3. Bile and urine samples were hydrolysed by standard methods: metabolites excreted included unconjugated material, glucuronides and substances hydrolysed by cold and hot acid; the major part of the excreted radioactivity was present as substances remaining water-soluble after all forms of hydrolysis.

4. When samples of bile were collected over periods of 1 hr. or less the proportion of radioactivity excreted as glucuronide always decreased in successive samples whereas water-soluble metabolites generally increased. Substances hydrolysed by cold acid remained virtually constant whereas those hydrolysed by hot acid showed no consistent change.

5. The relationship of these results to similar studies of progesterone metabolism in the cat and of other steroids in man and animals is discussed.

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The Metabolism of Ethanol in Rat-Liver Suspensions

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The work of Batelli & Stern (1910), in which minced horse liver was employed, gave only a very crude idea of the rate at which ethanol is metabolized, as the conditions used were not optimum. Leloir & Muñoz (1938) carried out a valuable series of experiments on the metabolism of liver slices in the presence of ethanol. The metabolism of ethanol by liver preparations has not been extensively studied, mainly because this is a relatively slow process, and the analytical methods available to the earlier workers lacked the precision necessary to measure the small changes in ethanol concentration that occur in the limited periods during which liver slices and suspensions are stable. Very accurate determination of the ethanol concentration is essential if the rate of metabolism is to be measured reliably within a period of 1 or 2 hr. The enzymic method for ethanol determination (Horecker & Kornberg, 1948; Bonnichsen & Theorell, 1951; Bücher & Redetzki, 1951) is sufficiently accurate and specific for this purpose.

The aim of the present work was to study the kinetics of ethanol metabolism in liver suspensions, to examine the possible role of catalase and to determine the influence of a number of substances which have been claimed to accelerate the metabolism *in vivo*. A preliminary account of the work has been given (Svendsen & Lundquist, 1960).

EXPERIMENTAL

Materials

NAD, cytochrome *c*, notatin (glucose oxidase) (activity 15 μmoles/min./mg.) and yeast alcohol dehydrogenase were preparations from C. F. Boehringer und Soehne, Mannheim, Germany; DL-glyceraldehyde and 3-amino-1,2,4-triazole were obtained from Fluka (Buchs, St Gallen, Switzerland); ATP was the crystalline disodium salt of Sigma Chemical Co. (St Louis, Mo., U.S.A.). Sodium pyruvate was prepared by neutralization of pyruvic acid that had been vacuum-distilled three times.

Experimental technique

White rats (150–200 g.) bred in this Laboratory for many generations were used. Unless stated otherwise they were starved for 24 hr. with free access to water before the experiment. The animals were killed by decapitation and the livers immediately placed in ice. Homogenates were prepared in ice-cooled Potter–Elvehjem glass homogenizers with 5 vol. of 0·1 M-potassium phosphate buffer, pH 7·1 (K_2HPO_4–HCl). To minimize nicotinamide–adenine dinucleotidase activity 1 M-nicotinamide was added to the buffer immediately before homogenization to give a final concentration in the suspension of 0·04 M. In some experiments tris–HCl was used as buffer either alone or together with phosphate. The choice of pH 7·1 was made to approximate to the intracellular pH. The experimental liver suspensions were in all cases nearly iso-osmotic with blood plasma. NAD (30 mM) was added to bring the concentration of endogenous NAD (approx. 0·05 mM) plus added NAD (0·15 mM) to a final value of about 0·2 mM.

The suspension (20–40 ml.) was shaken mechanically in a conical flask in a water bath at 21° for 10 min. and, after the addition of ethanol (to give a final concentration of about 11 mM), 1 ml. samples were removed at 10 min. intervals and deproteinized with 3 ml. of 12 % (w/v) metaphosphoric acid or of 5 % (w/v) perchloric acid. The samples were centrifuged at 0° after standing for 10 min. and the supernatant solution was used directly for the determination of ethanol and acetate. In experiments where the effects of various substances were examined, these were added after a control period of 1 hr. during which the ethanol metabolism was measured under normal conditions. Ethanol (3–4 mM) and NAD (0·05 mM) were then added again, together with the substance to be tested, bringing the concentrations of both these constituents up to the levels at the start of the first hour (compare Fig. 1). The ethanol metabolism was then followed again for 1 hr.

