Acta Med. Okayama, 2008
Vol. 62, No. 3, pp. 193–203

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Formate Excretion in Urine of Rats Fed Dimethylaminoazobenzene-rich Diets: The Possibility of Formate Formation from D-Lactate

Yuki Kitamura, Michi Kawase, and Shinji Ohmori*

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700–8530, Japan

This experiment was carried out to evaluate the possibility of degradation of d-lactate into formate and acetaldehyde. In order to induce hyperproduction of d-lactate in rats. Donryu male albino rats were fed diets containing 0.064% 3'-methyl-4-dimethylaminoazobenzene (3'-MDAB), 4'-methyl-4-dimethylaminoazobenzene (4'-MDAB) or 2-methyl-4-dimethylaminoazobenzene (2-MDAB) for 10 weeks. During the experiment, body mass, food and water intake and volume of urine were documented. Methylglyoxal, d-lactate and formate in the urine samples were determined. On the first day of the eleventh week, methylglyoxal, d-lactate, glutathione and enzymatic activities of demethylation and glyoxalase I and II in liver were measured. Methylglyoxal, d-lactate and clinical chemistry parameters of blood plasma were also measured. The levels of methylglyoxal and d-lactate in livers of rats fed 3'-MDAB were very high, while those of 2-MDAB fed-rats and the control group were the same. The fact that glyoxalase I activity and the level of glutathione, a cofactor of glyoxalase I, were high in the livers of the 3'-MDAB-fed rats can explain the elevated levels of methylglyoxal and d-lactate in the liver. The most striking results were the elevated formate levels in the urine of rats fed 3'- and 4'-MDAB in a precancerous state. The degradation of d-lactate, an end product of the methylglyoxal bypass, into acetaldehyde and formate was suggested as a possible way to explain the results.

Key words: formate, methylglyoxal, d-lactate, azo dyes, rat

Neuberg as well as Dakin and Dudley discovered the mixture of glyoxalase I and II in animal tissues and yeast that catalyzes the conversion of methylglyoxal into d-lactate [1, 2]. This reaction requires glutathione. For about 20 years after the discovery of the glyoxalases, methylglyoxal was thought to be an intermediate of glycolysis. After Lohmann demonstrated that muscle extracts formed l-lactate from glycogen without glutathione [3], the Embden Meyerhof pathway (glycolysis) was gradually accepted and, conversely, methylglyoxal and d-lactate were almost forgotten. However, in 1970, the work of Neuberg and his collaborators was vindicated when Cooper and Anderson showed that *E. coli* contained enzymes that converted dihydroxyacetone phosphate to pyruvate via methylglyoxal and named this route the methylglyoxal bypass (Fig. 1) [4]. The biochemical and physiological significance of d-lactate especially in mammals is unclear even today. We are interested in the biochemical and physiological question of d-lactate and have studied it for almost 20 years. We first developed quantification methods for d-lactate [5, 6]

Received October 2, 2007; accepted February 1, 2008.
*Corresponding author. Phone:+81–86–275–3838; Fax:+81–86–275–3838
E-mail:ohmori_shinji@yahoo.co.jp (S. Ohomori)

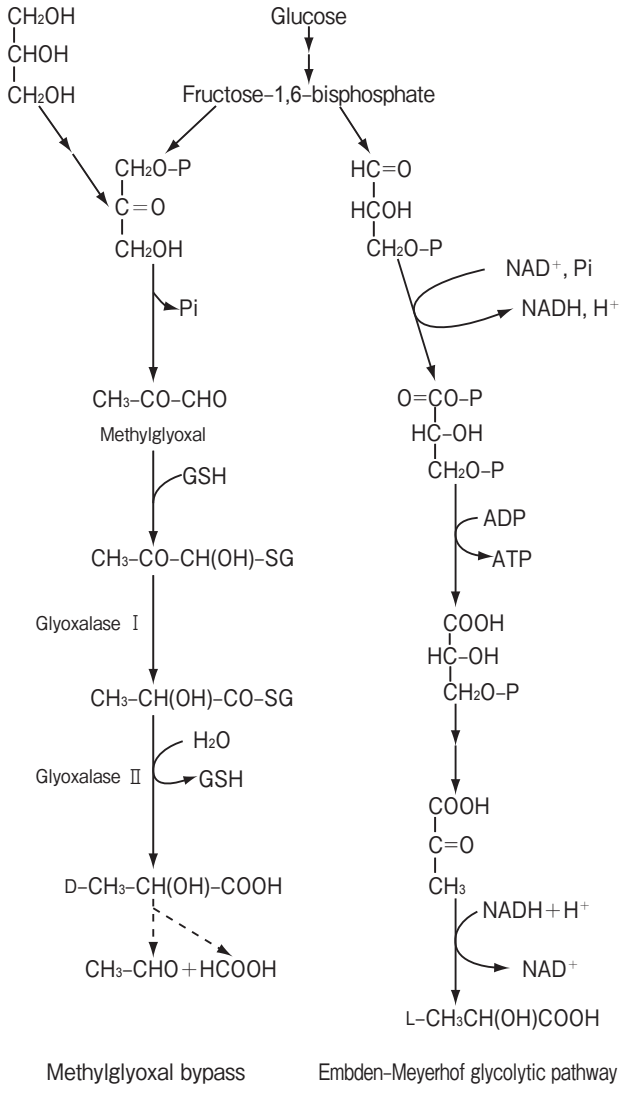

Fig. 1 The formation of d-lactate and l-lactate in rat liver.

and methylglyoxal [7, 8]. Thereafter, we investigated the biochemical and physiological questions of d-lactate and the methylglyoxal bypass. From our extensive studies, the physiological and biochemical role of d-lactate and the methylglyoxal bypass is beginning to be understood. For example, we found that the methylglyoxal bypass plays an important biochemical role for energy production in the octopus [9-11]. For mammals, the experimental results can be summarized as follows: d-Lactate is formed from methylglyoxal, which in turn is nonenzymatically formed from triose-phosphates [12]. In the normal rat liver, d-lactate concentration is one-sixth that of l-lactate [13]. Humans absorb d-lactate from their diet, for example from yoghurt [5], and barely detectable amounts of d-lactate are excreted into the urine of humans and rats [13, 14]. Higher animals do not have d-lactate dehydrogenase [9]. Incidentally, the presence of lactate isomerase has not been demonstrated in organisms. The isomerization reaction is thought to be unreasonable given the basic concepts of organic chemistry. From these facts, we think that d-lactate as an end product of the methylglyoxal bypass (Fig. 1) might be further degraded into formate and acetaldehyde in mammals. As a second idea, methylglyoxal may be degraded into acetyl-Co and formate. To test these hypotheses, we determined the amounts of formate in the urine of rats in a state of hyperproduction of d-lactate and methylglyoxal. As we have already reported, feeding rats with 3'-MDAB produces high hepatic levels of d-lactate and methylglyoxal [15]. In the study on urinary formate levels, the methyl isomers, 4'-MDAB and 2-MDAB, were also used as references.

