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The Metabolism of Glyoxylate by Human- and Rat-Liver Mitochondria

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The place of glyoxylate as an intermediate in glycine metabolism has been reviewed by Weinhouse (1955), although its importance in the intact animal is still under discussion (Neuberger, 1961; Liang, 1962*a, b*). Nakada & Sund (1958), who studied the decarboxylation of glyoxylate by washed liver homogenates and prepared a partially purified glyoxylate decarboxylase from liver mitochondria, suggested that glyoxylate and L-glutamate condense to form *N*-glyoxyl-L-glutamic acid (*N*-carboxymethylene-L-glutamic acid), which is either hydrolysed to glycine and 2-oxoglutarate or decarboxylated by glyoxylate decarboxylase to *N*-formyl-L-glutamic acid. According to this scheme formic acid and L-glutamic acid are produced by the subsequent hydrolysis of *N*-formyl-L-glutamic acid. Glyoxylate can also be oxidized enzymically to oxalate *in vitro* (Ratner, Nocoto & Green, 1944; Nakada & Weinhouse, 1953*a*; Richardson & Tolbert, 1961; Quayle & Taylor, 1961) and *in vivo* (Weinhouse & Friedmann, 1951).

The present work was designed to investigate the mechanism of glyoxylate metabolism in intact mammalian liver mitochondria. We also attempted to determine whether *N*-formyl-L-glutamic acid was a metabolic intermediate in this system as well as in Nakada & Sund's (1958) system. The cytochrome-oxidase activity of the mitochondria was used as an independent criterion of their biological activity.

Some of the results presented here have been the subject of preliminary communications (Crawhall & Watts, 1960, 1961).

MATERIALS AND METHODS

Melting points are uncorrected and microanalyses (C, H, N) were by Weiler and Strauss, Oxford, and by A. Bernhardt, Mülheim.

All chemicals were of A.R. grade, and water that had been deionized by passage through a mixed-bed ion-exchange column was used. ATP, ADP and cytochrome *c* were obtained from the Sigma Chemical Co., glyoxylic acid from L. Light and Co. Ltd., oxaloacetic acid and 2-oxoglutaric acid from C. F. Boehringer und Soehne (through Courtin and Warner Ltd.). [^{14}C]Glyoxylic acid, sodium [^{14}C]glyoxylate, 2-[^{14}C]oxoglutaric acid, DL-[^{14}C]glutamic acid and [2,3- $^{14}\text{C}_2$]fumaric acid were obtained from The Radiochemical Centre, Amersham, Bucks.

Synthesis of [2- ^{14}C]sodium glyoxylate. [2,3- $^{14}\text{C}_2$]Fumaric acid (1 m-mole) was oxidized with OsO_4 and KClO_3 , and potassium hydrogen [2,3- $^{14}\text{C}_2$]tartrate isolated (Weissbach & Sprinson, 1953). An aqueous solution of potassium hydrogen [2,3- $^{14}\text{C}_2$]tartrate was passed through a column (5 cm. $\times$ 1 cm.) of Dowex 50 (H^+ form) and the [2,3- $^{14}\text{C}_2$]tartaric acid in the eluate converted into sodium [2- ^{14}C]glyoxylate as described by Radin & Metzler (1955). Yield, based on [2,3- $^{14}\text{C}_2$]fumaric acid, was 30%. The product could be converted into the 2,4-dinitrophenylhydrazone in 95% yield, which agrees with the experience of Wild (1953) and Radin & Metzler (1955). Two batches of unlabelled sodium glyoxylate synthesized in this way gave Na 20.2 and 19.9% respectively (weighed as Na_2SO_4) ($\text{C}_2\text{H}_3\text{O}_4\text{Na}$ requires Na, 20.2%) and acid KMnO_4 titrations that corresponded to 102 and 101% of the theoretical value for $(\text{OHC}\cdot\text{CO}_2\cdot\text{Na})_2\text{H}_2\text{O}$.

Synthesis of N-formyl-L(-)-glutamic acid. Two batches of this material were prepared. On the first occasion we followed the procedure of Tabor & Mehler (1955), on a 45 m-moles scale, and obtained a yield of only 7%. The product melted at 108–109°. (Found: C, 41.4; H, 5.1; N, 8.0; $\text{C}_6\text{H}_9\text{O}_5\text{N}$ requires C, 41.4; H, 4.6; N, 8.0%). On the second occasion freshly redistilled acetic anhydride (30 ml.) was added dropwise over the course of about 0.5 hr. to formic acid (90 ml., 98/100%) in a three-necked flask fitted with a spiral condenser, a Hg-sealed stirrer and a dropping funnel. The condenser and dropping funnel were fitted with guard tubes containing Anhydron. Stirring was continued until the temperature of the reaction mixture, which rose initially, had returned to room temperature. The flask was then cooled in ice–NaCl freezing mixture, L(-)-glutamic acid (45 m-moles) was added and stirring continued for 1 hr. The remaining acetic anhydride and

formic acid were removed by distillation under reduced pressure at 45°, care being taken to avoid the ingress of traces of water vapour during this procedure. The residual oil crystallized after standing over flaked NaOH *in vacuo* for 48 hr. It was dissolved in the minimum volume of anhydrous ethanol, recrystallized by the addition of benzene and the crystals were dried *in vacuo* over flaked paraffin wax. Recrystallization twice more from ethanol-benzene (1:10) gave a product, m.p. 106–110°, $[\alpha]_D^{20} - 7.50 \pm 0.38^\circ$ (2%, w/v, in water) (Found: C, 41.8; H, 5.4; N, 7.7; $C_6H_5O_5N$ requires C, 41.4; H, 5.1; N, 8.0%). Yield 30%.

