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Simple and sensitive determination of 2,3-butanediol in biological samples by gas chromatography with electron-capture detection

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Abstract

2,3-Butanediol was quantitatively oxidized into diacetyl by reaction with MnO_4^- at 20°C for 30 min under neutral conditions. The reaction of diacetyl with 4,5-dichloro-1,2-diaminobenzene afforded 6,7-dichloro-2,3-dimethylquinoxaline (DCDMQ), which was extracted with *n*-hexane and determined by gas chromatography with electron-capture detection. As an internal standard 1,2-cyclohexanediol was used. The detection limit of DCDMQ (or 2,3-butanediol) was 10 fmol/μl in the extract, and the determination limit of DCDMQ (or 2,3-butanediol) was at least from 50 fmol/μl to 20 pmol/μl in the extract. Recoveries from normal rat urine and rat liver homogenate were 97.8 ± 3.4% and 98.4 ± 2.9%, respectively. The method is very simple and sensitive and is applicable to the determination of 2,3-butanediol in normal rat tissues.

1. Introduction

Extensive studies of 2,3-butanediol have been made in the fields of fermentation technology and food chemistry [1,2]. It has been detected in alcoholic beverages, such as wine [3,4] and beer [5]. However, little is known about the biochemistry of 2,3-butanediol, especially in animals, although it has been well known for a long time. Recently this compound has been studied in relation to alcoholism [6]. In order to study the formation and degradation of C_4 compounds such as diacetyl, acetoin and 2,3-butanediol in animals, we required a method for determining these C_4 compounds. In a previous paper, we reported methods for the determination of diacetyl and acetoin [7].

In this paper, a simple and sensitive method for the determination of 2,3-butanediol in biological samples is described using capillary gas chromatography (GC) with electron-capture detection (ECD), based on the reaction shown in Fig. 1.

2. Experimental

2.1. Chemicals

Diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone), 2,3-butanediol and *n*-hexane for GC–ECD were purchased from Wako (Osaka, Japan) and 4,5-dichloro-1,2-diamino-

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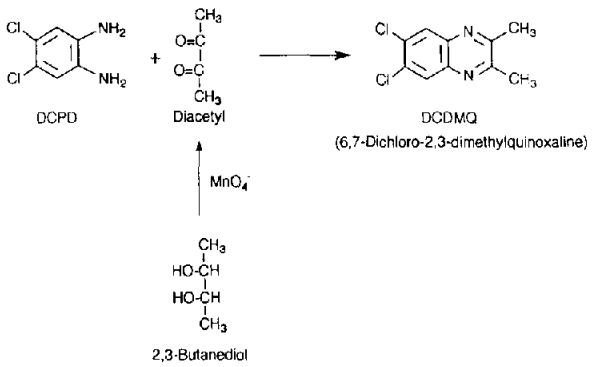

Fig. 1. Reaction scheme for the determination of 2,3-butanediol.

benzene (DCDB) and 1,2-cyclohexanediol from Aldrich (Milwaukee, WI, USA). DCDB was purified by recrystallization from hydrochloric acid after charcoal treatment. 6,7-Dichloro-2,3-dimethylquinoxaline (DCDMQ) was prepared as described previously [7]. 7,8-Dichloro-1,2,3,4-tetrahydrophenazine (DCTHP) was prepared by reaction of 0.25 g of DCDB and 0.1 g of 1,2-cyclohexanedione in 30 ml of 1 *M* HCl at 40°C for 2 h. The reaction mixture was allowed to stand overnight at room temperature and then extracted with 30 ml of ethyl acetate twice. The extract was dried with Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was recrystallized from ethanol in 89.4% yield, m.p. 189–191°C, as a yellowish brown powder. Analysis: calculated for $C_{12}H_{10}N_2Cl_2$, C 56.94, H 3.98, N 11.07, Cl 28.01; found, C 56.91, H 3.97, N 11.12, Cl, 28.00%.

2.2. Gas chromatography

All samples were analysed on a GC14A gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a ^{63}Ni electron-capture detector. A Hi-Cap CBP1-M25-025 capillary column (25 m × 0.25 mm I.D., film thickness 0.25 μm) (Shimadzu) was used. The electron-capture detector was maintained at 270°C. The column temperature was raised automatically from 60 to 200°C at 30°C/min, and from 200 to 260°C at 10°C/min. The injector block temperature was adjusted to 260°C. The flow-rate of carrier gas (nitrogen) was *ca.* 1.5 ml/min and its pressure was 1 kg/cm^2. The injection technique was splitless.

