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STUDIES OF CERTAIN ASPECTS OF INTERMEDIARY METABOLISM  
IN PHOTOSYNTHESIZING COTYLEDONS OF *RAPHANUS* WITH  
SPECIAL REFERENCE TO THE UTILIZATION OF  
SOME ONE AND TWO CARBON COMPOUNDS

by



ALMA CABALLERO (GREEN)

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled STUDIES OF CERTAIN ASPECTS OF INTERMEDIARY METABOLISM IN PHOTOSYNTHESIZING COTYLEDONS OF *RAPHANUS* WITH SPECIAL REFERENCE TO THE UTILIZATION OF SOME ONE AND TWO CARBON COMPOUNDS submitted by Alma Caballero (Green) in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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*To my husband*

"But", you may say, "none of this shakes my belief that 2 and 2 are 4". You are quite right, except in marginal cases - and it is only in marginal cases that you are doubtful whether a certain animal is a dog, or a certain length is less than a meter. Two must be two of something, and the proposition "2 and 2 are 4" is useless unless it can be applied. Two dogs and two dogs are certainly four dogs, but cases may arise in which you are doubtful whether two of them are dogs. "Well, at any rate there are four animals" you may say. But there are microorganisms concerning which it is doubtful whether they are animals or plants. "Well then, living organisms", you say. But there are things of which it is doubtful whether they are living organisms or not. You will be driven into saying: "Two entities and two entities are four entities". When you have told me what you mean by "entity", we will resume the argument.

Lord Bertrand Russell

*How to Become a Mathematician*

## ABSTRACT

The utilization of labelled  $\text{CO}_2$ , glyoxylate, glycine, serine and acetate by disks of radish cotyledons in light and dark has been investigated. Improved methods for the chromatographic separation of amino acids and organic acids from plant extracts, as well as a method for preservation of thin-layer chromatograms are presented.

When  $\text{C}^{14}\text{O}_2$  was supplied in the light to radish cotyledons, the labelled products obtained, particularly in pulse-chase experiments, indicated the presence of an active photosynthetic carbon reduction cycle. Furthermore, radioactive carbon dioxide was extensively metabolized. The intermediates of the glycollate pathway all contained considerable radioactivity.

When labelled glyoxylate, glycine and serine were supplied, further verification of the existence of a glycollate pathway was obtained. The data also demonstrated an active conversion of glycine to serine which implicates an active transfer of one-carbon fragments.

Supplying radioactive glyoxylate and glycine in light and dark showed that while labelling of lipids was light dependent, the overall operation of the glycollate pathway was not. In these experiments it was also shown that while the carbon-2 of glyoxylate was a better precursor of sugars, in both light and dark, the contribution of the carboxyl carbon of glyoxylate and glycine to this fraction was enhanced by light. This probably resulted from an efficient re-fixation

of  $C^{14}O_2$ .

When glyoxylate-2- $C^{14}$  was supplied in the presence of non-radioactive glycine, the incorporation of  $C^{14}$  into sugars was enhanced. This incorporation was light independent and plausible pathways to account for this are discussed.

When acetate-2- $C^{14}$  was fed in a pulse-chase experiment the major products were different from those obtained in the  $C^{14}O_2$  and glycine-2- $C^{14}$  experiments, indicating that acetate was metabolized by different pathways. As the lipid fraction and members of the tricarboxylic acid cycle were heavily labelled in these experiments, it is concluded that acetate was metabolized mainly via acetyl-CoA.

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## LIST OF ABBREVIATIONS

TPP	:	thiamine pyrophosphate
NADH	:	nicotinamide adenine dinucleotide (reduced)
NADPH	:	nicotinamide adenine dinucleotide phosphate (reduced)
RuDP	:	ribulose-1,5-diphosphate
PEP	:	phosphoenolpyruvate
PGA	:	phosphoglyceric acid
PPO	:	2,5-diphenyloxazole
POPOP	:	1,4- <i>bis</i> -2-(4 methyl-5-phenyloxazolyl)-benzene
ft. c.	:	foot candles

## INTRODUCTION

During the study of intermediary metabolism in plants, some low molecular weight metabolites have been shown to play a diversity of roles, many of them having a direct bearing on key processes within the plant cell. It is the purpose of this work to present a review of the most recent and significant research related to these compounds in different types of plant tissues and subsequently to apply and compare this accumulated knowledge to a tissue not very extensively studied, namely the photosynthesizing cotyledons of radish.

*Glycollate formation*

It has been repeatedly demonstrated that plant tissues are capable of the production of glycollic acid in both dark and light. Uniformly labelled glycollate accumulates in tissue extracts after short-time photosynthesis with  $C^{14}O_2$ . This accumulation was noticed even before the photosynthetic carbon reduction cycle had been fully worked out (Burris *et al.*, 1949; Benson and Calvin, 1950). Glycollic acid accumulation was also observed in the light, in the absence of  $CO_2$  (Schou *et al.*, 1950) and it was thus assumed that its precursors were some of the ketose intermediates of the Calvin cycle, by adduct formation of TPP with a 2-carbon fragment of these sugars (Tolbert, 1963; Bassham, 1964). Alternatively other theories have been offered regarding glycollate formation in light directly from  $CO_2$

by a *de novo* process which entails a non-specified carboxylation reaction different from that involving ribulose-1, 5-diphosphate (Stiller, 1962; Zelitch, 1965a).

Evidently, there are several pathways whereby glycollic acid may be formed. Thus, Milhaud *et al.*, (1956) fed hydroxypyruvate-2-C<sup>14</sup> to *Scenedesmus* for 40 minutes in the light and better than 85% of the label was recovered in the carboxyl carbon of glycollic acid. When D-ribose-1-C<sup>14</sup> was infiltrated into leaves of *Nicotiana rustica*, incorporation of label appeared in glycollic acid in light and dark (Griffith and Byerrum, 1959). Glyoxylic acid-C<sup>14</sup> feedings produced heavily labelled glycollic acid (Tolbert, 1958) and acetate-C<sup>14</sup> produced labelled glycollate in *Chlorella* by a pathway which was light driven (Lord and Merrett, 1968).

In kinetic experiments with *Chlorella* and its utilization of acetate in the light, after 10 seconds, glycollate was one of the primary products, containing up to 45% of the radioactivity incorporated (Goulding and Merrett, 1967b; Merrett and Goulding, 1968). This compound has been found in tissues other than those capable of photosynthesis, such as etiolated barley shoots (Elliott, 1954). So far, however, the most obvious source of glycollate is through the photosynthetic process when carried out under certain conditions, although the details of its formation and utilization are not yet fully elucidated.

Glycollate formation appears to depend upon a series of environmental factors. In an atmosphere of oxygen and in

light, considerably more radioactivity is incorporated into glycollate from  $C^{14}O_2$  in *Chlorella* (Bassham and Kirk, 1962) and *Scenedesmus* (Hess, Tolbert and Pike, 1967). On the other hand, and quite recently, Nelson *et al.* (1969) established that when glycollate or glyoxylate were added to dividing cells of *Scenedesmus* in the light, at normal  $CO_2$  tensions, the rate of oxygen evolved by these cells was stimulated by up to 300%. These authors proposed a scheme whereby glyoxylate served as a terminal hydrogen acceptor from NADPH produced by photosynthesis, thus stimulating oxygen evolution when carbon dioxide was not available in the necessary quantities. In addition, this acid may play a series of important roles in plant metabolism.

These roles have been studied and reviewed by several authors (Tolbert, 1963; Zelitch, 1958; Zelitch, 1964; Zelitch, 1965b). Thus, the existence of a permease system has been suggested, based on the reactions which interconvert glycollate and glyoxylate (Zelitch, 1958; Tolbert, 1963). Concurrently, the excretion of glycollate from chloroplasts may compensate with entry of bicarbonate ions, thus providing an anion exchange system. Zelitch and Walker (1964) and Zelitch (1965b) suggested that glycollate plays a role in the control of stomatal movements. This role is very strongly supported by data demonstrating that every factor affecting the formation of glycollate, such as light, temperature, anaerobiosis,  $CO_2$  tensions, specific inhibitors, etc., similarly affects the movement of

stomata (Mortimer, 1959).

*Metabolism of glycollic acid*

Tolbert, Claggett and Burris discovered in 1949, an enzyme of widespread occurrence in green plants, which catalyzed the oxidation of glycollic acid through glyoxylic acid, to formate and CO<sub>2</sub>. This enzyme was later isolated and purified from spinach leaves (Zelitch and Ochoa, 1953). Glycollate oxidase (*Glycollate:oxygen oxidoreductase*, EC 1.1.3.1) which catalyzes this reaction, is a flavoprotein having riboflavin phosphate as prosthetic group. Glyoxylate reductase (*Glycollate:NADP oxidoreductase*, EC 1.1.1.26) which catalyzes the reduction of glyoxylic acid to glycollic acid was isolated and studied by Zelitch (1953) from spinach leaves. NADH or NADPH were effective coenzymes. Glyoxylate reductase appeared to be confined to the chloroplasts, while the site of operation of glycollate oxidase was ascribed to the cytoplasm (Thompson and Whittingham, 1968). Accordingly, the glycollate carbons would enter the chloroplast after oxidation to glyoxylate by glycollate oxidase. An NAD-linked glyoxylate reductase present within the chloroplast would again produce glycollate. This last reaction has been shown to occur in isolated spinach chloroplasts (Zelitch, 1960; Chan and Bassham, 1967). Exogenously supplied glycollate-C<sup>14</sup> did not enter the chloroplasts, while similarly supplied glyoxylate-C<sup>14</sup> readily entered the organelles (Kearney and Tolbert, 1962). Thompson and Whittingham (1968) however, pointed out that glyoxylate

reductase has an equilibrium greatly in favor of glycollate formation, while the reverse reaction with glycollate oxidase has not yet been demonstrated; therefore glycollate movement across the membrane appeared to be unidirectional.

Glycollate oxidase plays an important role in light stimulated oxygen uptake and CO<sub>2</sub> evolution during photosynthesis. It may, in conjunction with the possible existence of a permease system, be responsible for photorespiration. Evidences for a light stimulated respiration have accumulated in recent years (Krotkov *et al.*, 1958; Forrester *et al.*, 1966; Tregunna *et al.*, 1966).

The magnitude of glycollic acid accumulation when its oxidation is blocked *in vivo* by inhibitors (Asada and Kasai, 1962) together with experiments on oxygen uptake and CO<sub>2</sub> evolution in the light, point to the possibility that both have the same oxygen requirements; therefore, both processes might be inter-dependent, glycollic acid serving as the substrate for photorespiration.

There is considerable variation among different species of plants in their rates of photorespiration, and this latter increases with greater temperatures and light intensities (Zelitch, 1966). More recent work, however, contributed extensive clarification to the intracellular distribution of the above mentioned enzymes, and the relationship existing between the metabolism of their substrates and the phenomena of photorespiration and oxygen evolution enhancement. Tolbert *et al.* (1968) isolated from spinach leaves a particulate fractions having

all the necessary enzymes for the operation of the glycollate pathway, and which provided a feasible explanation for photorespiration. These microbodies, designated by their discoverers as "peroxisomes" were definitely distinct from the mitochondrial and the chloroplastic fractions, both in their external appearance and shape, and in their enzymic contents. The peroxisomes have been shown to contain glycollate oxidase, NADPH-glyoxylate reductase, catalase, malate dehydrogenase and a glutamate-glyoxylate aminotransferase (Kisaki and Tolbert, 1969). Accordingly, glycine would be the end product of peroxisomal activity for the glycollate pathway, for no serine hydroxymethyltransferase was detected in these microbodies.

Since photorespiration was attributed to the oxidation of glycollate formed during photosynthesis, Tolbert *et al.* (1969) made a survey of plants for leaf peroxisomes. It was found that plants with photorespiration contained larger amounts of peroxisomes and the levels of activity of glycollate oxidase, glyoxylate reductase and catalase were considerably higher than in the leaves of plants with no photorespiration.

Kisaki and Tolbert (1969) finally proposed a working hypothesis to account for the distribution of the enzymes associated with glycollate metabolism and photorespiration. Thus, glycollate is biosynthesized during photosynthesis in the chloroplast, from where it is excreted. In the peroxisome, glycollate is oxidized to glyoxylate and  $H_2O_2$ . Next, catalase produces the break-down of hydrogen peroxide;

the oxygen taken up in the process would explain photorespiration. Glyoxylate, on the other hand, would be converted to glycine by the transaminase present in the peroxisomes. If excess glyoxylate left the peroxisomes and entered the chloroplasts, it would be either oxidized to  $\text{CO}_2$  or reduced to glycollate by NADPH:glyoxylate reductase. The glyoxylate thus reduced would be involved in a shuttle system for transfer of reducing power out of the chloroplasts. The same authors investigated the decarboxylation of glyoxylate to  $\text{CO}_2$  and formate within the chloroplast, and found it to be slow but effective. Although it is not known how this decarboxylation is achieved, it was found that this activity is firmly attached to the chloroplastic structures (Kisaki and Tolbert, 1969).

The oxidation of glycollic acid to glyoxylic acid marks the first of a series of reactions known as the glycollate pathway. This pathway as it was formulated by Tolbert (1963) is based on the work of many authors (e.g. Tolbert and Cohan, 1953; Rabson, Tolbert and Kearney, 1962; Jimenez *et al.*, 1962; Wang and Waygood, 1962).

The first experiments which led to the formulation of this pathway were carried out by isolation and identification of the labelled products obtained after administration of labelled glycollic acid to different plant tissues in the light. Tolbert (1963) proposed that the first compound in the pathway was phosphoglycollic acid, a 2-carbon fragment which was cleaved by a specific phosphatase to yield glycollic acid. This phosphoglycollate was considered by Tolbert's

(Vandor and Tolbert, 1968) and Bassham's groups (Jensen and Bassham, 1966) to arise from a glycolaldehyde-TPP adduct derived by transketolase action on ribulose diphosphate or other ketose intermediates of the photosynthetic carbon reduction cycle. When glycollate-2-C<sup>14</sup> is externally fed to plant tissues it produces glyoxylic acid and glycine labelled in carbon-2 as well (Rabson *et al.*, 1962). Breakdown of a glycine molecule will produce CO<sub>2</sub> and a molecule of formate-C<sup>14</sup> (Cossins and Sinha, 1965b; Cossins and Sinha, 1967). This formate, plus another molecule of glycine-2-C<sup>14</sup> will then produce serine 2,3-C<sup>14</sup> and glycerate similarly labelled, from which hexoses will finally be produced labelled in carbons 1 and 2, and 5 and 6. Leaves of wheat, coleus, soybean, maize, barley and of many other plants, were used. Intermediates with different labels were fed. In every case, the compounds obtained were labelled as expected (Jimenez *et al.*, 1962; Wang and Waygood, 1962).

The use of inhibitors specific for certain enzymes of this pathway also produced some interesting results. For example, Asada and Kasai (1962) observed that when  $\alpha$ -hydroxysulfonates were added to barley and wheat seedlings, tobacco leaves and *Chlorella* cells, CO<sub>2</sub> fixation in the light was inhibited. Also the amounts of C<sup>14</sup>O<sub>2</sub> incorporated into glycollate and alanine were increased, whereas the labelling of serine, malate, isocitrate and citrate were decreased. It was inferred that these changes were brought about by inhibition of glyoxylate formation with the subsequent diversion of the label through other pathways.

Clearly, several systems for the synthesis of sucrose or serine are present in the cytoplasm and in the chloroplast. The labelling of sucrose and serine formed in the chloroplast generally reflect the labelling of phosphoglycerate produced by carboxylation of RuDP, while the cytoplasmic serine is mostly derived from glycollate via the glycollate pathway (Bassham, 1965). Accordingly, the 3-phosphoglyceric acid and the sucrose synthesized in the cytoplasm via glycollate are labelled differently from those formed in the chloroplast (Bassham, 1965).

While the experiments mentioned above were carried out in the light, based on the assumption of a close relationship between glycollate production and photosynthesis, other workers used glycollate in experiments with non-green tissues with interesting results. That glycollic acid oxidase is not restricted to green tissues was demonstrated with fungi and bacteria (Kornberg and Gotto, 1959). Tanner and Beevers (1965) who found the enzyme in germinating castor beans, demonstrated that in the endosperm, although glycollate was actively metabolized, it did not contribute significantly to the synthesis of sugars.

Cossins and Sinha (1967) studied glycollate utilization by the same tissue and coupled their kinetic experiments with examination of the pertinent enzymes. Their results agreed with those of Tanner and Beevers, and further demonstrated that after the break-down of glyoxylic acid into formate and  $\text{CO}_2$ , the formate molecule would enter in the formation of serine.

That glycollic acid, produced in steady-state photosynthesis with  $C^{14}O_2$ , was excreted into the medium, was demonstrated initially with algae (Tolbert and Zill, 1956) and isolated spinach chloroplasts (Kearney and Tolbert, 1962). In *Scenedesmus* and *Chlorella* this accumulation of glycollate in the medium has been attributed to failure to metabolize glycollate- $C^{14}$ , when present or added exogenously in large amounts (Hess and Tolbert, 1967). Hence, glycollate excreted by algae may be an end product of photosynthetic carbon metabolism.

