Table 1. Effect of varying the interface area on the activation of bacterial β-glucuronidase by a constant volume of chloroform. Assay mixture, 0.5 ml of 0.2-percent glucuronidase; 0.5 ml of 0.075*M* phosphate buffer, *p*H 6.8; and 0.5 ml of 0.015*M* substrate. Volume of chloroform, 1 ml.

Interface area (approx.) (cm^2)	Activity (per gm)
0	25,500
0.38	61,500
0.95	78,000
2.0	88,000

Table 2. Activation of bacterial β-glucuronidase by 0.1 ml of chloroform present as a layer and as an emulsion. Assay mixture as in Table 1; incubation in 15 × 125-mm test tubes.

Condition of chloroform	Activity (per gm)
None	25,500
Layer	71,000
Emulsion (a)*	81,000
Emulsion (b)*	95,000

* The chloroform was more finely divided in (b) than in (a).

Co. (*6*) and is based on that of Talalay, Fishman, and Huggins (*7*). Phenolphthalein glucuronide is used as substrate, but incubation is in 0.075*M* phosphate buffer at *p*H 6.8. Action of the enzyme is stopped by the addition of 0.2*M* glycine buffer at *p*H 10.4. In our experiments the tubes containing the assay mixture and the solvent were restoppered after the addition of the glycine buffer and then shaken vigorously and centrifuged in order to ensure equilibration of phenolphthalein between the solvent and the alkaline aqueous phase.

When a constant volume of chloroform is incubated with the assay mixture in vessels of different dimensions, so as to vary the interface area between the two liquids, there is an increasing activation of the enzyme as the interface area increases (Table 1). This same increase in activation with a constant volume of chloroform can be achieved by merely shaking the vessel prior to incubation so as to emulsify the chloroform and thus increase the interface area (Table 2). When the vessel is thus shaken, the extent of activation is dependent on the degree of visible emulsification. Conversely, if the interface area is maintained constant while the volume of chloroform is varied, the degree of activation of the enzyme remains constant and independent of the volume of chloroform. Thus, when the assay mixture is layered over volumes of chloroform varying from 5 to 13 ml, with an interface area of 0.38 cm^2, the activity averaged 45,000 (range 42,000 to 47,000)—that is, less than 50 percent of that produced by 0.1 ml of chloroform present as an emulsion. The fact that this value is somewhat less than that for the same interface area in Table 1 is due to the day-to-day variation often observed in the assay of glucuronidase activity (*1*). However, its constancy with regard to the variations seen in Table 1 emphasizes that the interface area is the determinant.

These in vitro results suggest an action of the enzyme at the solvent/water interface and could be a reflexion of a fundamental phenomenon of enzyme action at lipid/water interfaces in the cell, as suggested by Robertson (*8*) and more recently by Danielli and Davies (*9*). This effect probably depends on some special physicochemical property of the bacterial enzyme not shared by the animal enzyme and may involve the concentration and possibly the orientation of the enzyme at the interface. The effect differs from that noted by Schulman (*10*), who attributed the difference in reaction rate in the hydrolysis of various esters by pancreatin to orientation of the substrate molecules at the ester/water interface. Wasteneys and Borsook (*11*) showed that the activity of pepsin in the synthesis of plasteins was increased by the addition of benzene or benzaldehyde. The observation by Doyle (*12*) that *Escherichia coli* glucuronidase can be split into two fragments, (i) an inactive butyl-alcohol-soluble fragment of undefined nature and (ii) a much less active protein fragment, is suggestive of a lipoprotein character for the enzyme and would not be incompatible with the notion of concentration at the solvent-water interface. Finally, in addition to this interface effect observed by us, the nature of the solvent plays an important role, as is seen in the varying activating power of different solvents observed by Gautney, Barker, and Hill and confirmed by us.

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Characterization of Endogenous Ethanol in the Mammal

Abstract. Ethanol has been isolated from the tissues of several animal species in amounts ranging from 23 to 145 μmole/100 gm of tissue. Intestinal bacterial flora appear to be excluded as a source of this ethanol. Radioactivity from pyruvate-2-C^{14} appeared in ethanol after incubation with liver slices; this finding indicates an endogenous synthesis.

Whether ethanol occurs endogenously in mammalian tissues has been a moot question for years. Although some investigators (*1*) have reported the presence of ethanol in amounts of from 2 to 5 mg per 100 gm of tissue or per 100 ml of plasma, as determined by chemical methods, other investigators (*2*) have questioned this finding on the basis of possible bacterial contamination of the tissues studied or inadequate isolation and assay procedures. This report describes some experiments designed to clarify this question.

Liver, kidney, heart, and skeletal muscles were removed rapidly from animals and homogenized immediately in a one-to-one mixture of ice-cold distilled water or 0.1*M* phosphate buffer at *p*H 7.4 in a Potter-Elvehjem homogenizer. The skeletal and heart-muscle samples were minced prior to homogenization. In some cases the homogenate was heated for 5 minutes in a water bath at 80° to 90°C in a sealed tube and then freeze-dried in a glass lyophilizer. In other cases there was no heating. The lyophilates were kept frozen until it was time to assay them for ethanol. Aliquots of the lyophilate were assayed by spectrophotometric measurement of diphosphopyridine nucleotide (DPN) reduction catalyzed by twice-recrystallized yeast alcohol dehydrogenase (*3*) according to the procedure described by Bonnichsen and Theorell (*4*).