2.3. High-performance liquid chromatography

A Shimadzu LC6A liquid chromatograph was used with a Shimadzu SPD-6A UV spectrophotometric detector. The separations of DCDMQ were performed on a Cosmosil 5C_{18}-AR column (150 mm × 4.6 mm I.D.) (Nacalai Tesque, Kyoto, Japan) with isocratic elution using 10 m*M* potassium phosphate (pH 2.1)–acetonitrile (50:50, v/v) at a flow-rate of 1.0 ml/min. DCDMQ was detected by UV absorption at 325 nm.

2.4. Determination of 2,3-butanediol in water

Various concentrations of 2,3-butanediol (10 μl of 0.1–20 m*M* 2,3-butanediol) were placed into 5-ml vials with screw-caps coated with PTFE, then 10 μl of 0.3 m*M* 1,2-cyclohexanediol as internal standard (I.S.) and 0.5 ml of 50 m*M* $KMnO_4$ were added to the vials. After reaction at 20°C for 30 min, 0.5 ml of saturated oxalic acid was added to eliminate MnO_4^- and the diacetyl formed was derivatized with DCDB [7] and extracted with *n*-hexane. The *n*-hexane layer was transferred into a 5-ml vial and dried with sodium sulphate and an aliquot (1 μl) was injected into the GC column.

2.5. Determination of 2,3-butanediol in biological samples

Male Wistar albino rats weighing 200–250 g were injected with 0.2 ml of 5% pentobarbital. After 5 min the abdomens were opened and 4 ml of blood were drawn through cannulation of the abdominal aorta. The blood samples were centrifuged at 1700 *g* for 15 min. From the same rats, the livers, kidneys, hearts and brains were immediately excised and rinsed in ice-cold physiological saline solution. Livers and kidneys were perfused with ice-cold saline solution. These organs were homogenized at 4°C for 1 min in a Teflon homogenizer. The homogenates (1 ml)

were placed in 5-ml test-tubes and equal volumes of 1.2 *M* perchloric acid were added. After allowing them to stand at 0°C for 30 min, they were centrifuged at 700 *g* for 5 min. The supernatants were neutralized with equal volumes of 2 *M* K_2HPO_4. The neutralized solutions (0.5 ml) were placed in 5-ml vials with screw-caps coated with PTFE, and 3 ml of acetonitrile containing 3 nmol of 1,2-cyclohexanediol as internal standard (I.S.) were added. The acetonitrile layer was transferred into another 5-ml vial and concentrated to a reduced volume (*ca.* 0.1 ml) by using a stream of nitrogen at room temperature. To the residue, 0.5 ml of 50 m*M* $KMnO_4$ was added. After reaction at 20°C for 30 min, 0.5 ml of saturated oxalic acid was added to eliminate MnO_4^- and the diacetyl formed was derivatized with DCDB [7]. The amounts of 2,3-butanediol were calculated by subtracting the values obtained from determination of endogenous acetoin. In this instance, it is not necessary to subtract the endogenous values of diacetyl, because it was removed by nitrogen during the evaporation procedure.

For normal human and rat urine, the samples were diluted fivefold with water and 0.5 ml of the diluted samples was analysed as described above.

