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A Detoxication Route for Acetaldehyde: Metabolism of Diacetyl, Acetoin, and 2,3-Butanediol in Liver Homogenate and Perfused Liver of Rats[1]

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The metabolism of diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone), and 2,3-butanediol, which are metabolites of acetaldehyde was quantitatively investigated using rat liver homogenate, liver perfusion, and *in vivo* experiments. Diacetyl and acetoin were reduced to 2,3-butanediol in these experiments, but acetoin and 2,3-butanediol were scarcely oxidized to diacetyl, indicating that the reduction reaction to 2,3-butanediol from diacetyl occurs actively in rat liver. The formation of acetoin from diacetyl required either NADH or NADPH as a reductant, while the reduction of acetoin to 2,3-butanediol required NADH. Acetoin and 2,3-butanediol were more readily accumulated than diacetyl in brain tissue.

Key words: acetoin, 2,3-butanediol, diacetyl, metabolism, rat.

Diacetyl, acetoin, and 2,3-butanediol have been thought to be by-products of the fermentation process and their metabolism has been studied mainly in microorganisms (*1-3*). However, these molecules are widely distributed not only in microorganisms but also in higher animals (*4, 5*). Acetaldehyde, which is a chemically reactive metabolite of ethanol, reacts with pyruvate enzymatically or nonenzymatically in the presence of thiamine to form acetoin (*6, 7*). Oxidation of acetoin would afford diacetyl and reduction would afford butanediol. Recently, the presence of 2,3-butanediol was detected in serum from alcoholics (*8-10*). The biochemistry of these C_4 compounds in animal tissues is still not well understood and the meaning of their presence remains to be elucidated, as will be discussed later.

We previously established sensitive and specific determination methods for these C_4 compounds (*11, 12*), in order to obtain evidence for our hypothesis that the accumulation of acetoin, diacetyl, and 2,3-butanediol may be responsible for so-called hangover after drinking alcohol. In this study, we examined the metabolism and the interconversion of these C_4 compounds in rat using the above determination methods to elucidate the biochemical basis of hangover, and to obtain clues to its possible treatment.

MATERIALS AND METHODS

Chemicals—Diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone), 2,3-butanediol, aldrin (standard grade), *n*-hexane, and benzene for GLC analysis were purchased from Wako Pure Chemicals (Osaka). 4,5-Dichloro-1,2-diaminobenzene and cyclohexanediol were obtained from Aldrich (Milwaukee, WI, USA). 4,5-Dichloro-1,2-diaminobenzene was purified by recrystallization from 2 M hydrochloric acid. 6,7-Dichloro-2,3-dimethylquinoxaline and 7,8-dichloro-1,2,3,4-tetrahydrophenazine were prepared by us (*11, 12*).

Animals—Male Wistar strain albino rats weighing from 200 to 220 g were purchased from Charles River Japan (Yokohama) and fed on standard rat cake MF (Oriental Yeast, Tokyo) for 7 days before use.

Activities of Diacetyl- or Acetoin-Reducing Enzymes in Rat Tissues—The liver, kidney, or brain of rat was homogenized in 4 volumes of 50 mM sodium phosphate (pH 7.4), and the homogenate was centrifuged at $700 \times g$ for 15 min. The activity in 10 μl of the supernatant was assayed at 30°C as the initial reaction rate in terms of the absorbance change at 340 nm in the presence of 10 mM diacetyl or acetoin, 0.9 mM NADH or NADPH, 2.5 mM nicotinamide, and 50 mM sodium phosphate (pH 7.4), in a total volume of 1 ml. One unit was defined as the amount of enzyme oxidizing 1 μmol of NADH or NADPH/min under these conditions. The reaction mixtures without the substrates were used as controls. The protein concentrations were determined by the biuret method (*13*).

