E., Gang, H., Misra, P.S. & Lieber, C.S. (1970) Inhibition of drug metabolism by acute ethanol intoxication: a hepatic microsomal mechanism. *American Journal of Medicine*, **49**, 801.

Sullivan, L.W. & Herbert, V. (1964) Suppression of hematopoiesis by ethanol. *Journal of Clinical Investigation*, **43**, 2048.

White, J.M. & Ali, M.A.M. (1973) Globin synthesis in sideroblastic anaemia. II. The effect of pyridoxine, δ-aminolaevulinic acid and haem, *in vitro*. *British Journal of Haematology*, **24**, 481.

White, J.M., Brain, M.C. & Ali, M.A.M. (1971) Globin synthesis in sideroblastic anaemia. I. α and β peptide chain synthesis. *British Journal of Haematology*, **20**, 263.

Zieve, L. (1958) Jaundice, hyperlipemia and hemolytic anemia: a heretofore unrecognized syndrome associated with alcoholic fatty liver and cirrhosis. *Annals of Internal Medicine*, **48**, 471.