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AN EFFECT OF ETHANOL ADMINISTRATION ON TRYPTOPHAN OXYGENASE IN THE PERFUSED RAT LIVER

DECREASED ENZYME SYNTHESIS DEPENDENT ON LOW PERFUSATE pH

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Abstract—The effect of ethanol on the isolated perfused rat liver was examined.

A gradual decrease in the activity of tryptophan oxygenase was observed when the ethanol concentration in the perfusate was kept at 30–40 mM for about 2 hr. During the perfusion with ethanol the pH of the perfusate dropped from about 7·2 to about 6·2. When this reduction of pH was prevented by adding NaOH to the perfusate, no decrease of tryptophan oxygenase activity occurred. It is concluded that the increase of H^+-ion concentration may play a central role in this effect of ethanol.

The biochemical mechanism responsible for the reduced enzyme activity was studied in experiments with perfused rat livers and liver homogenates. The homogenate experiments indicated that ethanol or metabolites did not cause enzyme inhibition or decreased enzyme activation. Furthermore, increased loss of tryptophan oxygenase to the perfusate or bile after ethanol treatment was not demonstrated. Perfused rat livers given ethanol, cycloheximide or both showed nearly the same enzyme activity.

We assume that the reduction of tryptophan oxygenase activity registered after ethanol administration to the perfused liver most likely is due to reduced amount of apoenzyme possibly caused by decreased enzyme synthesis. Ethanol treatment showed some specificity as the activity of tyrosine aminotransferase was not altered.

STUDIES on the metabolic effect of ethanol on liver have mostly been concerned with lipid and carbohydrate metabolism, and less information is available on its action on specific enzymes.[1] While an increase in the ratio between NADH and NAD^+ caused by ethanol oxidation seems to play a central role in the observed change of lipid and carbohydrate metabolism, it is not known whether ethanol itself or some of its metabolic alterations mediate the effect on enzyme activities.[1,2]

Long term treatment of rats with ethanol may cause some changes in liver enzyme activities.[2,3] A single dose of ethanol may cause rapid increases in the activities of tryptophan oxygenase,[4] glucose-6-phosphatase[5,6] and δ-aminolaevulinate synthetase.[7] These effects *in vivo* may be indirect since ethanol stimulates the secretion of adrenal steroid[4,8] capable of enhancing the activity of all these enzymes.[9–11]

Liver perfusion is a suitable technique for studying rapid effects of alcohol on the organ in the absence of hormones and other humoral factors,[12–15] although new errors may be introduced by this technique, such as accumulation of acid metabolites and unphysiologically reduced perfusate pH. In the present work we examined the effect of ethanol on the activity of tryptophan oxygenase (L-tryptophan: oxygen oxidoreductase, EC 1.13.1.12) in the perfused rat liver at different H^+-ion concentrations of the perfusate.

MATERIALS AND METHODS

Liver perfusion

Male Wistar rats (280–340 g) maintained on standard laboratory rat chow were fasted for 18–20 hr prior to experiments. Livers were isolated and perfused by a technique described previously.[16,17] Liver specimens for enzyme analysis were taken at the beginning of each perfusion and at times indicated. Perfusate samples for measurement of ethanol content and pH were taken from upper reservoir. Ethanol was infused and other additions were given into the lower reservoir. The standard administration of ethanol was a continuous infusion of 40 μl/min 6·4% (v/v) for 2 hr after an initial addition of 100 μl 96% ethanol (v/v) (75 mg). The total amount of ethanol added in this way was 320 mg.