Analytical methods

Ethanol was determined enzymically as described by Lundquist (1959), and acetate by the enzymic method of Lundquist, Fugmann & Rasmussen (1961); NAD was assayed as described by Lundquist, Fugmann, Kläning & Rasmussen (1959) and catalase activity as described by Bonnichsen (1955). The analytical methods for the determination of acetate and ethanol were controlled by the addition of known amounts of these substances to samples of the suspension removed before the addition of ethanol. The recoveries were satisfactory in all cases. The alcohol-dehydrogenase activity of homogenates was measured as follows. 0·1 M-Phosphate buffer, pH 7·1 (3 ml.), was mixed in a 10 mm. cuvette with 50 μl. of homogenate diluted approximately 10 times and 50 μl. of 0·5 % $NADH_2$. 0·2 M-Acetaldehyde (50 μl.) was added and the decrease in extinction at 340 mμ was measured at 30 sec. intervals for 3 min.

RESULTS

Kinetics of ethanol oxidation

In 27 experiments under the conditions described in the Experimental section the rate of ethanol metabolism was 0·86 mg./g. fresh wt. of liver/hr. (S.D. 0·077, range 0·74–1·12). In five experiments on rats which were not starved the rate was 0·79 mg./g. of liver/hr. (range 0·54–1·02). The small difference is not statistically significant. The concentration of NAD in fresh liver homogenates is sufficient to secure a nearly maximal initial velocity. Thus the addition of NAD to bring the concentration to about 0·2 mM as described in the Experimental section increased the velocity by about 10 % only (see Fig. 1). However, as shown in Table 1, the concentration of NAD decreases con-

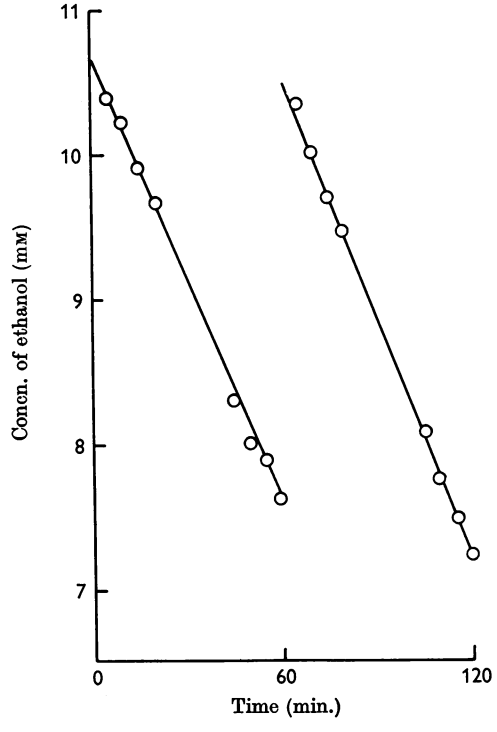

Fig. 1. Effect of the addition of nicotinamide–adenine dinucleotide on the rate of ethanol utilization in rat-liver suspension. Ethanol was added at zero time. At 60 min. NAD was added to give a final concentration of about 0·2 mM, and the ethanol concentration was readjusted to the initial level by the addition of 10 % (v/v) ethanol solution. The velocities in the two periods were respectively 0·84 and 0·92 mg. of ethanol/hr./g. fresh wt. of tissue.

siderably during the experiments in spite of the presence of nicotinamide, and may well be rate-limiting at the end of 1 hr.

To compare the results obtained with liver suspensions with the rate of ethanol utilization in the living rat a few experiments were made in which blood samples were taken from the tail vein of rats given 3 g. of ethanol/kg. body wt. by intraperitoneal injection. The first sample was taken 1 hr. after the injection, and the blood ethanol concentration measured at intervals of 1 hr. At blood ethanol concentrations of between 1·5 and 2·5 mg./ml. the decrease in concentration was about 0·25 mg./ml./hr.