2.6. Identification of DCDMQ derived from biological sample

In order to identify the DCDMQ peak, MS analysis must be carried out. However, the amount of DCDMQ in a peak is too small for MS analysis so the 2,3-butanediol must be previously concentrated from the biological sample, as follows. A dose of 5 mmol/kg ethanol (30%, v/v) was administered to a rat *per os*. After 1 h, the liver was removed and homogenized as described above. The homogenates (10 ml) were deprotenized by using equal volumes of 1.2 *M* perchloric acid. After allowing them to stand at 0°C for 30 min, they were centrifuged at 700 *g* for 5 min. The supernatants were neutralized with equal volumes of 2 *M* K_2HPO_4. The neutralized solutions (5 ml) and 30 ml of acetonitrile were added to a 100-ml round-bottomed flask. The acetonitrile layer was transferred into another 100-ml round-bottomed flask and concentrated to a small volume (*ca.* 1 ml) by using a rotary evaporator at room temperature. To the concentrate, 5 ml of 50 m*M* $KMnO_4$ were added. After reaction at 20°C for 30 min, 5 ml of saturated oxalic acid were added to eliminate MnO_4^- and the diacetyl formed was derivatized with DCDB [7]. The reacted solution was extracted with *n*-hexane and the *n*-hexane layer was evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml of 10 m*M* potassium phosphate (pH 2.1)–acetonitrile (50:50) and 100-μl aliquots were injected for HPLC. The peak corresponding to DCDMQ was collected from the end of the column. This fractionation procedure was repeated ten times. The combined fraction (*ca.* 10 ml) was concentrated to *ca.* 1 ml under reduced pressure and extracted with 5 ml of *n*-hexane. The extract (*ca.* 50 μl) was diluted 100-fold and an aliquot (1 μl) was injected into the GC–ECD system to test the purity. The chromatogram indicated a single peak of DCDMQ. The extract was concentrated to *ca.* 1 ml under reduced pressure and subjected to mass spectrometry.

3. Results

3.1. Mass spectra of DCDMQ

In Fig. 2, mass spectra of (a) authentic DCDMQ and (b) derivatized DCDMQ from ethanol-administered rat liver homogenate are shown. The fragmentations and the ratio of the peaks were identical in (a) and (b), indicating a parent peak (M^+) of *m/z* 226 on the chromatogram.

3.2. Reaction conditions for the oxidation of 2,3-butanediol

To a 5-ml vial with a screw-cap coated with PTFE, 0.1 ml of 10 μ*M* 2,3-butanediol was added and oxidized with 0.5 ml of 50 m*M* $KMnO_4$ at 20°C for various time intervals. The diacetyl formed was reacted with DCDB as

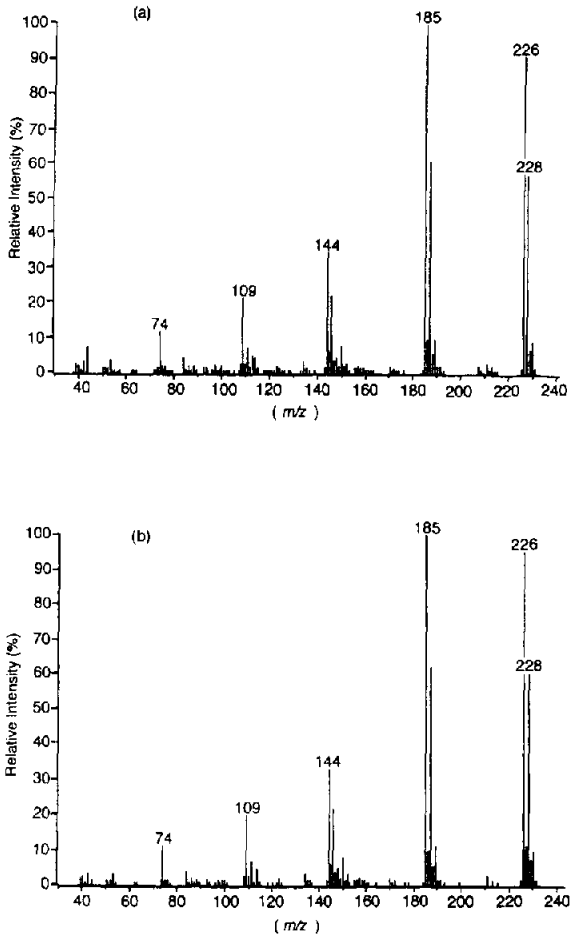

Fig. 2. Mass spectra of DCDMQ. (a) Authentic DCDMQ, 0.1 mg/ml; (b) DCDMQ derived from ethanol-administered rat liver homogenate: after the supernatant of the rat liver homogenate had been treated with DCPD, the DCDMQ formed was isolated by HPLC.

described previously [7] and extracted with *n*-hexane. 2,3-Butanediol could be quantitatively oxidized to diacetyl after 30 min, but the yield gradually decreased to 50% after 2 h of further oxidation. When other oxidizing reagents such as CrO_3 and Na_2CrO_7 were used, they could not oxidize 2,3-butanediol to diacetyl. Fe^{3+} oxidizes acetoin at 90°C for 2 h under acidic conditions, but 2,3-butanediol is not oxidized under the same conditions.