Metabolic Experiments on Diacetyl, Acetoin, and 2,3-Butanediol in Rat Liver Homogenates—Rat liver was homogenized in 4 volumes of 50 mM sodium phosphate (pH 7.4). The homogenate was centrifuged at $700 \times g$ for 15 min. Ten micromolar substrate (diacetyl, acetoin, or 2,3-butanediol), 0.9 mM NADH, 2.5 mM nicotinamide, 0.1 ml of the rat liver homogenate supernatant, and 50 mM sodium phosphate (pH 7.4) were incubated at 37°C for 10 min in a total volume of 0.5 ml. The reaction was stopped by adding 1 ml of 0.9 M $HClO_4$ to the reaction mixture. The sample was centrifuged at $10,000 \times g$ for 5 min and one-tenth of the supernatant was assayed for diacetyl or acetoin according to the method of Otsuka and Ohmori (*11*). Briefly, acetoin was oxidized by Fe^{3+} to diacetyl, which was converted to 6,7-dichloro-2,3-dimethylquinoxaline. The quinoxaline derivative was determined by GLC with

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electron capture detection. For the determination of 2,3-butanediol, 0.5 ml of the supernatant was diluted with an equal volume of 2 M K_2HPO_4, and centrifuged. The supernatant (0.5 ml) was analyzed by the method of Otsuka and Ohmori (*12*), which is based on the oxidation reaction of the diol to diacetyl by $KMnO_4$.

In Vivo Metabolic Experiments on Diacetyl, Acetoin, and 2,3-Butanediol—Diacetyl, acetoin, or 2,3-butanediol was dissolved in physiological saline to a final concentration of 1 M and given *per os* to rats (5 mmol per kg body weight). One hour after administration, rats were intraperitoneally injected with 0.2 ml of pentobarbital (50 mg/ml). Liver, kidney, and brain were immediately excised and rinsed in ice-cold physiological saline. Liver and kidney were perfused with ice-cold saline. The organs were homogenized in 4 volumes of physiological saline at 4°C in a glass homogenizer using a Teflon pestle. The homogenates were centrifuged at $700 \times g$ for 15 min. Diacetyl, acetoin, and 2,3-butanediol in the supernatants were measured as described in previous reports (*11, 12*). All these experiments were performed from 8:00 to 8:30 a.m. and rats given physiological saline were used as controls.

Liver Perfusion Experiments—The perfusion experiments were performed according to the method of Shiota *et al.* (*14*). The rats were intraperitoneally injected with 0.2 ml of pentobarbital (50 mg/ml), tied to a dissection board, and the abdomen opened. The perfusion was carried out from the portal vein to the inferior vena cava with Krebs-Ringer-Henseleit buffer saturated with a gas mixture of O_2 and CO_2 (95 : 5) at a flow rate of 4.3 ml/min/g liver and 37°C. After perfusion for 30 min, the substrate was added to the buffer to a concentration of 1 mM. After non-recirculating perfusion for 1 h, the liver was excised and 1 g of liver was homogenized, deproteinized, and analyzed for diacetyl, acetoin, and 2,3-butanediol as described in "*In vivo metabolic experiments.*" The perfusate (0.1 ml) was analyzed for diacetyl and acetoin, and 0.5 ml of the perfusate was used for the analysis of 2,3-butanediol.

RESULTS

Activities of Diacetyl and Acetoin Reducing Enzymes in Rat Organs—Diacetyl-reducing enzyme activities in rat tissue homogenates are listed in Table I. The total and specific activity were highest in the liver; either NADH or NADPH could act as a coenzyme. Little diacetyl-reducing activity was found in brain tissue. As shown in Table II, the total activity of NADH-linked acetoin-reducing enzyme activity was highest in the liver and lowest in the brain. The acetoin-reducing activity using NADPH as a coenzyme was generally low compared to that using NADH in these organs.

Interconversion of C_4 Compounds Added to Rat Liver Homogenate—Diacetyl, acetoin, or 2,3-butanediol was added at 10 nmol to the rat liver homogenate and the mixture was incubated at 37°C for 10 min. The results (Table III) indicate that the three compounds were interconvertible and became equilibrated at the molar ratio of about 0 : 3 : 7, respectively, in rat liver homogenate when diacetyl and acetoin were used as substrates.

Contents of C_4 Compounds in Liver, Kidney, and Brain after Oral Administration of These Compounds to Rats—The C_4 compounds were determined in liver, kidney, and brain 1 h after oral administration (5 mmol/kg body weight). The results are shown in Fig. 1, a-c, and indicate that the three C_4 compounds undergo interconversion. When diacetyl was administered (Fig. 1a), it was found in liver, kidney and brain as the unchanged molecule, but in a small amounts. The total amount of diacetyl in these tissues from treated rats corresponded to 0.03% of the administered dose. The diacetyl was reduced to acetoin, which was mainly found in brain tissue. Another reduced metabolite, 2,3-butanediol was found in comparatively high amounts in these organs, corresponding to 2.3% of administered diacetyl.