Table 1. *Concentration of nicotinamide–adenine dinucleotide in liver suspensions during ethanol metabolism*

Ethanol was added at zero time. The conditions used were as described in the Experimental section.

	Additions	Time (min.)	Concn. of NAD (μM)
Expt. 1	None	6	68
		60	51
	NAD added at 61 min.	66	209
		121	147
Expt. 2	NAD	5	159
		60	116
	New addition of NAD at 60 min.	65	162
		120	106

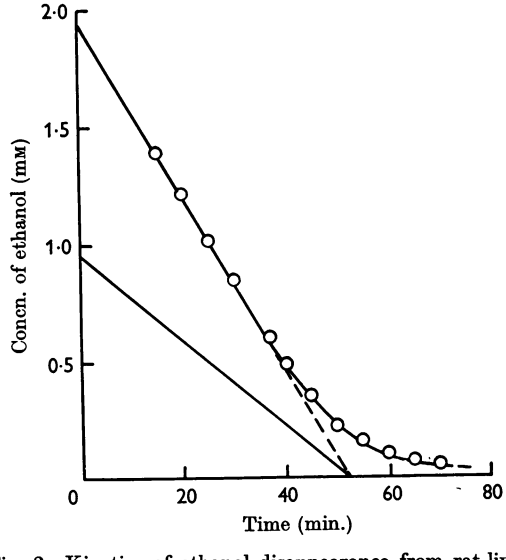

Fig. 2. Kinetics of ethanol disappearance from rat-liver suspension. The conditions used were as described in the Experimental section, except that the initial ethanol concentration was 2 mM. The concentration of NAD was readjusted to the initial value (approximately) at 30 min. The K_m is found as the concentration where the tangent of the curve has half the maximal slope. In this experiment K_m is about 0·3 mM.

The method used for the determination of ethanol makes it possible to follow the decrease in ethanol concentration in liver suspensions down to approx. 20 μM (Fig. 2). The half-maximum velocity was determined as shown in the Figure. In eight experiments a value of 0·3 mM (range 0·2–0·4 mM) was found for K_m.

Effect of possible cofactors and other metabolites

Cytochrome *c* (0·1 mM), Mg^{2+} ions (4 mM) and ATP (2 mM) were without influence on the rate of ethanol utilization, whether added separately or together. These cofactors were therefore omitted in most experiments. Some experiments were made at pH 7·4, where the metabolism of ethanol is about 10 % higher than at 7·1. Fructose (Stuhlfauth & Neumeier, 1951; Pletscher, Bernstein & Staub, 1952), pyruvate (Westerfeld, Stotz & Berg, 1942; Owens & Marshall, 1955) and L-alanine (LeBreton, 1934; Eggleton, 1940) have been observed to cause an increase in the rate of ethanol metabolism in animals and man. The effects of 28 mM-fructose, 28 mM-DL-glyceraldehyde, 22 mM-L-alanine and 17 mM-pyruvate on liver suspensions metabolizing ethanol were therefore examined. In no case was acceleration of ethanol metabolism observed. Similar experiments with rats which were not starved gave the same results. As the action of the accelerating substances *in vivo* might in some cases be explained through their ability to make available more NAD for the dehydrogenation of ethanol, a series of experiments was performed in which no extra NAD was added, so that the concentration of this coenzyme could be rate-limiting. Under these conditions also the four substances were without significant effect.

Ethanol utilization and acetate formation

Fig. 3 shows that the ethanol which disappears from the rat-liver homogenate is nearly quantitatively recovered as free acetate.

