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Carbon Sources for D-Lactate Formation in Rat Liver

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Carbon sources for D-lactate formation were investigated *in vitro* using 6,000×*g* supernatant of rat liver homogenate and by rat liver perfusion *in situ*. As carbon sources, L-threonine, glucose, glycerol, acetone, and acetoacetate were tested. Glycerol was the best substrate for D-lactate formation *via* methylglyoxal in rat liver. Glucose was the second most preferred substrate, while L-threonine, acetone, and acetoacetate were poor substrates for D-lactate formation. Glycerol was several times more effective than normal as a substrate of D-lactate in the supernatants of liver homogenates of diabetic and starved rats, while it was less effective as a substrate of L-lactate. The glycerol kinase [EC 2.7.1.30] activities in livers increased in the diabetic and starved states. These and other results can explain why the plasma concentration of D-lactate increases several-fold after running and why the D-lactate contents in plasma, liver, and skeletal muscle are markedly increases in diabetic and starved rats.

Key words: glycerol, D-lactate, L-lactate, liver perfusion, methylglyoxal.

Neuberg (*1*) and Dakin and Dudley (*2*) proposed that methylglyoxal is a key intermediate in catabolism of glucose in animals, plants, and microorganisms, and this concept had been widely supported for about 20 years. In the 1930s, the process of glycolysis had been studied and established by Embden and Meyerhof, and others (*3, 4*), and it was generally accepted that glucose is metabolized to L-lactate *via* triosephosphate (the right-hand side in Fig. 1).

In 1970 Cooper and Anderson showed that *Escherichia coli* contained enzymes which convert dihydroxyacetone phosphate to pyruvate *via* methylglyoxal and named this route the methylglyoxal bypass (the left-hand side in Fig. 1) (*5*). In the following year Hopper and Cooper purified methylglyoxal synthetase [EC 4.2.99.11] from *E. coli* (*6*).

Though it was recognized that glyoxalases I and II are widely distributed in animals and other organisms, the methylglyoxal bypass in animals has been disregarded. The degradation and formation of methylglyoxal and D-lactate are barely mentioned in most textbooks. It is even stated in the literature that D-lactic acid is not produced in the body, and that therefore L-lactic acid is the physiological form of lactic acid. In fact, D-lactate is constitutive and is always produced *via* methylglyoxal in animals: D-lactate levels were about one-sixth of L-lactate levels in rat liver (*7*) and interestingly, it was found by us that D-lactate levels were higher than those of L-lactate in some animals and plants. For example, the D-lactate/L-lactate concentration ratios in legs of octopus and squid as well as in onion were 30, 32, 10 (to be published).

Recently we reported that the plasma level of D-lactate increased 3.6 times after even 5 min running and methylglyoxal content in red blood cells markedly decreased after running (*8*). We also showed that the D-lactate level in plasma, liver, and skeletal muscle increased significantly in diabetic and starved rats (*7*). In starved rat, the methylglyoxal level was significantly elevated in plasma, but fell in liver and muscle (*7*).

Sensitive and specific determination methods of methylglyoxal (*9, 10*) and D-lactate (*11, 12*) were established by us. Those methods have enabled us to investigate the contents of methylglyoxal and D-lactate in biological samples and their physiological roles in animals.

In this report we describe the biosynthesis of D-lactate in rat liver, especially the carbon sources for D-lactate formation.

MATERIALS AND METHODS

Chemicals—Lithium D-lactate was purchased from Sigma Chemical (St. Louis, U.S.A.). D-Lactate dehydrogenase (D-LDH) [EC 1.1.1.28] from *Staphylococcus* sp. and diaphorase [EC 1.6.4.5] from *Clostridium kluyveri* were kindly supplied by Amano Pharmaceutical (Nagoya). β-NAD^+ was from Oriental Yeast (Tokyo). Methylglyoxal was prepared just before use by hydrolysis of the dimethyl acetal (Aldrich, Milwaukee, WI, U.S.A.) (*13*). D,L-6.8-Thioctamide and *o*-phenylenediamine were obtained from Tokyo Kasei Kogyo (Tokyo). L-Threonine, glycerol, hydrazine sulfate, streptozotocin, and all other chemicals were purchased from Wako Pure Chemicals (Osaka).

