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THE EFFECT OF ETHANOL CONSUMPTION ON FOLATE POLYGLUTAMATE BIOSYNTHESIS IN THE RAT

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Abstract—Contrary to previous studies, the folate polyglutamate chain length in the rat liver appears to be unaffected by ethanol ingestion for periods of 2–13 weeks. The appearance of short chain length folates after 13 weeks has been shown to arise as a result of increased *in vitro* folate polyglutamate breakdown during extraction due to a greater release of lysosomal conjugase in these animals.

Folate deficiency is frequently found in patients with a history of chronic alcoholism [1]. In addition to a poor diet, intestinal malabsorption of folates [2], altered hepatic folate metabolism [3] and more recently, altered enterohepatic folate circulation [4] and folate excretion [5] have been suggested as possible mechanisms of this ethanol-related folate deficiency.

Previous studies in this laboratory suggested that ethanol consumption for short periods of up to two weeks resulted in a decreased synthesis of folate polyglutamates in rats 24 hr after injection of a tracer dose [6]. In addition, there appears to be a decrease in the folate polyglutamate chain length in these rats [6]. In a different study, it was concluded that feeding ethanol for three days resulted in an increased hepatic synthesis of polyglutamyl folates [4].

More recently it has been shown that long-term feeding of ethanol, as 30% of the dietary caloric intake, significantly increased the rate of hepatic synthesis of labelled polyglutamyl folates in the rat [7]. This observation is in contrast to earlier work on chronically alcoholic monkeys [8]. In that study it was concluded that the synthesis of polyglutamyl folates was not affected by ethanol feeding for 2 years, though hepatic incorporation of labelled folates was significant decreased in the ethanol-treated group.

In view of these conflicting results, this study was designed to re-examine the initial findings in this laboratory [6], in terms of hepatic distribution of folate polyglutamates, in rats fed ethanol for periods of 2–13 weeks.

MATERIALS AND METHODS

Materials. [3′5′7,9-^{3}H]folic acid, potassium salt (29 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, U.K.). Whatman pre-swollen anion exchanger, DE-52 was purchased from Whatman Chemical Separation Ltd. (Maidstone, Kent, U.K). All other chemicals and solvents were Analar grade and were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Treatment of animals. Adult Wistar rats (150–200 g) were fed a normal diet with the drinking water replaced by 8% ethanol for various periods of time, e.g. 2 weeks, 3 weeks, 7 weeks and 13 weeks. Fluid intake was of the order of 15–20 ml per day and was similar in ethanol fed animals and controls.

Isolation and polyglutamate analysis of labelled folates. Rats were killed by cranial dislocation 24 hr after radiolabel injection. Tissue folates were extracted following inactivation of endogenous conjugase either by boiling [9] or by protein precipitation with trichloroacetic acid [10] and cleaved oxidatively [11]. Chromatography of the resulting para aminobenzoyl-glutamate derivatives was carried out as previously described [11].

Arylsulphatase measurement. The lysosomal enzyme, arylsulphatase, was assayed as described by Nicholls and Roy [12]. The enzyme preparation was obtained by homogenizing the tissue in 2 vol. of ice cold [0.05 M] potassium phosphate buffer, pH 8.5, followed by centrifugation of 20,000 *g* for 40 min. Activity was expressed as moles/min per g tissue.

Statistical analysis. All parameters were tested for statistically significant differences using Student's *t*-test.

RESULTS

Rat liver folate polyglutamates from control rats, and rats fed ethanol for 2, 3, 7 and 13 weeks were prepared and chromatographed as previously described [6, 9, 11]. A representative analysis is shown in Fig. 1. By utilizing the elution positions of the various polyglutamate standards from previously equilibrated columns [11] it was possible to identify the largest control liver peak as the pentaglutamate with lesser amounts of the hexaglutamate and the lower chain length polyglutamates (Fig. 1a). Rats fed ethanol for 2–7 weeks showed a similar profile (Fig. 1b). In animals fed ethanol for 13 weeks, however, the liver profile appeared to be predominantly the lower chain length polyglutamates with lesser amounts of the pentaglutamate and hexaglutamate (Fig. 1c).