Livers showing dark spots, low bile production or other abnormalities were discarded and the perfusions interrupted. The initial perfusion rate was 1·0–2·0 ml/min/g liver (7–14 ml/min). In all experiments the perfusion rate increased somewhat during the first hr, reaching its maximum after about 1 hr. Six ml/min was chosen as a lower limit and perfusions with slower rates were interrupted. All experiments were started about 12 noon to reduce influence from circadian variations in tryptophan and steroid levels and tryptophan oxygenase activity.[18]

Enzyme analysis

Tryptophan oxygenase was assayed in duplicate specimens by the procedure of Seglen and Jervell.[19] The mean enzyme activity was calculated as μmoles of kynurenine formed per hr at 37°/g liver tissue (wet wt.) and expressed here as percentage of values obtained at zero time (within 5 min after the beginning of perfusion). Some assay supernatants were examined for N-formylkynurenine and kynurenine metabolites according to Mehler and Knox[20] and Jago, Nelson and Rose.[21] Tyrosine aminotransferase was assayed by the method of Rosen *et al.*[22]

In some experiments the perfusate was assayed for tryptophan oxygenase activity by addition of 16 ml tryptophan oxygenase assay buffer (0·02 M phosphate buffer, pH 7·0, containing 2·5 mM of tryptophan and 8 μM of heme) to 4 ml samples of perfusate. Tryptophan oxygenase activity might be reduced rapidly in perfusates. Separate experiments were therefore performed to test this in the following way. We added liver homogenate with known tryptophan oxygenase activity to recirculating perfusates containing 40 mM ethanol (without liver). The enzyme activity of perfusate specimens was equal to that calculated for the first 30 min, thereafter the measured tryptophan oxygenase values were lower in the perfusate than expected, 50 per cent recovery after 2 hr.

The bile was analysed for tryptophan oxygenase activity by mixing two aliquots of 100 μl bile collected during the first 3 hr of perfusion with 19 ml assay buffer. As also bile might influence tryptophan oxygenase activity, we tested this in the following way. Some liver specimens with known tryptophan oxygenase activity were homogenized in bile from control as well as ethanol-treated livers and incubated for 30 min at 37°. These homogenates were then assayed for tryptophan oxygenase activity the usual way (dilution 1 : 100) and compared to the activity obtained from other liver specimens not pre-incubated with bile. The recovery of enzyme activity in the homogenates pre-incubated with bile was about 60 per cent.

Any greater loss of tryptophan oxygenase from the liver to the perfusate or bile during our liver perfusions would thus be detected if tryptophan oxygenase was released during perfusion in a manner comparable to this addition of whole liver homogenate.

Ethanol determination in perfusate

The ethanol concentration in the perfusate was measured in triplicate samples by a slight modification of the method of Bücher and Redetski.[23]

pH determinations

pH was measured in the perfusate entering the liver with a micro electrode unit (Radiometer, Copenhagen).

To measure the degree of intracellular acidity, samples of liver (100 mg) were rapidly homogenized in 10 vol. of ice-cold 145 mM KCl, 10 mM NaCl solution and pH determined by the micro electrode unit. pH–values obtained with this method in muscle were comparable to those obtained by the DMO-method.[24,25]

Chemicals

Ethanol, 96% (v/v) was obtained from A/S Vinmonopolet, Oslo. Albumin (Bovine fraction V), L(+) lactic acid (Grade L–III), cycloheximide and β-diphosphopyridine nucleotide, reduced form (β-NADH grade III) were purchased from Sigma. Pyrazole was from Fluka AG. Other chemicals were reagent grade.

RESULTS

Decreased tryptophan oxygenase activity after ethanol administration

Perfused rat livers infused with buffer (5–6 ml, pH 7·4) for 2 hr or given no additions showed fairly constant tryptophan oxygenase activity for at least 3 hr (Fig. 1). Ethanol (administered according to the standard procedure by a single dose of 100 μl 96% (v/v) at the beginning followed by a constant infusion of 40 μl 6·4% (v/v)/min for 2 hr) caused a significant decrease of tryptophan oxygenase activity (Fig. 1). The assay of tryptophan oxygenase is based upon the spectrophotometrical measurement of kynurenine at 365 nm. The reduced kynurenine formation measured was due to reduced tryptophan oxygenase activity and not to reduced kynurenine production by formamidase (Aryl–formylamin amidohydrolase, EC 3.5.1.9) or increased metabolism of kynurenine in the assay mixture. This was confirmed spectrophotometrically.[17] The immediate increase in enzyme activity after taking the samples[19] was not affected by ethanol treatment. The tryptophan oxygenase activity was about 50 per cent after 2 hr perfusion and about 30 per cent after 3 hr of the initial activity. After 6 hr perfusion the enzyme activity was not significantly different from that obtained at 3 hr. In one experiment a larger dose of ethanol was given initially (200 μl 96% (v/v)) followed by infusion of 40 μl 8% (v/v)/min for 2 hr. This administration did not increase the reduction of tryptophan oxygenase activity. By giving a smaller single dose of ethanol, 150 μl 96% (v/v) only at the start, control values were obtained.

