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1,4-BUTANEDIOL—A SUBSTRATE FOR RAT LIVER AND HORSE LIVER ALCOHOL DEHYDROGENASES*

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Abstract—1,4-Butanediol (1,4-BD) is oxidized, with the concomitant reduction of NAD, by a rat liver 100,000 *g* supernatant and by horse liver alcohol dehydrogenase. The apparent K_m for this reaction in the rat liver system is 0·61 mM as compared with an apparent K_m for ethanol of 1·3 mM in the same system. Pyrazole, an inhibitor of alcohol dehydrogenase, competitively blocks the oxidation of 1,4-BD by the rat liver supernatant. Administration *in vivo* of pyrazole to the rat prevents the appearance of γ-hydroxybutyrate, a 1,4-BD metabolite, in the blood and also prevents the neuropharmacological effects of 1,4-BD. In contrast, pyrazole prolongs the neuropharmacological effects of the related compound, γ-butyrolactone. 1,4-BD, as a normal component of the "diol lipids" of rat liver, might represent a physiological substrate for alcohol dehydrogenase. It is speculated that ethanol might competitively block 1,4-BD metabolism with accumulation in the liver of unesterified 1,4-BD and 1,4-BD esterified in the diol lipids.

1,4-BD† HAS BEEN reported to be a constituent of the "diol lipid" class of neutral fats from various microbial, plant, and animal sources, which included rat liver.[1–3] 1,4-BD has numerous industrial applications particularly in the plastics industry where it is used in the manufacturing of polyurethanes and in the synthesis of the plasma expander polyvinylpyrrolidone.[4–6] Utilization of this material in agriculture has also been discussed.[4] Additional commercial applications are represented by patents for use of 1,4-BD as a mold-inhibiting humectant for tobacco products,[7] and as a component of cigarette filters,[8] flavoring and perfume solutions,[9] and suppositories.[10] Considerations of manufacture and use of this substance have prompted one Russian investigator to express concern over its role as an environmental pollutant.[4] Thus, 1,4-BD as a component of the mammalian internal and external milieu is a compound of biochemical as well as toxicological interest.

Hinrichs *et al.*[11] observed, and it was subsequently confirmed,[12] that 1,4-BD produced intoxication in man and animals with deep narcosis, total areflexia, and death in acute poisoning from paralysis of vital centers. Sprince *et al.*[13] noted that the neuropharmacological effects of 1,4-BD "resembled to a considerable degree" those of GHB or GBL and assumed that 1,4-BD was metabolized to GHB (Fig. 1). Fishbein and Bessman[14] had demonstrated the reversible oxidation of GHB to succinic semi-

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† Abbreviations used: 1,4-BD, 1,4-butanediol; GBL, γ-butyrolactone (4-hydroxybutanoic acid lactone); and GHB, γ-hydroxybutyric acid (4-hydroxybutanoic acid).