The result of acetoin administration is shown in Fig. 1b. When acetoin was administered to rats, it was oxidized to diacetyl and reduced to 2,3-butanediol. The patterns of interconversion and accumulation in these organs were similar to those in the same organs after administration of diacetyl (Fig. 1a). After oral administration, the highest level of this compound was found in brain tissue. The total amount of 2,3-butanediol in these organs was equivalent to 2.6% of administrated acetoin.

Finally, 2,3-butanediol was oxidized, albeit in small amounts, to the other two compounds, which were accumulated in the liver (Fig. 1c). The diol itself was deposited in

TABLE I. **Activity of diacetyl-reducing enzyme.** Measurements and calculation were performed as described in "MATERIALS AND METHODS." Values are presented as means±SD for five rats.

Tissue	Total activity (U/organ)		Specific activity (mU/mg protein)	
	NADH	NADPH	NADH	NADPH
Liver	21.6±1.2	25.2±0.9	32.4±5.3	37.2±1.4
Kidney	3.1±0.8	2.0±0.7	10.4±2.5	7.1±2.1
Brain	0.2±0.1	0.7±0.3	5.1±2.4	9.3±2.9

TABLE II. **Activity of acetoin-reducing enzyme.** Measurements and calculation were performed as described in "MATERIALS AND METHODS." Values are presented as means±SD for five rats.

Tissue	Total activity (U/organ)		Specific activity (mU/mg protein)	
	NADH	NADPH	NADH	NADPH
Liver	11.8±1.2	0.8±0.1	18.4±1.5	2.2±0.4
Kidney	2.3±0.8	2.0±0.3	6.2±1.2	6.1±0.4
Brain	0.8±0.1	0.1±0.01	43.3±2.6	5.2±0.8

TABLE III. **Interconversion of C_4 compounds added to rat liver homogenate.** Rat liver was homogenized in 4 volumes of 50 mM sodium phosphate (pH 7.4). The homogenate was centrifuged at $700 \times g$ for 15 min. Twenty micromolar substrate (diacetyl, acetoin, or 2,3-butanediol), 0.9 mM NADH, 2.5 mM nicotinamide, and 0.1 ml of the supernatant were incubated at 37°C for 10 min in a total volume of 0.5 ml made up by adding 50 mM sodium phosphate (pH 7.4). After the reaction, 1 ml of 0.9 M $HClO_4$ was added to the incubation mixture, followed by centrifugation at $10,000 \times g$ for 5 min. For the determination of diacetyl and acetoin, 0.1 ml of the supernatant was used according to the previous method (*11*). For the determination of 2,3-butanediol, 0.5 ml of the supernatant was diluted with an equal volume of 2 M K_2HPO_4, followed by centrifugation and then the supernatant (0.5 ml) was used for the determination (*12*). Values are presented as means±SD for three rats. n.d., not detectable.

Added (10 nmol)	Found (nmol)			Total
Substrates	Diacetyl	Acetoin	2,3-Butanediol	
Diacetyl	n.d.	3.74±0.23	6.29±0.22	10.03±0.04
Acetoin	n.d.	3.13±0.13	7.07±0.33	10.20±0.39
2,3-Butanediol	n.d.	0.16±0.05	9.80±0.28	9.96±0.30

all three organs, the total amount being to 3% of the administered dose.

Contents of C_4 Compounds in Liver and Perfusate after Liver Perfusion—Rat livers were perfused for 1 h with Krebs-Ringer-Henseleit buffer which was saturated with O_2 and CO_2 (95 : 5), and contained 1 mM substrate. The

