Phytochemistry, 1972, Vol. 11, pp. 1587 to 1594. Pergamon Press. Printedi n England.

OXIDATION AND PHOSPHORYLATION ASSOCIATED WITH THE CONVERSION OF GLYCINE TO SERINE

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(*Received* 1 *February* 1972)

Abstract—Particles separated from extracts of tobacco leaves by differential centrifugation catalysed the conversion of two molecules of glycine to one molecule of serine and one of carbon dioxide. The catalytic system was inhibited by inhibitors of the mitochondrial electron transport system. The reaction was slow in the absence of oxygen but attained a maximum rate at 3% by vol. of oxygen in the atmosphere. Approximately one atom of oxygen was taken up for each molecule of serine formed. Added ADP increased the rate of reaction and was converted to ATP. Some ATP was formed and oxygen consumed in the absence of glycine but both processes proceeded faster when glycine was added. Isonicotinyl hydrazide, an inhibitor of serine synthesis, decreased phosphorylation more in the presence of glycine than in its absence. *Trans*-aconitate, a competitive inhibitor of aconitase, did not inhibit the phosphorylation caused by adding glycine but strongly inhibited phosphorylation occuring in the absence of glycine. Phosphorylation accompanying the conversion of glycine to serine probably involves the electron transport chain of mitochondria and associated phosphorylation sites. The conversion of glycine to serine is probably responsible for the evolution of carbon dioxide in photorespiration, so our results suggest that photorespiration causes ATP to be synthesized in mitochondria during photosynthesis.

INTRODUCTION

CALVIN *et al.*[1,2] showed that glycollate, glycine and serine were formed, in sequence, during photosynthesis by algae and higher plants. In algae, glycollate is excreted from the cells during photosynthesis[3] and in the presence of certain inhibitors, for example, α-hydroxysulphonates, leaves of higher plants accumulate glycollate.[4–6] Hence, attention was focussed on the mechanism of formation of glycollate and the role of glycollate oxidase in photosynthetic systems. Less attention was given to the subsequent reactions by which glycine and serine are formed from glycollate. Earlier experiments in which ^{14}C-labelled glycollate was supplied to green leaves in the light[7–12] demonstrated its conversion to glycine, serine and sucrose.

The importance of the two amino acids *in vivo* was confirmed by Whittingham *et al.*[13–16]; who found that adding isonicotinyl hydrazide (INH) resulted in [^{14}C]glycine accumulating

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in addition to glycollate during photosynthesis in [^{14}C]CO$_2$ whilst ^{14}C in serine was decreased. The conversion of the carbon assimilated during photosynthesis through glycollate to glycine, serine and then sucrose has become known as the glycollate pathway. Its operation results in an uptake of oxygen and evolution of carbon dioxide (photorespiration) accompanying photosynthesis.

Several attempts have been made to isolate the enzyme systems concerned. Cossins and Sinha[17] obtained a soluble protein fraction from wheat leaves that catalysed the slow conversion of glycine to serine only when tetrahydrofolate was added. Shah and Cossins[18] showed that chloroplasts isolated from pea leaves by the non-aqueous method contained the enzyme serine hydroxymethyltransferase (E.C.2.1.2.1) but the activity detected was not adequate to account for the rates of serine synthesis observed *in vivo*. Kisaki and Tolbert[19] showed that leaf disks decarboxylated glycine faster than glyoxylate, glycollate or serine. The reaction was inhibited by INH and not by α-hydroxysulphonates, but decarboxylation of [1-^{14}C]glycollate was inhibited by INH and by α-hydroxysulphonate, suggesting that glycollate was converted to glycine before decarboxylation took place. The carbon dioxide released during metabolism of glycine was shown to be derived from the carboxyl group. Serine was also formed and the results were consistent with the conversion of two molecules of glycine to one of serine and one of carbon dioxide. Kisaki and Tolbert[19] suggested that the CO$_2$ formed in the conversion of glycine to serine was sufficient to account for photorespiration. Kisaki *et al.*[20,21] found that glycine was decarboxylated in the presence of mitochondria from spinach leaves fast enough to account for observed rates of photorespiration. The products were serine, CO$_2$ and ammonia; NAD, pyridoxal phosphate and tetrahydrofolate were co-factors for the reaction. Treatments that disrupt mitochondria destroyed the activity. The reaction was slower under aerobic conditions than in nitrogen.

