STUDIES ON THE INTERMEDIARY METABOLISM OF GLYCINE

by

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GENERAL INTRODUCTION.

Glycine was the first amino acid shown by isolation to be present in a protein hydrolysate (Braconnot, 1820). Besides incorporation into proteins, peptide hormones and glutathione, the carbon atoms of glycine appear in other amino acids like serine and creatine, glycocholic acid, hippuric acid, purine and porphyrin rings. The methylene carbon atom of the glycine molecule also contributes significantly to the one carbon pool. Thus, although structurally the simplest amino acid, the metabolism of glycine presents a complex picture.

If carbohydrate and lipids are considered as fuels for the metabolic furnace, proteins may be regarded as forming not only the structural frame work, but also the gears and leavers of the machinery. The α-keto acids produced from amino acids function not only as the raw materials for the synthetic processes, but also as catalysts in the chanelling of the acetyl units derived from all classes of foodstuffs through the citric acid cycle. In vitro investigations of the metabolism of glycine leading to its integration with the intermediary metabolism of the organism will be reported in this thesis.

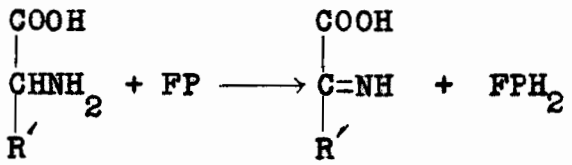
At the present time, many facets of glycine metabolism are known and a summary of those aspects that lead to or result in the oxidation of the molecule in the animal tissues will be presented.

Generally, the degradative metabolism of an amino acid involves the removal of the amino group and the subsequent breakdown of the carbon skeleton. The removal of the amino group occurs in the mammals usually by transamination and to a lesser extent by oxidative deamination.

Oxidative deamination.

The interconversion of amino acids and the analogous α-keto acids, in many animal species, was observed early in the century (Neubauer, 1909; Knoop, 1910). In 1932, Krebs reported that liver and kidney preparations can deaminate D and L-amino acids. Subsequently (Krebs, 1933; 1935), he was able to separate the D-amino acid oxidase activity from the L-amino acid oxidase activity. Various D-amino acid oxidases have been shown to require flavin adenine dinucleotide (FAD) for activity (Straub, 1938a; 1939b; Warburg and Christian, 1938a; 1938b). So far only one L-amino acid oxidase has been obtained from mammalian tissue (rat kidney) and it utilizes flavin mononucleotide (FMN) as a cofactor (Blanchard, Green, Nocito and Ratner, 1944; 1945). L-amino acid oxidase has also been isolated from snake venom (Zeller and Maritz, 1944; 1945). The coenzyme for this preparation is FAD (Singer and Kearney, 1950a; 1950b).

The general mechanism of action of the amino acid oxidase may be represented as follows:



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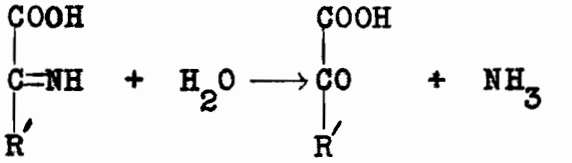

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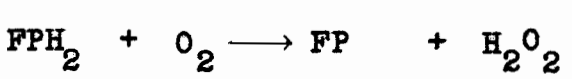
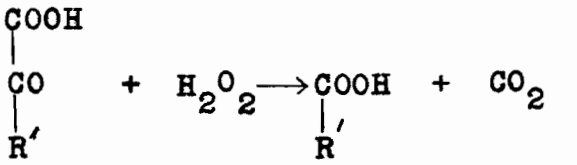


(3)

FP stands for the flavoprotein cofactor, either FAD or FMN.

Formation of the imino acid (Equation 1) has long been considered to be hypothetical but recently Meister, Wellner and Scott (1960) have put forward evidence for its formation with ophio-L-amino acid oxidase. Hydrolysis of the imino acid (Equation 2) is generally considered to be spontaneous. In the absence of catalase, the hydrogen peroxide formed during the oxidation of the reduced flavoprotein (Equation 3), can non-enzymically, oxidatively decarboxylate the α-keto acid (Equation 4).



(4)

The amino acid oxidases, as a rule attack a large number of their respective stereo isomers, though at different rates.

Again, enzymes from different sources apparently contain different apo-enzymes for they exhibit different substrate specificities. The mammalian amino acid oxidase has also the peculiar capacity of oxidizing the α-hydroxy acids at similar rates. An apparently different L-amino acid oxidase, more specific towards the basic amino acids has also been reported to occur in turkey (Boulanger and Osteux, 1955) and chicken liver (Struck and Sizer, 1960).

The metabolic significance of the amino acid oxidases is not clear. The L-amino acid oxidase of rat kidney has too low a turnover (approximately 6) to possess an important physiological role (Blanchard et al. 1944; 1945). In contrast, the D-amino acid oxidase from the same source has a turnover number of 1440 (Warburg and Christian, 1938a; 1938b), but the occurrence of the D-amino acids themselves is rare. It is curious that only D-amino acid oxidases of mammalian tissues and L-amino acid oxidases of snake venoms and certain molds which are very active, contain FAD, in contrast to the sluggish L-amino acid oxidases of the animal tissues which utilize FMN as coenzyme.

Glycine oxidase.

Glycine oxidase, which requires FAD as a cofactor, has been prepared from pig kidney. Besides catalyzing the reaction,

$$\begin{matrix} CH_2NH_2 \\ | \\ COOH \end{matrix} + \tfrac{1}{2}O_2 \longrightarrow \begin{matrix} CHO \\ | \\ COOH \end{matrix} + NH_3 \qquad (5)$$

the enzyme also attacks sarcosine to form glyoxylic acid and methylamine (Ratner, Nocito and Green, 1944). The metabolic importance of this enzyme has also been questioned (Arnstein, 1954; Weinhouse, 1955a), for it has an unusually high K_m value for glycine and is inactive at physiological pHs. Glycine oxidase preparations have both D-amino acid oxidase and catalase activities, but Ratner et al. (1944) have claimed that purified D-amino acid oxidase does not attack glycine. Contradictory to this, is the recent report by Neims and Hellerman (1962) that glycine oxidase and D-amino acid oxidase are one and the same enzyme.

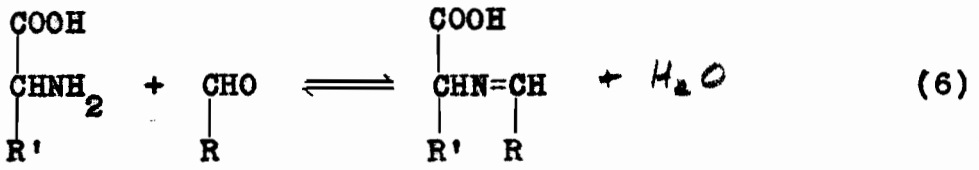
Transamination.

In contrast to oxidative deamination, the scope of enzymic transamination is very broad. Herbst and Engel (1934) first reported the non-enzymic transfer of the α-amino groups of amino acids to α-keto acids without the formation or participation of free ammonia. Working with pigeon breast muscle, Braunstein and Kritsmann (1937) demonstrated enzymic transamination of a number of amino acids.

Transaminases require vitamin B_6 as a co-factor. Snell (1944), first made the suggestion that pyridoxal and pyridoxamine might be interconverted during transamination, and subsequently (Snell, 1945) demonstrated it in non-enzymic

models. Nutritional studies provided the early experimental evidence for the participation of vitamin B_6 as a coenzyme in transamination reactions (Lichstein, Gunsalus, and Umbreit, 1945; Schlenk and Snell, 1945; Ames, Sarma and Elvehjem, 1947). Numerous investigations have provided additional support for these early studies and now it is generally accepted that pyridoxal phosphate or pyridoxamine phosphate is a necessary cofactor for transaminases (see Braunstein, 1960).

Transamination involves the reversible intermediate formation of a Schiff base between the reactants (Equation 6) followed by a shift in the double bond to form a new Schiff base (Equation 7). This is then hydrolyzed and results in the net transfer of the amino group (Equation 8).



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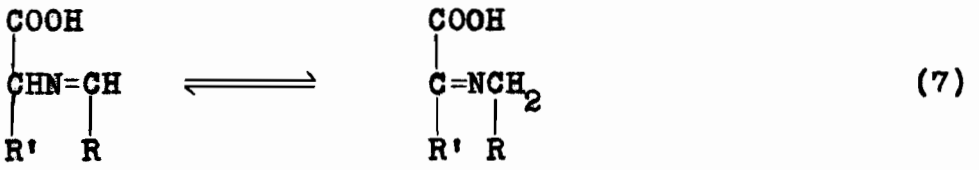

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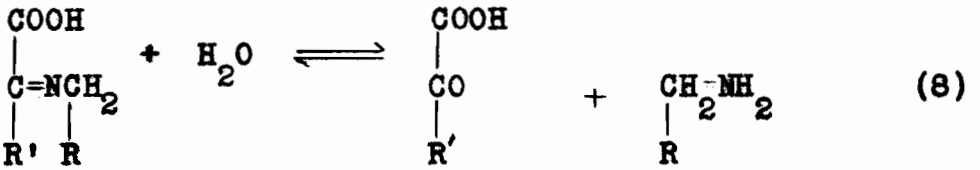
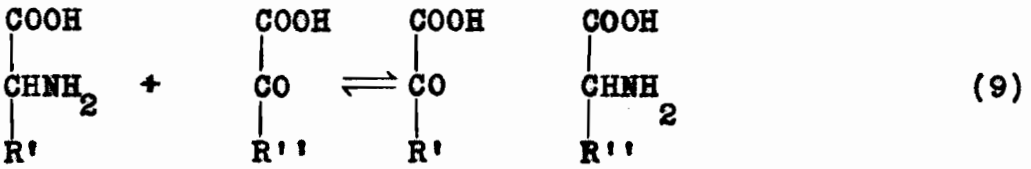


(8)

R-CHO and R'-$CHNH_2$-COOH stand for pyridoxal phosphate and a L-α-amino acid respectively.

A similar series of equations can be written for the reactions between pyridoxamine phosphate (RCH_2NH_2) formed (Equation 8) and another keto acid R'' CO-COOH resulting in the recovery of pyridoxal phosphate and the formation of a new amino acid (R''CH_2NH_2COOH). The sum of these reactions is:



(9)

Herbst first suggested this mechanism (Herbst, 1946) to explain the interconvertibility of α-amino and α-keto acids in boiling aqueous solutions (Herbst and Engel, 1934). A similar scheme was proposed by Braunstein (Braunstein, 1947) for the enzyme catalyzed transamination. A number of investigators have postulated mechanisms with minor modifications to include the strong catalytic influence of metal ions on the velocity of non-enzymic reactions and the influence of the apo-enzymes on the stereo-specificity of the enzyme-catalyzed reactions. The necessity of the metal ions in enzymic transaminations have not yet been proved unequivocally.

The Schiff base of various amino acids (Equation 6) with pyridoxal (Heyl, Harris and Folkers, 1948; Heyl, Luz, Harris and Folkers, 1948a; 1948b; Brandenberger and Cohen, 1953) and pyridoxal phosphate (Matsuo, 1957) have been isolated from solution in alcohol. The formation of two Schiff base intermediates as illustrated in Equation 7, has been shown by spectrophotometry (Eichhorn and Dawes, 1954) with pyridoxal-alanine system and an actual separation of them on paper has also been reported (Fasella, Silipraudi and Baglioni, 1955).

Although the participation of D-amino acids in transamination reactions occurs in bacteria (Molnar and Thorne, 1955; Thorne, Gomez and Housewright, 1955), such reactions are yet to be demonstrated in animal tissues.

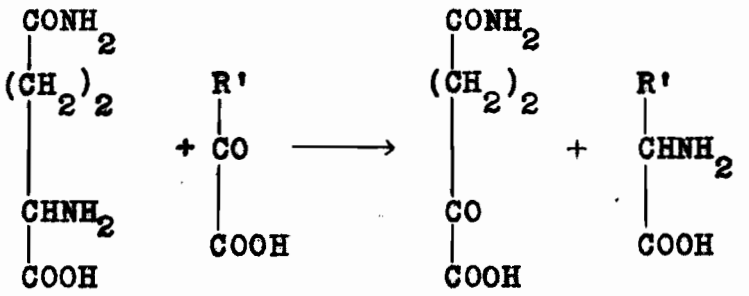
Glycine and Transamination Reactions.

Though early experiments failed to demonstrate the conversion of glyoxylic acid to glycine as measured by hippuric acid excretion in benzoic acid fed rabbits (Sassa, 1913), similar experiments conducted later with labelled glyoxylate in rats have provided convincing evidence for this transformation, presumably via transamination (Weinhouse and Friedmann, 1951; Chao, Dehriche and Greenberg, 1953). Glyoxylate has also been

shown to label the uric acid excreted by pigeon in a manner characteristic of glycine (Weissbach and Sprinson, 1953a). In vitro formation of glycine from glyoxylate, with glutamate as the amino-group donor in rat liver extracts (Meister, Sober, Tice and Fraser, 1952), in rat kidney and ventral prostrate (Awapara and Seale, 1952) and the reverse process of this conversion in rat liver extracts (Cammarata and Cohen, 1950) have also been shown to occur. Aspartate also transaminates with glyoxylate (Meister, et al. 1952). The equilibrium in all these transamination reactions favours the formation of glycine.

Transamination reactions involving amino acid amides.

Greenstein and Carter (1947) first observed that glutamine can undergo transamination in rat liver extracts. Glutamate exhibits little or no activity in this system (Meister, Fraser and Tice, 1954), although it is the α-amino group that is transferred during the reaction and the amide nitrogen is released as ammonia. The w-amidase that hydrolyses the α-ketoglutaramate (Equation 11) formed by the transaminase (Equation 10) has been isolated free from the transaminase activity (Meister, Levintow, Greenfield and Abendschein, 1955).



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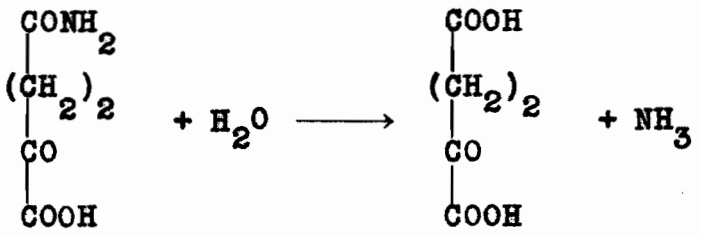


(11)

This transaminase system is active with a large number of α-keto acids (Meister et al. 1952; Meister, 1954a, 1954b; 1954c; Meister and Fraser, 1954; Meister et al. 1954). Asparagine can also take part in similar transamination-deamidation reactions; apparently the same w-amidase that hydrolyses α-ketoglutaramic acid hydrolyses α-ketosuccinamic acid, though the transaminase seems to be a different enzyme (Meister and Fraser, 1954). Since the affinity of w-amidase for α-ketoglutaramic acid is very great, the reaction is practically irreversible; in other words, these transaminases are probably concerned in the synthesis of α-amino acids from their keto analogues rather than in their deamination.

Amino acid amides are more active in transamination reactions with glyoxylic acid than are the amino acids. Both glutamine (Mardashev and Semina, 1950; Meister et al. 1952; Nakada and Weinhouse, 1953b; Meister, 1954a; 1954b) and asparagine (Meister et al. 1952) are active in this reaction and apparently the reaction is irreversible.

The w-amino group of ornithine can also undergo transamination in animal tissues (Quastel and Witty, 1951) and in fact it transaminates very rapidly with glyoxylate in rat liver extracts (Meister, 1954a).

It should be realized that although transamination has been demonstrated to occur with practically all the amino acids (see Meister, 1955), it is really an exchange reaction, and presumably other mechanisms exist for the removal of the amino group. As discussed before, (Page 4) amino acid oxidases do not seem to play a significant role in deamination. Braunstein and Bychkov (1939) first made the suggestion that glutamic dehydrogenase (Equation 13) may be the important link between amino acid metabolism and carbohydrate and fat metabolism. The enzyme is widely distributed in mammalian tissues, of which liver and kidney are found to be very active (Von Euler, Adler and Steennoff-Errickson, 1937; Damodaran and Nair, 1938). Since glutamic acid participates extensively in transamination

reactions (Equation 12) (Cammarata and Cohen, 1950; Awapara and Seale, 1952), coupling this reaction with that catalyzed by glutamic dehydrogenase (Equation 13) results in the net deamination of an amino acid (Equation 14).

L-α-amino acid + α-ketoglutaric acid $\rightleftharpoons$

L-glutamic acid + α-keto acid (12)

L-glutamic acid + $\frac{1}{2}O_2 \longrightarrow$ α-ketoglutaric acid + NH_3 (13)

Sum: L-α-amino acid + $\frac{1}{2}O_2 \longrightarrow$ α-keto acid + NH_3 (14)

Metabolism of glyoxylic acid.

Glyoxylic acid, formed either by oxidative deamination or transamination of glycine, can be metabolized in different ways. The general non-enzymic oxidation of keto acids by H_2O_2 (Equation 4) is extremely rapid with glyoxylic acid (Zelitch and Ochoa, 1953), since it is a highly reactive molecule. The reaction is also catalyzed by heavy metal ions (Kenten and Mann, 1952).

The glyoxylate cycle, proposed by Krebs and Kornberg (1957) operates in conjunction with the citric acid cycle; two reactions, (a) the condensation of succinic acid and glyoxylic acid to form D-isocitric acid catalyzed by isocitritase (Equation 15) and (b) the condensation of acetyl-CoA and glyoxylic acid to form malic acid catalyzed by malate synthetase (Equation 16), bring the glyoxylate

molecule into the general stream of metabolism.

$$\text{Succinate} + \text{Glyoxylate} \longrightarrow \text{D-isocitrate} \qquad (15)$$

$$\text{Acetyl CoA} + \text{Glyoxylate} \longrightarrow \text{Malate} + \text{CoA} \qquad (16)$$

Isocitritase was first shown to occur in Pseudomonas aeruginosa (Campbell, Smith and Eagles, 1953) and is present also in various mold species (Olson, 1954), in E. coli (Wong and Ajl, 1955) and in the castor beans (Kornberg and Beevers, 1957); the presence of malate synthetase in Pseudomonas (Kornberg and Madsen, 1957), in E. coli (Wong and Ajl, 1956) and in the castor beans (Kornberg and Beevers, 1957) has also been demonstrated. The metabolism of glyoxylic acid via the glyoxylate pathway in animal tissues is very unlikely for both the key enzymes are absent.

E. coli has also another enzyme system which catalyzes the following reaction (Krakow and Barkulis, 1956).

$$\begin{array}{l} \text{CHO} \\ | \\ \text{COOH} \end{array} + \begin{array}{l} \text{CHO} \\ | \\ \text{COOH} \end{array} \longrightarrow \begin{array}{l} \text{CH}_2\text{OH} \\ | \\ \text{CO} \\ | \\ \text{COOH} \end{array} + \text{CO}_2 \qquad (17)$$

Glyoxylic acid undergoes a dismutation in rabbit skeletal muscle under anaerobic conditions (Ratner et al. 1944).

$$\begin{array}{l}\text{CHO}\\|\\\text{COOH}\end{array} + \begin{array}{l}\text{CHO}\\|\\\text{COOH}\end{array} + H_2O \longrightarrow \begin{array}{l}CH_2OH\\|\\\text{COOH}\end{array} + \begin{array}{l}\text{COOH}\\|\\\text{COOH}\end{array} \qquad (18)$$

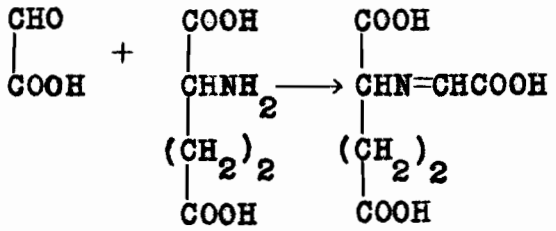
Xanthine oxidase oxidizes glyoxylic acid to oxalic acid (Ratner et al. 1944). Apparently pigeon liver contains a specific dehydrogenase which can catalyze this reaction (Nakada and Weinhouse, 1953b).

$$\begin{array}{l}\text{CHO}\\|\\\text{COOH}\end{array} + \tfrac{1}{2}O_2 \longrightarrow \begin{array}{l}\text{COOH}\\|\\\text{COOH}\end{array} \qquad (19)$$

Glycollic acid can be formed by the reduction of glyoxylic acid by the non-specific action of lactic dehydrogenase (Meister, 1952b; Nakada and Weinhouse, 1953b).

$$\begin{array}{l}\text{CHO}\\|\\\text{COOH}\end{array} + 2H \rightleftharpoons \begin{array}{l}CH_2OH\\|\\\text{COOH}\end{array} \qquad (20)$$

Nakada and Sund (1958) have demonstrated that rat liver mitochondrial extracts oxidatively decarboxylate glyoxylic acid. Purified preparations require specifically L-glutamic acid for maximal activity and N-formyl glutamic acid was identified by paper chromatography as a product of the reaction. The following scheme was proposed:



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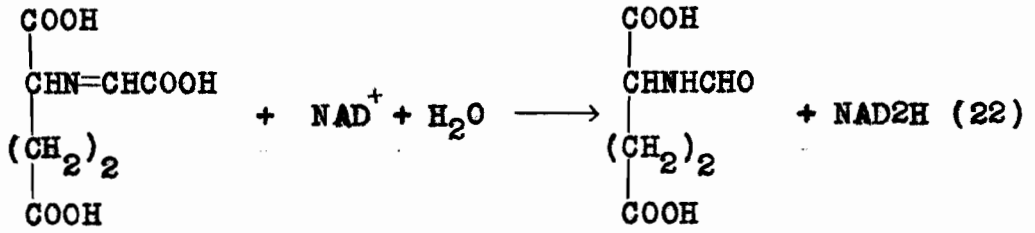
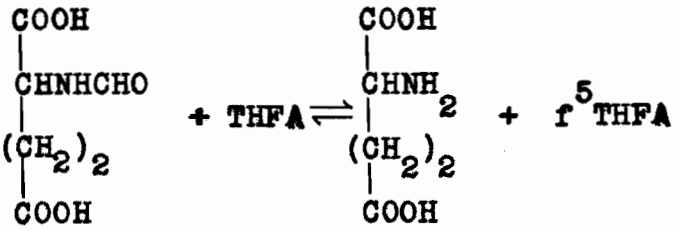


(22)

The N-formyl glutamic acid formed (Equation 22) could then be hydrolyzed to formic acid and L-glutamic acid or more probably formylates tetrahydro-folic acid (THFA) to form citrovorum factor (f^5THFA) (Silverman, Keresztesy, Koval and Gardiner, 1957) (Equation 23).



(23)

Rat liver mitochondria stored at -15°, have been shown recently to synergistically decarboxylate glyoxylic acid and L-glutamic acid (replaceable by α-ketoglutaric acid) (Crawhall and Watts, 1962). Though an absolute requirement for L-glutamic acid was shown for the glyoxylate decarboxylase, N-formyl glutamic acid was shown not to be a product of the reaction.

Glyoxylic acid has been shown to inhibit the respiration of tissue preparations (Kleinzeller, 1943). A complete inhibition of oxidation of citric acid by glyoxylic acid in presence of oxaloacetic acid has been reported to occur in rat liver homogenates (D'Abramo, Romano and Ruffo, 1957a; 1957b; 1958). Ruffo, Romano and Adinolfi (1959) have isolated a compound believed to be oxalomalic acid when glyoxylic acid and oxaloacetic acid were incubated in absence of any enzyme preparation and postulated it to be an inhibitor of the enzyme aconitase. Recent indications are that glyoxylic acid probably has a direct and nonspecific effect on the citric acid cycle enzyme systems (Ruffo, Adinolfi, Budillion and Capobianco, 1962).

Metabolism of formate.

The possible role of formic acid in animal metabolism has long been considered (Dakin, 1922), and recent indications on the formation and utilization of active one carbon fragments in a wide variety of metabolic processes show their great biochemical significance.

Among the potential sources of formic acid can be included (a) α-carbon atom of glycine (Sakami, 1948), (b) α and β-carbon atoms of serine (Arnstein, 1951), (c) α-carbon atoms of glycolic and glyoxylic acids

(Weinhouse and Friedman, 1952), (d) $-CH_3$ groups of methionine, choline, betaine and dimethyl aminoethanol (Stetten, 1941; Siekevitz and Greenberg, 1950; Soloway and Stetten, 1953), (e) $-CH_3$ groups of N-methyl glycines (Mackenzie, Sallach and Frisell, 1953), (f) α-carbon of threonine (Meltzer and Sprinson, 1950), (g) C-2 of tryptophan (Knox and Mehler, 1949), (h) one of the $-CH_3$ groups of acetone (Sakami, 1950a), (i) C-8 of purine rings (Rabinowitz and Pricer, 1956) and (j) C-2 of histidine (Miller and Waelsch, 1956).

Formic acid can be activated to N^{10} formyl tetrahydrofolic acid ($f^{10}THFA$) in the presence of ATP (Greenberg, 1954a; Greenberg, Jaenicke and Silverman, 1955). The reaction probably proceeds in two steps (Whiteley, Osborn and Hurennekens, 1958).

$$ATP + THFA \longrightarrow ADP + pTHFA \qquad (24)$$

$$pTHFA + HCOOH \longrightarrow f^{10}THFA + p_i \qquad (25)$$

f^5THFA, involved in the formylation of glutamic acid (Equation 23) can also be isomerized unidirectionally to $f^{10}THFA$ (Equation 26). The reaction is ATP dependent (Greenberg, 1954b).

$$f^5THFA + ATP \longrightarrow f^{10}THFA + ADP + p_i \qquad (26)$$

Methenyl-

Methylenetetrahydrofolic acid (f^{5-10}THFA) is formed from f^{10}THFA (Equation 27) by the enzyme cyclohydrolase (Rabinowitz and Pricer, 1956).

$$f^{10}\text{THFA} + H^+ \rightleftharpoons f^{5-10}\text{THFA} + H_2O \qquad (27)$$

The activated formate moiety (f^{10}THFA or f^{5-10}THFA) is incorporated into purines (C-2 and 8) by two different enzymes (Goldthwait and Greenberg, 1955). Apparently, 2-amino-N-ribosylacetamide-5-phosphate or glycinamide ribotide (GAR) transformylase (Equation 28) utilizes f^{5-10}THFA as a cofactor and 5-amino-1-ribosyl-4-imidazole-carboxamide-5-phosphate (AICAR) transformylase (Equation 29) utilizes f^{10}THFA (Hartman and Buchanan, 1959).

$$\text{GAR} + f^{5-10}\text{THFA} \longrightarrow \text{N-formyl-GAR} + \text{THFA} \qquad (28)$$

$$\text{AICAR} + f^{10}\text{THFA} \longrightarrow \text{N-formyl-AICAR} + \text{THFA} \qquad (29)$$

f^{10}THFA can be reduced to active formaldehyde, N^{5-10} methenyltetrahydro folic acid (h^{5-10}THFA) (Equation 30), by hydroxymethyl tetrahydrofolic acid dehydrogenase and NADP (Osborn and Huennekens, 1957).

$$f^{5-10}\text{THFA} + \text{NADP}^+ \rightleftharpoons h^{5-10}\text{THFA} + \text{NADPH} \qquad (30)$$

The de novo synthesis of h^{5-10}THFA from its components THFA and HCHO (Equation 31) has also been demonstrated (Osborn, Vercamer, Talbert and Huennekens, 1957).

$$THFA + HCHO \longrightarrow h^{5-10}THFA + H_2O \quad (31)$$

h^{5-10}THFA is the active hydroxymethyl donor in the synthesis of serine from glycine (Equation 32) by serine aldolase (Alexander and Greenberg, 1955) also called serine hydroxymethylase (Kisliuk and Sakami, 1955).

$$h^{5-10}THFA + H_2O + \text{glycine} \rightleftharpoons \text{L-serine} + THFA \quad (32)$$

Synthesis of the methyl groups of methionine (Nakao and Greenberg, 1955; Doctor, Trunnell and Awapara, 1956; Stevens and Sakami, 1958) and thymidine (Blakely, 1957b; Friedkin and Kornberg, 1957; Phear and Greenberg, 1957) also proceeds by hydroxymethylation with h^{5-10}THFA followed by reduction involving pyridine nucleotide (NAD^+ or $NADP^+$). The role of vitamin B_{12} in methyl group biosynthesis is probably related to this reduction step (Helleiner, Kisliuk and Woods, 1958; Kisliuk and Woods, 1958).

Enzymes capable of catalyzing the oxidation of formic acid had been reported, widely distributed in plant and animal tissues (Elliot, 1941). NAD^+ linked formic dehydrogenase is apparently specific for the plant kingdom (Mathews and Vennesland, 1950). Chance has shown that formic acid can be oxidized by a catalase-hydrogen peroxide complex (Chance, 1949; 1950). An ATP dependent formic oxidase has also been described by Mathews and Vennesland (1950) in animal tissues and the activity is very low in rat kidney

and liver. From a detailed study, Nakada and Weinhouse (1953a) concluded that the physiological oxidation of formic acid is mediated by catalase and hydrogen peroxide, at least in rat liver extracts.