Experimental Animals—Male Wistar strain albino rats were used for all experiments and fed on standard rat cake MF (Oriental Yeast, Tokyo) or starved for 72 h with water *ad libitum*. Animals weighing 180–200 g were used for *in vitro* and starvation experiments. The diabetic condition was induced in rats weighing 120–140 g by a single intraperitoneal injection of streptozotocin, which was dissolved in 0.05 M sodium citrate (pH 4.5) to make 5.25% (w/v) just before injection. The control group was injected with physiological saline. After 30 days, the animals were killed and their livers were used for experiments.

Homogenate Preparations—Rats were intraperitoneally injected with 0.2 ml of 5% pentobarbital. After 5 min, the livers were removed, perfused with ice-cold saline and

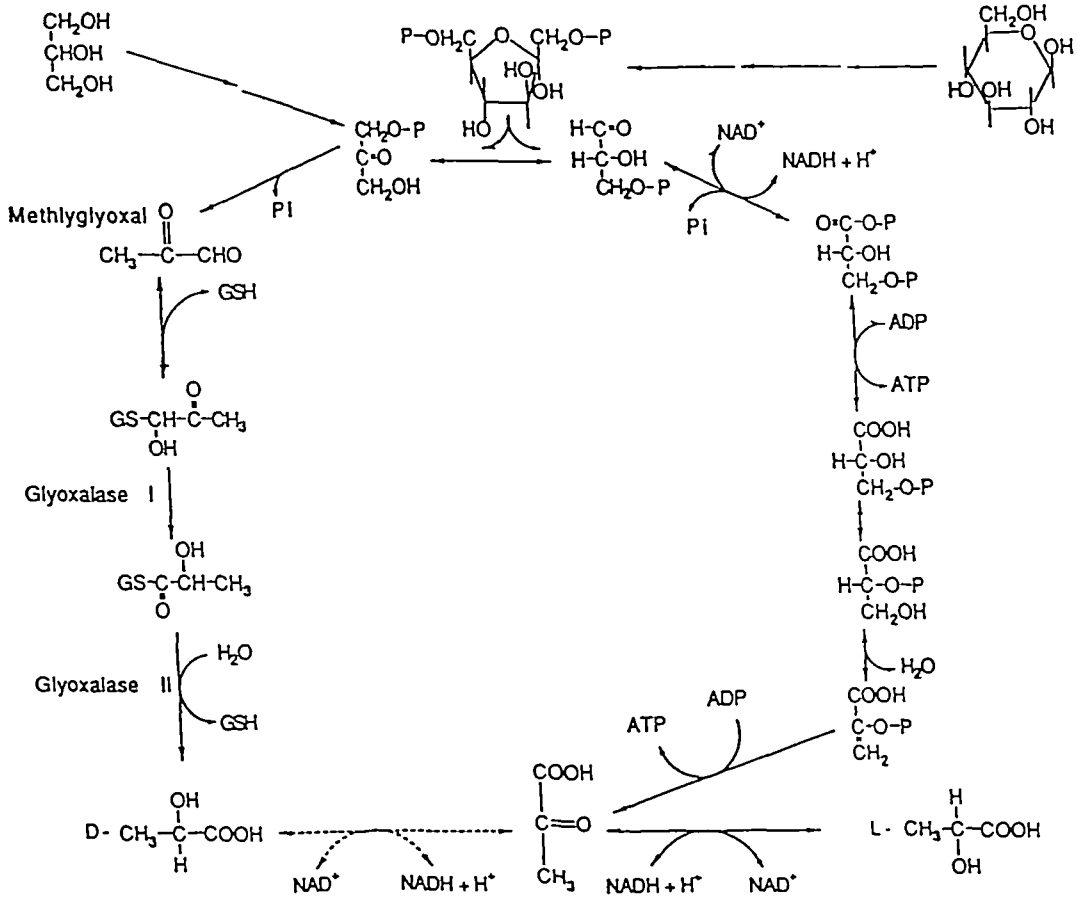

Fig. 1 **The Embden-Meyerhof glycolytic pathway (right-hand side) and methylglyoxal bypass (left-hand side).**