Decarboxylation of glycine and synthesis of serine were catalysed by mitochondria isolated from avian liver[22,23] and rat liver.[24] The catalytic system was made soluble in buffered aqueous media by treating mitrochondria from rat liver with acetone.[25] The soluble system required tetrahydrofolate and was stimulated by pyridoxal phosphate and NAD. Aerobic conditions were necessary for the reaction to proceed at a significant rate.[26] The enzyme system in micro-organisms which synthesizes serine from glycine contains at least four proteins[27–29] one of which contains pyridoxal phosphate and another FAD. The enzyme containing FAD has the properties of lipoamide dehydrogenase (E.C.1.6.4.3)[28] and was thought to be one of two protein electron carriers between glycine and NAD. Kochi and Kikuchi[29] found that conversion of glycine to serine, catalysed by an enzyme system from *Arthrobacter globiformis*, required oxygen but was almost unaffected by addition of tetrahydrofolate. Evidence was provided that a bound tetrahydrofolate took part in the reaction. Also, added NAD and pyridoxal phosphate were necessary.

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We report the properties of a particle-bound system from tobacco leaves that catalyses the synthesis of serine from glycine. It resembles the soluble systems from rat liver mitochondria and from *Arthrobacter globiformis* in requiring oxygen. It is inhibited by inhibitors of mitochondrial electron transport and couples the reaction forming serine from glycine to synthesis of ATP from ADP.

It can be calculated from data presented by Parks and Huffman[30] that the reaction:

$$\tfrac{1}{2}\,O_{2(g)} + 2\ \text{glycine} \rightarrow \text{serine} + CO_{2(g)} + NH_{3(g)} \qquad (1)$$

is accompanied by a free energy change ($\Delta\ G^0_{298}$) of $-42{\cdot}2$ kcal. This would be adequate for synthesis of three molecules of ATP for each molecule of serine formed.

RESULTS

Crude extracts, prepared by grinding fresh tobacco leaves (10 g) with 60 mM Tris buffer (10 ml; pH 8·0) containing 20 mM dithiothreitol, catalysed synthesis of serine from glycine but soon lost their activity. Activity was better preserved in media containing sucrose, phosphate, EDTA, tris buffer and sodium citrate[31,32] together with dithiothreitol or bovine serum albumin. The presence of citrate was essential in media used to extract leaves and for resuspending particles separated by centrifugation. Table 1 shows that, in the reaction catalysed by these preparations, two molecules of glycine were converted to one molecule each of serine and CO_2. We have not observed decarboxylation of glycine catalysed by any preparation from plants that was not accompanied by the synthesis of serine and we shall refer to the reaction as serine synthesis. It was convenient to measure activity of the system by assay of [^{14}C]CO_2 evolved from reaction mixtures containing [1-^{14}C]glycine. Oxygen was necessary for the reaction to take place but an oxygen concentration in the atmosphere of 3% by vol. gave maximum activity (Table 1).

TABLE 1. THE EFFECT OF OXYGEN TENSION ON THE SYNTHESIS OF SERINE FROM GLYCINE CATALYSED BY A PARTICULATE PREPARATION FROM TOBACCO LEAVES

Oxygen concn in the assay vessel % by vol.	CO_2 evolved	Serine formed (μmol/hr/g fr. wt)	Glycine utilized
0	1·11	1·13	2·67
0·52	2·87	3·23	5·91
1·06	4·77	4·71	8·58
1·56	4·85	4·94	8·22
2·18	5·52	5·03	12·45
2·67	6·23	6·14	12·93
3·13	6·39	7·11	13·71
3·74	5·04	5·01	9·78
4·22	5·21	4·83	10·08
4·75	5·88	4·62	9·90
5·18	4·70	4·35	8·80
10·90	4·49	4·41	7·89

Reaction mixtures contained 2 μmol (0·5 μCi) [1-^{14}C]glycine, 1 μmol NAD, 0·1 μmol pyridoxal phosphate and 0·5 ml of the suspension of particles. The total volume was 0·6 ml. The gaseous phase above the reaction mixtures was composed of pure O_2 and N_2 mixed together to give the composition shown.