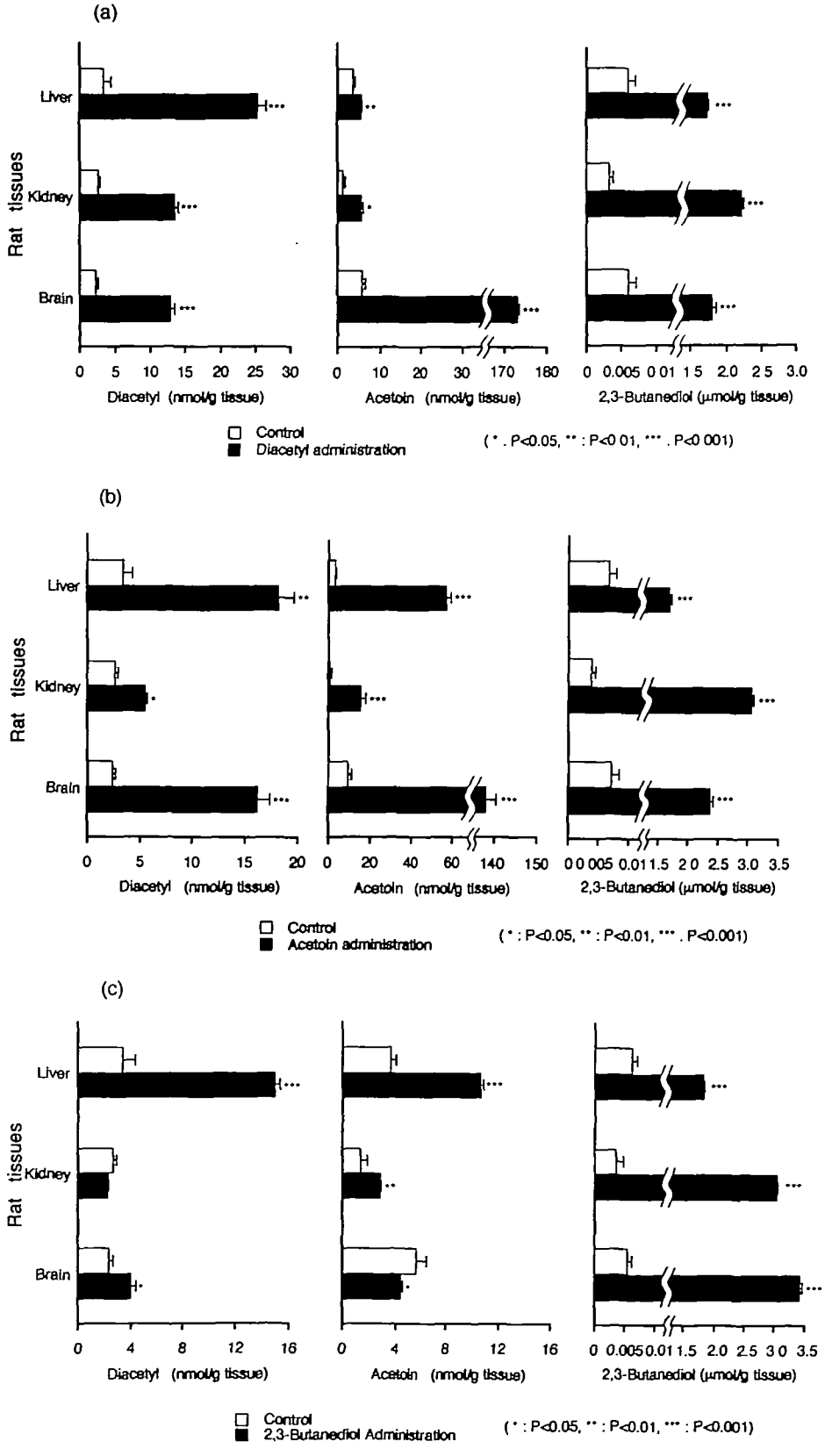


Fig. 1. **Contents of C_4 compounds in liver, kidney, and brain after oral administration of these compounds to rats.** Diacetyl (a), acetoin (b), or 2,3-butanediol (c) was dissolved in physiological saline at 1 M concentration and given *per os* to rats (5 mmol per kg body weight). One hour after oral administration, liver, kidney, and brain were immediately excised and rinsed in ice-cold physiological saline. Liver and kidney were perfused with 5 ml of ice-cold saline. The organs were homogenized and centrifuged at 700× *g* for 15 min. Diacetyl, acetoin, and 2,3-butanediol in the homogenates were measured as described in "*In vivo experiment.*" The rats given physiological saline were used as controls.