homogenized in 5 volumes of 10 mM potassium phosphate (pH 7.0) using a glass Teflon homogenizer. The homogenates were centrifuged at 6,000 × *g* and 4°C for 15 min. The supernatant was used as the enzyme source without dialysis. Glycerol, L-threonine, acetone, acetoacetate (0.5 mM), and glucose (5 mM) were incubated with 200 μl of the 6,000 × *g* supernatant of the rat liver homogenate in the presence of 2 mM ATP, GSH, $MgSO_4$, and 10 mM potassium phosphate (pH 7.0) in a total volume of 2 ml. As the control, substrate and cofactors were not added to the supernatant. The mixtures were incubated at 37°C for 30 min, then the reaction was terminated by addition 3 ml of methanol, and methylglyoxal, D-lactate, and L-lactate were determined as described under "Analytical Methods."

Liver Perfusion—Rats were intraperitoneally injected with 0.2 ml of 5% pentobarbital per 100 g body weight. After 5 min the abdomen was opened and the liver was perfused *in situ* at 32°C at constant flow rate (30 ml/ min) with oxygenated Krebs-Ringer-phosphate buffer in a flow-through mode. The buffer was continuously gassed with a humidified mixture of O_2-CO_2 (95:5) at 32°C. The perfusion experiment was carried out by the method of Sugano *et al.*, but *in situ* (*14*). The liver was perfused with the buffer for 30 min, followed by the buffer containing a substrate for 60 min. The perfusates were collected at 10-min intervals during perfusion and analyzed for methylglyoxal, D-lactate, and L-lactate.

Analytical Methods—D-Lactate was converted by D-lactate dehydrogenase into pyruvate, which was further converted into quinoxalinol by *o*-phenylenediamine in a one-vial reaction. The quinoxalinol was extracted and measured by high-performance liquid chromatography with UV or fluorimetric detection (*11, 12*). L-Lactate concentration was measured by the UV method (F-kit, Boehringer, Mannheim, Germany). Methylglyoxal was reacted with 4,5-dichloro-1,2-phenylenediamine. The 6,7-dichloro-2-methylquinoxaline formed was determined by gas chromatography with electron capture detection (*10*). Measurement of blood glucose was performed using an UV detection kit (Glucose C-Test Wako, Wako Pure Chemicals). The activity of glycerol kinase [EC 2.7.1.30] was assayed by the method of Bergmeyer *et al.* (*15*). Protein concentrations were measured by the biuret method (*16*).

RESULTS

Methylglyoxal and D-Lactic Acid Formation from Several Substrates in 6,000 × g Supernatant of Rat Liver Homogenate—Glycerol, L-threonine, glucose, acetone, and acetoacetate were examined as substrates for D-lactate formation. The reason why they were chosen as substrates will be described in "DISCUSSION." D-Lactate is known to be formed *via* methylglyoxal. As shown in Table I, the best substrate for D-lactate formation in the supernatant of rat liver homogenate was glycerol, from which 4.26 nmol of D-lactate per mg protein was formed in 30 min, 61 times

TABLE I **Methylglyoxal, D-lactate, and L-lactate formation from several substrates in 6,000×g supernatant of rat liver homogenate.** All values are expressed as nmol/mg protein of liver per 30 min and mean±SE (n=3-5).

	Control	Glycerol (0.5 mM)	L-Threonine (0.5 mM)	Glucose (5 mM)	Acetone (0.5 mM)	Acetoacetate (0.5 mM)
Methylglyoxal	0.04±0.01	0.04±0.01	0.04±0.02	0.05±0.01	0.04±0.01	0.04±0.01
D-Lactate	0.07±0.01	4.26±0.92	0.51±0.05	2.18±0.10	0.66±0.06	0.13±0.03
L-Lactate	0.42±0.11	2.47±0.18	1.12±0.31	2.53±0.12	0.52±0.08	0.45±0.21

TABLE II. **Methylglyoxal, D-lactate, and L-lactate contents in liver perfused with several substrates.** All values are expressed as nmol/g w.w. of liver per 60 min and mean±SE (n=3-6) The concentration of substrates were the same as in Table I As the control, liver was perfused with Krebs-Ringer-phosphate buffer at the flow rate of 30 ml/min After perfusion for 60 min, the livers were homogenized with 5 volumes of 10 mM potassium phosphate (pH 7.0). The homogenates were centrifuged at 6,000×g and 4°C for 15 min, and the supernatant was assayed for methylglyoxal, D-lactate, and L-lactate.