Separation and storage of liver mitochondria. Suspensions of mitochondria in 0.25M-sucrose were prepared from the livers of adult male or female Wistar-strain albino rats as described by Schneider (1948), except that the final centrifuging was performed at 7000g (measured at the closed end of the centrifuge tube), and stored at -15°. Sucrose solution (0.25M) was used for all stages of the preparation. Normal human-liver tissue was obtained at laparotomy, chilled in ice immediately, and the preparation of the mitochondrial suspension (Schneider, 1948) begun without further delay. The final suspensions of mitochondria were stored at -15°.

Assay of cytochrome oxidase. A spectrophotometric procedure (Hogeboom, Schneider & Striebig, 1952) was used. Some of these determinations were made with an automatic recording spectrophotometer (Unicam SP. 800) and the rate of oxidation of reduced cytochrome *c* was calculated from the change in extinction at 537 mμ during the first minute of the reaction phase.

Manometric assay of glyoxylate decarboxylase. Single-side-arm Warburg flasks (total volume of the manometric system about 10 ml.) were used. Sodium glyoxylate (0.25 ml., 7.5 μmoles, pH adjusted to 7.4) in 0.25M-sucrose, and sodium L-glutamate (0.25 ml., 7.5 μmoles, pH adjusted to 7.4) in 0.25M-sucrose, were placed in the main compartment of the flasks, with metaphosphoric acid (0.1 ml.; 10%, w/v, soln.) in the side arms. The mitochondrial suspension (0.75 ml., total N approx. 1.5 mg.) was added to the contents of the main compartment and the flasks were shaken in the Warburg bath at 37° for the appropriate length of time, usually 1 hr., with air as the gas phase. The metaphosphoric acid was added by tipping the flasks and the volume of CO₂ evolved was read after shaking for a further period of 1 hr. Control flasks containing only mitochondria and sodium L-glutamate in the main compartments and metaphosphoric acid in the side arms were run simultaneously, and the difference between the volume of CO₂ (μmoles/mg. of N/hr.) evolved in the presence and in the absence of glyoxylate was used as a measure of the glyoxylate-decarboxylase activity of the mitochondria. The volume of CO₂ evolved by a control system consisting of mitochondria, 0.25M-sucrose and glyoxylate was too small to be measured on this scale of work.

Estimation of oxygen uptake by mitochondria. Double-side-arm Warburg flasks were used. Mitochondrial suspension (2.0 ml., total N approx. 4.0 mg.) in 0.25M-sucrose, and sodium L-glutamate (0.66 ml., 20 μmoles, pH adjusted to 7.4) in 0.25M-sucrose, were placed in the main compartment of the flasks, with metaphosphoric acid (0.1 ml.; 10%, w/v, soln.) in one side arm and sodium glyoxylate (0.66 ml., 20 μmoles, pH adjusted to 7.4) in 0.25M-sucrose in the other. The flasks were shaken in the Warburg bath at 37° until equilibrium had been attained (approx. 5 min.).

The sodium glyoxylate was tipped into the main compartment of the flasks, which were then shaken for a further 1 hr., at the end of which the metaphosphoric acid was tipped from the second side arm into the main compartment of the flasks and shaking in the Warburg bath at 37° continued for a further 1 hr. Control flasks were set up containing 0.25M-sucrose soln. (0.66 ml.) in place of the sodium glyoxylate solution in the first side arm. This permitted the measurement of CO₂ evolution by mitochondria and L-glutamate in the absence of glyoxylate. The complete system (mitochondria, L-glutamate and glyoxylate) was set up in ten flasks, and the centre wells of six of these contained NaOH (0.1 ml.; 40%, w/v, soln.) for the measurement of uptake of O₂.

Investigation of the metabolism of [1-¹⁴C]glyoxylate under anaerobic conditions. Double-side-arm Warburg flasks were used. The main compartments contained mitochondria (total N 3.60 mg.) suspended in 0.25M-sucrose (2.66 ml., pH 7.4) containing sodium L-glutamate (19.8 μmoles). One side arm contained sodium [1-¹⁴C]glyoxylate (19.8 μmoles, 5900 counts/min. as BaCO₃/mg. of mitochondrial total N), dissolved in 0.66 ml. of 0.25M-sucrose; the second side arm contained metaphosphoric acid (0.2 ml.; 10%, w/v, soln.). The centre wells contained NaOH (0.1 ml.; 40%, w/v, soln.). Duplicate flasks were gassed with helium and shaken in the Warburg bath at 37° until equilibrium had been attained (approx. 5 min.). The sodium glyoxylate was tipped into the main compartments of the flasks, which were then shaken for 1 hr., after which the metaphosphoric acid was added from the second side arm into the main compartments of the flasks and shaking in the Warburg bath at 37° was continued for a further 1 hr. Two control flasks containing air as the gas phase were run simultaneously with the anaerobic flasks. The radioactive CO₂, glyoxylate, oxalate and glycine were isolated as described below, and their radioactivities compared. Carbon dioxide production under aerobic and anaerobic conditions was also determined manometrically simultaneously with the radiochemical experiments; the flasks were set up and incubated as described above except that NaOH was omitted, and control flasks containing the complete system except for glyoxylate were used.