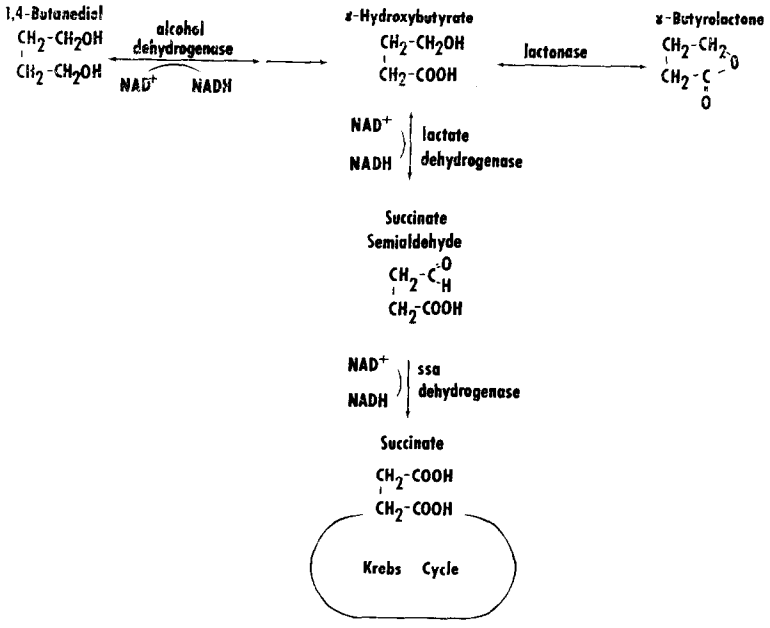


FIG. 1. Metabolic scheme for 1,4-BD and GBL.

aldehyde, which would then enter the Krebs cycle via succinate, and Gessner *et al.*[15] had shown in rabbits that, following 1,4-BD administration, succinate could be isolated from the urine in an amount representing 7 per cent of the 1,4-BD dose. Roth and Giarman[16] reported the presence of GHB in blood and urine following 1,4-BD injection and observed a correlation between brain GHB and loss of the righting reflex. Further indirect evidence for the concept of GHB as the neuropharmacologically active intermediate was provided by Gessa *et al.*,[17] with the observation that 1,4-BD, like GHB and GBL, produced a selective increase in brain dopamine. Additional biochemical information about this substance has been supplied by Ferguson *et al.*[18,19] with the histochemical demonstration of the NAD-coupled metabolism of 1,4-BD in trace amounts in rat liver, gut, uterus, corpora lutea, and ovarian interstitial tissue.

The utilization of 1,4-BD by micro-organisms has also been studied. Several strains of mycobacteria[20] and soil bacteria, strain 14B8,[21] have been observed to utilize 1,4-BD as a sole carbon source; the soil bacteria grown under this condition are able to form *O*-4-hydroxybutylhomoserine from this compound. Acetic acid bacteria appear to handle 1,4-BD in a manner similar to the mammal, oxidizing it to succinate and CO_2 via GHB.[22,23] In cell free systems from these micro-organisms, 1,4-BD is metabolized by the crude particle (cytoplasmic membrane) fraction, as well as by an NAD-linked, soluble, primary alcohol dehydrogenase.[23]

This report provides evidence that at least one step in the metabolism of 1,4-BD to GHB is mediated by alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) and that the neuropharmacological effects of 1,4-BD are prevented by blocking the enzymatic conversion at this step with pyrazole, a known inhibitor of alcohol dehydrogenase.[24]

MATERIALS AND METHODS

Reagents. Pyrazole was purchased from J. T. Baker, absolute ethanol from U.S. Industrial Chemicals, and NAD (grade III) and the sodium salt of GHB from Sigma Chemical Co. 1,4-BD and GBL were purchased from Matheson, Coleman & Bell. GBL was redistilled prior to use in all experiments; 1,4-BD was redistilled only for the experiments *in vitro*. Sigma bovine albumin (Fr V) was used as the standard for all protein determinations.

Experiments in vivo. Male Sprague–Dawley rats weighing approximately 150 g were used. In experiments measuring "sleeping time" (duration of loss of the righting reflex), rats were injected intraperitoneally according to the following dose schedule, with the pyrazole or saline injections preceeding the second member of the pair (H_2O, 1,4-BD or GBL) by 9 min: saline (2 ml/100 g of 0·9% NaCl) + H_2O (1 ml/100 g) (N = 8); pyrazole, 6·6 m-moles/kg (2 ml/100 g of a 2·245% solution in 0·9% NaCl) (N = 8); saline + 1,4-BD, 5·55 m-moles/kg (1 ml/100 g of 50 mg/ml) (N = 8); pyrazole + 1,4-BD (N = 8); saline + GBL, 5·55 m-moles/kg (1 ml/100 g of 47·75 mg/ml) (N = 12); pyrazole + GBL (initially 13 in this group but one died during course of experiment; therefore, for statistics N = 12). The pyrazole dose was that used by Lester and Benson.[25]