	Control	Glycerol	L-Threonine	Glucose	Acetone
Methylglyoxal	3.6±1.8	2.9±1.4	3.5±2.0	3.6±1.8	4.0±1.7
D-Lactate	82±14	754±136	103±11	260±51	99±12
L-Lactate	393±66	2,471±311	658±177	3,107±696	330±109

TABLE III. **Time course of methylglyoxal, D-lactate, and L-lactate formations from glycerol in perfusate.** The rat liver was perfused at 32°C for 30 min with Krebs-Ringer-phosphate buffer saturated with 95% O_2-5% CO_2 and then for 60 min with the buffer containing 0.5 mM glycerol. The perfusion rate was 30 ml/min The perfusate was not recirculated. Methylglyoxal, D-lactate, and L-lactate were determined in the perfusates for 1 min at the defined time after addition of the substrate to the buffer. The values are expressed as mean±SE (n=3-5). N.D., not detectable

	0 min	10 min	30 min	60 min
Control (μmol/min)				
Methylglyoxal	N.D	N.D.	N.D.	N.D.
D-Lactate	N.D	N.D	N.D	N.D.
L-Lactate	1.62±0.45	1.10±0.16	1.10±0.15	0.99±0.11
0.5 mM glycerol (μmol/min)				
Methylglyoxal	N.D.	N.D	N.D.	N.D.
D-Lactate	N.D	0.38±0.03	0.28±0.04	0.34±0.04
L-Lactate	1.53±0.67	6.61±1.29	3.15±0.71	2.81±0.39

TABLE IV. **D-Lactate and L-lactate formations from 0.5 mM glycerol and glycerol kinase activity in 6,000×g supernatant of liver homogenate of diabetic and starved rats.** The rats were starved for 72 h or injected with streptozotocin to induce diabetes. The diabetic rats were used 30 days after injection. The livers were homogenized with 5 volumes of 10 mM potassium phosphate (pH 7.0) The homogenates were centrifuged at 6,000×g at 4°C for 15 min. The reaction conditions and determination methods for D-lactate and L-lactate are described in Table I. The glycerol kinase activity in 6,000×g supernatant was assayed by the method of Bergmeyer. All values are expressed as nmol/mg protein of liver per 30 min and are means±SE $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (n=5)

	Normal	Diabetic	Starved
D-Lactate (nmol/mg protein)	4.2±0.9	21.3±3.2***	12.5±2.3**
L-Lactate (nmol/mg protein)	2.2±0.4	0.2±0.0***	1.7±0.7
Glycerol kinase activity (mU/mg)	4.3±0.5	22.2±7.4***	6.5±0.9*
Blood glucose (mg/dl)	150±5	460±16	130±10

higher than the control value. Glucose was also a good substrate in rat liver and afforded 2.18 nmol of D-lactate per mg protein in 30 min. Twice as much D-lactate was formed from glycerol as from glucose. L-Threonine, acetone and acetoacetate were not favorable as substrates for D-lactate formation. As shown in Table I, no difference was observed in methylglyoxal formation from them in the supernatant.

As for L-lactate formation, both glycerol and glucose were good substrates and produced 6 times as much as the control. L-Threonine was also a substrate, but acetone and acetoacetate were not available as substrates.

When methylglyoxal was incubated at 37°C for 30 min at the concentration of 30 and 100 μM with rat liver homogenate, 22.0 μM (73.3% yield) and 79.7 μM (79.7% yield) D-lactate was formed, respectively.