Attempted demonstration of the formation of N-[formyl-¹⁴C]formyl-L-glutamic acid from [2-¹⁴C]glyoxylate by mitochondria suspended in 0.25M-sucrose. Method 1. Rat-liver mitochondria (total N 3.0 mg.) suspended in 0.25M-sucrose (2.5 ml., pH 7.4) containing sodium [2-¹⁴C]glyoxylate (7.5 μmoles, 9280 counts/min. as BaCO₃) and sodium L-glutamate (7.5 μmoles) were incubated for 1 hr. at 37° in the main compartments of four Warburg flasks (A, B, C and D) simultaneously. The volumes of CO₂ evolved in flasks A and B were measured to confirm that mitochondrial metabolism had proceeded normally, and the CO₂ evolved in flask C was collected in NaOH (0.1 ml.; 40%, w/v) on filter paper in the centre well. Formate, oxalate, glyoxylate and glycine were isolated from the main compartments of flasks A and B, N-formyl-L-glutamic acid was isolated from the contents of the main compartment of flask D, and the formate and N-formyl-L-glutamic acid were degraded (see below).

Method 2 ('isotope-trapping experiment'). Rat-liver mitochondria (total N 1.27 mg.), suspended in 1.25 ml. of 0.25M-sucrose containing [2-¹⁴C]glyoxylate (7.5 μmoles, 3830 counts/min. as BaCO₃), sodium L-glutamate (7.5 μmoles) and sodium N-formyl-L-glutamic acid (8.2 μmoles)

were incubated at 37° in the main compartment of single-arm Warburg flasks for 10, 20, 30, 40 and 60 min. The reaction was stopped and *N*-formyl-L-glutamic acid isolated and degraded as described below.

Isolation of evolved carbon dioxide, glyoxylate, oxalate and glycine. Mitochondrial metabolism was stopped by the addition of 0.1 ml. of 10% (w/v) metaphosphoric acid from the side arm of the Warburg flask, and evolved CO₂ was collected in the centre wells of the flasks on strips of filter paper impregnated with 0.1 ml. of 25% (w/v) NaOH. These were soaked in 0.5 ml. of 2.0N-Na₂CO₃ for 18 hr., washed with water and the washings were added to the main bulk of the solution; BaCO₃ was precipitated by the addition of 0.5 ml. of a saturated BaCl₂ solution, washed three times with water and dried *in vacuo* over P₂O₅ (CO₂-free water was used for these manipulations). Carrier amounts (1 m-mole) of glyoxylic acid and oxalic acid and of glycine were added to the contents of the Warburg flask; the denatured mitochondrial protein was separated by centrifuging, washed and discarded, the washings being added to the supernatant solution.

To the supernatant solution was added 2,4-dinitrophenylhydrazine (109 ml.; 0.2% soln. in 2N-HCl); glyoxylic acid-2,4-dinitrophenylhydrazone crystallized and was purified to constant radioactivity by recrystallization from ethanol-water (1:10). The mother liquor was shaken with ethyl acetate to remove residual glyoxylic acid-DNP-hydrazone and calcium oxalate precipitated after the solution had been brought to pH 5.2 by the addition of 10N-NaOH. The calcium oxalate was washed twice with aq. 0.35N-NH₃ soln. and with water; reprecipitation and washing were repeated until the radioactivity of the dried product was unaltered by this procedure; its purity was confirmed by KMnO₄ titration and determination of calcium (as CaSO₄).

The supernatant solution was saturated with NaHCO₃ and evaporated to a dry residue, which was caused to react with 1-fluoro-2,4-dinitrobenzene and DNP-glycine isolated (Watts & Crawhall, 1959).

Isolation of formate. Carrier sodium formate (0.33 m-mole) was added with the other carrier compounds (see above). Formic acid was isolated by steam-distilling the supernatant from the calcium oxalate precipitation step after the addition of H₂SO₄ (1.0 ml. of approx. 18N) and excess of Ag₂SO₄ (calculated on the basis of the amount of HCl present; Weinhouse & Friedmann, 1952). DNP-glycine was isolated from the solution after the steam-distillation.

Isolation of N-formyl-L-glutamic acid. Mitochondrial metabolism was stopped by immersing the Warburg flask in a boiling-water bath. Carrier *N*-formyl-L-glutamic acid (1 m-mole) was added, and the denatured protein was separated by centrifuging and washed with three portions (1.0 ml.) of water; the washings were added to the supernatant solution and the combined supernatant and washings freeze-dried. The dry residue was dissolved in anhydrous ethanol and *N*-formyl-L-glutamic acid recrystallized by the addition of benzene. The product was recrystallized from anhydrous ethanol-benzene (1:10) until its melting point was constant and identical (when observed in the hot-stage melting-point apparatus) with that of the original carrier *N*-formyl-L-glutamic acid, and until its radioactivity was unchanged by further recrystallization.

Degradation of formate and N-formyl-L-glutamic acid. Sodium formate was degraded to BaCO₃ as described by

Weinhouse & Friedmann (1952). This method was also used to hydrolyse *N*-[formyl-¹⁴C]formyl-L-glutamic acid and to convert the formyl C atom into BaCO₃ without isolation of the liberated formic acid.

