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Incorporation of labelled amino acids into liver protein after acute ethanol administration

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Chronic consumption of ethanol was accompanied by reduced incorporation of labelled amino acids into rat liver protein measured *in vivo* [1–3] and in a perfused system [3, 4]. The present experiments were performed to study the effect of acute administration of ethanol on the incorporation of labelled amino acids into liver protein. Previous results on the effect of acute ethanol treatment on the synthesis of fixed liver proteins are controversial [5–8]. This could have been due to effects of ethanol on the uptake of amino acids [5, 9, 10], or on the hepatic metabolism of amino acids [11, 12] which could have changed the specific radioactivity of the labelled precursor used in those incorporation experiments. Therefore, in the present study the incorporation was studied in experiments which allowed us to assess if ethanol influenced the precursor pools.

Male albino Wistar rats (240–350 g final body wt) were fed either ordinary laboratory chow plus water or in long-term experiments a special diet [3, 4] providing approximately 11 g ethanol per kg rat and day for 4 weeks. In this diet, ethanol calories constituted about 25 per cent of the total calories consumed. In long-term control diets, sucrose replaced ethanol isocalorically. All animals were fasted 24 hr before sacrifice. Liver perfusions were conducted as described elsewhere [13, 14]. The pH of recirculating perfusates (40 ml) was maintained at 7·40 ± 0·02 by additions of 0·5 N $NaHCO_3$ from an autoburette monitored by a titrator (ABU-13, Titrator 11, Radiometer, Copenhagen) coupled to a pH-electrode (pH-meter 28, Radiometer, Copenhagen) reading perfusate pH continually. Ethanol (19·2%, v/v) was added after perfusion for 5 min followed by infusion of a 12·8 per cent solution to maintain the desired ethanol concentration, infusion rate varying from 15 to 30 μl/min. Equal amounts of perfusion buffer were added instead of ethanol to control perfusates. More 0·5 N $NaHCO_3$ was added to ethanol perfusates than to control perfusates to keep pH at 7·40 [15]. 0·5 M NaCl was therefore added to the latter perfusates to obtain an equal dilution. 50 μCi of [2,3-^{3}H] L-valine (TRK. 327, 39 Ci/m-mole, The Radiochemical Centre, Amersham) was added to the perfusates after 15 min and cycloheximide (Sigma) was added after perfusion for 1 hr, final concentration, 20 μg/ml. Plasma from 1 ml of perfusate was separated at times indicated and frozen at −20°. A liver lobe was ligated and removed after 1 hr, and several liver samples were taken at the end of perfusion. All samples were frozen in liquid nitrogen and stored at −20° until analysis. Ethanol (0·25 g/ml 0·15 M NaCl) 3·3 g/kg was injected intraperitoneally into some rats 4 hr before sacrifice, while controls were given an injection of saline only. All these rats received 5 μCi/kg of [U-^{14}C] L-leucine (CFB. 67, 311 mCi/m-mole, The Radiochemical Centre, Amersham) by the same route 3 hr later, 1 hr before decapitation, when liver samples were taken, frozen and stored as described above. Blood was collected from the neck vessels. Ethanol concentrations were determined by gas chromatography in perfusate (every 30 min) and in blood at sacrifice. The concentration of free unlabelled leucine in liver was measured* after precipitation of liver proteins with 3 vol ice-cold 10% (w/w) trichloroacetic acid (TCA). The supernatants were diluted with 9 vol 0·2 M sodium citrate pH 2·2 containing 0·5% (v/v) thiodiglycol and 0·01% (v/v) caprylic

* This determination was kindly performed by Dr. Hans Prydz, University of Tromsø.

Table 1. Effect of ethanol on distribution and incorporation into protein of [^{3}H]valine in perfused livers before and after administration of cycloheximide*

		1 hr (before cycloheximide)					2 hr 30 min (after cycloheximide)			
			TCA-soluble		TCA-precipitable		TCA-soluble		TCA-precipitable (liver)	
	No. of livers	Liver wt (g)	Liver (cpm/mg)	Perfusate (cpm/μl)	Liver (cpm/liver)	Perfusate† (cpm/g liver)	Liver (cpm/mg)	Perfusate (cpm/μl)	(cpm/liver)‡	(% of 1-hr cpm/liver)
Ethanol	7	6·29 ± 0·32	229 ± 5	305 ± 5	6·81 ± 0·63 × 10^6	6·62 ± 0·43 × 10^4	243 ± 15	291 ± 5	4·52 ± 0·36 × 10^6	66·5
Control	7	5·82 ± 0·35	234 ± 13	310 ± 9	5·76 ± 0·70 × 10^6	7·55 ± 0·37 × 10^4	233 ± 7	296 ± 8	4·19 ± 0·28 × 10^6	74·0
Significance	—	NS	NS	NS	NS	0·029	NS	NS	NS	NS

* Perfusate ethanol concentration was 0·15–0·20%. [^{3}H]valine, 50 μCi/perfusate was given after 15 min and cycloheximide at 1 hr, final concentration 20 μg/ml. The results are given ± S.E.M. NS = not significant according to Wilcoxon's test.