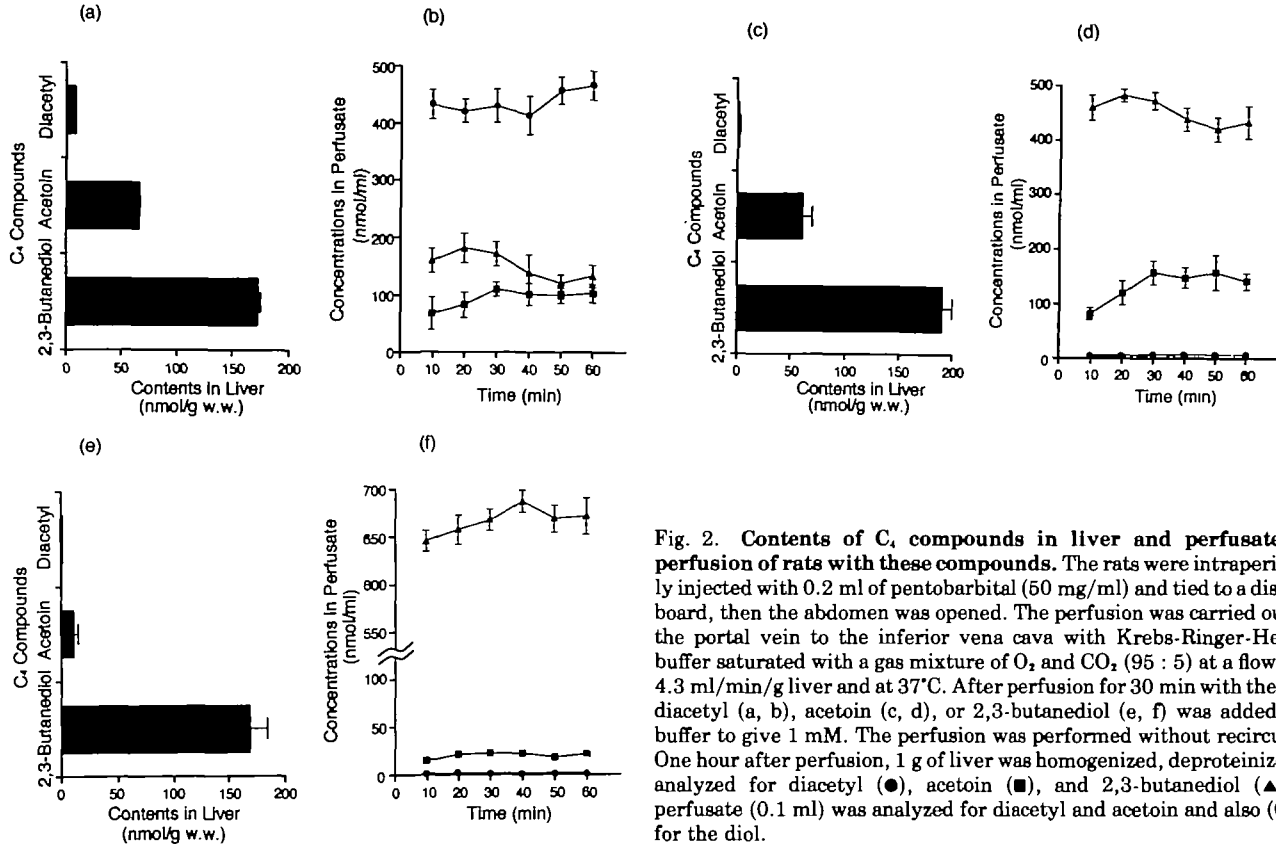


Fig. 2. **Contents of C_4 compounds in liver and perfusate after perfusion of rats with these compounds.** The rats were intraperitoneally injected with 0.2 ml of pentobarbital (50 mg/ml) and tied to a dissection board, then the abdomen was opened. The perfusion was carried out from the portal vein to the inferior vena cava with Krebs-Ringer-Henseleit buffer saturated with a gas mixture of O_2 and CO_2 (95 : 5) at a flow rate of 4.3 ml/min/g liver and at 37°C. After perfusion for 30 min with the buffer, diacetyl (a, b), acetoin (c, d), or 2,3-butanediol (e, f) was added to the buffer to give 1 mM. The perfusion was performed without recirculation. One hour after perfusion, 1 g of liver was homogenized, deproteinized, and analyzed for diacetyl (●), acetoin (■), and 2,3-butanediol (▲). The perfusate (0.1 ml) was analyzed for diacetyl and acetoin and also (0.5 ml) for the diol.

TABLE IV. **Non enzymatic acetoin formation from pyruvate and acetaldehyde in the presence of thiamine.**

0.1 M thiamine (ml)	0.1 M pyruvate (ml)	1 M acetaldehyde (ml)	pH	Acetoin found (μmol)
0.5	1.25	0.3	8.4	2.55
0.5	1.0	0.3	8.4	2.21
0.5	0.5	0.3	8.4	1.16
0.5	0.25	0.3	8.4	0.59
0.5	0	0.3	8.4	n.d.
0.5	1.0	0	8.4	0.48
0	1.0	0.3	8.4	n.d.
0.5	1.0	0.3	9.2	0.28

perfusates were collected at intervals of 10 min. After 1 h of perfusion, the livers were analyzed for diacetyl, acetoin and 2,3-butanediol. The results are shown in Fig. 2, a-f.