On the other hand, Lord and Menrett (1968) recently reported the isolation and partial purification of a glycollate oxidase in a different strain of *Chlorella pyrenoidosa*, while at the same time Zelitch and Day (1968) clearly demonstrated the presence of such enzyme in both *Chlorella* and *Chlamydomonas* with the reinforcing statement that in *Chlamydomonas* the activity of this enzyme is almost as high as in homogenates of tobacco leaves.

#### *The glyoxylate cycle*

This cycle is essentially a by-pass within the tricarboxylic acid cycle for the anabolic utilization of acetate.

The glyoxylate cycle, initially demonstrated in higher plants by Kornberg and Beevers (1957) who showed the rapid conversion of acetate- $C^{14}$  to sucrose via the intermediary formation of certain organic acids, has since been found in a wide variety of plant tissues (e.g. Marcus and Velasco,

1960; Johnson *et al.*, 1966; Firenzuoli *et al.*, 1968).

Beevers' group studied the glyoxylate cycle in tissues converting fats to carbohydrates (Beevers, 1961). These workers observed that the cycle enzymes are formed prior to the onset of gluconeogenesis from fats, and disappear after this process is completed (Beevers, 1961). Kinetic experiments showed that when acetate-2-C<sup>14</sup> was provided to such tissues, malate was labelled exclusively in carbon atoms 2 and 3 and the glucose moiety of sucrose was labelled in carbons 1, 2, 5 and 6 (Canvin and Beevers, 1961).

Attempts to study the intracellular localization of the glyoxylate cycle have been made with mitochondria isolated from germinating peanut cotyledons (Marcus and Velasco, 1960) and later with a particulate fraction called glyoxysomes (Breidenbach and Beevers, 1967). This latter particulate material, isolated from castor-bean endosperm and watermelon cotyledons, contained the key enzymes of the glyoxylate cycle (Breidenbach and Beevers, 1967).

The rhizobia and nodules of legumes such as lupine bush-bean and soy-bean (Johnson *et al.*, 1966), as well as certain gymnosperm seedlings such as *Pinus* (Firenzuoli *et al.*, 1968), also contain the glyoxylate cycle enzymes. Therefore, these two enzymes are not limited to fatty tissues, although they may be more active in such tissues.

#### *The role of glyoxylate in plant metabolism*

The large number of metabolic reactions in which glyoxylic acid participates in all organisms gives a

suggestion of its importance.

To be sure, glyoxylic acid acts as an intermediate in the glycollate pathway and the glyoxylate cycle. It is the immediate precursor of certain amino acids in both chloroplasts and the cytoplasm, and furthermore, shuttles freely across the chloroplastic membrane, therefore suggesting its participation in transport mechanisms (Tolbert, 1963).

The role of glyoxylate in several aspects of plant metabolism have been studied lately with increasing interest. Sinha and Cossins (1965) examined and compared the utilization of this acid to that of acetate in assorted germinating fatty seeds. Both glyoxylate-1,2-C<sup>14</sup> and acetate-2-C<sup>14</sup> were rapidly metabolized by these tissues, known to contain the enzymes of the glyoxylate cycle. Furthermore, glyoxylate-C<sup>14</sup> was incorporated rapidly into glycine and serine. In contrast, acetate labelled mostly those amino acids and amides usually formed via the tricarboxylic acid cycle and the glyoxylate cycle. Some differences were also noticed between tissues in the products formed from glyoxylate and acetate. For example, in germinating sunflower cotyledons both compounds were mainly incorporated into the organic acid and amino acid fractions. In castorbean endosperm instead, sugars were the main fraction labelled at all times from both substrates.

Additions of glyoxylate inhibited to a certain extent the fixation of carbon dioxide by chloroplasts isolated from spinach and pea leaves (Bucke *et al.*, 1966). This inhibitory

action of glyoxylate has also been demonstrated for certain reactions of the tricarboxylic acid cycle. For example, Zelitch and Barber (1960) observed that the oxidation of glycollic acid by mitochondrial fractions produced an inhibitory action on member acids of that cycle. This inhibition was apparently brought about by glyoxylate being formed from glycollate. The mechanisms of this inhibition have been studied by several investigators (Payes and Laties, 1963; Ruffo *et al.*, 1967; Adinolfi *et al.*, 1967; Ruffo, 1967). Glyoxylic acid in very low concentrations exerts a direct or indirect inhibition on certain important enzymes of the citric acid cycle and the Embden, Meyerhoff, Parnas pathway (Ruffo, 1967). These inhibitory characteristics of glyoxylic acid, place this compound as a potential regulator of respiratory metabolism. Also, glyoxylic acid, due to its aldehyde group, may participate in other reversible reactions with groups such as thiols and amino compounds (Ruffo *et al.*, 1967). These two reactions stress the biological importance of glyoxylic acid which therefore could react with compounds such as cysteine, glutathione, CoA, thiol enzymes and amino acids.

Three types of glyoxylic acid decarboxylation have been described, so far, in plant tissues:

1. by pyruvic decarboxylase (in wheat germ, Davies, 1967)
2. synergistic decarboxylation with  $\alpha$ -ketoglutarate, also in wheat germ, whereby carbon-1 of glyoxylate and of  $\alpha$ -ketoglutarate contribute to the  $\text{CO}_2$

evolved (Davies, 1967). The nature of this reaction is still not well known.

3. oxidative decarboxylation to formate and  $\text{CO}_2$  (Cossins and Sinha, 1965b) (Cossins and Sinha, 1967). This particular reaction acquires significance in relation to the glycollate pathway and to the biosynthesis of certain amino acids in plants.

*Amino acid biosynthesis and metabolism in plants in relation to glycollate and glyoxylate metabolism*

Several of the common amino acids present in the soluble and protein fractions of the plant cell may arise by entirely different biosynthetic pathways under different circumstances. A good example of this is the formation of glycine and serine.

In photosynthesis, glycine and serine become labelled. Accordingly serine will be formed before glycine if the sequence 3-phosphoglyceric acid  $\rightarrow$  3-phosphohydroxypyruvic acid  $\rightarrow$  3-phosphoserine  $\rightarrow$  serine, operates. This pathway, which will label the carbon-1 of each compound in succession, when  $\text{C}^{14}\text{O}_2$  is supplied, will be the preferred route at high  $\text{CO}_2$  tensions, (Bassham, 1965) while the formation of glycollate under these conditions is not favored.

Alternatively at low  $\text{CO}_2$  tensions, glycine will be formed prior to serine, via the glycollate pathway. Also, when glycollate- $\text{C}^{14}$  is provided exogenously, glycine will readily acquire label similar to that of the glycollate- $\text{C}^{14}$  fed. Thus, it is clear that the label shown by serine and

other products of this sequence, will depend in either case on the label in the compound originally fed.

Experiments with  $C^{14}O_2$  in light using a variety of plant leaves and times varying from 5 to 20 seconds, gave data as outlined above (Vernon and Aronoff, 1950; Saltman *et al.*, 1957; Rabson *et al.*, 1962; Smith *et al.*, 1961). For example, at 20 seconds alanine, serine and glycine were the major amino acids labelled. Alanine and serine were predominantly labelled in carbon-1. Glycine, however, was equally labelled in both carbons. In some cases (Rabson *et al.*, 1962) 3-phosphoglyceric acid, glycerate and alanine were found to be labelled mainly in carbon-1 but serine was uniformly labelled. When glycollate-2- $C^{14}$  was fed, serine-2,3- $C^{14}$  and glycerate-2,3- $C^{14}$  were the major products. Again glycine was uniformly labelled.

When glycollate was produced endogenously by photosynthesis in  $C^{14}O_2$  it was shown to be uniformly labelled, thus affecting the labelling of glycine (Hess and Tolbert, 1967) (Jensen and Bassham, 1966). The label found in serine will therefore be due to contributions from two separate pathways.

In short term experiments with *Chlorella* and *Chlamydomonas*, serine formed from photosynthesis in  $C^{14}O_2$  contained up to 80% of its activity in the carboxyl carbon. The same distribution was found in 3-phosphoglyceric acid (Hess and Tolbert, 1967). This accumulation of label in the carbon-1 of these compounds was attributed to the lack of formation of glycollic acid or alternatively to the absence

of a complete glycollate pathway in these organisms. In later experiments with double labelled acetate- $H^3$ -2- $C^{14}$  supplied to *Chlorella*, a basis was provided for an alternative pathway from glycollate through glycerate to serine (Merrett and Goulding, 1968).

The formation of sugars from serine in higher plants via the glycollate pathway was studied in leaves and whole wheat plants in the light (Jimenez *et al.*, 1962; Wang and Waygood, 1962).

Conversely, when ribose-1- $C^{14}$  was fed to detached tobacco leaves, the compound was metabolized in both dark and light to glycine, serine and alanine. The reactions were light independent, but illumination increased the total amount of  $C^{14}$  incorporated into the different compounds (Griffith and Byerrum, 1959). The intracellular localization of these compounds has been studied (Kearney and Tolbert, 1962; Tolbert *et al.*, 1968; Kasaki and Tolbert, 1969). Endogenously formed glycollate, once converted into glyoxylate in the peroxisomes may enter the chloroplast in both dark and light, is next aminated to glycine and as such it may be retained by the organelle for protein synthesis or converted to serine. Amination may also occur in the peroxisomes. With exogenously supplied glyoxylate, however, no synthesis of serine occurred within the chloroplast, which suggested that serine synthesis within the plastid, was either very slow or it arose from some other precursor than the glycollate pathway.

Using quantitative tracer studies during steady-state

photosynthesis with  $C^{14}O_2$ , Smith *et al.* (1961) demonstrated that *Chlorella pyrenoidosa* incorporated up to 30% of the label into amino acids. The rate of incorporation into alanine was faster than that of sucrose. Differences in the relative importance of the various pathways of carbon incorporation into amino acids, were noticed when *Chlorella* was exposed to  $C^{14}O_2$  in light and dark (Hiller and Whittingham, 1964). In light, glycine and serine were the main products. If these same cells were subsequently exposed to darkness, the activity in the sugar-phosphates which had accumulated during the light period, was rapidly transferred to aspartate, malate and alanine. In dark  $C^{14}O_2$  fixation, relatively little label accumulated in aspartate, glutamate or alanine and labelling of glycollate and its derivatives was extremely low. That alanine is formed by a different pathway than serine or glycine, for instance, has been supported by several authors (e.g. Mifflin *et al.*, 1966; Asada and Kasai, 1965). For example, it may be formed by reductive amination of phosphoenol pyruvate which in turn is derived from 3-phosphoglyceric acid via 2-phosphoglyceric acid (Smith *et al.*, 1961).

Further evidence for an origin of alanine different from that of serine has been provided by feeding serine-3- $C^{14}$  exogenously to photosynthesizing tissues (Rabson *et al.*, 1962). Glycerate-3- $C^{14}$  was formed, but the precursors of serine in the glycollate pathway were not labelled. When glycine-2- $C^{14}$  was supplied to pea leaves in the presence of excess non-radioactive serine, the amount of radioactiv-

ity going into glycerate and sucrose decreased, but the label incorporated into alanine was not altered.

When the oxidation of glycollate produced by photosynthesis in  $C^{14}O_2$  was blocked with  $\alpha$ -hydroxysulfonates, an increase in labelling of glycollic acid, and a decrease in that of serine was noted (Asada and Kasai, 1962). The labelling of alanine was not affected by these inhibitors.

The importance of glyoxylate in amino acid biosynthesis has been stressed by several authors. For example, Kretovich (1965) fed glyoxylate in light and dark, and observed an intensive synthesis of glycine and serine. This synthesis was stimulated by a variety of amino donors, particularly  $\gamma$ -aminobutyric acid and alanine. A variety of plant tissues including leaves, green seedlings, germinating cotyledons and storage tissues have been used in experiments to ascertain ability to metabolize glyoxylate- $C^{14}$  (Sinha and Cossins, 1965b). These studies showed that glyoxylic acid- $C^{14}$  was an active precursor of glycine and serine. The intramolecular distribution of label in serine showed that in sunflower cotyledons the label was almost equally distributed but in pea leaves the proportion was 3:1:1 for carbons 1, 2 and 3 respectively. More recently, King and Waygood (1968) reported the presence of a glyoxylate aminotransferase in wheat leaves, with serine as the amino donor, while the latest report on this subject involves a glutamate-glyoxylate aminotransferase present in leaf peroxisomes (Kisaki and Tolbert, 1969).

It is therefore clear that glyoxylate can enter a

series of different reactions including transaminations, one-carbon transfer reactions and oxidative decarboxylations.

*The role of one-carbon compounds in glycine and serine biosynthesis*

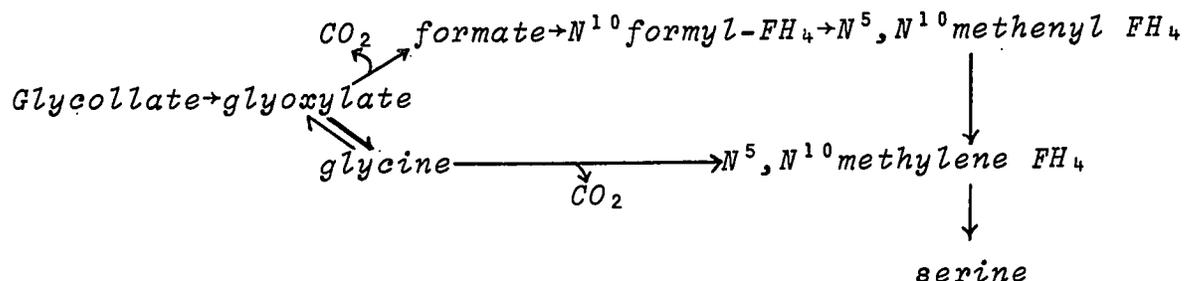
The transfer of one-carbon fragments constitutes an important class of metabolic reactions. Such transformations generally occur via coenzymes derived from tetrahydrofolic acid. During the course of their studies of one-carbon compounds in plants, Cossins (1964) and Cossins and Sinha (1965b) demonstrated that compounds such as methanol and formate either exogenously supplied or endogenously produced, play an important role in amino acids biosynthesis particularly in the synthesis of serine and methionine.

For example, methanol- $C^{14}$  was incorporated into the carbon-3 of serine in a variety of plant tissues. Evidence for such 1-carbon transfer reactions in wheat plants is derived from previous experiments with formate- $C^{14}$  and glycine-1- $C^{14}$  (McConnell and Bilinski, 1959). Green radish cotyledons also are capable of incorporating formate- $C^{14}$  into serine and methionine and their derivatives (Cossins and Sinha, 1965b). This incorporation was stimulated under anaerobic conditions. In the case of serine, labelling was increased by additions of inactive glycine. Clearly, 1-carbon transfer reactions take place quite readily in this tissue. The donors of such 1-carbon fragments may include glycollate, glyoxylate and glycine as all were found to be good precursors of formate when incubated with tissues such

as sunflower cotyledons (Cossins and Sinha, 1965b). The carbon-2 of glycollate, glyoxylate and glycine and the carbon-3 of serine have been demonstrated as efficient precursors for the methyl groups of nicotine and lignin in tobacco plants and of pectin in radish plants (Wu and Byerrum, 1959). A study of the key enzymes related to some of the reactions involving transfers of 1-carbon in metabolism has been carried out (Cossins and Sinha, 1967).

Glycine seems to be a key substrate for 1-carbon group donation in plants. This may occur either after conversion to glyoxylate or by direct cleavage of the glycine molecule. Both glyoxylate and glycine appear to be oxidized by  $\text{CO}_2$  and formate (Cossins and Sinha, 1965b; Cossins and Sinha, 1966; Cossins and Sinha, 1967). This formate would be activated by tetrahydrofolate enzymes and subject to oxidation-reduction reactions. Splitting of glycine and formation of an active formaldehyde has been reported in plant tissues, as an important part of the glycine-serine interconversion (McConnell, 1964; Cossins and Sinha, 1966).

The series of reactions that take place when glycollate or glycine are utilized in the production of serine, were schematically illustrated by Cossins and Sinha (1967) as follows:



$N^5, N^{10}$ -methylene-tetrahydrofolate dehydrogenase (5, 10-methylenetetrahydrofolate:NADP oxidoreductase, EC 1.5.1.5) has been shown to occur in plant tissues by Wong and Cossins (1966). The final transfer of the carbon-1 moiety to another molecule of glycine to form serine is accomplished by L-serine hydroxymethyl-transferase (*L-Serine:tetrahydrofolate 5, 10-hydroxymethyltransferase*, EC 2.1.2.1) which is extensively distributed in plant tissues (Cossins and Sinha, 1966; Schirch and Ropp, 1957; Mazelis and Liu, 1967).

#### *Interconversion of glycine and serine*

The conversion of glycine into serine by the above mentioned reactions in plant tissues was first reported by Wilkinson and Davies (1958). The reversibility of this reaction has been demonstrated. For example, glycine is readily formed by loss of the carbon-3 of serine, as "active formate" (Nath and McConnell, 1960; McConnell and Finlayson, 1961). In experiments with a variety of plant tissues, Cossins and Sinha (1966) studied the glycine-serine interconversion in dark and light. Under the conditions of their experiments, the reactions although freely reversible, favored serine formation. Partial purification of a protein fraction from wheat confirmed this. Only serine  $C^{14}$  was obtained *in vitro*; no labelled glyoxylate, glycollate or formate were detected. Additions of formaldehyde- $C^{14}$  to the mixture strongly stimulated the conversion of glycine into serine, with a concomitant increase in activity in the carbon-3 of serine.