† Results from four livers of each group only. After perfusion for 1 hr 15 min the results were: 9·74 ± 0·85 × 10^4 (ethanol) and 12·84 ± 0·67 × 10^4 (control).

‡ Values are corrected to 1 hr liver wt.

Table 2. Effect of ethanol on free hepatic [^{14}C]leucine specific radioactivity and incorporation into liver protein *in vivo**

Treatment							Free leucine			
Chronic	Acute	No. of rats	Rat wt (g)	Liver wt (g)	Blood ethanol (g/100 ml)	(cpm/ g liver)	(nmoles/g liver)†	(cpm/ nmole)‡	Protein (cpm/g liver)
Lab. chow	Ethanol	6	307 ± 18	8·60 ± 0·72	0·21 ± 0·02	2913 ± 204	248(211·5–285·2)	11·7	15·11 ± 1·01 × 10^3
Lab. chow	Control	6	319 ± 14	8·80 ± 0·61	—	1544 ± 137	136(127·0–145·2)	11·3	16·77 ± 1·14 × 10^3
Significance		—	NS	NS	α = 0·001	α = 0·001	—	—	NS
Ethanol diet	Ethanol	6	294 ± 16	8·18 ± 0·80	0·23 ± 0·03	3062 ± 215	261(253·8–267·2)	11·8	13·34 ± 1·21 × 10^3
Control diet	Control	6	336 ± 8	8·74 ± 0·53	—	1151 ± 163	170(147·0–192·5)	6·8	23·81 ± 1·46 × 10^3
Significance		—	α = 0·021	α = 0·021	α = 0·001	α = 0·001	—	—	α = 0·008

* Chronic ethanol and control treatment was given for 4 weeks (see text). Ethanol was given acutely i.p. (3·3 g/kg) while controls received saline i.p. [^{14}C]leucine was injected i.p. (5 μCi/kg) 3 hr later and 1 hr prior to sacrifice.

† Results from two animals in each group only: range is given.

‡ Specific radioactivity of the total leucine pool.

acid before analysis [16, 17]. Hepatic protein concentration was determined in all liver samples according to the method of Lowry *et al.* [18]. Samples of liver and perfusate plasma were homogenized in 20 vol 10% TCA and total TCA-soluble radioactivity (representing free amino acids) and TCA-precipitable radioactivity (representing proteins) were measured as detailed elsewhere [4], Wilcoxon's test was used to determine statistical significance, $\alpha > 0.05$ was considered as insignificant.

The presence of ethanol (perfusate concentration 0·15–0·20% w/w) did not affect the amount of [^{3}H]valine incorporated into protein of perfused livers after 1 hr (Table 1, 1 hr). Similar results were obtained after liver perfusion for 2 hr 30 min, and after perfusion for 1 hr at either higher (0·25–0·30%) or lower (0·05–0·15%) perfusate ethanol concentrations (data not shown). The proportion of label which remained in liver protein 1 hr 30 min after administration of cycloheximide was the same in ethanol-treated livers (perfusate concentration 0·15–0·20%) and in control livers (Table 1), indicating that the rate of protein degradation was unaffected by ethanol. The release and leakage of labelled proteins from these livers to the perfusate was not increased within the period during which cycloheximide was present. Valine used for protein synthesis in the perfused liver exists in one separate intracellular pool, which equilibrates readily with the extracellular valine pool [19]. Acute administration of ethanol did not affect either the distribution of labelled valine between perfusate and liver, or the amount of free [^{3}H]valine present in the liver after perfusion for 1 hr or 2 hr 30 min (Table 1). In separate experiments, we found that the amount of [^{3}H]valine present in the liver 5 min after the addition of label was unchanged by the presence of ethanol. The specific radioactivity of valine in the pool used for protein synthesis could therefore be considered to be similar in perfusions with and without ethanol. This implied that the rate of synthesis of stationary proteins in the perfused liver was uninfluenced by ethanol. Ethanol has been reported to reduce the incorporation into proteins of the perfused liver [5]. The uptake of the amino acid precursors used in that experiment was, however, reduced by ethanol, and it is therefore uncertain whether protein synthesis was affected. To our knowledge, that report is the only one published until now concerning the effect of ethanol on the incorporation of labelled amino into protein of perfused livers.

The radioactivity found in perfusate proteins was, however, reduced by ethanol (Table 1). After perfusion for 1 hr, 5·6 per cent of the radioactivity present in hepatic proteins was found in perfusate proteins of ethanol-treated livers, while 7·1 per cent of the label present in control liver proteins was found in control perfusates. This suggested that ethanol either reduced the synthesis or the release of perfusate proteins. This observation seems related to the reported reduction of albumin synthesis in the perfused liver [20] and the intact animal [21, 22] after short-term ethanol treatment.