Methylglyoxal and D-Lactic Acid Formation from Several Substrates in Liver by Rat Liver Perfusion—As shown in Table II, when glycerol (0.5 mM) was perfused for 60 min through rat liver, the highest concentration of D-lactate found in the perfused liver was 754 nmol per g wet weight of liver, which is 9.3 times higher than the control. As in the experiment on the homogenate level, glucose was the second most favorable substrate for D-lactate formation in the perfused liver. L-Threonine and acetone played a little role in D-lactate formation. Whatever substrate was used, the production of methylglyoxal was at the control level. On the other hand, for L-lactate formation, glucose was the best substrate, affording 8 times the control level. The second most favorable substrate was glycerol. L-Threonine was only slightly effective and acetone was not available. Perfusion with acetoacetate as the substrate was not performed, because neither D-lactate nor L-lactate was formed from it in the rat liver homogenate.

When the liver was perfused at the slower flow rate of 2 ml/min for 25 min, 823±150 nmol of D-lactate was formed per g wet weight of the liver from 5 mM glycerol and 252±40 nmol from 5 mM glucose. A comparison with Table II indicates that the perfusion rate did not alter the D-lactate level in the liver at the same concentration (5 mM) of glucose or when the concentration of glycerol was increased 10 times. However, when 5 mM glycerol or glucose was perfused at the flow rate of 2 ml/min, the methylglyoxal levels in liver were increased 3 and 2.3 times, respectively. When L-threonine or acetone was used as the substrate, neither methylglyoxal nor D-lactate level increased significantly.

Methylglyoxal, D-Lactate and L-Lactate in the Perfusate with Glycerol as the Substrate by Rat Liver Perfusion—With glycerol as the substrate, the formation rates of methylglyoxal, D-lactate and L-lactate in the perfusates were measured. The results are summarized in Table III.

Although methylglyoxal was found in the perfused liver or the supernatant of liver homogenate with glycerol as the substrate, it was not detected in the perfusate. Since as little as 10 fmol of it can be measured, we conclude that it is not released from the cells.

L-Lactate was found in the perfusate, even when only the phosphate buffer was perfused. This implies that L-lactate must be formed from glycogen in the liver. Glycerol was also used as a substrate for L-lactate, but there was lag period of 10 min in the time course, on subtracting the control values (Table III).

As for D-lactate, it seems likely glycogen may not have served as the substrate while glycerol was available. There was also a lag period of 10 min. When the liver was perfused at the flow rate of 2 ml/min with 5 mM glycerol, D-lactate was found at 0.02, 0.12, 0.56, 0.80, and 0.67 μmol/min in the perfusates ($n=5$) at 5, 10, 15, 20, and 25 min, respectively. On the other hand, as shown in Table III, when the liver was perfused at a higher rate, the formation rate of D-lactate in the perfusate was almost the same.

D-Lactate Formation from Glycerol in 6,000 × g Supernatant of Liver Homogenate of Diabetic and Starved Rats—As shown in Table IV, when glycerol (0.5 mM) was incubated with 6,000 × g supernatants of liver homogenates of diabetic and starved rats, D-lactate was synthesized from it in the largest amounts in diabetic rats, corresponding to 5 times the control (normal rat liver). D-Lactate was also produced from glycerol in the starved rats at 3 times the control value. In this experiment the supernatant was used as the enzyme source without dialysis, because glycerol kinase loses its activity during dialysis. In contrast to D-lactate, L-lactate was formed to a lesser extent from glycerol in the supernatant of the homogenates of diabetic and starved rats than in those of normal rats. The differences between the modes of D-lactate and L-lactate formation are of particular interest.

In connection with glycerol metabolism, glycerol kinase activities in the supernatants were measured. The results are listed in Table IV, indicating that the order of the activity was related to that of D-lactate formation from glycerol.

DISCUSSION

First we will briefly review studies of the carbon sources of methylglyoxal. The pathways of biosynthesis of D-lactate are depicted in Fig. 2.