Materials and Methods

Chemicals. Hydrazine sulfate was purchased from Wako Pure Chemicals (Osaka, Japan). dl-6, 8-Thioctamide, *o*-phenylenediamine, 4'-methyl-4-dimethylaminoazo benzene (4'-MDAB) [Chemical abstracts R. No. 3010-57-9] and 2-methyl-4- dimethylaminoazobenzene (2-MDAB) [R. No. 54-88-6] were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 3'-Methyl-4-dimethylaminoazobenzene (3'-MDAB) [R. No. 55-80-1] was synthesized in our laboratory according to the method of Giese and coworkers [16]. Dimethylacetal of methylglyoxal and 4, 5-dichloro-1, 2-phenylenediamine (DCPD) were obtained from Aldrich (Milwaukee, WI, USA). Methylglyoxal was prepared just before use by hydrolysis of the dimethylacetal [17]. S-Lactoylglutathione was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The F-Kit used for determining formate was a product of Boehringer Mannheim Co. Ltd. (Mannheim, Germany). d-Lactate dehydrogenase (d-LDH) from *Staphylococcus sp* and diaphorase from *Clostridium kluyveri* were kindly supplied by Amano Pharmaceutical (Nagoya, Japan). Glutathione was supplied by Senju Pharmaceutical (Osaka, Japan). β-NAD^+ was purchased from Oriental Yeast Co.

(Tokyo, Japan).

Animals. Five-week-old male Donryu/Crj (SPF/VAF)-strain albino rats were obtained from Charles River Japan (Yokohama, Japan) and allowed to adapt for 1 week. Twenty rats were divided into 4 equal groups. Three groups were fed rat cake MF (Oriental Yeast, Tokyo, Japan) containing 0.064% 3'-, 4'- or 2-MDAB. The fourth group received the standard rat cake MF. All rats received water *ad libitum* and were housed at 25℃.

Instruments. d-Lactate was determined by a Shimazu LC-3A HPLC system (Kyoto, Japan) equipped with a SPD-2A, and a C-R6A data module was used. A Cosmosil-packed column 5 C_{18}AR-II (4.6 × 150 mm) (Nacalai Tesque, Kyoto, Japan) was used. The chromatography was run at 40℃, and detection was performed at 334 nm. The column was eluted with 10 mM potassium phosphate (pH 2.1) containing 13% acetonitrile [5].

For spectrophotometric measurements (activities of glyoxalase I and II and demethylation, and concentration of glutathione and protein), a Shimadzu double-beam UV spectrometer (UV-180) and a Shimadzu UV-visible recording spectrometer (UV-160A) were used. A Shimadzu gas chromatograph model GC-4CMPFE (Shimadzu, Kyoto, Japan), equipped with a ^{63}Ni electron-capture detector, was used for the determination of methylglyoxal [8]. The glass column (2 m × 3 mm I. D.) was packed with 1.5% Silicon OV-17 on Shimalite W, 80–100 mesh (Shimadzu). The temperature of the detector and injector block was maintained at 270℃, and the column was run at 200 ℃. The flow rate of the carrier nitrogen gas was 50 ml/min.

Urine collection. Urine samples from the 4 groups of rats were collected for 40 h starting from 5 : 00 p.m. of the first day of experimental weeks 0, 2, 4, 6, 8 and 10. A small amount of toluene was added to the samples. The collected samples were separated from toluene by a separatory funnel and the urine layers were centrifuged at 1,700 × g for 15 min.

Blood sampling and analysis of clinical chemistry parameters of plasma. Rats were injected intraperitoneally with 2.0 ml of 2.5% pentobarbital per kg of body weight at week 11. After about 5 min, the abdomens were opened and about 4 ml blood was drawn through cannulation of the abdominal aorta. All needles and syringes were heparinized. Blood samples were centrifuged for 15 min at 1,700 × g. The blood sampling was carried out between 10 : 00 and 11 : 00 a.m. Two milliliters of the plasma were analyzed for clinical chemistry parameters in the Okayama Blood Examination Center where Olympus AU 5000, AU 700 and AS-300 autoanalyzers were used. The remainder was used for the determination of methylglyoxal and d-lactate.

Liver homogenate. After the blood samples were drawn, the livers of rats were immediately excised, rinsed and perfused with ice-cold physiological saline. For glutathione determination, a portion of the liver was frozen in liquid nitrogen and stored until the determination was carried out. The remainder was homogenized at 0℃ for 30 sec in 5 volumes of ice-cold 10 mM potassium phosphate (pH 7.0) in a glass Teflon homogenizer. The homogenates were centrifuged at 6,000 × g and 4℃ for 15 min. The supernatant was used for the determination of methylglyoxal and d-lactate levels, the activities of glyoxalase I and II, and demethylation.

Determination of metabolites. Methylglyoxal was determined according to the method of Ohmori *et al.* [8]. Methylglyoxal in samples was reacted with DCPD and converted to 6, 7-dichloro-2-methylqunoxaline, which was determined by gas chromatography with electron capture detection.

d-Lactate was converted by d-LDH into pyruvate, which was further converted into 2-methylquinoxalinol by *o*-phenylenediamine in a one-vial reaction [5]. The quinoxalinol formed was extracted with ethyl acetate. The extract was evaporated to dryness. The residue was dissolved into the column equilibration solution, and an aliquot was analyzed by HPLC. Since the amount of 2-methylquinoxalinol was determined to be the sum of the amounts of intrinsic pyruvate and pyruvate derived from d-lactate, the value of d-lactate was obtained by subtracting the amount of intrinsic pyruvate from the amount of 2-methylquinoxalinol. Formate in urine samples was determined using a formate F-kit (Boehringer Mannheim). Reduced glutathione was determined by our previously described method [18]. Cysteine was measured by an HPLC method developed in our laboratory [19].