The rat liver polyglutamates were also prepared by trichloroacetic acid (TCA) precipitation of protein as described by Tyerman *et al.* [10], prior to oxidative cleavage of the C_9–N_{10} bond [11]. Using this method

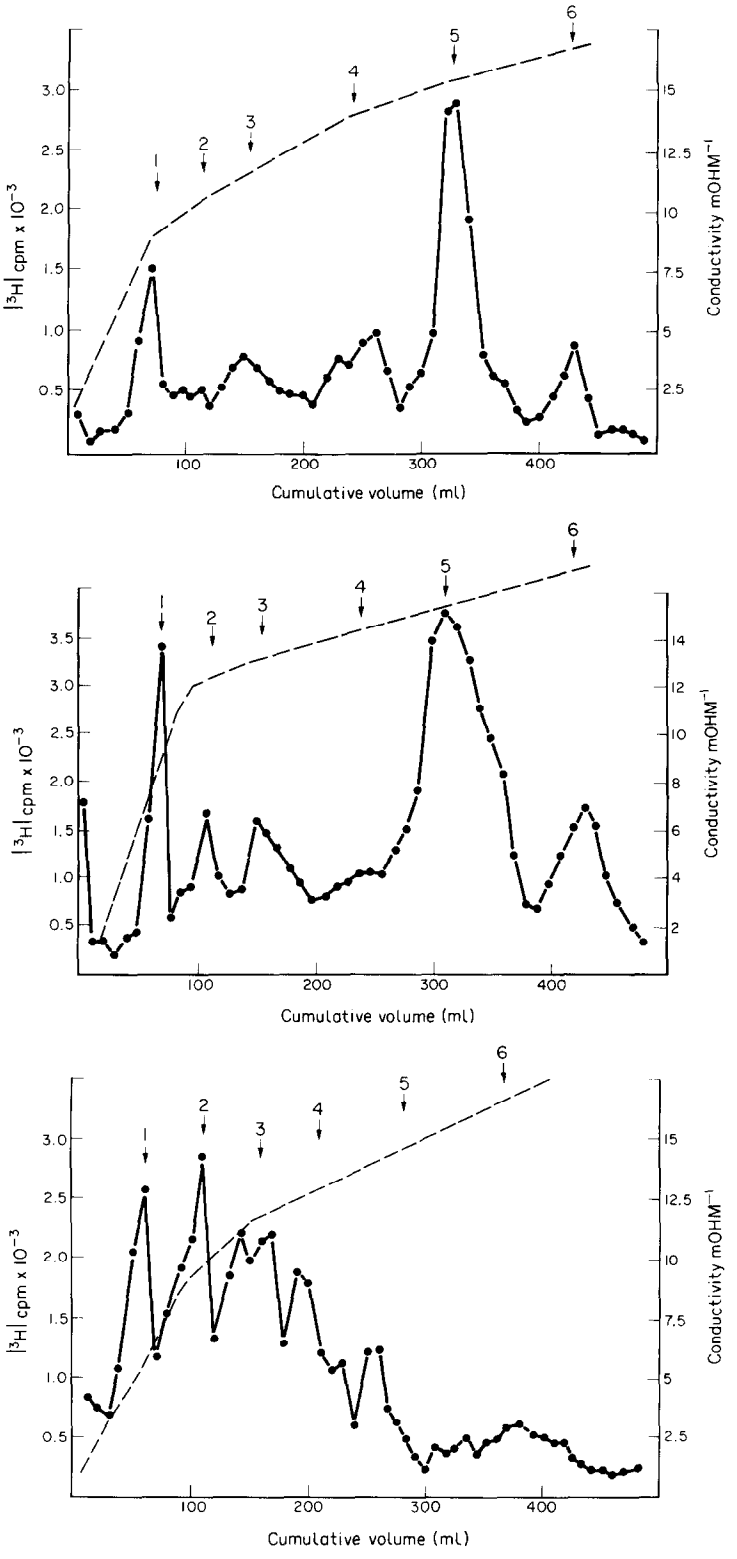

Fig. 1. Representative chromatographic analysis of liver folate polyglutamates from a control rat (a), a rat fed ethanol for 2 weeks (b), and a rat fed ethanol for 13 weeks (c). Rats were injected intraperitoneally 24 hr prior to sacrifice with (3′5′7′9-[^{3}H]PteGlu). Ion exchange chromatography was performed on Whatman DE-52, on the rat liver folate polyglutamate extract, oxidatively cleaved with permanganate [11]. Elution was affected by a non-linear salt gradient (– – –) of 525 ml Tris/HCl (5 mmoles/l) pH 7.0 and 305 ml of Tris/HCl (5 mmoles/l) pH 7.0 containing KCl (1.1 mmoles/l) at a flow rate of 0.5 ml/min. Endogenous folate conjugates was inactivated by boiling for 5 min. Numbers indicate the approximate elution positions of polyglutamate standards, pABGlu [1] and pABGlu$_x$ (x = 2–6) from similar columns.