On the other hand, it is of interest to note that other workers have suggested that a completely different pathway may convert serine to glycine (Wang and Burris, 1963, 1965a). When labelled serine was used in these feeding experiments, the label incorporated in glycine did not correspond to that expected if the hydroxymethyltransferase reaction had been reversible. Instead, label similar to that of the serine fed appeared in compounds such as 3-phosphoglyceric acid, phosphoserine and later in glycine. The direct conversion of serine to glycine was extremely sluggish, and the glycine formed entered the glyoxylate pool. This discrepancy in results have been explained as produced by differences in age and nature of the tissues used in the different experiments, and an alternative route has also been offered, in the form of a choline or ethanolamine cycle, for the formation of glycine from serine (McConnell, 1964; Sinha and Cossins, 1964). The sequence of this cycle could explain the observed increase in activity of the carbon-2 of glycine. It involves a decarboxylation of serine to yield ethanolamine which, after deamination to glycolaldehyde, would yield glycollate, glyoxylate and finally glycine. Thus, carbon-3 of serine could become the carbon-2 of glycine.

The interconversions involving glyoxylate, glycine and serine seem to be affected by a number of factors (Mifflin *et al.*, 1966). High partial pressures of CO<sub>2</sub> in light inhibited the transfer of label from glycine-C<sup>14</sup> to sucrose in pea leaves presumably by increasing the amount

of non-active 3-phosphoglyceric acid and sugar phosphates, due to the faster cycling of the photosynthetic carbon reduction cycle intermediates under these conditions. Thus, the formation of sucrose from glycine and serine would be significant only at partial pressures of CO<sub>2</sub> close to those normally found in air. In other words, while partial pressures of CO<sub>2</sub> will affect the metabolism of glycine and serine formed during photosynthesis, that factor will not have any effect on these compounds when they or their immediate precursors are exogenously supplied.

Glycine-2-C<sup>14</sup> fed to pea leaves, produced labelled sucrose, aspartic acid, alanine, glyceric acid, 3-phosphoglyceric acid, serine and insoluble compounds in light, but only the latter two compounds were formed in the dark (Mifflin *et al.*, 1966). When labelled glycine or serine were supplied to wheat leaves, much activity was incorporated into the organic acid fraction. In the light, glyceric and malic acid were the heaviest labelled acids, while in the dark, glycollic acid predominated (Wang and Burris, 1965b).

Sinha and Cossins (1964) also studied the metabolism of glycine-C<sup>14</sup> in a variety of tissues; a great amount of the activity in the dark was found in the acidic amino acids and in malate, succinate, citrate and pyruvate.

The metabolism of serine when exogenously supplied has also been studied under different experimental conditions. For example, radioactive serine supplied to tobacco leaves was only metabolized in the light to produce sucrose and

some protein (Ongun and Stocking, 1965a). Metabolism of serine was faster in the chloroplasts of these tissues than in the cytoplasm. Hence, although metabolic pathways may be similar for exogenous and endogenous substrates, the metabolism of each may take place at sites or pools physically separated within the cell, such as chloroplasts during photosynthesis and within the cytoplasm in darkness.

*Synthesis of terpenoids in relation to intermediates of the glycollate pathway.*

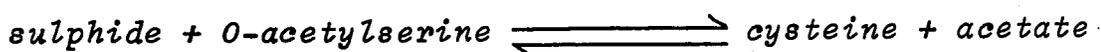
Shah *et al.* (1967) obtained data which demonstrated that glyoxylate-2-C<sup>14</sup>, glycine-2-C<sup>14</sup> and L-serine-U-C<sup>14</sup> were incorporated into chloroplastic  $\beta$ -carotene. Glyoxylate-1-C<sup>14</sup> or glycine-1-C<sup>14</sup> were not as effective precursors in this biosynthesis. This data suggests that acetyl-CoA is an obligatory intermediate in chloroplast terpenoid biosynthesis, and in these cases seemed to be formed within the chloroplast, probably via the glycollate pathway to serine and then to pyruvate and acetyl-CoA. Support for this scheme was derived from isotope dilution experiments. Addition of both serine and glycine reduced the incorporation of label from glyoxylate-2-C<sup>14</sup> into  $\beta$ -carotene by up to 80%, while L-serine almost totally suppressed the incorporation of label from glycine-2-C<sup>14</sup> into  $\beta$ -carotene. In contrast, addition of L-serine did not suppress the incorporation of label from either glyoxylate or glycine-C<sup>14</sup> into extrachloroplastic sterols.

*Additional information concerning green radish cotyledons.*

It is usually assumed that green epigeal cotyledons behave in general, as leaves would. This assumption is however, largely conjectural, since there are many aspects of plant metabolism for which this tissue has not been examined.

Some information regarding diverse experiments using radish cotyledons have been mentioned throughout the present work. Additional information specific to this tissue is summarized below.

Sulfur metabolism is very active in radish plants, including its cotyledons. Sulfur-35 enters not only into a series of amino acids, but also in some substances peculiar to this crucifer, which are responsible for the characteristic odour and taste of its tissues. For example, Sinha and Cossins (1963) found that ten day old green radish cotyledons rapidly incorporated  $S^{35}O_4$  into the amino acid fraction, in both dark and light. Incorporation was practically linear with time. Considerable activity was also found in the ether soluble fraction. Cysteine and cysteic acid were the first labelled compounds detected and at 30 minutes, activity appeared in taurine, methionine and its derivatives. Both cysteine and methionine have been detected in rather large amounts in radish cotyledons by other workers (Thompson and Moore, 1968) who determined serine and acetyl-serine as the more or less direct precursors of cysteine. The reaction would operate *in vitro* as follows:



Other effective substrates for this reaction were demonstrated: o-phospho-L-serine and D-serine. It seems clear that in these cases, serine and acetate play important roles.

Raphanin, sulphoraphane and sulphoraphene are common names of sulfur compounds of the isothiocyanate group contained in seeds and plants of radish. All these compounds have shown to possess certain antibacterial activities. The most recent work on this particular subject has been reviewed by Fowden (1967).

Studies of the regulatory mechanisms involved in the assimilation of nitrate by radish cotyledons (Ingle *et al.*, 1966) demonstrated that the incubation of this tissue in nitrate induced the specific formation of nitrate reductase (*NADH:nitrate oxidoreductase*, EC 1.6.6.1) and indirectly nitrite reductase (*nitric oxide:(acceptor)oxidoreductase*, EC 1.7.99.3), without affecting the general metabolism of the cotyledons.

This *de novo* synthesis of enzymes, implies nucleic acid and protein synthesis, and Ingle (1968) proceeded to investigate these syntheses in the same tissue. The results obtained show that synthesis of total ribonucleic acid and protein took place in cotyledons varying in age. Ribonucleic acid metabolism in radish cotyledons was shown to be more complex than in the non-green or etiolated tissues which have been commonly used in other studies. Two additional low-molecular-weight and three additional high-molecular-weight types of ribonucleic acid were found to be present in the

green cotyledons and appeared to be restricted to the chloroplasts. Fractionation of the nucleic acids from radish cotyledons showed the presence of a soluble-ribonucleic acid, a light ribosomal-ribonucleic acid, a heavy ribosomal-ribonucleic acid and a deoxyribonucleic acid fraction.

Significantly, the induction of nitrate reductase was maximal at those stages of development when the cotyledons might be expected to have low general metabolic activity, that is when their full growth had been attained, and as a consequence no net protein synthesis would be expected to occur (Ingle, 1968). The induction of nitrate reductase in the radish cotyledons involved only a very small percentage of the protein, either on the basis of total protein present or on the protein synthesized during the induction period. This was established by incorporation of radioactivity into the different protein fractions and by purification of the nitrate reductase.

Enzymes which catalyze the formation of compounds common to the pathways concerned with carbohydrate biosynthesis and metabolism, have been found present in radish cotyledons. These enzymes are glyceraldehyde-3-phosphate dehydrogenase (*D-glyceraldehyde-3-phosphate:NADP oxidoreductase*, EC 1.2.1.9), aldolase (*ketose-1-phosphate aldehyde-lyase*, EC 4.1.2.7) and glucose-6-phosphate dehydrogenase (*D-glucose-6-phosphate:NADP oxidoreductase*, EC 1.1.1.49) (Ingle, 1968).

The formation of sterols during germination in radish cotyledons, and the influence of light on this process

(Dupéron, 1968) were studied. In the dark, dry matter diminished regularly, with parallel decreases in the quantities of nitrogenous compounds and lipids. The sterols content in the dark was negligible at all stages of growth. In the light, growth was promoted with increases in dry matter, amino compounds and a dramatic increase in total sterols. However, total lipids remained constant in both dark and light.

Considering the large amount of information that has accumulated regarding the metabolism of glyoxylate and related compounds in higher plants, it is surprising that no detailed studies have been carried out with photosynthesizing cotyledons. As pointed out above, the cotyledons of radish are highly active metabolically. Furthermore, recent data regarding different aspects of protein synthesis in this tissue gave strong indications of an active nitrogen metabolism centered on the amino acids. For these reasons, radish cotyledons were chosen as the experimental material for the present studies with the object of extending the present knowledge of amino acid biosynthesis and also of further understanding the overall patterns of metabolism in this organ.

## MATERIALS AND METHODS

*Plant tissue*

Seeds of *Raphanus sativus* L. c. var. "Red Boy, Short Top", were sown in flats packed loosely with sterilized slightly sandy soil of near neutral pH. The flats were placed in growth chambers at a constant temperature of 65°F with alternating periods of dark and light of 12 hours each. The soil was kept moist by watering daily with distilled water.

Eleven days after planting, the cotyledons were fully grown and without any apparent trace of senescence and the primary leaves had just started to develop. The size of the cotyledons at this stage varied in width from 30 to 39 mm and the seedlings had average lengths of 85 mm (Appendix, Plate 2).

The cotyledons were excised from such seedlings as quickly as possible after removal of the flats from the growth chambers. The tissues were washed with distilled water, placed on moist paper towels to keep the material from wilting, and disks were then cut with a sharp, clean cork borer, avoiding large veins and margins. After cutting, the disks were immediately used in the experiments. The time between cutting the disks and the actual start of the experiments never exceeded 60 seconds.

*Labelled compounds*

Glyoxylate-1,2-C<sup>14</sup> and glycine-1-C<sup>14</sup> were purchased from Atomic Energy of Canada Ltd., Ottawa. Sodium carbonate-C<sup>14</sup>, glycine-2-C<sup>14</sup>, glyoxylate-1-C<sup>14</sup>, glyoxylate-2-C<sup>14</sup> and serine-3-C<sup>14</sup> were obtained from the Radio Chemical Centre, Amersham, England. Sodium acetate-2-C<sup>14</sup> was supplied by Nuclear Chicago, Des Plaines, Illinois, U.S.A. In all cases, these radioisotopes were diluted with carrier to obtain solutions having the specific activities mentioned in the Results Section.

*Feeding experiments*

There was one basic type of experiment, and variables were methodically introduced in the design of the various experiments. The variables were presence or absence of light, time of incubation in the labelled substrates (pulse) and time of incubation in unlabelled substrates (post-pulse).

The basic procedure followed in these experiments is summarized below.

0.5 ml of 0.1 M potassium phosphate buffer (pH 5.0) was placed in the main compartment of a 25 ml Warburg flask fitted with a single side arm. Twenty plant disks, with an average fresh weight of 0.100 gm were then carefully placed in the flask and distributed so that they formed a single layer with the underside of the cotyledon in contact with the buffer solution. In this manner differences in photosynthetic capacities and assimilation rates of the leaf disks were avoided (Sesták and Vodová, 1964). When

CO<sub>2</sub> evolved during the experimental period was collected, 0.5 ml of 20% (w/v) potassium hydroxide solution was placed in the centre well of the flask. The absorbed carbon dioxide was converted to barium carbonate, plated onto glass-fibre filter paper disks by means of vacuum filtration and dried overnight at 90°C. Carbon-14 contents were then determined by means of a Nuclear Chicago gas flow counter Model D47. The counts were corrected for self-absorption and background. With the exception of the C<sup>14</sup>O<sub>2</sub> experiments, in all other experiments the radioactive compounds were added directly into the flasks over the plant tissues, and even distribution was achieved by occasional gentle shaking. When sodium carbonate-C<sup>14</sup> was used, 0.1 ml of the labelled solution was carefully pipetted into the side arm, and an equal amount of distilled water added to increase the efficiency of the transfer of C<sup>14</sup> to the main compartment of the flask. A 0.1 ml aliquot of distilled water was added in all other feeding experiments to the buffer in the flask, to ensure that the volume of the feeding solution was in all cases 0.7 ml.

All the experiments were performed at room temperature (22-25°C) and in duplicate or triplicate. When, for reasons of space, only one value is given per experiment, this represents the average of the values obtained from at least two identical experiments carried out simultaneously. Variations in the above standard procedures were as follows.

*a. Experiments in the dark*

The flasks were wrapped in aluminum foil, and once the

plant material had been introduced, they were kept in total darkness for 30 minutes. After this time, aliquots of glyoxylate-1-C<sup>14</sup>, glyoxylate-2-C<sup>14</sup> or glycine-1-C<sup>14</sup> were carefully added to each flask. The tissues were then incubated in darkness with intermittent shaking, for a period of 30 minutes.

*b. Experiments with illumination*

The majority of the feeding experiments were of this type. Illumination was provided by a series of photo-flood bulbs. The light intensity received by the tissue was 2,500 ft. candles with the arrangement shown in Appendix, Plate 1. The ambient temperature remained relatively constant ( $\pm 3^{\circ}\text{C}$ ).

The addition of labelled compounds in all the experiments in light was preceded by a ten minute pre-illumination period, after which it was assumed that a photosynthetic steady-state had been reached.

*c. Variations in incubation time and Pulse-chase experiments*

In several experiments the time of incubation of the tissues with the labelled substrate was varied. At the end of each experimental period the tissues were routinely removed and killed as described below. In other experiments the labelled substrate was supplied for 30 minutes (pulse period) followed by rapid transfer of the tissue to similar flasks containing equimolar amounts of the unlabelled substrate. Such tissues were then incubated for varying periods in the light (post-pulse period).

*Analytical methods*

At the end of the experimental periods, the tissues were rapidly killed by boiling for 5 minutes in 3-5 ml of 80% ethanol. In some experiments the media in which the tissues had been incubated were removed before addition of ethanol.

The tissues were then finely homogenized by means of an all glass homogenizer and centrifuged at 9,000 g. for twenty minutes to separate the alcohol soluble fraction from insoluble residual material. The insoluble residue was then successively washed with 15 ml of 50% ethanol followed by 15 ml of distilled water, and the centrifugation repeated after each wash. The supernatants were combined and evaporated to dryness under reduced pressure, at 38°C using a Buchler Rotary Flask evaporator, Model FE-2 (Buchler Instruments, Inc.). The dried material was washed three times with small amounts (2 ml) of anhydrous diethyl ether to remove ether soluble materials, leaving a lipid-free residue. The ether extracts were washed twice with 2 ml aliquots of distilled water and the ether layer was made up to 10 ml. Aliquots were then taken for assay of radioactivity as described later.

The insoluble residue left from the repeated centrifugations of the whole sample, was resuspended in 20 ml of 6 N hydrochloric acid, transferred to a 100 ml Quickfit tube, covered and boiled for 20 hours to achieve hydrolysis of proteins, polysaccharides and any other hydrolyzable compounds. The contents of the tube were then filtered

and any solid residue collected on glass fibre filter disks followed by drying and assay of radioactivity in the gas-flow counter.

The hydrolyzates were evaporated to dryness *in vacuo* and, in order to ensure a complete removal of hydrochloric acid, two or three washes with distilled water followed by further evaporation were made. The dry hydrolyzates were then fractionated by ion exchange chromatography.

#### *Ion exchange chromatography of plant extracts*

The ethanol soluble material and the acid hydrolyzates were then redissolved in 5 ml of distilled water at room temperature, and separated into three main fractions by the use of ion exchange resins as previously described (Canvin and Beevers, 1961; Cossins and Beevers, 1963).

The three main fractions obtained were:

1. Basic compounds, largely amino acids and their derivatives.
2. Acidic compounds, largely organic acids, phosphate esters and nucleotides.
3. Neutral compounds, mainly sugars.

#### *Ion exchange resins*

Two types of resins were obtained from California Corporation for Biochemical Research, Los Angeles:

Cation exchange resin, Dowex 50W-X8 (hydrogen form)  
200-400 mesh.

Anion exchange resin, Dowex 1-X10 (chloride form)

200-400 mesh.

*Preparation of anion exchange resins*

The cation exchange resin needed no further preparation, except for washing with distilled water until the effluent was close to neutral. The anion exchange resin required conversion to the formate or acetate forms before use. The conversion of the chloride form of the resin into the formate form, was achieved by washing with 1M sodium formate until the effluent gave a negative test for chlorides. The resin was then washed with 0.1N formic acid (50ml/5ml resin) followed by distilled water until the effluent was close to neutral (Canvin and Beevers, 1961).

