Alcoholysis of the Endogenous Phosphate Esters in Rats Treated with Large Doses of Ethanol

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1. Monoethyl phosphate was isolated from the liver of rats treated with large doses of ethanol. The ^{14}C - and ^{32}P -labelled products were obtained when $[2\text{-}^{14}\text{C}]$ ethanol and $[^{32}\text{P}]\text{orthophosphate}$ respectively were used as the radioactive precursors. 2. The isolated ethyl phosphate preparations were identified by their chemical properties, chromatographic behaviour and enzymic hydrolysis, which, for the ^{14}C -labelled substrate, resulted in a partial recovery of the administered $[^{14}\text{C}]$ ethanol. 3. The possibility of artifact formation of ethyl phosphate was excluded by suitable control experiments. 4. It is concluded that ethyl phosphate formed *in vivo* may be a product of phosphate-catalysed alcoholysis of various phosphate esters. The physiological significance of the possible substitution of water by ethanol in reactions catalysed by hydrolytic enzymes under conditions of acute body intoxication with the alcohol is emphasized.

While the main path of ethanol metabolism and physiological effects accompanying administration of the drug are well established, little attention is given to the possibility of its direct participation in degradative processes. It is known, however, that as a water analogue ethanol may be substituted for water in reactions catalysed by some hydrolytic enzymes *in vitro* (Heppel & Whitfeld, 1955; Bender & Glasson, 1960; Findlay *et al.*, 1962; Sluyterman, 1967; Yang *et al.*, 1967; Tomaszewski & Buchowicz, 1971). These reactions would be of a great physiological importance if they occurred *in vivo*, since ethyl analogues of various metabolites might be formed in the place of usual products of hydrolytic processes.

To test the validity of this assumption, ethyl phosphate formation was investigated in rats treated with ethanol. Such an index of enzymic alcoholysis *in vivo* was chosen as an especially convenient one in view of the fact that ethyl phosphate might be formed as a product common to ethanolytic dephosphorylations of a number phosphate esters occurring in tissues in relatively large amounts. Some accounts of this work, with a special reference to the toxic effects of ethanolysis products, have been presented in a preliminary form (Tomaszewski & Buchowicz, 1972).

Materials and Methods

Treatment with ethanol

Female albino rats of the Wistar strain, weighing about 250g and given a standard laboratory diet and water *ad libitum*, were used. The animals were divided randomly into six groups for treatments with ethanol

under different regimes. Either $[2\text{-}^{14}\text{C}]$ ethanol or $^{32}\text{P}_i$ was used to label products formed in ethanol-treated rats. The doses per animal and sequence of treatments were as follows: group 1, 50 μCi of $[^{14}\text{C}]$ ethanol of high specific radioactivity (20.9 $\mu\text{Ci}/\mu\text{mol}$); group 2, 50 μCi of the radioactive alcohol diluted with 17 mmol of unlabelled ethanol; group 3, untreated; group 4, 1 mCi of carrier-free $^{32}\text{P}_i$ plus 17 mmol of non-labelled ethanol; group 5, as group 4 but ethanol was given 2 h after the administration of $^{32}\text{P}_i$; group 6, 17 mmol of unlabelled ethanol. All precursors were injected intraperitoneally (in 5 ml of 0.9% NaCl) under ether anaesthesia. The rats were killed by decapitation 2 h after the ethanol injection and the livers removed within several seconds and frozen immediately. The livers of the untreated rats (group 3) were similarly removed at the same time.

Isolation of ethyl phosphate

The whole liver was minced and homogenized with 25 ml of 0.3 M-HClO₄. With livers derived from animals that were not injected with radioactive precursor, either 50 μCi of $[^{14}\text{C}]$ ethanol (group 3) or 1 mCi of $^{32}\text{P}_i$ (group 6) was added to the homogenate to control artifact formation of radioactive ethyl phosphate. In each case 0.5 mmol of unlabelled ethyl phosphate was added as the carrier. The liver homogenate was centrifuged at 6000g for 15 min and the supernatant obtained was treated with 200 mg of activated charcoal. The suspension was filtered and molybdate and 3-methylpropan-1-ol-benzene (1:1, v/v) was added to the filtrate (Buchowicz & Belozersky, 1958). The aqueous phase, free of P_i , was then

separated and neutralized with KOH. After removal of KClO_4 , the solution was treated with $\text{Ba}(\text{OH})_2$ to precipitate ethyl phosphate as described by Butcher & Westheimer (1955). The barium salt was dissolved in water and transformed into the free acid form by passing the solution through a Dowex 50 (H^+ form) column. Ethyl phosphate was recovered from the effluent, identified and determined quantitatively as described by Buchowicz & Lesniewska (1970). About 70% of the added carrier was finally recovered.

