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EFFECT OF ETHANOL METABOLISM ON INITIATION OF PROTEIN SYNTHESIS IN RAT HEPATOCYTES

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Abstract—Rat liver parenchymal cells were incubated in the presence and absence of ethanol (80 mM). Polysomes were isolated and analysed on sucrose gradients. Ethanol was shown to (1) inhibit the incorporation of ^{14}C-valine into proteins, (2) result in a shift in the distribution of polysomes towards smaller sizes, (3) inhibit the formation of 40S initiation complexes, and (4) diminish the concentration of glucose-6-phosphate in the hepatocytes. Addition of 4-methylpyrazole (0.5 mM) partially prevented the inhibition of protein synthesis and completely restored the polysomal distribution. It is concluded that ethanol inhibits protein synthesis partly by a mechanism linked to ethanol metabolism. This effect takes place at the level of initiation and may be mediated by a reduced gluconeogenesis.

The effect of ethanol on protein metabolism in isolated parenchymal cells has been studied by several groups [1–6], and it is generally accepted that ethanol inhibits the incorporation of radioactive amino acids into proteins. Two of the reports, however, claim that inhibition takes place only at very high concentrations of ethanol, 100–300 mM, corresponding to 0.5–1.6 vol.% [2, 6]. Effects on incorporation have earlier been shown to reflect effects on protein synthesis [1, 7]. The inhibitory effect of low concentrations of ethanol (8 mM) appears to be a result of ethanol metabolism, while the effect of higher concentrations (16–65 mM) seems to be caused also by ethanol *per se* [7]. Since regulation of protein synthesis in many systems takes place at the level of initiation [8–10], we found it to be of interest to ascertain whether this is also the case for ethanol in its effect on hepatocytes, and whether the metabolic component of its effect and the direct effect in this respect were similar or different. In the present study this problem was approached through a study of the effect of ethanol on labelled valine incorporation, polysome distribution and 40S initiation complex formation. 4-MP, an inhibitor of alcohol dehydrogenase (EC 1.1.1.1, alcohol: NAD^+ oxidoreductase), was present in some of the experiments, in order to separate direct and metabolic effects of ethanol.

MATERIALS AND METHODS

Animals. Male Wistar rats, 200–280 g, were maintained on a 12-hr light–12-hr dark cycle and fasted for 24 hr before the experiment.

Chemicals. Collagenase Type 1 (EC 3.4.24.3, clostridiopeptidase A), albumin (A-6003 fatty-acid free), CTAB, 4-MP and sucrose were obtained from Sigma. Other reagents were analytical grade.

Cell preparations. Primary suspensions of liver cells were prepared after perfusion of the liver with a collagenase solution according to Seglen [11] as described previously [12], omitting antibiotics in the media. Amino acids at approximately 1.5 times physiological concentrations [1] and 0.8 mM Mg^{2+} were present in all solutions. After preincubation for 20 min in a buffered salt solution containing 2% albumin [12], the cell suspension was filtered through a nylon mesh, the cells sedimented by centrifugation and resuspended in the same medium to a concentration of 3–8.10^6 cells per ml.

Cell incubation. Volumes of 30 ml cell suspension were transferred to 1 l. flasks and incubated in a shaking incubator at 37° for 85 min. Control and experimental cell suspensions were derived from the same liver and incubated in parallel at an equal cell concentration. In two experiments the pH of one control suspension was lowered to 7.18. L-[U-^{14}C]-valine (New England Nuclear, Boston, MA), final concentration 0.1 μCi/ml, 2.2 mM, specific radioactivity 46 mCi/mol, was added after incubation for 1 min. Ethanol was added after incubation for 15 min as a 10% (v/v) solution in 0.9% saline to a final concentration of 80 mM, saline alone to the control suspensions. In some experiments 4-MP was added to a final concentration of 0.5 mM immediately after the addition of ethanol or saline. Samples of cell suspensions were taken for analysis several times during the incubation as indicated in the text and tables.