Milligan and Baldwin reported the oxidative decarboxylation of acetoacetate to methylglyoxal in sheep heart extracts in the presence of oxygen and Mn^{2+}, and identified the catalytic activity as being that of myoglobin (*17*) (depicted as A in Fig. 2). We repeated their experiments and found the reaction proceeded optimally at pH 5.0, but did not proceed above pH 6.5 (*18*). Conversion into methylglyoxal did not occur below millimolar Mn^{2+} concentration. However, Mn^{2+} concentrations of liver, muscle,

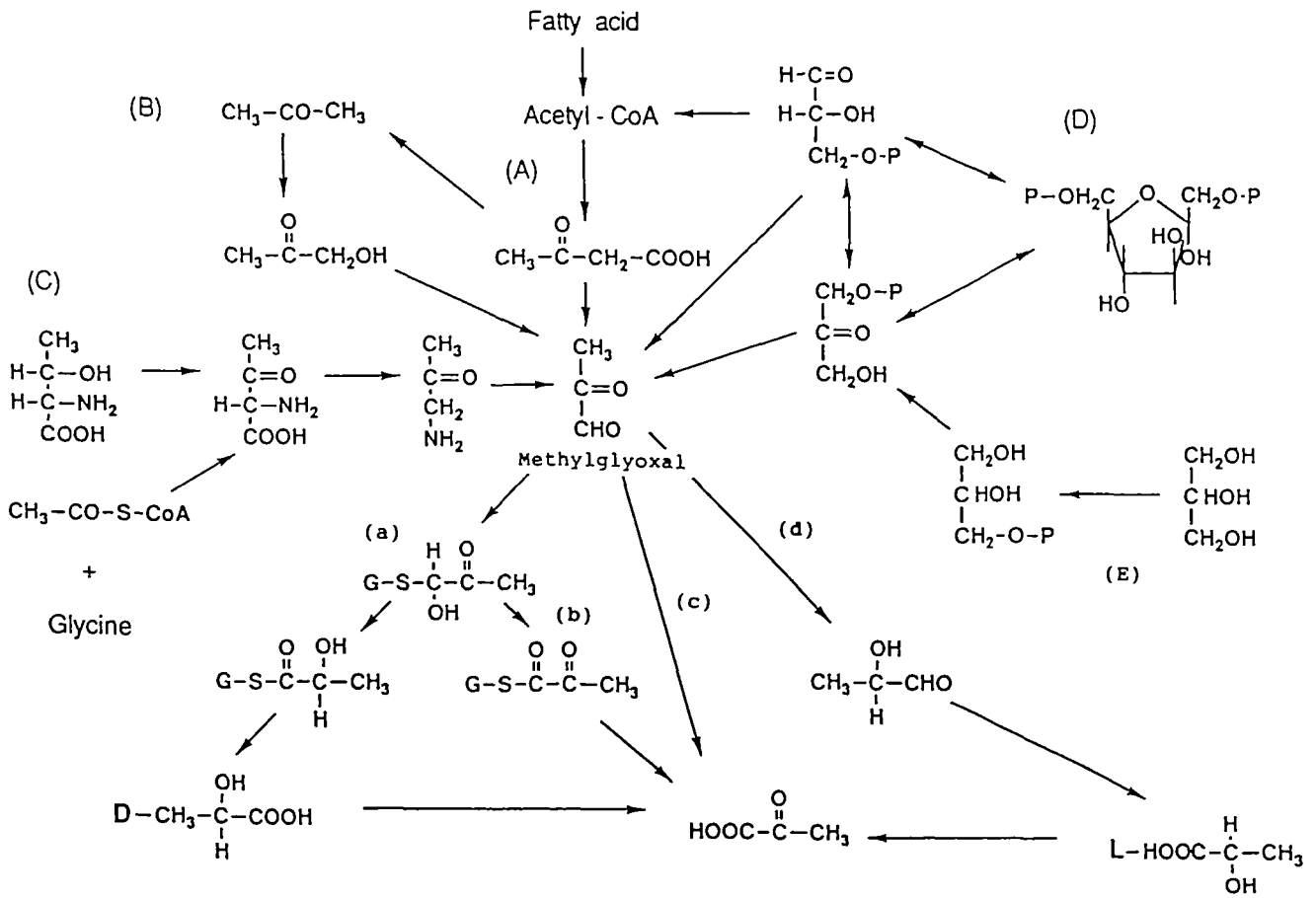

Fig. 2. **Biosynthesis and metabolism of methylglyoxal.**

and blood were reported to be at the μM level. Thus, we concluded that the methylglyoxal formation from acetoacetate by myoglobin was not physiological but chemical (*18*). As shown in Table I, acetoacetate was hardly available as a substrate in normal rat liver homogenate. Acetoacetate is decarboxylated to acetone, which is metabolized to acetol by a monooxygenase, although acetone was formerly considered to be a biochemically stable substance in animals. Acetol is oxidized by a monooxygenase to methylglyoxal (*19-21*) (depicted as B in Fig. 2). As indicated in "RESULTS," acetone was a poor substrate in rat liver homogenate and perfused liver.