The standard administration of ethanol, resulted in perfusate ethanol concentrations between 30 and 40 mM (0·15–0·18%) for the first 2 hr. When the ethanol infusion was stopped, the concentration declined to zero during the following hr.

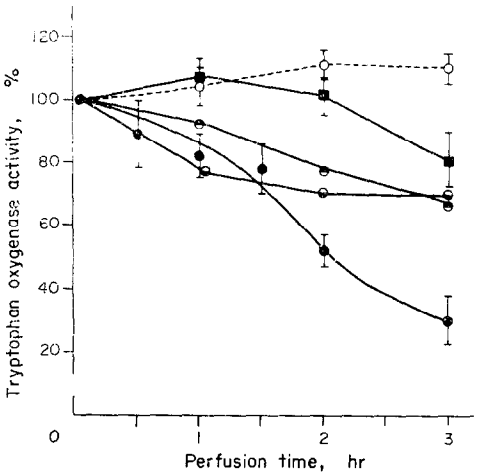

FIG. 1. Effect of ethanol on tryptophan oxygenase activity in perfused rat liver at different perfusate pH. Tryptophan oxygenase activity is expressed as percentage of the initial activity. The mean activity at start in eight control livers was 6·3 ± 2·3 (S.D.) units and in 12 livers to be ethanol treated: 8·4 ± 2·5 (S.D.). Each point represents the mean of values from 2–9 perfusion experiments with ± S.E.M. (standard error of the mean) indicated as vertical bars. ○- - -○ control perfusions; in the other perfusions ethanol was added as an initial dose of 100 μl 96% (v/v), then infused continuously for 2 hr at a rate of 40 μl 6·4% (v/v)/min: ●——● with no correction of perfusate pH; ◒——◒ pH was allowed to reach 6·4 and was then kept constant; ◓——◓ pH was allowed to reach 6·75 and was kept there; ■——■ pH was kept between 7·2 and 7·4 for the whole perfusion.

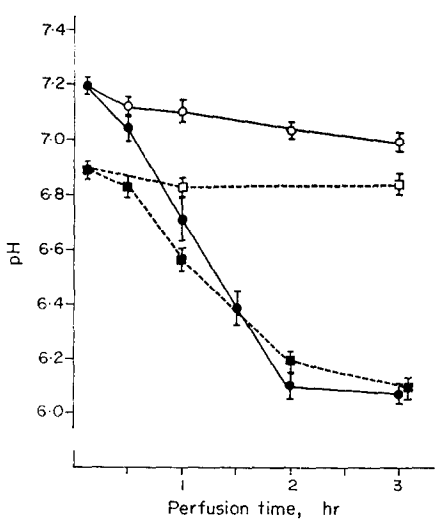

FIG. 2. The effect of ethanol on perfusate pH and intracellular acidity. Each point represents the mean value of at least three experiments. Vertical bars indicate ± S.E.M. Ethanol was administered as given in legend to Fig. 1. ●——● perfusate pH, ■- - -■ pH in liver homogenates after ethanol administration. ○——○ perfusate pH, □- - -□ liver homogenate pH in control experiments.

pH during perfusion

pH of the perfusate was initially about 7·2 in all perfusions. In control perfusions pH declined slowly as shown in Fig. 2. When ethanol was given by the standard procedure, perfusate pH was between 6·0 and 6·2 after 2 hr. The administration of a larger dose of ethanol gave no greater increase of H^+-ion concentration in the perfusate.

pH determinations in freshly prepared liver homogenates (see Methods) (Fig. 2) revealed nearly constant intracellular acidity during the control perfusions (6·8–6·9), close to values of intracellular pH of perfused livers reported by others.[26] After ethanol treatment there was a marked reduction of liver homogenate pH (6·1–6·2), which was lower than the corresponding perfusate pH for the first hr.

pH was always 7·0 in the tryptophan oxygenase assay homogenates (1:100) regardless of the pH recorded during perfusion.