Measurement of ^{14}C-valine incorporation and protein. Incorporation of ^{14}C-valine into protein was determined in samples of cell homogenates and media precipitated with 10% TCA on filter-paper discs [13]. The discs were washed and extracted to remove unincorporated activity, dried and counted in a liquid scintillation counter. Protein was determined according to Lowry *et al.* [14].

Isolation of polysomes. Polysomes were isolated largely according to Dickson and Pogson [15]. At the end of the incubation, samples of 8 ml cell suspension were transferred to cold centrifuge tubes and left on

Abbreviations: 4-MP, 4-methylpyrazole; CTAB, cetyltrimethylammonium bromide.

ice for a few minutes. All subsequent operations were performed at 0–2°. Glassware was acid washed and sterilized and solutions autoclaved. The cells were sedimented at 1000 g_{av} for 1 min and homogenized (Potter–Elvehjem 15 strokes) in 2.5 ml buffer A of Dickson and Pogson, containing 3/4 volume of a rat liver high speed supernatant [16], 1.33% Triton X-100 and 0.44% sodium deoxycholate. After centrifugation at 12,000 g_{av} for 5 min the postmitochondrial supernatants were layered on discontinuous sucrose gradients (0.6 ml 1 M sucrose overlaid by 1.1 ml 0.4 M sucrose in Dickson and Pogson buffer B). The gradients were centrifuged at 56,000 rpm (g_{av} 308,000) for 25 min in a Beckman SW 56 rotor, and the polysomal pellets either frozen and stored overnight in liquid nitrogen or resuspended in 200 μl Dickson and Pogson buffer C. The suspensions were clarified by centrifugation at 400 g_{av} for 3 min. 1.5–2.0 A_{260}-units of polysomes were analysed on 4 ml convex exponential (isokinetic) sucrose gradients (15–28%) made up in buffer C [17]. Centrifugation took place at 50,000 rpm (g_{av} 246,000) for 35 min. The gradients were analysed in a Gilford 252 photometer and the A_{260}-profiles recorded and integrated. For calibration of the gradients, a rabbit reticulocyte lysate was used, with sharp peaks in the range monosomes to hexasomes. The sedimentation distances were proportional to $n^{2/3}$ [18], and this was used for extrapolation to higher numbers n of ribosomes in the polysomes. The polysome distribution was expressed as the area corresponding to polysomes greater than trisomes in the per cent of the total ribosomal area (subunits excluded). In one series of experiments we also calculated the areas corresponding to polysomes greater than nonasomes as per cent of total area.

The significance of the differences between the means was evaluated by the Student t-test.

Analysis of ^{35}S-Met-tRNA$_f$-40 S initiation complexes. ^{35}S-Met-tRNA-40 S initiation complexes were analysed in cell suspensions incubated in the presence of ^{35}S-methionine. After preincubation the cells were washed once and resuspended in medium lacking methionine. 125 μCi ^{35}S-methionine (Amersham International Ltd, Amersham, Bucks., U.K.) and ethanol or saline were added to the cell suspensions (20 ml) and incubation continued for 30 min. The suspensions were quickly cooled on ice, and the cells were sedimented and homogenized (Potter–Elvehjem 12 strokes) in 75 mM Tris–HCl pH 7.2, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1.33% Triton X-100 and 0.44% Na-deoxycholate containing 1/2 volume of a rat liver high speed supernatant. The postmitochondrial supernatants were layered on a 4 ml isokinetic sucrose gradient (15–28% sucrose in 10 mM Na-cacodylate pH 6.0, 300 mM KCl and 3 mM $MgCl_2$) and centrifuged at 56,000 rpm for 90 min. The gradients were analysed in a photometer with flowcell as above and the A_{260} profiles recorded. Fractions of 0.25 ml were collected. ^{35}S-Met-tRNA$_f$ was precipitated with CTAB [19], collected on glass-fibre filter and counted in a liquid scintillation counter.

Determination of glucose-6-phosphate. The concentration of glucose-6-phosphate in the hepatocytes was measured enzymatically at the end of the incubation. Twenty-five ml cell suspension was quickly cooled on ice, and the cells sedimented and extracted with 0.6 M $HClO_4$. The supernatants were neutralized and analysed for glucose-6-phosphate with glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. NADH produced was determined fluorimetrically in a Perkin–Elmer 204 S fluorescence spectrophotometer.