Glycine-serine interconversion.

Knoop (1914) first proposed that serine and other hydroxy α-amino acids are converted to glycine by cleavage at the β-carbon atom. By feeding N^{15} labelled serine labelled with C^{13} in the carboxyl group together with benzoic acid to rats and guinea pigs and isolating the glycine of the hippuric acid excreted, Shemin (1946) demonstrated that the N^{15}-C^{13} ratio was essentially unaltered. The efficient utilization of L-serine for heme synthesis in ducks also suggests a rapid conversion of the two amino acids (Shemin, London and Rittenberg, 1950). Direct in vivo evidence for the interconversion of glycine and serine has also been obtained. Thus, labelled serine has been isolated after administration of labelled glycine (Sakami, 1949a; 1953; Goldworthy, Winnick and Greenberg, 1949; Elwyn and Sprinson, 1954) and labelled glycine after administration of labelled serine (Aqvist, 1951; Arnstein and Neuberger, 1953).

The one-carbon moiety needed for the synthesis of serine from glycine by serine aldolase (Equation 32) can originate from any one of the contributors to the one-carbon pool. Thus, it has been demonstrated that the β-carbon atom of serine can be labelled by formaldehyde (Siegel and Lafaye, 1950; Mitoma and Greenberg, 1952), formic acid (Sakami, 1948; Siekevitz and Greenberg, 1949; Kruhoffer, 1951; Mitoma and Greenberg, 1952), and the methyl groups of choline (Sakami, 1949b; Siekevitz and Greenberg, 1950), methionine (Siekevitz and Greenberg, 1950), acetone (Sakami, 1950b), dimethyl glycine (Mackenzie et al. 1953) and sarcosine (Mitoma and Greenberg, 1952; Mackenzie et al. 1953; Frisell, Meech and Mackenzie, 1954). The methylene carbon atom of glycine itself can be a precursor of the β-carbon atom of serine to give doubly labelled serine (Sakami, 1948; 1949a; Siekevitz et al. 1949; Siekevitz and Greenberg, 1949; 1950; Mitoma and Greenberg, 1952; Vohra, Lantz and Kratzner, 1956; White, 1958; Sanadi and Bennett, 1960; Richert, Amberg and Wilson, 1962). Apparently B_{12} is also involved in the incorporation of the α-carbon atom of glycine into the β-carbon atom of serine (Sanadi and Bennett, 1960).

Involvement of pyridoxal phosphate in the glycine-serine conversion was first indicated by the studies of Lascelles and Woods (1950). This cofactor requirement has been amply

confirmed with enzyme preparations (Alexander and Greenberg, 1955; Blakely, 1955; Jaenicke, 1956; Wright and Stadtman, 1956; Hatefi, Osborn, Kay and Huennekens, 1957). A mechanism based on the formation of a Schiff base followed by tautomeric rearrangements has been proposed by Metzler, Ikawa and Snell (1954) and is probably applicable to the enzyme catalyzed reaction.

The one carbon fragment involved in the serine synthesis is carried by THFA at the oxidation level of formaldehyde. Growth studies in bacteria (Holland and Meinke, 1949) and metabolic experiments in animals (Elwyn and Sprinson, 1950; Plaut, Betheil and Lardy, 1950; Totter, Kelley, Day and Edwards, 1950; Kelley, 1951) demonstrated the participation of folic acid in the interconversion of serine and glycine. The reaction catalyzed by serine aldolase had been studied in detail by many investigators in purified preparations of rabbit (Blakely, 1957a), pigeon (Kisliuk and Sakami, 1955; Jaenicke, 1956; Greenberg and Jaenicke, 1957), guinea pig, sheep (Alexander and Greenberg, 1955), rat (Alexander and Greenberg, 1955; 1956) and beef (Huennekens, Hatefi and Kay, 1957) livers and microorganisms (Wright, 1955; Wright and Stadtman, 1956). There is evidence suggesting the intermediary formation of a THFA-serine complex in the reaction (Equation 32), possibly catalyzed by separate enzymes (Jaenicke, 1956;

Kisliuk, 1956). The reaction (Equation 32) thus proceeds in two steps (Equations 33 and 34).

$$h^{5\text{-}10}\text{THFA} + \text{glycine} + H_2O \rightarrow \text{THFA-serine} \quad (33)$$

$$\text{THFA-serine} \rightarrow \text{Serine} + \text{THFA} \quad (34)$$

Metabolism of serine.

L-serine is not attacked by the L-amino acid oxidase of rat kidney (Blanchard et al. 1944; 1945). It is also a poor substrate for transaminase(s) (Cammarata and Cohen, 1950; Awapara and Seale, 1952). More recently, transamination of hydroxypyruvic acid by a specific transaminase for which only L-alanine can act as the amino group donor has been described in preparations of dog, rabbit (Sallach, 1955; 1956) and human liver (Hedrick and Sallack, 1960). Rat liver has also been shown to catalyze the transamination of hydroxypyruvic acid or 3-phosphohydroxypyruvic acid with glutamic acid (Ichihara and Greenberg, 1955; 1957; Ballou and Hesse, 1956).

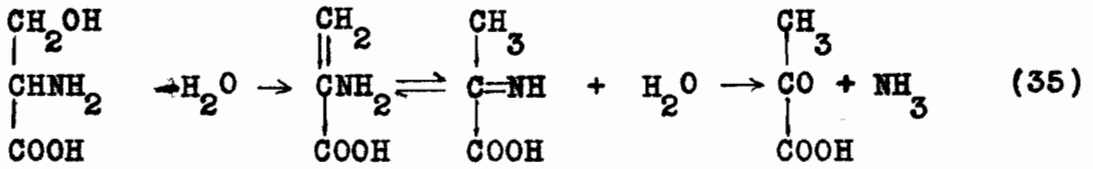
Whether these reactions are sufficiently reversible to effect significant deamination of serine for its further metabolism is doubtful. Hydroxypyruvic acid and 3-phosphohydroxypyruvic acid can be reduced to glyceric acid and phosphoglyceric acid as rapidly as pyruvic acid itself, by the nonspecific action of lactic dehydrogenase (Meister,

1952b; Ballou and Hesse, 1956; Ichihara and Greenberg, 1957). As a result, they can give rise to a distribution of the labelling of the glucose moiety of glycogen in a manner predictable by the reversal of the glycolytic reactions (Dickens and Williamson, 1959). However, the labelling pattern observed with L-serine is always more randomized and resembles more closely that obtained with pyruvic acid, suggesting the formation of a symmetrical four carbon intermediate before incorporation into glucose (Minthorn, Mourkides and Koeppe, 1959a; 1959b; Nadkarni, Friedmann and Weinhouse, 1960). Leibman and Fellner (1962) have provided direct evidence to show that the keto acid formed during the deamination of L-serine by rat liver homogenates is chiefly pyruvic acid (at least 90%).

On the other hand hydroxypyruvic acid seems to be the major metabolite of D-serine (Sprinson and Chargaff, 1946; Holzer and Holldorf, 1957). D-serine is incorporated into glucose more rapidly than L-serine and the labelling pattern is also similar to that observed with hydroxypyruvic acid (Elwyn, Ashmore, Cahill, Zottu, Welch and Hastings, 1957; Dickens and Williamson, 1959; Minthorn et al. 1959a; 1959b; Nadkarni et al. 1960). D-serine is a substrate for D-amino acid oxidase (Bernheim and Bernheim, 1935; Krebs, 1935; Klein and Handler, 1941) and apparently this is the

enzyme responsible for the formation of hydroxypyruvic acid rather than a transaminase.

Chargaff and Sprinson (1943) first suggested that the formation of pyruvic acid from L-serine involves dehydration (Equation 35).



(35)

The reaction catalyzed by serine dehydrase is irreversible; if the pyruvic acid is formed via reduction of hydroxypyruvic acid, the steps would be expected to be reversible and pyruvic acid should give rise to serine with the carbon chain intact. However, pyruvic acid-3-C^{14} injected to rats yields serine labelled equally in carbons 2 and 3 (Nyc and Zabin, 1955) suggesting that the serine-pyruvic acid system proceeds along different metabolic pathways when moving in different directions.

Glycine-aminoethanol cycle.

The oxidation of glycine could be effected by the following series of reactions:

Glycine→serine→aminoethanol→glycolaldehyde→glycollic acid→glyoxylic acid→glycine.

The formation of serine from glycine (Equation 32) had already been discussed. Stetten (1942) first demonstrated that N^{15} labelled serine is converted to N^{15} labelled aminoethanol by decarboxylation. Numerous groups of investigators have provided conclusive evidence for the conversion of serine to aminoethanol (see Levine and Tarver, 1950; Weissbach, Elwyn and Sprinson, 1950; Arnstein, 1951; Pilgeram, Sassenrath and Greenberg, 1953; Elwyn and Sprinson, 1954; Elwyn, Weissbach, Henry and Sprinson, 1955). It has also been shown that the formation of aminoethanol from glycine takes place only after these compounds are converted to serine and serine labelled in the 3 position with C^{14} and deuterium forms aminoethanol with little change in the C^{14}-H^{2} ratio (Elwyn et al. 1955). Although this excludes the possibility of reduction of glycine directly to aminoethanol, the mechanism of aminoethanol formation from serine still remains obscure.

The lack of formation of free aminoethanol from labelled serine (Nemer and Elwyn, 1960; Wilson, Gibson and Udenfriend, 1960) and the fact that the early workers isolated aminoethanol from phospholipids of the reaction mixture, suggest that the decarboxylation of serine actually represents interconversions of phospholipids. Turnover studies by Bremer, Figaurd and Greenberg (1960), indicate

neither phosphoaminoethanol nor cytidine phosphoaminoethanol are intermediates in the formation of phospholipid aminoethanol from serine. On the other hand, no intermediates are known in the conversion of serine to phosphatidyl serine (Dils and Hubscher, 1959). Recently, Borkenhagen, Kennedy and Fielding (1961) suggested the following mechanism for the decarboxylation of serine (equation 36 and 37).

L-serine + Phosphatidyl aminoethanol $\rightleftharpoons$ Phosphatidyl serine + aminoethanol (36)

Phosphatidyl serine $\rightarrow$ Phosphatidyl aminoethanol + CO_2 (37)

Phosphatidyl aminoethanol is formed by the following series of reactions (Equation 38) (Kennedy and Weiss, 1956).

Aminoethanol $\rightarrow$ phosphoaminoethanol $\rightarrow$ cytidine diphospho-aminoethanol $\rightarrow$ phosphatidyl aminoethanol. (38)

Though Weissbach and Sprinson (1953b) suggested that glycolaldehyde is a possible intermediate in the conversion of aminoethanol to glycine and it is possible to visualize the reaction catalyzed by an amine oxidase, it is not an active substrate for the presently known amine oxidases (Zeller, 1951; Blaschko and Duthie, 1945).

Glycolaldehyde can be oxidized to glycollic acid by the non-specific action of liver aldehyde oxidase (Gorden, Green and Subrahmanyan, 1940) or milk xanthine oxidase (Booth, 1938), and glycollic acid to glyoxylic acid by lactic dehydrogenase (Equation 20). Glyoxylic acid can be transaminated to glycine to complete the cycle. The fact that glycolaldehyde does not participate in a transketolase type of reaction but is converted to glycogen via the glycine-serine system (Friedmann, Levin and Weinhouse, 1956), provides additional support for the series of reactions, glycolaldehyde $\rightarrow$glycolic acid $\longrightarrow$glyoxylic acid $\rightarrow$glycine.

The only evidence available for the operation of the glycine-aminoethanol cycle is the result of Weissbach and Sprinson (1953b). With differentially labelled aminoethanol these workers were able to demonstrate that the amino carbon of aminoethanol is converted to the amino carbon of glycine of hippuric acid in the intact rat.

Succinate-glycine cycle.

Shemin and Rittenberg (1945; 1946) first reported that the nitrogen atoms of glycine appear in the heme of hemoglobin. It was also demonstrated that although the four nitrogen atoms of protoporphyrin are derived from glycine (Shemin and

Rittenberg, 1945; 1946; Wittenberg and Shemin, 1948; 1949; Muir and Neuberger, 1949), eight carbon atoms of the protoporphyrin molecule are also derived from the α-carbon atom of glycine (Muir and Neuberger, 1950; Radin, Rittenberg and Shemin, 1950; Wittenberg and Shemin, 1950). The carboxyl carbon atom of glycine is not at all utilized (Radin et al. 1950) and the remaining twenty-six carbon atoms of protoporphyrin originate from active-succinate (Shemin and Kumin, 1952).

During a study of this mechanism, Shemin and Russel (1953) proposed a pathway of glycine metabolism called the succinate-glycine cycle.

Glycine + succinyl-CoA $\longrightarrow$ α-amino-β-ketoadipic acid $\xrightarrow{CO_2}$ δ-aminolevulinic acid $\longrightarrow$ α-ketoglutaraldehyde $\longrightarrow$ succinic + acid + (HCHO).

It was postulated that active-succinate condenses with glycine to form α-amino-β-ketoadipic acid which loses carbon dioxide to form δ-aminolevulinic acid. The latter can then be oxidised to α-ketoglutaraldehyde and the aldehyde carbon atom of α-ketoglutaraldehyde converted to a one-carbon fragment or oxidised to carbon dioxide. The resultant molecule is succinic acid, which is reactivated and once more enters the cycle with another molecule of glycine.

Nutritional studies with pantothenic acid deficient ducks (Schulman and Richert, 1957) provided evidence to show that active-succinate is succinyl-CoA. Direct involvement of succinyl-CoA in the synthesis of δ-aminolevulinic acid has been demonstrated in a number of purified preparations (Brown, 1958; Gibson, Laver and Neuberger, 1958; Kikuchi, Kumar, Talmage and Shemin, 1958). The participation of pyridoxal phosphate in this reaction, presumably to activate the methylene carbon atom of glycine, has also been demonstrated (Schulman and Richert, 1957; Brown, 1958; Kikuchi et al. 1958).

Intraperitoneal injection of the diethyl ester of α-amino-β-ketoadipic acid into a rat has led to the urinary excretion of porphobilinogen, indicating that the theoretically expected α-amino-β-ketoadipic acid is indeed the condensation product formed from succinyl-CoA and glycine and an obligatory intermediate in porphyrin synthesis (Weliky and Shemin, 1955; 1957). The formation of α-amino-β-ketoadipic acid itself has not been demonstrated, and Laver, Neuberger and Scott (1958) have observed that it spontaneously loses carbon dioxide rapidly under physiological conditions.

It has been shown that δ-aminolevulinic acid decreases the extent of incorporation of radioactivity from succinic acid or glycine into porphyrin (Shemin and Russell, 1953;

Shemin, Russel and Abramsky, 1955). It has also been shown that δ-aminolevulinic acid is a more active precursor of the porphyrin molecule than is glycine or succinic acid (Shemin, Abramsky and Russell, 1954); and that all the carbon atoms of porphyrin originate from δ-aminolevulinic acid (Schiffman and Shemin, 1957). These findings have also been confirmed by reports from other laboratories (Dresel and Falk, 1953; Neuberger and Scott, 1953). Net synthesis of δ-aminolevulinic acid has also been shown to occur in purified preparations (Brown, 1958; Laver et al. 1958).

δ-aminolevulinic acid-5-C^{14} has been shown to be a more efficient precursor than glycine-2-C^{14} in labelling guanine and formic acid in intact ducks and rats, respectively. The labelling pattern of uric acid isolated from a pigeon injected with δ-aminolevulinic acid-5-C^{14} is similar to that of glycine-2-C^{14}. The radioactivity found in urinary formic acid and exhaled carbon dioxide is of the same magnitude when α-ketoglutaraldehyde-5-C^{14} or glycine-2-C^{14} is injected into rat (Nemeth, Russell, and Shemin, 1957). δ-aminolevulinic acid-5-C^{14} can also label the β-carbon atom of serine (Gatt and Shemin, 1955) and the methyl group of methionine (Foster and Shemin, 1955). Thus it is seen that the metabolic pattern obtained with δ-aminolevulinic acid-5-C^{14} or α-ketoglutaraldehyde-5-C^{14} resembles that of glycine-2-C^{14},

suggesting the possibility that the succinate-glycine system is a mechanism by which the α-carbon atom of glycine can be detached from the carboxyl group.

The conversion of δ-aminolevulinic acid to α-keto-glutaraldehyde seems to be irreversible, since neither hemolyzed duck erythrocytes nor intact pigeons can utilize α-ketoglutaraldehyde for heme synthesis. δ-aminolevulinic acid-1,4-C^{14} injected together with malonic acid leads to the excretion of succinic acid-1,4-C^{14} in the rat, showing that the postulated reactions are of a cyclic nature (Nemeth et al. 1957).

The ability of δ-aminolevulinic acid or α-ketoglutaraldehyde to contribute to the one carbon pool or to be converted to succinic acid has not been demonstrated in vitro.

<u>Acetate-glycine cycle</u>.

A cycle similar to the succinate-glycine cycle of Shemin has been proposed by Urata and Granick (1961) for the metabolism of glycine in which acetyl CoA replaces succinyl CoA.

glycine + Acetyl CoA $\rightarrow$ α-aminoacetoacetic acid $\rightarrow$ amino acetone $\rightarrow$ methyl glyoxal $\rightarrow$ acetic acid + (HCHO).

α-aminoacetoacetic acid formed by the condensation of acetyl CoA and glycine, upon decarboxylation would yield amino acetone which could be converted to methyl glyoxal by the action of amino oxidase. Methyl glyoxal could either be converted to acetyl CoA and carbon dioxide via lactic and pyruvic acids or could be hydrolytically cleaved to acetic acid and a one carbon fragment (HCHO).

Shemin (1955a; 1955b) first suggested that active-acetate may compete with active-succinate for condensation with glycine. Later it was shown that δ-aminolevulinic acid synthetase would also catalyse the condensation of acetyl CoA and glycine and amino acetone was identified as a product of this reaction (Gibson et al, 1958; Kikuchi, Kumar and Shemin, 1959). Guinea pig liver mitochondria had been shown to form small amounts of aminoketones (unidentified) when incubated with glycine and a number of α-keto acids (Urata and Granick, 1961).

It had been observed that $CH_3COC^{14}H_2NH_2$ is converted to carbon dioxide, formic acid and ureido groups of uric acid more readily than glycine-2-C^{14} (Shemin, 1955a; 1955b; Nemeth et al. 1957). The oxidation of amino acetone to methyl glyoxal in ox plasma had been demonstrated (Elliot, 1960). Methyl glyoxal could readily be converted to pyruvic

acid via D-and L-lactic acids by the actions of glyoxalase (Neuberg, 1913a; 1913b; Dakin and Dudley, 1913a; 1913b), lactic racemase (Huennekens, Mahler and Nordmann, 1951) and lactic dehydrogenase. Oxidative decarboxylation of pyruvic acid to acetyl CoA would complete the cycle with the concommitant oxidation of one molecule of glycine.

MATERIALS AND METHODS.

Animals.

Hooded male rats of a local inbred strain, weighing approximately 150 to 200 grams were used. The animals had free access to food and water till they were sacrificed. White Rock female chicken were bought from the market as required.

Chemicals.

All common chemicals were of reagent quality and used without further purification. The following radioactive chemicals used in the investigations were obtained from commerical sources as shown:

Glycine-1-C^{14}	Picker X-Ray and Engineering Montreal.
Glycine-2-C^{14}	Picker X-Ray and Engineering Montreal.
DL-serine-3-C^{14}	Radiochemical Centre, Amersham.
L-serine-U-C^{14}	Radiochemical Centre, Amersham.
Succinic acid-2,3-C^{14}	Radiochemical Centre, Amersham.
Decanoic acid-1-C^{14}	Merck and Co., Montreal.

Glycine-1-C^{14}, DL-serine-U-C^{14} and succinic acid-2,3-C^{14}, used as reference compounds in chromatograms, moved as single spots.

Preparation of solutions.

All solutions were made up at 5 or 10 times higher concentrations than the final concentrations required and pH adjusted to approximately 7.4, whenever necessary. Solutions of organic compounds were usually stored at -20°C and others at +5°C. Radioactive solutions were prepared by dissolving the radioactive compounds with the required amount of carriers.

Tissue preparations.

(a) Rat brain cortex slices.

The rats were killed by vertebral dislocation (achieved by a sharp blow on the back of the animal with the handle of a hammer), decapitated, the brain removed quickly and dropped into ice-cold Krebs Ringer salt solution contained in a beaker surrounded by cracked ice. Slices were cut from the cerebral hemispheres using a Stadie-Riggs tissue slicer, and only the first slices were used. Two slices from the same brain were weighed quickly on a torsion balance ($\pm$1 mg) and pushed into the incubation medium contained in Warburg vessels kept in crushed ice.

(b) Rat kidney cortex slices.

Rats were stunned and killed instantly by decapitation, the kidneys removed and dropped into cracked ice. Slices were prepared as described for brain. The first slice was

rejected and the next two or three slices were used, care being taken not to cut into the medulla. The slices were randomised before weighing.

(c) Rat liver slices.

Rats were stunned, killed instantly by decapitation, and blood drained. Liver was removed, rinsed by passing it through running cold water tap, and dropped into a beaker containing cracked ice and water. Slices were prepared as described before from a small piece of liver, obtaining all the pieces required from the same piece.

(d) Rat liver homogenates.

Homogenates of liver were prepared by passing the tissue through a tissue press and throughly homogenising, at high speed, for 3-4 minutes, so as to break up as many intracellular components as possible. Krebs-Ringer solution was used as medium.

(e) Rat liver mitochondria.

Liver mitochondria were isolated essentially according to the method of Schneider (1948). Liver was removed from the animal as described above, kept in cracked ice and water for one minute to cool it completely. Connective tissue was removed by passing the tissue through a precooled tissue press, collecting the exudate in a tared beaker and the weight of the tissue noted. The calculated amount of ice-cold 0.25M sucrose solution was added to give a 20% suspension and a homogenate prepared in a Potter-Elvehjem homogenizer and a Teflon

pestle kept in crushed ice. The coarse particles were broken up by passing the pestle through the suspension kept in the homogenizer, once by plunging the pestle through the suspension with the hand, and twice with the motor at low speed. Cell nuclei and debris were removed by spinning the homogenate for 10 minutes at 600 g using a PR_2 International Centrifuge with high speed attachment. Mitochondria were sedimented from the supernatant by centrifuging for 10 minutes at 8000 g and resuspended once in 0.25 M sucrose solution and spun down again at the same speed. The mitochondrial pellet was suspended in a known volume of 0.25 M sucrose (when a 3 ml incubation mixture was used) or in the mitochondrial media described below (for use with 1.5 ml incubation mixture). All operations were carried out in the cold.

(f) Chicken erythrocytes.

Chicken were bled by opening the jugular vein and the blood was collected in a small beaker containing a little heparin as anticoagulant. Plasma and the white cells were removed by centrifugation and the red cells were washed once with isotonic phosphate buffer at pH 7.4, using a PR_2 International Centrifuge with the high speed attachment.

Incubation methods.

The Warburg apparatus and conventional incubation techniques were used. The incubation medium used with rat brain and kidney cortex slices, rat liver slices and homogenates was Krebs Ringer salt solution of the composition noted below with sodium phosphate buffer added separately

NaCl, 145 mM; $CaCl_2$, 3.1 mM; KCl, 5.8 mM; KH_2PO_4, 1.5 mM; $MgSO_4$, 1.5 mM; sodium phosphate buffer pH 7.4, 10 mM.

The incubation media used with rat liver mitochondria was essentially that described by Judah and Rees (1953). It contained

$MgSO_4$, 6.7 mM; KCl, 33 mM; potassium adenylate (muscle), 1.0 mM; cytochrome c, 167 µg/ml; and sodium phosphate buffer pH 7.4, 10 mM.

Chicken erythrocytes were incubated in isotonic sodium phosphate buffer pH 7.4 (100 mM).

All incubations were carried out in the Warburg apparatus, at 37°C, in an atmosphere of oxygen when rat brain and kidney cortex slices and rat liver slices were used and in an atmosphere of air when rat liver homogenates and mitochondria and chicken erythrocytes were used.

Assay of $C^{14}O_2$

The carbon dioxide formed during the reaction was trapped in KOH soaked filter papers kept in the centre wells of the Warburg vessels. The reaction was stopped by tipping 0.2 ml of a 30% trichloroacetic acid (TCA) and incubation continued for 10 more minutes to release any carbon dioxide trapped in the medium. When TCA interfered with other assays needed of the medium, the reaction was stopped by plunging the vessels in cracked ice and kept the vessels stoppered till the filter papers were removed. The filter papers were removed from the centre wells and dropped into centrifuge tubes containing a known amount of 1.34% sodium carbonate as carrier (usually 0.3 ml) to give a final weight of 10-12 mg of $BaCO_3$. The centre wells were washed four times with fresh distilled water (CO_2 free) and the washings added to the tubes. The tubes were tightly stoppered and left overnight to elute the carbonate adsorbed on the filter papers. On the next day, the papers were removed, rinsed with distilled water, squeezed out and discarded. 0.2 ml of 2 M ammonium chloride and 0.5 ml of 20% barium chloride were added to each tube. The precipitated barium carbonate was washed once with distilled water, once with acetone in a centrifuge and resuspended in

0.3 ml acetone. The suspension was transferred quantitatively to tared aluminium planchets, using one more 0.3 ml aliquot of acetone to rinse out the last traces of barium carbonate, from the centrifuge tubes. The planchets were dried, reweighed and counted.

Isolation and estimation of hemin.

The procedure of Anson and Mirsky (1930) was used with little modification. After incubation, the reaction was stopped by cooling the vessels in ice, the contents of the vessels transferred to centrifuge tubes, the vessels rinsed out twice with ice cold isotonic phosphate buffer, and the washings transferred to the centrifuge tubes. The red blood cells were centrifuged down in the cold, washed once with ice cold isotonic phosphate buffer in the centrifuge and lysed by adding 1.5 ml distilled water and shaking thoroughly. The lysate was added dropwise, with constant stirring, to ten volumes of acetone containing 1.2% concentrated hydrochloric acid. The hemin solution was filtered and the precipitated proteins were washed once with 10 ml acetone-HCl solution. The total volume of the hemin solution was noted, an aliquot taken and hemin estimated by measuring optical density at 510 mμ and comparing to an appropriate dilution of standard hemin solution made up in acetone-HCl. An aliquot of the

standard solution containing 10 mg of hemin was mixed with a measured volume of the experimental hemin solution and the mixed hemin precipitated by adding an equal volume of water. The precipitated hemin was filtered, dried and dissolved in the minimum amount of pyridine and chloroform (3:1) and crystallized from glacial acetic acid. The yield was usually 30 to 40%. No recrystallization was carried out, but hemin crystallized by this procedure has been reported to have a constant specific activity upon repeated recrystallization (Kassenaar, Morell and London, 1957). The isolated hemin was dissolved in the minimum volume of pyridine, precipitated by adding water, and the suspension plated on weighed aluminium planchets, dried, weighed and counted.

Chromatography of the amino acids.

The reaction was stopped by plunging the vessels into crushed ice and the medium transferred to centrifuge tubes. The vessels were rinsed twice with 3 ml of distilled water, and the washings were added to the centrifuge tubes. The proteins were precipitated by heating the centrifuge tubes in a boiling water-bath, and centrifuged down. The supernatant was added to Dowex-50 X 12 resin (H^+ form, 200-400 mesh) contained in 0.75 cm diameter micro funnels fitted with sintered glass filters, packed to 1.0 cm height.

The protein precipitate was washed once with distilled water and the washings added to the column. The resin was washed thoroughly with 5 X 2 ml aliquots of distilled water and eluted with 10 ml of 2 N ammonium hydroxide. The eluate was dried under vacuum in a dessicator over concentrated sulfuric acid and redissolved in 0.6 ml of 50% ethanol-water and 0.1 ml spotted on the chromatogram and 0.03 ml plated on a planchet for counting.

Spots of standard radioactive compounds were also spotted together with the experimental spots and the chromatogram run with methylethyl ketone, tertiary butanol, ammonia and water (50:50:15:25) in a single dimension. The chromatograms were run for 24 hours, the solvent being allowed to drip through the serrated end of the chromatogram. Radioactive spots were identified by leaving the chromatograms in close contact with Kodak No-Screen X-Ray films, the radioactive spots marked with pencil and counted with a Geiger-Müller tube, shielding other areas of the chromatogram with lead sheets. The radioactive spots corresponded to ninhydrin positive spots.

Isolation of glyoxylic acid.

The filtrate from the Dowex-50 columns was neutralized and added to a similar column packed with Amberlite-IR-400 (OH form). The column was washed with 10 column volumes of distilled water and the adsorbed acids eluted with 20 column volumes of 2 M ammonium carbonate. The eluate was concentrated in a vaccum desicator, redissolved in 0.6 ml distilled water and 0.05 ml spotted on the paper. The chromatogram was run with ethanol, water and concentrated ammonia (50: 45: 5). The radioactive spots were identified as for amino acid chromatograms. Reference spots of glyoxylic acid were run side by side and were identified by spraying with bromcresol blue indicator which gives a yellow colour for the acid spots.