*Fractionation of plant extracts*

The ethanol soluble material, and the acid hydrolyzate were passed through 4 x 1 cm columns of Dowex-H<sup>+</sup> (hydrogen form) resin. The effluent, containing all the negatively charged and neutral compounds, was collected. The column was subsequently washed with distilled water to ensure complete removal of compounds not adsorbed. The column effluents were combined, concentrated and designated as the organic acid-sugar fraction. The amino acids were then eluted by washing the column with approximately 100 ml of 2 N ammonium hydroxide solution, and the effluent collected and evaporated to dryness.

The organic acid-sugar fraction was then applied to a 3 x 1 cm column of Dowex anion exchange resin in the formate

form. The sugars passed through the column and were collected, followed by a 50-100 ml rinse of distilled water. The organic acids, which had been retained by the resin, were eluted with 100 ml of 4 N formic acid. Both fractions were evaporated to dryness, and redissolved in 5 ml of distilled water for radioactivity determination.

#### *Determination of the radioactivity*

With the exception of evolved  $C^{14}O_2$ , recovered as  $BaC^{14}O_3$ , and the solid residues, all other samples were measured for radioactivity by means of scintillation counting, using a Nuclear Chicago Scintillation Counter, Model Unilux II.

Aliquots of the aqueous samples obtained from the columns were placed in scintillation vials containing 20 ml of dioxane scintillation cocktail (0.5 gm POPOP, 12 gm PPO, 125 ml anisole, 125 ml dimethoxyethane, 750 ml dioxane).

Extensive tests with solutions of known activity showed that the above procedure gave efficiencies of up to 70% when no more than 100  $\mu$ l of clear aqueous samples were used. All radioactivity data presented are the average of at least two separate determinations on each sample. Radioactivities were corrected for background.

#### *Separation of the individual amino acids*

An aliquot of the amino acid fraction, containing approximately 20,000 to 50,000 cpm was used for chromatographic separation of the component amino acids. When this

aliquot proved to be too large (>50  $\mu$ l) its volume was reduced in a stream of cold air. Separation of each individual amino acid was carried out by a modification of the method of Bielecki and Turner (1963).

Thin layer plates 20 x 20 cm were prepared using a Desaga spreader (Desaga, Heidelberg, West Germany) to give a 300  $\mu$  thick layer of cellulose:silica gel in the proportions of 12.5 gm cellulose:5 gm silica gel. A two dimensional ascending technique was used. To avoid irregularities in solvent front, a 5 mm band of the layer was scraped off the edges. In order to avoid inconsistencies, the second solvent was always run in the same direction as the spreader.

Fifteen different pairs of solvent systems were examined in order to find a system which would give satisfactory separations with good reproducibility and a minimum of loss of the amino acids. The pair finally selected had been previously used by Fink *et al.* (1963). The composition of these solvent systems was t-butanol:methylethylketone:formic acid:water, 40:30:15:15 by volume and phenol:water, 75:25 w/v. Several preliminary runs were made with this solvent pair, using mixtures of known amino acids and protein hydrolyzates. The separations achieved are presented in Appendix, Plate 3.

When considering recoveries of various amino acids it was observed that glutamine and glutamic acid underwent the greatest amount of loss, probably by spontaneous deamidation and subsequent cyclisation to pyrrolidone-5-carboxylic acid which is known to occur in the presence of phenol (Bielecki

and Turner, 1962). Losses, however, were never greater than 20% for any amino acid examined.

Samples varying from 1 to 50  $\mu$ l were applied at the lower left corner of the plates at a distance of 25 mm from each edge. Each sample was run in duplicate. A plate containing authentic amino acids was run with each group of six plates, in order to detect differences due to each particular run. This reference plate included at least six amino acids in known concentration.

The plates were then placed vertically on a metal rack and introduced into a 30 x 30 x 30 cm Shandon all glass chromatotank (Colab Canada Ltd., Ont., Can.). An adequate volume of the first solvent was placed in the tank and allowed to ascend 150 mm from origin. At this point, the rack was removed from the tank, and dried overnight in a fume-hood.

The second solvent treatment was carried out in a similar manner, at right angles to the direction of the first solvent. When the plates were free of any obvious traces of phenol, the labelled amino acids were located by radioautography, using 20.3 x 25.4 cm sheets of Kodak No-screen X-ray film. The film was placed directly on top of the thin layer, sandwiched by another clean glass plate and secured in place with cellulose tape. The thin layer was not disturbed by this procedure and the cellulose tape did not leave ghost-shadows in the film, as occurred with other materials such as masking tape, metal clips, etc. In the samples of low radioactivity (<20,000 cpm) the films

were exposed for 10 days, while in samples with higher activity (50,000 cpm) four to six days were sufficient to detect all of the important compounds.

After development of the films, the radioactive areas on the thin layer plate were circled with a sharp instrument. A purified pyroxylin, marketed by Mallinkrodt Chemical Works under the name of Parlodion, was dissolved into a mixture of absolute ethanol and anhydrous diethylether (1:1 v/v) to make a 1.5 % solution. Of this, a small amount was poured close to and along one edge of the plate and then spread over the entire surface of the plate with the aid of a clean glass rod. When the plastic film had dried, the labelled areas could be very easily cut out and lifted from the glass with the help of a sharp razor blade. In this way, the thin layer material was quantitatively removed from the glass plate. The areas were then placed intact into scintillation vials together with 20 ml of dioxane scintillation fluor, and their activities determined. Efficiencies of up to 70% were obtained in most cases. Shaking or breaking of the samples within the vial, or in cases where the samples were lying flat on the bottom of the vial, did not alter the counting efficiency.

The duplicate plate of each sample along with the reference plate were sprayed with a solution of 0.5% ninhydrin in acetone. Permanent records of the chromatograms were obtained by application of three successive coats of Parlodion solution. The resulting film, when thoroughly dried, was flexible enough to allow removal from the glass

plate and filing for future reference (Appendix, Plate 3). It is interesting to note that the characteristic colours with ninhydrin were also preserved.

*Separation of the component organic acids*

Further fractionation of the organic acid fraction eluted from the formate resin was achieved by a modification of the method of Nygaard (1967). The modification involved use of a high-voltage electrophoresis technique for separation of the organic acids on thin layers of cellulose.

Cellulose powder MN 300 (Macherey, Nagel & Co.) mixed with water (17.5 gm to 100 ml) was spread on glass plates to a thickness of 350  $\mu$ . The plates were dried at room temperature and prepared as described by Nygaard (1967). Samples of 10  $\mu$ l were applied whenever possible without accelerated drying. The plates were cooled to 4°C in the refrigerator. The electrophoresis chamber, (Gelman Instruments Co. Deluxe model) was also placed in the refrigerator, partially filled with cold 0.5 M acetic acid buffer adjusted to pH 4.0 with pyridine, and connected to an external power supply (voltage and current regulated DC Buchler Power Supply, Model 3-1014 A).

The plates with samples were quickly and evenly sprayed with the above mentioned buffer, and immediately positioned in the chamber so that the sample was as close as possible to the cathode. Chamber and refrigerator were closed and a potential of 1,000V was applied for 30 minutes. The rest of

the procedure for the separation of organic acids was as described by Nygaard (1967) (Appendix, Plate 4).

Reference plates, containing individual and mixtures of the organic acids most likely to be found labelled in the feeding experiments, were also run, with concentrations of each organic acid varying from 0.025  $\mu$ mole to 0.2  $\mu$ mole, in total volumes of 1, 5 and 10  $\mu$ l respectively.

The experimental samples were concentrated to the desired level of radioactivity as described for the amino acids. Volumes ranging from 2 to 10  $\mu$ l with total activities of 11,000 to 20,000 cpm were used.

Although recoveries of the common organic acids were good, considerable losses occurred with most of the keto-acids, particularly glyoxylic and pyruvic.  $\alpha$ -Ketoglutaric and glycollic acid were also only partially recovered but oxaloacetic acid was not detected at all.

After separation and identification of the main organic acids on each plate, radioactivity determinations were carried out as described for the amino acids.

## RESULTS

*The utilization of CO<sub>2</sub> by radish cotyledons in the light.*

Radish cotyledons, as any other photosynthesizing tissue, have the ability to fix CO<sub>2</sub> in the light. For example, since commencement of the present work, Black, Gordon and Williams (1968) have shown that certain parasitic infections affect the photosynthetic rate of these tissues. Coincidentally, they used 11 day old cotyledons which had been grown under conditions similar to those of the present studies. They determined that the maximum net CO<sub>2</sub> uptake in healthy cotyledons occurred at that age, and further, that while a light intensity of 800-1,000 ft. c. was required to maintain a net carbon accumulation, light saturation of photosynthesis was between 1,200 and 1,400 ft. c. At this light intensity the net CO<sub>2</sub> fixation was approximately 18  $\mu$ l/hr/mgm dry weight; respiratory CO<sub>2</sub> evolution in the dark was 3.4  $\mu$ l/hr/mgm dry weight. An attempt was therefore made, in the present work, to determine the products of CO<sub>2</sub> fixation of this tissue in the light and to compare these with the products of photosynthesis in the leaves of higher plants.

*Carbon dioxide utilization by illuminated disks of radish cotyledons as a function of time. Preliminary experiment.*

Labelled CO<sub>2</sub> was provided to disks of radish cotyledons in the light, in the form of NaHC<sup>14</sup>O<sub>3</sub>, with a specific activity of 20  $\mu$ c/ $\mu$ mole/0.1 ml. of solution. The results

of this experiment are summarized in Table I. After 30 seconds of illumination, the bulk of the radioactivity was distributed almost equally between the neutral and basic amino acid and the organic acid fractions. These fractions accounted for 80% of the total  $C^{14}$  incorporated after 30 seconds. Practically all of the remaining 20% was found to be distributed between sugars and the ethanol insoluble residue.

As the time of incubation was increased, the percentage of total activity incorporated in the organic acid fraction steadily decreased. At the end of 20 minutes, the percentage of label present in that fraction was less than half of that found at the end of the 30 seconds experiment. The percentage of activity present in the neutral and basic amino acid fraction reached a maximum at 3 minutes, followed by a decreasing trend. On the other hand, the acidic amino acids and amides showed a steady but small increase with time.

The insoluble residue showed a pattern of incorporation which was similar to that of the neutral and basic amino acid fraction, attaining a maximum after 3 minutes followed by a steady decrease. Sugars and lipids, showed a linear increase with time throughout the experiment.

After 20 minutes of illumination, the bulk of the radioactivity, expressed as percentage of the total  $C^{14}$  incorporated, was still in the neutral and basic amino acid fraction, while the activity found in organic acids had decreased and that of the sugars increased. Both the

neutral and basic amino acid and the sugar fractions accounted for 64.6% of the total  $C^{14}$  incorporated at the end of the 20 minute period. Of the remaining radioactivity, 17% was present in the organic acid fraction. The results of this  $C^{14}O_2$  feeding experiment are summarized graphically in Fig. 1.

Chromatography and autoradiography of the organic acid and amino acid fractions showed that most  $C^{14}$  was present in malate, citrate and various sugar-phosphates. Malate was in all cases more active than citrate.  $\alpha$ -Ketoglutaric acid, was labelled after 10 min. of  $C^{14}O_2$  fixation. Serine, glutamate, glycine, aspartate and  $\alpha$ -alanine were also labelled, the levels of  $C^{14}$  being greatest in serine and decreasing in the order given. This pattern of activity was essentially unaltered during the 20 min. experimental period.

Clearly, from the results obtained in the above experiment, the tissue has the ability to fix  $CO_2$  in the light, and metabolize such carbon by different pathways. The heavy labelling of malic acid in the shortest time experiment, may indicate incorporation of  $CO_2$  via PEP and oxaloacetate. The amino acids labelled in this experiment are those expected from photosynthesis.

In order to examine these pathways in more detail a second experiment was carried out in which  $C^{14}O_2$  was pulse fed for a longer period followed by incubation in  $C^{12}O_2$ .

TABLE I

*The utilization of  $C^{14}O_2$  by illuminated disks  
of radish cotyledons as a function of time*

Samples of disks (100 mgm) from eleven-day old radish cotyledons were incubated at room temperature in Warburg flasks containing 0.5 ml of 0.1 M  $KH_2PO_4$  buffer at pH 5.0, 0.1 ml of  $NaHC^{14}O_3$  (20  $\mu c/\mu mole/0.1$  ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and extracted as described in the text.

Note: No activity was detected at 30 seconds in the solid residue.

TABLE I

Incubation time	30 sec.	1 min.	2 min.	3 min.	5 min.	10 min.	20 min.
Lipids	1,450 (0.78)	4,390 (1.17)	6,530 (1.30)	12,580 (1.99)	22,310 (3.07)	53,180 (4.88)	133,800 (8.26)
Acid hydrolyzates	17,870 (9.47)	37,250 (9.93)	62,470 (12.47)	78,250 (12.40)	78,180 (10.77)	117,250 (10.76)	144,900 (8.95)
Solid residue	---	230 (0.06)	400 (0.08)	700 (0.11)	1,120 (0.15)	2,640 (0.24)	4,240 (0.26)
Sugars	15,680 (8.26)	31,910 (8.51)	51,450 (10.27)	74,780 (11.85)	100,900 (13.91)	212,920 (19.53)	495,610 (30.59)
Organic acids	74,780 (39.56)	131,430 (35.04)	146,890 (29.32)	167,420 (26.55)	179,050 (24.67)	238,000 (21.84)	276,320 (17.06)
Neutral and basic amino acids	81,620 (42.95)	167,970 (44.80)	231,890 (46.29)	294,510 (46.67)	341,080 (46.98)	462,200 (42.41)	550,980 (34.01)
Acidic amino acids	270 (0.14)	930 (0.25)	1,040 (0.21)	1,550 (0.25)	1,900 (0.26)	3,280 (0.30)	5,280 (0.33)
Amides	150 (0.08)	340 (0.09)	490 (0.10)	760 (0.12)	1,080 (0.15)	1,990 (0.18)	4,750 (0.29)
Total C <sup>14</sup> incorporated	191,820	374,450	501,160	630,550	725,620	1,091,550	1,615,880
	202,900	359,620	512,920	612,470	738,450	1,100,050	1,637,610

Figures in brackets represent percentages of the total C<sup>14</sup> incorporated.  
Data are expressed as cpm.

FIGURE 1

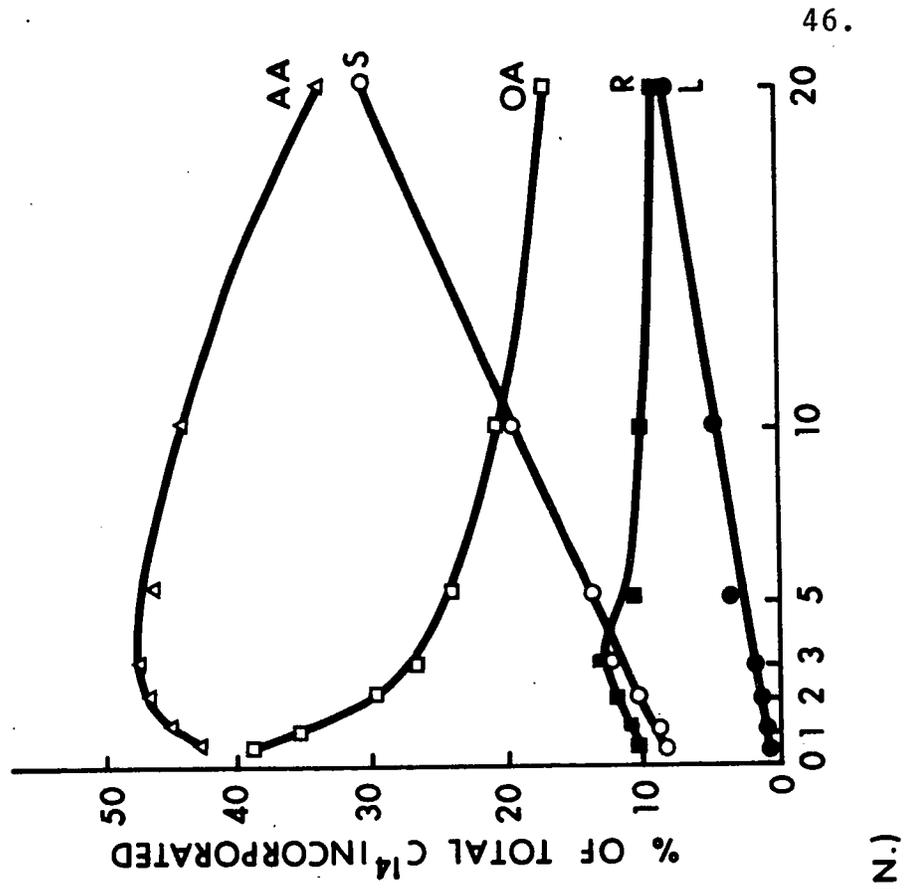
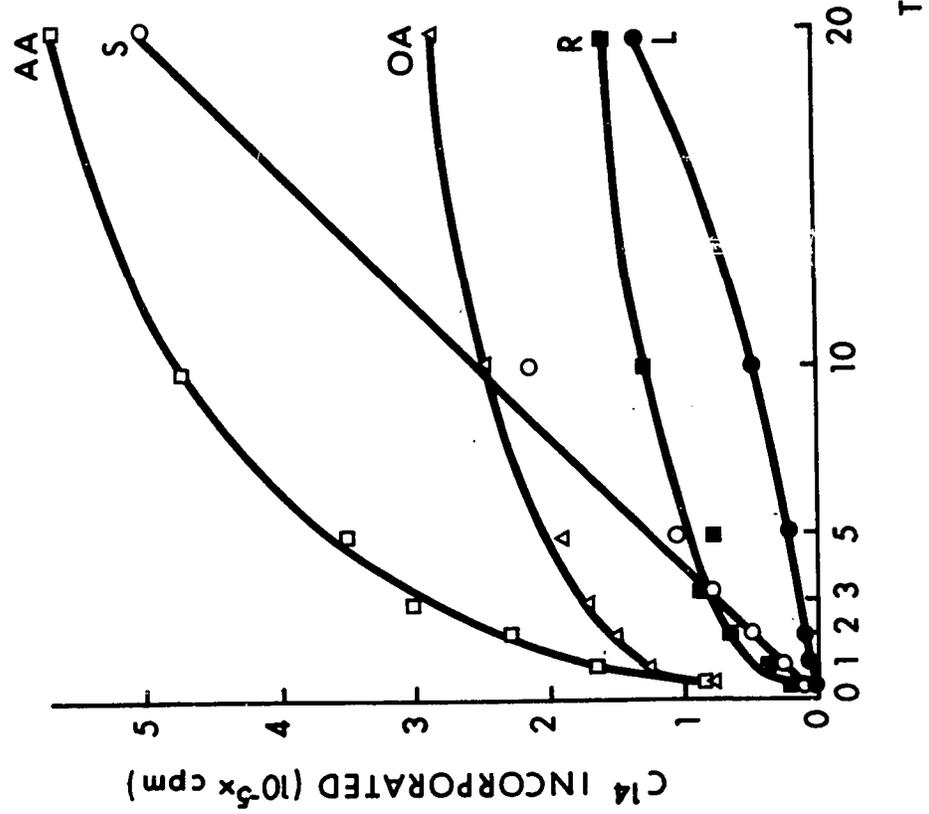
*Incorporation of  $C^{14}O_2$  by illuminated disks of  
radish cotyledons as a function of time.*

The data presented are obtained from Table 1.

