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Formation of Malate from Glyoxylate in Animal Tissues

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1. Incubation of rat liver homogenate with [1-^{14}C]glyoxylate, ATP and acetate shows a rapid sequential incorporation of radioactivity into malate, oxaloacetate and citrate. 2. In liver from normal rats the rate of the formation of each substance in question is higher than that in liver from thiamin-deficient rats. 3. The net accumulation of malate is greater with liver from thiamin-deficient rats. Its further metabolism is retarded, it is suggested, by inhibitors formed by a condensation of glyoxylate and oxaloacetate.

Wong & Ajl (1956) first detected in *Escherichia coli* the malate synthase that catalyses the condensation of acetyl-CoA with glyoxylate to form malate. It has been shown to be formed in organisms adapted to grow on acetate (Kornberg & Beevers, 1957) and in the parts of plants where the active breakdown of fat is taking place (Carpenter & Beevers, 1959). It has also been shown to form malate via glyoxylate in some bacteria living in glycine–glutamate culture (Tsuiki & Kikuchi, 1962).

Isocitrate lyase catalyses a reaction with the formation of succinate and glyoxylate, which replenishes the citrate-cycle intermediates in organisms utilizing acetate as their sole carbon source. These findings led Kornberg & Krebs (1957) to formulate the 'glyoxylate bypass' in lower organisms. However, this bypass has not yet been found in animals (Smith & Gunsalus, 1957). Ganguli & Chakraverty (1961) reported that malate synthase metabolized glyoxylate into malate in animal tissues. It seems that lower organisms, plants and animals all possess malate synthases that can form malate from glyoxylate.

The detection of glyoxylate in thiamin-deficient rats (Liang, 1962*a*) may mean that it plays a specific metabolic role in thiamin-deficient conditions. A possible precursor for glyoxylate is glycine, arising from excessive breakdown of tissue protein (Liang, 1962*b*). Glyoxylate may be formed by transamination between glycine and 2-oxoglutarate in liver (Cammarate & Cohen, 1950), by oxidation of glycine by a glycine oxidase found in liver and kidney (Ratner, Nocito & Green, 1944), by reversible cleavage of 4-hydroxy-2-oxoglutarate to pyruvate and glyoxylate in liver (Dekker & Maitra, 1962; Kuratomi & Fukunaga, 1963) and as an intermediate in the degradation of allantoin (quoted by Kolesnikov, 1962). Glyoxylate has also been detected in human urine (Hockaday, Frederick, Clayton & Smith, 1965; van der Horst, 1960). The role of glyoxylate in animal tissue is still not completely known. Brief reviews of interconversion of glyoxylate with other substances have been given by Kolesnikov (1962) and Stewart & Quayle (1967).

Glyoxylate may condense with oxaloacetate to form inhibitors in the citrate cycle (Ruffo, Testa, Adinolfi & Pelizza, 1962; Payes & Laties, 1963*a*; Ruffo, 1967; Shiio & Ozaki, 1968). This might result in an increase of citrate in the body of the thiamin-deficient rats (Liang, 1962*c*) and in some pathological conditions (Ruffo, 1967).

The present work provides evidence for the entrance of glyoxylate into the citrate cycle by condensation with acetate to form malate in the animal tissues.

MATERIALS AND METHODS

Animals. Male and female albino rats (4 months old) of Sprague–Dawley strain weighing from 250–300g were used. They were fed on thiamin-containing and thiamin-deficient foods as described by Liang (1962*a*).

Determination of malate, oxaloacetate and citrate formed during incubation of liver homogenate. Homogenates were prepared from freshly obtained samples of liver in a glass Potter-type homogenizer, with 1 part of tissue in 9 parts of Krebs–Ringer phosphate medium, pH7.4 (0.154M-NaCl, 0.154M-KCl, 0.11M-$CaCl_2$, 0.154M-$MgSO_4$,$7H_2O$, 0.1M-phosphate buffer, pH7.4). Portions (10ml) of freshly prepared liver homogenate (containing 1g of liver) were placed in separate suction flasks (50ml) and incubated at 37°C in a thermostat with shaking at a rate of 60 times/min. Flasks contained the following ingredients: (*a*) homogenate + ATP (2mM) + sodium glyoxylate (40mM) + sodium acetate (20mM); (*b*) homogenate + ATP (2mM) + sodium glyoxylate (40mM); (*c*) homogenate + ATP (2mM) +