Counting techniques.

Appropriate dilutions of the standard radioactive solutions were made and counted under identical conditions along with experimental planchets and served as a check on the counter. Formic acid-C^{14} standard planchets were counted along with 0.1 ml. of 1% sodium carbonate solution to prevent the formate from volatilizing. Background was also counted daily and subtracted from the counts of the samples. All samples were counted for at least 2,500 counts

to give a statistical counting error of less than ± 2%.

Barium carbonate planchets (approximately 2 mg per cm^2) were corrected to infinitely thin layers with no absorption with the use of an absorption curve prepared for the counter used. The curve was essentially similar to that obtained by Calvin, Heidelberger, Reid, Tolbert and Yankwich (1949).

Standard spots run on the paper chromatograms served to estimate the self-absorption of the chromatographic paper. The self-absorption varied between 80 and 75%. The recovery of the counts put on the origin (counted on both sides of paper and average used for calculation) to that of the total obtained by adding the radioactive spots varied between 75 to 110%.

Paper chromatograms were counted with an end window Geiger-Müller tube attached to a Nuclear-Chicago scaler. Hemin samples were counted with a windowless flow counter (Nuclear-Chicago). All other countings were performed in a thin window gas flow counter attached to a Baird Atomic 123 scaler.

Representation of results

All Q values reported in this Thesis represent µl of oxygen consumed per 100 mg wet weight tissue per hour

in the case of slices, homogenates and chicken erythrocytes and µl of oxygen consumed per hour by mitochondria obtained from 1 g of fresh rat liver, unless otherwise specified. $C^{14}O_2$ values represent mµmoles of carbon dioxide produced from the labelled carbon(s) under the conditions specified for Q values. Other values are as described in the text or tables.

The data are averages of duplicates of a typical experiment, usually the experiments repeated more than once. Results obtained with mitochondria showed a variation of less than $\pm$ 3% and with other tissues $\pm$ 10% between duplicates. Day to day variation was as high as 50% with mitochondria, and usually less with the slices.

C H A P T E R I

OXIDATION OF GLYCINE IN VARIOUS TISSUES.

Introduction.

Nakada and Weinhouse (1953a) determined the rate of oxidation of glycine in various tissues of the rat. A preliminary study of a similar nature was conducted in the present investigation, with the aim of selecting an adequate system in which to study the oxidation of glycine.

Oxidation of glycine by different tissues.

The rates of oxidation of glycine by rat brain and kidney cortex slices, rat liver slices and homogenates and chicken erythrocytes are shown in Table I. It may be seen from the Table that various tissues have widely different capacities to oxidize glycine. For instance, kidney cortex slices convert glycine-1-C^{14} to $C^{14}O_2$ at a rate which is about a thousand times greater than that observed with chicken erythrocytes. A rough parallelism also exists between the ability of these tissues to consume oxygen and their ability to oxidize glycine.

Further, all the tissues examined, except liver, oxidize glycine-1-C^{14} approximately ten times faster than they oxidize glycine-2-C^{14}. In the case of liver, the ratio is approximately two.

T A B L E I.

OXIDATION OF GLYCINE BY DIFFERENT TISSUE PREPARATIONS.

Tissue Preparations	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Rat Brain Cortex Slices	214	72	208	4.8
Rat Kidney Cortex Slices	344	928	340	98.5
Rat Liver Slices	164	64	162	36.0
Rat Liver Homogenates	20	42	17	6.1
Chicken Erythrocytes	8.4	0.98	6.9	0.12

Q - µl. of oxygen consumed per 100 mg. wet weight tissue per hour.

$C^{14}O_2$ - mµmoles of $C^{14}O_2$ produced per 100 mg. wet weight tissue per hour.

2 mM. labelled substrate (with 10 mM. glucose in the case of brain cortex slices) was incubated in a total volume of 3 ml. for 60 minutes, but in the case of chicken erythrocytes where the incubation period was 240 minutes, under the standard incubation conditions.

The effects of other substrates on the rate of oxidation of glycine-1-C^{14} by chicken erythrocytes.

The rate of oxidation of glycine by chicken erythrocytes is extremely low (Table I). The effects of other substrates were investigated with the aim of selecting one which increases the metabolic activity of the chicken erythrocytes and thereby increases the oxidation of glycine. A limited number of these exogenous substrates increase the respiratory activity of these cells, but none affected the production of $C^{14}O_2$ from glycine-1-C^{14} (Table II).

The effects of different metabolites on the rate of oxidation and incorporation of glycine-2-C^{14} into hemin by chicken erythrocytes.

The oxidation and incorporation of the labelled carbon atom into hemin was investigated, using high specific activity glycine-2-C^{14} (115 cpm/mμmole). The oxidation of glycine-2-C^{14} is not affected significantly on the addition of glucose, α-ketoglutarate, formate or δ-aminolevulinate (Table III). δ-aminolevulinate, but not the other metabolites, decreases the specific activity of the hemin isolated from the incubation medium by more than 50%. In none of the other systems tested (rat brain and kidney cortex slices and rat liver slices, homogenates and mitochondria) was there an inhibition

T A B L E II.

EFFECT OF CO-SUBSTRATES ON THE OXIDATION OF GLYCINE-1-C^{14} BY CHICKEN ERYTHROCYTES.

Additions (5 mM)	Q	$C^{14}O_2$	Additions (5 mM)	Q	$C^{14}O_2$
Nil	5.8	0.83	Nil	5.2	0.70
Glucose	5.4	0.89	Adenosine	7.7	0.69
Ribose	5.3	0.76	Guanosine	5.9	0.67
Pyruvic acid	6.0	0.74	Cytidine	4.9	0.65
Oxaloacetic acid	6.3	0.87	Inosine	6.2	0.64
α-Ketoglutaric acid	5.6	0.87	Adenylic acid (Muscle)	6.0	0.68

Q and $C^{14}O_2$ values as described in Table I.

1 ml packed chicken erythrocytes were incubated with 2 mM glycine-1-C^{14} with additions as shown above in a total volume of 3 ml, for 240 minutes under the standard incubation conditions.

TABLE III

EFFECTS OF GLUCOSE, α-KETOGLUTARIC ACID, FORMIC ACID AND δ-AMINOLEVULINIC ACID ON THE OXIDATION AND INCORPORATION OF GLYCINE-2-C^{14} INTO HEMIN BY CHICKEN ERYTHROCYTES.

Additions	Q	$C^{14}O_2$	HEMIN	
			Total (mg)	Specific activity (cpm mg)
Nil	6.9	0.12	4.50	279
Glucose (5mM)	6.7	0.10	4.95	273
α-Ketoglutaric acid (2mM)	6.6	0.10	5.10	305
Formic acid	6.4	0¢12	4.52	317
δ-aminolevulinic acid	6.5	0.12	4.85	129

Q and $C^{14}O_2$ values as described in Table I.

1 ml of packed chicken erythrocytes were incubated with 2mM glycine-2-C^{14} with additions as shown above, in a total volume of 3ml, for 240 minutes under the standard incubation conditions.

of glycine-2-C^{14} oxidation by δ-aminolevulinic acid.

Attempts to demonstrate the incorporation of the glycine molecule into the nucleosides or nucleotides as measured by the radioactivity adsorbed on Norite, in the presence or absence of glucose, formate, succinate or glutamine added singly or together, were unsuccessful in chicken erythrocytes. This is consistent with the observation of Bishop (1960) that none of the chicken blood systems carries out a de novo synthesis of purines.

The effects of other substrates on the rate of oxidation of glycine-2-C^{14} by rat brain cortex slices.

The effects of pyruvic, succinic, L-glutamic and L-aspartic acids, in the presence and in the absence of glucose, on the rate of oxidation of glycine-2-C^{14} by rat brain cortex slices are shown in Table IV. Glucose (10 mM) increases the rate of oxidation of glycine-2-C^{14} at least twofold and a parallel increase in the oxygen consumption is also observed. None of the other substrates tried is able to stimulate the oxidation of glycine-2-C^{14}. Pyruvic, succinic, L-glutamic and L-aspartic acids stimulate the respiration of rat brain cortex slices in the presence of glucose. However, these substrates inhibit the oxidation of glycine-2-C^{14}.

TABLE IV

EFFECTS OF CO-SUBSTRATES ON THE OXIDATION OF GLYCINE-2-C^{14} BY RAT BRAIN CORTEX SLICES

Other additions	Pyruvate (mM)		Succinate (mM)			L-glutamate (mM)			L-aspartate (mM)		
	Nil	2.5	Nil	1.5	10.0	Nil	1.5	10.0	Nil	1.5	5.0
Nil											
Q	23.3	56.0	29.5	39.3	57.0	28.5	35.5	42.5	23.3	24.5	22.0
$C^{14}O_2$	2.45	2.10	3.55	3.10	4.60	2.60	2.05	1.65	2.55	1.75	1.70
Glucose (10mM)											
Q	54.0	58.0	57.5	69.5	78.5	54.0	62.0	60.5	56.0	55.5	59.5
$C^{14}O_2$	4.95	2.35	7.95	7.05	5.25	5.95	2.75	1.40	5.05	2.80	2.15

Q and $C^{14}O_2$ values as described for Table I.

Rat brain cortex slices (ca. 70mg) were incubated with 2mM glycine-2-C^{14} and additions as shown above, in a total volume of 3ml for 60 minutes under the standard incubation conditions.

The effects of L-serine on the rate of oxidation of glycine by rat liver slices and homogenates.

Neither the respiration nor the production of $C^{14}O_2$ from glycine-1-C^{14} or glycine-2-C^{14} by rat liver slices is affected on addition of 10 mM L-serine (Table V). However, the production of $C^{14}O_2$ from glycine-1-C^{14} by rat liver homogenates is decreased by approximately 50% on the addition of 10 mM L-serine. Rat liver homogenates used in these experiments were prepared by homogenizing fresh rat liver in a Potter-Elvehjem homogenizer at high speed for 3-4 minutes in such a way as to break up nuclei and mitochondria. Most of the respiratory activity, the glycine-2-C^{14}-oxidase activity and a considerable portion of the glycine-1-C^{14}-oxidase activity are lost during this procedure.

Localization of the glycine-oxidase activity of rat liver.

An attempt was made to localize the glycine-oxidase activity associated with rat liver. A 20% fresh rat liver homogenate was prepared in 0.25 mM sucrose and fractionated to yield nuclei, mitochondria and a supernatant containing the soluble and the microsomal fractions, by differential centrifugation (details given in the section, Materials and Methods). More than 90% of the glycine-1-C^{14}-oxidase activity and all the glycine-2-C^{14}-oxidase activity resided

TABLE V

EFFECT OF L-SERINE ON THE OXIDATION OF GLYCINE BY RAT LIVER SLICES AND HOMOGENATES.

L-serine (mM)	LIVER SLICES				LIVER HOMOGENATES			
	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}		GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	41.0	64	40.5	36	5.0	42.0	4.1	6.1
10.0	38.5	76	42.0	25	6.1	21.2	5.2	5.2

Q and $C^{14}O_2$ values as described in Table I.

Rat liver slices (ca. 150mg) or rat liver homogenates (200mg equivalent of fresh wet tissue) were incubated with 2mM labelled glycine with addition of L-serine as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

in the mitochondrial fraction. The supernatant fraction exhibited a small amount of glycine-1-C^{14}-oxidase activity and the nuclear fraction had a small amount of glycine-2-C^{14}-oxidase activity. Addition of the nuclear or the soluble fraction did not alter the oxidation of glycine by the mitochondrial fraction significantly.

Discussion

A survey of the rates of oxidation of glycine by various tissues indicates that a rough parallelism exists between the metabolic activity of the system, as measured by the oxygen uptake, and the glycine-oxidase activity. The conversion of the carboxyl-carbon atom of glycine to carbon dioxide takes place 5-10 times faster than the oxidation of the methylene carbon atom of glycine.

Though the oxidation of glycine by chicken erythrocytes is very small, attempts were made to investigate the possible operation of the succinate-glycine cycle in this system. The oxidation of the methylene carbon atom of glycine is not affected by the addition of δ-aminolevulinic acid. δ-aminolevulinic acid is an obligatory intermediate in the oxidation of the methylene carbon atom of glycine via the succinate-glycine cycle (see General Introduction). The failure of δ-aminolevulinic acid to decrease the oxidation of the methylene carbon atom of glycine indicates that the

succinate-glycine cycle does not participate in the oxidation of glycine in chicken erythrocytes to a significant extent. δ-aminolevulinic acid causes a significant drop in the radioactivity of glycine-2-C^{14} incorporated into hemin, showing that the added δ-aminolevulinic acid is in equilibrium with the metabolic pool of δ-aminolevulinic acid. On the basis of similar results obtained in all the other systems employed in the present investigations, it was concluded that the succinate-glycine cycle is not a major pathway for the oxidation of glycine.

The oxidation of the methylene carbon atom of glycine by rat brain cortex slices is decreased on the addition of pyruvic, succinic, L-glutamic and L-aspartic acids, in spite of a considerable increase in the oxygen consumption. Though it is reasonable to assume that these metabolites have a direct effect on the oxidation of glycine-2-C^{14}, the possibility that they affect the active transport of glycine cannot be ignored. It has been shown by Sved (1958) that the oxidation of glycine in rat brain cortex slices is dependent upon the concentration of glycine inside the cell, and Abadom and Scholefield (1962) have shown that these metabolites cause a decrease in the concentrative uptake of glycine by rat brain cortex slices.

Experiments conducted with rat liver slices and homogenates

indicate that L-serine affects the oxidation of glycine only in the homogenates. It may be that L-serine does not enter the cells at a sufficiently rapid rate to affect the oxidation of glycine by the slices.

These results indicate two drawbacks in using slices for studying the oxidation of glycine. (a) The ability of an added metabolite to influence the transport of the labelled substrate and hence cause an effect which is not related to the metabolic reactions involved. (b) The inability of an added metabolite to influence the metabolism of the labelled substrate because of the selective permeability of the cell-membrane. In order to obviate such difficulties, it was decided to localize the glycine-oxidase activity of rat liver. Rat liver was chosen because of its ability to oxidize both carbon atoms of glycine at a rapid rate.

Fractionation of the intracellular components of rat liver cells by differential centrifugation, shows that mitochondria possess most of the glycine-oxidase activity. The small amount of activity observed with the nuclear fraction could be due to contamination by mitochondria and the activity observed with the supernatant fraction could be due to the enzymes escaped out of the damaged mitochondria. No further attempts were made to solubilize or concentrate the enzyme(s) involved, for damaging the mitochondria led to loss of activity (Table V).

Summary.

(1) Different tissue systems have widely different capacities to oxidize glycine. All of them produce carbon dioxide from the carboxyl carbon 5-10 times faster than from the methylene carbon atom of glycine.

(2) δ-aminolevulinic acid decreases the radioactivity of glycine-2-C^{14}, incorporated into hemin in chicken erythrocytes, though the oxidation of the labelled carbon atom is not affected.

(3) The enzymes involved in the oxidation of glycine by rat liver are located in the mitochondrial particles.

C H A P T E R II

THE ROLE OF GLYOXYLIC ACID IN THE CATABOLISM OF GLYCINE

Introduction

The discovery of glycine oxidase in rat kidney preparations by Ratner et al. (1944) followed by the demonstration that rat liver preparations catalyze transamination reactions between glyoxylic acid and amino acid amides (Mardashev and Semina, 1950; Meister et al. 1952; Nakada and Weinhouse, 1953a; Meister, 1954a; Meister, 1954b; Meister et al. 1954) has implicated glyoxylic acid as an intermediate in the catabolism of glycine

Nakada and Weinhouse (1953b) have shown that the rate of oxidation of glycine-2-C^{14} by washed rat liver homogenates is reduced on addition of non-labelled glyoxylic acid and they also isolated glyoxylic acid-2-C^{14} from the reaction mixture. Nakada and Sund (1958) prepared rat liver mitochondrial extracts which oxidatively decarboxylate glyoxylic acid via the intermediate formation of N-glyoxyl and N-formyl glutamic acids (Equations 21 and 22). Formate was visualized as being oxidized by the peroxidative function of catalase (Nakada and Weinhouse, 1953b).

In view of the significant role glyoxylic acid plays in the terminal oxidations, at least in the microbial metabolism,

it was of interest to investigate the formation of glyoxylic acid from glycine.

Effect of glyoxylic acid on the oxidation of glycine by rat brain cortex slices.

The effects of varying concentrations of glyoxylic acid on the rate of oxidation of glycine by rat brain cortex slices are shown in Table VI. A definite lowering of the rate of oxidation of glycine occurs on the addition of glyoxylic acid. However, a simultaneous decrease in the oxygen consumption is also noticed when glyoxylic acid is present in the incubation mixture. Since previous studies have indicated the possible relation between the metabolic activity and glycine-oxidase activity (Table I), it was suspected that the effect of glyoxylic acid on the rate of glycine oxidation is a secondary effect caused by the decreased respiration of brain cortex slices. This proved to be true since the rate of oxidation of glycine, when represented in terms of oxygen consumed under similar conditions, was more or less same, with a slight tendency to increase.

Effect of glyoxylic acid on the oxidation of glycine by rat liver mitochondria.

Previous workers have presented evidence for the operation of the glyoxylate pathway of glycine oxidation in rat liver preparations (Nakada and Weinhouse, 1953a; 1953b; Nakada and Sund, 1958; Crawhall and Watts, 1962). Since the glycine-

T A B L E VI

EFFECT OF GLYOXYLATE ON THE OXIDATION OF GLYCINE BY RAT BRAIN CORTEX SLICES

Sodium glyoxylate (mM)	GLYCINE-1-C^{14}			GLYCINE-2-C^{14}		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
Nil	55.5	69	1.2	59.5	4.9	0.08
0.25	49.5	61	1.2	54.0	5.4	0.10
0.75	51.5	67	1.3	58.0	6.2	0.11
1.50	42.0	52	1.2	51.5	4.9	0.10
3.00	39.0	56	1.4	48.0	4.3	0.09
5.00	32.0	43	1.4	39.0	4.0	0.10

Q and $C^{14}O_2$ values as described in Table I.

Rat brain cortex slices (ca. 70mg) were incubated with 2mM labelled glycine and 10mM glucose, with addition of sodium glyoxylate as shown above, in a total volume of 3ml for 60 minutes, under the standard conditions of incubation.

oxidase activity of the rat liver has now been shown to be associated with the mitochondrial particles (see Chapter I), efforts were made to determine the extent of participation of glyoxylic acid in the oxidation of glycine by rat liver mitochondria.

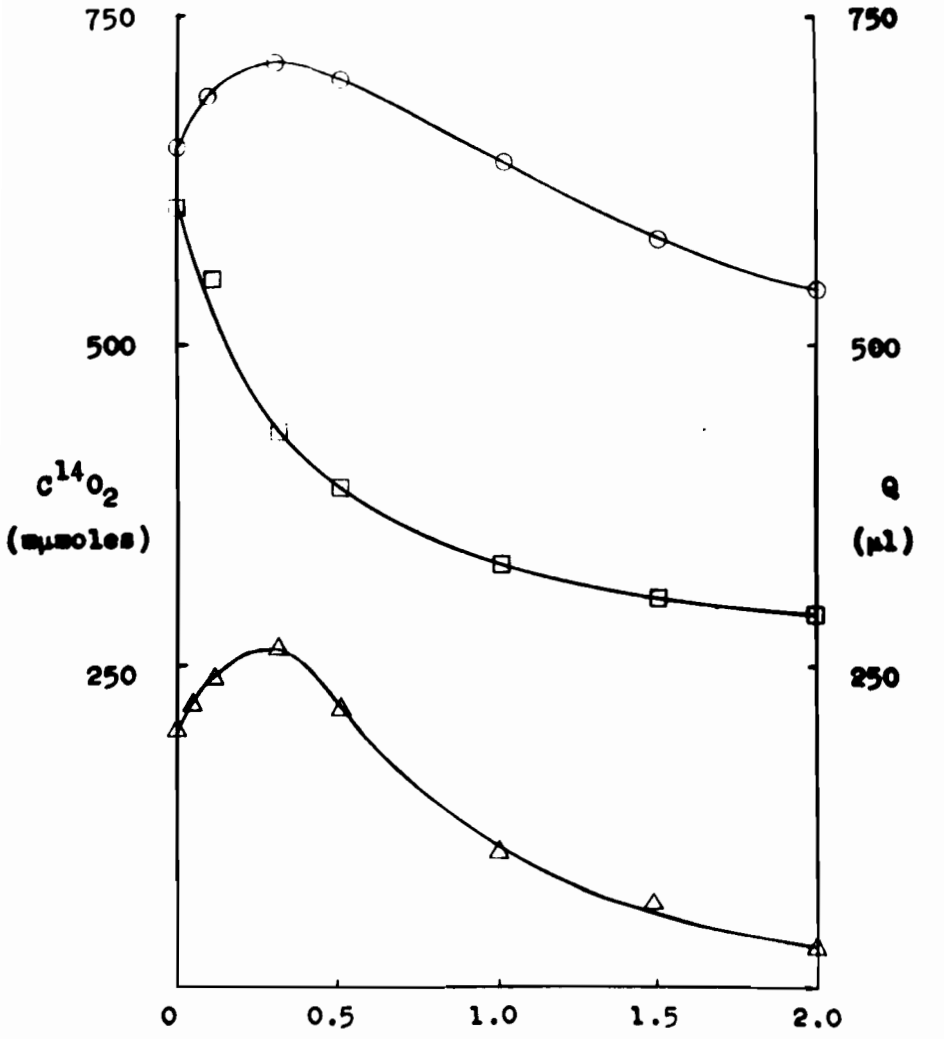
The effects of glyoxylic acid on the respiration and oxidation of glycine by rat liver mitochondria are shown in Figure I. The respiratory activity of the particles is sensitive to glyoxylic acid, but low concentrations of glyoxylic acid, though inhibitory to respiration, were repeatedly found to stimulate the oxidation of glycine before exhibiting an inhibitory effect. With glycine-2-C^{14} as substrate,,the maximum percentage stimulation obtained with glyoxylic acid is very pronounced compared to that observed with glycine-1-C^{14} as substrate (approximately 50% and 15% respectively). However, the maximum stimulation is the same in terms of absolute units (mμmoles of the labelled carbon converted to $C^{14}O_2$) irrespective of whether the glycine molecule is labelled in the carboxyl or the methylene carbon.

The effects of amino group donors on the glyoxylic acid stimulated oxidation of glycine-2-C^{14}.

The peculiar effect of glyoxylic acid on glycine oxidation could be understood if one assumes that glyoxylic acid can transaminate with glycine. Such an exchange of amino group between labelled glycine and non-labelled glyoxylic acid will result in the formation of labelled

FIGURE I

THE EFFECTS OF GLYOXYLIC ACID ON THE RATE OF OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA



Mitochondria from 1g of fresh rat liver were incubated with 5mM sodium succinate and 2mM labelled glycine with additions of sodium glyoxylate as shown, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions

-$C^{14}O_2$ (Glycine-1-C^{14}) △ -$C^{14}O_2$ (Glycine-2-C^{14}) □ -Q

glyoxylic acid, which can be further oxidised independent of the oxidation of glycine. If this is true the presence of another amino group donor should diminish the stimulation of the oxidation of glycine observed in the presence of glyoxylic acid.

Results presented in Table VII, show that 0.5mM sodium glyoxylate increases the rate of oxidation of glycine-2-C^{14} from 259 mμmoles per hour to 433 mμmoles per hour; i.e. an increase of 174 mμmoles. When L-glutamine (10mM) is added, 0.5mM sodium glyoxylate increases the rate of oxidation of glycine-2-C^{14} from 233 mμmoles per hour to 260 mμmoles per hour; i.e. an increase of only 27 mμmoles. Similarly, small stimulations of 25 and 11 mμmoles per hour are obtained with 0.5mM sodium glyoxylate, if L-leucine (10mM) or L-ornithine (10mM) are present. Thus, in the presence of other amino-group donors, the stimulation of glycine oxidation observed with glyoxylic acid is greatly diminished.

<u>The effects of L-serine and formic acid on glyoxylic acid stimulated oxidation of glycine-2-C^{14}.</u>

Table VIII shows the effects of L-serine and formic acid, added seperately or together, on the glyoxylic acid stimulated oxidation of glycine-2-C^{14}.

A large inhibition of the rate of oxidation of glycine is observed when L-serine is added to the reaction mixture. However, it is seen that glyoxylic acid can cause approximately

T A B L E VII

EFFECTS OF L-SERINE AND SODIUM FORMATE ON THE GLYOXYLATE-STIMULATED OXIDATION OF GLYCINE-2-C^{14} BY RAT LIVER MITOCHONDRIA.

Additions (mM)	CONTROL		0.5mM GLYOXYLATE		GLYOXYLATE STIMULATION	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$	mμmoles	%
Nil	626	232	412	350	118	-
L-serine (2mM)	575	89	427	196	107	-
Sodium formate (2mM)	694	130	383	210	-	61
Sodium formate (2mM) and L-serine (2mM)	662	59	425	94	-	59

Q - μl of oxygen consumed by the mitochondrial preparation obtained from 1g of fresh rat liver per 60 minutes.

$C^{14}O_2$ - mμmoles of $C^{14}O_2$ produced from the labelled substrate by the mitochondrial preparation obtained from 1g of fresh rat liver per 60 minutes.

Mitochondria from 1g fresh rat liver were incubated with 2mM glycine-2-C^{14} and 5mM sodium succinate with additions as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

TABLE VIII

EFFECTS OF AMINO GROUP DONORS ON THE GLYOXYLATE-STIMULATED OXIDATION OF GLYCINE-2-C^{14} BY RAT LIVER MITOCHONDRIA.

Additions (10mM)	CONTROL		0.5mM GLYOXYLATE	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	679	259	493	433
L-glutamine	627	233	660	260
L-leucine	622	220	657	245
L-ornithine	648	155	656	166

Q and $C^{14}O_2$ values as described in Table VII.

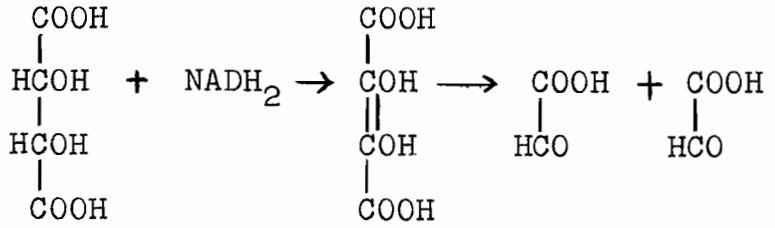
Mitochondria from 1g of fresh rat liver were incubated with 2mM glycine-2-C^{14} and 5mM sodium succinate with additions as shown above, in a total volume of 3ml, for 60 minutes under the standard incubation conditions.

the same amount of stimulation in absolute units, even in the presence of L-serine, suggesting that glyoxylic acid-2-C^{14} is formed by an exchange reaction between glycine-2-C^{14} and it is oxidised independently of the presence of L-serine.

The glyoxylate stimulated oxidation of glycine-2-C^{14} proceeds via the intermediate formation of formic acid-C^{14}. If a large pool of non-labelled formic acid is added together with glyoxylic acid, a certain percentage of the formate pool can be expected to be labelled causing a stimulation, which in percentwise is of the same magnitude of the control value and again remains unaffected by the presence of L-serine.

Effect of meso-tartaric acid on the rate of oxidation of glycine by rat liver mitochondria.

Meso-tartaric acid has been shown to be oxidised rapidly by liver mitochondria and among other products glyoxylic acid is formed by the following series of reactions (Kun, 1956).



(39)

The effect of meso-tartaric acid on the rate of oxidation of glycine was investigated in rat liver mitochondria, with the aim of providing a metabolic source of glyoxylic acid. These data are shown in Table **IX**. Though meso-tartaric acid is rapidly oxidised by mitochondria as shown by the increased

T A B L E IX

EFFECT OF SODIUM MESO-TARTARATE ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Addition	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	226	522	236	202
Meso-tartarate (10mM)	324	562	300	198

Q and $C^{14}O_2$ values as described in Table VII.

Mitochondria from 1g of fresh rat liver were incubated with 2mM labelled glycine and 5mM choline chloride with addition of sodium meso-tartarate as shown above, in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

oxygen consumption, there is no significant effect on the production of labelled carbon dioxide from either glycine-1-C^{14} or glycine-2-C^{14}.

Effect of ethanol on the rate of glycine oxidation by rat liver mitochondria.

Ethanol is known to be oxidised by catalase and hydrogen peroxide (Keilin and Hartree, 1945). Formic acid, the intermediate in the oxidation of the methylene carbon atom of glycine by the glyoxylate pathway, is also postulated to be oxidised by catalase and hydrogen peroxide (Nakada and Weinhouse, 1953b). Addition of ethanol to a system oxidising glycine via the glyoxylate pathway can be predicted to inhibit the oxidation of the methylene carbon atom with little or no effect on the rate of oxidation of the carboxyl carbon atom. Results presented in Table X show that there is a small inhibition of the rate of glycine oxidation when ethanol is added. However, the percentage inhibition is the same for both carbon atoms of glycine, suggesting that the effect is probably due to a non-specific action of the large amount of ethanol used.