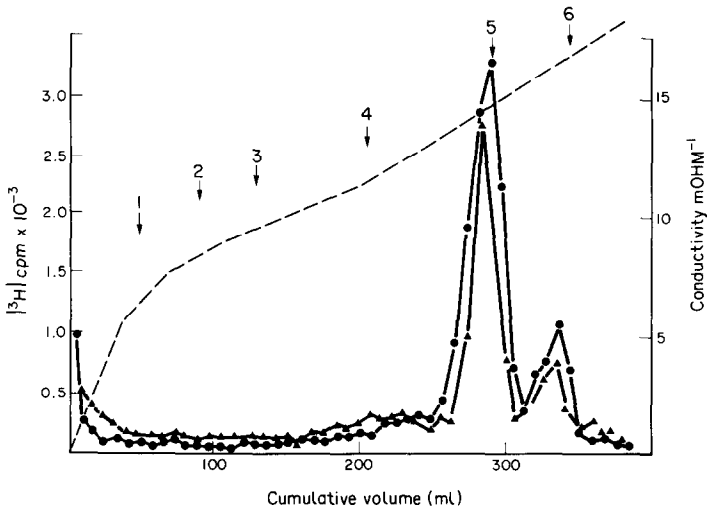

Fig. 2. Chromatographic analysis of rat liver folate polyglutamates from a control (—●—) and a rat fed ethanol for 13 weeks (—▲—). Analysis was performed as described in Fig. 1, with the exception of endogenous conjugase inactivation, which was effected by tissue homogenization in 3 vol. 6.7% trichloroacetic acid [10].

there appeared to be no difference between the control profile, that of the 2–7 week animals (result not shown) and those fed ethanol for 13 weeks (Fig. 2).

To investigate whether the difference in polyglutamate profiles, when prepared by boil-inactivation of the endogenous conjugase, might be an artefact of preparation, i.e. a failure to inactivate this enzyme rapidly enough, thereby resulting in the degradation of the intact liver polyglutamates, labelled polyglutamates were obtained following protein precipitation of endogenous conjugase with TCA [10], and a portion chromatographed as described [11] (Fig. 3). A further portion of these labelled polyglutamates were added to a control and an alcoholic (13 weeks) liver phosphate buffer homogenate prior to polyglutamate isolation by boil-inactivation of conjugase [9].

This procedure resulted in the production of a monoglutamate and diglutamate peak with con-