A similar dose schedule, excluding the GBL groups, was followed in the experiments measuring GBL following 1,4-BD administration, with the exception that the saline and pyrazole control groups were not given a water injection (N = 4 for all groups except the 60-min pyrazole control group, where N = 3). Animals were decapitated and exsanguinated at either 60 or 120 min after injection with 1,4-BD, or the equivalent time, 69 or 129 min, after pyrazole or saline in the control groups. Blood proteins were precipitated with 3 vol. of a 10 per cent metaphosphoric acid solution. The sum of GHB and GBL was determined by a modification of the method of Guidotti and Ballotti,[26] and is expressed as micromoles of GBL per gram of whole blood.

Rat liver supernatant. All preparative operations were carried out at 4°. For each experiment, a male Sprague–Dawley rat weighing approximately 150 g was decapitated and the liver rapidly removed. A 20 per cent homogenate was prepared in a Potter–Elvehjem homogenizer (5 min) with 0·01 M potassium phosphate buffer, pH 8·0. The homogenate was centrifuged at 100,000 *g* for 1 hr at 4° in an International preparative ultracentrifuge, B-60. The 3-ml reaction mixtures contained 0·3 ml of 0·25 M potassium phosphate buffer, pH 7·2; 0·1 ml of 100,000 *g* supernatant; 1 mg NAD added in 0·1 ml; and 1,4-BD, ethanol and pyrazole in the appropriate concentrations. Reactions were carried out at 30° and were initiated by the addition of NAD. Reduction of NAD was followed at 340 nm in a Gilford 240 recording spectrophotometer. Maximal linear rates following an initial lag period were plotted according to Lineweaver and Burk.[27] Protein concentration was determined by the Biuret method.[28]

Crystalline equine liver alcohol dehydrogenase. Equine liver alcohol dehydrogenase was purchased from CalBiochem (A grade) as a crystalline suspension in 0·02 M phosphate buffer containing 10 per cent ethanol. This suspension (0·4 ml) was diluted by addition of 1·6 ml of 0·1 M potassium phosphate buffer, pH 7·8, and was dialyzed against 1 l. of the 0·1 M phosphate buffer, pH 7·8, at 4° for 57 hr (changed once, at 24 hr) to remove ethanol.[29] Protein content was measured by the method of Lowry *et al.*[30]

Enzyme activity was assayed at 23·5° according to Dalziel:[31] 1·85 ml glycine–NaOH buffer (0·1 M, pH 10·0), 1 ml NAD solution (1 mg/ml), and 0·15 ml substrate solution (180 mM, final concentration 9 mM) in a 1 cm cuvette. The reaction was initiated by addition of 5–20 μl of dialyzed alcohol dehydrogenase to the reaction mixture, and the reaction was followed at 340 nm. Activity was calculated from measurements of the time required for an optical density change of 0·200 at 340 nm.