Additional procedures. Cell counts were determined with a Bürker chamber and the viability of the cells estimated by the exclusion of trypan blue. Viability was also tested by assay of ATP content, using a Sigma kit. Lactate and pyruvate were analysed by kits from Boehringer Mannheim.

RESULTS AND DISCUSSION

Preparation of polysomes

Two earlier reports on the distribution of polysomes in hepatocytes claim that polysomes in these cells consist of monomers, dimers and smaller aggregates [20, 21]. Recently, however, Dickson and Pogson described a method which yielded highly aggregated polysomes from such cells [15]. The crucial features of their method were the inclusion in the homogenizing medium of a rat liver high speed supernatant which contains a potent RNAse inhibitor, and sedimentation of the polysomes through a two layer discontinuous sucrose gradient. Using this isolation procedure, but displaying on isokinetic rather than linear sucrose gradients, we obtained profiles as shown on Fig. 1. Incubation of the cells for 85 min led to some run off (Fig. 1A). Reduction in the total area may be due to dissociation of monomers formed. Dickson and Pogson describe the polysome conserving effect of glucose, insulin and amino acids in the perfusion buffer. We found well organized polysomes without insulin (Fig. 1A, upper trace). After incubation for 85 min, the proportion of larger polysomes is similar for cells prepared and incubated in 20 mM glucose and 10× physiological concentrations of amino acids (Fig. 1A, lower trace) and cells prepared and incubated without glucose and with normal concentrations of amino acids (Fig. 1B). All experiments described below are of the latter type as these conditions gave a more pronounced effect of ethanol.

Effect of ethanol on protein synthesis

When cell suspensions were incubated in the presence of 80 mM ethanol, the rate of incorporation of ^{14}C-valine into cell proteins was reduced and the distribution of polysomes shifted towards smaller sizes (Table 1). The magnitude of the effect on protein synthesis, 35% reduction, was as observed before [7].

In previous work with hepatocyte preparations identical to those of the present study [1], it was shown that the changes in valine incorporation elicited by ethanol were not a result of changes in valine pools but reflected changes in the rate of protein synthesis. For low external concentrations of amino acids protein degradation supplies most of the amino acids used in protein synthesis. This degradation was not enhanced in the presence of ethanol. The fall in specific radioactivity of valine during the