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sodium acetate (20mM); (*d*) homogenate + sodium glyoxylate (40mM); (*e*) homogenate alone; (*f*) boiled homogenate (5min at 100°C) + ATP (2mM) + sodium glyoxylate (40mM)+sodium acetate (20mM). During incubation the homogenates were gassed continuously through the side arm of the suction flask with O_2 passing through a moisture bottle at the same temperaure, so that the incubated homogenates were under an atmosphere of O_2. After 1h 5ml of 10% (v/v) trichloroacetic acid was added to stop the reaction and also to precipitate the proteins. The mixtures were then centrifuged at 2000*g* for 15min. Measured samples of the clear supernatant were used for the determination of free glyoxylate by the method of McFadden & Howes (1960), citrate by the method of Stern (1957), oxaloacetate by the method of Kalnitsky & Topley (1959) and malate by the method of Hummel (1949). For this last method the sample was first heated at 100°C for 20min with 1M-HCl to hydrolyse the residual glycogen, which might otherwise interfere with the determination of malate.

Determination of the sequence of the formation of the intermediates with added glyoxylate. Liver homogenate was prepared as described above but with 20mM-tris–HCl buffer, pH7.4, instead of Krebs–Ringer phosphate medium. [Thompson & Richardson (1968) claimed that tris forms a complex with glyoxylate as a Schiff base, but at pH7.4 and with the tris/glyoxylate ratio 5:1 there is actually little possibility of such interference.] A preliminary test was carried out by incubating the liver homogenate in both tris–HCl buffer and Krebs–Ringer phosphate medium and both gave the same results. The choice of the tris–HCl buffer has the advantage that it gives a better chromatographic separation of the spots on paper strips, without tailing.

A 90ml portion of the homogenate was warmed to 37°C in a suction flask (300ml) in the thermostat with a shaking device and gassed through the side arm with O_2 as described above. The reaction was initiated by adding 10ml of tris–HCl buffer containing ATP (2mM), sodium glyoxylate (20mM) containing 2.5μCi of sodium [1-^{14}C]-glyoxylate and sodium acetate (20mM) to the suspension. Samples (about 2ml) of the incubation mixture were withdrawn at intervals with an automatic pipette and each was delivered quickly into a graduated centrifuge tube containing 6ml of ethanol at 60°C (so that the final concentration of ethanol was approx. 75%, v/v) to stop the reaction. The volume of the mixture was measured and the exact volume of the homogenate was obtained by the difference of the total suspension volume and the volume of ethanol. Immediately after delivery the mixture was heated at 80°C for 5min to complete the precipitation of the protein. After centrifugation at 2000*g* for 15min, the supernatant was transferred to a test-tube and evacuated to dryness in a desiccator under reduced pressure over P_2O_5 and paraffin chips. The dry residue was extracted repeatedly with HCl–ether (made by shaking ether with a small volume of 1M-HCl) until no radioactivity could be detected in the residue. The pooled ether extract was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of propan-2-ol–water (1:1, v/v) and separated by ascending chromatography on Whatman 3MM filter paper in phenol–6M-formic acid–water (500:13:167, w/v/v) for 20h at room temperature. The paper strips were then dried in air for 2 days. The radioactivity (c.p.m.) after background correction was determined at 5mm intervals, the strip being placed on a shielded slide (30mm × 5mm) under an end-window Geiger–Müller counter (Panax GM10). The counting efficiency was 78% with a background count of 27c.p.m. and the counting rate of radioactive sodium glyoxylate was 38700c.p.m./0.1g of liver. The substance in question was determined by co-chromatography with known substances: glyoxylate, citrate, malate, oxaloacetate, fumarate+succinate and creatinine. These form definite spots on the paper strip with R_F 0.31, 0.44, 0.53, 0.65, 0.76 and 0.92 respectively. The yield of radioactivity in different substances at different intervals was plotted on graph paper. Apart from these known substances, some lipids with radioactivity that could be completely extracted by ether were also found on the paper strip with low R_F values in the range 0.0–0.17. Their exact nature was not determined.