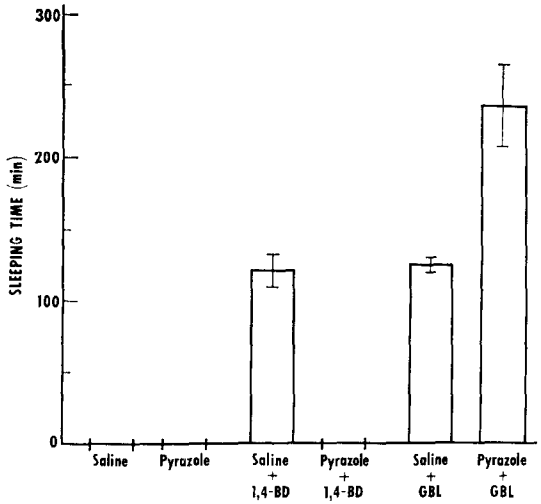


FIG. 2. Effect of pyrazole (6·6 m-moles/kg) on sleeping time (duration of loss of righting reflex) in minutes (mean ± S.E.M.) following injections with 1,4-BD (5·55 m-moles/kg) and GBL (5·55 m-moles/kg). Pyrazole preinjection prevents loss of the righting reflex following 1,4-BD which, when compared with the saline + pyrazole control, is a difference with $P \ll 0{\cdot}001$. Pyrazole + GBL prolongs the sleeping time relative to the saline + GBL control ($P < 0{\cdot}001$). ($N = 8$ for the saline, pyrazole, saline + 1,4-BD, and pyrazole + 1,4-BD groups; $N = 12$ for the saline + GBL and pyrazole + GBL groups.)

RESULTS

Initially, the effects of 1,4-BD on the sleeping time of rats with and without pyrazole were studied. Pyrazole preinjection at a dose of 6·6 m-moles/kg prevents the loss of the righting reflex following 1,4-BD injection ($P \ll 0{\cdot}001$) (Fig. 2); the mean sleeping time of 121 min in the absence of pyrazole corresponds closely to that observed by Roth and Giarman,[16] using a similar dose of 1,4-BD. Pyrazole at this dose prolongs the sleeping time following GBL injection from a mean of 125–237 min ($P < 0{\cdot}001$) (Fig. 2).

It appeared that pyrazole might have prevented the metabolism of 1,4-BD to a centrally acting product. In order to investigate this question further, the combined contents of GHB and GBL in the blood were measured at 60 and 120 min (Fig. 3). These substances were absent following pyrazole preinjection at both of these times, but attained levels of 4·8 and 3·6 μm/g (at 60 and 120 min respectively) in the absence of pyrazole ($P \ll 0{\cdot}001$). These blood levels are also similar to those seen by Roth and Giarman.[16]

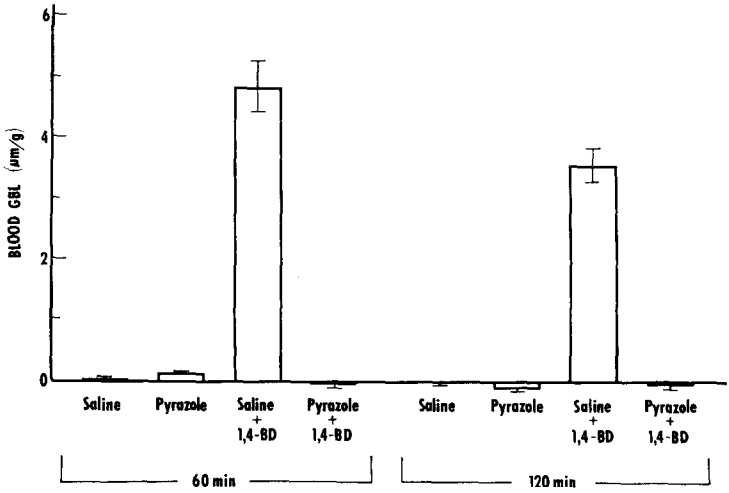


FIG. 3. Effect of pyrazole (6·6 m-moles/kg) on blood GBL + GHB (measured as GBL, micro-moles per gram of blood, reported as mean ± S.E.M.) 60 and 120 min following 1,4-BD injection (5·55 m-moles/kg). At both 60 and 120 min, pyrazole preinjection is associated with the absence of GBL + GHB in blood, a significant difference relative to the saline + 1,4-BD controls ($P \ll 0{\cdot}001$). (N = 4 for all groups except the 60-min pyrazole control group, where N = 3.)