Reactions responsible for ethanol utilization

The addition of *o*-phenanthroline (5 mM), which inhibits zinc-containing dehydrogenases (Hoch, Williams & Vallee, 1958), caused total inhibition of ethanol utilization in liver suspensions, in agreement with the assumption that alcohol dehydrogenase is the responsible enzyme. In separate experiments it was ascertained that the concentration of *o*-phenanthroline employed did not inhibit the liver catalase, which brings about a considerable peroxidatic oxidation of ethanol provided that hydrogen peroxide is formed continuously at a suitable rate (Keilin & Hartree, 1945).

The addition of 3-amino-1,2,4-triazole, which inhibits liver catalase (Heim, Appleman & Pyfrom, 1955; Margoliash & Novogrodsky, 1958), to liver suspensions at a concentration of 50 mM gave rise to a progressive but variable inhibition of catalase activity. After 3 hr. at 21° the catalase activity had decreased to 40–60 % of the initial value. Control experiments showed no change in catalase activity even after 6 hr. When alcohol-dehydrogenase activity was measured in homogenates incubated with 3-amino-1,2,4-triazole an inhibition of about 10 % was constantly found, independent of the incubation period. Experiments with 3-amino-1,2,4-triazole and sodium cyanide are shown in Table 2. As apparently some deterioration of the homogenate took place on standing for 3–4 hr. at 21° (Table 2, Expt. 1), an attempt was made to 'revive' the preparation by the addition of methylene blue to circumvent the cytochrome-oxidase step. This brought the rate of ethanol utilization near to that found in fresh homogenates (Expts. 2 and 3). 3-Amino-1,2,4-triazole caused a much greater inhibition of ethanol utilization than could be accounted for through inhibition of catalase, even if catalase were the only enzyme responsible for ethanol metabolism. Cyanide caused about 25 % inhibition of ethanol oxidation at a concentration at which catalase was nearly completely blocked.

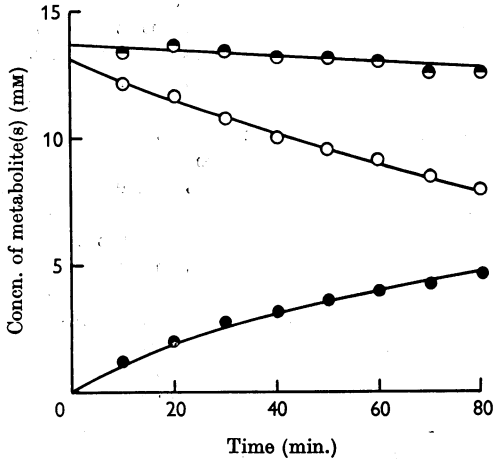

Fig. 3. Ethanol utilization and acetate formation in rat-liver suspension. The conditions used were as described in the Experimental section. ○, Concn. of ethanol; ●, concn. of acetate; ◓, concn. of ethanol + concn. of acetate.

The possibility that substrates yielding hydrogen peroxide were not available in sufficient quantity was tested by examining the rate of ethanol utilization after the addition of substances known to be oxidized enzymically with the formation of hydrogen peroxide. However, xanthine, hypoxanthine or DL-alanine, at concentrations of 10 mM, did not affect the rate of ethanol metabolism. Catalase is, however, present in sufficient quantity to cause a considerable oxidation of ethanol. This was shown in experiments in which a hydrogen peroxide-forming enzyme (glucose oxidase) was added to the liver homogenate.

To a liver suspension (30 ml.) which had been equilibrated for 10 min. were added 0·2 ml. of 1·7 M-ethanol and 0·4 ml. of 15 mM-NAD. Ethanol concentrations were determined at 10 min. intervals for 1 hr. (control period). To the 20 ml. of suspension remaining at this stage were added 0·05 ml. of 1·7 M-ethanol and 0·25 ml. of 15 mM-NAD to re-establish the initial concentrations. Glucose (80 mg.) and notatin (5 mg.) were then added and

Table 2. *Influence of the catalase inhibitors 3-amino-1,2,4-triazole and cyanide on catalase activity, alcohol-dehydrogenase activity and the rate of ethanol metabolism in rat-liver suspensions*

The homogenate was divided into two equal parts and to one was added inhibitor (at zero time). The two suspensions were kept at 21° and samples were removed at the times stated for the determination of catalase activity and alcohol-dehydrogenase activity. The rate of ethanol metabolism (see the Experimental section) of both suspensions was measured at 21° during a period of 1 hr. starting at the times given.