Measurements of ¹⁴C. All samples were mounted at infinite thickness on tared nickel planchets (area 1.76 cm.²) and gently pressed down with a stainless-steel rod of the same diameter as the internal diameter of the planchets. Thin end-window (0.2 mg./cm.²) Geiger-Müller counters were used and sufficient counts were recorded to give a total count with a probable error not greater than ±2.5%. All the specimens were counted at least twice and the count rates were corrected for the 'background' count and for day-to-day fluctuations in counter sensitivity. The differences between the molecular weights of the substances counted and that of BaCO₃ were allowed for by multiplying the observed count rate by the factor (mol.wt. of compound counted/mol.wt. of BaCO₃) and the final count rates expressed in counts/min. as BaCO₃ at infinite thickness.

Total nitrogen. This was determined by a micro-Kjeldahl procedure (Francis, Mulligan & Wormall, 1959).

RESULTS

Glyoxylate-decarboxylase and cytochrome-oxidase activities of the mitochondria. The glyoxylate-decarboxylase activities of different batches of both rat- and human-liver mitochondria varied between 1.3 and 2.6 μmoles of carbon dioxide/mg. of N/hr. We obtained values of between 7.3 and 9.5 μmoles of cytochrome c/mg. of N/min. for the cytochrome-oxidase activity of our rat-liver mitochondria, but the values for human-liver mitochondria were considerably less than this (Table 1). Hogeboom *et al.* (1952) quote 10.1 μmoles of cytochrome c/mg. of N/hr. as the cytochrome-oxidase activity of rat-liver mitochondria.

The cytochrome-oxidase and glyoxylate-decarboxylase activities of the mitochondria were unaffected by storage in 0.25M-sucrose at -15° for as long as 71 days. The cytochrome-oxidase activity was also unaltered by the incubation procedure used for glyoxylate-decarboxylase determinations and for the experiments with [¹⁴C]glyoxylate, or by three cycles of rapid freezing to -70° and rethawing to 15°, although a fourth cycle of rapid freezing and thawing decreased the cytochrome-oxidase activity by about 25%.

Table 1. *Glyoxylate-decarboxylase and cytochrome-oxidase activities of mitochondrial fractions from human liver*

No. of mitochondrial preparation	Glyoxylate- decarboxylase activity (μmoles of CO ₂ /mg. of N/hr.)	Cytochrome- oxidase activity (μmoles of cyto- chrome c/mg. of N/min.)
1	1.3	1.75
2	2.6	1.05
3	2.5	2.10

The effects of some inorganic ions, of ADP and ATP on the glyoxylate-decarboxylase activity of the mitochondria are shown in Table 2. Phosphate ions were inhibitory and this precluded the use of a phosphate buffer in our system.

Biosynthesis of glycine and oxalate from glyoxylate and the conversion of C-1 atoms of glyoxylate into carbon dioxide. The time-courses of the incorporation of the C-1 atom of glyoxylate into carbon dioxide, oxalate and glycine by rat- and human-liver mitochondria are shown in Fig. 1; the results presented in Table 3 show that the corresponding non-enzymic reactions made a negligible contribution to the labelling of these metabolites under our experimental conditions.

Relationship between the metabolism of glyoxylate and that of L-glutamate. The amount of carbon dioxide formed in a system consisting of mitochondria, 0.25 M-sucrose and equimolar amounts of glyoxylate and L-glutamate was decreased by 90 % if the glyoxylate was omitted. This effect was demonstrated both manometrically and radiochemically.

Effect of varying the concentration of L-glutamate on the incorporation of C-1 atoms of glyoxylate into carbon dioxide, glycine and oxalate. Decreasing the concentration of L-glutamate in our system decreased the conversion of [1-¹⁴C]glyoxylate into [¹⁴C]carbon dioxide and [¹⁴C]glycine in non-linear manner but [¹⁴C]oxalate formation was unaltered

(Fig. 2). In the absence of added L-glutamate, the amount of [¹⁴C]carbon dioxide formed was about 10 % of the amount formed when the concentrations of added L-glutamate and [1-¹⁴C]glyoxylate were equimolar, although no glyoxylate-decarboxylase activity had been detected when no L-glutamate

Fig. 1. Incorporation of C-1 atoms of sodium [1-¹⁴C]-glyoxylate into CO₂, glycine and oxalate by (a) human- and (b) rat-liver mitochondria over the course of 1 hr. The main compartment of each Warburg flask contained mitochondria (total N 0.266 mg. and 0.408 mg. for human- and rat-liver mitochondria respectively) suspended in 0.59 ml. of 0.25 M-sucrose soln. containing sodium [1-¹⁴C]glyoxylate (2.1 μmoles; 2090 counts/min. as BaCO₃) and sodium L-glutamate (2.1 μmoles). Metaphosphoric acid (0.1 ml.; 10%, w/v, soln.) was added from the side arms of the flasks at the times shown, the corresponding flasks belonging to each half of the experiment being 'tipped' simultaneously. O, CO₂; □, oxalate; △, glycine.