Isolation of labelled glyoxylic acid.

A small amount of radioactivity appeared in glyoxylic acid isolated by paper chromatography, only when 2mM glyoxylic acid was included in the reaction mixture metabolising glycine-2-C^{14}. Though the labelled carbon dioxide production

TABLE X

EFFECT OF ETHANOL ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Additions	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	598	492 (100)	575	117 (100)
Ethanol (10mM)	573	486 (99)	585	111 (95)
Ethanol (100mM)	534	400 (81)	629	97 (83)

Q and $C^{14}O_2$ values as described in Table VII.

Values in parentheses refer to percentages.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 5mM sodium succinate with addition of ethanol as shown above, for 60 minutes, in a total volume of 3ml, under the standard incubation conditions.

is decreased under these conditions by 80%, only approximately 10% of the missing radioactivity could be accounted by the labelling of glyoxylic acid. Again, the radioactivity found in the glyoxylic acid spot is nearly same whether the incubation mixture contained respiring mitochondria or mitochondria heated in a boiling water-bath for five minutes. With boiled mitochondria, there is no oxygen consumption, nor is there any labelling of the carbon dioxide.

Discussion.

It is known that glyoxylic acid inhibits respiration of tissues (Kleinzeller, 1943). Recently, an explanation has been offered for this phenomena. Glyoxylic acid and oxaloacetic acid combine non-enzymatically to form a compound believed to be oxalomalic acid, which inhibits the enzyme, aconitase (D Abramo et al. 1957a; 1957b; 1958; Ruffo et al. 1959). Since glycine oxidation is apparently related to the metabolic activity of the system (see Table I), the glyoxylic acid inhibition of the rate of glycine oxidation is probably due to the effect of glyoxylic acid on respiration rather than due to isotopic dilution. When the $C^{14}O_2$ production is represented per unit oxygen consumption, glyoxylic acid does not show an inhibition of the rate of oxidation of glycine.

The conversion of the methylene carbon atom of glycine to an active one carbon fragment also does not seem to proceed via the intermediate formation of glyoxylic acid. Labelling of the β-carbon atom of serine and the C-2 and 8 of purines

(involving the participation of active-formate) in intact rats and ducks respectively, seems to proceed faster with glycine-2-C^{14} than with glyoxylic acid-2-C^{14} (Weissbach and Sprinson, 1953b). In vitro studies with chicken liver preparations, synthesising doubly labelled serine from glycine-2-C^{14}, have shown that glyoxylic acid is unable to reduce the formation of serine (Sanadi and Bennett, 1960; Richert et al. 1962).

Glyoxylic acid has been shown to be converted to glycine in the intact rat (Weinhouse and Friedmann, 1951; Chao et al. 1953). In vitro studies have demonstrated the conversion of glyoxylic acid to glycine (Cammarata and Cohen, 1950; Mardashev and Semina, 1950; Awapara and Seale, 1952; Meister et al. 1952; Nakada and Weinhouse, 1953a; Meister, 1954a; 1954b; Wilson, King and Burris, 1954; Pitts, Stewart and Crosbie, 1960). Non-enzymatic conversion of glyoxylic acid to glycine has also been demonstrated (Nakada and Weinhouse, 1953a; Meister et al. 1954b; Fleming and Crosbie, 1960). Metzler et al. (1954) have calculated the ΔF for the reaction,

L-glutamic acid + glyoxylic acid $\longrightarrow$ α-ketoglutaric acid + glycine

to be —2000 Cals. The reaction is apparently irreversible.

Though the formation of glutamic acid from glycine and α-ketoglutaric acid has been reported (Cammarata and Cohen, 1950; Wilson et al. 1954), the formation of glyoxylic acid

as a product of this reaction is yet to be demonstrated (Meister, 1957). Pitts et al. (1960), from their non-enzymic studies on glyoxylic acid-glycine interconversion (Fleming and Crosbie, 1960) have questioned the validity of the isotopic evidence adduced by Nakada and Weinhouse (1953a; 1953b) in support of the involvement of glyoxylic acid in the formation of formic acid from glycine-2-C^{14}.

The aldehyde groups of pyridoxal and glyoxylic acid are analogous in electronic properties due to the strong electron attraction of the contiguous carboxyl group in the latter compound (Braunstein, 1960). For this reason, glyoxylic acid undergoes transamination readily. The absence of labelling of glyoxylic acid from glycine, unless a pool of glyoxylic acid is added, as reported by Nakada and Weinhouse (1953b) and confirmed by Richert et al. (1962) and in the present work, suggests that the labelling of glyoxylic acid is due to an exchange reaction between glycine and glyoxylic acid. The present results also indicate that the labelling of glyoxylic acid from glycine is much lower than could be calculated from the inhibition of the oxidation of glycine and is uninfluenced whether the mitochondrial preparation is inactivated by boiling or not. Richert et al. (1962), have recently reported that the labelling of glyoxylic acid from chicken, pigeon and duck liver particulate preparations oxidising glycine, is the same even when the liver preparation is not included in the incubation mixture.

The initial stimulation of the rate of glycine oxidation observed with low concentrations of glyoxylic acid and the ability of amino-group donors to abolish this stimulation provides additional support for the suggestion of an exchange reaction between glyoxylic acid and glycine.

Glyoxylate dehydrogenase of rat liver mitochondrial extracts, which oxidises glyoxylic acid to formic acid and carbon dioxide, exhibits an absolute dependence for L-glutamic acid (Nakada and Sund, 1958; Crawhall and Watts, 1962). By inference, the oxidation of glycine via glyoxylic acid should proceed only in the presence of L-glutamic acid. However, the oxidation of glycine by rat liver mitochondria is inhibited by the addition of L-glutamic acid (Chapter IV, Table XXIV).

Results presented in Table X indicate that the oxidation of the methylene carbon atom of glycine does not proceed via formic acid. Similar conclusions were drawn by Schulman and Richert (1959) on the basis of results obtained by a different technique. These workers demonstrated that the rats kept on molybdenum deficient diets oxidise both carbon atoms of glycine normally, but oxidised formic acid at a reduced rate, both in vivo and in vitro. Presumably the oxidation of the methylene carbon atom of glycine proceeds via a pathway not involving formic acid.

Summary.

(1) The oxidation of glycine by rat brain cortex slices

is inhibited by glyoxylic acid; the inhibition is considered to be due to an inhibition of the metabolic activity of rat brain cortex slices.

(2) Labelled glyoxylic acid is isolated by incubating labelled glycine and glyoxylic acid, even in the presence of boiled mitochondria which do not oxidise glycine.

(3) The oxidation of glycine by rat liver mitochondria shows an initial stimulation followed by inhibition on the addition of glyoxylic acid. The stimulation is reduced by the addition of amino-group donors, L-glutamine, L-leucine or L-ornithine. The glyoxylate-stimulated oxidation of glycine is not affected by the presence of L-serine (in absolute units) and by the presence of formic acid (in percentage).

(4) Meso-tartaric acid has no effect on the oxidation of glycine by rat liver mitochondria.

(5) Inhibition studies with ethanol indicate that the oxidation of the methylene carbon atom of glycine does not proceed via formic acid.

C H A P T E R III

THE INCORPORATION OF GLYCINE INTO SERINE.

Introduction.

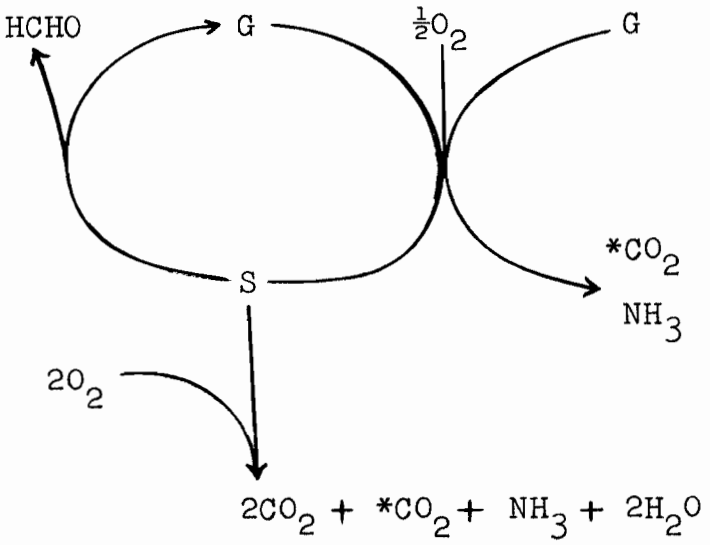
The rapid interconversion of glycine and serine in animal tissues is well established. Serine can be converted to pyruvic acid and by the reversal of the glycolytic reactions may provide the glucose units for glycogenesis. Glycine is also a glycogenic amino acid. It is possible to visualize that glycine is converted to glycol aldehyde via the intermediate formation of glyoxylic acid and glycolic acid and that the glycol aldehyde is converted to glycogen by a transketolase type of reaction. However, studies with glycol aldehyde-2-C^{14} have shown that the transformation involves the intermediate formation of glycine, serine and pyruvic acid (Friedmann et al. 1956) and presumably serine and pyruvic acid are obligatory intermediates in the conversion of glycine to glycogen. The glyoxylate pathway of glycine catabolism is either absent or insignificant in isolated rat liver mitochondria (vide Chapter II). The glycogenic pathway of glycine metabolism may provide an alternate route for the oxidation of glycine, since the pyruvic acid formed can be oxidised rapidly by liver mitochondria.

The methylene carbon atom of glycine can serve as the active one carbon fragment needed for the synthesis of serine from glycine and this necessitates the prior conversion of the carboxyl carbon atom of one of the two glycine molecules

to carbon dioxide. The rate of oxidation of the serine in which all the carbon atoms originated from glycine will also determine the rate of oxidation of the methylene carbon atom of glycine. A slower rate of oxidation of serine in which glycine has been incorporated will also result in a higher rate of production of carbon dioxide from the carboxyl carbon atom of glycine as is observed in all the systems examined (Table I).

The methylene carbon atom of glycine, appearing as the β-carbon atom of serine, can also be converted into an active one carbon unit which may be donated to any one of the single carbon fragment acceptors. This will result in the regeneration of glycine with the concomittant breakdown of another molecule of glycine, the carboxyl carbon of which appears as carbon dioxide and the methylene carbon atom assumes a synthetic role. The simultaneous operation of such a scheme along with the further oxidation of serine, will also result in a higher rate of production of carbon dioxide from the carboxyl carbon atom of glycine.

These two mechanisms by which a differential rate of conversion of the two carbon atoms of glycine to carbon dioxide is made possible, are schematically represented as shown on the next page.



G and S represent glycine and serine respectively. $*CO_2$ originates from the carboxyl carbon of glycine.

Experiments whose aim was the exploration of the role played by serine in the catabolism of glycine, are reported in this chapter.

The effects of serine on the rate of oxidation of glycine in rat brain and kidney cortex slices.

The effects of different concentrations of L-serine on the rate of the oxidation of glycine-1-C^{14} and glycine-2-C^{14} by rat brain cortex slices are shown in Table XI. The amount of oxygen utilized under identical conditions is also shown in the same table. The rate of $C^{14}O_2$ production is approximately twenty times faster with glycine-1-C^{14} than with glycine-2-C^{14}. No significant change in the oxygen consumed is observed when L-serine is added to the respiring rat brain cortex slices. However,

TABLE XI

EFFECT OF L-SERINE ON THE OXIDATION OF GLYCINE BY RAT BRAIN CORTEX SLICES.

L-serine (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	54.5	90 (100)	55.0	4.60 (100)
0.5	55.0	70 (78)	52.0	4.15 (90)
1.0	51.0	56 (62)	48.0	3.80 (83)
2.0	52.5	50 (56)	50.0	3.65 (79)
3.0	55.0	42 (47)	-	-
3.3	-	-	49.5	3.00 (65)
5.0	52.5	40 (44)	50.5	2.55 (55)
7.5	54.5	36 (40)	48.0	2.05 (45)
10.0	62.0	36 (40)	50.5	1.95 (42)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat brain cortex slices (ca. 70mg) were incubated with 2mM labelled glycine and 10mM glucose with additions of L-serine as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

increasing concentrations of L-serine cause a progressive decrease in the rate of production of labelled carbon dioxide from both glycine-1-C^{14} and glycine-2-C^{14}. The maximum inhibition obtained is approximately 60% in both cases.

The results obtained in similar experiments conducted with rat kidney cortex slices are presented in Table XII. The respiration of the rat kidney cortex slices is affected very little by the presence of L-serine. Approximately twelve times more carbon dioxide is produced from the carboxyl carbon atom of glycine than from the methylene carbon atom of glycine. L-serine inhibits the rate of the oxidation of glycine-1-C^{14} and glycine-2-C^{14} in a similar manner. However, compared to rat brain cortex slices, the rate of oxidation of glycine by the rat kidney cortex slices is less influenced by L-serine. The maximum inhibition observed is approximately 30% for both carbon atoms of glycine.

<u>The effects of various substrates on the oxidation of glycine by isolated rat liver mitochondria.</u>

Previous results (Chapter I, Table V) have indicated that damaging the mitochondria results in the loss of glycine-oxidase activity. Since rapidly oxidizable substrates are necessary for mitochondrial integrity, preliminary investigations were conducted with various substrates to find the appropriate conditions necessary for studying the oxidation of glycine in rat liver

TABLE XII

EFFECT OF L-SERINE ON THE OXIDATION OF GLYCINE BY RAT KIDNEY CORTEX SLICES.

L-serine (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	79.5	802 (100)	76.5	68 (100)
0.5	88.0	815 (101)	-	-
1.0	88.5	840 (105)	82.5	67 (98)
2.0	85.0	745 (93)	72.5	51 (75)
3.0	85.0	760 (95)	76.0	54 (79)
5.0	81.5	620 (77)	75.5	51 (75)
7.5	80.0	560 (70)	-	-
8.0	-	-	80.0	50 (74)
10.0	84.0	560 (70)	78.5	52 (76)
15.0	-	-	81.0	50 (74)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat kidney cortex slices (ca. 60mg) were incubated with 2mM labelled glycine and additions of L-serine as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

mitochondria.

Table XIII shows the rates of $C^{14}O_2$ production from glycine-1-C^{14} and glycine-2-C^{14} by rat liver mitochondria along with the amount of oxygen utilized under identical conditions for various substrates. The values correspond to, or are recalculated for, a reaction period of one hour. With few exceptions, the carboxyl carbon atom of glycine is oxidised approximately 3-4 times faster than the methylene carbon atom of glycine, with all the substrates tried.

Both carbon atoms of glycine are converted to carbon dioxide most rapidly when choline chloride of either 2.5 or 10mM concentration is used as substrate. The higher concentration of choline chloride increases the oxygen consumption over that obtained with the lower concentration, but the rate of the oxidation of glycine is not significantly altered. The oxygen consumed is small, when compared to the high values obtained with the citric acid cycle substrates.

Sodium salts of succinic, fumaric, oxaloacetic and pyruvic acids, when used at 5mM concentrations maintain good respiratory activity of rat liver mitochondria. However, the rate of $C^{14}O_2$ produced from glycine-1-C^{14} is approximately half of that is obtained with choline chloride as substrate. The oxygen consumption is very high with 5mM sodium α-ketoglutarate but the conversion of

T A B L E XIII

EFFECTS OF CO-SUBSTRATES ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Additions	Concentration (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
		Q	$C^{14}O_2$	Q	$C^{14}O_2$
*					
Choline chloride	2.5	210	1114	228	332
	10.0	255	1119	309	322
β-hydroxy butyrate	2.5	168	600	168	133
	10.0	393	500	348	59
Fumarate	5.0	373	666	360	182
Oxaloacetate	5.0	491	568	489	173
Pyruvate	5.0	494	531	530	113
Succinate	5.0	540	490	548	112
α-ketoglutarate	5.0	578	383	613	79
Hexanoate	5.0	601	394	672	19

Q and $C^{14}O_2$ values as described in Table VII. * Incubated for 25 minutes.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine with additions as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

glycine-1-C^{14} to $C^{14}O_2$ is decreased to about a third of the value obtained with choline chloride as substrate. The rate of oxidation of the methylene carbon atom of glycine is also inhibited markedly, approximately to half with fumaric and oxaloacetic acids, to one third with pyruvic and succinic acids and to one fourth with α-ketoglutaric acid as substrates when compared to the value obtained with choline chloride.

The respiration of the mitochondria is at its peak with 5mM potassium hexanoate and the rate of $C^{14}O_2$ production from glycine-1-C^{14} is comparable to that obtained with 5mM sodium α-ketoglutarate as substrate. However, it is interesting to observe that the oxidation of the methylene carbon atom of glycine is almost completely abolished. When pyruvic and hexanoic acids were used as substrates, 0.5mM sodium fumarate was also added to stimulate the operation of the citric acid cycle.

The respiration of liver mitochondria is at its minimum with 2.5mM sodium β-hydroxybutyrate as substrate. The rate of labelled carbon dioxide production from both carbons of glycine is approximately half of that which is observed with choline chloride. The oxygen consumption is more than doubled if the concentration of sodium β-hydroxybutyrate is increased from 2.5mM to 10mM. With this higher concentration of sodium β-hydroxybutyrate, there is a slight inhibition of the $C^{14}O_2$ production from glycine-1-C^{14} but a strikingly greater inhibition of the oxidation of the methylene carbon

atom of glycine.

Time course studies on the rate of oxidation of glycine by rat liver mitochondria.

The rate of oxygen consumption observed with the various citric acid cycle intermediates gave a possible clue to the low rate of oxidation of glycine obtained with these substrates. It is seen from Table XIII that, when citric acid cycle intermediates are used as substrates, a rough inverse relationship exists between the amount of oxygen consumed and the amount of $C^{14}O_2$ produced from labelled glycine, but for a few exceptions with glycine-2-C^{14}. Subsequent experiments showed that when sodium α-ketoglutarate, sodium succinate sodium pyruvate and sodium oxaloacetate were used at a final concentration of 1mM the rate of glycine oxidation was very rapid. The amount of $C^{14}O_2$ produced from glycine-1-C^{14} and glycine-2-C^{14} was estimated for different time intervals, with both 1mM and 5mM sodium α-ketoglutarate as substrates. The results of these time course experiments are presented graphically in Figures IIa and IIb.

The respiration observed with 5mM sodium α-ketoglutarate is high and linear with time. With 1mM substrate the rate of oxygen consumption decreases with time and after 115 minutes reaches a value approximately equal to half of that observed with 5mM substrate.

The rate of production of labelled carbon dioxide from

FIGURE IIa

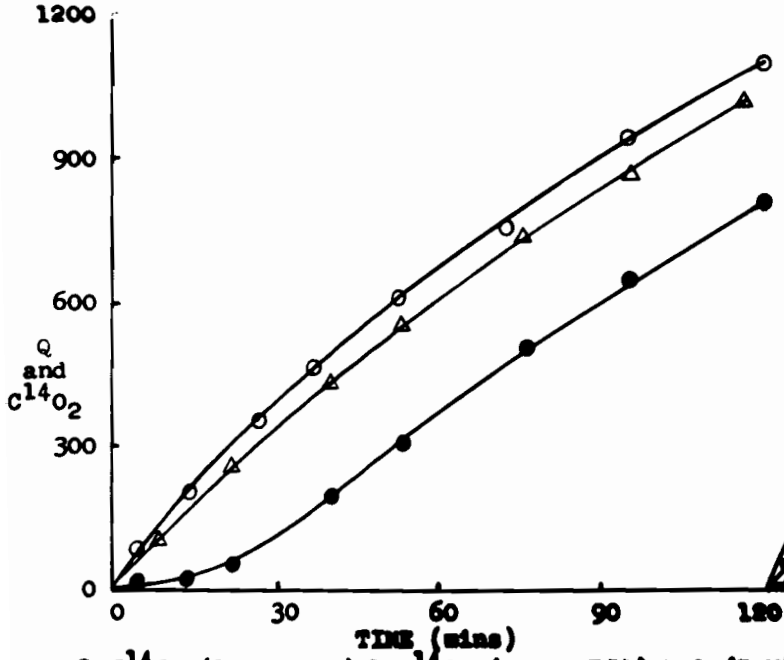
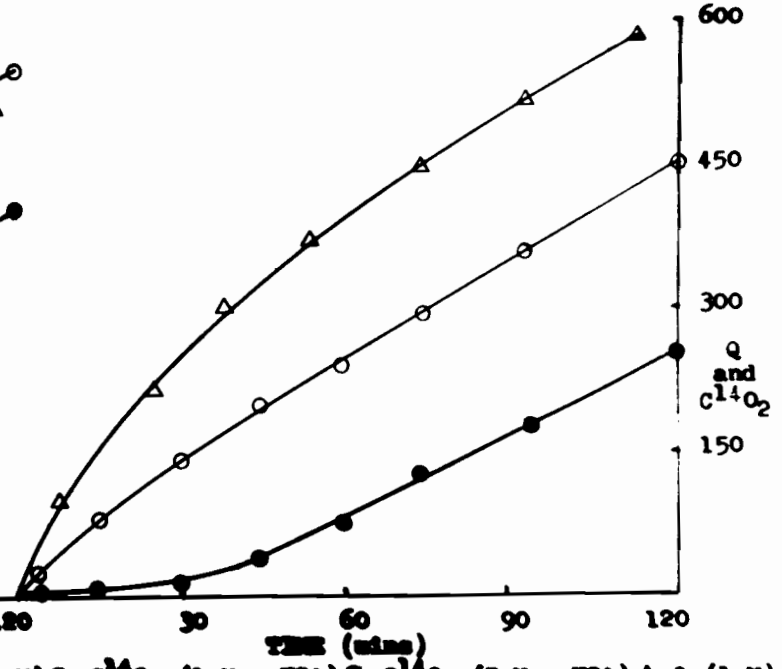


FIGURE IIb



○-$C^{14}O_2$ (1mM α-KGA) ●-$C^{14}O_2$ (5mM α-KGA) △-Q (1mM) ○-$C^{14}O_2$ (1mM α-KGA) ●-$C^{14}O_2$ (5mM α-KGA) △-Q (1mM)

Mitochondria from 1g fresh [illegible] incubated with 5mM glycine-1-C^{14} (Figure IIa) and glycine-2-C^{14} (Figure IIb) [illegible] α-ketoglutarate (as indicated) in a total volume of 3ml, under the standard [illegible] conditions.

both glycine-1-C^{14} and glycine-2-C^{14} is high and proceeds without any observable lag period, if 1mM sodium α-ketoglutarate is used as substrate. However, with 5mM sodium α-ketoglutarate there is a pronounced inhibition of the rate of oxidation of glycine-1-C^{14} and glycine-2-C^{14} for approximately 20 and 40 minutes respectively. The rate of oxidation of glycine is linear with time thereafter, but still proceeds at a much slower rate.

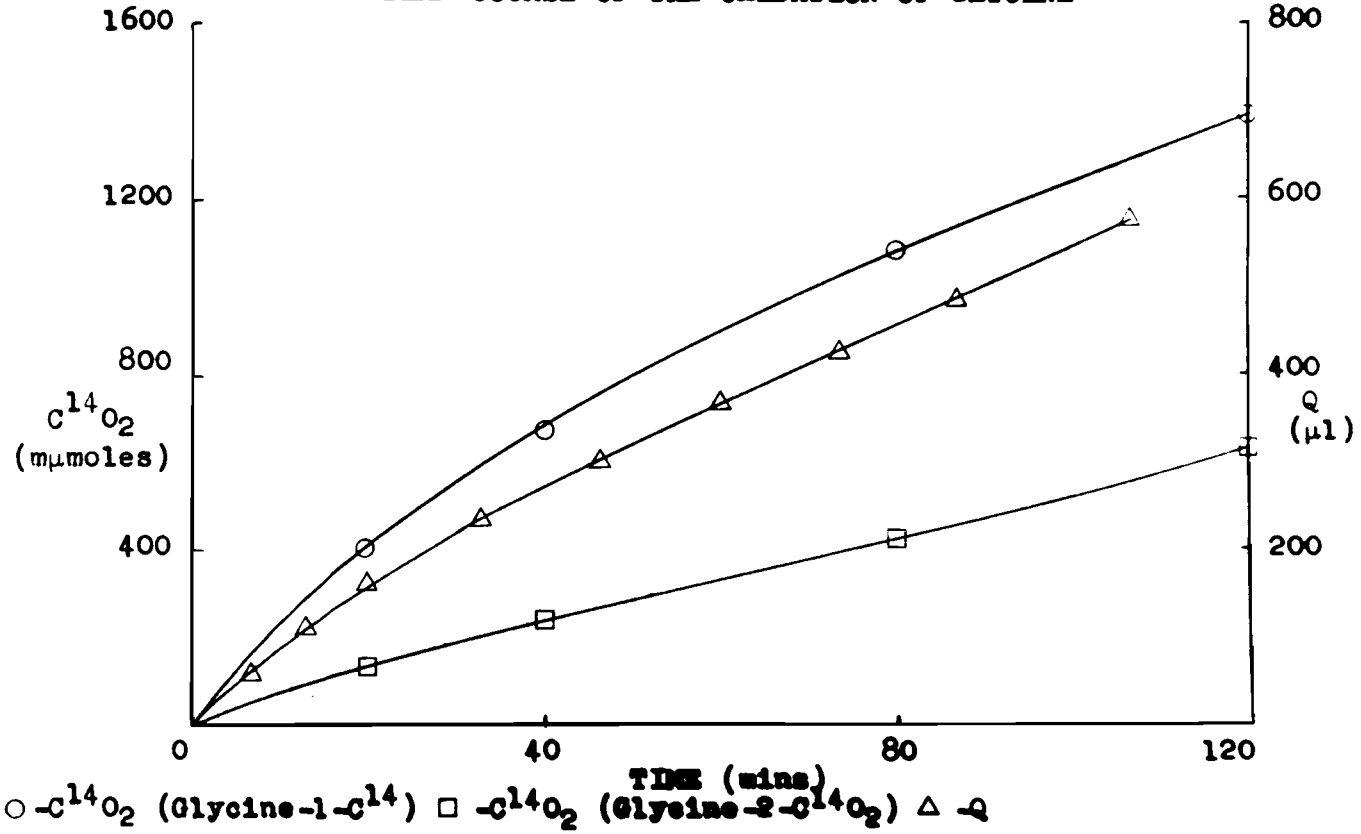
Experiments similar to those described above were also conducted with 10mM choline chloride instead of α-ketoglutarate as substrate. These results are shown in Figure III. In contrast to the results obtained with 5mM sodium α-ketoglutarate, the rate of the production of labelled carbon dioxide from glycine-1-C^{14} and glycine-2-C^{14} with choline chloride shows no initial inhibition. The rate of oxygen consumption shows only a slight decrease with time under these conditions.

Effect of malonic acid on the rate of glycine oxidation.

The time course experiments reported above show that a high rate of oxidation of the citric acid cycle substrates is inhibitory to the oxidation of glycine. Additional proof was also obtained by a different approach. It was argued that malonic acid, which can slow down the operation of the citric acid cycle, should stimulate the rate of the oxidation of glycine, when high concentrations of citric acid cycle intermediates are used as substrates. The rate of production of labelled carbon dioxide from glycine-1-C^{14} and glycine-2-C^{14}

F I G U R E III

TIME COURSE OF THE OXIDATION OF GLYCINE



Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 10mM choline chloride in a total volume of 1.5ml, under the standard incubation conditions.

with increasing concentrations of sodium malonate, when 5mM sodium α-ketoglutarate is used as substrate, is given in Table XIV. The rate of oxygen consumption which is also shown decreases considerably with increasing concentrations of sodium malonate. As predicted, there is a great increase in the rate of production of labelled carbon dioxide from both glycine-1-C^{14} and glycine-2-C^{14} when sodium malonate is added.

Results obtained for similar experiments conducted with 10mM choline chloride as substrate (Table XV) show that sodium malonate causes a slight inhibition of the oxidation of glycine with choline chloride as substrate.

The effects of L-serine on the rate of oxidation of glycine by rat liver mitochondria.

Table XVI shows the results obtained with increasing concentrations of L-serine on the rate of oxidation of glycine by rat liver mitochondria. The production of labelled carbon dioxide from glycine-1-C^{14} is only slightly inhibited by L-serine, but there is a large inhibition of the rate of oxidation of the methylene carbon atom of glycine. As much as 50% inhibition is observed with 2mM L-serine. L-serine has little effect on the oxygen consumption of the rat liver mitochondria.

Michaelis constants for the oxidation of glycine-1-C^{14} and glycine-2-C^{14} by rat liver mitochondria.

T A B L E XIV

EFFECT OF SODIUM MALONATE ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA IN PRESENCE OF α-KETOGLUTARATE.