Radioactivities of the isolated ethyl phosphate preparations were measured in a Packard liquid-scintillation counter. The results of these determinations were used for calculations of the molar quantities of ethyl phosphate formed. Dilution effects were neglected for counts of ^{14}C found. When, however, the calculations were based on counts of ^{32}P , a dilution of administered $^{32}\text{P}_i$ with endogenous P_i was taken into account. The results are given as $\mu\text{mol/liver}$ (weighing on average 7.9 g) and no further evaluations were necessary for the conclusions made.

Reagents

Monoethyl phosphate, barium salt, was prepared according to the method of Butcher & Westheimer (1955) and converted into the ammonium salt before use. $[2\text{-}^{14}\text{C}]\text{Ethanol}$ was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Acid phosphatase of wheat germ was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. $^{32}\text{P}_i$, in dilute HCl, and spectrally pure ethanol were of local origins.

Results

The results of ethyl phosphate determinations in the liver of experimental and control rats are summarized in Table 1. When the animals were treated with $[^{14}\text{C}]\text{ethanol}$ of high specific radioactivity (group 1), no

ethyl phosphate formation could be observed. When however, the same amount of the radioactive precursor was diluted with a large excess of unlabelled ethanol (group 2), a considerable amount of ^{14}C -labelled ethyl phosphate was formed. The isolated product apparently did not represent an artifact, since no radioactive ethyl phosphate was formed in control animals (group 3), where $[^{14}\text{C}]\text{ethanol}$ was added at the initial stage of the preparation procedure. Further data on ethyl phosphate formation come from experiments carried out with $^{32}\text{P}_i$. When this precursor and non-labelled ethanol were administered simultaneously (group 4) little radioactive ethyl phosphate was formed. On the other hand, when $^{32}\text{P}_i$ was allowed to be incorporated for 2 h before the ethanol introduction (group 5) highly radioactive ethyl phosphate could be isolated at the end of the experiment. Clearly, P_i was an indirect precursor of ethyl phosphate. No radioactive ethyl phosphate appeared when $^{32}\text{P}_i$ was added to the liver homogenate derived from animals treated with unlabelled ethanol (group 6). Similarly, no artifact formation of ethyl phosphate could be observed when livers, derived from animals that were treated in parallel either with $^{32}\text{P}_i$ or with non-radioactive ethanol (not shown in Table 1), were minced and pooled together for the homogenization.

Although the standard identification procedure (chromatographic behaviour and resistance to acid and alkaline hydrolysis) indicated unequivocally that the ^{14}C - and ^{32}P -labelled ethyl phosphate preparations isolated from rat liver did not differ from the reference sample, further evidence for their identity was thought to be desirable. This was gained from studies on the kinetics of their degradation by acid phosphatase and identification of the hydrolysis products. It was found that ethyl phosphate was hydrolysed by acid phosphatase at a relatively low rate. This ester was dephosphorylated approximately 10-fold more slowly than other phosphatase substrates, e.g. UMP (Fig. 1). When ^{32}P -labelled ethyl phosphate

Table 1. *Ethyl phosphate formation in the liver of rats treated with ethanol*

Radioactivities actually found are given. Molar quantities were calculated from the radioactivity data after their correction for a 100% recovery. Details of treatments are given in the Materials and Methods section.

Animal group no.	Treatment	Ethyl phosphate formed	
		Radioactivity (d.p.s.)	Amount ($\mu\text{mol/liver}$)
1	$[^{14}\text{C}]\text{Ethanol}$	<1	<0.001
2	$[^{14}\text{C}]\text{Ethanol} + \text{ethanol}$	355	4.5
3	None	<1	<0.001
4	$^{32}\text{P}_i + \text{ethanol}$, simultaneously	251	0.4
5	$^{32}\text{P}_i$, followed by ethanol	1995	2.7
6	Ethanol	<1	—

isolated from rat liver was mixed with the non-radioactive reference sample both P_i and $^{32}P_i$ were liberated at exactly the same rate (Fig. 1). Further, when the ^{14}C -labelled ethyl phosphate preparation was treated with phosphatase radioactive ethanol was formed in addition to the liberation of P_i . The labelled ethanol thus released could be recovered by a micro-distillation at $79^\circ C$ after a dilution of the incubation mixture with commercial non-radioactive ethanol (Table 2). The low final recovery was due to imperfections of the technique used, not to the poor yield of ethanol, as compared with the P_i liberation on the phosphatase treatment. Similar losses were observed in a model experiment, where a known amount of $[2-^{14}C]$ ethanol was added at zero incubation time.