Enzyme activities. The activities of glyoxalase I and II were determined spectrophotometrically at 240 nm as described in the literature (Racker) [20]. The measurement of the demethylation of N-dimethyl

groups in rat liver microsomes was based on the methods of Mannering [21] and Poland and Nebert [22]. Briefly, the reaction mixture containing 0.1 ml 10 mM aminopyrine, 0.1 ml 1 mM EDTA, 0.45 ml 0.2 M potassium phosphate (pH 7.4), 0.1 ml 0.3 mM NADPH and a 0.1-ml microsomal fraction (10 mg protein/ml), adjusted to a 1.0-ml volume by adding water, was incubated for 20 min at 37℃. The reaction was stopped by adding 0.1 ml 10% TCA. After the mixture was centrifuged at 3,000 × g for 15 min, 1.0 ml of the supernatant was reacted with 2.0 ml of Nash reagent [23]. The mixture was incubated at 37 ℃ for 30 min and the absorbance at 412 nm was measured. The microsomal fractions from livers of rats fed the respective azo dyes were also tested for demethylation activity using the respective azo dyes as the substrate.

Protein determination. Protein concentration was determined by the biuret method (Beisenherz *et al.*) [24].

Results

Changes in body mass and intake amounts of food and water by rats fed azo dyes. As shown in Fig. 2, the mass of rats in all groups increased during the experiment. However, the body mass increase of the 3'-MDAB group was the slowest: its body mass was only 81% of that of the control group after 10 weeks of the 3'-MDAB-enriched diet. The body mass of the 2-MDAB group also showed a slower

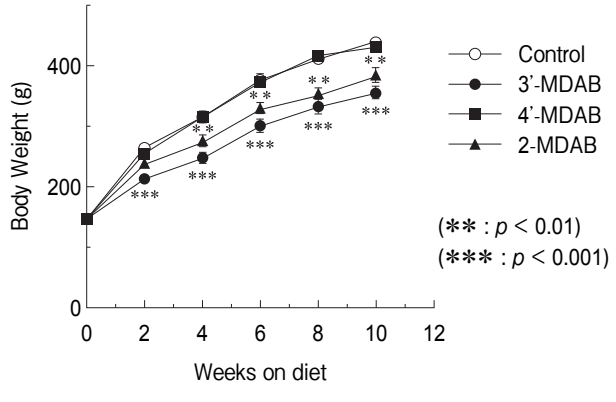

Fig. 2 Body weights of rats fed 0.064% azo dyes Values are means ± S.E.M. n = 5. Statistical significant differences from controls were tested using the Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

increase, reaching only 88% of that of the control group at week 10. The body mass of the 4'-MDAB group was the same as that of the control group.

The curves of the increased dietary intake by the 2- and 4'-MDAB groups were similar to that of the control group, which had increased its intake 16% by week 10. On the other hand, the intake of the 3'-MDAB-fed group was lower than that of the control group: At weeks 2, 4, 6, 8 and 10, its intakes were 66, 58, 75, 76 and 92% of those of the control, respectively. The amounts of intake of 3'-MDAB reflected the increase of body mass.

The amount of water drunk increased with growth for all groups. The amount of water drunk by the control group increased linearly and by week 10 was 2.3 times more than at the start of the experiment. The amount of water drunk by the 4'-MDAB-fed group increased at the same rate as the control group. The 3'- and 2-MDAB groups drank less water compared to the control and 4'-MDAB groups. At week 2, 4, 6, 8 and 10, the amounts of water drunk by the 3'-MDAB group were 56, 64, 57, 53 and 48% of those by the controls, respectively. The respective amounts of water drunk by the 2-MDAB group were 83, 74, 70, 50 and 63% of the control amounts.

Rat liver weight after administering MDAB for 11 weeks. Rat liver weights of the 3'-MDAB and 4'-MDAB groups at 11 weeks were 24 and 15% heavier than that of the control group, respectively. No difference was found in liver weight between the 2-MDAB-fed group and the control. The shape and color of livers of the 3 groups were normal.

Clinical chemistry parameters of rat blood. Blood samples from the rats at week 11 were examined for 28 parameters (data not shown). No distinct differences were observed between the MDAB-fed groups and the control group, except for γ-GTP and direct and indirect bilirubin. The values of serum γ-GTP were 4.1, 3.3 and 1.7 times higher for the 3'-MDAB-, 4'-MDAB- and 2-MDAB-fed groups than that of the control group, respectively. Serum indirect bilirubin levels for 3'-, 4'- and 2-MDAB-fed groups were 80, 43 and 67% of the control values, respectively. The direct bilirubin value of the 3'-MDAB group was 1.6 times higher than that of the control group.

Plasma levels of methylglyoxal and D-lactate of rats. When rats were fed with MDAB, the

plasma levels of methylglyoxal in all groups were lower than that of the control;, however, the differences were not statistically significant. On the other hand, the d-lactate plasma level of rats fed 3'-MDAB was 34% higher than that of the control, while those of the 4'- and 2-MDAB groups were almost the same as the control level.

Contents of metabolites in livers of rats fed azo dyes. Methylglyoxal is a precursor of d-lactate. The levels of methylglyoxal in the livers of rats administered 4'- and 2-MDAB and the control were almost the same (Fig. 3A). In contrast, the methylglyoxal level in the livers of rats fed 3'-MDAB was 4 times higher than that of the control group (Fig. 3A). In addition, the d-lactate content in the liver of this group of rats was dramatically higher than those of the other 3 groups, which all had comparable levels (Fig. 3B). The pyruvate content in the liver of the normal group was 0.83 ± 0.06 nmol/mg protein, while that of the 3'-MDAB group was 1.51 ± 0.08 nmol/mg protein, or 1.8 times higher than that of the control group. The hepatic pyruvate contents of rats fed with 4'-MDAB and 2-MDAB were 0.71 ± 0.09 and 0.75 ± 0.03 nmol/mg protein, respectively.