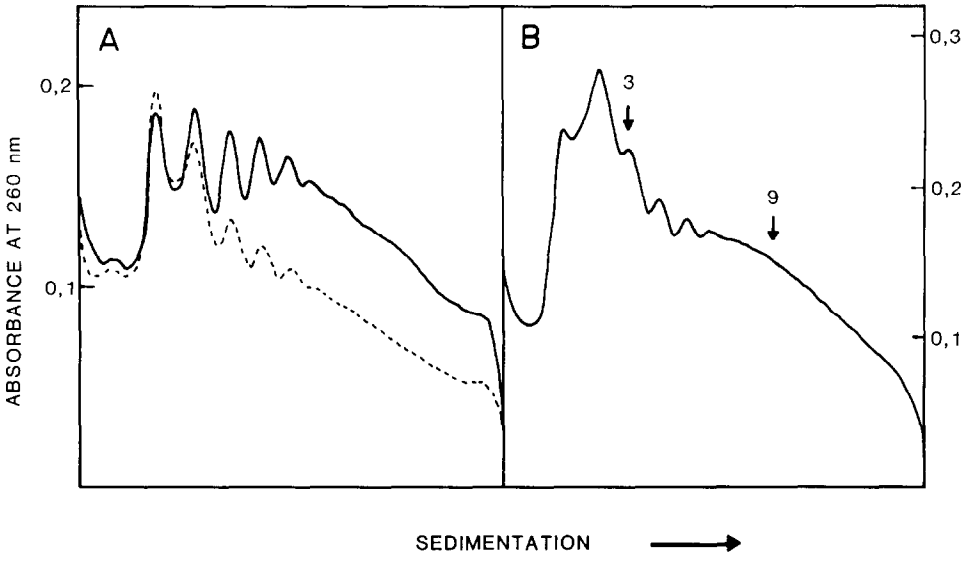

Fig. 1. Polysomal profiles from isolated rat liver hepatocytes. Polysomes were isolated and analysed on 4 ml isokinetic sucrose gradients as described in Materials and Methods. (A) Cells incubated in the presence of 20 mM glucose and 10× physiological concentration of amino acids for 14 min (———) and 85 min (– – –). Polysomes from equal number of cells were used in this experiment. (B) Cells incubated without glucose and in the presence of 1.5× physiological concentration of amino acids for 85 min. Two A_{260}-units of polysomes were loaded on the gradients.

incubation was the same with and without ethanol [1]. The effect of ethanol on incorporation of valine was the same without amino acids added to the external medium, with amino acids added in physiological concentration [1], and with 2, 5 or 10 mM valine added, 2 mM valine giving nearly maximal incorporation (J. Mørland, unpublished observations). In the present experiments ethanol did not affect the concentration of acid-soluble radioactivity at 15 min or later (data not shown). We assume on this background that effects on incorporation also in this study reflect effects on protein synthesis.

The pH of the medium dropped somewhat in the cell suspensions where ethanol had been added, indicating that ethanol had been metabolized (Table 1). No inhibition of incorporation of amino acids was, however, seen in control suspensions (two experiments) in which pH had been lowered to 7.18 (data not shown). This is in accordance with an earlier report [7], and it shows that the effect on protein synthesis is not simply an effect of reduced pH in the medium. The drop in pH was somewhat greater in the series of experiments reported in Table 1 than in Table 2. This was probably a result of a somewhat higher cell concentration in the former case. The average number of cells in the two series of experiments were $5.0 \cdot 10^6$ and $3.9 \cdot 10^6$ cells per ml respectively. The viability of the cells, measured by trypan blue exclusion, was 92.5 ± 0.8% for controls, 91.5 ± 1.1% for ethanol treated cells (mean of 14 experiments ± S.E.). ATP contents were 2.36 ± 0.09 and 2.38 ± 0.06 μmol/g wet weight respectively. There was thus no effect of ethanol on viability.

Effect on protein synthesis initiation

The change observed in polysome distribution (Table 1) is in the direction expected if initiation is inhibited more than elongation [22]. Since some of the larger polysomes under these circumstances would be shifted into the range between nonasomes and trisomes, one would expect the effect to be

Table 1. Effects of ethanol on incorporation of amino acids, distribution of polysomes and medium pH

Group	^{14}C-val incorporation into cell protein (pmoles/mg/min)	Distribution of polysomes		Δ pH*
		% > trisomes	% > nonasomes	
1 Control	61.2 ± 2	54.4 ± 0.4	19.1 ± 0.5	0.04 ± 0.02
2 Ethanol	39.8 ± 2	50.1 ± 0.7†	16.0 ± 0.4	0.25 ± 0.01
Ethanol/control	65%	92%	84%‡	

The incorporation values are means ± S.E.M. of 14 determinations in each group. For some cell suspensions two parallel analyses of polysomes were performed, and the polysomal values are of means of 26 determinations.

* Difference in pH measured after 16 min and 85 min incubation.

† Significant difference ($P < 0.001$) vs control.

‡ Significant difference ($P < 0.001$) vs % > trisome.

greater on the fraction larger than nonasomes than on the fraction larger than trisomes. This was indeed observed.

In order to test more directly whether ethanol inhibits the initiation of protein synthesis, the formation of ^{35}S-Met-tRNA$_f$-40 S initiation complexes were analysed in cell suspensions incubated in the presence and absence of ethanol. As shown in Fig. 2, the presence of ethanol inhibited the formation of 40 S initiation complexes by about 35%. The peak of ^{35}S-Met-tRNA$_f$ activity was found slightly higher in the gradient than the 40 S ribosomal peak. A similar effect has been noted by Trachsel *et al.* [23]. This could possibly be the result of a pressure-induced dissociation of Met-tRNA$_f$ from the ribosomal subunit during ultracentrifugation [24].