In previously normal rats, injection of ethanol *in vivo* increased free leucine in the livers to 183 per cent and free leucine counts to 189 per cent of the respective control values, but did not influence the incorporation into hepatic proteins (Table 2, 3 upper lines). When ethanol was given i.p. to rats which had been given ethanol in their diets for 4 weeks, free leucine concentration and radioactivity increased to 153 and 263 per cent respectively of their corresponding control values. The incorporation of label into protein was markedly reduced, however, to approximately 55 per cent of that found in controls (Table 2, 3 lower lines). Ethanol given acutely *in vivo* thus affected the amount of labelled leucine present in the liver in both of these experiments. If the specific radioactivity of the total intrahepatic free leucine pool measured after the labelling period, was considered to reflect the specific activity prevailing during most of this period, this activity (Table 2) could be used to correct the incorporation data. It was then found that acute ethanol treatment alone had still no effect on the incorporation of leucine into protein, and that the effect of long-term plus acute ethanol treatment on apparent protein synthesis was even greater than when it was estimated from uncorrected data. When ethanol is given acutely *in vivo* as described here, plasma corticosterone concentration increases [23–25], to the same extent (6-fold) in both previously normal and long-term ethanol-treated rats.* The lack of effect of acute ethanol administration on protein synthesis (Table 1) as well as the inhibitory effect of long-term ethanol consumption on liver protein synthesis, shown previously [3, 4], was thus not changed by this acute rise of endogenous corticosterone levels.

The tissue levels of amino acids were estimated only once in most of the present experiments, which therefore do not allow any definite conclusions to be drawn concerning the rate of amino acid uptake. The results obtained could, however, indicate that the reduction of hepatic amino acid uptake reported for alanine and α-aminoisobutyric acid in liver slices and perfused livers [5, 9, 10] does not apply to all amino acids. Ethanol might therefore exert different effects on the various carrier systems for amino acids (for references see Christensen *et al.*) [26]. The increase of free hepatic leucine observed *in vivo* in this report may point at an effect of ethanol on amino acid uptake which could have been a consequence of increased steroid hormone levels [27, 28]. Reduced hepatic oxidation of leucine [29] could also at least partially explain the accumulation of this amino acid.

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* J. Mørland, unpublished observation.

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Oxidative cleavage of the ethylenic linkage of stilbene by rabbit liver microsomes

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A variety of ethylenic compounds such as stilbene [1], safrole [2], isosafrole [2], and quinine [3] have long been known to undergo oxidative cleavage in the animal body and excreted as carboxylic acids, e.g. in 1939 Stroud [1] demonstrated benzoic acid to be the major urinary metabolite of stilbene in the rabbit. Similarly, double bonds of carcinogenic hydrocarbons with olefinic character such as $C_{1,2}$ of acenaphthylene [4] and the K-region of dibenz(*a,h*)anthracene [5] are also known to be oxidatively cleaved into dicarboxylic acids *in vivo*. In microorganisms, aromatic double bonds such as those of benzene, naphthalene, and anthracene are cleaved by a dioxygenase via catechols [6]. Although nothing is known as yet of the cleavage mechanism of ethylenic double bonds, including that of stilbene, an attempt to approach this problem has been made by Hopkins [7] by using acenaphthene-1,2-diols as substrates. During the course of the investigation on hepatic microsomal interconversion of *cis*- and *trans*-acenaphthene-1,2-diols both of which are urinary metabolites of acenaphthylene and of either *cis*- or *trans*-acenaphthene-1,2-diol, a hypothetical ketol intermediate was thought to be unstable and spontaneously rearrange to the chemical equivalent, 1,8-naphthalic aldehyde, being enzymatically [8] or non-enzymatically [9] converted to 1,8-naphthalic acid. Further investigations carried out by Drummond *et al.* [10], however, failed to detect the aldehyde in their microsomal reaction system. The procedure used by Hopkins for the isolation of the dicarboxylic acid was found later to be so drastic that acenaphthene quinone formed from the diols by the microsomes was autoxidized and failed to prove whether or not the acid formation was enzymatic [10, 11]. In view of oxidative cleavage mechanism involving a new system other than dioxygenation, the Hopkins' assumption is worthy of reconsideration since a wide variety of olefins [12–15] and arenes [16, 17] have recently been shown to be oxidized by hepatic microsomal monooxygenase to epoxides and subsequently hydrolyzed to glycols. This metabolic reaction is strongly suggested to be closely related to a carcinogenesis mechanism involving aromatic hydrocarbons such as benzo(*a*)pyrene, dibenz(*a,h*)anthracene, and 7-methylbenzanthracene which are converted by hepatic microsomes into the corresponding K-region epoxides as proximal active carcinogens [18–21].

Very recently, we have demonstrated that *cis*- and *trans*-stilbenes are also converted by rabbit liver microsomes stereospecifically to *threo*- and *meso*-1,2-diphenyl-1,2-ethanediols, respectively, via the corresponding epoxides [22, 23]. Furthermore, it was shown that a 9000 *g* supernatant of a rabbit liver homogenate catalyzed the interconversion of the *threo*- and *meso*-glycols [23]. This fact would reasonably indicate that the ketol, benzoin (benzoylphenylcarbinol) which is considerably stable, is an intermediate for the interconversion. A promising approach to the problem of the oxidative cleavage of ethylenic double bonds by hepatic microsomes could be to use the stilbene glycols as model substrates and to prove the intermediacy of the ketol and whether it is a precursor of benzoic acid or not.

For the isolation and identification of the intermediate,