AA: amino acid fraction; L: lipid fraction;

OA: organic acid fraction; R: ethanol insoluble  
fraction; S: sugar fraction.

FIGURE 1



*Utilization of carbon dioxide by illuminated disks of radish cotyledons. Pulse-chase experiment.*

This experiment was performed in the manner described in the Materials and Methods section. The labelled  $C^{14}O_2$  was provided in the form of 0.1 ml of  $NaHC^{14}O_3$  solution (specific activity 20  $\mu c/\mu mole/0.1$  ml. of solution). Since many workers (e.g. Ongun and Stocking, 1965b; Bassham *et al.*, 1968) have reported that compounds formed either by photosynthesis or other processes leach out into the medium even in short time experiments, the medium in which the present tissues were incubated was collected at the end of each incubation period and examined for labelled components.

Table II and Fig. 2 show the distribution of radioactivity in the different fractions at the end of each incubation period expressed in cpm. It was found that the amount of activity present in the medium at the end of each period was considerable. As the post-pulse incubation was extended to 4 hours, the total radioactivity in the medium rose to 30% of the total  $C^{14}$  incorporated. During this time the radioactivity present in the sugar fraction showed a pronounced decrease suggesting that a rapid turnover of sugars was occurring. A direct relationship between labelling of the soluble amino acid fraction, the protein amino acid fraction and the organic acid fraction was apparent during the post-pulse incubation. All three fractions showed maximal incorporation of  $C^{14}$  between 30 and 60 minutes of post-pulse incubation.

The activity incorporated into the lipids and its increase with time, extends the finding of the preliminary experiment. It is well known that the incorporation of lipids into chloroplastic membranes is stimulated by light, particularly in growing tissues (Stumpf, 1965). Microscopic observation of the radish cotyledons used in the present work showed a mesophyll abundant in chloroplasts, and it is possible that synthesis of chloroplastic membranes is still continuing at this age. Also, incorporation into cytoplasmic sterols, which is independent of light, might account for the slow but steady incorporation of the label into the lipid fraction.

When the alcohol insoluble residue was hydrolyzed, most of the radioactivity was solubilized (Table II, Fig. 3a). Fractionation of this hydrolyzate yielded two fractions with characteristic patterns of  $C^{14}$  incorporation as shown in Table IIIa and Fig. 3b. The protein amino acid fraction became increasingly radioactive during the post-pulse period whereas organic acids liberated by acid hydrolysis showed a decrease during this period.

Chromatography of the protein amino acids revealed heavy labelling of glycine and serine together with smaller amounts of  $C^{14}$  in glutamate, aspartate and  $\alpha$ -alanine (Table IIIb, Fig. 3c).

These amino acids were also important labelled components of the soluble amino acid fraction (Table IV, Fig. 4). It is also of interest to note that in

addition,  $C^{14}$  was detected in glutamine and in certain sulphur amino acids like cysteine.

Tables Va and Vb summarize the distribution of  $C^{14}$  within the organic acid fraction. As in the preliminary  $C^{14}O_2$  feeding experiment, malate was the major labelled compound in this fraction. Labelling of malate was considerably increased during the post-pulse period in contrast to the labelling of the other acids which reached a maximum and then declined over this period (Tables Va and Vb, Figs. 5a and 5b).

As the medium in which the tissues had been incubated contained considerable levels of radioactivity (Table II) it was of importance to determine the nature of the labelled compounds involved. Labelled components in the medium were separated by thin layer chromatography as described in the Materials and Methods. The results of these analyses are summarized in Table VI, Fig. 6. Sucrose, the principal labelled component in the medium at the end of the experiment, rapidly accumulated during the post-pulse period. Similar trends were shown by asparagine, glutamine and serine. Smaller levels of radioactivity were also detected in glutamate, citrate and aspartate, each accumulating in the medium as the experimental period was extended.

TABLE II

*The incorporation of  $C^{14}O_2$  and turnover of  
labelled compounds during a post-pulse  
incubation*

Samples of disks (100 mgm) of eleven day old radish cotyledons were incubated for 30 minutes with illumination at room temperature in Warburg flasks containing 0.5 ml. of 0.1M  $KH_2PO_4$  buffer at pH 5.0, 0.1 ml. of  $NaHC^{14}O_3$  (20  $\mu c/\mu mole/0.1$  ml) and 0.1 ml. of distilled water in a total volume of 0.7 ml. (pulse). The tissues were subsequently transferred to Warburg flasks containing 0.5 ml. of 0.1M  $KH_2PO_4$  buffer and 0.2 ml. of distilled water, and incubated for periods ranging from 10 minutes to 4 hours (post-pulse). The tissues were killed and extracted as described in the text.

TABLE II

Incubation Time (min.)	Medium	Lipids	Amino acids	Organic acids	Sugars	Ethanol insoluble residue	
						Solids	Hydrolyzates
<i>Pulse</i>							
30	854	243	1,889	2,270	10,679	8	352
<i>Post-Pulse</i>							
10	1,202	290	2,491	4,531	8,407	10	391
20	1,332	412	3,013	4,809	9,581	15	475
30	1,456	438	2,992	4,730	8,710	16	605
60	1,618	479	2,575	4,521	7,070	19	707
120	2,118	527	2,148	3,929	5,929	21	732
180	3,024	648	1,847	3,559	4,988	22	512
240	4,199	862	1,457	3,085	3,689	23	439

Data are expressed as cpm.

Values presented are one thousandth of those obtained in the experiment.

FIGURE 2

*The incorporation of  $C^{14}O_2$  and turnover of  
labelled compounds during a post-pulse  
incubation.*

The data presented are obtained from Table II and are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities), and plotted as a function of time during the post-pulse period.

AA: amino acid fraction; L: lipid fraction;  
M: medium; OA: organic acid fraction; S: sugar  
fraction.

FIGURE 2

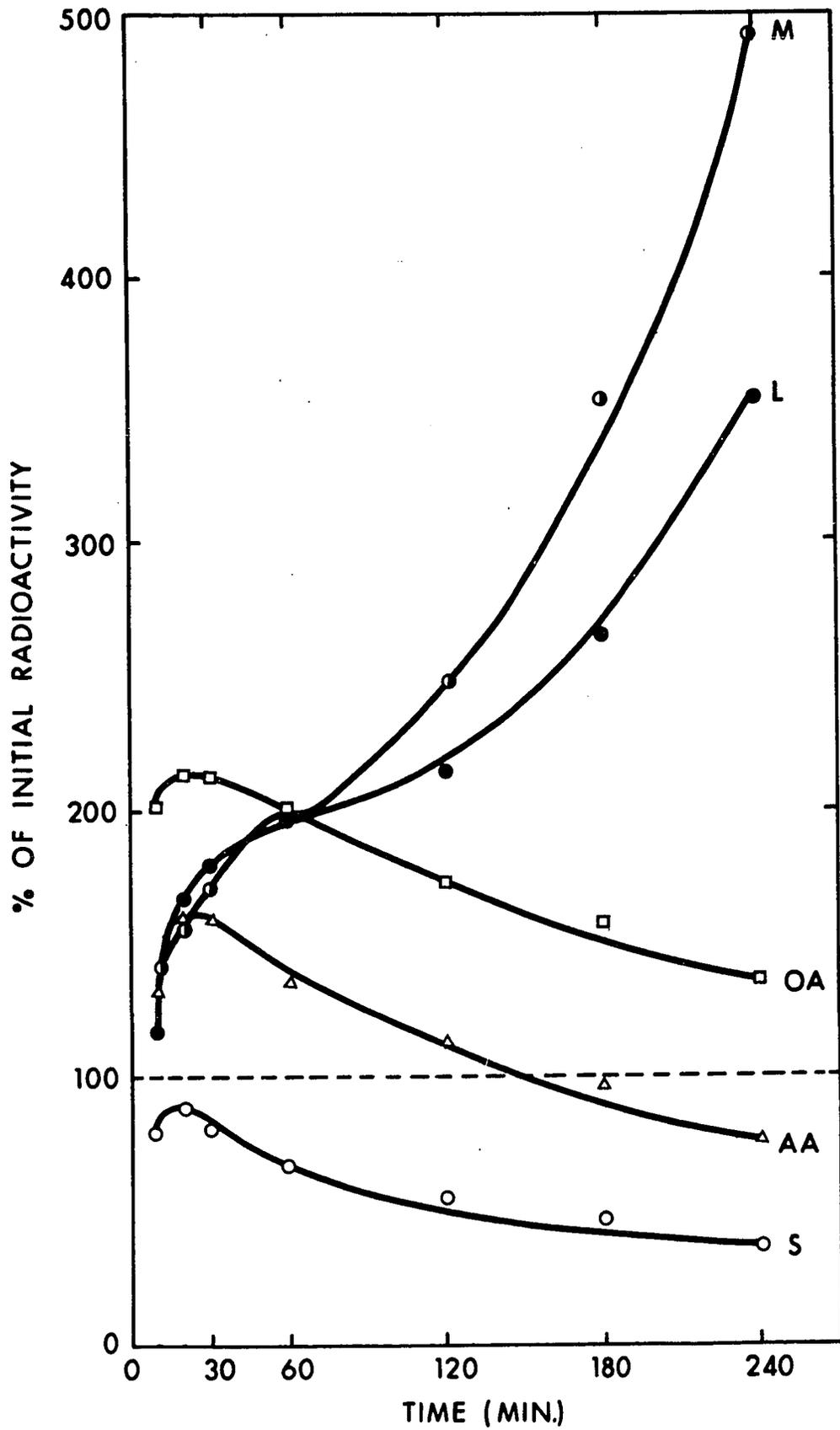


TABLE IIIa

*Incorporation of  $C^{14}O_2$  into the alcohol insoluble residue. Changes in radioactivity during the post-pulse incubation.*

TABLE IIIb

*Distribution of  $C^{14}$  from  $C^{14}O_2$  in protein amino acids. Pulse-chase experiment.*

Samples of the alcohol insoluble residue were acid hydrolyzed and the component amino acids were separated by thin-layer chromatography as described in the text.

TABLE IIIa

Incubation Time (min.)	Amino acids	Sugar and Organic acid fractions
<i>Pulse</i>		
30	70,750	281,250
<i>Post-Pulse</i>		
10	121,760	269,240
20	105,040	368,960
30	228,450	376,550
60	280,470	426,530
120	357,610	376,400
180	287,950	224,050
240	272,490	166,510

Data are expressed as cpm

TABLE IIIb

Incubation Time (min.)	Glycine	Serine	Glutamate	Aspartate	$\alpha$ -Alanine
<i>Pulse</i>					
30	5,460	6,550	1,100	1,220	1,250
<i>Post-Pulse</i>					
10	10,920	11,050	1,140	1,360	1,380
20	11,500	12,880	1,700	2,500	2,640
30	12,710	14,480	3,450	2,630	3,180
60	14,610	15,790	5,770	3,790	3,810
120	15,420	16,670	7,600	4,720	4,890
180	18,830	19,980	9,550	5,490	5,490
240	20,050	21,310	11,130	5,880	6,840

Data are expressed as cpm.

FIGURE 3

*a - Products of hydrolysis of the ethanol insoluble residue*

The data presented are obtained from Table II.\*

S1: solids; H: hydrolyzates

*b - Distribution of  $C^{14}$  into the products of fractionation of the hydrolyzate*

The data presented are obtained from Table IIIa.\*

AA: amino acid fraction; OA: organic acid fraction

*c - Distribution of  $C^{14}$  from  $C^{14}O_2$  into some of the different protein amino acids*

The data presented are obtained from Table IIIb.\*

Ala: alanine; Asp: aspartate; Gly: glycine; Glu: glutamate; Ser: serine.

\*The data are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

FIGURE 3

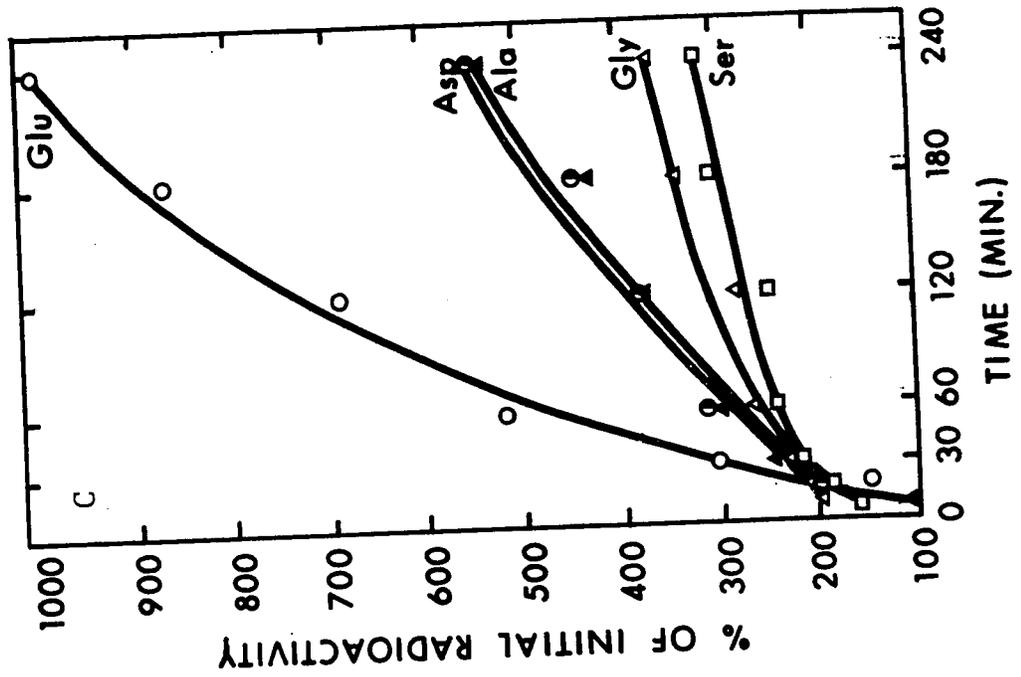
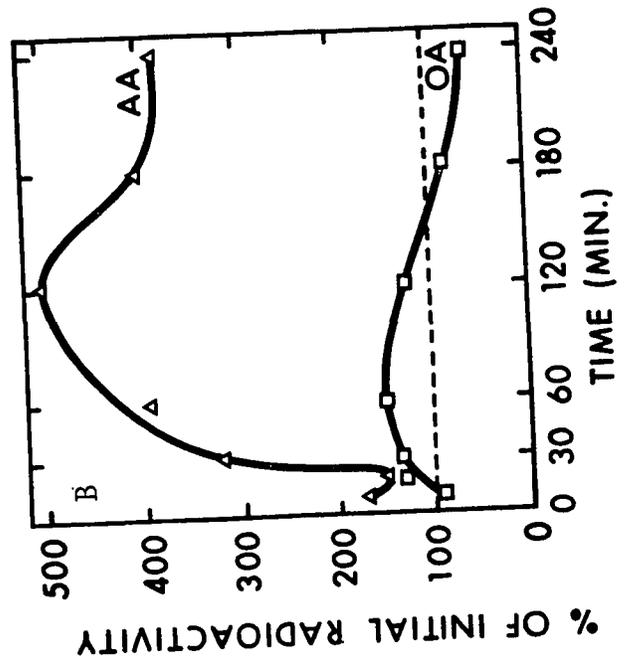
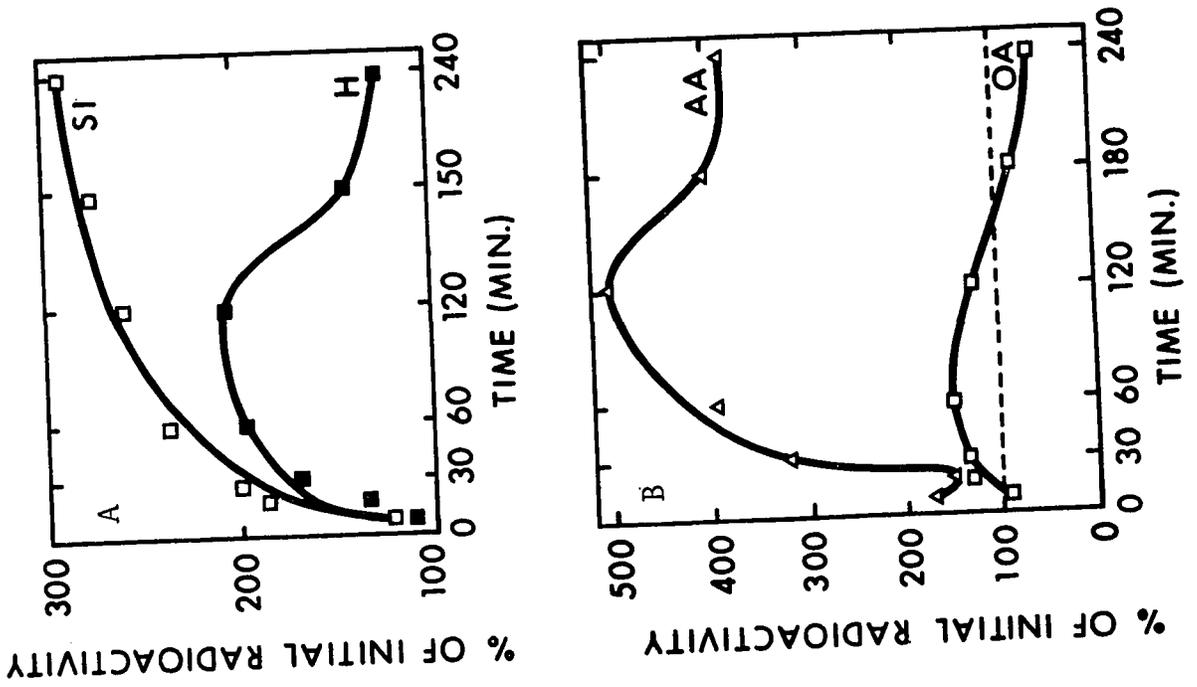