When diacetyl was added to the perfusion solution, it was reduced to acetoin and 2,3-butanediol in liver. The mole ratio of diacetyl, acetoin, and 2,3-butanediol in liver was 5 : 39 : 100 (Fig. 2a). In the perfusate, diacetyl, 2,3-butanediol, and acetoin were found in amounts equivalent to about 45, 15, and 10% of the added diacetyl, respectively (Fig. 2b). As shown in Fig. 2, a and b, the total amount of the C_4 compounds found in perfused liver corresponds to 0.1% of perfused diacetyl, while the total amount of the C_4 compounds found in the perfusate is *ca.* 70% of perfused diacetyl, and consequently, *ca.* 30% of perfused diacetyl must be metabolized or subjected to glucuronidation in the liver. Diacetyl added to the perfusion buffer was readily reduced to acetoin and the diol in the rat liver.

When acetoin was added to the perfusion buffer, it was mostly reduced to the diol, with a smaller portion oxidized to diacetyl. Diacetyl, acetoin, and the diol were found in the molar ratio of about 1 : 38 : 100 in liver and 1 : 15 : 45 in the perfusate (Fig. 2, c and d). In analogy with the perfusion experiment with diacetyl, the total amount of C_4 compounds found in liver corresponds to *ca.* 0.1% of the total amount of perfused acetoin, while total amount of the C_4 compounds found in the perfusate was 70%, so that the remaining 30% of perfused acetoin must be metabolized or conjugated in the liver. It seems that added acetoin was more quickly reduced to the diol in the first 30 min than in the next 30 min (Fig. 2d).

As shown in Figs. 1c, 2e, and 2f, oxidation of 2,3-butanediol to the other two compounds seem to be more difficult than the reduction from diacetyl or acetoin to diol. After subtracting the total amount of C_4 compounds found in the liver and the perfusate from that of perfused 2,3-butanediol, we presume that the remaining 33% of the perfused diol may be metabolized or conjugated. In addition, when only the buffer was perfused, no C_4 compounds were found in the perfusate.

Acyloin Condensation—Various amounts of pyruvate and acetaldehyde were reacted at 40°C for 24 h in the presence of thiamine, according to the method of Mizuhara *et al.* (*7*). After the reaction, acetoin formed in the reaction mixture was determined by our method (*11*). The results are listed in Table IV. Acetoin was formed from acetaldehyde and pyruvate, and its yield depended on the pyruvate concentration. After one molecule of pyruvate has been nonenzymatically decarboxylated, the active aldehyde

formed reacts nonenzymatically with a second molecule of pyruvate to form acetoin. However, no acetoin is formed in a from the mixture of thiamine and acetaldehyde or from pyruvate and acetaldehyde. The highest yield was only 2% from pyruvate.

DISCUSSION

Ethanol is detoxified by alcohol and aldehyde dehydrogenase or other enzymes in mammals. Small amounts of alcohol are quantitatively oxidized to acetaldehyde and then to acetate in the human body, supplying energy and causing no problem. However, when fairly large quantities of alcohol are imbibed, acetaldehyde is formed and accumulated in body. This occurs particularly in about 50% of Mongolian people, who are deficient in aldehyde dehydrogenase 2 (*15*); acetaldehyde accumulates readily in their bodies after alcohol has been taken.

Acetaldehyde is chemically active, reacting easily with amino and thiol groups to form a Schiff's base or hemithioacetal. It reacts with cysteine and catecholamine, for example, to form thiazolidine and tetrahydroisoquinoline rings (*16*). For these reasons, acetaldehyde is toxic. As shown in Table IV, acetaldehyde also reacts nonenzymatically with pyruvate in the presence of thiamine to form acetoin, though a higher yield is obtained in the presence of enzyme.