Effect of 4-methylpyrazole

4-MP was added to cell suspensions in order to see whether the effects of ethanol on protein synthesis and polysomal distribution were caused by its metabolism. As shown in Table 2, 4-MP partially prevented the inhibition of ^{14}C-valine incorporation into proteins caused by ethanol. This is in agreement with earlier experiments by Mørland *et al.* [7] which indicated that ethanol affects protein synthesis in two ways. The effect of low concentrations of ethanol was linked to ethanol metabolism, while in addition ethanol *per se* inhibited protein synthesis at higher ethanol concentrations. 4-MP completely relieved the shift in the distribution of polysomes caused by ethanol (Table 2). When added to control suspensions, 4-MP resulted in a slight disaggregation of polysomes and the values in Table 2, group 3, have been corrected accordingly.

It thus appears that the preferential effect of ethanol on the initiation of protein synthesis is related to its metabolism. The additional effect, caused by ethanol *per se*, must be of a nature affecting both initiation and elongation in a balanced manner, yielding an unchanged polysomal distribution. A marked disaggregation of polysomes caused by ethanol metabolism has earlier been reported for perfused rabbit liver [25]. The disaggregation observed was largely prevented by the presence of 4-MP, most efficiently when fasted donors were used.

Effect of ethanol on glucose-6-phosphate levels

The metabolic effects of ethanol is generally accepted to be a consequence of the lowered NAD^+/NADH ratio. A lower NAD^+/NADH ratio in itself does not, however, appear to be sufficient, since in a previous study sorbitol did not inhibit protein synthesis, although a reduction of NAD^+ took place [7]. Several authors have pointed to the possible inhibitory effect of ethanol on gluconeogenesis. This would explain the hypoglycemia observed in fasting alcoholics [26]. Krebs *et al.* have in a study of perfused rat liver demonstrated that inhibition of gluconeogenesis does indeed take place, and that it is a consequence of a reduced pyruvate concentration [27].

A link may well exist between gluconeogenesis and initiation of protein synthesis. Ernst *et al.* [28] have shown that inhibition elicited by oxidized glutathione or lack of hemin in reticulocyte lysates can

Table 2. Effects of ethanol and 4-MP on incorporation of amino acids, distribution of polysomes and medium pH

Group	^{14}C-val incorporation into cell protein (pmoles/mg/min)	(% of control)	Distribution of polysomes: % > trisomes	% of control	Δ pH
1 Control	60.5 ± 3	100	50.9 ± 1.6	100	0.07 ± 0.02
2 Ethanol	40.7 ± 1	67	45.3 ± 1.6†	89	0.19 ± 0.02
3 Ethanol + 4-MP‡	49.6 ± 2	82	51.9 ± 1.9*	102	0.11 ± 0.02

The values are means ± S.E.M. of 6 determinations in each group. For some cell suspensions two parallel analyses of polysomes were performed, and the polysomal values are means of 11 determinations in groups 1 and 2 and 8 determinations in group 3.

* Significant difference ($P < 0.02$) vs group 2.

† Significant difference ($P < 0.05$) vs group 1.

‡ Values are corrected for the slight effect of 4-MP on control suspensions.

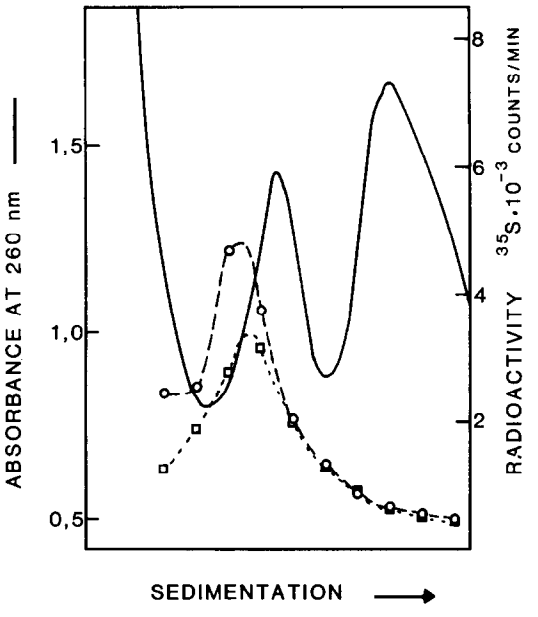

Fig. 2. Formation of ^{35}S-Met-tRNA$_f$-40 S initiation complexes in hepatocytes in the presence and absence of ethanol. Cell suspensions were incubated with ^{35}S-methionine and postmitochondrial supernatants prepared and analysed on sucrose gradients as described under Materials and Methods. ^{35}S-methionine radioactivity in the absence O– – –O, and presence □– – –□ of ethanol.