TABLE IV

*Incorporation of  $C^{14}O_2$  into the soluble amino acids and changes in their radioactivities during a post-pulse incubation*

Separation of the component amino acids was obtained by thin-layer chromatography as described in the text.

TABLE IV

Incubation Time (min.)	Glycine	Serine	Glutamate	Aspartate	$\alpha$ -Alanine	Glutamine
<i>Pulse</i>						
30	8	680	190	240	87	18
<i>Post-Pulse</i>						
10	105	1,100	202	280	95	26
20	190	1,773	280	190	120	48
30	251	1,496	340	105	135	77
60	207	1,150	334	70	140	87
120	135	1,085	357	58	152	97
180	112	840	403	24	164	70
240	90	530	420	---	210	--

Incubation Time (min.)	Cysteine	Cystine	Cysteic acid	Phosphoserine	$\gamma$ -Amino butyrate	S-methyl cysteine
<i>Pulse</i>						
30	--	--	41	100	33	--
<i>Post-Pulse</i>						
10	10	16	25	75	26	--
20	18	26	38	60	20	--
30	25	30	18	20	--	--
60	37	43	--	--	--	51
120	69	50	--	--	--	21
180	75	--	--	--	--	13
240	--	--	--	--	--	--

Data are expressed as cpm.

Values presented are one thousandth of those obtained in the experiment.

Note: Dash-lines in the table stand for "not detected".

#### FIGURE 4

*Incorporation of  $C^{14}O_2$  into the soluble amino acids.  
Changes in their radioactivities during a post-pulse  
incubation.*

The data presented are obtained from Table IV. Only the major radioactive amino acids of Table IV are plotted. The data are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

Ala: alanine; Asp: aspartate; Gly: glycine; Glu: glutamate;  
Gln: glutamine; Ser: serine.

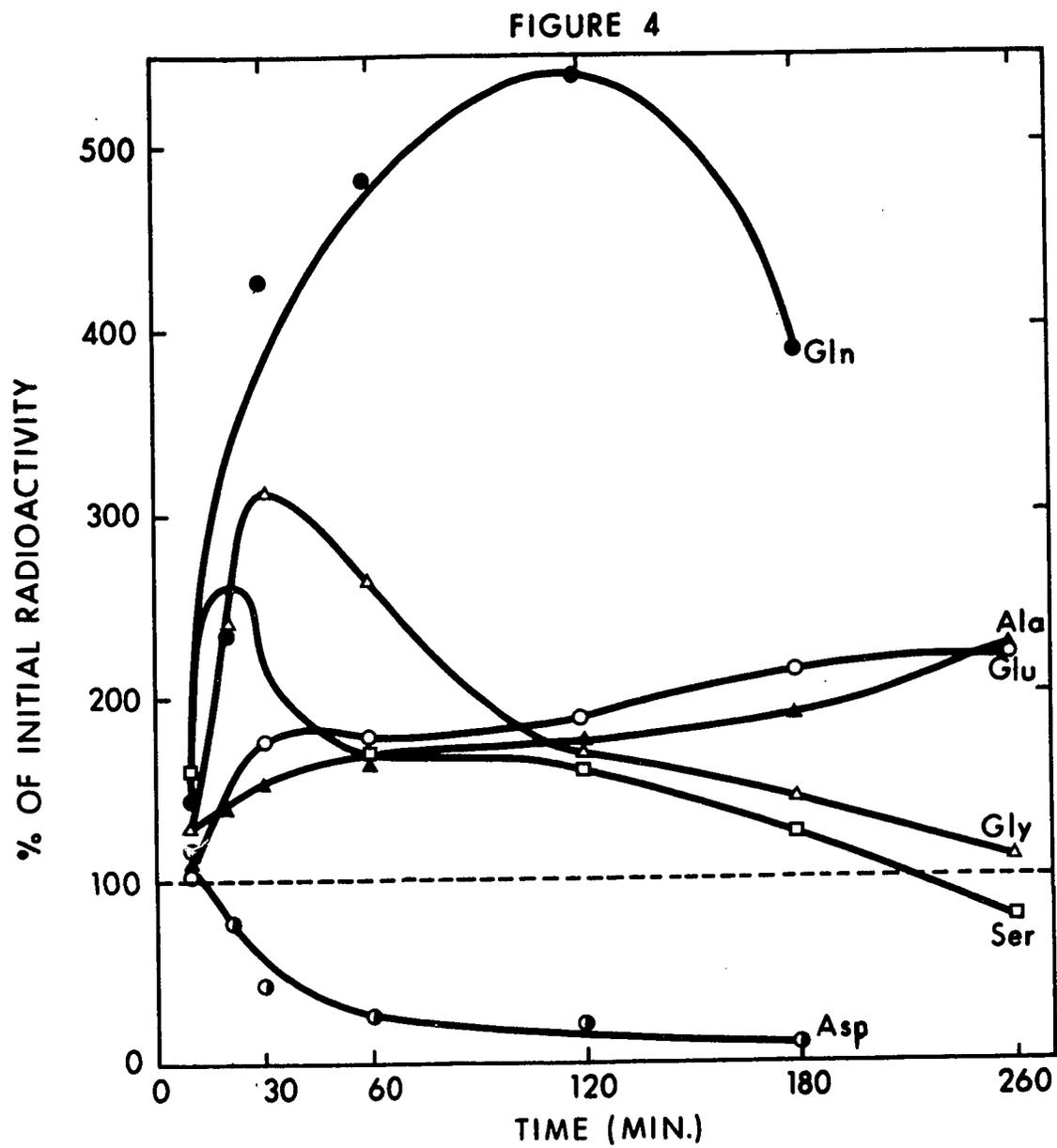


TABLE V  
a and b

*Incorporation of  $C^{14}O_2$  into the components of the organic acid fraction, and changes in their radioactivities during a post-pulse incubation.*

Separation of the component organic acids was obtained by thin-layer chromatography and high-voltage electrophoresis as described in the text.



TABLE Vb

Incubation Time (min.)	Glycollate	Glyoxylate	Phosphoenol- pyruvate	Phospho- glycerate	Unidentified sugar-phosphates			
					1	2	3	
<i>Pulse</i>								
30	6,730	597	3,200	2,800	40,010	48,910	13,180	
<i>Post-Pulse</i>								
10	1,305	1,980	1,800	900	31,330	39,240	12,450	
20	3,450	2,150	10,000	12,100	37,230	48,630	13,522	
30	6,680	2,890	14,000	18,900	44,780	55,040	15,580	
60	11,340	3,520	15,000	14,000	52,910	75,040	16,530	
120	9,720	3,270	15,300	8,200	31,280	34,159	8,820	
180	7,100	1,710	16,900	5,800	25,520	29,400	7,510	
240	4,230	1,500	17,250	4,200	17,530	20,250	4,230	

Data are expressed as cpm.

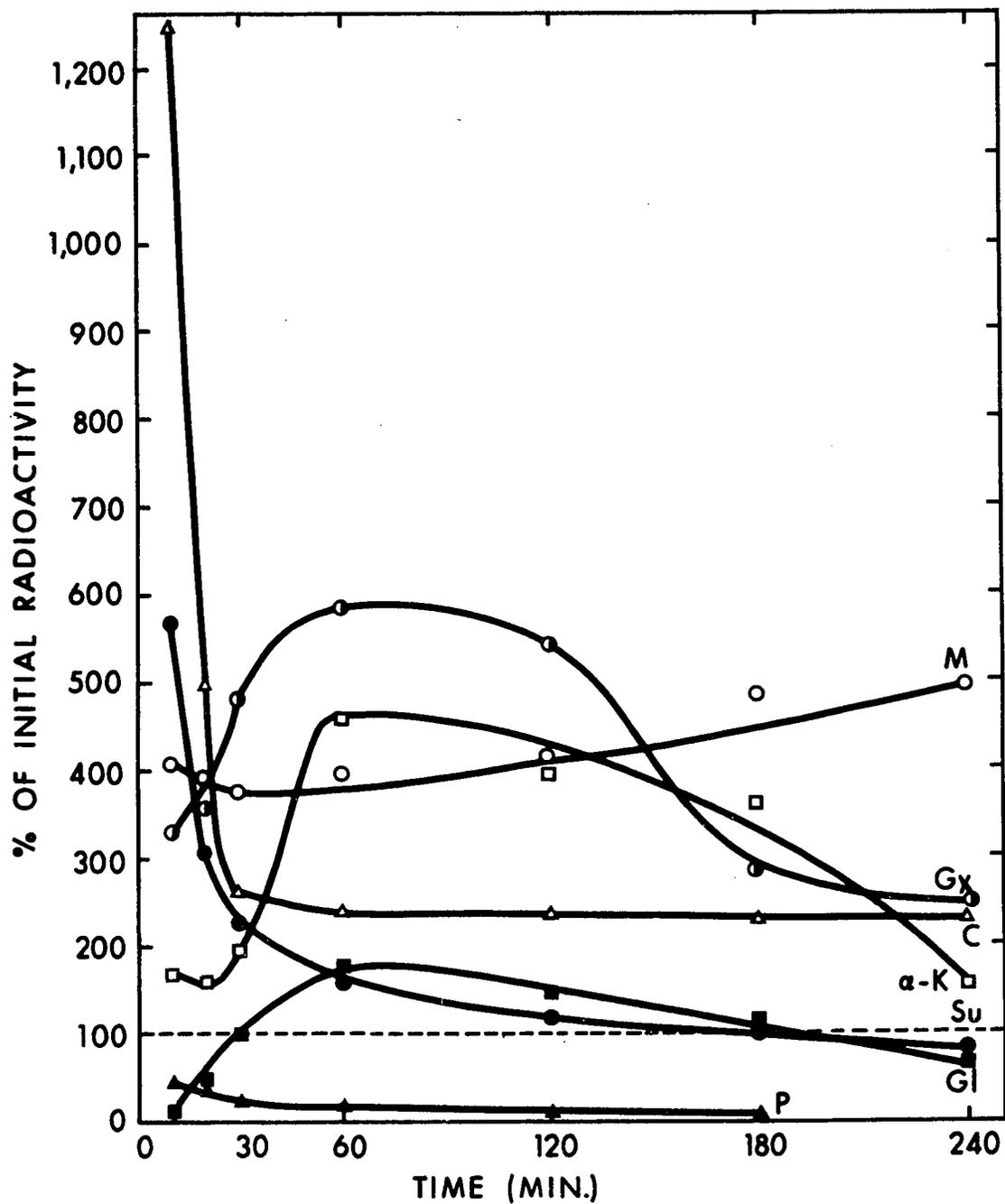
FIGURE 5a

*Incorporation of  $C^{14}O_2$  into some of the non-phosphorylated organic acids. Changes in their radioactivities during post-pulse.*

The data presented are obtained from Table V (a and b) and are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

C: citrate; G1: glycollate; Gx: glyoxylate;  $\alpha$ -K:  $\alpha$ -keto-glutarate; M: malate; P: pyruvate; Su: succinate.

FIGURE 5a



## FIGURE 5b

*Incorporation of  $C^{14}O_2$  into some of the phosphorylated compounds of the organic acid fraction. Changes in their radioactivities during post-pulse.*

The data presented are obtained from Table V (a and b) and are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

PEP: phosphoenolpyruvate; PGA: phosphoglyceric acid;

SP1, SP2, SP3: unidentified sugar-phosphates.

FIGURE 5 b

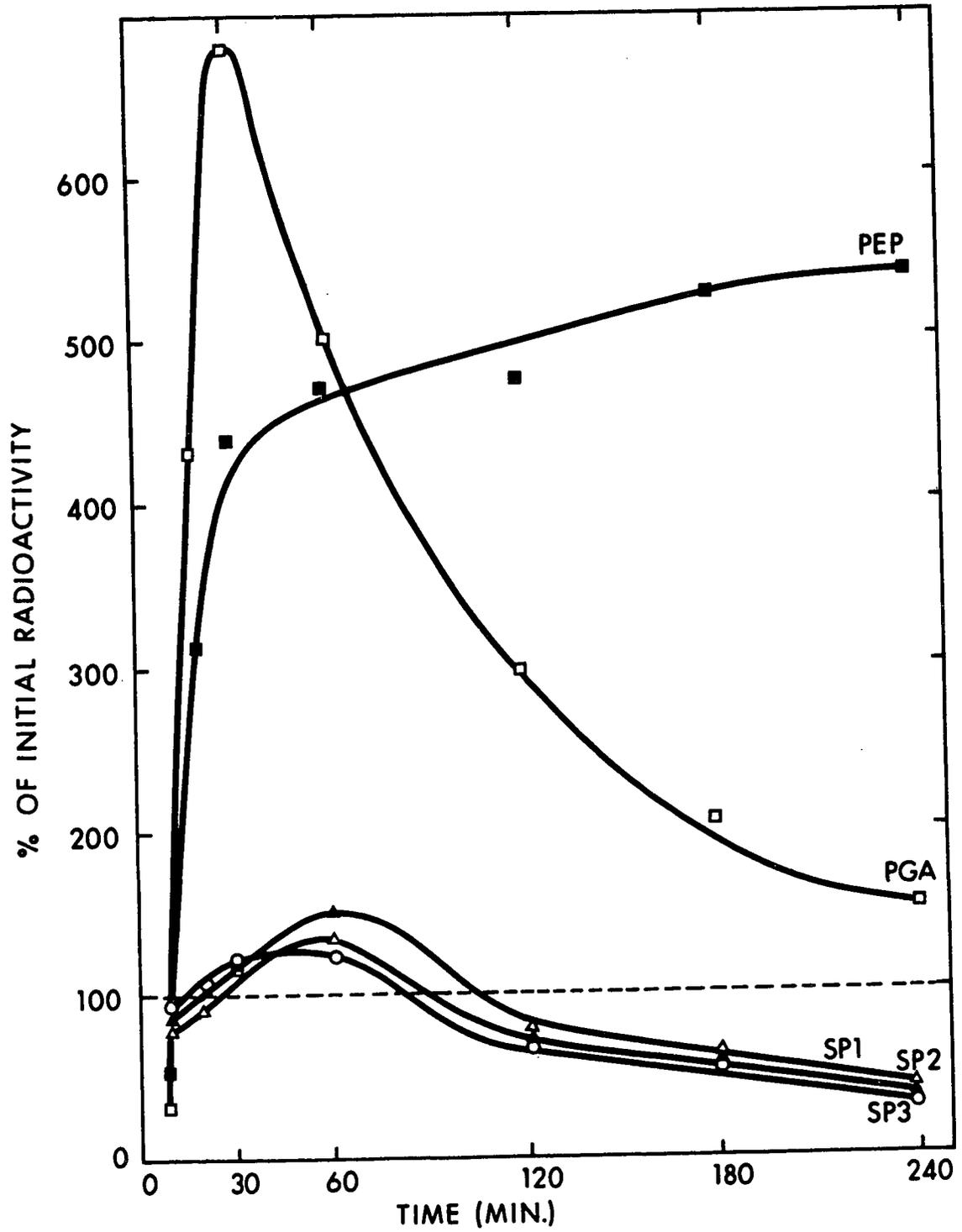


TABLE VI

*Appearance of products of  $C^{14}O_2$  fixation in the incubation medium and changes in their  $C^{14}$  contents during a post-pulse incubation.*

Separation of the components was obtained by thin-layer chromatography as described in the text.