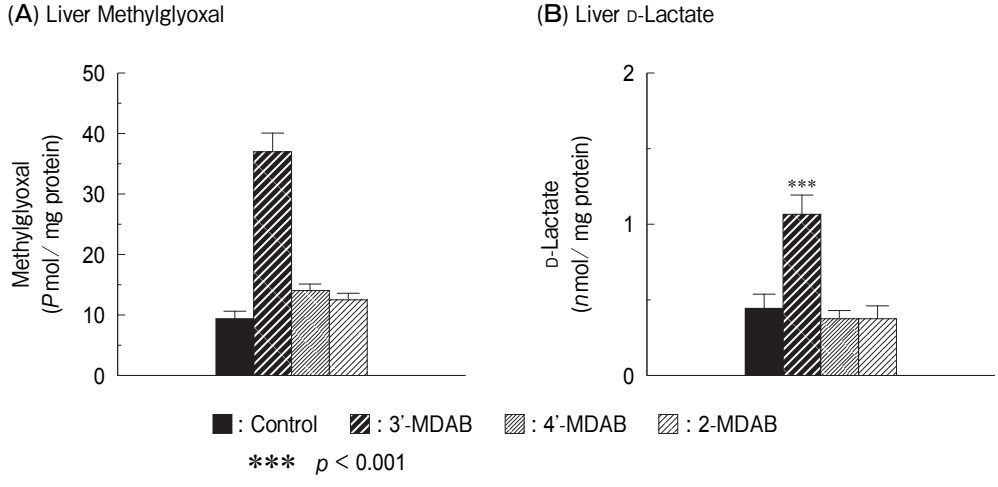

Fig. 3 Contents of methylglyoxal (A) and d-lactate (B) in livers of rats fed 0.064% azo dyes Means ± S.E.M, n = 5.

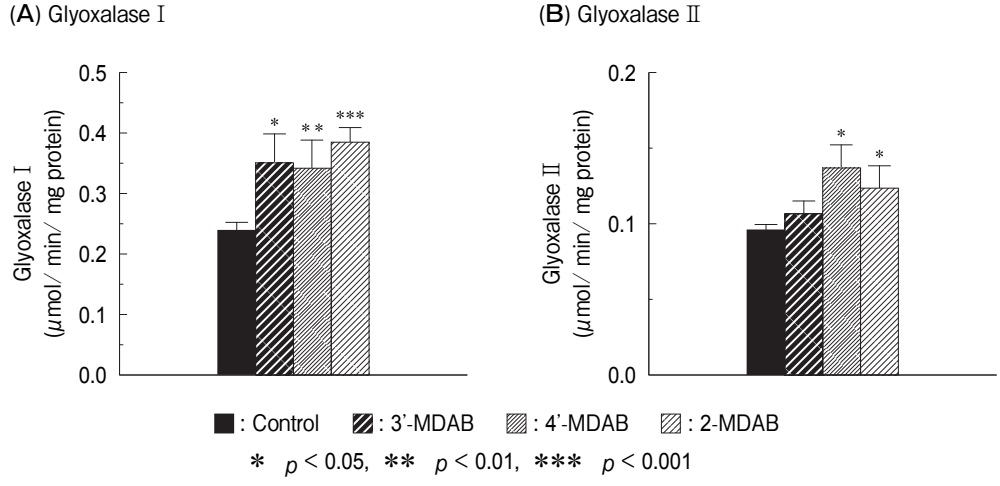

Fig. 4 Activities of glyoxalase I (A) and II (B) in livers of rats fed 0.064% azo dyes. Means ± S.E.M., n = 5.

Enzymatic activities of glyoxalase I and II in livers of rats fed azo dyes. Glyoxalase I catalyzes the isomerization reaction of thiohemiacetal, which is formed nonenzymatically from methylglyoxal and glutathione (Fig. 1). Glyoxalase II catalyzes the hydrolytic reaction of the d-lactate thioester of glutathione to d-lactate and glutathione (Fig. 1). The reaction is rate-limiting. As shown in Figs. 4A and B, azo dye-fed rats all had high glyoxalase activities in the liver. At the end of the experiment glyoxalase I activities for 3'-, 4'- and 2-MDAB-fed rats were 1.6, 1.5 and 1.7 times higher than that of the control animals, respectively. Glyoxalase Ⅱ activities of 4'- and 2-MDAB-fed rats were 1.4 and 1.3 times higher than that of the control rats, respectively. The hepatic glyoxalase Ⅱ activity of the 3'-MDAB group was almost the same as that of the control group.

Hepatic levels of glutathione and cysteine. Glutathione is a cofactor for the glyoxalase reaction. As shown in Fig. 5, the hepatic glutathione level at week 11 was 1.2-fold higher in the 3'-MDAB group, while the level in the 2-MDAB-fed group was 78% of that of the control group. The glutathione level in 4'-MDAB-fed rats was comparable to that of the control group. The hepatic level of cysteine at week 11 was determined in order to ascertain the hepatic glutathione level. In a previous paper, we reported that elevated synthesis of glutathione was associated with increased levels of cysteine [25]. The cysteine content in the livers of the 3'-MDAB group was 0.39 μmol/g wet weight, while that of the control group was 0.12 μmol/g wet weight. The hepatic cysteine contents of the 4'-MDAB- and 2-MDAB-fed groups were 0.24 and 0.2 μmol/g wet weight, respectively.

Demethylation activity in the liver of rats fed azo dyes. As shown in Fig. 6, when 2-MDAB was used as a substrate, the liver microsomal fraction of rats fed 2-MDAB contained the highest demethylation activity. In other words, much larger quantities of formaldehyde were formed from 2-MDAB in the microsomal fraction of 2-MDAB-fed rats (25.6 nmol HCHO/mg protein/20 min). When 4'-MDAB was used as a substrate, the microsomal fraction from the 4'-MDAB-fed rats showed the lowest demethylation activity (14.1 nmol HCHO/mg protein/20 min). When 3'-MDAB was used as a substrate, the hepatic microsomal fraction from rats fed 3'-MDAB had 63% of the demethylation activity of the 2-MDAB-fed rats (16.2 nmol HCHO/mg protein/20 min). These results are very important and will be discussed later.

When aminopyrine was used as the substrate, the enzymatic demethylation activity in the liver microsomal fraction of 3'-MDAB-fed rats was 70% that of the control (31.0 nmol HCHO/mg protein/20 min), while the activities of the liver microsomal fractions of rats fed with 4'- and 2-MDAB were 30.0 and 26.9 nmol HCHO/mg protein/20 min, respectively.