3.3. ECD gas chromatograms of DCDMQ derived from 2,3-butanediol

In Fig. 3, gas chromatograms of DCDMQ derived from 2,3-butanediol in (a) urine and (c) liver of a normal rat are shown. The chromatograms of the blanks (DCDB absent) for urine and liver samples are shown in Fig. 3b and d, respectively. Peaks of DCDMQ and DCTHP derived from 1,2-cyclohexanediol as internal standard appeared at 8.05 and 10.9 min, respectively. From these results it was concluded that 2,3-butanediol is present in normal rat urine and liver.

3.4. Intra- and inter-assay precision and accuracy

Aliquots (0.5 ml) of supernatants of a rat liver homogenates prepared as described under Experimental were assayed five times for 2,3-butanediol on the same day. The mean value ± S.D. was 6.20 ± 0.07 nmol/g tissue (C.V. = 1.2%). When the same samples were analysed using the same procedure on other days, the result was 6.32 ± 0.14 nmol/g tissue (C.V. = 2.2%) for 2,3-butanediol.

3.5. Calibration graphs and limits of determination

Various amounts of 2,3-butanediol in aqueous solution were oxidized and reacted with DCDB, the DCDMQ formed was extracted with *n*-hexane and an aliquot of the *n*-hexane layer was injected into the GC column. When 2,3-butanediol was determined at concentrations from 0.1 to 1 nmol/ml, the peak-height ratio between DCDMQ and the I.S. (y) was directly proportional to the 2,3-butanediol concentration (x, nmol/ml): $y = 1.91x + 0.01$ ($r = 0.9998$, $n = 5$). When 2,3-butanediol was determined at