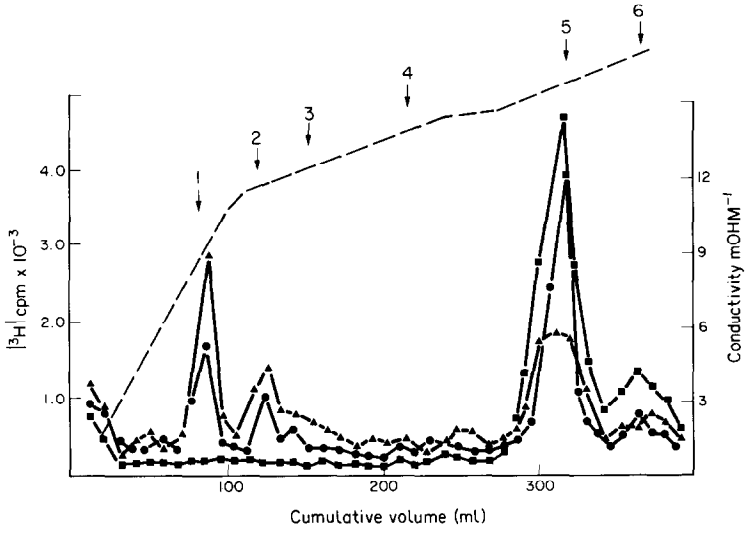

Fig. 3. Chromatographic analysis of rat liver folate polyglutamates. Folate polyglutamates were obtained from a rat injected intraperitoneally 24 hr prior to sacrifice with (3′5′7′9-[^{3}H]PteGlu), and were prepared by conjugase inactivation with TCA [10]. Following TCA extraction into diethyl ether [10] a portion of the tritiated polyglutamate preparation was chromatographed as described [11] (—■—) and a further portion added to the liver homogenizing phosphate buffer (0.05 m, pH 8.5) of a control rat (—●—) and a rat fed ethanol for 13 weeks (—▲—) prior to homogenization and activation of endogenous conjugase by boiling [9]. Ion exchange chromatography of the extracted folate polyglutamates [9] was performed as described in Fig. 1, following cleavage of the C_9—N_{10} bond with permanganate.

comitant decrease in the pentaglutamate both in the control and the ethanol fed rat livers (Fig. 3). Approximately 30% of the control rat liver preparation (and 50% of the alcoholic liver preparation) was recovered as polyglutamates of chain length of less than five glutamates.

It was thought that the degradation of the polyglutamates might have been due to the release of lysosomal enzymes including conjugase, which were released in preparation and not inactivated rapidly enough by boiling. The lysosomal marker enzyme, arylsulphatase, was measured in the crude polyglutamate supernatant. The activity of the enzyme in the control preparation (6.78 ± 0.89 μmoles/min per g tissue mean ± S.E.M.; N = 6) was significantly less than the 13 week ethanol fed rat liver preparation (17.13 ± 1.2, $P < 0.001$), but not in the two week ethanol fed rat liver preparation (7.42 ± 0.90 μmoles/min per g tissue).

This would suggest that the polyglutamate profile prepared by boil-inactivation of the endogenous conjugase may not be the true profile, and that the increase in the lower chain polyglutamates in the ethanol fed rat liver might have been as a result of an increase in the availability of the lysosomal enzyme, conjugase.

DISCUSSION

When rat liver folate polyglutamates were extracted as described by Brown *et al.* [6], i.e. following heat inactivation of endogenous conjugase, the rat liver polyglutamate profile of rats fed ethanol for 13 weeks appeared to be very different to that of the control rat, though not at 2 weeks as ethanol feeding had been previously suggested [6]. In the subsequent study the inactivation of endogenous conjugase by TCA precipitation of protein yielded a conflicting result, i.e. that alcohol ingestion does not in fact affect this liver polyglutamate profile.

Measurement of the release of the lysosomal enzyme, arylsulphatase, confirmed the suggestion that some leakage of lysosomal enzymes occurs in polyglutamate extraction from the liver, and that the difference in polyglutamate profile in the control liver, prepared by heat inactivation as against TCA precipitation, of endogenous conjugase may be due to failure of the boiling step to inactivate this enzyme rapidly enough. This results in the degradation of the higher polyglutamates, which alone are in evidence following the inactivation of conjugase with TCA. Assuming such to be the case, alcohol ingestion exacerbates this effect. This appears to be due to an increased availability of the lysosomal enzyme, folate conjugase as evidence by an increased level of the marker enzyme, arysulphatase, in rat liver homogenates from the alcohol fed rats.

There is now considerable evidence that concentration of ethanol of the same order as those achieved *in vivo* by intoxication, increased the fluidity of biomembranes close to their surfaces [13, 14]. It seems reasonable, therefore, that alcohol ingestion may in fact affect lysosomal fragility, and that the homogenization of livers from alcohol fed rats may result in the rupture of the lysosomes with subsequent release of lysosomal enzymes, including folate polyglutamate conjugase. Alcohol consumption does not affect the *in vivo* morphology of the lysosomes as evidenced by electron-microscopy (results not shown).

Our results indicate that TCA precipitation causes an immediate inactivation of the enzymes present in the extract. Thus, even if as is indicated by raised levels of the lysosomal marker enzyme, arylsulphatase, long term alcohol treatment has resulted in increased release of lysosomal folate conjugase, this latter enzyme would be rapidly inactivated. The resultant chromatographic analysis of the folate polyglutamates present in such TCA extracts would thus appear to represent the true profile of what was present in the liver *in vivo*. By comparing such TCA extracts in the ethanol treated animals to controls, it seems clear that the feeding of ethanol for periods of up to 13 weeks produces no change in the pattern of conversion of folate into its polyglutamyl forms.

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