Sodium malonate (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	666	376	635	89
1.0	644	484	611	95
2.0	638	535	682	161
3.5	627	665	643	220
5.0	581	675	629	321
7.5	595	735	558	376
10.0	488	730	494	405

Q and $C^{14}O_2$ values as described in Table VII.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 5mM sodium α-ketoglutarate with additions of sodium malonate as shown above, in a total volume of 3ml for 60 minutes, under the standard incubation conditions.

T A B L E XV

EFFECT OF SODIUM MALONATE ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA IN PRESENCE OF CHOLINE CHLORIDE.

Sodium malonate (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	278	760	262	276
5.0	192	632	190	264
10.0	170	578	188	250
15.0	183	544	170	244

Q and $C^{14}O_2$ values as described in Table VII.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 2.5mM choline chloride with additions of sodium malonate as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

T A B L E XVI

EFFECT OF L-SERINE ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

L-serine (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.00	132	1080	188	228
0.25	158	1076	188	212
0.75	152	1050	194	158
1.25	156	1028	204	139
2.00	166	1020	192	130

Q and $C^{14}O_2$ values as described in Table VII.

Mitochondria from 1g of fresh rat liver were incubated with 2mM labelled glycine and 2.5mM choline chloride with additions of L-serine as shown above, in a total volume of 1.5ml, for 30 minutes, under the standard incubation conditions.

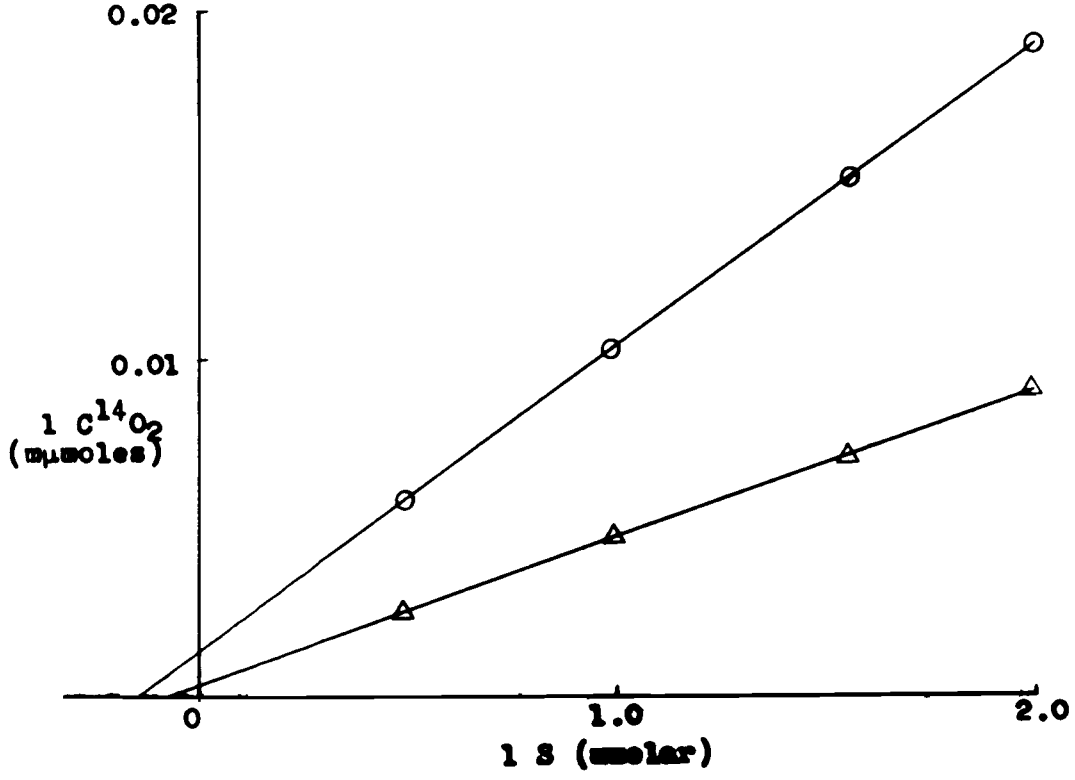
The effects of different concentrations of glycine on the rate of oxidation of glycine-1-C^{14} and glycine-2-C^{14} by the isolated rat liver mitochondria were studied to find out the influence of the substrate concentration on the velocity of the reaction. The reciprocal of the substrate concentration versus the reciprocal of the velocity are plotted according to the method of Lineweaver and Burk (Figure IV). The apparent K_m values for glycine-1-C^{14} and glycine-2-C^{14} are approximately 5mM and 12mM respectively. The different K_m values for the different carbon atoms of glycine indicate that the two carbon atoms of glycine depend upon different enzyme system(s) for their conversion to carbon dioxide.

The effects of D-serine on the rate of the oxidation of glycine by rat liver mitochondria.

The effects of D-serine on the rate of oxidation of glycine are shown in Table XVII. There is a definite increase in the amount of oxygen consumed in the presence of D-serine. The oxygen consumption increases as much as 50µl with 10mM D-serine whereas L-serine has little effect on the rate of oxygen consumption (Table XVI). However, in contrast to the results obtained with L-serine, there is no significant effect on the rate of the production of labelled carbon dioxide from either glycine-1-C^{14} or glycine-2-C^{14}.

The effects of sodium formate on the rate of oxidation of glycine by rat liver mitochondria.

FIGURE IV



△ -$C^{14}O_2$ (Glycine-1-C^{14}) ○ -$C^{14}O_2$ (Glycine-2-C^{14})

Mitochondria from 1g fresh rat liver were incubated with different concentrations of labelled glycine and 10mM choline chloride in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

T A B L E XVII

EFFECT OF D-SERINE ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

D-serine (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	330	526	305	186
1.0	340	554	352	200
3.0	368	600	395	208
10.0	438	586	416	180

Q and $C^{14}O_2$ values as described in Table VII.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 10mM choline chloride with additions of D-serine as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

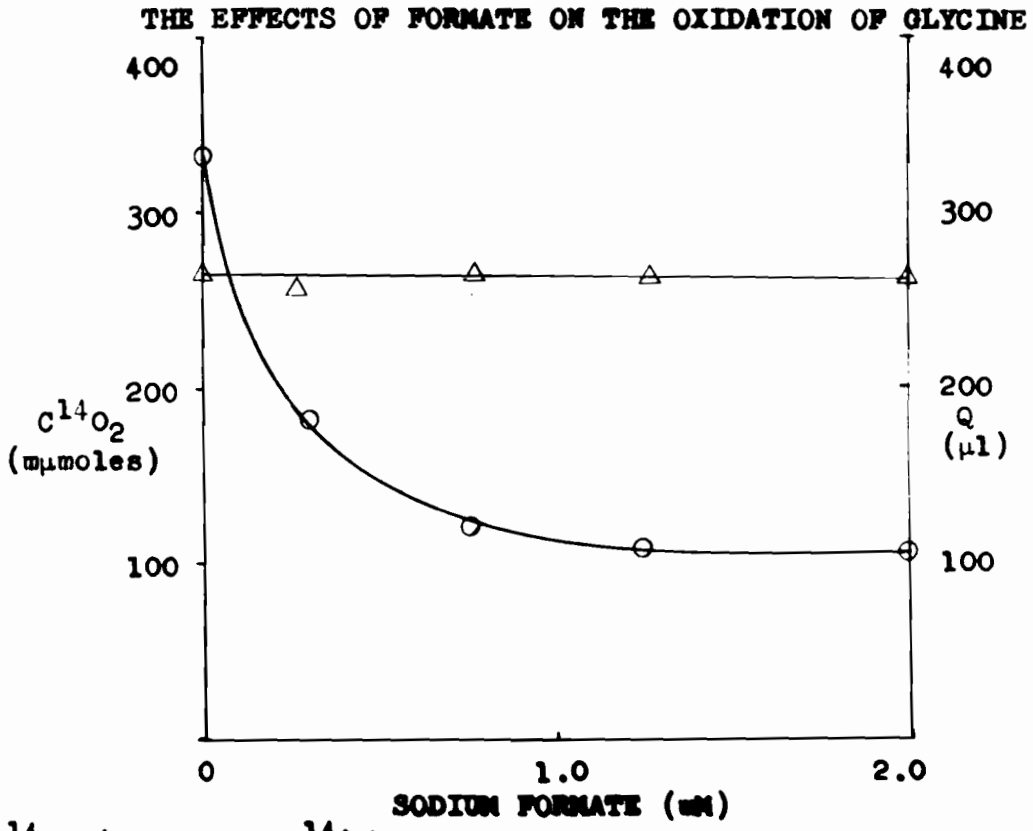
The effects of increasing concentrations of sodium formate on the rate of oxidation of glycine-2-C^{14} are shown graphically in Figure V. Less than 50% inhibition is observed with 0.75mM sodium formate and further increase in the concentration of sodium formate has no additional effect on the rate of oxidation of glycine-2-C^{14}. No significant effect on the oxygen consumption is observed when sodium formate is added to the incubation mixture. Sodium formate, even at a concentration of 5mM, had no observable effect on the rate of conversion of glycine-1-C^{14} to $C^{14}O_2$.

Isolation of labelled serine.

With the help of ion exchange chromatography and paper chromatography (as described in the section Materials and Methods), radioactive serine was isolated from the incubation mixture metabolising glycine-1-C^{14} or glycine-2-C^{14}. Little radioactivity escaped the Dowex 50 columns, indicating the absence of the accumulation of non-amino compounds during the reaction.

Figure VI shows a representative reproduction of a radioautogram in which the effect of L-serine added to trap the radioactivity incorporated into serine, to an incubation mixture metabolizing glycine-1-C^{14} is seen. Besides the glycine and serine spots, small amounts of radioactivity also appeared in a spot corresponding to the glutamic-aspartic acid region of the radioautogram, when glycine-2-C^{14} was used.

F I G U R E V



○ -$C^{14}O_2$ (Glycine-2-C^{14}) △ -Q

Mitochondria from 1g fresh rat liver were incubated with 2mM glycine-2-C^{14} and 10mM choline chloride in a total volume of 3ml, for 30 minutes, under the standard incubation conditions

F I G U R E VI

INCORPORATION OF GLYCINE INTO SERINE.

Mitochondria from 1g of fresh rat liver were incubated with 2mM glycine-1-C^{14} and 10mM choline chloride, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

Additions:

(1) and (3) Nil.

(2) and (4) L-serine (2mM).

GLU
GLY
ALA
SER

No measurable amount of radioactivity accumulated in any other region of the radioautogram.

Table XVIII shows the extent of the incorporation of the different carbon atoms of glycine into serine (in mμ atoms of the labelled carbon as calculated from the radioactivity of the serine spot on the radioautogram). The methylene carbon atom of glycine is incorporated into serine approximately twice as fast as the carboxyl carbon atom of glycine. When reduced glutathione is added to the incubation mixture to increase the metabolism of glycine, the radioactivity of the serine spot increases more than twofold; however, the ratio of the radioactivity from glycine-2-C^{14} to glycine-1-C^{14} remains unaltered at two. Added L-serine also increases the radioactivity of the isolated serine considerably, but the presence of exogenous L-serine decreases the ratio of the methylene carbon atom of glycine to the carboxyl carbon atom of glycine incorporated into serine.

Experiments conducted with high specific activity glycine -2-C^{14}, showed that very little radioactivy was associated with the protein fraction. The lipids were also isolated from the incubation mixture, but showed no radioactivity.

Discussion.

In the previous chapter, the possibility was considered that glycine is oxidised via the glyoxylate pathway. However, the results obtained indicate that glyoxylic acid is not an

T A B L E XVIII

INCORPORATION OF GLYCINE INTO SERINE BY RAT LIVER MITOCHONDRIA.

Additions (10mM)	mμ atoms of labelled carbon incorporated into serine		
	Glycine-1-C^{14}	Glycine-2-C^{14}	G-2-C^{14}/G-1-C^{14}
Nil	49	89	1.8
Reduced glutathione	147	300	2.0
L-serine	241	331	1.4

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 10mM choline chloride with additions as shown above, in a total volume of 1.5ml, for 60 minutes, under the standard incubation conditions.

intermediate in the metabolism of glycine. Evidence was also obtained to show that formic acid is not an obligatory intermediate in the oxidation of the methylene carbon atom of glycine (see also Chapter IV). The other possibility, namely, that L-serine is an intermediate in the catabolism of glycine merits attention. Direct and indirect evidence has now been obtained for the participation of L-serine in the oxidation of glycine.

Surviving rat kidney and brain cortex slices are known to concentrate amino acids against a concentration gradient and a concentration ratio as high as ten has been reported for glycine in rat brain cortex slices (Abadom and Scholefield, 1962). The rate of oxidation of glycine in rat brain cortex slices has been shown by Sved (1958) to be dependent upon the concentration of glycine inside the cell to a large extent. This observation has been confirmed in the present investigation and extended to rat kidney cortex slices. Any agent that reduces the concentration of glycine inside the cell can, therefore, be expected to inhibit the oxidation of glycine. Glycine and L-serine are probably transported by the same carrier system and hence can inhibit the active transport of each other by competing for the carrier (Abadom and Scholefield, 1962). A major part of the decreased rate of oxidation of glycine by rat kidney and brain cortex slices, in the presence of increasing concentrations of L-serine, can thus be explained as a secondary effect of L-serine.

The oxidation of glycine by rat liver mitochondria is dependent upon mitochondrial integrity and a rapidly oxidisable substrate is necessary to maintain this integrity. Again, the oxidation of glycine via serine and pyruvic acid is related to the operation of the citric acid cycle. Therefore, it is to be expected that, in the presence of a citric acid cycle intermediate, added to maintain mitochondrial integrity, inhibition of the oxidation of glycine will occur.

In studying the influence of co-substrates, it is found that choline chloride permits the highest rate of oxidation of glycine, by rat liver mitochondria. Since the oxidation of choline in liver mitochondria does not interfere with the operation of the citric acid cycle the oxidation of glycine proceeds at the maximal rate with this substrate.

It has been shown that liver mitochondria do not oxidise the methyl groups of choline or betaine, though choline is oxidised to betaine (Mackenzie, Johnston and Frisell, 1952; 1953). Isolation of the amino compounds formed during the reaction shows that neither choline, dimethyl aminoethanol, monomethyl aminoethanol nor amino ethanol acquire radioactivity, though a significant amount of radioactivity appears in serine. The phospholipids isolated from the incubation mixture are not radioactive. Incorporation of serine into phospholipids is necessary for the decarboxylation of serine to amino ethanol (Equation 37). Presumably, then, serine is not metabolised by decarboxylation in liver mitochondrial

preparations used in the present investigations. This and the conclusion that glyoxylic acid is not an intermediate in the metabolism of glycine (vide Chapter II) indicate that the glycine-amino ethanol cycle does not operate to any significant extent in rat liver mitochondria.

The rate of conversion of glycine-1-C^{14} to labelled carbon dioxide in the presence of various citric acid cycle intermediates is approximately half of that observed with choline chloride as substrate. The inhibition could be due to one or more of the following reasons. (a) The rapid oxidation of the citric acid cycle substrates probably maintains a high steady state concentration of oxaloacetic acid which can be decarboxylated to pyruvic acid. This source of pyruvic acid can compete and dilute the pyruvic acid-1-C^{14} formed from glycine-1-C^{14} via serine-1-C^{14}. (b) α-ketoglutaric acid either added or formed during the operation of the citric acid cycle could compete with pyruvic oxidase for one or more of the cofactors common to both α-keto oxidase systems (i.e. diphosphothiamine, NAD^+, coenzyme A and lipoic acid). (c) The rapid oxidation of the citric acid cycle substrates could cause an unfavourable equilibrium in NAD^+/NADH ratio for the dehydrogenase associated with the formation of serine from two glycine molecules as represented in Equation 40.

The oxidation of the methylene carbon atom of glycine is also inhibited in a more or less similar manner by the presence of a high concentration of a citric acid cycle substrate.

Probably one or more of the reasons given above also applies to the decreased rate of oxidation of glycine-2-C^{14}. An exceptionally low rate of oxidation of the methylene carbon atom of glycine is observed with hexanoic acid and with the higher concentration of β-hydroxybutyrate. These substrates produce a very high concentration of acetyl CoA which probably dilutes and competes with the labelled acetyl CoA formed from glycine-2-C^{14}.

Malonic acid competes with succinic acid for the enzyme succinic dehydrogenase. Used at low concentrations, in the presence of 5mM α-ketoglutaric acid, it slows down the operation of the citric acid cycle. Under these conditions, the rate of oxidation of glycine by rat liver mitochondria is stimulated for the inhibitory effect of the high concentration of α-keto-glutaric acid is reversed. With choline chloride as substrate, malonic acid does not show a stimulation of the rate of oxidation of glycine as observed with α-ketoglutaric acid as substrate.

The malonate-stimulated oxidation of glycine, is not due to an increased rate of oxidation of glycine by the succinate-glycine cycle. Inhibition of the citric acid cycle with malonic acid increases the concentration of succinic acid but not that of succinyl CoA which is necessary for the operation of the succinate-glycine cycle. It is possible that the increased concentration of succinic acid facilitates an increased formation of succinyl CoA by an activating enzyme or more probably by a CoA transferase enzyme from acetyl CoA.

However, this is not true under the experimental conditions employed, for the rate of oxidation of glycine observed with 5mM sodium succinate is much lower than that observed with 5mM sodium α-ketoglutarate and malonate. Again no radioactive δ-aminolevulinic acid can be isolated from the reaction mixture containing glycine-2-C^{14} by amino acid chromatography, nor is there an inhibition of the rate of oxidation of glycine-2-C^{14} when non-labelled δ-aminolevulinic acid is added to the incubation medium.

Time course experiments conducted with 1mM and 5mM sodium α-ketoglutarate provide direct evidence to show that the higher concentration of the citric acid cycle intermediate is inhibitory to the oxidation of glycine. On the one hand, there is a large inhibition of $C^{14}O_2$ production for approximately 20 and 40 minutes from glycine-1-C^{14} and glycine-2-C^{14} respectively, with the higher concentration of sodium α-ketoglutarate, the oxygen consumption being linear with time. On the other hand, with 1mM sodium α-ketoglutarate the oxidation of glycine is linear with time and also considerably more rapid. The results of the time course experiments conducted with 10mM choline chloride are similar to those obtained with 1mM sodium α-ketoglutarate as substrate.

Isotopic dilution studies with L-serine indicate that the carboxyl carbon atom of glycine is converted to carbon dioxide only to a small extent via L-serine. Presumably, most of the labelled carbon dioxide produced from glycine-1-C^{14}

is released prior to the formation of serine. L-serine decreases the labelled carbon dioxide production from glycine-2-C^{14} to a large extent. In contrast to L-serine, the oxygen consumption of rat liver mitochondria increases considerably with the addition of D-serine. The oxidation of D-serine is probably due to the highly active D-amino acid oxidase associated with animal tissues. Apparently D-serine does not lie on the pathway of the oxidation of glycine, for it does not influence the production of $C^{14}O_2$ from either glycine-1-C^{14} or glycine-2-C^{14}.

The maximum inhibition of the rate of oxidation of the methylene carbon atom of glycine obtainable with sodium formate is less than 50%. The observed effect of sodium formate is probably not due to isotopic dilution. An isotopic dilution curve should be hyperbolic tending towards 100% inhibition at higher concentrations of the diluent. Data represented in Figure V definitely show that the curve flattens at about 45% inhibition with 0.75mM sodium formate and increasing the concentration of sodium formate does not increase the inhibition. The isolation of doubly labelled serine from glycine-2-C^{14} and the results discussed in Chapters II and IV, show that formic acid is not an obligatory intermediate in the oxidation of the methylene carbon atom of glycine. Formic acid is activated by liver mitochondria, and the β-carbon atom of L-serine is easily converted to the active one carbon fragment. Therefore, the addition of non-radioactive formate to a system synthesising doubly labelled serine

from glycine-2-C^{14} will result in an exchange of the isotope of the β-carbon atom of L-serine resulting in the formation of singly labelled L-serine. If glycine-2-C^{14} is solely oxidised via doubly labelled serine, formic acid can be predicted to inhibit the oxidation of glycine-2-C^{14} by not more than 50% by this exchange reaction. The results shown in Figure V are in agreement with such an hypothesis. The conversion of glycine-1-C^{14} to $C^{14}O_2$ is not related to the metabolism of formic acid, for the production of labelled carbon dioxide is not affected by the addition of even 5mM sodium formate.

The isotopic exchange reaction between formic acid and L-serine will be discussed in greater detail in Chapter IV, in connection with the oxidation of formic acid.

It has been well established by in vivo and in vitro investigations that the methylene carbon atom of glycine itself can serve as the active one carbon unit necessary for the serine synthesis (Sakami, 1948; 1949a; Siekevitz and Greenberg, 1949; 1950; Siekevitz et al. 1949; Mitomo and Greenberg, 1952; Vohra et al. 1956; White, 1958; Sanadi and Bennett, 1960; Richert et al. 1962). In the absence of an exogenous source of the active one carbon unit, the glycine-oxidase activity associated with isolated rat liver mitochondria, probably proceeds by a similar reaction.

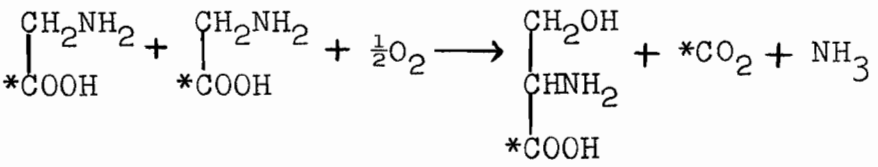
Isolation of the serine formed by incubating labelled glycine with rat liver mitochondria, yields radioactive serine.

The radioactivity of the serine formed from glycine-2-C^{14} is approximately twice as radioactive as the serine formed from glycine-1-C^{14}. Since the experimental conditions were identical but for the location of the label in the glycine molecule, the amount of serine synthesised in both cases should be the same. Hence, the higher radioactivity of the serine formed from glycine-2-C^{14} is not due to a larger amount of synthesis of radioactive serine, but due to a larger number of the radioactive carbon atoms finding their way to the serine molecule. Increasing the rate of the oxidation of glycine with reduced glutathione leads to a large increase in the radioactivity of the isolated serine, but still the ratio of the radioactivity incorporated into serine from glycine-2-C^{14} to that of glycine-1-C^{14} is two.

Glyoxylic acid is not an intermediate in the metabolism of glycine (see Chapter II). In vitro investigations with avian liver extracts that synthesise serine from glycine have shown that glyoxylic acid is not an intermediate in the reaction (Sanadi and Bennett, 1960; Richert et al. 1962).

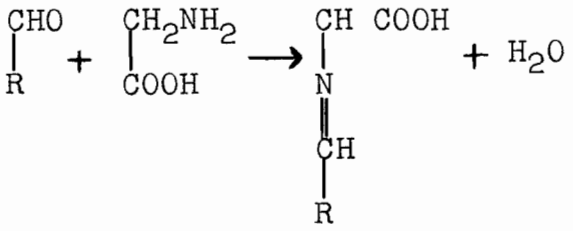
Rat liver mitochondria, employed in the present investigations, probably form serine from glycine by a reaction similar to, but not identical with, the one that is catalysed by serine aldolase (Equation 32).

The overall reaction may be written as shown on the next page.

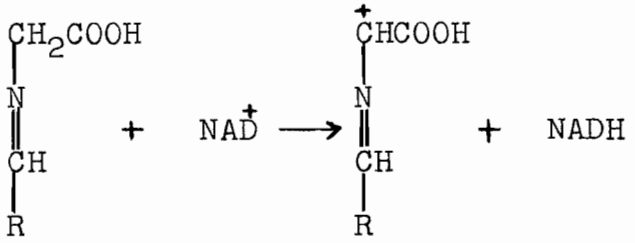


(40)

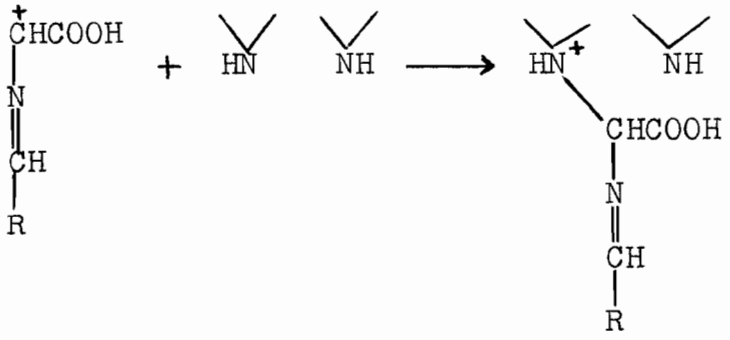
The reaction may be visualised to proceed through the following steps: (Adapted from Richert et al. 1962).



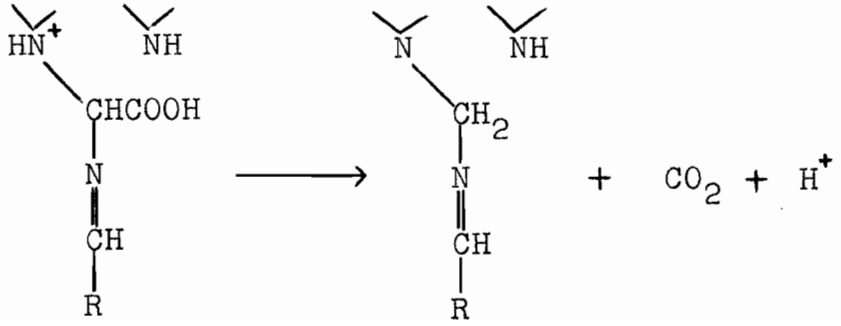
(A)



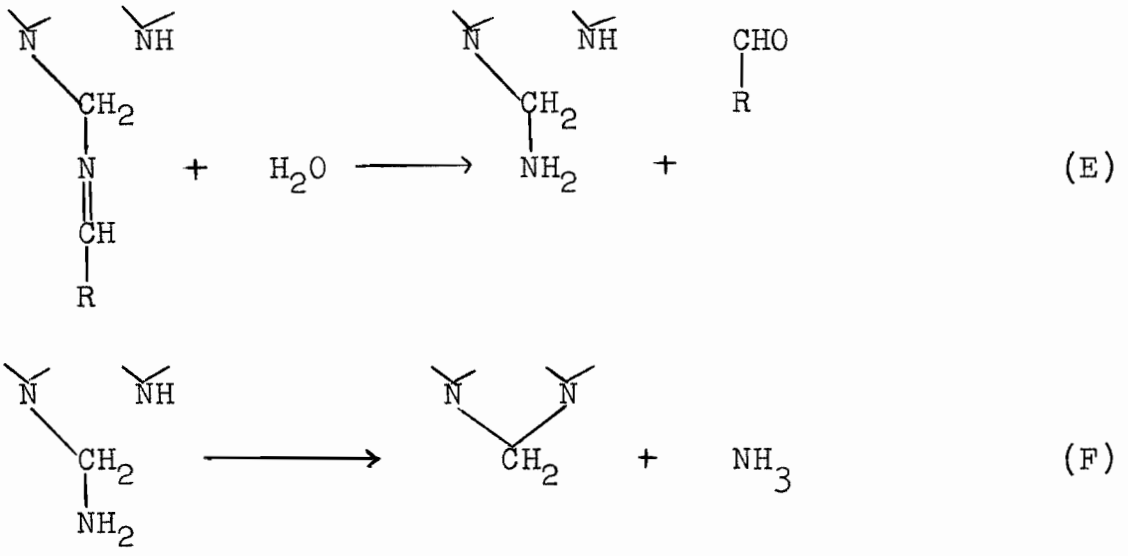
(B)



(C)



(D)

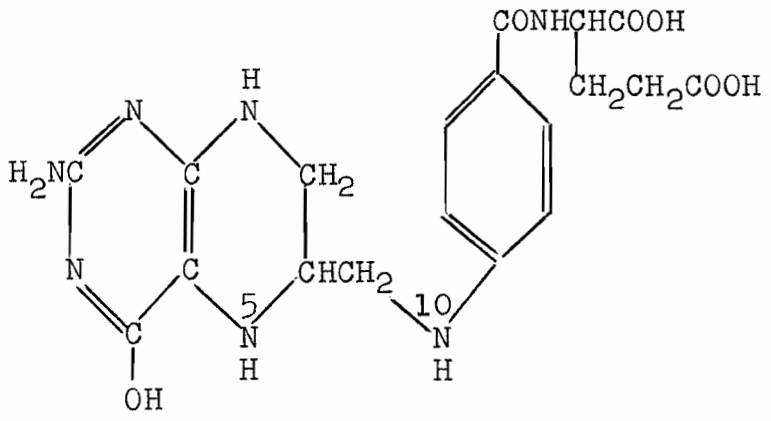


(E)

(F)

R-CHO and $\overset{\diagdown}{N}\ \overset{\diagdown}{N}$ are used to represent pyridoxal-5-phosphate and N_5 and N_{10} (without prejudice) of the THFA, respectively. NAD^+ is used to represent the cofactor linked with the dehydrogenase, though this need not be true. The reactions are shown to proceed unidirectionally towards $h^{5\text{-}10}$THFA synthesis but it is possible that all the reactions (but the one shown in Equation D) are reversible.