Casazza has reviewed the presence, source, metabolism, and toxicity of 2,3-butanediol (*17*). The presence and source of the diol, which was detected in blood from alcoholic men (*8-10, 18*), remain controversial, mainly because of the lack of specific and sensitive determination methods for acetaldehyde and C_4 compounds in biological samples and of fundamental enzymic studies. The determination methods for diacetyl, acetoin, and 2,3-butanediol, that we have developed are sufficiently sensitive to detect 50 fmol/μl, and C_4 compounds are measurable in even 0.1 ml of normal human urine or blood. It is noteworthy that no simple, sensitive and specific determination method for acetaldehyde in biological samples was available until we recently developed one (*19*), though the biochemical and physiological importance of acetaldehyde has long been known.

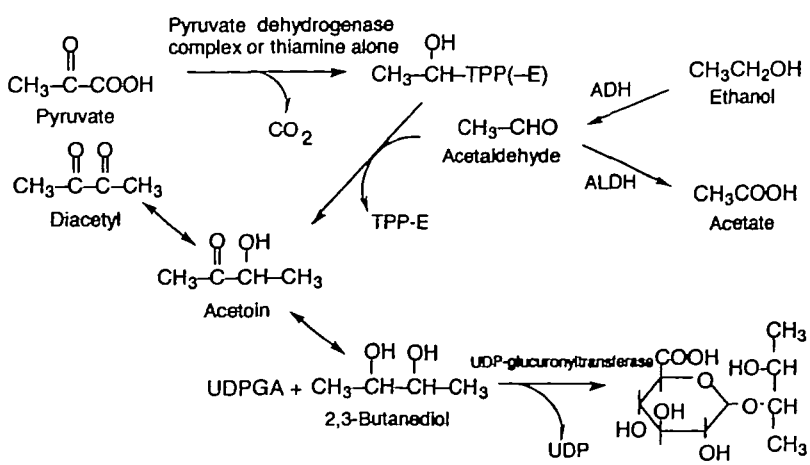
When rats were given 5 mmol/kg of ethanol *per os*, 0.75 μmol of total C_4 compounds (mainly 2,3-butanediol) was found in the liver (data not shown). When acetoin was given orally as a single dose of 5 mmol/kg, 15 μmol of total C_4 compounds was found in the liver after 1 h. In these experiments, the amounts of ethanol and acetoin administered were equimolar. In practice, however, equimolar intake of ethanol and C_4 compounds do not occur. Since, in general, alcoholic beverages contain C_4 compounds amounting to 0.003% of ethanol (*11*), the contribution of diacetyl and acetoin in alcoholic beverages to the elevated hepatic level of 2,3-butanediol must be very small.

When a student volunteer with a defect of aldehyde dehydrogenase 2 drank chemically pure alcohol (0.8 g/kg), 40 nmol of 2,3-butanediol was found in 1 ml of blood. This concentration was 2-3 times higher than that of a volunteer with intact aldehyde dehydrogenase 2. These experimental results, which will be reported in more detail elsewhere, indicate that 2,3-butanediol in blood dose arise from ethanol *via* acetaldehyde. These results resolve the arguments presented by Kuehle *et al.* (*18*).

As also shown in this report, about two-thirds of added diacetyl, acetoin, or diol was recovered as the total C_4 compounds in liver perfusion experiments. This may be due to formation of glucuronide. During the preparation of this paper, a butanediol-glucuronide has been found and identified in rat and human urine after administration of 2,3-butanediol or alcohol. The glucuronide is a new substance and will be reported later. We propose that acetaldehyde is converted to acetoin, which is reduced to 2,3-butanediol, which is then conjugated with uridine diphosphate glucuronide, followed by excretion into urine. This would be a detoxication route for acetaldehyde (Fig. 3).

As can be seen from Fig. 1, a and b, when diacetyl or acetoin was orally administered to rats, acetoin was accumulated at much higher concentration in brain than in liver or kidney, and 2,3-butanediol was accumulated in brain. The accumulation is thought to be caused by low levels of diacetyl- and acetoin-reducing activity in brain (Tables I and II), in combination with the permeability of brain to acetoin and 2,3-butanediol and their affinity for brain tissue (Fig. 1).

Finally, it can be seen from Tables I and II that enzyme



TPP : Thiaminepyrophosphate
E : Pyruvate dehydrogenase complex

Fig. 3. Biosynthesis and metabolism of C_4 compounds.

activity for reduction of diacetyl and acetoin is mainly present in liver and requires NADH or NADPH as a coenzyme. We have purified a diacetyl-reducing enzyme from rat liver. This enzyme has a molecular weight of 39 kDa and requires only NADPH. These data will be reported elsewhere in detail.

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