Reduction of tryptophan oxygenase activity dependent on the H^+-ion concentration of perfusate

Correction of pH. In a series of experiments with the standard ethanol administration, pH of the perfusate was either kept between 7·2 and 7·4 by dropwise addition of 1 N NaOH (usually about 1 ml) to the system, or at a lower level by adding a smaller amount NaOH at a later stage of perfusion (Fig. 1). The reduction of tryptophan oxygenase activity was smaller when pH was kept higher by addition of NaOH, and for at least the first 2 hr of perfusion the reduction disappeared when pH was between 7·2 and 7·4. Furthermore Fig. 3, representing results from several ethanol perfusions, shows that the magnitude of the decrease of tryptophan oxygenase activity at 2 hr perfusion is correlated to the degree of pH reduction at that time.

The correction of perfusate pH to 7·2 after standard ethanol treatment was delayed

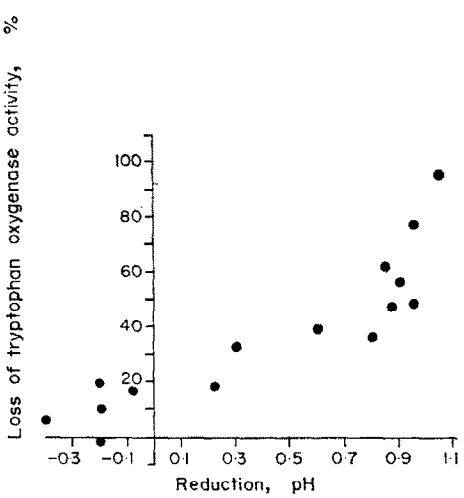

FIG. 3. Loss of tryptophan oxygenase activity at 2 hr of perfusion as a function of the corresponding fall in pH of the perfusate. The loss of tryptophan oxygenase activity is expressed as the difference in enzyme activity (as per cent of initial value) between control and ethanol treated livers. This value is plotted against the reduction of perfusate pH (difference from control experiment) recorded in the same experiment. (Same experiments as shown in Fig. 1.)

to 2 hr of perfusion in one preliminary experiment. Tryptophan oxygenase activity did not return to original values, but remained about 50 per cent of control level.

Addition of both HCl and ethanol. Livers perfused at pH 6·2, due to HCl additions to the perfusate, showed a decrease of tryptophan oxygenase activity resembling the reduction after ethanol treatment. Experiments in which a small single dose of ethanol (150 μl 96% (v/v)) was added to perfusate already at pH 6·2 (by means of HCl), revealed no further reduction of tryptophan oxygenase activity.

No influence on tryptophan oxygenase activity by other metabolic alterations due to ethanol

Effect of lactate, acetate and NADH. Several perfusion experiments with infusion of sodium acetate or sodium L (+) lactate were performed at pH 7·2–7·4. Even high concentrations of acetate (200 mM) or L (+) lactate (100 mM) did not reduce the activity of tryptophan oxygenase significantly.

The addition of acetate (10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} M) or lactate (5.10^{-5}, 5.10^{-4} and $2{\cdot}5.10^{-3}$ M) to the tryptophan oxygenase assay mixture prepared from normal, fasted rats, did not cause any inhibition of the enzyme activity. Likewise did NADH (10^{-5}, 10^{-4} and 10^{-3} M) or ethanol (5.10^{-3}, 10^{-2}, 10^{-1} and 2.10^{-1} M) not cause any enzyme inhibition in the assay mixture. The highest concentrations tested far exceeded what can be expected to be found in the assay mixtures from livers metabolizing ethanol.