The structure of THFA is shown below.



The possibility of direct hydrogen transfer (Equation B) has long been recognised (see for instance, Westheimer, 1954). The charge on the resulting carbonium ion will facilitate the complex formation with THFA (Equation C), which can be decarboxylated with the simultaneous expulsion of a H^+ (Equation D). The Schiff's base can be hydrolysed at this stage (Equation E) forming formamino THFA. An enzyme similar to the formimino THFA cyclodeaminase of the rabbit liver (Tabor and Rabinowitz, 1956) probably forms f^{5-10}THFA (Equation F) which can react with one more molecule of glycine to form serine (Equation 32). Thus serine aldolase may form part of this system.

Summary:

(1) L-serine inhibited the rate of oxidation of glycine by rat brain and kidney cortex slices. The inhibition was mainly due to a decrease in the uptake of glycine by the slices.

(2) The influence of various substrates on the oxidation of glycine by rat liver mitochondria was studied. The highest rate of oxidation of glycine took place with choline chloride as substrate.

(3) High concentrations of the citric acid cycle intermediates inhibited the oxidation of glycine by rat liver mitochondria. This inhibition was relieved by malonic acid.

(4) The enzyme system(s) oxidising glycine-1-C^{14} and

glycine-2-C^{14} have different affinities for the substrate(s). Apparent K_m values of 5mM and 12mM were obtained for glycine-1-C^{14} and glycine-2-C^{14} respectively in rat liver mitochondria.

(5) The oxidation of the methylene carbon atom of glycine was inhibited by formic acid by 50%. This inhibition probably results from an isotopic exchange reaction between formic acid and doubly labelled serine formed from glycine-2-C^{14}.

(6) L-serine decreased the rate of oxidation of the methylene carbon atom of glycine considerably and had a small effect on the $C^{14}O_2$ production from glycine-1-C^{14}, whereas D-serine had no effect on the rate of oxidation of glycine-1-C^{14} or glycine-2-C^{14}.

(7) Serine isolated from the reaction mixture was doubly labelled with the methylenecarbon atom and singly labelled with the carboxyl carbon atom of glycine. A scheme has been proposed for the reactions involved in the incorporation of glycine into serine.

C H A P T E R IV

OXIDATION OF GLYCINE VIA SERINE.

Introduction.

In Chapter II, it was concluded that glyoxylic acid is unlikely to be an intermediate in the metabolism of glycine. The other possibility, namely that serine is an obligatory intermediate in the catabolism of glycine was next considered and direct and indirect evidence was obtained implicating L-serine in the oxidation of glycine (Chapter III). The oxidation of serine proceeds by deamination of L-serine to pyruvic acid, followed by the oxidation of pyruvic acid by the well-known pyruvic oxidase and citric acid cycle enzyme systems. Investigations on the glycogenic action of serine have definitely established pyruvic acid as an intermediate in the process of glycogen formation (see General Introduction). Experiments reported in this chapter were designed to obtain evidence for the oxidation of glycine and serine via pyruvic acid, by the isotopic dilution technique.

The methylene carbon atom of glycine contributes significantly to the active one carbon pool. Experiments reported in the last chapter have shown that formic acid is also in equilibrium with the active one carbon pool. Hence, it was of interest to investigate the oxidation of formic acid by rat liver mitochondria and the possible influence of

formic-oxidase activity of liver mitochondria on the oxidation of glycine.

The effects of pyruvic acid on the rate of oxidation of glycine by rat brain cortex slices.

The effects of increasing concentrations of sodium pyruvate on the production of labelled carbon dioxide from both glycine-1-C^{14} and glycine-2-C^{14} by rat brain cortex slices are shown in Table XIX. It has been shown that the rate of oxidation of glycine by rat brain cortex slices is increased twofold by the presence of glucose, and the effect approximately parellels the change in the rate of oxygen consumption (Sved, 1958). For this reason, when rat brain cortex slices were used, 10mM glucose was always added to the incubation mixture; also, whenever a significant change was observed in the oxygen consumption of the tissue preparation, the $C^{14}O_2$ produced per unit volume of oxygen consumed was also calculated.

It is evident from the data presented in Table XIX, that the rate of oxidation of the methylene carbon atom of glycine is sensitive to the presence of pyruvic acid in the incubation medium. The oxidation of glycine-2-C^{14} is almost abolished in the presence of 15mM sodium pyruvate. However, the conversion of glycine-1-C^{14} to labelled carbon dioxide is affected very little. Actually, a slight stimulation of the production of labelled carbon dioxide from glycine-1-C^{14} occurs, which is probably related to the increased metabolic

TABLE XIX

EFFECT OF PYRUVIC ACID ON THE OXIDATION OF GLYCINE BY RAT BRAIN CORTEX SLICES.

Sodium pyruvate (mM)	GLYCINE-1-C^{14}			GLYCINE-2-C^{14}		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
0.0	53.0	69.5	1.32 (100)	55.0	5.20	0.096 (100)
0.3	53.5	67.5	1.26 (95)	57.0	2.60	0.046 (48)
0.6	53.5	74.0	1.38 (104)	53.0	2.65	0.050 (52)
1.2	52.5	79.5	1.50 (114)	58.0	2.35	0.040 (41)
2.5	52.0	75.0	1.44 (109)	50.0	1.70	0.034 (35)
4.0	62.0	82.0	1.32 (100)	55.0	1.55	0.028 (29)
6.0	65.0	74.0	1.14 (86)	54.0	1.35	0.026 (27)
10.0	61.5	77.5	1.26 (95)	58.0	1.22	0.022 (23)
15.0	63.5	76.0	1.20 (91)	57.0	0.85	0.008 (17)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat brain cortex slices (ca. 70mg) were incubated with 2mM labelled glycine and 10mM glucose with additions of sodium pyruvate as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

activity of the tissue when pyruvic acid is added. If the conversion of glycine-1-C^{14} to $C^{14}O_2$ is represented in terms of the amount of oxygen consumed, a slight inhibition amounting to approximately 10% is observed.

The effects of succinic acid on the rate of oxidation of glycine by rat brain cortex slices.

Table XX shows the effects of increasing concentrations of sodium succinate on the rate of oxidation of glycine by rat brain cortex slices. The amount of oxygen consumed by the respiring rat brain cortex slices increases when the medium concentration of succinate is increased. The conversion of glycine-1-C^{14} to labelled carbon dioxide is also increased by the presence of sodium succinate. When the results are expressed as a ratio of the labelled carbon dioxide produced per unit volume of oxygen consumed, a constant approximately equal to 1.05 is obtained, for all concentrations of succinate used. When similarly expressed, the rate of oxidation of the methylene carbon atom of glycine is decreased considerably and an inhibition of approximately 60% is observed with 10mM sodium succinate.

The effects of pyruvic acid on the rate of oxidation of glycine by rat kidney cortex slices.

The effects of increasing concentrations of sodium pyruvate on the production of labelled carbon dioxide from glycine-1-C^{14} and glycine-2-C^{14} by rat kidney cortex slices were also investigated. The results obtained (Table XXI)

T A B L E XX

EFFECT OF SUCCINIC ACID ON THE OXIDATION OF GLYCINE BY RAT BRAIN CORTEX SLICES.

Sodium succinate (mM)	GLYCINE-1-C^{14}			GLYCINE-2-C^{14}		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
0.0	53.0	58	1.10	58.0	4.9	0.086 (100)
1.0	54.0	59	1.10	59.5	2.5	0.042 (48)
3.0	59.5	58	0.98	68.5	3.2	0.046 (52)
6.0	60.5	61	1.00	68.0	2.8	0.021 (48)
10.0	65.5	69	1.06	67.5	2.4	0.018 (41)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat brain cortex slices (ca. 70mg) were incubated with 2mM labelled glycine and 10mM glucose with additions of sodium succinate as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

T A B L E XXI

EFFECT OF PYRUVIC ACID ON THE OXIDATION OF GLYCINE BY RAT KIDNEY CORTEX SLICES.

Sodium pyruvate (mM)	GLYCINE-1-C^{14}			GLYCINE-2-C^{14}		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
0.0	78.0	600	7.6 (100)	80.0	98.5	1.24 (100)
0.5	85.0	705	8.2 (108)	88.0	85.0	0.96 (77)
1.0	82.5	630	7.6 (100)	80.5	65.5	0.82 (66)
1.5	91.5	700	7.6 (100)	94.0	56.8	0.60 (48)
3.0	102.0	655	6.4 (84)	94.5	44.0	0.46 (37)
6.0	101.5	675	6.6 (86)	102.0	34.7	0.34 (27)
10.0	106.5	710	6.6 (86)	101.0	31.0	0.30 (24)
15.0	107.5	740	6.8 (89)	113.0	35.6	0.32 (26)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat kidney cortex slices (ca. 60mg) were incubated with 2mM labelled glycine with additions of sodium pyruvate as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

are similar to those obtained in similar experiments conducted with rat brain cortex slices. The oxygen consumption of rat kidney cortex slices also increases on the addition of sodium pyruvate. The rate of oxidation of the methylene carbon atom of glycine per unit volume of oxygen consumed is inhibited by approximately 75%, when the sodium pyruvate concentration of the medium is 6mM and above. Under similar conditions, the conversion of glycine-1-C^{14} to labelled carbon dioxide is inhibited by only 15%.

The effects of pyruvic acid on the rate of oxidation of L-serine-U-C^{14} by rat kidney and brain cortex slices.

The effects of sodium pyruvate on the rate of oxidation of L-serine-U-C^{14} was also investigated. The results obtained with rat brain and kidney cortex slices are presented in Table XXII. L-serine-U-C^{14} is oxidised much faster than glycine-2-C^{14} by either tissue. Thus, the production of labelled carbon dioxide is approximately 5 times faster by rat kidney cortex slices and 12 times faster by rat brain cortex slices than is the production of labelled carbon dioxide from glycine-2-C^{14} by the same tissues. As shown in the previous experiments, sodium pyruvate increases the oxygen consumption of these tissues and so, the labelled carbon dioxide produced per unit volume of oxygen consumed was used to evaluate the effect of pyruvic acid on the rate of oxidation of L-serine-U-C^{14}. There is a considerable decrease in the rate of oxidation of L-serine-U-C^{14} by both

T A B L E XXII

EFFECT OF PYRUVIC ACID ON THE OXIDATION OF L-SERINE-U-C^{14} BY RAT BRAIN AND KIDNEY CORTEX SLICES.

Sodium pyruvate (mM)	RAT BRAIN CORTEX SLICES			RAT KIDNEY CORTEX SLICES		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
0.0	51.5	58.5	1.15 (100)	93.5	501	54.0 (100)
1.0	51.0	57.0	1.12 (97)	119.0	369	31.2 (58)
1.5	53.5	57.0	1.17 (93)	91.0	300	33.0 (61)
2.0	48.0	54.0	1.13 (98)	108.0	345	31.8 (59)
2.5	45.0	42.0	0.94 (82)	118.5	321	27.0 (50)
3.0	51.0	45.0	0.87 (77)	-	-	-
3.5	-	-	-	112.5	246	28.2 (41)
4.0	68.5	40.5	0.83 (72)	-	-	--
5.0	-	-	-	121.5	243	19.8 (37)
5.5	61.0	37.5	0.68 (60)	-	-	-
7.5	61.0	35.5	0.64 (56)	90.0	147	16.2 (30)
10.0	55.0	33.0	0.53 (46)	122.0	185	15.0 (28)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat brain cortex slices (ca. 70mg) or rat kidney cortex slices (ca. 60mg) were incubated with 2mM L-serine-U-C^{14} and additions of pyruvic acid as shown above in a total volume of 3ml, for 60 minutes, under the standard incubation conditions. (10mM glucose was also added to the incubation mixture when brain cortex slices were used)

rat brain and kidney cortex slices when sodium pyruvate is added to the incubation mixture. The inhibition of the rate of oxidation of L-serine-U-C^{14} by rat kidney cortex slices in the presence of sodium pyruvate is almost equal in magnitude to the inhibition of the rate of oxidation of glycine-2-C^{14} (Table XXI) observed in the presence of sodium pyruvate. Rat brain cortex slices, however, show a decreased rate of oxidation of L-serine-U-C^{14} amounting to 46% with 10mM sodium pyruvate, as compared to a value of 26% observed with glycine-2-C^{14}.

The effects of glycine on the oxidation of L-serine-U-C^{14} by rat brain and kidney cortex slices.

It was shown in the last chapter (Tables XI and XII) that the rate of oxidation of glycine by rat brain cortex slices and to a lesser extent, rat kidney cortex slices, is decreased on the addition of L-serine. The inhibition was regarded to be due to a decreased transport of glycine into the cell, in the presence of L-serine. It was therefore decided to investigate whether the presence of glycine can adversely affect the rate of oxidation of L-serine-U-C^{14} by rat brain and kidney cortex slices. The oxygen consumption of both rat brain and kidney cortex slices is not influenced by the presence of glycine. The rate of oxidation of L-serine-U-C^{14} by rat kidney cortex slices is not significantly affected when glycine is added to the incubation medium. However, glycine decreases the rate of

oxidation of L-serine-U-C^{14} by rat brain cortex slices considerably, an inhibition of approximately 50% being observed with 10mM glycine (Table XXIII).

The influence of varying concentrations of substrate on the rate of oxidation of L-serine-U-C^{14} by rat brain cortex slices.

The influence of substrate concentration on the velocity of the reaction catalysed by the serine-oxidase system of rat brain cortex slices was studied. The results of this experiment are shown in Figure VII. The rate of oxidation of L-serine-U-C^{14} by rat brain cortex slices is almost linear up to a concentration of 10mM, the rate decreasing only slightly with the higher concentrations. The oxygen consumption of the rat brain cortex slices in this experiment was approximately 52µl per 100mg wet weight of tissue per hour and did not change with increasing concentrations of L-serine.

The influence of varying conentrations of substrate on the rate of oxidation of L-serine-U-C^{14} by rat liver mitochondria.

Direct evidence showing that L-serine is oxidised by rat liver mitochondria at a sufficiently rapid rate to implicate it as an intermediate in the oxidation of glycine is presented in Figure VIII. The curve in this figure shows the amount of labelled carbon dioxide produced from different concentrations of L-serine-U-C^{14}. The rate of oxidation of

T A B L E XXIII

EFFECT OF GLYCINE ON THE OXIDATION OF L-SERINE-U-C^{14} BY RAT KIDNEY AND BRAIN CORTEX SLICES.

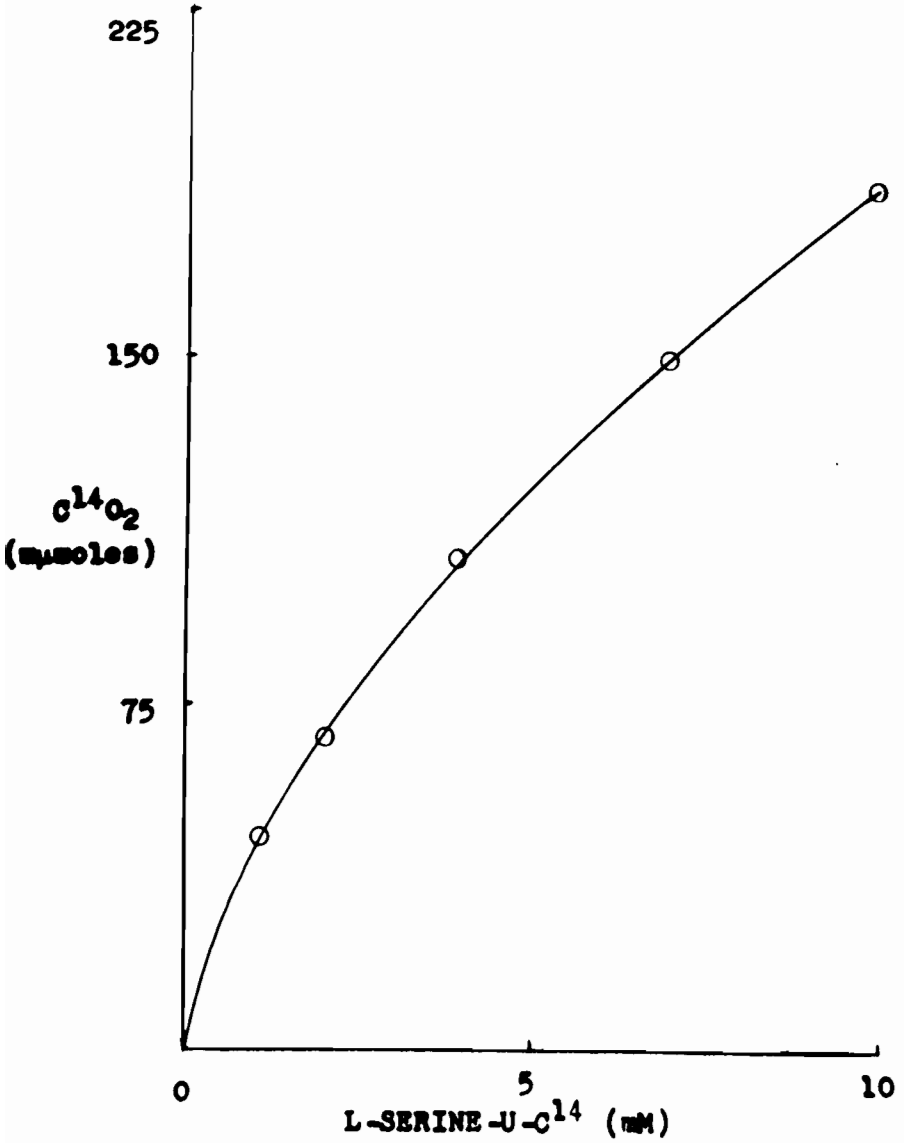
Glycine (mM)	RAT KIDNEY CORTEX SLICES		RAT BRAIN CORTEX SLICES	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	86.0	390	54.0	60.0 (100)
0.5	79.0	390	54.5	55.5 (93)
1.0	79.0	402	52.0	52.5 (87)
1.5	75.5	405	49.5	51.0 (85)
2.0	79.0	420	53.0	45.0 (75)
3.0	79.0	430	52.5	42.0 (70)
5.0	76.0	399	59.0	36.0 (60)
7.5	79.0	405	60.0	37.5 (62)
10.0	79.5	375	59.0	30.0 (50)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat kidney cortex slices (ca. 60mg) or rat brain cortex slices (ca. 70mg) were incubated with 2mM L-serine-U-C^{14} and additions of glycine as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions. (10mM glucose was also added to the incubation mixture when rat brain cortex slices were used)

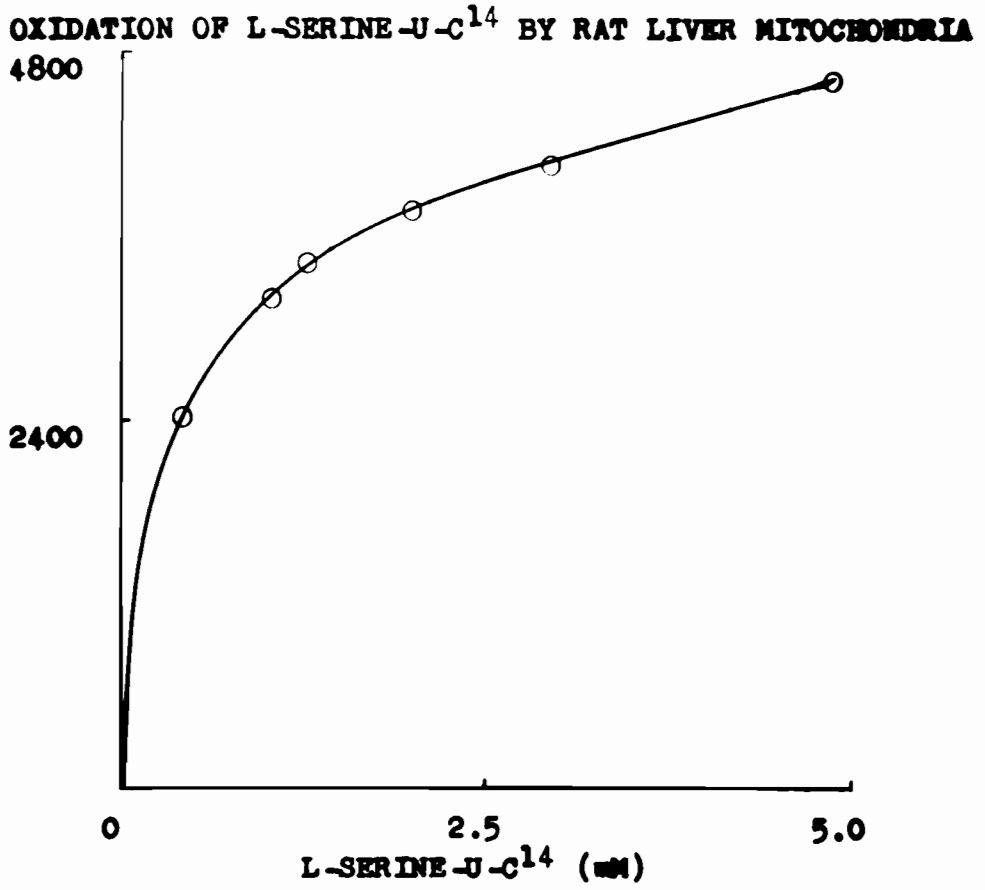
F I G U R E VII

OXIDATION OF L-SERINE-U-C^{14} BY RAT BRAIN CORTEX SLICES



Rat brain cortex slices (ca. 70mg) were incubated with different concentrations of L-serine-U-C^{14} and 10mM glucose for 60 minutes, under the standard incubation conditions.

F I G U R E VIII



Mitochondria from 1g of fresh rat liver were incubated with different concentrations of L-serine-U-C^{14} and 10mM choline chloride in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

L-serine-U-C^{14} increases with increase in the concentration of L-serine, the rise of the curve being very steep at the lower concentrations. The oxygen consumption of rat liver mitochondria in this experiment was approximately 400µl and did not show any significant change in the presence of different concentrations of L-serine in the medium.

The effects of pyruvic, succinic, α-ketoglutaric and hexanoic acids on the oxidation of glycine-1-C^{14}, glycine-2-C^{14} and L-serine-U-C^{14} by rat liver mitochondria.

The oxygen uptake of rat liver mitochondria increases by 50 to 100% on the addition of pyruvate, succinate, α-ketoglutarate or hexanoate. There is a considerable decrease in the production of $C^{14}O_2$ from glycine-1-C^{14} on addition of these metabolites, the largest inhibition being observed with α-ketoglutarate (approximately 65%) and the smallest inhibition being observed with sodium succinate (approximately 45%). In contrast to their effect on the rate of $C^{14}O_2$ production from glycine-1-C^{14}, these metabolites cause far greater inhibition of the rate of oxidation of the methylene carbon atom of glycine. The inhibitions in all these cases are more than 75%, potassium hexanoate being the most effective, decreasing the production of $C^{14}O_2$ from glycine-2-C^{14} by approximately 85%. The rate of oxidation of L-serine-U-C^{14} is also decreased by these metabolites, the decrease in the oxidation amounting to approximately 65% with potassium hexanoate and sodium succinate and approximately 70% with sodium pyruvate (**Table XXIV**).

T A B L E XXIV

EFFECTS OF PYRUVIC, SUCCINIC, HEXANOIC AND α-KETOGLUTARIC ACIDS ON THE OXIDATIONS OF GLYCINE-1-C^{14}, GLYCINE-2-C^{14} AND L-SERINE-U-C^{14} AND THE EFFECT OF GLYCINE ON THE OXIDATION OF L-SERINE-U-C^{14} BY RAT LIVER MITOCHONDRIA.

Additions	Conc.	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}		L-SERINE-U-C^{14}	
		Q	$C^{14}O_2$	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	-	428	994 (100)	384	276 (100)	470	5050 (100)
Pyruvate	4mM	760	555 (56)	700	62 (22)	680	1092 (29)
Succinate	4mM	720	651 (66)	671	74 (27)	647	1374 (34)
Hexanoate	4mM	735	510 (51)	700	45 (16)	765	1392 (34)
α-ketoglutarate	4mM	744	462 (46)	761	53 (19)	-	-
Nil	-	444	800 (100)	468	292 (100)	380	3450 (100)
L-glutamate	5mM	660	512 (64)	652	92 (31)	-	-
Glycine	2mM	-	-	-	-	397	3260 (98)

Q and $C^{14}O_2$ values as described in Table VII. Values in parentheses refer to percentages.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled substrate and 10mM choline chloride with additions as shown above, in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

The effects of hydroxy pyruvic acid on the rate of oxidation of glycine by rat liver mitochondria.

The effects of different concentrations of sodium hydroxy pyruvate on the production of $C^{14}O_2$ from glycine-1-C^{14} and glycine-2-C^{14} by rat liver mitochondria are shown in Table XXV. The oxygen consumption of rat liver mitochondria shows a slight increase with low concentrations of sodium hydroxy pyruvate but drops again with 5mM sodium hydroxy pyruvate. The production of $C^{14}O_2$ from glycine-1-C^{14} is affected very little, a decrease of approximately 10% being observed in the presence of 5mM hydroxy pyruvate. However, the rate of oxidation of the methylene carbon atom of glycine is inhibited by approximately 45% under identical conditions. The effect of hydroxy pyruvate on the rate of oxidation of glycine by rat liver mitochondria resembles closely the effect produced by L-serine (Table XVI).

The effects of glycine and serine on the rate of oxidation of formic acid by rat liver mitochondria.

The effects of increasing concentrations of glycine and L-serine on the rate of oxidation of sodium formate-C^{14} by rat liver mitochondria is shown in Figure IX. The oxygen consumption of rat liver mitochondria in this experiment was approximately 360µl in one hour and did not alter appreciably in the presence of different concentrations of glycine or L-serine.

T A B L E XXV

EFFECT OF HYDROXY PYRUVIC ACID ON THE OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

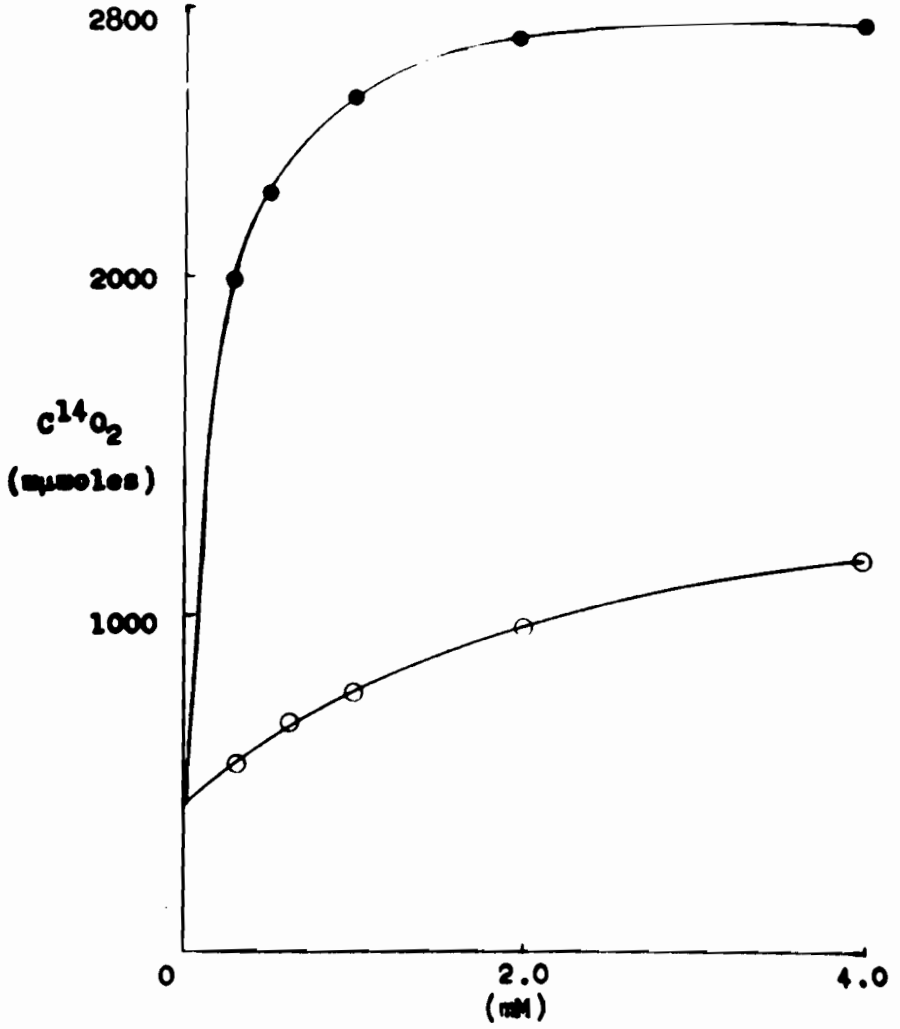
Hydroxy pyruvic acid (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.0	491	697	480	286
1.0	536	638	534	101
3.0	574	622	526	103
5.0	484	646	494	126

Q and $C^{14}O_2$ values as described in Table VII.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 10mM choli ne chloride with additions as shown above, for 30 minutes, in a total volume of 1.5ml, under the standard incubation conditions

F I G U R E IX

THE EFFECTS OF GLYCINE AND L-SERINE ON THE OXIDATION OF FORMATE-C^{14} BY RAT LIVER MITOCHONDRIA



○-○ $C^{14}O_2$ (, Glycine) ●-● $C^{14}O_2$ (, L-serine)

Mitochondria from 1g of fresh rat liver were incubated with 2mM sodium formate-C^{14} and 10mM choline chloride with additions as shown in a total volume of 1.5ml, for 30 minutes, under the standard incubation conditions.