Note: Dash-lines in the table stand for "not detected".

TABLE VI

Incubation Time (min.)	Glycine	Serine	Glutamate	Aspartate	$\alpha$ -Alanine	Glutamine
<i>Pulse</i>						
30	--	21	2.5	1.3	--	25
<i>Post-Pulse</i>						
10	4	32	5	3	--	66
20	9	45	6	5	3	163
30	14	48	14	8	3	183
60	40	76	18	11	4	284
120	11	83	20	12	6	433
180	11	200	33	19	15	668
240	10	352	43	23	16	1,140

Incubation Time (min.)	Asparagine	Sucrose	Citrate	Phosphoserine	Cystine	S-methyl cysteine
<i>Pulse</i>						
30	25	--	3.5	--	--	--
<i>Post-Pulse</i>						
10	65	14	4	--	--	--
20	145	298	7	3.5	--	--
30	152	490	14	5	--	--
60	256	424	16	14	9	10
120	330	750	17	35	19	14
180	618	1,107	17	33	41	21
240	958	1,489	34	30	33	32

Data are expressed as cpm.

Values presented are one thousandth of those obtained in the experiment.

## FIGURE 6

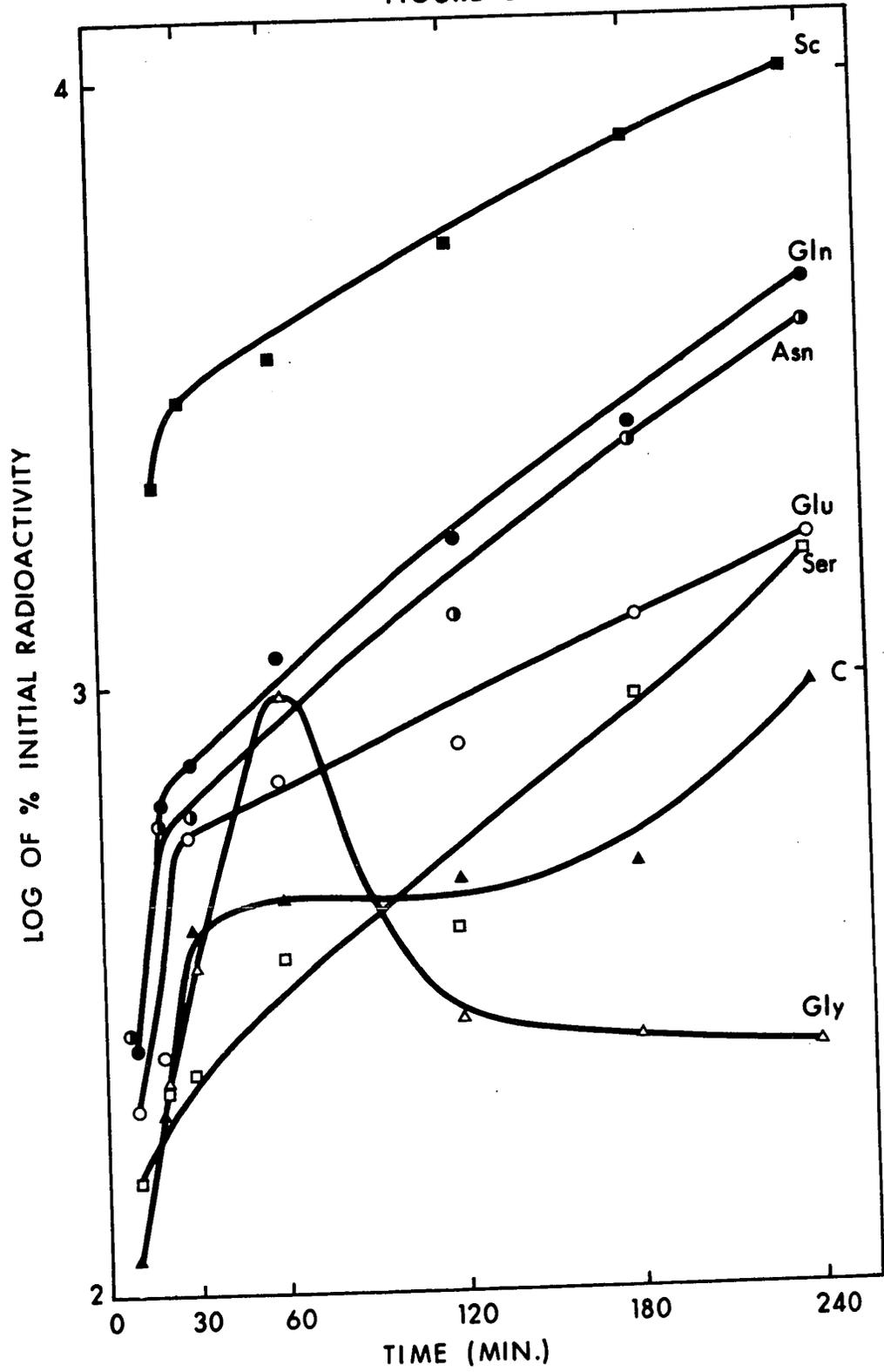
*Appearance of products of  $C^{14}O_2$  fixation in the incubation medium. Relative trends and changes in their  $C^{14}$  contents during a post-pulse incubation.*

Only the major labelled components of the medium are plotted. The data presented are obtained from Table VI and are expressed as the logarithm of the percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) as a function of time during the post-pulse period.

Logarithms are used in order to accomodate the considerable increases in radioactivities shown by some of the compounds during the post-pulse incubation.

Asn: asparagine; C: citrate; Gly: glycine; Glu: glutamate;  
Gln: glutamine; Ser: serine; Sc: sucrose.

FIGURE 6



*Utilization of glyoxylate-1,2-C<sup>14</sup> by illuminated disks of radish cotyledons. Preliminary experiment.*

It is clear from the results of the C<sup>14</sup>O<sub>2</sub> feeding experiments that radish cotyledons utilize CO<sub>2</sub> for synthesis of glycine and serine. The pathways for these syntheses may include glycollate and glyoxylate or three carbon precursors such as phosphohydroxypyruvate as intermediates. It is of interest to note that these acids were all labelled during C<sup>14</sup>O<sub>2</sub> feeding. In order to determine the possible importance of glyoxylate in the synthesis of glycine and serine, further feeding experiments were carried out using glyoxylate-1,2-C<sup>14</sup>.

Glyoxylic acid was supplied in the form of sodium glyoxylate-1,2-C<sup>14</sup> solution with a specific activity of 1  $\mu\text{C}/\mu\text{mole}/0.1 \text{ ml.}$  solution. Aliquots of 0.1 ml. of the labelled solution were fed to each sample of tissue with illumination and CO<sub>2</sub> collection. The tissues were killed after 5, 15, 30 and 60 minutes of incubation in the labelled solution and then fractionated and examined for distribution of radioactivity. Table VII and Fig. 7 show the distribution of the activity present in the different major fractions. The high levels of C<sup>14</sup> in the organic acid fraction possibly reflects the presence of glyoxylate-C<sup>14</sup> which had not been utilized by the tissues. The percentage of the total radioactivity recovered in CO<sub>2</sub> was comparatively small at all times. Amino acids, particularly those of the neutral and basic fraction accounted for the greatest

## TABLE VII

*The utilization of glyoxylate-1,2-C<sup>14</sup> by illuminated disks of radish cotyledons as a function of time.*

Samples of disks (100 mgm) from eleven-day old radish cotyledons were incubated at room temperature in Warburg flasks containing 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of glyoxylate-1,2-C<sup>14</sup> (1 μc/μmole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and fractionated as described in the text.

TABLE VII

Fraction	5 min.	15 min.	30 min.	60 min.
CO <sub>2</sub>	1,148 (0.45)	1,274 (0.49)	1,377 (0.53)	1,658 (0.64)
Solids	114 (0.05)	127 (0.05)	136 (0.05)	185 (0.07)
Hydrolyzates	4,612 (1.80)	7,434 (2.84)	9,752 (3.76)	11,408 (4.38)
Lipids	5,400 (2.11)	12,050 (4.61)	17,150 (6.61)	26,958 (10.35)
Sugars	8,280 (3.24)	10,340 (3.95)	16,260 (6.26)	36,491 (14.01)
Organic acids	195,240 (76.48)	170,275 (65.10)	132,480 (51.04)	93,690 (35.97)
Neutral and basic amino acids	40,000 (15.67)	59,125 (22.60)	81,140 (31.26)	89,861 (34.50)
Acidic amino acids	401 (0.16)	720 (0.28)	955 (0.37)	1,263 (0.49)
Amides	105 (0.04)	200 (0.08)	336 (0.13)	668 (0.26)
Total C <sup>14</sup> recovered	255,300	262,465	259,586	262,042

Data expressed as cpm.

Numbers in brackets indicate percentages of total C<sup>14</sup> recovered.

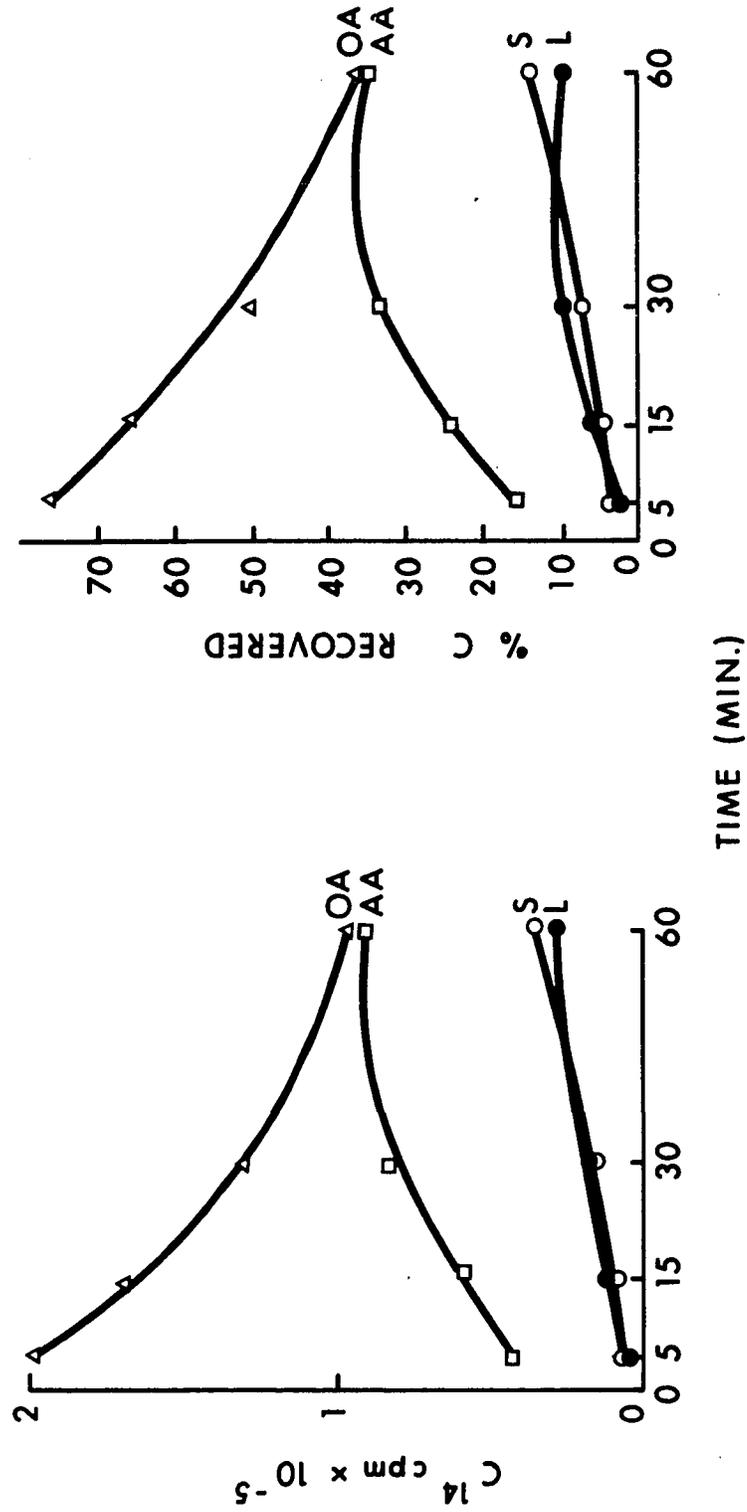
FIGURE 7

*The utilization of glyoxylate-1,2-C<sup>14</sup> by illuminated disks of radish cotyledons as a function of time.*

The data presented are obtained from Table VII.

AA: amino acid fraction; L: lipid fraction; OA: organic acid fraction; S: sugar fraction.

FIGURE 7



incorporation throughout the experiment. For example, after 60 minutes the neutral and basic amino acids accounted for 34% of the activity supplied. This fraction was found to contain large amounts of labelled glycine and serine. Unfortunately, the components of the organic acid fraction were not separated but this fraction decreased in activity during the experimental period. Smaller amounts of  $C^{14}$  were also detected in the lipid and sugar fractions.

As a result of these experiments it is clear that the tissue can utilize exogenously supplied glyoxylate for the formation of glycine and serine. To investigate the role of glycine as a precursor of serine further feeding experiments were conducted with glycine- $C^{14}$ .

*Utilization of glycine-1- $C^{14}$  by illuminated disks of radish cotyledons. Preliminary experiment.*

In order to examine the above possibilities, a glycine-1- $C^{14}$  feeding was carried out under the same experimental conditions as the preliminary glyoxylate- $C^{14}$  experiment. Glycine-1- $C^{14}$  was supplied to the samples in the form of an aqueous solution (0.1 ml. to each sample) with a specific activity of 2  $\mu\text{c}/\mu\text{mole}/0.1$  ml. solution.

Glycine, when supplied under these conditions, was actively metabolized by the tissue (Table VIII, Fig. 8). After 60 minutes, approximately 35% of the activity recovered was present in the organic acid fraction. Chromatography revealed the presence of labelled glyoxylic, glycollic, malic and citric acids and sugar-phosphates. The greatest

TABLE VIII

*The utilization of glycine-1-C<sup>14</sup> by illuminated disks of radish cotyledons as a function of time.*

Samples of disks (100 mgm) from eleven-day old radish cotyledons were incubated at room temperature in Warburg flasks containing 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of glycine-1-C<sup>14</sup> (2 µc/µmole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and fractionated as described in the text.

TABLE VIII

Fraction	5 min.	15 min.	30 min.	60 min.
CO <sub>2</sub> as BaCO <sub>3</sub>	1,114 (0.23)	4,860 (0.98)	11,857 (2.18)	19,156 (4.36)
Lipids	4,920 (1.00)	16,990 (3.43)	67,010 (12.34)	91,500 (20.86)
Sugars	9,180 (1.88)	15,640 (3.16)	25,940 (4.78)	64,699 (14.75)
Organic acids	31,880 (6.54)	85,580 (17.27)	146,160 (26.92)	169,600 (38.67)
Neutral and basic amino acids	436,848 (89.59)	367,521 (74.15)	286,146 (52.70)	194,350 (44.31)
Acidic amino acids	3,300 (0.67)	4,310 (0.87)	5,000 (0.92)	5,080 (1.16)
Amides	350 (0.07)	776 (0.16)	899 (0.17)	1,750 (0.40)
Total C <sup>14</sup> recovered	487,592	499,279	543,012	438,636
		509,005	509,670	525,756

Data expressed in cpm.

Numbers in brackets indicate percent of total C<sup>14</sup> recovered.