In vitro formation of ethanol was studied in liver slices which were incubated in Warburg flasks in an atmosphere of nitrogen at 38°C; about 300 mg of slices per milliliter of Krebs-Ringer phosphate medium were used. At the end of these experiments, the tissues and medium were prepared for analysis in the manner specified above, except that heating was omitted.

Since a number of alcohols and related compounds act as substrates for yeast alcohol dehydrogenase to varying degrees (*5*), it seemed important to characterize the substrate isolated from tissue as ethanol. The following criteria were used to establish the identity. First, the isolation procedure permits complete recovery of known amounts of ethanol, and the assay is sensitive to 0.1 μmole of ethanol. Second, other possible reactive substrates (*5*) have been assayed under standard experimental conditions and eliminated as contributors to the reduction of DPN in the presence of yeast alcohol dehydrogenase. Third, acetaldehyde has been detected qualitatively as a product of alcohol dehydrogenase activity in the presence of a tissue lyophilate by reaction with sodium nitroprusside and piperidine (*6*), and it has been measured by iodine titration of the bisulfite complex. In two experiments, about 50 percent of the acetaldehyde expected on the basis of the degree of DPN reduction was recovered after distillation of a reaction mixture and iodine titration of aldehyde trapped as the aldehyde-bisulfite complex. Fourth, the 3,5-dinitrobenzoate of ethanol was isolated and was identified (i) by its mobility on paper in 20:80 dioxane: water, R_f=0.08 (*7*), and in 30:70 pyridine:water, R_f=0.77, and (ii) by its behavior on a silicic acid–Super-Cel column (*8*) with and without added authentic ethyl dinitrobenzoate. The chromatographic procedures failed to reveal measurable amounts of the lower alcohols, such as propanol and butanol.

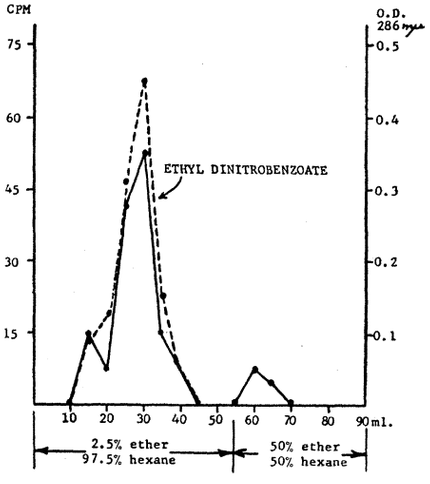

Fig. 1. Chromatography of 3,5-dinitrobenzoates obtained from pyruvate-2-C^{14} incubation with rat-liver slices. Unlabeled ethanol (5 mg) was added as a carrier at the end of 1 hour of incubation. Dimensions of column, 0.5 by 14 cm. Solid line, counts per minute; broken line, optical density of derivative.

Table 1. Concentration of ethanol in mammalian tissues.

Tissue	Concn. of ethanol (μmole per 100 gm of tissue or per 100 ml of plasma)
Rat liver	106
Rat liver (rats maintained on purified diet)	119
Rat plasma	49
Rabbit liver	67
Human liver	145
Rat kidney	23
Rat skeletal muscle	38
Rat heart	106

Although ethanolamine was detected in a low amount in the lyophilate prepared from rat liver, by its reaction with Ninhydrin (*9*), at this level it failed to reduce DPN in the presence of alcohol dehydrogenase under the experimental conditions employed. Furthermore, ethanolamine bisdinitrobenzoate was found to behave differently from ethyl dinitrobenzoate on the silicic acid–Super-Cel column.

After the presence of ethanol in liver had been established, the levels of ethanol in various tissues from several species were determined with yeast alcohol dehydrogenase. These data are presented in Table 1. The highest levels were found in rat liver and heart and in human liver. Since ethanol is distributed in body water (*10*), these differences may reflect in part differences in the ratio of tissue mass to water content. The contribution of intestinal bacterial flora to the observed ethanol levels was studied by feeding eight rats a complete synthetic diet (*11*) and eight rats the same diet supplemented with 2 percent of succinylsulfathiazole to suppress bacterial flora (*12*). After 3 weeks on an ad libitum feeding regime, the two groups were sacrificed and the livers were assayed for ethanol. No significant difference was apparent in the levels of ethanol found in the two groups, and it is therefore unlikely that bacterial metabolites contribute significantly to the formation of endogenous ethanol.

The metabolic origin of this ethanol was next explored. One possible route leading to its formation could involve the participation of pyruvic oxidase and pyruvic acid (*13*). Juni and Heym (*14*) showed that muscle pyruvic oxidase catalyzed the production of some free acetaldehyde. In their system, acetoin appeared as the major product. Under strongly reductive conditions in the presence of alcohol dehydrogenase, free acetaldehyde would be readily reduced to ethanol (*15*). Preliminary results have been obtained which are consistent with the operation of this pathway, although other routes of synthesis are not excluded. The incorporation of pyruvate-2-C^{14} (205,530 count/min per 11.05 μmole) into ethanol was studied in liver slices anaerobically in Krebs-Ringer phosphate medium at *p*H 7.4. After 1 hour, the slices plus the medium were homogenized and lyophilized after the addition of unlabeled carrier ethanol, and the 3,5-dinitrobenzoates were isolated and chromatographed. As shown in Fig. 1, ethyldinitrobenzoate was the principal compound found by silicic acid–Super-Cel chromatography (*8*). It contained essentially all of the radioactivity; this finding corresponds to the formation of 35.7 mμmole of ethanol from pyruvate. Experiments are in progress to obtain further evidence on the mechanism of this synthesis and to investigate the contribution of other possible precursors of endogenous ethanol (*16*).

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