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Differential and isopycnic density gradient centrifugation were used to fractionate particles from tobacco leaves. The catalytic activity was associated with fractions containing mitochondria. Only mitochondria from cells containing chloroplasts were active. The serine-synthesizing activity of particles isolated from turnip and cauliflower, per unit of cytochrome oxidase (E.C. 1.9.3.1) activity used as a measure of the mitochondria present, was only 8 and 12% respectively of that in particles from tobacco leaves. Etiolated leaves of wheat or broad bean contained, per unit of cytochrome oxidase, only 15% of the serine synthesizing activity found in green leaves of the same age. Age of the leaf material also influenced activity and stability. The most satisfactory particles were obtained from leaves half-way up tobacco plants that were about to flower. Some younger leaves gave more active particles but their activities decreased during storage at 0°. Leaves situated between those at the base and those half-way up the stalk gave particles with activity that increased during storage at 0°. Very young leaves and old senescent leaves catalysed serine synthesis from glycine slowly.

Known inhibitors of mitochondrial electron transport, namely antimycin A, *o*-phenanthroline and potassium cyanide, strongly inhibited synthesis of serine from glycine (Table 2). Glycine could not be replaced by glycollate and NAD and pyridoxal phosphate could be omitted from the reaction mixtures with little effect on the rate of reaction. Adding tetrahydrofolate to other reaction mixtures had little or no effect on the rate of serine synthesis.

TABLE 2. THE EFFECT OF INHIBITORS AND COFACTORS ON THE SYNTHESIS OF SERINE FROM GLYCINE

Additions (+) and omissions (−) to the basic reaction mixture	Serine synthesized (μmol/hr/g fr. wt)
Complete	10·3
+ Antimycin A (10^{-5} M)	1·0
+ *o*-phenanthroline (10^{-2} M)	1·8
+ KCN (10^{-3} M)	5·5
− NAD	9·0
− Pyridoxal phosphate	11·5
− NAD–pyridoxal phosphate	9·2
Complete with [U-^{14}C]glycollate instead of [1-^{14}C]glycine	0·6

Reaction mixtures contained 10 μmol (0·5 μCi) [1-^{14}C]glycine, 1 μmol NAD, 0·1 μmol pyridoxal phosphate, 2 μmol ADP, and 0·5 ml of the suspension of particles. The total volume was 0·64 ml.

Serine was synthesized from glycine faster when ADP was added to the reaction mixtures (see Table 5). Added ADP was phosphorylated and its phosphorylation was speeded by adding glycine (Table 3). Inhibitors were used to determine whether the increased rate of phosphorylation was coupled to the conversion of glycine to serine or was caused by increased use of other substrates present. Isonicotinyl hydrazide, which inhibits the conversion of glycine to serine *in vivo*[15] inhibited the glycine-stimulated phosphorylation but not phosphorylation which occurred in the absence of glycine. Sodium arsenite and semicarbazide inhibited serine synthesis catalysed by soluble protein from rat liver mitochondria.[26] These two inhibitors also had a much greater effect on the increased rate of phosphorylation caused by adding glycine than on the rate of phosphorylation without

TABLE 3. THE EFFECT OF INHIBITORS ON PHOSPHORYLATION OF ADP ASSOCIATED WITH SYNTHESIS OF SERINE FROM GLYCINE

Inhibitor	Concn	Glycine in the reaction mixture (μmol)	Serine synthesized	$[^{32}P]PO_4^{3-}$ incorporated into ATP (μmol/hr/g fr. wt)	Molar ratio: phosphate incorporated/serine synthesized
None	—	10	9·70	19·04	0·9
None	—	0	—	10·32	—
INH	10 mM	10	3·80	14·54	1·2
INH	10 mM	0	—	10·00	—
Arsenite	50 μM	10	2·86	12·98	1·1
Arsenite	50 μM	0	—	9·76	—
Semicarbazide	0·5 mM	10	1·38	10·56	1·2
Semicarbazide	0·5 mM	0	—	8·92	—
Trans-aconitate	60 mM	10	7·74	11·52	1·1
Trans-aconitate	60 mM	0	—	3·26	—

Reaction mixtures contained 10 μmol (0·5 μCi) [1-^{14}C]glycine, 1 μmol NAD, 0·1 μmol pyridoxal phosphate, 2 μmol ADP, 0·5 ml of the suspension of particles, 0·1 μmol (2 μCi) of ^{32}P labelled KH_2PO_4 (making a total of 5·1 μmol of orthophosphate in each reaction mixture) and the inhibitors shown. The total volume was 0·66 ml.

added glycine (Tables 3 and 4). *Trans*-aconitate, which is a competitive inhibitor of aconitase[33] (E.C. 4.2.1.3) had the opposite effect and inhibited the synthesis of ATP supported by substrate present in the suspension of particles but had only a small effect on the amount of additional ATP synthesized when glycine was added. The ratio, phosphorylation in the presence of glycine minus that in its absence: serine synthesized, was not greatly affected by the presence of the inhibitors. Table 5 confirms that the CO_2 evolved was still stoichiometrically related to serine formed when inhibitors were present.