Table 2. *Effect of phosphate and magnesium ions and of adenosine di- and tri-phosphate on the glyoxylate-decarboxylase activity of rat-liver mitochondria*

Six Warburg flasks (A, B, C, D, E and F) were employed in each experiment. The main compartments of flasks A and B contained mitochondria, 1.5 ml. of 0.25 M-sucrose, 7.5 μmoles of sodium glyoxylate, 7.5 μmoles of sodium L-glutamate and the substances being tested. Flasks C and D contained mitochondria, 1.5 ml. of 0.25 M-sucrose and 7.5 μmoles of sodium L-glutamate in their main compartments. The main compartments of flasks E and F contained mitochondria, 1.5 ml. of 0.25 M-sucrose, 7.5 μmoles of sodium glyoxylate and 7.5 μmoles of sodium L-glutamate.

Addition	Glyoxylate-decarboxylase activity of mitochondria (μmoles of CO ₂ /mg. of N/hr.)	
	Without addition	With addition
Phosphate buffer, pH 7.4 (final concn. of PO ₄ ³⁻ ions, 16 mM)	1.1	0.5
MgCl ₂ (6.0 mM)	1.1	1.2
Phosphate buffer, pH 7.4 (final concn.) of PO ₄ ³⁻ ions, (16 mM) and MgCl ₂ (6.0 mM)	1.9	0.7
ATP (0.1 mM)	2.0	2.1
ADP (0.1 mM)	2.0	1.8
CaCl ₂ (8.6 mM)	1.8	1.8

Table 3. *Non-enzymic reactions of [1-¹⁴C]glyoxylate*

The main compartment of each Warburg manometer flask contained boiled rat-liver mitochondria (total N 1.20 mg.) suspended in 1.25 ml. of 0.25 M-sucrose soln., sodium L-glutamate (8.6 μmoles) and sodium [1-¹⁴C]glyoxylate (8.6 μmoles, 19100 counts/min. as BaCO₃).

Duration of incubation (hr.)	Radioactivity (counts/min. as BaCO ₃)		
	CO ₂	Glycine	Oxalate
1	56	220	164
2	104	320	178
3	118	332	163

was added in preliminary manometric experiments.

Fraction of the respiratory carbon dioxide derived from C-1 atoms of glyoxylate and L-glutamate. A comparison of the radioactivity of the evolved carbon dioxide and that of $[1-^{14}\text{C}]$ glyoxylate or $\text{L}-[1-^{14}\text{C}]$ glutamate in the incubation medium indicated that about one-half to two-thirds of the total carbon dioxide evolved during the metabolism of equimolar amounts of glyoxylate and L-glutamate by mitochondria was derived from the C-1 atoms of L-glutamate (Table 4).

Specificity of L-glutamate with respect to the glyoxylate-decarboxylase activity of mitochondria. The glyoxylate-decarboxylase activity of our mitochondrial suspensions was abolished when the L-glutamate in the medium was replaced by an equimolar amount of aspartate, alanine, *N*-formyl-L-glutamate, isocitrate, succinate, DL-malate, fumarate or pyruvate, but it was enhanced (e.g. from

2.04 to 4.36 μmoles of carbon dioxide/mg. of N/hr.), when an equimolar amount of 2-oxoglutarate was substituted for the L-glutamate. The proportion of the respiratory carbon dioxide that was derived from C-1 atoms of glyoxylate in an experiment in which $[1-^{14}\text{C}]$ glyoxylate was used and the L-glutamate was replaced by 2-oxoglutarate was of the same order (37%) as in the comparable experiments in which L-glutamate was present.

Metabolism of $[1-^{14}\text{C}]$ glyoxylate by liver mitochondria in the presence of 2-oxoglutarate. In view of the ability of 2-oxoglutarate to replace L-glutamate in the manometric assay of glyoxylate decarboxylase, we investigated the metabolism of $[1-^{14}\text{C}]$ glyoxylate by mitochondria in the presence of different concentrations of this compound in place of L-glutamate (Fig. 3). The conversion of C-1 atoms of glyoxylate into carbon dioxide was enhanced and glycine formation was grossly diminished by substituting 2-oxoglutarate for L-glutamate, but there was no direct quantitative relationship between carbon dioxide formation and

Fig. 2. Effect of different relative concentrations of glyoxylate and L-glutamate on the metabolism of $[1-^{14}\text{C}]$ -glyoxylate. The main compartment of each Warburg flask contained mitochondria (total N 0.52 mg.) suspended in 0.59 ml. of 0.25M-sucrose containing sodium $[1-^{14}\text{C}]$ -glyoxylate (2.1 μmoles ; 4540 counts/min. as BaCO_3 /mg. of mitochondrial total N) in each flask and sodium L-glutamate. Metaphosphoric acid (0.1 ml.; 10%, w/v, soln.) was added from the side arms of the flasks after incubation for 1 hr. ○, CO_2 ; □, oxalate; △, glycine.

Table 4. Fraction of the evolved carbon dioxide derived from the C-1 atoms of glyoxylate and L-glutamate during the metabolism of glyoxylate by liver mitochondria in the presence of an equimolar amount of L-glutamate

Double-side-arm Warburg flasks were used. The main compartments contained rat-liver mitochondria (total N 3.90 mg.) suspended in 0.25M-sucrose (3.32 ml., pH 7.4). For the determination of the fraction derived from glyoxylate C-1 atoms non-radioactive sodium L-glutamate (19.8 μmoles) was added to the contents of the main compartment; one side arm contained sodium $[1-^{14}\text{C}]$ glyoxylate (19.8 μmoles , 20000 counts/min. as BaCO_3 in 0.66 ml. of 0.25M-sucrose, pH 7.4), and this was added to the main compartment after the flasks had been equilibrated in the Warburg bath; incubation was continued for 1 hr. and HPO_3 (0.1 ml.; 10%, w/v, soln.) added from the second side arm. The volume of CO_2 evolved was determined in duplicate; in three of the flasks, the CO_2 was converted into BaCO_3 and counted as described in the text. The radioactivity that the CO_2 would have possessed had it all been derived from the metabolism of glyoxylate C-1 atoms in the presence of L-glutamate was calculated and the observed radioactivity expressed as a fraction of this. The fraction derived from L-glutamate C-1 atoms was determined simultaneously by using sodium DL- $[1-^{14}\text{C}]$ glutamate and non-radioactive glyoxylate. It was assumed that D-glutamate was not decarboxylated under these conditions (Wilson & Koeppel, 1961).