Urine volume of rats fed azo dyes. In the case of the 4'-MDAB and the control group, the urinary volume increased almost linearly during growth and reached 4.2-fold the initial volume by week 10.

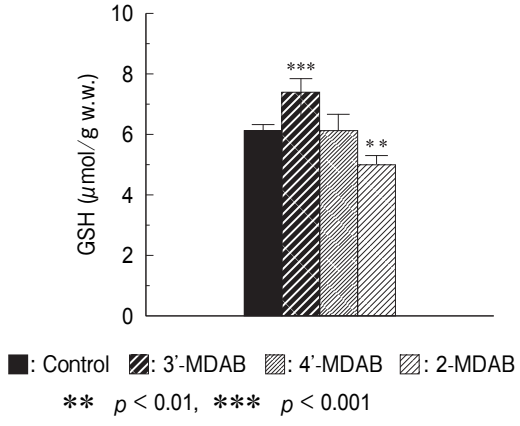

Fig. 5 Contents of glutathione in livers of rats fed 0.064% azo dyes. Means ± S.E.M., n = 5.

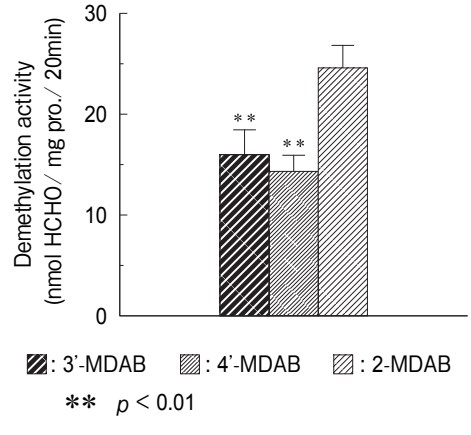

Fig. 6 Demethylation activities in livers of rats fed 0.064% azo dyes. The microsomal fractions from rat livers of rats fed 3'-, 4'- and 2-MDAB were tested for the activities using the respective MDAB as a substrate. Means ± S.E.M., n = 5.

The excreted urine of 3'-MDAB-fed rats increased linearly and reached 2-fold the initial volume at week 8 and then decreased to the starting level by week 10. When rats were fed 2-MDAB, the excretion of urine increased linearly, but more slowly than the control and reached only 75% of that of the control animals at week 10. The increase of urine volume paralleled the volume of water drunk. That is, the 3'-MDAB-fed rats drank the smallest amounts of water and hence, produced the smallest quantity of urine.

Amount of methylglyoxal in rat urine. The amount of methylglyoxal excreted by the rats of the control group was fairly constant, between 140 and 188 nmol/40 h throughout the experiment. The concentration was about 6 nmol/ml. The 3'-MDAB-fed rats excreted less methylglyoxal than the control rats. The amount of methylglyoxal excreted at week 4 was 60% that of the control. The 4'-MDAB fed-rats excreted linearly increasing amounts of methylglyoxal and at week 10, the level was 1.4 times higher than that of the control rats. When rats were fed 2-MDAB, the amount of methylglyoxal excreted was similar to that of the control animals.

D-Lactate content in rat urine. The control group excreted d-lactate at a rate of about 1.5 μmol/40 h throughout the 10 weeks. The concentration was about 60 nmol/ml. Rats fed azo dyes excreted d-lactate in the urine at higher levels than the controls for the duration of the experiment. When 3'-MDAB was fed, the excretion increased until a maximum (4.1 μmol/40 h) was reached at week 4. The excretion returned to the control level at week 8. When 4'-MDAB was administered to the rats, the amounts of d-lactate in urine increased gradually from the 4 th week and reached 3.9 μmol/40 h at week 10. In the case of the 2-MDAB-fed group, d-lactate was excreted at about the control level throughout the experiment.

The amounts of formate excreted in urine after administration of azo dyes. As shown in Figs. 7A, B and C, the amounts of formate excreted by the control group remained constant at about 80 μmol per 40 h throughout the experiment. The formate levels in the urine of the rats fed 3'-MDAB and 4'-MDAB increased dramatically during the administration (Figs. 7A and B), while that of rats fed 2-MDAB was similar to that of the control (Fig. 7C).

Discussion

In 1937, Kinoshita showed that *p*-dimethylaminoazobenzene (butter yellow) produced hepatomas in rats [26]. Miller and Baumann fed 11 derivatives of butter yellow to rats for periods up to 240 days and a normal diet after that. They reported that 3'-MDAB caused hepatomas in 5 of 8 rats after 3 months and in 7 of 8 rats after 4 months, while 4'-MDAB produced only 1 tumor in 10 rats after 10 months. No tumors were observed in 2-MDAB-fed rats after 10 months [27].

We have studied which organisms have high levels of d-lactate in their bodies and are suitable for biochemical studies of d-lactate. Furthermore, we have also been interested in knowing under which physiological conditions these animals have high levels of d-lactate. Earlier, we found that when rats were given 3'-MDAB, the hepatic levels of d-lactate and methylglyoxal increased considerably from the start of the administration to the 20 th week when compared to the control group [15]. In order to substantiate the hypothesis that d-lactate is degraded into formate and acetaldehyde, at first we induced hyperproduction of d-lactate in rats by administering azo dyes; then, formate levels in urine were determined. The experiments on rats given 4'-MDAB and 2-MDAB were done to compare results with those of rats given 3'-MDAB.

We first ascertained the general physiological state of the experimental rats. As mentioned in the results, body mass, food, water intake and urine volume were documented and clinical chemistry parameters in blood plasma were examined. It is noteworthy that the rats given 3'-MDAB showed either the highest or lowest physiological and biochemical values among the groups given the 3 types of MDAB. As mentioned above, 3'-MDAB has the most potent carcinogenic activity. [27]. However, it is known that rats fed these three types of MDAB do not develop hepatomas within 10 weeks as mentioned above.

