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PRELIMINARY COMMUNICATIONS

ETHANOL STIMULATES 5-METHYLTETRAHYDROFOLATE ACCUMULATION IN ISOLATED RAT LIVER CELLS

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The megaloblastic anemia associated with chronic alcohol consumption is most frequently seen in malnourished alcoholics. This condition is seen only infrequently in well-fed alcoholics. Thus, it appears that a decreased dietary intake of folic acid (PteGlu) is necessary for alcoholics to develop PteGlu deficiency (for further discussion and references see Ref. 1). However, ethanol ingestion does play an important role in aggravating PteGlu deficiency, as first shown by Sullivan and Herbert.[2] They found that alcohol ingestion prevents the haematological response to small doses of PteGlu, either oral or parenteral, in three patients with megaloblastic anemia. The mechanism by which ethanol interferes with folate utilization is still unclear. Folate malabsorption in the alcoholic has been implicated as a contributing factor.[3-5] However, the malabsorption of folates seen in alcoholics may be caused by intestinal mucosa lesions resulting from a deficiency of folates and not by alcohol, *per se*.[2,5-8] Another mechanism whereby alcohol may influence folate utilization is suggested by the finding that alcohol apparently interferes with folate polyglutamate synthesis.[9] Alcohol induces a fall in the serum level of 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) in subjects receiving either a folate-deficient diet or a high-folate diet.[10,11] These and a subsequent study with radiolabeled PteGlu and 5-CH_3-H_4PteGlu[12] were consistent with the conclusion that ethanol interferes with the release of 5-CH_3-H_4PteGlu from tissue stores. In a further study Hillman *et al.*[13] found that ethanol apparently blocks release of folates derived from PteGlu, but not of 5-CH_3-H_4PteGlu, into the bile. Since the liver is the main storage depot for folates[14] this hypothesis should best be studied in this organ. Our interest in transport of folates in isolated rat liver cells led us to investigate the effect of alcohol on the transport process. We now report evidence that ethanol *in vitro* results in an apparent

increased accumulation of 5-CH_3-H_4PteGlu in freshly isolated hepatocytes. This may help to explain the decreased serum folate level after *in vivo* administration of ethanol.[10,11]

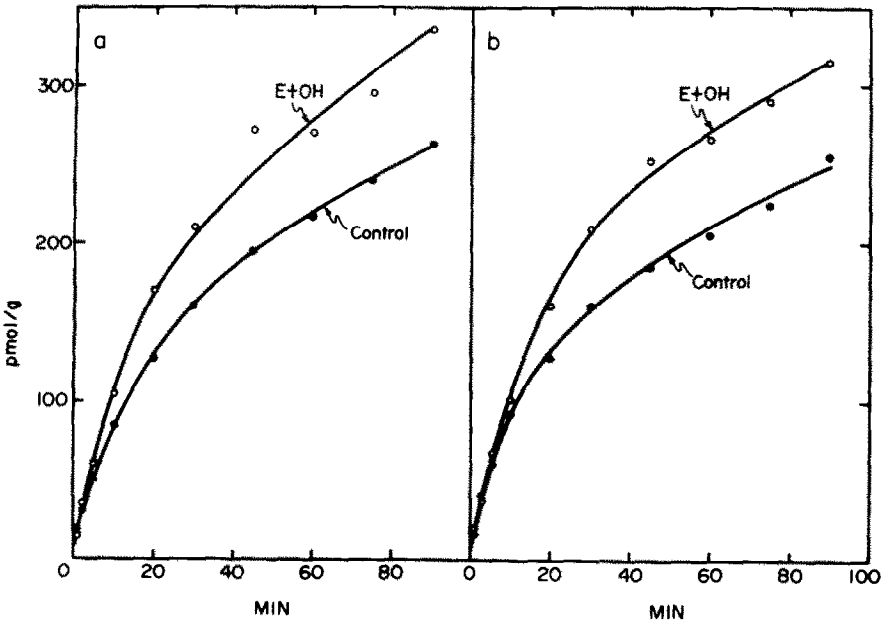

Fig. 1. Time course of uptake of 5-CH_3-H_4PteGlu in control cells (●) and in cells incubated in medium containing 40 mM ethanol (o). 5-CH_3-H_4[G-^{3}H]PteGlu (0.25 μM) and/or ethanol (40 mM) were added to the hepatocyte suspension in 125 ml plastic flasks open to the air. The cell suspension was incubated at 37°C with shaking and aliquots were removed at the indicated times for measuring uptake of 5-CH_3-H_4PteGlu.[14] Panel *a*. Rats received ethanol (4g/kg) by stomach tube one hour prior to hepatocyte isolation. Panel *b*. No pretreatment with oral ethanol. The results represent the average uptake by hepatocytes from two rats for each treatment.