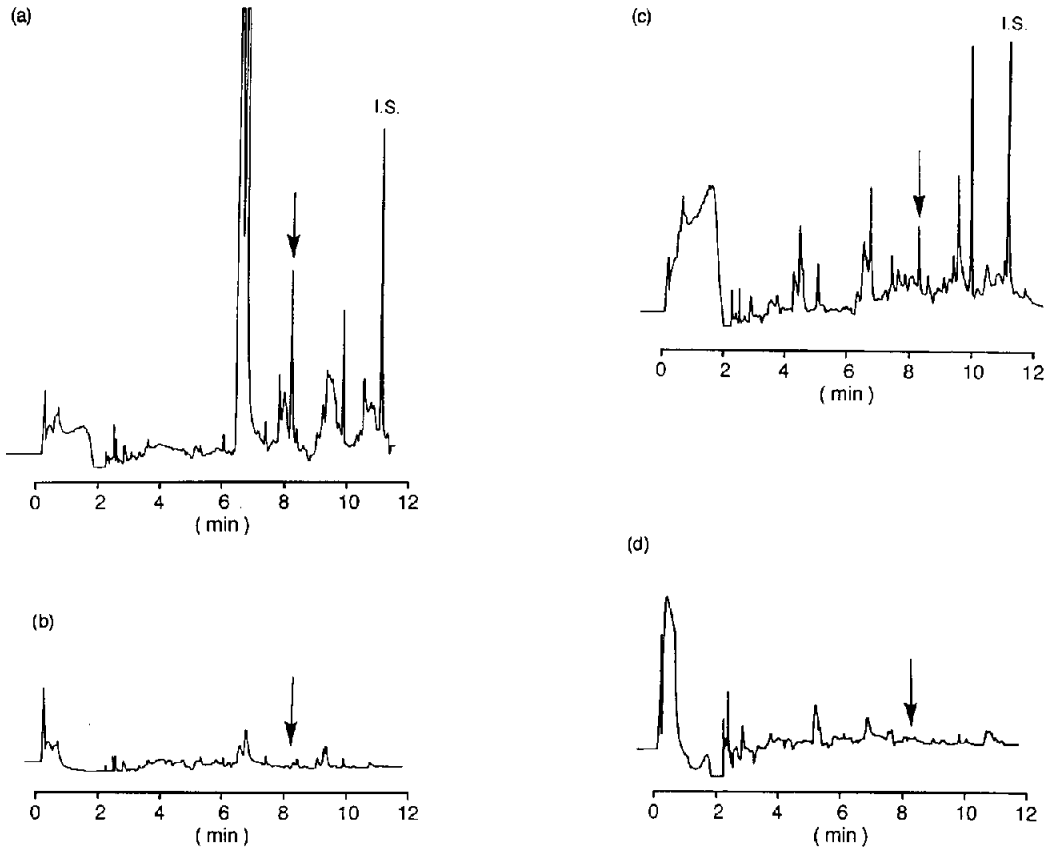

Fig. 3. Gas chromatograms of DCDMQ derived from 2,3-butanediol in biological samples. (a) To 1 ml of diluted (× 5) rat urine, 3 ml of acetonitrile containing 10 μM 1,2-cyclohexanediol as I.S. were added. The solution was stirred and the acetonitrile layer was transferred into another vial. After evaporation of acetonitrile under nitrogen, 0.5 ml of 50 mM $KMnO_4$ was added and allowed to stand for 30 min at 20°C. After addition of 0.5 ml of saturated oxalate, 1 ml of 12 mM DCDB in 1 M HCl was added. The mixture was allowed to stand at 40°C for 90 min and then extracted with 2.5 ml of hexane. An aliquot of the hexane layer (1 μl) was analysed by GC. (b) A 1-ml volume of diluted (× 5) rat urine was treated in the same manner as in (a), but without adding DCDB. An aliquot of the benzene layer (1 μl) was analysed by GC as the blank of (a). (c) Rat liver (1 g) was homogenized with four volumes of physiological saline in a homogenizer at 4°C for 1 min. To 1 ml of the homogenate, 1 ml of 1.2 M $HClO_4$ was added and the mixture was centrifuged at 700 g for 15 min. The supernatant was neutralized with equal volumes of K_2HPO_4. After extraction with 3 ml of acetonitrile containing 10 μM 1,2-cyclohexanediol, the acetonitrile layer was transferred into another vial. Acetonitrile was evaporated under nitrogen, 0.5 ml of 50 mM $KMnO_4$ was added to the residue, the mixture was allowed to stand at 20°C for 30 min and 0.5 ml of saturated oxalate was added. The diacetyl formed was determined as in (a). (d) Deprotenized supernatant (0.5 ml) of rat liver homogenate was treated in the same manner as in (c), but without DCDB. An aliquot of the benzene layer (1 μl) was analysed by GC as the blank of (c). The arrows show the peak of DCDMQ, and I.S. is the internal standard DCTHP.

concentrations from 1 to 100 nmol/ml, a plot of the peak-height ratio (y) *versus* 2,3-butanediol concentration (x, nmol/ml) was linear: $y = 1.82x + 0.46$ ($r = 0.9998$, $n = 5$). The detection limit of DCDMQ (or 2,3-butanediol) was 10 fmol/μl in the extract and the determination limit of DCDMQ (or 2,3-butanediol) was 50 fmol/μl.

Table 1
Contents of 2,3-butanediol in various rat tissues (means ± S.D. for five rats)

Rat tissues	2,3-Butanediol (nmol/g)
Liver	6.21 ± 0.33
Kidney	4.36 ± 0.24
Brain	7.59 ± 0.57
Heart	1.32 ± 0.11
Skeletal muscle	0.57 ± 0.09
Plasma	4.10 ± 0.38[a]
Urine	7.86 ± 0.55[b]

[a] nmol/ml.
[b] nmol/mg of creatinine.

3.6. Recovery of 2,3-butanediol from normal human urine and rat liver homogenate

Various amounts (5–20 nmol) of 2,3-butanediol were added to 0.5 ml of fivefold diluted normal human urine or supernatant of rat liver homogenates, centrifuged at 700 *g* for 15 min and analysed as described above. The recoveries were 97.8 ± 3.4% ($n = 5$) and 98.4 ± 2.9% ($n = 5$), respectively.

3.7. 2,3-Butanediol in tissues, plasma and urine

2,3-Butanediol in normal male rat tissues, plasma and urine was determined and the results are given in Table 1. It was found that 2,3-butanediol is present in all rat tissues, and especially larger amounts were observed in liver and brain. 2,3-Butanediol was also found in normal human urine at a concentration of 31.1 nmol/mg of creatinine.