Effect of glucose. Since ethanol may increase the glucose level during liver perfusions,[27] two perfusions with a very high initial glucose concentration (1000 mg/100 ml) were performed. No alteration of tryptophan oxygenase activity appeared in these experiments.

Pyrazole. Tryptophan oxygenase activity was inhibited in perfusions as well as in the assay mixture by pyrazole (10^{-2} M or higher). This inhibitor of alcohol dehydrogenase which has been used in order to separate metabolic from direct effects of ethanol[28] could therefore not be used in our ethanol experiments.

Biochemical mechanism responsible for the reduced enzyme activity. Tryptophan oxygenase is an enzyme with rapid turnover[29,30] and complex activation system.[31–36] By the following experiments we therefore tried to examine if the reduced enzyme activity after ethanol treatment was due to either decreased activity of existing enzyme molecules or reduced amount of apoenzyme.

Mixed assay homogenate experiments. In six experiments a freshly prepared assay homogenate from a liver perfused with ethanol (30–40 mM) was mixed with a homogenate from an untreated liver. The tryptophan oxygenase activity of the mixture was almost identical with the calculated activity in every experiment.

Kinetics of kynurenine formation during the incubation. The reaction product, kynurenine, is usually formed at the highest rate between 20 and 40 min incubation in a tryptophan oxygenase assay mixture, which is the assay period routinely used. Figure 4 shows that the rate of kynurenine production in the homogenates from ethanol treated livers is reduced by almost a constant proportion of the control values throughout at least 90 min of incubation.

Tryptophan oxygenase activity in perfusate and bile. In three experiments the perfusates from control and ethanol treated livers were assayed for tryptophan oxygenase activity. Low enzyme activities were found after 1 or 3 hr of perfusion in experiments

with ethanol as well as in control experiments. With a recovery of 50 per cent these low activities could maximally account for a loss of 10 per cent of total liver tryptophan oxygenase activity to the perfusate. Even smaller amounts of tryptophan oxygenase were released into the bile from control as well as ethanol-treated livers. We could therefore not detect any greater loss of tryptophan oxygenase to the perfusate or bile from livers subjected to ethanol than from control livers.

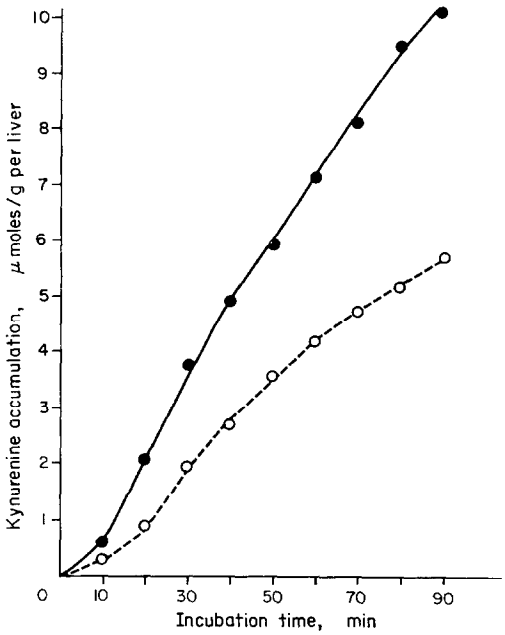

FIG. 4. Kynurenine accumulation during tryptophan oxygenase incubation. The results of a typical experiment are given. Each point represents the mean value of two or three samples. The reaction in the incubation mixture was stopped at the times indicated. ●——● liver given no additions, ○- - -○ the same liver given ethanol for 2 hr of perfusion.

Perfusions with cycloheximide. In a series of experiments 20 μg/ml cycloheximide was added after 3 min to the perfusate with or without the standard ethanol administration. In neither of these experiments did cycloheximide change pH of the perfusate, bile production, perfusion rate, or ethanol concentration of the perfusate from that recorded without cycloheximide. The drug did not cause any alterations in gross liver appearance. Figure 5 presents the tryptophan oxygenase activity in livers given ethanol alone, cycloheximide alone or the combination. We observed no significant difference in the enzyme activities resulting from these different treatments.