Aminoacetone was demonstrated to be formed in animals from acetyl-CoA and glycine by Gibson *et al.* (*22*) or from L-threonine by Bird *et al.* (*23*) and Ray and Ray (*24*) (depicted as C in Fig. 2). They showed that aminoacetone is deaminated by amine oxidase to methylglyoxal (*25, 26*). As shown in Tables I and II, L-threonine was poor substrate.

Some research has been done on the formation of methylglyoxal from glucose (*27, 28*) (depicted as D in Fig. 2). We also surveyed several sugars and their phosphates as substrates for methylglyoxal formation in the dialyzed homogenates of rat tissues and found that the best substrate was fructose-1,6-bisphosphate, while glucose, fructose, galactose and xylitol were poor substrates (*18*).

It has not been established whether glycerol (denoted as E in Fig. 2), can act as a substrate of methylglyoxal and D-lactate in animals (route E).

From this background, we examined what is the substrate for methylglyoxal and D-lactate formations in rat liver, especially by the liver perfusion method. In liver homogenate and perfused liver, glycerol was the best substrate for the D-lactate formation. The second best substrate was glucose, while L-threonine and acetone were poor substrates. Thus, it is considered that L-threonine and acetone play minor roles in D-lactate formation in the liver.

Change in the concentrations of glycerol, L-threonine, glucose, and acetone in the perfusion buffer hardly altered the rate of D-lactate formation. However, the concentrations of glycerol and glucose affected methylglyoxal formation. Higher levels of substrates led to higher formation of methylglyoxal (data not shown).

In a previous paper (*18*), we reported that methylglyoxal was not formed from glycerol by the dialyzed $700 \times g$ supernatant of rat liver. That result is apparently in conflict with the present report. This is due to the fact that glycerol kinase of rat liver homogenates loses its activity during dialysis at 4°C for 12 h. The experiments in Tables I and IV were carried out by incubation of the supernatant of the rat liver without dialysis. We also found that glyoxalase I [EC 4.4.1.5] in the supernatant of the liver homogenate lost its activity during dialysis.

Next we would like to discuss the relationship between D-lactate and glycerol levels in the physiological state. In previous papers, we reported that the plasma concentration of D-lactate increased several-fold after running (*8*) and that diabetic and starved rats had significantly higher levels of D-lactate in plasma, liver, and skeletal muscle (*7*).

Similarly, plasma glycerol concentration increased during exercise and reached 5 times the resting value (*29*). Hepatic glycerol content markedly increased in diabetic and starved rats (*7*). The arterial glycerol concentration was consistently higher in the diabetic patients than in healthy subjects (*30*). The physiological glycerol level described above can thus account for the findings of the higher levels of D-lactate in diabetic and starved rats, as well as those after running.

As indicated in Table IV, the evidence that the glycerol kinase activity is increased in diabetic and starved conditions can also explain the increased formation of D-lactate in those states.

Finally, enzyme activities related to the Embden Meyerhof pathway and the D-lactate formation will be discussed. In the previous study, we measured the activities of pyruvate kinase, phosphofructokinase, aldolase, and glyoxalase I in livers of diabetic and starved rats (*7*). No marked differences between diabetic or starving and normal rats were found except in pyruvate kinase, which decreased to 20% of the normal level. Gunn and Taylor reported that glucokinase and pyruvate kinase activities in liver decreased markedly in starving and diabetic animals, whereas the activity of phosphofructokinase changed little in these animals (*31*). According to Greenbaum *et al.*, only the levels of fructose-1,6-bisphosphate increased and other glycolytic metabolites decreased in liver of diabetic and starving animals (*32*). For these reasons, we concluded that triose phosphates derived from fructose-1,6-bisphosphate flow readily into the MG bypass in those states (Fig. 1).

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