4. Discussion

Several methods for the determination for 2,3-butanediol have been developed [8–13]. In 1979, Hommes *et al.* [8] reported the presence of 2,3-butanediol in the volatile fraction of urine collected from babies having a disease, identified by GC–MS. 2,3-Butanediol, however, was not detected in normal urine by their method. The results were qualitative rather than quantitative.

In 1982, Needham *et al.* [9] developed a capillary GC method based on the derivatization of the diol with *p*-bromophenylboric acid. They determined a reasonable level of 2,3-butanediol in plasma from an alcoholic patient. Because of the detection limit of 5 μM, they could not detect 2,3-butanediol levels in samples from non-alcoholic subjects. We failed to determine the diol in biological samples using the above method because of the contamination of the capillary column by the reagent or its derivatives. In 1989, Mills and Walker [10] detected urinary excretion of 2,3-butanediol and acetoin by neonates, most of whom were born prematurely and were in a special care unit. In their method, 2,3-butanediol was converted into a trimethylsilyl derivative, which was measured by capillary GC with flame ionization detection (FID). The detection limit of 2,3-butanediol was not reported and must be too poor to measure amounts of the compound in normal urine samples, because of the use of FID. Kezic *et al.* [11] reported the GC determination of 2,3-butanediol isomers in normal urine. The limit of the detection was 1 nmol/ml. They modified the method of Needham *et al.* [9] using *p*-bromophenyl borate as the derivatizing reagent and FID. We applied this method to our biological samples, but it was impossible to analyse a large number of samples because of the contamination of both the detector and the capillary column by the *p*-bromophenyl borate or its derivatives. Montgomery *et al.* [13] reported the normal blood concentration of 2,3-butanediol as 1.2 nmol/ml by using GC–MS. The sensitivity was much lower than that of our method. Anyhow, the methods described above were not satisfactory for determining 2,3-butanediol in biological samples and it was therefore necessary to develop a precise method for the determination of 2,3-butanediol for studies associated with the metabolic pathways of ethanol [6,14] and acetone [15,16].

The method presented here was very sensitive and useful for determining 2,3-butanediol in biological samples. In addition, the recoveries of 2,3-butanediol from biological samples and the

intra- and inter-assay precision and accuracy were satisfactory. The amounts of 2,3-butanediol were calculated by subtracting the values obtained from the determination of endogenous acetoin. In this instance, it is not necessary to subtract the endogenous values of diacetyl, as it was removed by nitrogen during the evaporation procedure because the b.p. of diacetyl (88°C) is much lower than that of 2,3-butanediol (181.7°C) and acetoin (143.2°C). In order to confirm that diacetyl was completely eliminated from the biological samples, and aqueous solution of diacetyl (50 nmol in 0.5 ml) was extracted with 3 ml of acetonitrile and evaporated under a nitrogen stream. After evaporation, only 0.1% of diacetyl remained. Further, the contents of diacetyl in biological samples were more less than those of 2,3-butanediol. On this account we could determined trace amounts of 2,3-butanediol in normal rat tissues and plasma and in human urine. The method, however, does not allow one to distinguish isomers of 2,3-butanediol.

In a previous study [7], benzene was used for extraction of DCDMQ. Recently, we found that *n*-hexane was much better than benzene and it was therefore adopted in the present method.

In general, a 1,2-glycol is quantitatively oxidized by lead tetraacetate and periodic acid under mild conditions into the aldehyde or ketone. In the present method, $KMnO_4$ was used to oxidize 2,3-butanediol to diacetyl. The suitable oxidation conditions and the concentration of $KMnO_4$ must be observed, because, for example, the yield gradually decreased to 50% after 2 h of further oxidation. This quantitative oxidation reaction of diol compounds into diketone compounds is very useful in the field of organic chemistry.

This is believed to be the first paper to report concentrations of 2,3-butanediol in normal rat tissues. We are studying the metabolism of these C_4 compounds using rat liver homogenate, perfused rat liver, rat liver hepatocytes and *in vivo* experiments. It is interesting that when diacetyl and acetoin were administered *per os* to rats, most of them were converted into 2,3-butanediol and a reasonable amount of acetoin accumulated in the brain. These results will be reported elsewhere.

5. References

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