Tyrosine aminotransferase activity during perfusion. In order to test the effect of ethanol administration on another liver enzyme with rapid turnover, the activity of tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5) was measured. Ethanol was given in three perfusion experiments and pH of the perfusate was not corrected. There were no significant differences in enzyme activities (recorded at 2, 4 or 6 hr of perfusion) between ethanol-treated and control livers.

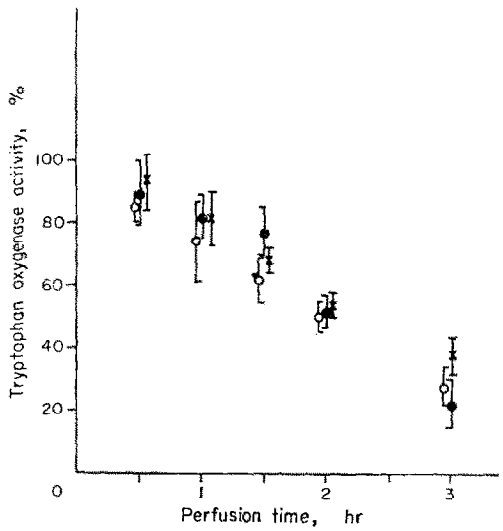

FIG. 5. Effect of ethanol and cycloheximide on tryptophan oxygenase activity in the perfused liver. Tryptophan oxygenase activity is expressed as percentage of initial activity. Each point represents the mean value of 5 perfusion experiments ± S.E.M. indicated as vertical bars. Ethanol was administered as given in legend to Fig. 1 with no correction of pH. Cycloheximide was given as an initial single dose (20 μg/ml perfusate). ○—ethanol and cycloheximide; ×—cycloheximide alone; ●—ethanol alone.

DISCUSSION

In our perfusion system ethanol administration was followed by reduced pH of the perfusate. This increased H^+-ion concentration has an intracellular origin as indicated by the measurements of intracellular acidity (Fig. 2). The increased acidity most probably reflects accumulation of acid metabolites due to ethanol oxidation.[37] The degree of perfusate pH reduction caused by ethanol treatment will depend on the composition and volume of the perfusate. The latter is only about 30 ml in our experiments and this may contribute to the large pH reduction registered.

The reduction of tryptophan oxygenase activity after ethanol administration was dependent on the H^+-ion concentration of the perfusate. In the experiments reported here no reduction of the enzyme activity was found for the first 2 hr of perfusion when perfusate pH was between 7·2 and 7·4,* while reduction of enzyme activity occurred at pH 6·8 or lower. Ethanol does not therefore, seem to be a direct cause for the decrease of tryptophan oxygenase activity, which rather may be caused either by the direct or metabolic effect of the increased H^+-ion concentration alone or in combination with ethanol. The absence of additive effects of ethanol and H^+-ions indicates that the increase of H^+-ion concentration plays the central role in this effect of ethanol.

Could a reduction of tryptophan oxygenase activity similar to that reported here occur *in vivo*? Certainly acidosis comparable to that measured in our perfusates does not occur *in vivo* after ethanol intake. Slight pH reductions of longer duration might perhaps be able to affect the enzyme activity, and there are some observations indicat-

* The addition of NaOH to the perfusate could *per se* reduce tryptophan oxygenase activity in perfused livers (J. Mørland, T. Christoffersen, J. B. Osnes and K. F. Jervell, unpublished observations) This might explain why livers perfused at 7·2–7·4 with the standard ethanol dose showed a slight reduction of enzyme activity at 2 hr of perfusion and a more marked reduction at 3 hr of perfusion (Fig. 1).

ing a mild metabolic acidosis after ethanol in man and rat.[38–41] Another possibility is that local pH reductions occur in the liver after ethanol intake. If not buffered properly *in vivo* such pH decreases might be of a degree sufficient to reduce tryptophan oxygenase activity.

Biochemical mechanism responsible for the reduced enzyme activity

Reduction of tryptophan oxygenase activity may be due to, (a) inhibition of the existing apoenzyme molecules, or lack of activators for the enzyme, or (b) reduced amount of apoenzyme.