The precipitate containing protein obtained from the ethanol treatment also possessed radioactivity derived from the added radioactive glyoxylate. The total radioactivity was determined, but not the detailed distribution in each amino acid.

Determination of ^{14}C *in* C-1 *and* C-4 *of malate formed from* [1-^{14}C]*glyoxylate.* Radioautographs of the paper strip were made on X-ray film. The area of the radioactive malate was marked against the shadow of the radioautogram and the cut-out was made. This was then eluted with HCl-acidified ether. The eluate was dried by evaporation under reduced pressure and subjected to degradation reactions (Utter, 1951; Schultz, 1944) to determine the relative amounts of radioactivity in C-1 and C-4 of malate.

RESULTS

Table 1 shows the quantity of malate, oxaloacetate and citrate formed in homogenates of liver from normal and thiamin-deficient rats after incubation for 1h. No new formation of these substances occurred in boiled homogenates (*f*), their concentrations remaining the same as before the incubation and being only slightly lower than those in (*c*), without glyoxylate, so that the possibility of non-enzymic formation of these substances was excluded. In general, liver from normal rats can metabolize more added glyoxylate than that from the thiamin-deficient rats, confirming the report of Liang (1962*b*).

With liver from normal rats the addition of ATP greatly enhanced malate formation in the presence of either glyoxylate (*b*) or glyoxylate+acetate (*a*). The addition of acetate alone increased the malate to only a small extent (*c*). However, in the absence of ATP the addition of glyoxylate did not cause an additional increase of malate, oxaloacetate and citrate (*d*). In (*e*), which represented the endogenously formed substances, there was some decrease in the amount after incubation for 1h.

With liver from thiamin-deficient rats the addition of glyoxylate caused a greater increase of these

Table 1. *Formation of malate, oxaloacetate and citrate from added glyoxylate by homogenates of liver from normal and thiamin-deficient rats*

Yields of malate, oxaloacetate and citrate are given in μmol/g fresh wt. of liver and as percentage formation from added glyxoylate after incubation of the homogenate (1 g of liver in 9 ml of Krebs–Ringer phosphate medium) plus the following: (*a*) ATP+glyoxylate+acetate; (*b*) ATP+glyoxylate; (*c*) ATP+acetate; (*d*) glyoxylate+acetate; (*e*) none; (*f*) boiled homogenate+ATP+glyoxylate+acetate (see the Materials and Methods section). Net formation of each substance from added glyoxylate is the difference of the quantity in (*a*), (*b*), (*d*) or (*e*) from that in (*c*). Percentage formation of each substance from added glyoxylate is the net formation of each substance divided by the quantity of glyoxylate removed. Results were obtained from six normal rats and eight thiamin-deficient rats (35 days on the diet described by Liang, 1962*a*) and are given as means ±S.E.M.

	Normal rats								
Incubation mixture	Glyoxylate removed	Malate formation			Oxaloacetate formation			Citrate formation		
		Total	Net	%	Total	Net	%	Total	Net	%
(*a*)	5.44±0.97	0.682±0.102	+0.355	+6.53	0.383±0.068	+0.221	+4.07	0.486±0.053	+0.332	+6.10
(*b*)	3.08±0.88	0.481±0.053	+0.154	+5.00	0.232±0.073	+0.070	+2.26	0.308±0.058	+0.154	+5.13
(*c*)		0.327±0.030			0.162±0.073			0.154±0.058		
(*d*)	2.63±0.76	0.327±0.038	0	0	0.160±0.062	−0.002	−0.07	0.152+0.060	−0.002	−0.07
(*e*)		0.288±0.031	−0.039		0.148±0.058	−0.014		0.145±0.050	−0.009	
(*f*)	0	0.319±0.035			0.158±0.035			0.150±0.038		
	Thiamin-deficient rats									
(*a*)	3.72±0.78	1.235±0.093	+0.825	+22.16	0.936±0.078	+0.387	+10.35	1.268±0.088	+0.636	+17.80
(*b*)	3.13±0.75	0.770±0.066	+0.360	+11.50	0.785±0.056	+0.236	+7.53	0.973±0.070	+0.341	+10.90
(*c*)		0.410±0.037			0.549±0.080			0.632±0.085		
(*d*)	1.85±0.33	0.576±0.166	+0.166	+8.98	0.642±0.085	+0.093	+5.03	0.686±0.081	+0.054	+2.92
(*e*)		0.212±0.025	−0.198		0.365±0.047	−0.184		0.478±0.062	−0.154	
(*f*)	0	0.388±0.031			0.519±0.068			0.586±0.076		