When we studied changes in metabolite concentrations and enzyme activities related to d-lactate at week 11, the most striking findings were high hepatic levels of methylglyoxal and d-lactate in the rats fed 3'-MDAB, as shown in Fig. 3A and B. Interestingly, the hepatic methylglyoxal and d-lactate levels of the 4'- and 2-MDAB group showed no significant difference from those of the control. Furthermore, the increases of methylglyoxal and d-lactate in the 3'-MDAB-fed

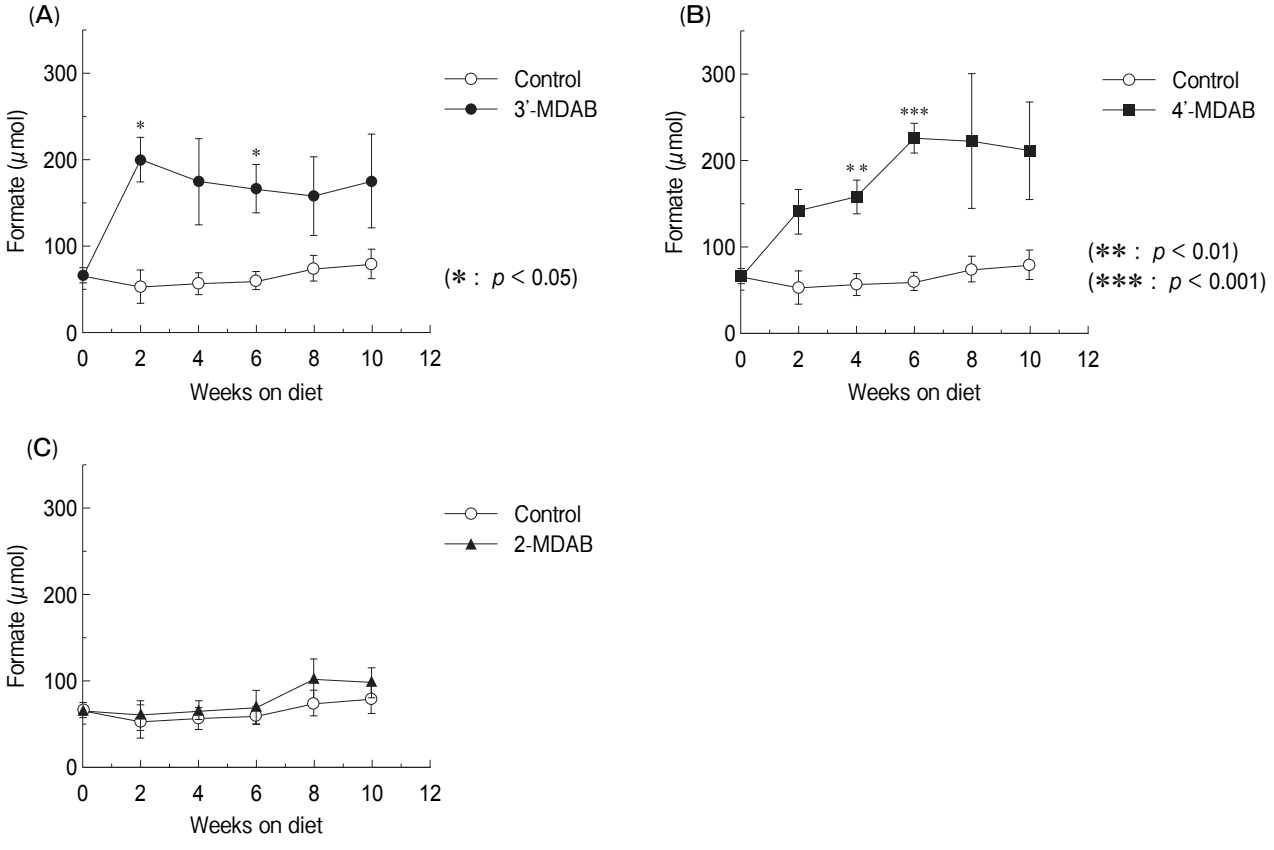

Fig. 7 Total formate contents in urine samples of rats fed 0.064% 3'-MDAB (A), 4'-MDAB (B) and 2-MDAB (C). The urine samples were collected for 40 h starting at 5.00 p.m. of the first day of the experiment week.

rats' livers were not reflected in their blood plasma levels.

As shown in Figs. 4A and B, the administration of azo dyes seemed to affect the activities of glyoxalase Ⅰ and Ⅱ. The increase of the activity of glyoxalase Ⅰ together with the increase of the hepatic glutathione level (Fig. 5) appears to be the cause of the increase of the hepatic contents of d-lactate in the 3'-MDAB-fed rats.

The main objective in this report was to determine the amount of formate in rat urine from intake of different MDAB isomers. As shown in Figs. 7A and B, the rats fed 3'- and 4'-MDAB excreted large quantities of formate from week 2 to the end of the experiment. However, formate was excreted only on the control level by the 2-MDAB group (Fig. 7C). The normal group also excreted considerable amounts of formate. The concentration was 3.3 mM. It is clinically worth noting that the elevation of urinary formate occurs in rats in the precancerous state.

As a working hypothesis, we expected that the amounts of formate in the urine of rats given different MDAB isomers must reflect their relative carcinogenic activities. However, the result was not in accordance with what would be expected; that is, formate was excreted in relatively large amounts in urine of rats given 4'-MDAB. Theoretically, the amount of formate in urine must be dependent upon 1) d-lactate formation, 2) glutathione concentration, 3) activity of glyoxalase I and II, 4) degradation rate of d-lactate in liver, and 5) efflux rate of d-lactate from liver. Here, factor 5 can be neglected due to the fact that no efflux of d-lactate into blood could be observed. The amount of formate excreted by the 3'-MDAB-fed group can be well explained by the factors mentioned above. The amount of urinary formate of rats given 4'-MDAB may be explained by factor 4, because the liver function of 4'-MDAB group must be more active

than that of the 3'-MDAB group, as shown in the results for the clinical chemistry parameters.

The origin of the formate in rats given azo dyes is a topic of some controversy. The first hypothesis is that the formate arises from the N-methyl group. Since N-dimethyl groups are subjected to a demethylation reaction in the microsomal fraction [28, 29], some of the formate in urine may be attributable to the methyl group from this process. However, if the origin of the urinary formate were the methyl group, all 3 groups of rats would excrete nearly equal amounts of formate. As shown Fig. 6, the demethylation activity for 3'-MDAB in the liver microsomal fraction of the 3'-MDAB group was somewhat less than the activity for 2-MDAB in the microsomal fraction of the 2-MDAB group. These experimental results suggest the possibility that urinary formate is formed not from the N-dimethyl group of MDABs but from d-lactate or methylglyoxal.