The metabolism of 1,4-BD by a 100,000 *g* rat liver supernatant fraction was shown to be dependent on NAD and inhibited by pyrazole. The reaction kinetics followed a linear relationship in a Lineweaver–Burk plot over a concentration range similar to that described by Reynier[32] for ethanol, 0·63–7·4 mM 1,4-BD. The apparent K_m was 0·54 mM in the experiment illustrated in Fig. 4, which was the median of five determinations; the mean value was 0·61 (S.D. = ±0·16 mM, N = 5). The apparent K_m for ethanol in this system was 1·3 mM; this value is of the same order of magnitude as that for 1,4-BD and is similar to the values determined by others with ethanol as the varied substrate, Reynier[32] obtaining a value of 0·5 mM using 10-fold purified rat liver alcohol dehydrogenase, and Markovic *et al.*[33] determining a value of 2·13 mM

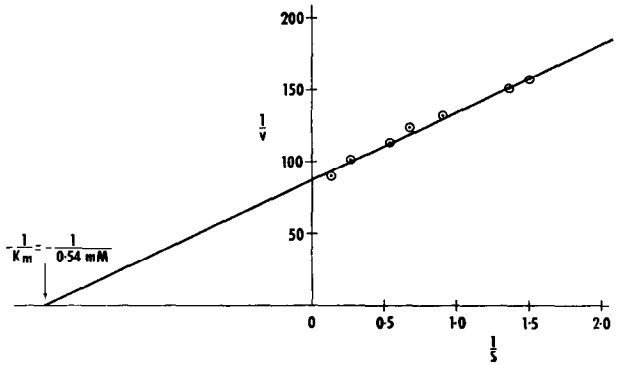


FIG. 4. Lineweaver–Burk plot of the NAD-coupled oxidation of 1,4-BD by a 100,000 *g* rat liver supernatant. This K_m of 0·54 mM was the median of five determinations; mean = 0·61 mM.

with electrophoretically homogeneous (107-fold purified) rat liver alcohol dehydrogenase. The V_{max} for ethanol in our system was 69 per cent of that for 1,4-BD.

Evidence that GHB is produced from 1,4-BD by this NAD-coupled reaction *in vitro* is provided by measurements of GHB colorimetrically[26] following incubation of a 45,000 *g* rat liver supernatant with 1,4-BD (74 mM) and NAD (1·3 mg/ml). GHB production was observed and was enhanced by addition of an NADH regenerating system.

Pyrazole at a concentration of 25·0 μm appears to be a competitive inhibitor for the rat liver reaction *in vitro*, with a 1,4-BD as the varied substrate, at high substrate concentrations (1·8–37 μM). The K_i is in the range of 3–15 $\times$ 10^{-6} M. The value determined by Reynier[32] on 10-fold purified rat liver alcohol dehydrogenase was 4·2 $\times$ 10^{-6} M.

TABLE 1. SPECIFIC ACTIVITY OF EQUINE LIVER ALCOHOL DEHYDROGENASE WITH VARIOUS SUBSTRATES

Substrates	Sp. act.* (mμmoles/min/mμg)
Ethanol	3·78
1,4-BD	2·44
GHB	0
GBL	0

* Activity measured according to Dalziel,[31] using substrate concentrations of 9 mM.

The activity of a crystalline horse liver alcohol dehydrogenase preparation was determined for ethanol, 1,4-BD, GHB and GBL (Table 1). The activity toward 1,4-BD was 65 per cent of the ethanol activity under the assay conditions. Neither GHB nor GBL exhibited activity as substrates for this enzyme.

DISCUSSION

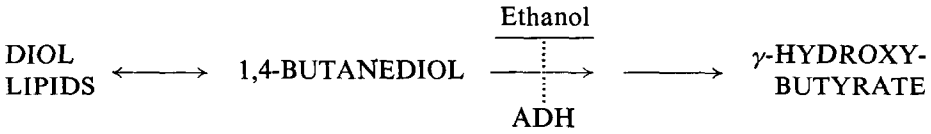
The sleeping time data from this report provide direct evidence that the neuropharmacological effects following 1,4-BD administration are due to a metabolite subsequent to the pyrazole-inhibited step.