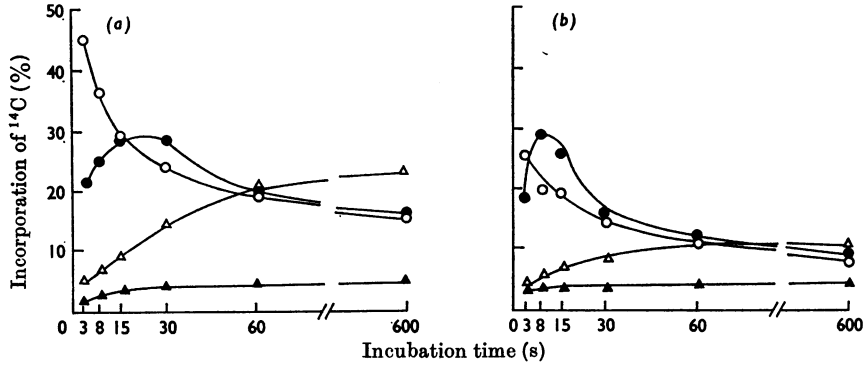

Fig. 1. Time-course for the incorporation of ^{14}C from [1-^{14}C]glyoxylate into malate (○), oxaloacetate (●), citrate (△) and succinate+fumarate (▲) extracted from the incubation mixtures of homogenate of liver from normal (*a*) and thiamin-deficient (*b*) rats. Experimental details are given in the Materials and Methods section. The incorporation of ^{14}C is the radioactivity of each substance expressed as a percentage of the total radioactivity of the added [1-^{14}C]glyoxylate at zero time.

Table 2. *Distribution of radioactivity in* C-1 *and* C-4 *of malate obtained during incubation of homogenate of normal rat liver with* [1-^{14}C]*glyoxylate*

Experimental details of the incubation and the determination of radioactivity are given in the Materials and Methods section.

Incubation time (s)	Radioactivity in eluted malate (c.p.m.) Total	At C-1	At C-4	C-1/C-4 radioactivity ratio
3	3138	2670	470	85/15
9	1476	915	560	62/38
30	1140	630	515	55/45
600	340	180	160	53/47

intermediates (*a* and *b*). Increase of malate, oxaloacetate and citrate occurred when acetate or acetate +glyoxylate was added (*c* and *d*). This may be due to some glyoxylate already formed in the tissue (Liang, 1962*a*). In (*d*) there was a greater formation of these substances than in (*c*), even though no ATP was added. It seemed to be affected by the increased residual acetyl-CoA (C.-C. Liang & L.-C. Ou, unpublished work) present in the liver from the thiamin-deficient animals.

Though the difference in the yield of malate from added glyoxylate (*b*) compared with that from glyoxylate+acetate (*a*) was small, the difference is statistically significant: $P<0.005$ for the normal rats and $P<0.01$ for the thiamin-deficient rats. The highest yield of malate in liver from the normal rats was about 6.53% and in that from thiamin-deficient rats 22.16% of the total glyoxylate removed after incubation for 1h. The percentage of malate formed was small, but liver from thiamin-deficient rats has about 1.81 times as much malate, 2.44 times as much oxaloacetate and 2.6 times as much citrate as that from normal rats.

Fig. 1 shows that in livers from both normal and thiamin-deficient rats there is a rapid incorporation of the radioactivity into citrate-cycle intermediates. Malate seemed to be one of the earliest products of ^{14}C incorporation, followed by oxaloacetate and then citrate.

Distribution of radioactivity in C-1 and C-4 of malate during the incubation is given in Table 2. After 3s C-1 and C-4 contained about 85% and 15% respectively of the total radioactivity in malate. After 10min the amounts became almost equal (53 and 47%).