(a) Inhibition of tryptophan oxygenase can occur *in vivo* or only in the assay mixture. Ethanol and some of the metabolic alterations due to ethanol (increased concentration of acetate, lactate and NADH) had no inhibitory effect in the assay. Thus the reported[42] NADH inhibition of tryptophan oxygenase does not seem to be of importance in our assay system. The effect of low sample pH on the assay can also be excluded since all incubation mixtures were buffered at pH 7·0. The results of the mixed homogenate experiments make the existence of tryptophan oxygenase inhibitors less likely. Such inhibitors would reduce the enzyme activity of the mixture unless the inhibitors combine completely with the enzyme (with no excess). As we measured reduced tryptophan oxygenase activity throughout 90 min incubation (Fig. 4), these inhibitors also had to resist this incubation and dilution (1 : 100).

Tryptophan oxygenase is activated during most assays by different processes requiring cofactor (heme), substrate and reductant to reach full activity.[19,33,35] Our enzyme assay system is claimed to activate tryptophan oxygenase maximally.[19] However, after ethanol administration to the perfusates, the possibility exists that another factor than the amount of tryptophan oxygenase (protein molecules) may become rate limiting. If this was the case, mixture of control and homogenates with low activity should yield an enzyme activity higher than expected—this was, however, not found. Long-term pre-incubation may compensate for lack of activator.[35] When homogenates from ethanol-treated livers were incubated as long as 90 min; they showed almost a constant proportion of enzyme activity of control homogenates (Fig. 4), making a loss of activator an unlikely explanation for the measured reduced enzyme activity.

(b) We find it more likely that the decrease of tryptophan oxygenase activity is due to reduced amount of apoenzyme. Ethanol administration could provoke increased enzyme loss from the liver cells during perfusion as shown for other enzymes *in vivo*.[43,44] Our results do not support this explanation as no considerable tryptophan oxygenase activity could be detected in perfusate or bile from experiments where ethanol was given.

A reduction of the amount of apoenzyme can be brought about by increased enzyme degradation, but also by reduced synthesis since tryptophan oxygenase has a rapid turnover with half-life of about 2 hr.[29,30] Administration of cycloheximide and ethanol separately or combined reduced the enzyme activity to the same degree (Fig. 5). The conclusions which may be drawn from these results depend upon the assumptions made. If cycloheximide and ethanol in combination keep their ability to influence tryptophan oxygenase exactly the same way as they do when administered separately, ethanol treatment is likely to reduce tryptophan oxygenase activity by the same mechanism as cycloheximide does. Cycloheximide is a well known inhibitor of protein

synthesis,[45,46] and there is no support for interference of cycloheximide with tryptophan oxygenase degradation[47,48] confirmed in our experiments (Fig. 5). Therefore, inhibition of enzyme synthesis can be a possible explanation of the reduced tryptophan oxygenase activity registered after ethanol administration to the perfused liver, if as mentioned, ethanol and cycloheximide treatment acts independently of each other when given together.

Tyrosine aminotransferase has about the same turnover as tryptophan oxygenase.[49,50] Cycloheximide (besides inhibiting synthesis) inhibits the degradation of this enzyme *in vivo*[47,51] and may do so in the perfused liver.[52] Therefore the effects of cycloheximide on tyrosine aminotransferase activity will depend on the rates of enzyme synthesis and degradation at the actual time as well as the cycloheximide sensitivity of these processes. Under experimental conditions resembling ours, the basal activity of tyrosine aminotransferase is reduced to levels below controls by cycloheximide,[53] while ethanol treatment in the experiments reported here did not reduce tyrosine aminotransferase activity significantly. Our observation thus indicates some enzyme specificity of the pH-dependent action of ethanol. One would assume relatively general disturbances in cellular function to accompany the large reduction of pH observed in our experiments. Therefore this selectivity of enzyme reduction is somewhat surprising, but it is noticed that carbon tetrachloride and endotoxin, which both have general effects on the liver, inhibit tryptophan oxygenase synthesis without any inhibition of tyrosine aminotransferase synthesis.[54,55]

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