Discussion

Although artifact formation of ethyl phosphate has been described (Hems & Rodnight, 1966; Buchowicz & Lesniewska, 1970; and references quoted therein) there is, to our knowledge, no report of its occurrence in living organisms except for anaerobically grown yeast cells (Szulmajster *et al.*, 1953). The present experiments indicate that ethyl phosphate may be formed in rat liver *in vivo*. This unusual product for animal tissues certainly resulted from ethanol metabolism as (i) it was formed only in animals treated with ethanol, (ii) labelled ethanol was extensively incorporated into ethyl phosphate and (iii) radioactive ethanol could be recovered from ethyl phosphate isolated from animals treated with $[2-^{14}C]$ ethanol. This obvious conclusion is, however, inconsistent with the current knowledge of the metabolic fate of ethanol, which is known to be rapidly oxidized *in vivo*. It is, however, well known that alcohol dehydrogenase cannot effectively remove ethanol from the circulation when the concentration of this potentially toxic substance exceeds some critical value (Krebs & Perkins, 1970). Apparently,

under such conditions ethanol may be channelled into a pathway leading to the formation of ethyl phosphate.

It is now suggested that ethanol may enter the cellular metabolism as a water analogue, being directly involved in degradative processes catalysed by hydrolytic enzymes. By analogy with hydrolysis

Fig. 1. Rates of phosphatase-catalysed liberation of orthophosphate from UMP and ethyl phosphate

Either UMP or an equimolar mixture of the isolated ^{32}P -labelled ethyl phosphate (specific radioactivity 300 d.p.m./ μmol) and non-radioactive reference sample of ethyl phosphate was used as the enzyme substrate. Incubation conditions: substrate, $2\mu\text{mol}$; acid phosphatase, 0.2 mg; sodium acetate buffer, 0.2 mmol (pH 4.8); total volume, 1 ml; temp., $37^\circ C$. Δ , P_i released from UMP; $\circ$, P_i released from ethyl phosphate; $\bullet$, ^{32}P radioactivity found in the released P_i .

Table 2. Recovery of radioactive ethanol from ethyl phosphate isolated from the liver of rats treated with $[2-^{14}C]$ -ethanol

The indicated substances were treated with acid phosphatase under conditions similar to those given in Fig. 1. After 4 h incubation the mixture was diluted with 2 ml of unlabelled 96% (v/v) ethanol and distilled at $79^\circ C$. The distillate was used for radioactivity determination and the remaining part of the mixture tested for P_i .

Treated substances	P_i liberated (mmol)	Radioactivity of distillate (d.p.s.)	
		Expected	Found
$[^{14}C]$ Ethyl phosphate (270 d.p.s.; 0.3 mmol)	0.10	90	53
$[^{14}C]$ Ethyl phosphate (270 d.p.s.; 0.3 mmol)*	<0.01	0	<1
Ethyl phosphate (0.3 mmol) + $[^{14}C]$ ethanol (90 d.p.s.)	0.10	90	62

* Phosphatase omitted.

these processes are referred to as alcoholysis. The occurrence of alcoholysis *in vivo* is indicated by the formation of ethyl phosphate in animals treated with ethanol. This product was shown to be formed in the place of orthophosphate when the hydrolytic mechanism of enzymic dephosphorylations is replaced by the alcoholytic one (Tomaszewski & Buchowicz, 1971). In full accordance with the proposed interpretation ethyl phosphate formation could be observed only in animals treated with large doses of ethanol. The dose should be large enough to overcome the saturation level of alcohol dehydrogenase. The remaining excess of ethanol should still be relatively great to make it possible for it to compete with water for participation in phosphatase-catalysed dephosphorylations. The efficient incorporation of $^{32}\text{P}_i$ into ethyl phosphate in animals injected with this precursor before the ethanol treatment, as compared with the scant utilization of $^{32}\text{P}_i$ when introduced simultaneously with the alcohol, seems to indicate that ethyl phosphate resulted from alcoholysis of the newly synthesized phosphate esters rather than from a direct interaction between orthophosphate and ethanol.

The physiological importance of alcoholytic processes cannot be neglected. It has been shown here that phosphatase-catalysed alcoholysis may result in the formation *in vivo* of ethyl phosphate, a compound structurally related to the most poisonous alkyl phosphates (Johnson, 1969). Other ethyl analogues might be similarly formed by various other hydrolytic enzymes. In particular, the alcoholytic activity of enzymes involved in nucleic acid metabolism (Heppel

& Whitfeld, 1955; Findlay *et al.*, 1962) may contribute to the pathological and heritable changes accompanying acute intoxications with ethanol.

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