Expt. no.	From glyoxylate C-1 atoms	From L-glutamate C-1 atoms
1	0.45	0.45
2	0.28	0.55
3	0.34	0.68
4	0.42	0.62
Mean	0.37	0.57

2-oxoglutarate concentration. Oxalate formation was unrelated to the 2-oxoglutarate concentration and was virtually the same as when L-glutamate was used.

Investigation of the possible conversion of C-5 atoms of 2-oxoglutarate into respiratory carbon dioxide. To determine whether 2-oxoglutarate was further metabolized in such a way as to yield carbon dioxide from the C-5 carbon atom, we incubated mitochondria with equimolar amounts of glyoxylate and 2-[5- 14 C]oxoglutarate in the usual manner. The evolved carbon dioxide was not radioactive.

Effect of inhibitors of the tricarboxylic acid cycle on glyoxylate-decarboxylase activity of liver mitochondria. The glyoxylate-decarboxylase activity of a rat-liver mitochondrial suspension, initial activity 1.14 μ moles of carbon dioxide/mg. of N/hr. in the presence of equimolar (7.5 μ moles/flask) concen-

trations of L-glutamate and glyoxylate, was abolished by adding 7.5 μ moles of sodium malonate (5 mM) to the system. An experiment in which the effect of different concentrations of malonate on the metabolism of [1- 14 C]glyoxylate was investigated confirmed that this reagent inhibited carbon dioxide formation, and showed that the amination of glyoxylate and oxalate formation were not inhibited (Fig. 4). The mechanism of the inhibition of carbon dioxide production was investigated by studying the 14 C content of the evolved carbon dioxide obtained by incubating mitochondria with different concentrations of [1- 14 C]glyoxylate and two different concentrations of malonate. Plotting $1/V$ (V , radioactivity of the evolved carbon dioxide) against $1/S$ (S , glyoxylate concentration) showed that the inhibition was competitive (Fig. 5), the Michaelis constant being 3.70×10^{-3} M.

Concentrations of sodium fluoroacetate between 0.22 and 3.56 mM did not influence the incorporation of the C-1 atom of [1- 14 C]glyoxylate into carbon dioxide, oxalate and glycine. The amounts of fluoroacetate used corresponded to between 0.063 and 1.0 'molar equivalents' (see Fig. 5) of the amounts of glyoxylate and L-glutamate present.

Oxygen requirement for the metabolism of glyoxylate by liver mitochondria. The volume of oxygen taken up during the metabolism of glyoxylate was about 7% of the volume of carbon dioxide evolved.

Anaerobic conditions diminished the conversion of [1- 14 C]glyoxylate into [14 C]carbon dioxide, [14 C]oxalate and [14 C]glycine by approximately 20,

Fig. 3. Effect of different relative concentrations of glyoxylate and 2-oxoglutarate on the metabolism of [1- 14 C]glyoxylate. The main compartment of each Warburg flask contained mitochondria (total N 0.52 mg.) suspended in 0.59 ml. of 0.25 M-sucrose containing sodium [1- 14 C]glyoxylate (2.1 μ moles; 4290 counts/min. as BaCO₃/mg. of mitochondrial total N in each flask) and sodium 2-oxoglutarate. Metaphosphoric acid was added from the side arms of the flasks after incubation for 1 hr. at 37°. O, CO₂; □, oxalate; △, glycine.

Fig. 4. Effect of sodium malonate on the metabolism of sodium [1- 14 C]glyoxylate by liver mitochondria. The main compartment of each Warburg flask contained rat-liver mitochondria (total N 0.415 mg.) suspended in 0.6 ml. of 0.25 M-sucrose soln. containing sodium [1- 14 C]glyoxylate (2.1 μ moles, 2120 counts/min. as BaCO₃), sodium L-glutamate (2.1 μ moles) and sodium malonate. The reaction was stopped after incubation for 1 hr. at 37°. O, CO₂; □, oxalate; △, glycine.

40 and 10% respectively. The manometrically determined carbon dioxide production by the system under these conditions was also decreased by 20% from the corresponding value under aerobic conditions.

Effect of repeated freezing and thawing of the mitochondria on the metabolism of [1-¹⁴C]glyoxylate. Three portions of a freshly prepared batch of mitochondria that had been rapidly frozen to -70° and thawed to 15° once, twice and thrice, had the same ability to convert [1-¹⁴C]glyoxylate into [¹⁴C]-carbon dioxide, [¹⁴C]oxalate and [¹⁴C]glycine as a control portion of the same mitochondrial preparation that had been kept at 0° while the freezing and thawing operations were being carried out.