The second hypothesis is that d-lactate is split into acetaldehyde and formate. For the background of this hypothesis, the experimental results of Poulos *et al.* are presented here. Poulos *et al.* reported that formic acid is a product of the α-oxidation of fatty acid having a 3-methyl group. Since phytanic acid has a 3-methyl group, it theoretically cannot be degraded by β-oxidation. Phytanic acid in food is first oxidized at the 2-position, and the 2-hydroxyacid formed is then degraded into formic acid and a long chain aldehyde [30]. However, the configuration of the 2-hydroxy acid was not shown by the authors.

Finally, there is the hypothesis presented by Argilés (1989) that methylglyoxal is degraded into formate [31]. He reported that mammalian pyruvate dehydrogenase complex catalyzed the oxidation of methylglyoxal to acetyl-CoA and formate in the presence of Coenzyme A, NAD^+ and cofactors. However, methylglyoxal is not thought to be the appropriate precursor for formate, because methylglyoxal is a competitive inhibitor of the complex for pyruvate and the Km value for methylglyoxal was 1.89 mM, while the methylglyoxal concentration in the rat liver was calculated to be about 4 μM.

Previously, we reported that fairly large amounts of formate are always found in the urine of humans, rats and mice under normal conditions [32]. The concentrations are about 5 mM for humans and 10 mM for rats. Many papers have documented the origins of formaldehyde, a precursor of formate, in animals. Formaldehyde can arise from methylguanidine (Kawata *et al.* 1983) [33], tertiary butyl alcohol (Cederbaum *et al.* 1983) [34], nitrosodimethylamine, methyl hydrazine and 1, 1-dimethyl hydrazine (Godoy *et al.* 1983) [29], aminopyrine, dimethyl sulfoxide and *t*-butanol (Cederbaum and Dicker 1983) [35], N-nitroso-N-methylbutylamine (Suzuki *et al.*) [36], and methanol (Ohmori *et al.* 1988) [32]. However, the occurrence of formate in normal urine cannot be explained by these sources, since they are artificial and xenobiotic. If formaldehyde, which is toxic and chemically reactive, is formed in organisms, it is oxidized to harmless formate by formaldehyde dehydrogenase, which we purified from rat liver cytosol [37].

There is no direct evidence for the formation of formate from d-lactate in animals as mentioned above. We present only the possibility of the degradation of d-lactate. However, the report by Brant and associates (1984) that the rate of oxidation of d-lactate exceeded that of l-lactate in the rat liver has particularly encouraged us in our study [38]. In any event, d-lactate metabolism must actively occur in mammals.

As more circumstantial evidence of the cleavage, we may present the existence of acetaldehyde in the organs of normal animals. We tried to measure the acetaldehyde concentration in several organs of rats using sensitive and specific determination methods of ethanol and acetaldehyde and reported that normal rats have considerable amounts of ethanol and acetaldehyde in their organs [39, 40]. At that time we questioned where the acetaldehyde comes from. We now think that the origin of acetaldehyde in normal mammals may be d-lactate. Incidentally, there is another possible origin of acetaldehyde. In biochemical textbooks, it is described that threonine is catabolized through 3 routes: threonine-3-dehydrogenase, threonine dehydratase, and threonine aldolase. Threonine aldolase catalyzes cleavage to acetaldehyde and glycine, However, we reported that the activity of threonine aldolase in the rat liver was so low that its role in threonine metabolism was negligible [41]. If the split reaction takes place, it is possible to explain the reaction mechanism by the reverse reaction of acyloin condensation.

Acknowledgments. We would like to thank Prof. T. Ubuka and

Dr. N. Masuoka of the Department of Biochemistry, Okayama University Medical School for their guidance in the maintenance and treatment of animals.