Hepatocytes were isolated by the collagenase-perfusion technique and transport experiments were performed as described previously[15] in an incubation medium of Krebs-Ringer salts solution buffered at pH 7.4 with 25 mM Hepes (Calbiochem). The isolated hepatocytes were judged to be viable on the basis of trypan blue dye exclusion. Over 95% of the cells excluded dye at the beginning of the experiments. From 70 to 80% of the cells excluded dye after 90 min incubation. No differences in viability were seen between control cells and those receiving *in vitro* ethanol. Previous studies[16] indicated an apparent Km for transport of about 0.9 μM for 5-CH_3-H_4PteGlu; therefore uptake experiments were performed at 0.25 μM

(ℓ)-L-5-CH_3-H_4[G-^{3}H]PteGlu (synthesized by the method of Horne et al.[17]) in order to reflect any changes in the carrier-mediated transport process. Uptake was measured at various time intervals in hepatocytes isolated from rats administered ethanol (4g/kg) by stomach tube one hr prior to beginning cell isolation procedures (Fig. 1a) and in hepatocytes from rats which received no oral ethanol (Fig. 1b). By comparing the control curves of panels a and b it can be seen that prior oral administration of ethanol had no appreciable effect on the intracellular accumulation of 5-CH_3-H_4PteGlu. However, in both cases in vitro ethanol at 40 mM (curves marked ETOH in panels a and b) caused an increased accumulation of 5-CH_3-H_4PteGlu when compared to the respective control cells. The maximal increase was about 30% in each case.

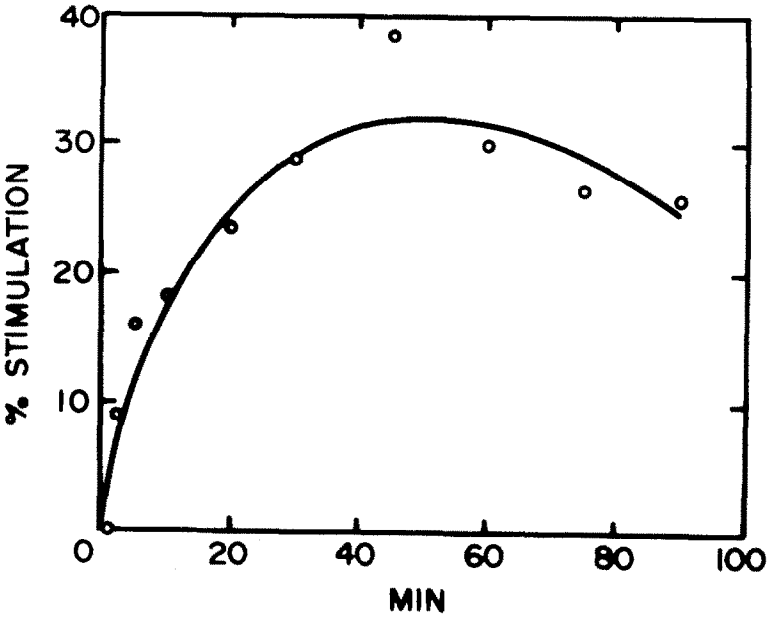

Fig. 2. Time course of ethanol stimulation of 5-CH_3-H_4PteGlu transport. The data of Fig. 1 have been recalculated to show the % stimulation of 5-CH_3-H_4PteGlu accumulation at each time point. The average of results in panels a and b is plotted as a function of time.

The time course of the ethanol effect on 5-CH_3-H_4PteGlu transport is shown in Fig. 2. It can be seen that the apparent stimulation of uptake increases with time of incubation up to about 45 min and declines slowly thereafter. The fact that this effect increases with time up to a maximum and then declines suggests that an accumulation of ethanol metabolite(s) may

be responsible for the stimulation. Preliminary experiments carried out to determine whether pyrazole, in vitro, would block the ethanol stimulated uptake of 5-CH_3-H_4PteGlu were unsuccessful because pyrazole at 2 mM inhibited 5-CH_3-H_4PteGlu uptake 25 to 30% in control cells.