Metabolism of glycollate by liver mitochondria. Incubation of equimolar amounts (7.5 μ moles) of glycollate and L-glutamate (pH 7.4) with rat-liver mitochondria (total N 0.95 mg.) resulted in the evolution of carbon dioxide at the rate of 0.40 μ mole/mg. of N/hr., compared with 1.35 μ moles of carbon dioxide/mg. of N/hr. in the identical control Warburg flasks that contained equimolar amounts of glyoxylate and L-glutamate (7.5 μ moles) and which were run simultaneously.

Attempted demonstration of the formation of N-[formyl-¹⁴C]formyl-L-glutamic acid from [2-¹⁴C]-glyoxylate by mitochondria suspended in 0.25 M-sucrose. By the use of method 1 (above) the mean

count rates of the isolated formate, oxalate and glycine were 248, 566 and 2475 counts/min. respectively, the evolved carbon dioxide contained 81 counts/min. and the formyl C atom of N-formyl-L-glutamic acid was not radioactive. With method 2 the formyl C atom of N-formyl-L-glutamic acid did not become labelled.

DISCUSSION

Transamination, decarboxylation and oxalate formation accounted for 90% of the [1-¹⁴C]glyoxylate metabolized by an aerobic system consisting of previously frozen mitochondria, 0.25 M-sucrose and equimolar amounts of glyoxylate and L-glutamate at pH 7.4 and 37° . Only a minimal amount of non-enzymic transamination occurred under these conditions (cf. Nakada & Weinhouse, 1953b; Fleming & Crosbie, 1960a, b). Repeated freezing and thawing of the mitochondria did not inhibit these metabolic activities, which suggested that they do not depend upon the preservation of the normal internal structure of the mitochondria.

Glyoxylate decarboxylation was almost completely abolished if added L-glutamate (or 2-oxoglutarate) was omitted from the system (Figs. 2 and 3), and very little carbon dioxide was evolved by a system consisting of mitochondria, 0.25 M-sucrose and L-glutamate only, although the C-1 atom of L-glutamate contributed a large proportion of the total amount of carbon dioxide that was formed in the complete system. It appeared therefore that neither glyoxylate nor L-glutamate could be decarboxylated efficiently by our mitochondrial preparations in the absence of an added amount of the other member of the pair. Only 2-oxoglutarate among a series of tricarboxylic acid-cycle intermediates and related compounds tested could replace L-glutamate in the manometric glyoxylate-decarboxylase system. In experiments in which the relative concentrations of L-glutamate and glyoxylate, and of 2-oxoglutarate and glyoxylate, were varied the curves relating [¹⁴C]carbon dioxide formation from [1-¹⁴C]glyoxylate with the concentrations of added L-glutamate and 2-oxoglutarate respectively were similar. The rate of glyoxylate amination to glycine was greater than the rate of carbon dioxide formation at all the concentrations of L-glutamate used. 2-Oxoglutarate is formed by transamination between L-glutamate and glyoxylate, so that the rate of 2-oxoglutarate formation was consistently greater than the rate of glyoxylate decarboxylation. We suggest that intact liver mitochondria effect a synergistic decarboxylation of glyoxylate and L-glutamate but that the L-glutamate may undergo preliminary transamination with glyoxylate, the 2-oxoglutarate formed reacting with a second molecule of glyoxylate to

Fig. 5. Lineweaver-Burk plot for the inhibition by malonate of the mitochondrial decarboxylation of glyoxylate. The main compartment of each Warburg flask contained mitochondria (total N 0.52 mg.), sodium L-glutamate (2.1 μ moles), [1-¹⁴C]glyoxylate (2280 counts/min. as BaCO₃/flask) and 0.66 ml. of 0.25 M-sucrose. The total glyoxylate concentration was expressed in terms of the number of 'molar equivalents' of glyoxylate present (i.e. 1 molar equivalent = 2.1 μ moles). The reaction was stopped after incubation for 1 hr. at 37° , and the CO₂ collected and counted as described in the text. Concentration of malonate: ●, 1.95 mM; ×, 1.26 mM; ○, nil.

form carbon dioxide from the C-1 atoms of both glyoxylate and 2-oxoglutarate. The further details of the mechanism whereby the C-1 atom of glyoxylate is converted into carbon dioxide are obscure, and the fate of the C-1 atom of 2-oxoglutarate could not be investigated directly because of the difficulty of preparing 2-[1-¹⁴C]oxoglutarate. The reaction was inhibited competitively by malonate but not by fluoroacetate; also 2-[5-¹⁴C]oxoglutarate did not yield [¹⁴C]carbon dioxide, confirming that there was no radiochemically detectable tricarboxylic acid-cycle activity in our system, and we do not think that malonate is acting by inhibition of succinic dehydrogenase. It is also well known that freezing mitochondria destroys their 2-oxoglutarate-oxidase and oxidative phosphorylation activities.

The mechanism of glyoxylate decarboxylation and transamination in our system appears to differ from that proposed by Nakada & Sund (1958) for a washed liver homogenate. These workers established that one step in their reaction sequence [the decarboxylation of *N*-(carboxymethylene)-L-glutamate to *N*-formyl-L-glutamate] was catalysed by an enzyme, which they prepared in a partially pure form from rat-liver mitochondria. We were unable to demonstrate that *N*-formyl-L-glutamate was an intermediate in our system.