The amount of labelled carbon dioxide produced from sodium formate-C^{14} increases steadily with increasing concentrations of glycine, reaching a value of 1100 mμmoles with 4mM glycine. The addition of L-serine causes a sharp increase in the rate of oxidation of sodium formate-C^{14} reaching a maximum value of approximately 2700mμmoles at 2mM L-serine and changes little on further increase in the concentration.

The incorporation of formic acid into serine by rat liver mitochondria.

The amino compounds formed during the metabolism of sodium formate-C^{14} by rat liver mitochondria in the presence or absence of 4mM glycine or 2mM L-serine, were isolated by ion exchange and paper chromatography as described in the section on Materials and Methods. No radioactive spots appeared on the paper chromatograms in the absence of these amino acids. However, in the presence of glycine or L-serine radioactive serine was shown to be present on the paper chromatograms (Figure X). The results were semiquantitative and no inference could be drawn about the steady state levels of the radioactive serine formed during the reactions. However, under identical conditions, the radioactivity incorporated from sodium formate-C^{14} into serine, was approximately 4 times greater when L-serine was present in the medium than when glycine was present.

The effects of hexanoic acid and ethanol on the rate of

F I G U R E X

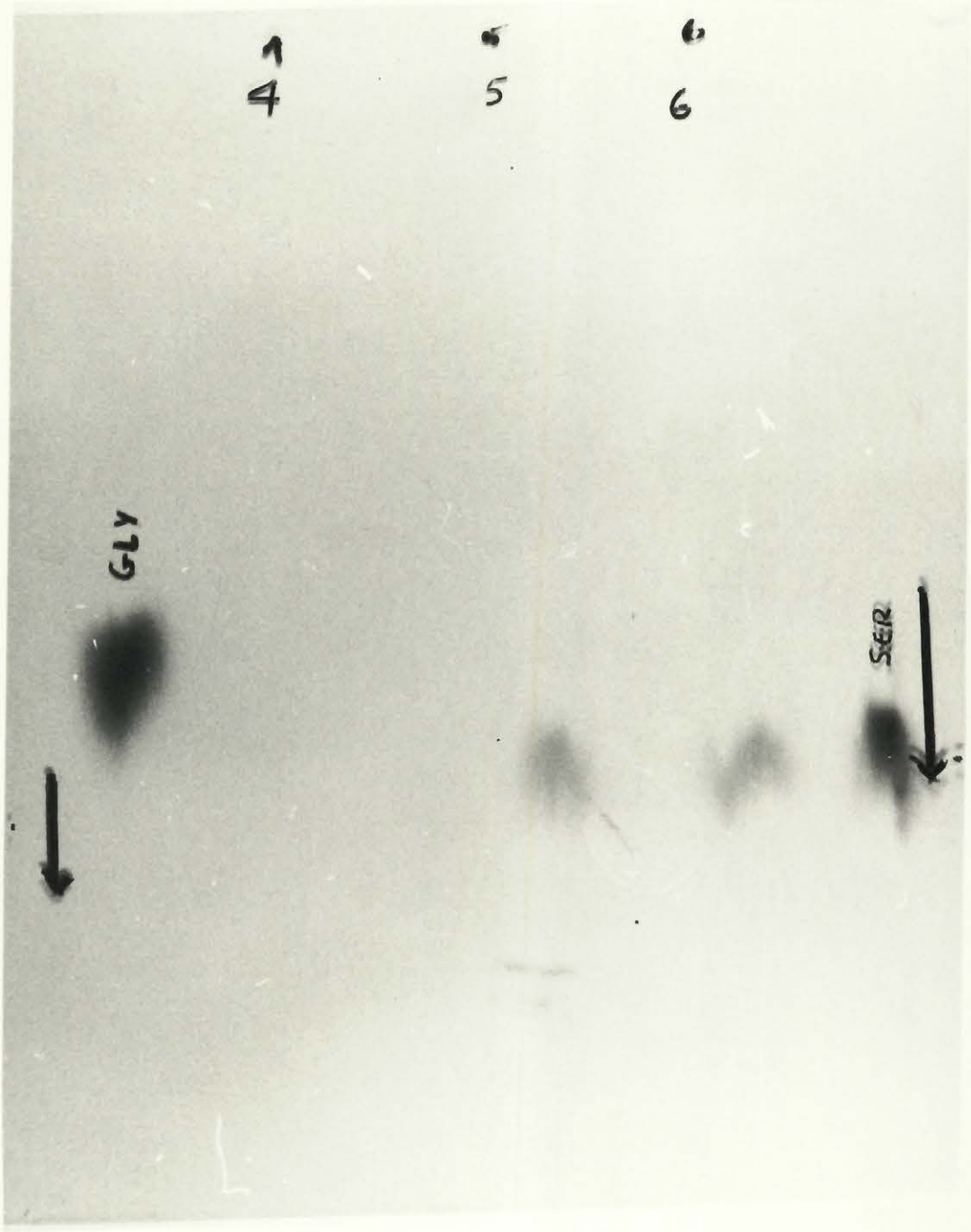
INCORPORATION OF FORMIC ACID-C^{14} INTO SERINE

Rat liver mitochondria obtained from 1g of fresh liver were incubated with 10mM choline chloride and 2.0mM sodium formate-C^{14} with additions as shown below, for 30 minutes, in a total volume of 1.5ml, under the standard incubation conditions.

4 - Nil.

5 - Glycine (10mM).

6 - L-serine (2mM).


4
5
6
GLY
SER

oxidation of formic acid by rat liver mitochondria in the presence and absence of glycine and L-serine.

The effects of 4mM potassium hexanoate and 100mM ethanol on the rate of oxidation of sodium formate-C^{14} by rat liver mitochondria, added singly or together, are shown in Table XXVI. The results obtained in similar experiments in the presence of 10mM glycine and 4mM L-serine are included in the same table. The presence of 4mM potassium hexanoate increases the oxygen consumption of rat liver mitochondria more than twofold. However, the oxidation of sodium formate-C^{14} is decreased by 66mμmoles. When 100mM ethanol is added to the incubation mixture, there is little effect on the oxygen consumption of rat liver mitochondria, but the amount of labelled carbon dioxide production from sodium formate-C^{14} is decreased by 124mμmoles. Potassium hexanoate and ethanol, when present together, decrease the oxidation of sodium formate-C^{14} by 166mμmoles.

The effects of potassium hexanoate and ethanol were also studied on the glycine-stimulated oxidation of sodium formate-C^{14}. The glycine-stimulated oxidation of sodium formate-C^{14} is inhibited strikingly by the presence of potassium hexanoate, a decrease of 698mμmoles being observed under these experimental conditions. The inhibition caused by 100mM ethanol is 86mμmoles. When present together, potassium hexanoate and ethanol have an additive effect.

T A B L E XXVI

EFFECTS OF GLYCINE, L-SERINE, HEXANOIC ACID AND ETHANOL ON THE OXIDATION OF FORMIC ACID BY RAT LIVER MITOCHONDRIA.

Further additions	Nil		Glycine (10mM)		L-Serine (2mM)	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	274	310	360	850	330	1800
Hexanoate (4mM)	580	244 (66)	532	152 (698)	562	488 (1312)
Ethanol (100mM)	286	186 (124)	310	764 (86)	344	1800 (0)
Hexanoate (4mM) and Ethanol (100mM)	430	144 (166)	556	54 (796)	552	422 (1378)

Q and $C^{14}O_2$ values as described in Table VII. Figures in parentheses refer to inhibition compared to control (Top horizontal row)

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled sodium formate with 10mM choline chloride and additions as shown above in a total volume of 1.5ml, for 30 minutes, under the standard incubation conditions.

As shown before (Figure IX), the presence of L-serine increases the rate of oxidation of sodium formate-C^{14}. Again, the presence of potassium hexanoate causes a great inhibition of the serine-stimulated oxidation of formate, a decrease of 1312mμmoles being observed. The addition of 100mM ethanol has no observable effect on L-serine-stimulated oxidation of sodium formate-C^{14}. However, ethanol increases the inhibition produced by the presence of potassium hexanoate by 66mμmoles. The absence of an inhibition by ethanol comparable to its effect under other conditions (presence or absence of glycine, presence of L-serine and potassium hexanoate, glycine and potassium hexanoate) is probably within the experimental error, since the expected inhibition is 105mμmoles and the control value is 1800mμmoles (6%).

Oxidation of formic acid by different pathways under the influence of glycine or L-serine.

The inhibitions of the rate of oxidation of sodium formate-C^{14} caused by the presence of potassium hexanoate or ethanol are shown in parentheses in Table XXVI. These values and the amount of oxidation of sodium formate-C^{14} that is insensitive to the combined actions of these two agents, are recompiled in Table XXVII. The amount of oxidation of sodium formate-C^{14} that is affected by potassium hexanoate, presumably proceeds by oxidation via the citric acid cycle, the amount affected by the

T A B L E XXVII

DIFFERENT PATHWAYS OF OXIDATION OF FORMIC ACID BY RAT LIVER MITOCHONDRIA.

Pathways	ADDITIONS		
	Nil	Glycine (10mM)	L-Serine (4mM)
Via catalase	124	86	105
Via citric acid cycle	66	698	1312
Via THFA	144	54	422
Total	334 (310)	838 (850)	1839 (1800)

All figures refer to $C^{14}O_2$ values recompiled from Table XXVI as described in the text. Figures in parentheses refer to observed total.

presence of ethanol occurs as a result of the peroxidase activity of catalase and the extent of oxidation which is uninfluenced by either of these compounds probably occurs via a THFA intermediate.

The oxidation of sodium formate-C^{14} via the catalase pathway seems to proceed with little change in the presence of glycine and probably also in the presence of L-serine. Approximately 20% of the oxidation of sodium formate proceeds via the citric acid cycle. However, in the presence of glycine the oxidation of sodium formate-C^{14} via the citric acid cycle increases approximately tenfold, and twentyfold in the presence of L-serine. Normally, the THFA catalysed pathway accounts for almost 50% of the oxidation of sodium formate-C^{14}. However, in the presence of glycine the oxidation of sodium formate-C^{14} by this pathway is decreased to almost a third and in the presence of L-serine increased threefold.

The sums of these assumed pathways of oxidation of sodium formate-C^{14} by rat liver mitochondria are shown in parentheses in Table XXVII. The experimentally observed values of the same are also taken from Table XXVI and given side by side. The calculated values and the observed values are in close agreement.

Discussion.

The isolation of doubly labelled serine during the

metabolism of glycine-2-C^{14} was discussed in the last chapter. Isotopic dilution experiments also indicated that the production of $C^{14}O_2$ from glycine-1-C^{14} was little affected by L-serine indicating that the first step in the oxidation of glycine can be represented by Equation 40. According to this representation the conversion of the carboxyl carbon atom of glycine to carbon dioxide occurs prior to the formation of serine and neither serine nor any of the metabolites formed from serine should affect this process. However, the oxidation of the methylene carbon atom of glycine should be inhibited by any metabolite that affects the oxidation of serine. The results reported in this chapter are in agreement with such a hypothesis.

The production of labelled carbon dioxide from glycine-1-C^{14} by rat brain and kidney cortex slices is inhibited to the extent of only 10 to 15% by pyruvic acid. However, the oxidation of the methylene carbon atom of glycine is inhibited approximately 75% by pyruvic acid in these tissues. The oxidation of glycine-2-C^{14} presumably proceeds via the formation of the obligatory intermediate, pyruvic acid. The effect of succinic acid on the oxidation of glycine-2-C^{14} by rat brain cortex slices provides additional evidence. The acetyl CoA formed from glycine via serine and pyruvic acid will be labelled only by the methylene carbon atom of glycine; and a citric acid cycle intermediate can be predicted to inhibit the oxidation of glycine-2-C^{14} by isotopic dilution,

with little effect on the production of labelled carbon dioxide from glycine-1-C^{14}. The results obtained with rat brain cortex slices are in complete agreement with this prediction.

The oxidation of L-serine-U-C^{14} by rat brain and kidney cortex slices proceeds at a much faster rate than the oxidation of glycine-2-C^{14}. The oxidation of L-serine is inhibited by pyruvic acid to approximately the same extent in rat kidney cortex slices and to a lesser extent in rat brain cortex slices than the oxidation of glycine-2-C^{14}.

Weinhouse (1955b) has reported that rat liver slices oxidise serine much faster than glycine and that a large amount of the radioactivity from glycine-2-C^{14} is found in the serine isolated from the incubation mixture. However, since there was no accumulation of labelled acetoacetate when serine-3-C^{14} was being oxidised by rat liver slices, he suggested that the oxidation of serine proceeds via glycine. These results are not in conformity with the recently reported results of Leibman and Fellner (1962) that the acid formed during the metabolism of L-serine in rat liver homogenates is atleast 90% pyruvic acid. The results reported in this chapter are in agreement with those reported by Leibman and Fellner (1962) and hence do not support the conclusions drawn by Weinhouse (1955b).

The oxidation of L-serine-U-C^{14} by rat kidney cortex

slices or by rat liver mitochondria is not affected by glycine. An inhibition of approximately 50% is, however observed with 10mM glycine, in the oxidation of L-serine-U-C^{14} by rat brain cortex slices. Under similar conditions, it has been reported that the concentration of L-serine inside the tissue is decreased to approximately 50% (Abadom and Scholefield, 1962). The influence of substrate concentrations on the rate of oxidation of L-serine-U-C^{14} shows that the rate of oxidation of L-serine-U-C^{14} by rat brain cortex slices is largely dependent on the concentration of L-serine. The inhibition of the oxidation of L-serine-U-C^{14} by glycine is probably a reflection of a decrease in the concentration of L-serine inside the cell under these conditions. These results are not in agreement with those reported by Nakada and Weinhouse (1955b) These workers have reported that the oxidation of serine-3-C^{14} by rat liver homogenates is inhibited by approximately 60% on addition of a mixture of glycine and glyoxylic acid. If the oxidation of serine proceeds via glycine and glyoxylic acid, as proposed by these workers, the radioactivity from serine-3-C^{14} will appear in the one carbon fragment and will not find its way to glycine or glyoxylic acid. The inhibition of the oxidation of serine by glycine and glyoxylic acid observed by these workers is, therefore due to the non-specific effect of the aldehyde (glyoxylic acid), which is known to interfere with the operation of the citric acid cycle.

The results obtained with rat liver mitochondria are

similar to those obtained with rat brain and kidney cortex slices. Thus, it was shown that the oxidation of the methylene carbon atom of glycine was inhibited much more than the conversion of the carboxyl carbon atom of glycine to carbon dioxide, by pyruvic, succinic, hexanoic, α-keto-glutaric and L-glutamic acids. Higher concentration of β-hydroxy butyric acid has already shown to increase the oxygen consumption of rat liver mitochondria. Because of its ability to produce acetyl CoA during oxidation, β-hydroxy butyric acid inhibits the oxidation of the methylene carbon atom of glycine with little effect on the production of carbon dioxide from the carboxyl carbon atom of glycine (Chapter III, Table XIII).

The results obtained with hydroxypyruvic acid on the oxidation of glycine by rat liver mitochondria show that the effect observed is very similar to that obtained with L-serine. Pyruvic acid is more effective in inhibiting the oxidation of glycine than is hydroxypyruvic acid. The oxidation of glycine probably proceeds via the formation of the intermediate pyruvic acid, and the observed inhibition with hydroxypyruvic acid could be explained by its prior conversion to L-serine by transamination. D-serine, though rapidly oxidised by rat liver mitochondria (presumably to -hydroxypyruvic acid by D-aminoacid oxidase) is not involved in the oxidation of glycine (see Chapter III, Table XVII). Serine dehydrase (Chargaff and Sprinson, 1943; Sayre and Greenberg, 1956) is probably the enzyme

concerned with the production of pyruvic acid from L-serine (Equation 35).

The ability of formic acid to replace the methylene carbon atom of glycine in the synthesis of the β-carbon atom of serine was demonstrated in the last chapter. Since the incorporation of formic acid into serine could provide an alternate pathway for the oxidation of formic acid via the serine-pyruvate system, the oxidation of formic acid by rat liver mitochondria was investigated in detail. As expected, glycine stimulated the oxidation of formic acid. It was interesting to observe also that L-serine could produce a much greater stimulation than could glycine and at a much lower concentration.

The stimulation of formic acid oxidation by glycine in rat liver mitochondria increases with increasing concentrations of glycine. This is consistent with the idea that the presence of glycine increases the systhesis of serine from glycine and the active one carbon fragment derived from formic acid (Equation 32). However, the presence of L-serine also increases the oxidation of formic acid, the stimulation being approximately twice as great as that obtained with glycine and reaching a maximum value at approximately 1.5mM L-serine. These results indicate the possibility of an exchange reaction between formic acid and L-serine whereby the labelled carbon atom of formic acid is transferred to L-serine. The exchange reaction may be represented as

follows:

$$\text{Gly-F} + \text{Enz} \rightleftharpoons \text{Enz-Gly-F} \rightleftharpoons \text{F} + \text{Enz-Gly} \qquad (43)$$

Gly-F, Enz, and F are used in this equation to represent L-serine, enzyme and the active one carbon fragment respectively.

This exchange reaction as written in Equation 43, has the interesting feature that glycine cannot participate in it. The glycine moiety of L-serine is probably attached to the enzyme by the amino and/or carboxyl group(s), leaving the one carbon fragment free for exchange and the glycine moiety is released from the enzyme only as L-serine (Gly-F) and not as free glycine. If glycine can participate in this exchange reaction, performing the experiment with labelled glycine and non-labelled serine, should lead to a stimulation of the oxidation of glycine by the increased radioactivity that now appears in serine, similar to the stimulation observed with radioactive formic acid and L-serine. However, it has been shown (Chapter III) that L-serine inhibits the oxidation of glycine under similar conditions.

Serine aldolase, catalysing the reaction represented by Equation 32, should be able to decompose the Enz-Gly complex reversibly. This implies that if the reaction is catalysed by serine aldolase, glycine also will be able to participate in the exchange reaction. Since glycine does not participate in this reaction (as discussed above),

the exchange reaction is catalysed by an enzyme other than serine aldolase.

The incorporation of radioactive formic acid into serine in the presence of glycine or serine as predicted above was also demonstrated by chromatographic isolation and resolution of the amino-compounds formed during the incubation (Figure IV).

Chance (1949; 1950) has shown that the catalase-hydrogen peroxide complex can oxidise formic acid; Nakada and Weinhouse (1953a) have demonstrated the oxidation of formic acid in model systems, by the coupled action of xanthine oxidase and catalase. Ethanol is also known to be dehydrogenated by the catalase-hydrogen peroxide complex (Keilin and Hartree, 1945). The oxidation of formic acid by the peroxidase activity of the catalase of dialyzed supernatants of liver homogenates has been shown to be prevented by ethanol (Oro and Rappoport, 1959). The oxidation of formic acid by rat liver mitochondrial preparations used in the present studies was investigated using ethanol to inhibit the oxidation of formic acid via catalase and using hexanoic acid to inhibit the oxidation via serine, pyruvic acid and the citric acid cycle.

Ethanol inhibits the oxidation of formic acid by 124 mμmoles. The presence of glycine or L-serine does not change this value significantly. The amount of inhibition caused by ethanol is found to be additive to the inhibition caused by hexanoic acid both in the presence and in the

absence of glycine or L-serine. Apparently, the rat liver mitochondria used in these experiments oxidise only approximately 100mμmoles of formic acid via a pathway catalysed by catalase.

The glycine-stimulated oxidation of formic acid by rat liver mitochondria is almost abolished by hexanoic acid. Even in the absence of added glycine or L-serine, oxidation of formic acid is inhibited by approximately 30%, suggesting that a significant proportion of the oxidation proceeds via the citric acid cycle. A very high portion (78%) of the L-serine-stimulated oxidation of formic acid is also inhibited by hexanoic acid.

It has been shown previously that the inhibition by ethanol of the oxidation of formic acid is independent of the presence or absence of glycine or L-serine and additive to the inhibition caused by hexanoic acid. However, there is a significant amount of oxidation of formic acid that is not affected by the combined action of ethanol and hexanoic acid. Possibly this represents a third pathway for the oxidation of formic acid, and the active one carbon fragment is assumed to be the substrate for this system. The amount of formic acid oxidised via this pathway decreases (from 156 to 54) in the presence of glycine, presumably because serine aldolase decreases the concentration of the active one carbon fragment by utilizing it for increased serine synthesis. However, L-serine increases

the concentration of the hot active one carbon fragment, after formic acid-C^{14} is incorporated into L-serine by the exchange reaction and hence the oxidation of formic acid by the THFA pathway is increased in the presence of L-serine. Neither the nature of the THFA complex nor the mechanisms involved in this pathway are known.

The extent of operation of these different pathways for the oxidation of formic acid under various conditions by rat liver mitochondria is shown in Table XXVII. The sums of these values are in close agreement with the observed total oxidation of formic acid under the respective conditions suggesting that the assumptions made are valid.

Summary.

(1) The oxidation of glycine-2-C^{14} by rat brain and kidney cortex slices is inhibited greatly by pyruvic acid, whereas pyruvic acid has little effect on the production of $C^{14}O_2$ from glycine-1-C^{14}.

(2) The oxidation of glycine-2-C^{14} by rat liver mitochondria is inhibited by pyruvic, hexanoic, α-keto-glutaric, L-glutamic and succinic acids more than the oxidation of glycine-1-C^{14}.

(3) Succinic acid inhibits the production of $C^{14}O_2$ from glycine-2-C^{14} but not from glycine-1-C^{14}, by rat brain cortex slices.

(4) Rat brain and kidney cortex slices and liver

mitochondria oxidise L-serine-U-C^{14} much faster than they oxidise glycine-2-C^{14}.

(5) Glycine has no effect on the oxidation of L-serine-U-C^{14} by rat kidney cortex slices or liver mitochondria.

(6) The oxidation of formic acid by rat liver mitochondria is stimulated to a large extent by glycine or L-serine. Serine isolated from the incubation mixture under these conditions, acquires radioactivity.

(7) Inhibition studies with ethanol and hexanoic acid have shown that three different pathways may operate in the oxidation of formic acid by rat liver mitochondria.

C H A P T E R V

INHIBITORS AND COFACTORS OF THE OXIDATION OF GLYCINE.

Introduction.

The results reported in the previous chapters have shown that the oxidation of glycine does not involve the participation of glyoxylic acid. The formation of serine as an obligatory intermediate during the oxidation of glycine was demonstrated and further evidence also was adduced to show that serine (and hence glycine also) is oxidized via pyruvic acid. The mechanism by which the methylene carbon atom of glycine is detached from the carboxyl carbon atom and converted to an active one carbon fragment for the synthesis of serine has been discussed. Investigations were also carried out with metabolic inhibitors and cofactors with the view that they might possibly throw additional light on the proposed pathway of the oxidation of glycine.

The effects of methyl glyoxal on the rate of oxidation of glycine by rat brain cortex slices.

Data on the effects of increasing concentrations of methyl glyoxal on the rate of oxidation of glycine-1-C^{14} and glycine-2-C^{14} by rat brain cortex slices are shown in Table XXVIII. Methyl glyoxal is inhibitory to the

TABLE XXVIII

THE EFFECTS OF METHYL GLYOXAL ON THE RATE OF OXIDATION OF GLYCINE BY RAT BRAIN CORTEX SLICES.

Methyl glyoxal (mM)	GLYCINE-1-C^{14}			GLYCINE-2-C^{14}		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
0.00	56.0	82.5	1.48 (100)	60.0	4.4	0.073 (100)
0.25	-	-	-	59.0	4.4	0.074 (101)
0.50	55.5	75.5	1.38 (92)	58.0	4.3	0.074 (101)
0.75	51.5	75.5	1.38 (92)	-	-	-
1.00	55.0	66.0	1.20 (81)	56.0	2.8	0.050 (69)
1.50	48.0	58.0	1.20 (81)	54.0	1.7	0.032 (45)
2.00	47.0	50.0	1.06 (72)	54.5	1.3	0.024 (33)
2.50	43.0	49.0	1.14 (77)	48.5	1.2	0.022 (30)
3.00	47.5	49.0	1.04 (70)	45.5	1.0	0.022 (30)

Q and $C^{14}O_2$ as described in Table I. Values in parentheses refer to percentages.

Rat brain cortex slices (ca.70mg) were incubated with 2mM labelled glycine and 10mM glucose with additions of methyl glyoxal as indicated, in a 3ml total volume, for 60 minutes, under the standard conditions.

respiration of rat brain cortex slices and since the oxidation of glycine by this tissue preparation is related to the respiration, low concentrations were used. The oxidation of the methylene carbon atom of glycine is inhibited by methyl glyoxal more than the oxidation of the carboxyl carbon, an inhibition of approximately 70% being observed with 3 mM methyl glyoxal, whereas the production of $C^{14}O_2$ from glycine-1-C^{14} is inhibited by only 30%.

The effects of methylglyoxal on the rate of oxidation of glycine and L-serine-U-C^{14} by rat kidney cortex slices.

Experiments, similar to those described above, were also conducted with rat kidney cortex slices and the results of these experiments are presented in Table XXIX. Up to a concentration of 5 mM methylglyoxal does not affect the $C^{14}O_2$ produced from glycine-1-C^{14} per unit volume of oxygen consumed. However, under similar conditions, the oxidations of glycine-2-C^{14} and L-serine-U-C^{14} are inhibited by approximately 40%.

The effects of cinnamate, arsenite and azide on the rate of oxidation of glycine by rat liver mitochondria.

Sodium cinnamate, at a concentration of 10 mM, inhibits the oxidation of glycine only slightly. Experiments

T A B L E XXIX

THE EFFECTS OF METHYL GLYOXAL ON THE RATE OF OXIDATION OF GLYCINE-1-C^{14}, GLYCINE-2-C^{14} AND L-SERINE-U-C^{14} BY RAT KIDNEY CORTEX SLICES.

Methyl glyoxal (mM)	GLYCINE-1-C^{14}			GLYCINE-2-C^{14}			L-SERINE-U-C^{14}		
	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$	Q	$C^{14}O_2$	$C^{14}O_2/Q$
0.00	85.5	780	9.1	86.0	69	0.80 (100)	89.0	450	0.51 (100)
0.25	80.5	780	9.7	82.5	61	0.74 (93)	-	-	-
0.50	-	-	-	-	-	-	100.0	480	0.48 (94)
0.75	92.0	860	9.2	96.5	63	0.66 (83)	90.0	375	0.42 (82)
1.00	-	-	-	-	-	-	91.0	321	0.35 (69)
1.25	97.5	855	8.8	84.5	50	0.60 (75)	-	-	-
2.00	92.5	780	8.4	94.0	53	0.60 (75)	91.5	315	0.34 (67)
3.00	94.0	750	8.0	94.5	54	0.58 (73)	85.0	324	0.39 (76)
3.50	-	-	-	-	-	-	83.5	270	0.32 (63)
4.00	90.0	750	8.5	73.0	38	0.52 (65)	82.0	252	0.31 (61)
5.0	68.0	635	9.2	65.0	32	0.48 (60)	72.0	210	0.29 (57)

Q and $C^{14}O_2$ values as described in Table I. Values in parentheses refer to percentages.

Rat kidney cortex slices (ca. 60mg) were incubated with 2mM labelled substrate and additions of methyl glyoxal as shown above, in a total volume of 3ml, for 60 minutes, under the standard incubation conditions.

conducted with different concentrations of glycine and sodium cinnamate, were not decisive as to the nature of the inhibition - competitive or non-competitive. The maximum inhibition observed on the $C^{14}O_2$ production from 0.5 mM glycine-1-C^{14} and glycine-2-C^{14} was only 25% and 45% respectively with 30 mM sodium cinnamate. Sodium azide, at the concentrations used does not affect the oxidation of glycine by rat liver mitochondria significantly. Sodium arsenite, however, is highly inhibitory, the $C^{14}O_2$ produced from glycine-1-C^{14} and glycine-2-C^{14} being reduced by 61% and 73% respectively by 0.3 mM sodium arsenite (Table XXX).

The effects of reduced glutathione (GSH), dinitrophenol and glucose-hexokinase on the rate of oxidation of glycine by rat liver mitochondria.

Table XXXI shows the effects of these agents on the rate of oxidation of glycine by rat liver mitochondria. GSH stimulates the oxidation of both carbon atoms of glycine approximately threefold. Dinitrophenol also increases the production of $C^{14}O_2$ from glycine-1-C^{14} and glycine-2-C^{14}. Qualitatively, similar results were obtained by draining off the ATP with glucose and hexokinase, though the stimulatory effect in this case is less pronounced compared to dinitrophenol.