The prolongation of sleeping time with GBL, following pyrazole preinjection, suggests that the effective metabolite of 1,4-BD is GBL or GHB. Neither GBL nor its metabolite, GHB,[34,35] is a substrate for equine liver alcohol dehydrogenase, and thus the increased duration of loss of the righting reflex following pyrazole plus GBL cannot be explained by pyrazole inhibition of the metabolism of GHB or GBL by liver alcohol dehydrogenase. However, other enzymes which are involved in the metabolism of these compounds and which might be inhibited by pyrazole include the γ-lactonase[34,35] and lactate dehydrogenase[14] (L-lactate: NAD oxidoreductase, EC 1.1.1.27) (Fig. 1). Though Reynier[32] has shown that pyrazole is not an inhibitor of the reaction of lactate with rabbit muscle lactate dehydrogenase, this does not rule out the possibility of the inhibition by pyrazole of the reaction of GHB with this

enzyme in the rat. An analogy to this proposal is to be found in the demonstration by Lester and Benson[25] that pyrazole more effectively inhibits ethanol oxidation than the oxidation of longer chain primary alcohols, explained by a decrease in K_m with increasing chain length. Other possible explanations of the prolongation of sleeping time following pyrazole plus GBL might be a synergy[36] between GBL and the low level of ethanol found by Krebs and Perkins[37] in the peripheral blood following pyrazole administration or an interaction of GBL directly with the toxic pyrazole.[38]

We have demonstrated that 1,4-BD is oxidized by a rat liver supernatant enzyme and by crystalline horse liver alcohol dehydrogenase. The rat liver system is inhibited by pyrazole *in vitro* and *in vivo*. Therefore, it may be concluded that alcohol dehydrogenase is involved in one step of the metabolic conversion of 1,4-BD to GHB, the second step probably being mediated by aldehyde dehydrogenase (aldehyde: NAD oxidoreductase, EC 1.2.1.3). The conclusion as to the extent of involvement of alcohol dehydrogenase in the rat must be based on what is known concerning the specificity of pyrazole as an inhibitor of this enzyme. Various zinc-containing enzymes have been studied and of these pyrazole inhibited only the horse liver, rat liver, and yeast alcohol dehydrogenases.[32] Rubin *et al.*[39] have recently shown that pyrazole inhibits the hepatic microsomal drug metabolizing system. While the possibility of microsomal inhibition may influence interpretation of the data *in vivo*, this is not a consideration in our studies *in vitro*, in view of the fact that our rat liver supernatant was free of microsomes (100,000 *g* for 60 min).

The similarity of the kinetics for the reaction of alcohol dehydrogenase with ethanol and 1,4-BD suggests the following possible roles of these substances in intermediary metabolism:



1,4-BD and ethanol, with similar K_m values for alcohol dehydrogenase, might be expected to be competitive inhibitors of this enzyme. Thus, in the presence of high ethanol concentrations the metabolism of 1,4-BD to GHB might be inhibited. We may further speculate that by inhibiting this enzymic step ethanol might produce a more extensive perturbation of metabolism, altering the steady state balance of unesterified 1,4-BD and 1,4-BD esterified in diol lipids, with the accumulation of these substances. In this connection it has been observed that chronic treatment with 1,4-BD was accompanied by the development of "fatty dystrophy and areas of sclerotic growth" in the liver,[4] though analysis of the accumulated fats for 1,4-BD content was not carried out. 1.2-Propanediol, another component of the diol lipids[1–3] and glycerol are also metabolized by mammalian alcohol dehydrogenases[40–43] and the above argument could be extended to include these substances, also. While thus providing an explanation of another physiological role for liver alcohol dehydrogenase,[37] this would also indicate the possible accumulation of the diol lipids as a component in the development of fatty liver following ethanol ingestion.

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