The oxidation of glyoxylate to oxalate did not require the presence of L-glutamate or 2-oxoglutarate but was more dependent on the presence of molecular oxygen than decarboxylation or transamination. Ratner *et al.* (1944) and Nakada & Weinhouse (1953*a*) comment on the catalysis of this reaction by xanthine oxidase, and Quayle & Taylor (1961) described a coenzyme A- and NADP-dependent glyoxylic acid dehydrogenase in *Pseudomonas oxalaticus* (OXI). Ratner *et al.* (1944) also mention the anaerobic dismutation of glyoxylate to oxalate and glycollate by a rabbit skeletal-muscle enzyme, and the occurrence of a reaction of this type as well as an aerobic reaction might account for the incomplete inhibition of oxalate formation in our system by anaerobiosis.

Under our conditions, only about 1% of the ¹⁴C of the [2-¹⁴C]glyoxylate/mg. of mitochondrial total nitrogen could be isolated as formate after incubation for 1 hr., the corresponding value for the conversion of the C-1 atom of glyoxylate into carbon dioxide being 29%. There was no evidence for the extensive conversion of [2-¹⁴C]glyoxylate into [¹⁴C]carbon dioxide, so that further attempts to identify the terminal metabolic products of the C-2 atom of glyoxylate in this system are needed; the demonstration of serine synthesis from glycine by mitochondrial fragments (Sanadi & Bennett, 1960) and by liver homogenates (Richert, Amberg & Wilson, 1962) is of interest in this connexion.

SUMMARY

1. The metabolism of [1-¹⁴C]glyoxylate by rat- and human-liver mitochondria suspended in 0.25M-sucrose (pH 7.4) has been studied in the presence of an equimolar amount of L-glutamate. Decarboxylation, oxidation to oxalate and amination to glycine account for virtually all of the glyoxylate that is metabolized. Glyoxylate-decarboxylase activity and cytochrome-oxidase activity were also studied. Human-liver mitochondria had a lower cytochrome-oxidase activity than rat-liver mitochondria, but no other appreciable species differences were detected. Non-enzymic reactions did not contribute significantly to glyoxylate degradation under our experimental conditions.

2. L-Glutamate was needed for decarboxylation and formation of glycine but not for production of oxalate. In the presence of equimolar amounts of glyoxylate and L-glutamate, about 40% of the evolved carbon dioxide was derived from the C-1 atom of glyoxylate, and the remainder was formed from the C-1 atom of L-glutamate. Decarboxylation occurred if L-glutamate was replaced by 2-oxoglutarate (but not by other tricarboxylic acid-cycle intermediates or related compounds), and the glyoxylate-decarboxylase activity of the mitochondria was enhanced by this substitution. 2-[5-¹⁴C]Oxoglutarate did not yield [¹⁴C]carbon dioxide.

3. Phosphate ions inhibited, but Mg²⁺ ions, adenosine diphosphate, adenosine triphosphate and Ca²⁺ ions did not influence the glyoxylate-decarboxylase activity of the mitochondria. Malonate inhibited the decarboxylation of [1-¹⁴C]-glyoxylate competitively but did not influence oxalate or glycine formation. The metabolism of [1-¹⁴C]glyoxylate was unaffected by fluoroacetate or by three cycles of freezing and thawing of the mitochondria. Anaerobiosis depressed decarboxylation, glycine formation and oxalate formation from [1-¹⁴C]glyoxylate by approximately 20, 10 and 40% respectively.

4. Some glyoxylate-decarboxylase activity was demonstrated when glycollate was substituted for glyoxylate.

5. We have been unable to demonstrate that *N*-formyl-L-glutamate is an intermediate in glyoxylate metabolism under our experimental conditions.

6. When [2-¹⁴C]glyoxylate was used, the proportion of the C-2 atom of glyoxylate that was recovered as formate or carbon dioxide was considerably less than the proportion of the C-1 atom of glyoxylate incorporated into carbon dioxide in the experiments with [1-¹⁴C]glyoxylate.

7. It appears that glyoxylate and L-glutamate (or a derivative thereof, which we suggest is 2-oxo-

glutarate) are decarboxylated synergistically in this system. The details of the mechanism by which this is effected have not, however, been elucidated.

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A Chromatographic and Electrophoretic Study of Sarcoplasm from Adult- and Foetal-Rabbit Muscles

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Most of the studies which have so far been carried out on the proteins of foetal muscle have been concerned primarily with the development of the proteins of the contractile system, the myofibril. The protein system of the soluble cytoplasm of muscle, the sarcoplasm, is complex and, although this fraction does not appear to play a direct role in contraction, its composition reflects certain aspects of the metabolic activity of the tissue. As it contains the enzymes of the glycolytic and other systems of special importance to muscle as well as those of a more general significance (see Perry, 1956, 1960a, for reviews), sarcoplasm has frequently been used as the starting material for enzyme preparations. Nevertheless, relatively

little systematic attempt has been made to develop methods for the fractionation and identification of the individual components which could be of value in comparative studies. Boundary-electrophoretic investigations (Jacob, 1947; Dubuisson, 1950) have indicated the presence of 10–12 distinct migrating boundaries with which certain enzymes have been identified by indirect evidence. Further work by others has shown that the electrophoretic pattern of sarcoplasm from a given muscle is distinctive for the species (see Perry, 1960a, for references) and in a given species for the particular muscle from which it is derived (Crepax, 1952).

The present paper presents the results of a study of the fractionation of sarcoplasm by chromato-