TABLE 4. DIFFERENCES IN THE RESPONSE TO INHIBITORS OF INCREASED PHOSPHORYLATION DUE TO ADDED GLYCINE AND PHOSPHORYLATION DEPENDENT ON OTHER SUBSTRATES

Inhibitor	Concn	% Inhibition of serine synthesis	% Inhibition of phosphorylation supported by substrates other than glycine	% Inhibition of increased phosphorylation caused by added glycine
INH	10 mM	61	3	48
Arsenite	50 μM	70	5	63
Semicarbazide	0·5 mM	86	14	81
Trans-aconitate	60 mM	20	68	0·5

Reaction mixtures as Table 3.

The uptake of oxygen associated with the synthesis of serine was approximately one atom per molecule of serine and CO_2 formed. For a preparation that catalysed the synthesis

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TABLE 5. AMOUNTS OF GLYCINE UTILIZED, SERINE SYNTHESIZED, CO_2 EVOLVED AND ADP PHOSPHORYLATED IN THE ABSENCE OR PRESENCE OF VARIOUS INHIBITORS

ADP added	Inhibitor	Glycine utilized	Serine synthesized	CO_2 evolved	ADP phosphorylated
		(μmol/hr/g fr. wt)			
None	None	16·64	7·36	7·74	0·24
2 μmol	None	25·48	12·80	11·86	20·84
2 μmol	INH (10 mM)	18·12	8·92	7·20	18·70
2 μmol	Arsenite (50 μM)	10·96	4·28	3·98	14·52
2 μmol	*Trans*-aconitate (60 mM)	24·88	9·56	8·86	13·76

Reaction mixture as Table 3.

of serine at 10·54 μmol/hr/g fr. wt, the increased oxygen uptake caused by adding glycine was 4·78 μmol/hr/g fr. wt.

DISCUSSION

The fact that the conversion of glycine to serine requires oxygen and the effect on the conversion of inhibitors of mitochondrial electron transport, suggest that the reaction involves the cytochrome system in mitochondria. Further, the reaction was stimulated by ADP and the ADP was phosphorylated faster in the presence than in the absence of glycine. Isonicotinyl hydrazide, arsenite and semicarbazide inhibited serine synthesis and the additional phosphorylation caused by adding glycine. *Trans*-aconitate had little effect on serine synthesis, and the phosphorylation associated with it, but strongly inhibited phosphorylation supported by substrates present in the suspension of particles. We conclude that ATP is synthesized at the expense of free energy derived from reaction (1).

In contrast to the particles from leaves described by Kisaki *et al.*,[20,21] the particles from tobacco leaves do not require the cofactor NAD to convert glycine to serine and catalyse the reaction at least six times faster under aerobic than under anaerobic conditions.

Although NAD was added to the reaction mixtures we have no evidence that it was reduced, and indeed even with an adequate amount of NAD, serine was synthesized only slowly under anaerobic conditions. An electron acceptor in mitochondria that transfers electrons to the mitochondrial electron transport chain must be an essential component of the catalytic system converting glycine to serine. At least one phosphorylating site must be included between the point of entry of the electrons into the chain and the terminal oxidase.

The number of ATP molecules obtained for each molecule of serine formed can be deduced from observations made in the presence of some inhibitors (Table 3). The molar ratio of serine synthesized to ATP formed was close to 1. Serine synthesis does take place without added ADP, at rates as fast as 65% of the rate in the presence of ADP (Table 5) but this may represent, in part, serine formation uncoupled from ATP synthesis because the particles were damaged during isolation. If this is so, the ratio of coupled serine synthesis to ATP formed is 1:3; thermodynamically this is possible.

Our results suggest that one of the main functions of the photorespiratory pathway is to allow the synthesis of ATP in the light in the mitochondria. A mechanism for transferring energy from the chloroplast to the cytoplasm in the light was suggested by Stocking and

Larson.[34] This involved the reduction of phosphoglycerate to triose phosphate, in the chloroplast in the light, the transport of triose phosphate to the cytoplasm and its re-oxidation there to phosphoglycerate with concomitant synthesis of $NADH_2$ from NAD and ATP from ADP and orthophosphate. However, Ongun and Stocking[35] and Roberts *et al.*,[36] found that glycine and serine were labelled outside of the chloroplast during photosynthesis in air containing $[^{14}C]CO_2$ faster than were the phosphate esters. Hence, in the cytoplasm during photosynthesis, there is a greater flow of carbon through glycine and serine than through phosphate esters.