	Time (hr.)	Additions	Catalase activity (% of control)	Alcohol-dehydrogenase activity (% of control)	Ethanol oxidation (mg./g. of liver/hr.) Control	Plus inhibitor
Expt. 1	0	50 mM-Aminotriazole	100	92	—	—
	3		62	—	0·37	0·15
	4		40	—	—	—
	5		15	93	—	—
Expt. 2	0	50 mM-Aminotriazole	100	—	—	—
	2·8		57	86	—	—
	3·2		45	—	0·66*	0·06*
	4·8		34	83	—	—
Expt. 3	0–1	0·1 mM-NaCN	8	—	0·61*	0·45*

* Methylene blue added (0·2 mg./ml.).

the ethanol concentrations were determined again at 10 min. intervals. In a typical experiment the control period showed a rate of ethanol utilization of 0·79 mg./g. of liver/hr., whereas the corresponding value for the notatin period was 1·21 mg./g. of liver/hr. The rate of oxidation was constant also after the addition of glucose plus glucose oxidase within the range of ethanol concentrations examined (8–12 mM).

Attempts were made to measure the Michaelis constant, K_m, for the oxidation of ethanol by catalase and glucose oxidase by the technique indicated in Fig. 2. The curve obtained was corrected by means of a control experiment on the same homogenate in which glucose and glucose oxidase were omitted. The experiments were not sufficiently accurate to allow a reliable estimation of K_m, but they suggest a value of about 5 mM.

DISCUSSION

The rate of ethanol metabolism observed in liver suspensions is in good agreement with the results obtained by Leloir & Muñoz (1938) with liver slices, if the difference in temperature is taken into account. The experiments on living rats gave values for the rate of disappearance of ethanol from the blood which were slightly lower than those obtained by Owens & Marshall (1955) and Aull, Roberts & Kinard (1956). If it is assumed that the liver weight of the rats is 3·5 % of the body weight the results of the experiments with liver suspensions account for only half the total rate of ethanol utilization by the rats. The energy metabolism of the rat per kg. body weight is, however, nearly 20 times that of man, and consequently the loss of ethanol through the lungs (provided equilibrium is attained for ethanol between blood and alveolar gases) is very considerable. A rough calculation shows the loss at a blood ethanol concentration of 2 mg./ml. to be between one-third and one-half of the total elimination. It therefore appears reasonable to assume that the oxidation of ethanol by liver suspensions proceeds at about the same rate as in the living animal.

Pathways of ethanol metabolism

It is generally believed that the zinc-containing NAD-requiring alcohol dehydrogenase present in the liver is responsible for at least the larger part of the ethanol metabolism in man and animals (Jacobsen, 1952; Bonnichsen & Theorell, 1951). However, it has been suggested (Keilin & Hartree, 1945; Chance, 1947) that catalase might be responsible for part of the ethanol metabolism through peroxidation by means of hydrogen peroxide formed by autoxidizable flavoproteins. Such processes have been demonstrated *in vitro* (Keilin & Hartree, 1945). Our experiments show that liver preparations can carry out the oxidation of ethanol by means of catalase, provided that a hydrogen peroxide-forming enzyme such as glucose oxidase is added. However, the spontaneous formation of hydrogen peroxide seems to be too slow to account for any measurable part of the ethanol metabolism, even after the addition of high concentrations of substrates of those oxidizing enzymes which produce hydrogen peroxide. It appears improbable, therefore, that substrates producing hydrogen peroxide are present in the liver in sufficient quantity to influence measurably the rate of ethanol oxidation under normal conditions. The inhibition experiments with *o*-phenanthroline confirm this conclusion, though our attempts to inhibit the catalase present in liver suspensions without inhibiting other enzymes did not give conclusive results.