The amount of radioactivity decreased from 100% to 34% in 1h in homogenate of normal rat liver and from 100% to 79% in that of liver from thiamin-deficient rats. This loss of radioactivity was presumably due to complete oxidation of the ^{14}C. Of the remaining radioactivity after incubation for 1h about 12–18% was found in the lipid fraction and 14–19% in the protein fraction in liver from both normal and thiamin-deficient rats. Only 16.7% (malate+oxaloacetate+citrate) as citrate-cycle intermediates was found in liver from normal

rats and 50.31% in that from thiamin-deficient rats.

DISCUSSION

The formation of the citrate-cycle intermediates after the addition of glyoxylate was extremely rapid. It is known that there are many ways open to the metabolism of glyoxylate in animals (Kolesnikov, 1962; Stewart & Quayle, 1967; Ganguli & Chakraverty, 1961). The yield of malate obtained in the normal and the thiamin-deficient rat livers represented only that amount in the dynamic state of the transformation after incubation for 1h, i.e. 6.53 and 22.16% respectively of the glyoxylate removed from the medium.

It has been shown by Payes & Laties (1963*b*) that in plants α-hydroxy-γ-oxoglutarate can arise from glyoxylate+oxaloacetate. This substance can give rise to malate. It is not known whether or not the animal system can do the same. If it can, this might account for a small amount of malate formation from a part of the glyoxylate disappearance. Judged from the rapid formation of malate the quantity of malate first formed could be higher than that found after incubation for 3s (Fig. 1).

It should be recognized that most of the added glyoxylate underwent further metabolism with the 1h incubation period to form CO_2 and water and through other pathways not completely known to form protein, lipids and other substances. Accumulation of pyruvate has previously been shown in birds (Peters, 1936), patients suffering from beriberi (Platt & Lu, 1956) and in liver of thiamin-deficient rats (Liang, 1962*a*). A higher concentration of citrate has also been found in thiamin-deficient animals (Liang, 1962*b*) and in man (Shigematsu, 1959). In the present study higher citrate and oxaloacetate concentrations were found in homogenate of liver from thiamin-deficient rats.

Ruffo, Adinolfi, Budillon & Capobianco (1962) studied the effect of glyoxylate on the oxidation of citrate-cycle intermediates by rat liver mitochondria and found inhibition of the oxidation of both citrate and pyruvate. This may account for the accumulation of pyruvate and oxaloacetate in thiamin-deficient animals. The inhibition of the citrate cycle produced by glyoxylate is comparable with that produced by malonate. Payes & Laties (1963*a*) isolated and characterized α-hydroxy-γ-oxoglutarate, a product of the non-enzymic condensation of glyoxylate and oxaloacetate, from plants. This compound has a remarkable inhibitory effect on citrate oxidation, specifically on the enzymes aconitase and isocitrate dehydrogenase. Shiio & Ozaki (1968) stated that in animals the inhibition by glyoxylate+oxaloacetate, or a condensation product, was competitive with respect to isocitrate; the nature of the inhibitors here is not yet known, but the fact of inhibition is clear.

Theoretically the C-1 of malate is directly derived from C-1 of glyoxylate, and C-4, which is derived from acetate, should not contain radioactivity. However, within 3s C-4 of malate contained 15% of the total radioactivity of the malate, probably owing to the randomization with C-1 (presumably because of the presence of fumarase activity). The gradual change of the C-1/C-4 radioactivity ratio might, in addition to the randomization, come from other sources, so that an equal mixture of 1-^{14}C- and 4-^{14}C-labelled malate was obtained after a longer period of incubation.

From all these findings it is clear that in rat liver a portion of glyoxylate, either administered or formed in the body, can be converted into malate and then further into oxaloacetate and citrate. The combination of glyoxylate and oxaloacetate may form compounds that inhibit the oxidation of citrate and pyruvate. The quantity of glyoxylate formed depends on the nutritional state of the animals (Liang, 1962*a*) and also on the various ways of metabolism open to it (Stewart & Quayle, 1967; Kolesnikov, 1962).

The incubation conditions used in this study may have been suboptimum for the conversion of glyoxylate into malate since the presumed actual substrate for reaction with glyoxylate is acetyl-CoA and the reaction studied was presumably dependent on endogenous CoA. In fact, it would appear that the present results may greatly underestimate the capacity of rat liver to promote this conversion.

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