It follows that, in the light, synthesis of ATP external to the chloroplast may depend more on the conversion of glycine to serine than on the transfer of phosphate esters from within the chloroplast to outside. This would provide a biological significance for the process of photorespiration.

EXPERIMENTAL

Preparation of particles from leaves. Plants of *Nicotiana tabacum* (var. White Burley) grown in a greenhouse in natural daylight supplemented with artificial light were kept in darkness for 48 hr before use. Selected leaves were washed with H_2O and the mid-ribs removed. All operations were at 0–5°. Lamina (10 g) was ground in a mortar for 2 min with 6 g of acid-washed sand and 40 ml of medium (pH 7·7) containing sucrose (0·4 M), KH_2PO_4 (10 mM), Na citrate (20 mM), EDTA (5 mM), Tris (0·2 M), and crystalline bovine serum albumin (1%). The homogenate was filtered through two layers of muslin and through a pad of absorbent cotton wool. The filtrate was centrifuged at 100 *g* for 10 min. Particles sedimenting from the supernatant liquid when it was centrifuged again at 38,000 *g* for 10 min were resuspended in medium (pH 7·7) containing sucrose (0·4 M), KH_2PO_4 (10 mM), Na citrate (20 mM), $MgCl_2$ (4 mM) and crystalline bovine serum albumin (1%). The final volume of suspension was made equal to the volume of supernatant liquid from which the particles were obtained. Each reaction mixture, containing 0·5 ml of the suspension, would therefore contain 200 μmol of sucrose, 5 μmol KH_2PO_4, 10 μmol Na citrate, 2 μmol $MgCl_2$, 5 mg bovine serum albumin and particles from 0·1 g fr. wt of tissue.

Assay for serine synthesis—decarboxylation of glycine. Reaction mixtures were incubated in 50 ml flasks having removable centre-wells and Suba-seal stoppers. The resuspended leaf particles were mixed with 0·1 μmol pyridoxal phosphate, 1 μmol NAD and other reagents shown in the tables, in the outer compartment of the flasks. Reaction was started by adding 10 μmol (0·5 μCi) of $[1\text{-}^{14}C]$ glycine and the $[^{14}C]CO_2$ evolved was collected in 0·1 ml of 2 N NaOH. Trichloroacetic acid (TCA, 10%; 0·5 ml) was used to stop the reaction. Flasks were shaken during the reaction period and for 30 min after adding the TCA. Radioactivity in the NaOH solution was determined using triton–toluene scintillation fluid.[37]

Determination of ^{14}C *in glycine and serine.* Denatured protein was removed from the reaction mixture by centrifugation and the TCA extracted from the supernatant liquid into Et_2O. Amino acids in the aqueous layer were absorbed on a column of cation exchange resin and eluted with 2 N NH_4OH. Excess NH_3 was removed by evaporation and the amino acids separated by PC using n-BuOH–Me_2CO–Et_2NH–H_2O (20:20:2:10, by vol.) as the solvent. The separated compounds were eluted from the paper with H_2O and their ^{14}C content determined by scintillation counting.

Phosphorylation and determination of ATP, ADP and AMP. A modification[38] of the method of Nielsen and Lehninger[39] was used to measure incorporation of ^{32}P into organic compounds. The results were confirmed by measuring the $[^{32}P]\ PO_4^{3-}$ incorporated into ATP after separation of individual nucleotides by ion-exchange chromatography.[40] Over 90% of the ^{32}P incorporated was in ATP and the rest in ADP. Amounts of nucleotides were also estimated from measurements of the UV absorption of fractions obtained from the ion-exchange columns. Some AMP was formed (probably resulting from myokinase (E.C. 2.7.4.3) activity) for which allowance was made in calculating true ATP synthesis. All three measurements of ATP synthesis were in good agreement.

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Oxygen uptake. O_2 uptake was measured with an O_2 electrode (Rank Bros.). The effect of O_2 concentration was determined in reaction flasks filled with O_2-free N_2 and a measured volume of O_2.

Tetrahydrofolate. Tetrahydrofolate was prepared fresh for each experiment by reduction of folic acid with Na dithionite.[41]

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Key Word Index—Nicotiana tabacum; Solanacceae; serine biosynthesis; glycine; photorespiration; ADP phosphorylation.