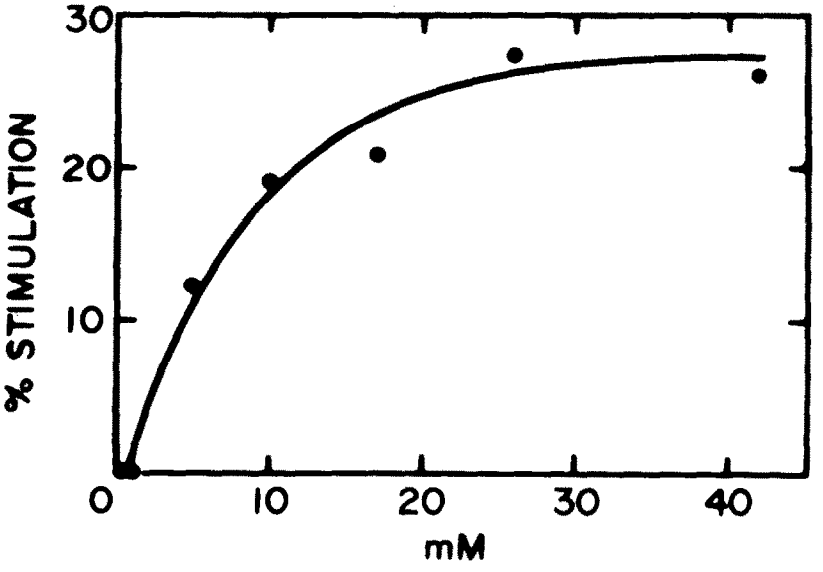

Fig. 3. Concentration dependence of ethanol stimulation of 5-CH_3-H_4 PteGlu transport. Ethanol at the concentrations indicated and 5-CH_3-H_4[G-^{3}H]PteGlu at 0.25 μM were added to 2 ml of hepatocyte suspension in 20 ml plastic scintillation vials open to the air. The samples were incubated with shaking at 37°C for 75 min and duplicate samples were taken to estimate 5-CH_3-H_4PteGlu uptake. The results are expressed as % stimulation of uptake as compared to controls without ethanol and represent the average data from two rats. Although it is not shown in the figure ethanol at 0.05, 0.1 and 0.25 mM as well as 0.5 and 1.0 mM (shown) was without effect on uptake.

Fig. 3 shows the ethanol concentration dependence of the apparent stimulation of 5-CH_3-H_4PteGlu transport. Although not all of the lower concentrations tested are shown in Fig. 3, at 0.05, 0.1, 0.25 and 1 mM there was no effect on transport. At higher ethanol concentrations the intracellular level of 5-CH_3-H_4PteGlu increased and reached a plateau at about 25 to 30 mM. These data show that there is a threshold level of ethanol below which no effect is seen and further show that the ethanol effect is concentration dependent above this

threshold level. Similar levels of ethanol added *in vitro* to isolated hepatocytes have been shown to inhibit the oxidation of palmitate to CO_2 and stimulate the incorporation of palmitate into glycerolipids.[18] Concentrations of ethanol as high as 65 mM have been used *in vitro* with isolated hepatocytes with no evidence of cellular damage.[19] In further experiments we have found that uptake of α-aminoisobutyric acid was inhibited about 24% when incubated with 40 mM ethanol for 60 min, the same conditions which produced a stimulation of 5-CH_3-H_4PteGlu uptake. Thus the ethanol effect seen with 5-CH_3-H_4PteGlu as substrate is not a general phenomenon due to nonspecific interactions of ethanol with the plasma membrane.

The mechanism whereby ethanol results in increased accumulation of 5-CH_3-H_4PteGlu is unclear. Ethanol (or a metabolite) might stimulate the entry of 5-CH_3-H_4PteGlu into the hepatocytes. However, in previous experiments[16] we have shown that an apparent stimulation of 5-CH_3-H_4PteGlu uptake in hepatocytes by sodium azide can be accounted for, at least in part, by inhibition of the efflux of 5-CH_3-H_4PteGlu from the cells. In this respect, it is of interest to note that azide is a potent inhibitor of mitochondrial electron transport, and that acetaldehyde has been reported to adversely affect oxidative phosphorylation at site I.[20] Thus ethanol via acetaldehyde may inhibit efflux of 5-CH_3-H_4PteGlu in a manner similar to azide. The oxidation of ethanol, however, leads to complex metabolic interactions. Among other effects, the level of reduced pyridine nucleotides is increased, acetaldehyde levels increase greatly, mitochondrial functions are altered and fatty acid oxidation decreases.[21]

Whether the effects of ethanol on 5-CH_3-H_4PteGlu transport are exerted primarily on influx or efflux and whether they are mediated through acetaldehyde formation or by altering the NADH/NAD ratio in the cell must await more detailed studies. Although it is difficult to extrapolate from these *in vitro* experiments to the *in vivo* situation, nevertheless these data obtained from experiments performed at the cellular level may provide a mechanism to explain the decreased serum levels observed upon *in vivo* ethanol administration.[10,11]

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