The nearly quantitative formation of free acetate from ethanol is in agreement with the results obtained by Forsander, Räihä & Suomalainen (1960), who found that very little radioactive carbon dioxide is formed by perfused rat liver metabolizing ^{14}C-labelled ethanol. The formation of acetate from ethanol by the human liver *in vivo* has been demonstrated by Lundquist, Tygstrup, Winkler, Mellemgaard & Munch-Petersen (1962). The fact that free acetate and not acetyl-coenzyme A is formed is in agreement with our previous findings with acetaldehyde (Lundquist *et al.* 1959). The possibility that the free acetate might arise through hydrolysis of acetyl-coenzyme A is a remote one, as free acetate is not formed in the presence of other substrates metabolized via acetyl-coenzyme A.

Attempts to increase the rate of ethanol metabolism by the addition of substances which are known to have this effect in living animals were all negative. With fructose, Holzer & Schneider (1955) have suggested that glyceraldehyde formed from fructose 1-phosphate by means of aldolase might be able to oxidize the $NADH_2$–alcohol dehydrogenase complex directly, and thus circumvent the rate-limiting step in the dehydrogenation of ethanol, which according to Theorell & Chance (1951) is the dissociation of the $NADH_2$–enzyme complex. The failure to find any accelerating effect of fructose or glyceraldehyde in liver suspensions argues against this hypothesis.

Michaelis constant for ethanol oxidation. The value of the K_m for ethanol oxidation measured in our experiments is a little lower than any observed with pure liver alcohol dehydrogenase. Thus Theorell & Bonnichsen (1951) found a K_m of 1·2 mM at pH 6·8; Theorell, Nygaard & Bonnichsen (1955) found 0·59 mM in phosphate buffer, I 0·1, pH 7·15, and

2·1 mM in the same buffer but with 0·15 M-sodium chloride also present; Lundquist & Wolthers (1958), using the same temperature (23°) and a medium of approximately physiological composition with respect to inorganic substances, found a K_m of 2·4 mM.

The conditions in our experiments for the determination of K_m values are ideal insofar as the concentration of the reaction product (acetaldehyde) is kept at the extremely low level of about 0·2 μg./ml. or less (U. Fugmann, H. Rasmussen & F. Lundquist, unpublished work), and the concentration of NAD is large enough to ensure that this factor is not rate-limiting. The velocities measured can be considered initial velocities, and inhibition by reaction products is avoided. It may therefore be assumed that the dehydrogenation step is rate-limiting in liver suspensions and that the K_m measured is comparable with the value found for pure alcohol dehydrogenase.

Lundquist & Wolthers (1958) have tried to determine the K_m for ethanol oxidation *in vivo* and found a value about 2 mM. This result is hardly compatible with the present measurements, nor with the finding of Larsen (1959) that hepatic venous blood is nearly devoid of ethanol when the amount of ethanol offered to the organ is less than its maximal metabolic capacity. The value 0·3 mM found in liver suspensions for the K_m for ethanol oxidation in the present work, on the other hand, is compatible with the physiological experiments of Larsen. The results of Lundquist & Wolthers (1958) may be explained if the absorption from the intestine of the ethanol ingested were not complete despite the fact that 2 hr. was allowed for this process.

SUMMARY

1. The metabolism of ethanol in rat-liver suspensions takes place at a rate comparable with that in the living animal. The concentration of ethanol at which the reaction rate was half the maximal value was about 0·3 mM under the experimental conditions.

2. Ethanol was converted almost quantitatively into acetate.

3. Catalase was not involved in any measurable part of the ethanol metabolism unless a hydrogen peroxide-producing enzyme–substrate system was added to the liver preparation. In such cases the rate of the reaction increased by more than 50 %. It is concluded that under normal conditions there are no substrates available in sufficient quantity to cause a measurable peroxidation of ethanol by catalase present in the liver.

4. A number of substances, which have been shown to have an accelerating effect on the ethanol metabolism in living animals, were devoid of effect in rat-liver suspensions.

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