be prevented or reversed by physiological concentrations of glucose-6-phosphate or sugar phosphate easily converted to this metabolite. Conditions which depleted glucose-6-phosphate in lysates gave rise to inhibition. Similar effects have been observed by Lenz *et al.* [29] for extracts of various mammalian cells. It was therefore of interest to measure hepatocyte glucose-6-phosphate levels in the absence and presence of ethanol. As shown in Table 3, the presence of ethanol during incubation of the cells resulted in a 65% reduction of the glucose-6-phosphate concentration. The ratio of lactate to pyruvate was also measured in some of the incubations. The table shows that the ratio was increased, as one would expect for cells metabolizing ethanol. Ethanol-induced increases in lactate/pyruvate in hepatocytes have been reported earlier, and addition of 4-MP restored the ratio back to control values [4, 7].

Ethanol may thus conceivably inhibit protein synthesis initiation through a lowering of the hexose-6-phosphate concentration in hepatocytes. We observed, in line with this hypothesis, that inclusion of 20 mM glucose in the incubation medium reduced the inhibitory effect of 80 mM ethanol on protein synthesis from 34% to 13% (mean of 12 experiments). Dich and Tønnesen [3] likewise found that glucose lowered the inhibition in hepatocytes from fasted rats, in their case from 42 to 14%. The ethanol effect was, as one would expect, less pronounced in cells from fed animals [3, 30]. This mechanism may also explain why sorbitol failed to inhibit protein synthesis [7]. Sorbitol is converted by sorbitol dehydrogenase to fructose which may then become phosphorylated. When xylitol largely removed the inhibitory effect of ethanol [3], this may again have been a consequence of gluconeogenesis.

The exact mechanism of the restorative effects of phosphorylated hexoses on initiation of protein synthesis is not known. It is likely, however, that factor eIF$_2$ and/or its kinase is involved. Oxidized glutathione or lack of hemin inhibits initiation in reticulocyte lysates through phosphorylation of factor eIF$_2$ [8, 9]. Hexose phosphate could bind to eIF$_2$ and change its reactivity in phosphorylation/dephosphorylation, a possibility suggested by the observations of Wu [31]. Alternatively, glucose-6-phosphate could generate NADPH, acting to keep eIF$_2$ in a reduced and more active state [32], or acting to prevent formation of the active kinase (J. M. Sierra, O. Vicente, C. Palomo and S. Ochoa, in *Proc. 13th Linderström Lang Conference on Translational and Post-Translational Events*, Humana Press, New Jersey, in press). Microsomal ethanol oxidation might contribute further to a lowering of NADPH.

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Table 3. Effects of ethanol on concentration of glucose-6-phosphate and lactate/pyruvate ratio in the cell suspensions after 80 min incubation

Group	Glucose-6-phosphate (nmoles per g dry cells)	Lactate/pyruvate
1 Control	44.0 ± 1.8	15.9 ± 0.3
2 Ethanol*	15.6 ± 2†	60.4 ± 2

Cell suspensions derived from 6 different rat livers were used, and 2–4 determinations of glucose-6-phosphate performed on each suspension. The S.E.M.s were calculated after a normalization in which the glucose-6-phosphate concentrations in the controls for different hepatocyte preparations were put equal to the mean value, 44 nmoles/g. The observed range of control values was 16.1–75.6 nmoles/g. The lactate/pyruvate ratios are means ± S.E.M. for 2 determinations in each group.

* The mean value of the inhibition of ^{14}C-val incorporation by ethanol was 72% compared to control values in these experiments.

† Significant differences $P < 0.001$ vs group 1.

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