TABLE XXX

THE EFFECTS OF SODIUM CINNAMATE, AZIDE AND ARSENITE ON THE RATE OF OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Additions	Conc. (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
		Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	-	310	650 (100)	296	288 (100)
Sodium cinnamate	10	278	576 (89)	300	236 (82)
Sodium azide	1	254	592 (91)	270	234 (81)
	3	228	670 (103)	228	262 (91)
Sodium arsenite	0.3	254	254 (39)	250	40 (27)

Q and $C^{14}O_2$ values as described in Table VII. Figures in parentheses refer to percentages.

Mitochondria from 1g of fresh rat liver were incubated with 2mM labelled glycine and 10mM choline chloride with additions as shown above, in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

TABLE XXXI

THE EFFECTS OF GSH, DNP AND HEXOKINASE AND GLUCOSE ON THE RATE OF OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Additions	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	268	400	298	232
GSH (10mM)	280	1444	277	664
Nil	356	666	328	246
DNP (0.005mM)	324	744	344	282
DNP (0.025mM)	276	1266	300	482
DNP (0.125mM)	282	1544	278	610
Nil	314	578	324	222
Hexokinase (100γ) glucose (10mM)	370	766	376	282

Q and $C^{14}O_2$ values as described in Table VII. Values in parentheses refer to percentages.

Mitochondria from 1g of fresh rat liver were incubated with 10mM choline chloride and 2mM labelled glycine with additions as shown above in a total volume of 3.0ml, for 30 minutes, under the standard conditions.

The effects of semicarbazide on the rate of oxidation of glycine by rat liver mitochondria.

The effects of increasing concentrations of semicarbazide on the rate of oxidation of glycine by rat liver mitochondria are shown in Table XXXII. Though 2 mM semicarbazide does not inhibit the respiration of rat liver mitochondria, the production of $C^{14}O_2$ from glycine-1-C^{14} and glycine-2-C^{14} is inhibited by approximately 95%.

The effects of Amytal on the rate of oxidation of glycine by rat liver mitochondria.

Amytal (5-ethyl-5-isoamylbarbiturate) at a concentration of 1 mM inhibits the production of $C^{14}O_2$ from glycine-1-C^{14} and glycine-2-C^{14} by 27% and 57% respectively (Table XXXIII). The oxygen consumption of rat liver mitochondria also decreases by approximately 50%. Higher concentrations of Amytal were found to stop the oxidation of glycine completely, but the respiration of the rat liver mitochondria was also abolished.

The effects of iproniazide and pyridoxine-5-phosphate on the rate of oxidation of glycine by rat liver mitochondria.

1.5 mM iproniazide (isonicotinic acid 2-isopropylhydrazide) decreases the $C^{14}O_2$ production by rat liver mitochondria

TABLE XXXII

THE EFFECTS OF SEMICARBAZIDE ON THE RATE OF OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Semicarbazide (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	240	611 (100)	240	353 (100)
0.125	233	324 (53)	235	240 (68)
0.500	243	115 (19)	240	68 (19)
1.000	223	65 (11)	235	37 (11)
2.000	219	33 (6)	225	20 (6)

Q and $C^{14}O_2$ values as described in Table VII. Figures in parentheses refer to percentages.

Mitochondria from 1g of fresh rat liver were incubated with 2mM labelled glycine and 10mM choline chloride with additions of semicarbazide as shown above in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

TABLE XXXIII

THE EFFECTS OF AMYTAL, IPRONIAZIDE AND PYRIDOXINE-5-PHOSPHATE ON THE RATE OF OXIDATION OF GLYCINE BY RAT LIVER MITOCHONDRIA.

Additions	Conc. (mM)	GLYCINE-1-C^{14}		GLYCINE-2-C^{14}	
		Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	-	330	526 (100)	296	190 (100)
Amytal	1.0	150	382 (73)	150	82 (43)
Nil	-	486	697 (100)	490	330 (100)
Iproniazide	1.5	510	296 (42)	514	108 (33)
Iproniazide +Pyridoxine-5-phosphate	1.5 3.0	456	506 (73)	424	344 (105)

Q and $C^{14}O_2$ values as described in Table VII. Values in parentheses refer to percentages.

Mitochondria from 1g fresh rat liver were incubated with 2mM labelled glycine and 2.5mM choline chloride with additions as shown above in a total volume of 1.5ml, for 30 minutes, under the standard incubation conditions.

from glycine-1-C^{14} and glycine-2-C^{14} by 58% and 67% respectively (Table XXXIII) and no significant effect on the oxygen consumption is observed. Complete reversal of iproniazide-inhibition of the $C^{14}O_2$ production from glycine-2-C^{14} and a partial reversal in the case of glycine-1-C^{14} is achieved on addition of 3.0mM pyridoxine-5-phosphate.

The effects of aminopterin on the rate of oxidation of glycine-2-C^{14} and DL-serine-3-C^{14} by rat liver mitochondria.

The oxidation of glycine-2-C^{14} by rat liver mitochondria decreases by 58% with the addition of 0.6mM aminopterin (N-p-2,4-diamino-6-pteridylmethyl-amino-benzoylglutamic acid). However, the oxidation of DL-serine-3-C^{14} proceeds unaffected in presence of 0.8mM aminopterin. Aminopterin has little effect on the oxygen consumption of rat liver mitochondria in these concentrations (Table XXXIV).

The effects of malonic acid on the rate of oxidation of glycine, succinic acid and decanoic acid by rat liver mitochondria.

Results reported in Chapter III (Table XV) show that the oxidation of glycine by rat liver mitochondria

T A B L E XXXIV

THE EFFECTS OF AMINOPTERIN ON THE RATE OF OXIDATION OF GLYCINE-2-C^{14} AND DL-SERINE-3-C^{14} BY RAT LIVER MITOCHONDRIA.

Additions	Conc. (mM)	GLYCINE-2-C^{14}		DL-SERINE-3-C^{14}	
		Q	$C^{14}O_2$	Q	$C^{14}O_2$
Nil	-	490	330 (100)	502	992 (100)
Aminopterine	0.6	406	139 (42)	-	-
Aminopterine	0.8	-	-	490	997 (101)

Q and $C^{14}O_2$ values as described Table VII. Values in parentheses represent percentages.

Mitochondria from 1g of fresh rat liver were incubated with 2mM labelled substrate and 10mM choline chloride, with additions as shown above, in a total volume of 1.5ml, for 30 minutes, under the standard incubation conditions.

is inhibited only to a small extent by sodium malonate. The effects of 10 mM sodium malonate on the rate of oxidation of low concentrations of sodium succinate-2,3-C^{14} by rat liver mitochondria are shown in Table XXXV. A marked inhibition of the oxidation of succinate-2,3-C^{14} is observed with malonate, which amounts to more than 95%. However, the inhibition of the oxidation of potassium decanoate-1-C^{14} is not so pronounced (Table XXXVI). As high a concentration as 60 mM sodium malonate is found to block only 70% of the oxidation of 0.2 mM potassium decanoate.

Discussion.

Experiments conducted with methylglyoxal to show possible implication of it with the metabolism of glycine were not successful. There is a considerable inhibition of the oxidation of glycine, probably due to the non-specific effect of the aldehydic nature of methylglyoxal. Methylglyoxal has been shown to inhibit tissue respiration in vitro (Kisch, 1932) and the effect has been attributed to the ability of methylglyoxal to combine with enzyme proteins requiring -SH groups for activity (Kun, 1950). The higher inhibition observed with the oxidation of glycine-2-C^{14} suggests that methylglyoxal probably acts by prior conversion to

T A B L E XXXV

THE EFFECTS OF MALONIC ACID ON THE RATE OF OXIDATION OF SUCCINIC ACID-2,3-C^{14} BY RAT LIVER MITOCHONDRIA

Sodium succinate (mM)	CONTROL		SODIUM MALONATE (mM)	
	Q	$C^{14}O_2$	Q	$C^{14}O_2$
0.05	252	102	216	4.8 (4.7)
0.07	374	136	186	6.2 (4.5)
0.10	370	276	190	10.2 (3.7)
0.20	412	532	186	18.0 (3.4)
0.50	400	1160	174	51.8 (4.5)
0.85	436	1840	206	102.2 (5.5)

Q and $C^{14}O_2$ values as described in Table VII. Values in parentheses refer to percentages.

Mitochondria from 0.5g of fresh rat liver were incubated with 2.5 mM choline chloride and additions as shown above in a total volume of 1.5ml, for 60 minutes, under the standard incubation conditions.

T A B L E XXXVI

THE EFFECTS OF MALONIC ACID ON THE RATE OF OXIDATION OF DECANOIC ACID-1-C^{14} BY RAT LIVER MITOCHONDRIA.

Sodium malonate (mM)	Q	$C^{14}O_2$
0.0	391	249 (100)
5.0	274	104 (42)
10.0	264	91 (37)
20.0	238	79 (32)
40.0	254	74 (30)
60.0	272	74 (30)

Q and $C^{14}O_2$ values as described in Table VII. Values in parentheses refer to percentages.

Mitochondria from 1g of fresh rat liver were incubated with 0.2mM potassium decanoate-1-C^{14} and 10mM choline chloride with additions of sodium malonate as shown above in a total volume of 3ml, for 30 minutes, under the standard incubation conditions.

pyruvic acid via lactic acid (compare Tables XXVIIIand XXIX with Tables XIX and XXI).

The cinnamate ion has been shown to be a competitive inhibitor of D-amino acid oxidase (Frisell, Lowe and Hellerman, 1956). Recently, basing their results on the competitive inhibition observed with glycine oxidase preparations, Neims and Hellerman (1962) have shown that D-amino acid oxidase and glycine oxidase are one and the same enzyme. The results reported in this chapter show that the inhibition of the oxidation of glycine by cinnamate is more pronounced with glycine-2-C^{14} than with glycine-1-C^{14}. The inhibitory effect of cinnamate on the oxidation of glycine is probably related to the ability of cinnamate to inhibit the oxidation of fatty acids (Quastel and Wheatley, 1933).

GSH stimulates the oxidation of glycine by rat liver mitochondria. This effect could be attributed to possible effects of GSH on SH enzymes (Racker, 1954) e.g. preventing their oxidation or by protecting them against toxic heavy metals. The stimulatory effect of GSH on the oxidation of glycine could be more specific, for GSH has been shown to be required by serine dehydrase (Sayre and Greenberg, 1956). The inhibitory effect of arsenite on the oxidation of glycine by rat liver mitochondria also indicates the

involvement of an SH enzyme in the oxidation of glycine.

Glucose-hexokinase and dinitrophenol stimulate the oxidation of glycine by rat liver mitochondria. These reagents are known to decrease the ATP concentration (Kielley and Kielley, 1951; Loomis and Lipmann, 1948). Mitochondrial oxidation of added substrates is low in the absence of phosphate acceptor (Lardy and Wellman, 1952). It is believed that ADP is the primary phosphate acceptor and that the concentration of this compound controls the level of mitochondrial oxidations (Chance and Williams, 1955). The stimulation of the oxidation of glycine by rat liver mitochondria, by these reagents is probably due to their uncoupling effects on mitochondria.

Semicarbazide, at concentrations that do not affect the oxygen consumption, inhibits the oxidation of glycine by rat liver mitochondria completely. The oxidation of glycine is also inhibited by iproniazide. Iproniazide inhibits the pyridoxal dependent enzymes and direct evidence is available for the formation of a hydrazone between iproniazide and pyridoxal (Davidson, 1956). Iproniazide inhibition of the oxidation of glycine is reversed by pyridoxine-5-phosphate. These results indicate the possible involvement of vitamin B_6 in the oxidation of glycine.

Amytal inhibits the respiration and the oxidation of glycine by rat liver mitochondria. Among other effects, amytal is known to be a powerful inhibitor of the oxidation of NADH (Ernster, Jalling, Low and Lindberg, 1955; Ernster, Low and Lindberg, 1955) and probably the observed inhibition of glycine oxidation is a secondary effect of amytal.

The oxidation of glycine-2-C^{14} is inhibited by aminopterin, whereas the oxidation of DL-serine-3-C^{14} is unaffected by aminopterin. Aminopterin is a competitive inhibitor of dihydrofolic acid reductase which converts folic acid to THFA (Osborn, Freeman and Huennekens, 1958). Nakada et al. (1955) have suggested that the oxidation of glycine-2-C^{14} proceeds via the formation of formic acid as an intermediate and the oxidation of serine proceeds via the obligatory formation of glycine as the intermediate. Inhibition studies with aminopterin reported in this chapter, however, show that the oxidation of glycine-2-C^{14}, but not that of DL-serine-3-C^{14} is dependent upon THFA. Presumably, serine aldolase (Equation 32) is involved in the oxidation of glycine-2-C^{14} and not in the oxidation of DL-serine-3-C^{14}.

Malonate (10mM) inhibits the oxidation of succinate-2,3-C^{14} (0.85mM) by approximately 95%. However, the oxidation

of decanoic acid-1-C^{14} (0.2 mM) is inhibited only 70% with a high concentration of malonate (60 mM). The oxidation of glycine and L-serine are inhibited only slightly under similar conditions.

These apparently contradictory results can probably be attributed to the highly complex structural organization of the mitochondrion (Yagi and Nagatsu, 1960). Soluble preparations of succinic dehydrogenases have been shown not to have the same properties as the endogenous form of the enzyme (Guiditta and Singer, 1959; Keilin and King, 1960). Discussing the mitochondrial function in relation to the precise structure which underlies this function, Green (1959) has observed that understanding of "... mitochondrial function cannot be achieved merely by degrading the mitochondrion into a collection of soluble enzymes and studying them in detail any more than the structure of a protein can be deduced by studying the individual amino acids after hydrolysis of the protein ..."

Though the oxidation of succinate can be completely stopped by malonate in the mitochondrial preparation used in the present investigations (Table XXXV), the oxidation of 375 mμmoles of acetyl CoA (derived from 75 mμmoles of decanoate, assuming that all the carbon atoms of the fatty acid are oxidized at the same rate proceeds unaffected by

the presence of high concentrations of malonate (Table IX). The oxidation of 150 mμmoles of acetyl CoA can account for the approximately 300 mμmoles of $C^{14}O_2$ produced from glycine-2-C^{14} (since the acetyl units derived from glycine-2-C^{14} are doubly labelled) under identical conditions, the observed oxidation of glycine-2-C^{14} probably can proceed unaffected by malonate.

Summary.

(1) Methylglyoxal inhibits the oxidation of glycine-2-C^{14} by rat brain and kidney cortex slices, probably by being converted to pyruvic acid via lactic acid.

(2) The oxidation of glycine by rat liver mitochondria is inhibited by arsenite and stimulated by GSH, suggesting the involvement of an SH enzyme.

(3) Decreasing the ATP concentration with dinitrophenol or glucose-hexokinase stimulates the oxidation of glycine by rat liver mitochondria.

(4) Semicarbazide inhibits the oxidation of glycine by rat liver mitochondria, probably by binding pyridoxal.

(5) Iproniazide inhibits the oxidation of glycine by rat liver mitochondria and the inhibition is relieved by pyridoxine-5-phosphate.

(6) Amytal inhibits the oxidation of glycine by rat liver mitochondria, probably by preventing the oxidation of NADH.

(7) Aminopterin inhibits the oxidation of glycine-2-C^{14} by rat liver mitochondria, implying that THFA is a cofactor in the process. Oxidation of DL-serine-3-C^{14} is not affected by aminopterin.

(8) Malonate does not inhibit the oxidation of glycine markedly though the oxidation of succinate is stopped by malonate.

GENERAL DISCUSSION

Previous workers have implicated glyoxylic acid as the obligatory intermediate in the oxidation of glycine (Nakada and Weinhouse, 1953a; 1953b). This inference is based on their observations that glyoxylic acid, when added to rat liver extracts oxidising labelled glycine inhibits the production of labelled carbon dioxide and the incorporation of radioactivity into glyoxylic acid. Glyoxylate dehydrogenase which oxidises glyoxylic acid to formic acid exhibits an absolute dependance on L-glutamic acid (Nakada and Sund, 1958; Crawhall and Watts, 1962). Formic acid is further oxidised to carbon dioxide by the peroxidase activity of catalase (Weinhouse, 1955b).

Friedmann and Weinhouse (1951) have studied the glycogenic pathway of glycine. Isotopic evidence has ruled out the possibility of a transketolase type of reaction involving glycolaldehyde which could be formed by reduction of glyoxylic acid. Conversion of glycine to serine appears to be obligatory for glycogenesis. Since, L-serine is known to be converted to pyruvic acid (Leibman and Fellner, 1962), the conversion of glycine to serine can provide an alternate route for the catabolism of glycine via serine and pyruvic acid.

Results obtained in the present investigations indicate that glyoxylic acid is not a major intermediate in the catabolism of glycine. The following observations

lead to this conclusion.

The inhibition of the oxidation of glycine by rat brain cortex slices on addition of glyoxylic acid, can be directly related to the simultaneous inhibition of respiration. Recently, glyoxylic acid has been shown to have a direct and non-specific inhibitory effect on the citric acid cycle enzyme systems (Ruffo et al. 1962) presumably because of the aldehydic nature of glyoxylic acid.

Further, labelled glyoxylic acid is isolated from the incubation mixture, only if a pool of glyoxylic acid is added. Very little radioactivity appears in glyoxylic acid compared to the large inhibition of oxidation of glycine. Again, more or less the same amount of radioactivity appears in glyoxylic acid even if the mitochondria are inactivated by boiling. The aldehyde groups of pyridoxal and glyoxylic acid are analogous in electronic properties due to the strong electron attraction of the contiguous carboxyl group in the latter compound. For this reason, glyoxylic acid undergoes transamination readily and the isotopic evidence presented by Nakada and Weinhouse (1953a; 1953b) to prove the participation of glyoxylic acid in the oxidation of glycine may only be the end result of a non-enzymic result of a formation of an intermediate metabolite.

The oxidation of glycine by rat liver mitochondria is inhibited on the addition of L-glutamic acid. In view of the fact that glyoxylate dehydrogenase of rat liver

mitochondrial extracts exhibit an absolute requirement for L-glutamic acid, the inhibition in place of the expected stimulation of the oxidation of glycine on addition of L-glutamic acid, indicates that glyoxylic acid is not involved in the oxidation of glycine.

The oxidation of the methylene carbon atom of glycine is not inhibited more than 50% on the addition of formic acid. Since formic acid can exchange with the β-carbon atom of serine, the observed inhibition probably indicates a decrease in the formation of doubly labelled serine from glycine-2-C^{14} and not the participation of formic acid in the oxidation of glycine-2-C^{14}.

Inhibition studies with ethanol also do not support the view that formic acid is involved in the oxidation of glycine-2-C^{14} by rat liver mitochondria. Oxidation of formic acid by the peroxidase activity of catalase has been shown to be prevented on the addition of ethanol (Oro and Rappoport, 1959).

Glycine-2-C^{14} on being metabolised via glyoxylate pathway will form formate-C^{14} and the oxidation of both glycine-2-C^{14} and formate-C^{14} should be affected in a similar manner by an added metabolite. However, the oxidation of glycine-2-C^{14} by rat liver mitochondria is inhibited by approximately 60% on the addition of 2mM L-serine, whereas the addition of 2mM L-serine, under identical conditions, stimulates the oxidation of formate-C^{14} sixfold. Presumably,

the oxidation of glycine-2-C^{14} does not proceed via the formation of formate.

Nakada and Weinhouse (1957) have suggested that the oxidation of serine proceeds via the formation of glycine which is further oxidised via glyoxylic acid. Absence of formation of acetoacetate in the reaction mixture metabolizing serine, led these workers to this suggestion. Further, they have demonstrated a large inhibition of the oxidation of serine-3-C^{14} on addition of a mixture of glycine and glyoxylic acid. Their conclusion that glycine is an intermediate in the oxidation of serine from this observation is based on the assumption that the labelled carbon from serine-3-C^{14} will find its way to glycine, which is not true. The inhibition found by these workers is probably due to the non-specific effect of glyoxylic acid which was included in the reaction mixture.

Results obtained in the present work indicate that the oxidation of serine proceeds via the formation of pyruvic acid as demonstrated by Leibman and Fellner (1962) rather than via the formation of glycine. The following arguments may be made in support of this inference.

The oxidation of L-serine-U-C^{14} by rat kidney cortex slices and rat liver mitochondria is not affected on the addition of glycine. Presumably, glycine does not lie on the pathway of oxidation of L-serine-U-C^{14}. Moreover, the oxidation of L-serine-U-C^{14} by rat brain and kidney cortex slices and by rat liver mitochondria is greatly inhibited

on addition of pyruvic acid. Serine dehydrase which converts serine to pyruvic acid is probably the enzyme responsible for the oxidation of serine.

Finally, the oxidation of DL-serine-3-C^{14} was not inhibited by aminopterin under conditions in which an inhibition of the oxidation of glycine-2-C^{14} could be demonstrated. This suggests that serine aldolase is not involved in the oxidation of DL-serine-3-C^{14}.

The methylene carbon atom of glycine itself can serve as the active one carbon fragment necessary for the formation of L-serine from glycine. The formation of doubly labelled serine from glycine-2-C^{14} as formulated in Equation 40, will result in the release of the carboxyl carbon atom of one of the two glycine molecules, as carbon dioxide. Avian liver preparations have been shown recently to catalyse such a reaction (Sanadi and Bennett, 1960; Richert et al. 1962). Glyoxylic acid does not seem to be an intermediate in the formation of the active one carbon unit necessary for the systhesis of serine from glycine, again indicating that glyoxylic acid is not a normal metabolite of glycine. A scheme has been proposed for the formation of doubly labelled serine from glycine-2-C^{14} (see Discussion, Chapter III).

The following observations support the view that the oxidation of glycine proceeds chiefly via the obligatory formation of L-serine into which two methylene carbon atoms and one carboxyl carbon atom of glycine is

incorporated.

Serine isolated from the incubation mixture metabolising labelled glycine, is twice as radioactive when glycine-2-C^{14} is used than when glycine-1-C^{14} is used. This suggests that the serine formed is doubly labelled with the methylene carbon atom and singly labelled with the carboxyl carbon atom of glycine.

The oxidation of glycine-2-C^{14} to $C^{14}O_2$ by rat brain and kidney cortex slices is inhibited greatly on the addition of pyruvic acid and only a small inhibition is observed on the production of $C^{14}O_2$ from glycine-1-C^{14}. The oxidation of L-serine-U-C^{14} is also inhibited considerably on the addition of pyruvic acid.

Moreover, the oxidation of glycine-2-C^{14} to $C^{14}O_2$ by rat liver mitochondria is greatly decreased on addition of β-hydroxybutyric, hexanoic, pyruvic, α-ketoglutaric, L-glutamic and succinic acids compared to the production of $C^{14}O_2$ from glycine-1-C^{14}. The oxidation of L-serine-U-C^{14} is also inhibited greatly on the addition of pyruvic, hexanoic and succinic acids. Apparently the oxidation of glycine-2-C^{14} and L-serine-U-C^{14} takes place via the citric acid cycle.

The operation of the glyoxylate pathway will convert the methylene carbon atom of glycine to formic acid. Chance (1945; 1946) has shown that the catalase-hydrogen

peroxide complex can oxidise formic acid. Nakada and Weinhouse (1953b) have demonstrated the oxidation of formic acid in model systems by the coupled actions of xanthine oxidase and catalase. Oro and Rappoport (1959) have reported the oxidation of formic acid by catalase-hydrogen peroxide complex by dialysed supernatants of rat liver homogenates. Rat liver mitochondria used in the present investigations oxidise formic acid at a similar or slightly faster rate than they oxidise glycine-2-C^{14}. However, the oxidation of formic acid is increased three fold on the addition of 10mM glycine and sixfold on the addition of 2mM L-serine. It is beleived that formic acid is oxidised via three different pathways, i.e. via catalase, via the citric acid cycle after formate has been incorporated into serine and via the THFA-complex formed from serine into which formate has been incorporated.

Ethanol inhibits the oxidation of formic acid by approximately 100mμmoles. The same amount of inhibition is observed even when the oxidation of formic acid is stimulated by glycine or L-serine, with or without the further addition of potassium hexanoate. Ethanol is known to be oxidised by the catalase-hydrogen peroxide complex (Keilin and Hartree, 1945) and is known to prevent the catalase catalysed oxidation of formic acid by substrate competition (Oro and Rappoport, 1959). The ethanol inhibited oxidation of formic acid is, therefore, presumed

to proceed via catalase.

Increasing concentrations of glycine increase the oxidation of formic acid. High concentrations of glycine may drive the reaction catalysed by serine aldolase towards serine synthesis. This is demonstrated by the fact that the serine isolated from the incubation mixture acquires radioactivity under these conditions. The stimulation is abolished on the addition of potassium hexanoate.

L-serine also stimulates the oxidation of formic acid. A sixfold increase is observed with 1.5mM L-serine and further increase in the concentration of L-serine causes no additional stimulation. These results suggest the occurrence of an exchange reaction between formic acid and L-serine. Serine, isolated by chromatography under these conditions is nearly four times more radioactive than serine isolated in the presence of glycine. Most of the serine-stimulated oxidation of formic acid is abolished on the addition of potassium hexanoate. Presumably the acetyl CoA formed from hexanoate competes with the labelled acetyl CoA formed from serine for entry into the citric acid cycle. The potassium hexanoate-inhibited oxidation of formic acid is believed to proceed via the citric acid cycle.

Approximately 200mμmoles of formate is oxidised without being affected by the combined actions of ethanol and

potassium hexanoate, in the presence of L-serine. Under the influence of serine aldolase, L-serine can provide a large quantity of active one carbon units. The oxidation of formic acid, uninfluenced by the combined actions of ethanol and potassium hexanoate is assumed to proceed by the oxidation of the active one carbon unit.

It is concluded that the oxidation of formic acid by isolated rat liver mitochondria via the catalase-hydrogen peroxide complex occurs only to a small extent. Glycine and L-serine stimulate the oxidation of formic acid to a great extent, but the stimulated-oxidation takes place chiefly via the operation of the citric acid cycle, after formic acid has been incorporated into serine.

Most of the production of carbon dioxide from the carboxyl carbon atom of glycine occurs during the condensation of two molecules of glycine to form L-serine. The oxidation of the methylene carbon atom of glycine takes place mainly by the oxidation of acetyl CoA via the citric acid cycle, both carbon atoms of acetyl CoA originating from the methylene carbon atom of glycine via the intermediate formation of serine and pyruvic acid.

CLAIMS TO ORIGINAL RESEARCH

(1) δ-aminolevulinic acid did not inhibit the oxidation of glycine-2-C^{14} by chicken erythrocytes under conditions where an inhibition of the radioactivity incorporated into hemin could be demonstrated.

(2) Pyruvic acid inhibited the oxidation of glycine-2-C^{14} and L-serine-U-C^{14} by rat brain and kidney cortex slices with little effect on the production of $C^{14}O_2$ from glycine-1-C^{14}.

(3) The oxidation of L-serine-U-C^{14} by rat kidney cortex slices and rat liver mitochondria was not affected on addition of glycine.

(4) The oxidation of glycine-2-C^{14} and L-serine-U-C^{14} by rat liver mitochondria was inhibited much more than the production of $C^{14}O_2$ from glycine-1-C^{14} on addition of hexanoic acid, pyruvic acid and succinic acid.

(5) L-serine inhibited the $C^{14}O_2$ production from glycine-2-C^{14} by rat liver mitochondria with little effect on the $C^{14}O_2$ production from glycine-1-C^{14}.

(6) Neither D-serine nor hydroxypyruvic acid influenced the oxidation of glycine by rat liver mitochondria.

(7) Radioactivity was incorporated twice as rapidly from glycine-2-C^{14} into the serine isolated from the

incubation mixture than from glycine-1-C^{14}.

(8) Formic acid inhibited the oxidation of glycine-2-C^{14} by rat liver mitochondria by a maximum of 50%.

(9) A scheme had been proposed for the oxidation of glycine by rat liver mitochondria.

(10) The oxidation of formic acid by rat liver mitochondria is stimulated in a progressive manner by increasing concentrations of glycine. L-serine stimulated the oxidation of formic acid more than glycine does and at much lower concentrations. The glycine and serine stimulated-oxidation of formic acid is greatly decreased on the addition of hexanoic acid.

(11) Serine isolated from an incubation mixture containing formic acid-C^{14} and either glycine or L-serine, is radioactive.

(12) Formic acid is incorporated into serine four times faster in the presence of L-serine than in the presence of glycine.

(13) Amytal inhibited the oxidation of glycine by rat liver mitochondria.

(14) Aminopterin did not inhibit the oxidation of DL-serine-3-C^{14} under conditions where an inhibition of the oxidation of glycine-2-C^{14} could be demonstrated.

(15) Iproniazide inhibited the oxidation of glycine by rat liver mitochondria and the inhibition was reversed on addition of pyridoxine-5-phosphate.

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