References

1. Neuberg C: Weitere Untersuchung über die biochemische Umwandlung von Methylglyoxal in Milchsäure nebst Bemerkung über die Entstehung der verschiedenen Milchsäuren in der Natur. Biochem Z (1913) 51: 484–508.
2. Dakin HD and Dudley HW: On glyoxalase. J Biol Chem (1913) 14: 423–431.
3. Lohmann K: Beitrag zur enzymatischen Umwandlung von synthetischem Methylglyoxal in Milchsäure. Biochem Z (1932) 254: 332–354.
4. Cooper RA and Anderson A: The formation and catabolism of methylglyoxal during glycolysis in *Escherichia coli*. FEBS Let, (1970) 11: 273–276.
5. Ohmori S and Iwamoto T: Sensitive determination of d-lactic acid in biological samples by high-performance liquid chromatography. J Chromatogr (1988) 431: 239–247.
6. Ohmori S, Nose Y, Ogawa H, Tsuyama K and Hirota T; Fluorimetric and high-performance liquid chromatographic determination of d-lactate in biological samples. J Chromatogr (1991) 566: 1–8.
7. Ohmori S, Mori M, Kawase M and Tsuboi S: Determination of methylglyoxal as 2-methylquinoxaline by high-performance liquid chromatography and its application to biological samples. J Chromatogr (1987) 414: 149–155.
8. Ohmori S, Kawase M, Mori M and Hirota T: Simple and sensitive determination of methylglyoxal in biological samples by gas chromatography with electron-capture detection. J Chromatogr (1987) 415: 221–229.
9. Ohmori S, Ohsaki Y, Akagi S, Kondoh C, Kawase M and Nagai T: d-Lactate is present in much larger amount than l-lactate in cephalopods and gastropods. Zool Sci (1997) 14: 429–434.
10. Akagi S and Ohmori S: Threonine is the best substrate for d-lactate formation in octopus tentacle. Amino Acids (2004) 26: 169–174.
11. Fujisawa T, Akagi S, Kawase M, Yamamoto M and Ohmori S: d-Lactate metabolism in starved *Octopus ocellatus*. J Exp Zool (2005) 303A: 489–496.
12. Ohmori S, Mori M, Shiraha K and Kawase M: Biosynthesis and degradation of methylglyoxal in animals; Enzymology and molecular biology of carbonyl metabolism (Vol. 2). Weiner H and Flynn TG eds: Alan Liss, New York (1989) pp 397–412.
13. Kondoh Y, Kawase M, Kawakami Y and Ohmori S: Concentrations of d-lactate and its metabolic intermediates in liver, blood, muscle of diabetic and starved rats. Res Exp Med (1992) 192: 407–414.
14. Kondoh Y, Kawase M and Ohmori S: d-Lactate concentration in blood, urine and sweat before and after exercise. Eur J Appl Physiol (1992) 65: 88–93.
15. Kawase M, Tada M, Akagi S and Ohmori S: Changes in concentrations of methylglyoxal, d-lactate and glyoxalase activities in liver and plasma of rats fed a 3'-methylaminobenzene-rich diet. Res Exp Med (1996) 196: 251–259.
16. Giese JE, Miller JA and Baumann CA: The carcinogenicity of *m'*-methyl-*p*-dimethyl-aminoazobenzene and *p*-monomethylaminoazobenzene. Cancer Res (1945) 5: 337–346.
17. Kellum MW, Oray B and Norton SJ: A convenient quantitative synthesis of methylglyoxal for glyoxalase I assays. Anal Biochem (1978) 85: 586–590.
18. Matsumoto S, Teshigawara M, Tsuboi S and Ohmori S: Determination of glutathione and glutathione disulfide in biological samples using acrylonitrile as a thiol-blocking reagent. Anal Sci (1996) 12: 91–95.
19. Ohmori S, Kawase M, Higashiura M, Chisaka Y, Nakata K and Yamasaki Y: High-performance liquid chromatographic method to analyze picomole levels of glutathione, cysteine and cysteinylglycine and its application to pre-cancerous rat livers. J Chromatogr B (2001) 762: 25–32.
20. Racker E: Glyoxalases; in Methods in Enzymology Vol. 1, Colowick S P and Kaplanm N O eds. Academic Press, New York City, (1955) pp. 454–460.
21. Mannering GJ: Measurement of effect of interferon on drug metabolism; in Methods in Enzymolog Vol. 119, Colowick S P, Kaplan N O eds, Academic Press, New York (1986) pp 718–725.
22. Poland AP and Nebert DW: A sensitive radiometric assay of aminopyrine N-demethylation. J Pharmacol Exp Ther (1973)184: 269–277.
23. Nash T: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J (1953) 55: 416–421.
24. Beisenherz G, Boltze HJ, Bücher Th, Czok R, Carbade KH, Meyer-Arendt E and Pfleiderer G: Diphosphofructose aldolase, phosphoglyceraldehyde dehydrogenase, lactic acid dehydrogenase, glycerophosphate dehydrogenase and pyruvate kinase from rabbit muscle in one process. Z Naturforsch (1953) 8b: 555–577.
25. Ohmori S, Nawata Y, Kiyono K, Murata H, Tsuboi S, Ikeda M, Akagi R, Morohashi K and Ono B: *Saccharomyces cerevisiae* cultured under aerobic and anaerobic conditions: air-level oxygen stress and protection against stress. Biochim Biophy Acta (1999) 1472: 587–594.
26. Kinoshita R: Studies on the carcinogenic chemical substances. Transact Jap Path Soc (1937) 27: 665–727.
27. Miller JA and Baumann CA: The carcinogenicity of certain azo dyes related to *p*-dimethylaminoazobenzene. Cancer Res (1945) 5: 227–234.
28. Stevenson ES, Dobriner K, Rhoads CP: The metabolism of dimethylamonoazobenzene in rats. Cancer Res (1942) 2: 160–167.
29. Godoy HM Gómez MID and Castro JA: Metabolism and activation of 1, 1-dimethylhydrazine and methylhydrazine, two products of nitrosodimethylamine reductive biotransformation, in rats. J Natl Cancer Inst (1983) 71: 1047–1051.
30. Poulos A, Sharp P, Singh H, Johnson DW, Carey WF and Easton C: Formic acid is a product of the α-oxidation of fatty acids by human skin fibroblasts: deficiency of formic acid production in peroxisome-deficient fibroblasts. Biochem J (1993) 292: 457–461.
31. Argilés JM: The oxidation of methylglyoxal by mammalian pyruvate dehydrogenase. Arch Biochem Biophys (1989) 273: 238–244.
32. Ohmori S, Sumii I, Toyonaga Y, Nakata K and Kawase M: High-performance liquid chromatographic determination of formate as benzimidazole in biological samples. J Chromatogr (1988) 426: 15–24.
33. Kawata S, Sugiyama T, Imai Y, Minami Y, Tarui S, Okamoto M and Yamano T: Hepatic microsomal cytochrome P-450-dependent N-demethylation of methylguanidine. Biochem Pharmac (1983) 32: 3723–3728.
34. Cederbaum AI, Qureshi A and Cohen G: Production of formaldehyde and acetone by hydroxyl-radcal generating systems during the metabolism of tertiary butyl alcohol. Biochem Pharmac (1983) 32: 3517–3524.
35. Cederbaum AI and Dicker E: Inhibition of microsomal oxidation of

alcohols and hydroxyl-radical-scavenging agents by the iron-chelating agent desferrioxamine. Biochem J (1983) 210: 107–113.
36. Suzuki E, Mochizuki M, Takeda K and Okada M: α-Hydroxylation and mutagenicity of unsymmetraical N-nitrosodialkylamines with a butyl group. Jpn J Cancer Res (*Gann*) (1985) 76: 184–191.
37. Tsuboi S, Kawase M, Takada A, Hiramatsu M, Wada Y, Kawakami Y, Ikeda M and Ohmori S: Purification and characterization of formaldehyde dehydrogenase from rat liver cytosol. J Biochem (1992) 111: 465–471.
38. Brandt RB, Waters MG and Rispler MJ: d- and l-Lactate catabolism to CO_2 in rat tissues. Proc Soc Exp Biol Med (1984) 175: 328–335.
39. Ohata H, Otsuka M and Ohmori S: Determination of acetaldehyde in biological samples by gas chromatography with electron-capture detection. J Chromatogr B (1997) 693: 297–305.
40. Otsuka M, Harada N, Itabashi T and Ohmori S: Blood and urinary levels of ethanol, acetaldehyde and C_4 compounds such as diacetyl, acetoin and 2, 3-butanediol in normal male students after ethanol ingestion. Alcohol (1999) 17: 119–124.
41. Akagi S, Sato K and Ohmori S: Threonine metabolism in Japanese quail liver. Amino Acids (2004) 26: 235–242.