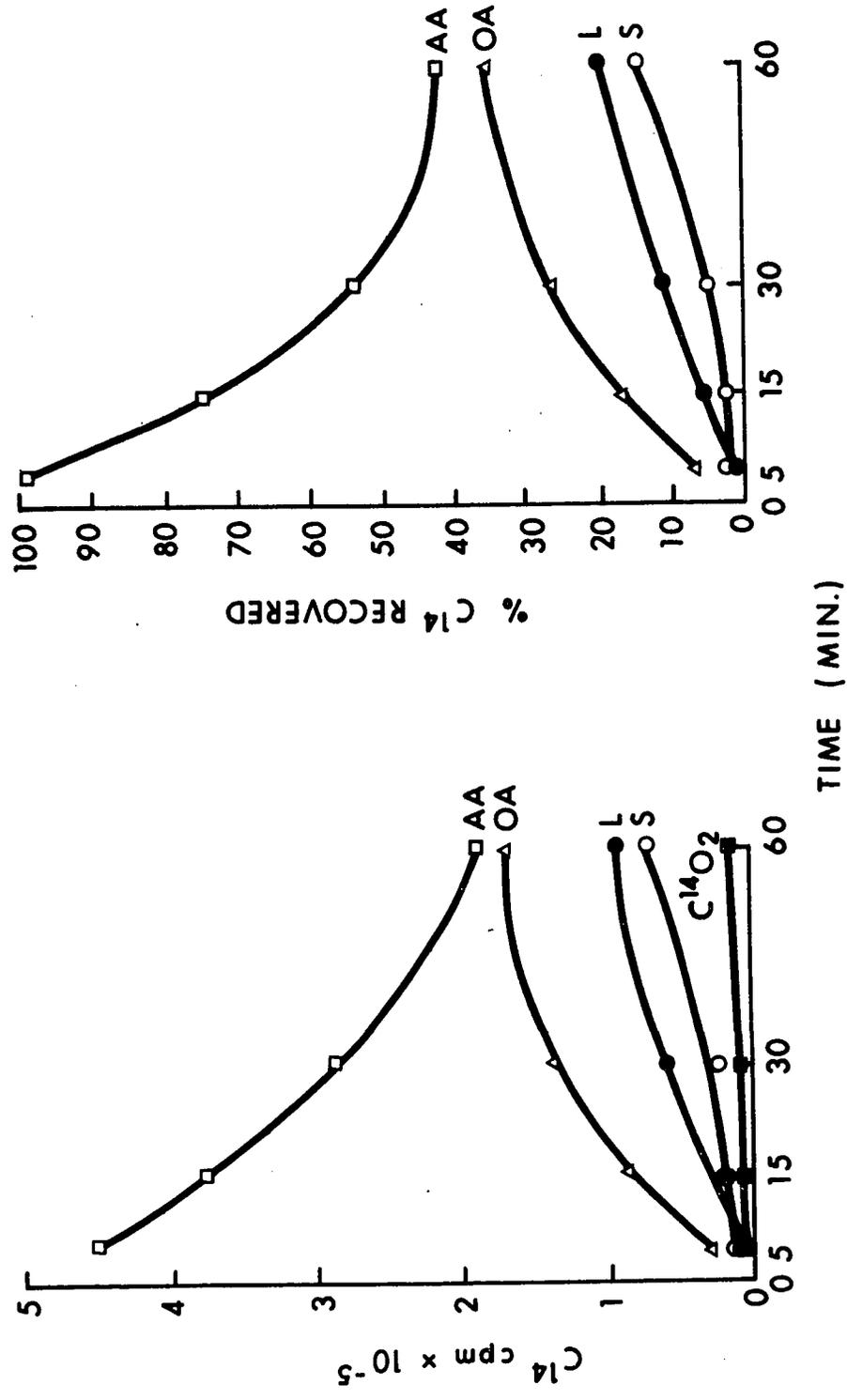
FIGURE 8

*The utilization of glycine-1-C<sup>14</sup> by illuminated disks  
of radish cotyledons as a function of time.*

The data presented are obtained from Table VIII.

AA: amino acid fraction; L: lipid fraction; OA: organic  
acid fraction; S: sugar fraction; C<sup>14</sup>O<sub>2</sub>: C<sup>14</sup>O<sub>2</sub> recovered  
as BaC<sup>14</sup>O<sub>3</sub>.

FIGURE 8



activities were shared by the sugar phosphates and malic acid. Serine was also very heavily labelled and  $C^{14}$  was detected in the  $CO_2$  evolved. The distribution of activity between lipids and sugars during glycine-1- $C^{14}$  utilization shows a rather similar pattern to that of the previous glyoxylate experiment.

There was also a small incorporation of activity into the acidic amino acids. The results obtained in both the glyoxylate-1,2- $C^{14}$  and glycine-1- $C^{14}$  experiments therefore clearly show that there is an extensive conversion of glyoxylate and glycine to serine.

*Utilization of glycine-2- $C^{14}$  by illuminated disks of radish cotyledons. Pulse-chase experiment.*

In order to determine the degree of turnover of glycine and serine within the tissues, further experiments were conducted in which labelled glycine was pulse-fed. Samples of tissue were incubated with 0.1 ml. of glycine-2- $C^{14}$  solution (specific activity 1  $\mu c/1\mu mole/0.1 ml$ ) as shown in Table IX and Fig. 9.

At the end of the 30 minute pulse, only 13% of the supplied glycine-2- $C^{14}$  had been taken up by the tissue. Examination of this medium by chromatography and radioautography showed a single large very active spot of glycine. Of the  $C^{14}$  taken up, however, 44% remained in the amino acids after the pulse period, while the corresponding figures for the sugar and organic acid fractions were 31% and 16% respectively. After 10 minutes of post-pulse

TABLE IX

*The incorporation of C<sup>14</sup> from glycine-2-C<sup>14</sup> and turnover of labelled compounds during a post-pulse incubation.*

Samples of disks (100 mgm) of eleven-day old radish cotyledons were incubated for 30 minutes with illumination in Warburg flasks containing 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of glycine-2-C<sup>14</sup> (1 µc/µmole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml (pulse). The tissues were subsequently transferred to Warburg flasks containing 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer and 0.2 ml of glycine solution (0.5 µmole/0.1 ml) and incubated for periods of time as indicated (post-pulse). The tissues were killed and fractionated as described in the text.

TABLE IX

Incubation Time (min.)	Medium	Lipids	Amino acids	Organic acids	Sugars	Solids	Hydrolyzates
<i>Pulse</i>							
30	1,408,000	13,000	96,000	30,000	68,000	172	11,000
<i>Post-Pulse</i>							
10	3,600	11,000	66,000	27,000	77,000	186	12,000
20	4,000	12,000	76,000	47,000	61,200	200	14,000
30	4,700	13,000	53,000	40,000	54,000	247	13,000
60	6,900	15,000	33,000	35,000	50,000	251	12,000
120	7,800	17,000	26,000	30,000	47,000	253	10,000
180	8,800	18,000	23,000	26,000	43,000	271	9,000
240	11,600	19,000	20,000	21,000	37,000	285	8,000

Data are expressed as cpm.

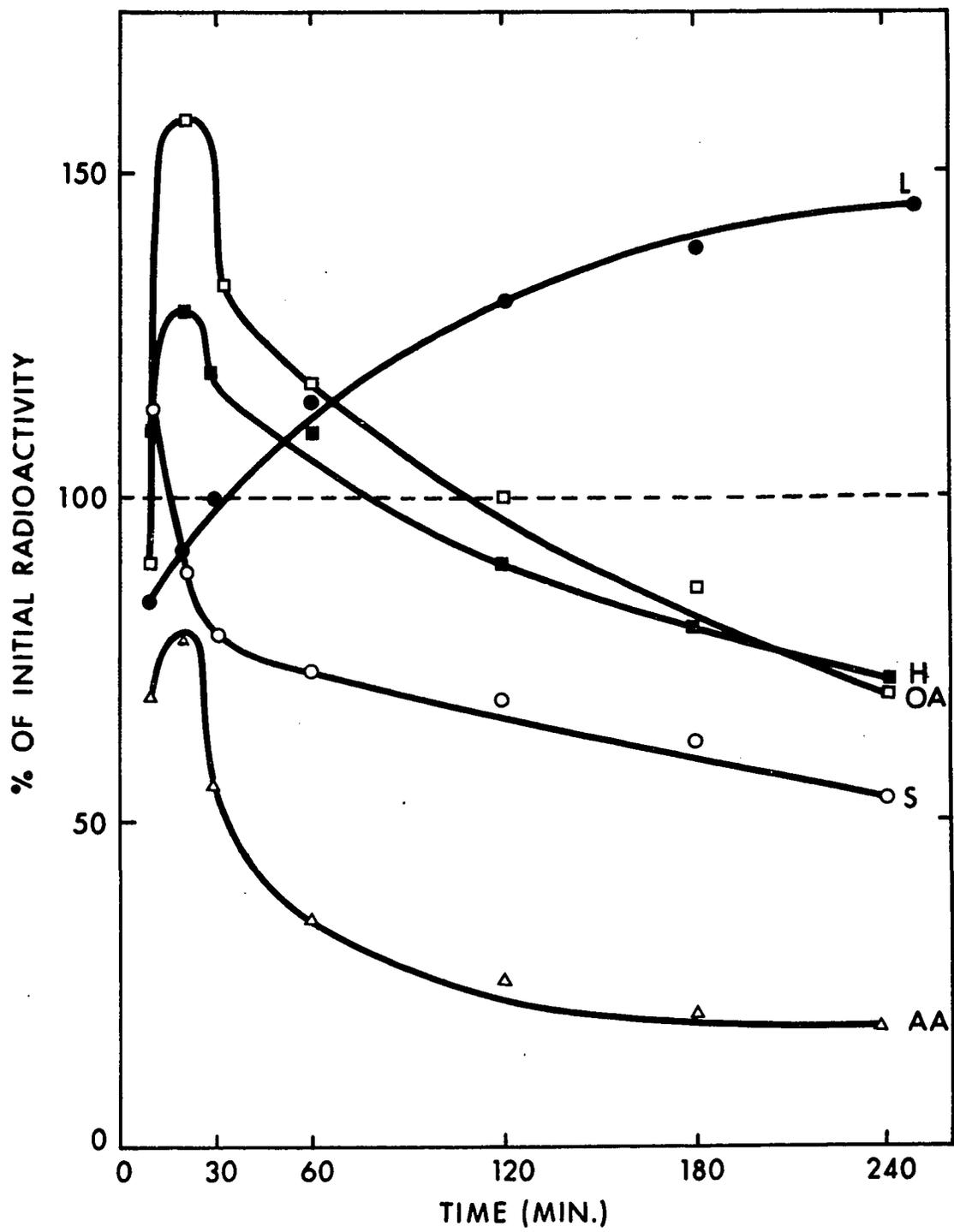
## FIGURE 9

*The incorporation of  $C^{14}$  from glycine-2- $C^{14}$  and the turnover of labelled compounds during a post-pulse incubation.*

The data presented are obtained from Table IX and are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

AA: amino acid fraction; H: hydrolyzate fraction; L: lipid fraction; OA: organic acid fraction; S: sugar fraction.

FIGURE 9



incubation, the sugars were the major labelled fraction. This was followed by a decrease which was continued as the post-pulse period was extended. A similar trend was shown by the amino acid fraction with maximum incorporation of  $C^{14}$  after 20 minutes of post-pulse incubation, followed by a decline. The organic acid fraction and the ethanol insoluble material showed a pattern similar to the amino acids but were of a lower activity throughout. Lipids were the only fraction which showed an increasing trend, almost doubling the initial incorporation value at the end of the experiment. When the media from the post-pulse incubation were examined for radioactivity, it was found that only two compounds could be detected, namely, glycine and serine. Initially, glycine was the most active, but by the end of one hour only serine was detected, and this compound kept increasing in activity with time. Clearly as interconversion of glycine and serine occurred, both of these compounds passed out of the tissues into the medium.

As in the previous experiments, serine contained the greatest amount of  $C^{14}$  (Table X, Fig. 10). At the end of the pulse period, 85% or more of the total activity in the amino acids was present in serine. The other amino acids labelled were glutamic acid and  $\alpha$ -alanine. During the post-pulse period labelling of serine and glycine rapidly declined while the other labelled amino acids increased in activity (Table X). In contrast to the  $C^{14}O_2$  experiments the glycine carbon was poorly incorporated into aspartate,

TABLE X

*Incorporation of  $C^{14}$  from glycine-2- $C^{14}$  into the soluble amino acids and changes in their radioactivities during a post-pulse incubation.*

Separation of the component amino acids was obtained by thin-layer chromatography as described in the text.

TABLE X

Incubation Time (min.)	Glycine	Serine	Glutamate	Glutamine	$\alpha$ -Alanine	Aspartate	Cysteine
<i>Pulse</i>							
30	10,941	81,265	354	--	120	--	--
<i>Post-Pulse</i>							
10	6,743	53,164	521	326	141	--	--
20	9,074	60,954	840	427	250	111	--
30	5,132	40,692	1,097	580	410	132	156
60	2,414	25,607	1,318	707	570	173	241
120	1,507	17,540	1,670	850	808	310	390
180	1,050	11,230	2,060	1,590	1,650	590	1,160
240	890	9,490	2,510	1,970	2,350	1,130	--

Data are expressed as cpm.

Note: Dash-lines in the table stand for "not detected".

FIGURE 10

*Incorporation of  $C^{14}$  from glycine-2- $C^{14}$  into the soluble amino acids. Changes in their radioactivities during a post-pulse incubation.*

The data presented are obtained from Table X. Only the major radioactive amino acids of Table X are plotted.

The data are expressed as the logarithms of the percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

Logarithms are used in order to accomodate the considerable increases in radioactivities shown by some of the compounds during the post-pulse incubation.

Ala: alanine; Gly: glycine; Glu: glutamate; Gln: glutamine; Ser: serine.

FIGURE 10

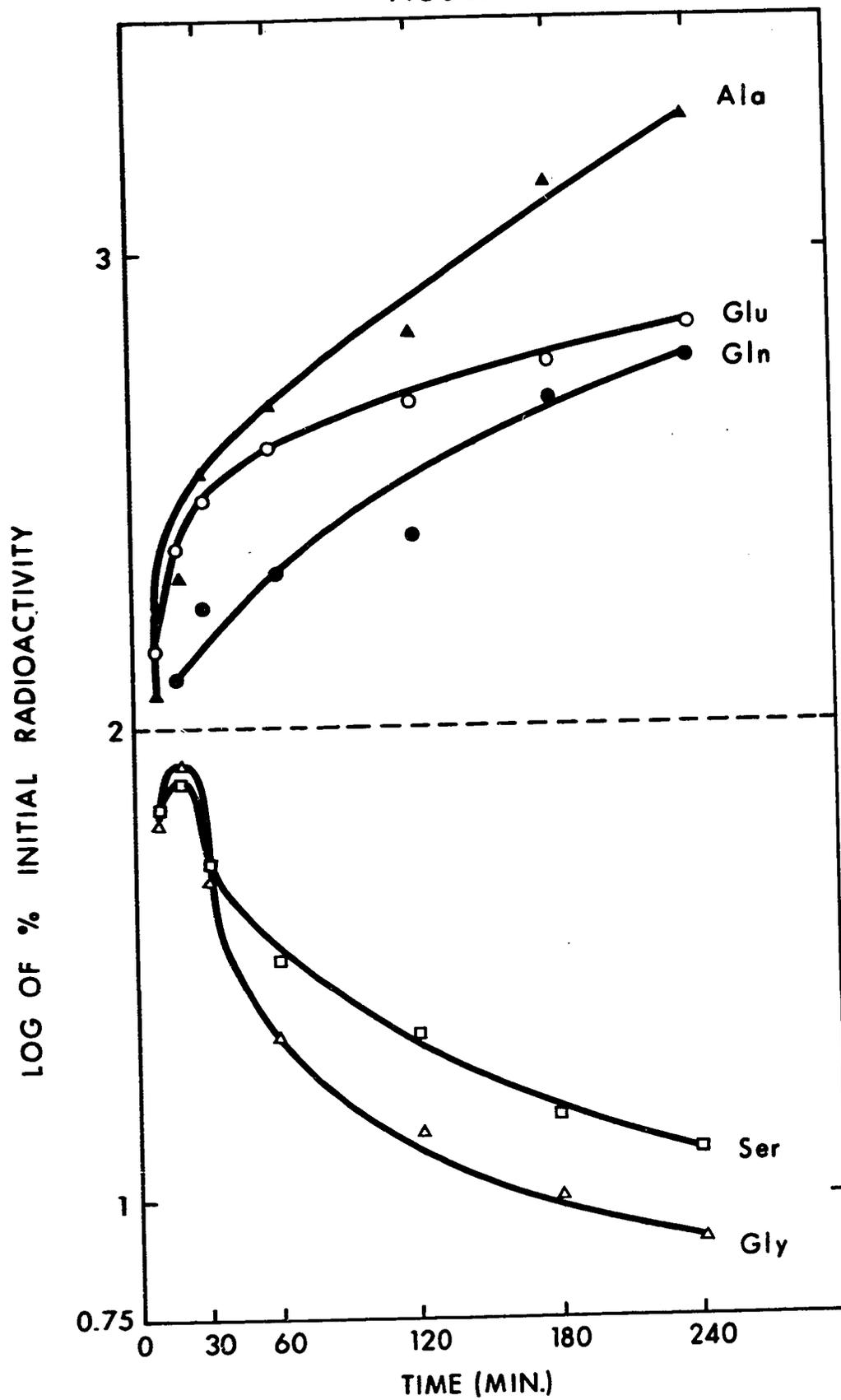


TABLE XI

*Incorporation of C<sup>14</sup> from glycine-2-C<sup>14</sup> into some of the components of the organic acid fraction and changes in their radioactivities during a post-pulse incubation.*

Separation of the component organic acids was obtained by thin-layer chromatography and high-voltage electrophoresis as described in the text.

Note: Dash-lines in the table stand for "not detected".

TABLE XI

Incubation Time (min.)	Succinate	Malate	Citrate	Sugar-phosphates, PEP and PGA	Glycollate
<i>Pulse</i>					
30	1,820	4,580	3,980	8,680	1,080
<i>Post-Pulse</i>					
10	940	5,920	2,590	11,100	640
20	2,340	9,700	5,300	21,500	920
30	1,900	12,500	4,520	14,300	4,501
60	1,620	9,000	2,450	11,350	--
120	1,400	7,820	1,380	9,560	--
180	600	6,160	890	7,100	--
240	--	4,300	500	5,800	--

Data are expressed as cpm.

## FIGURE 11

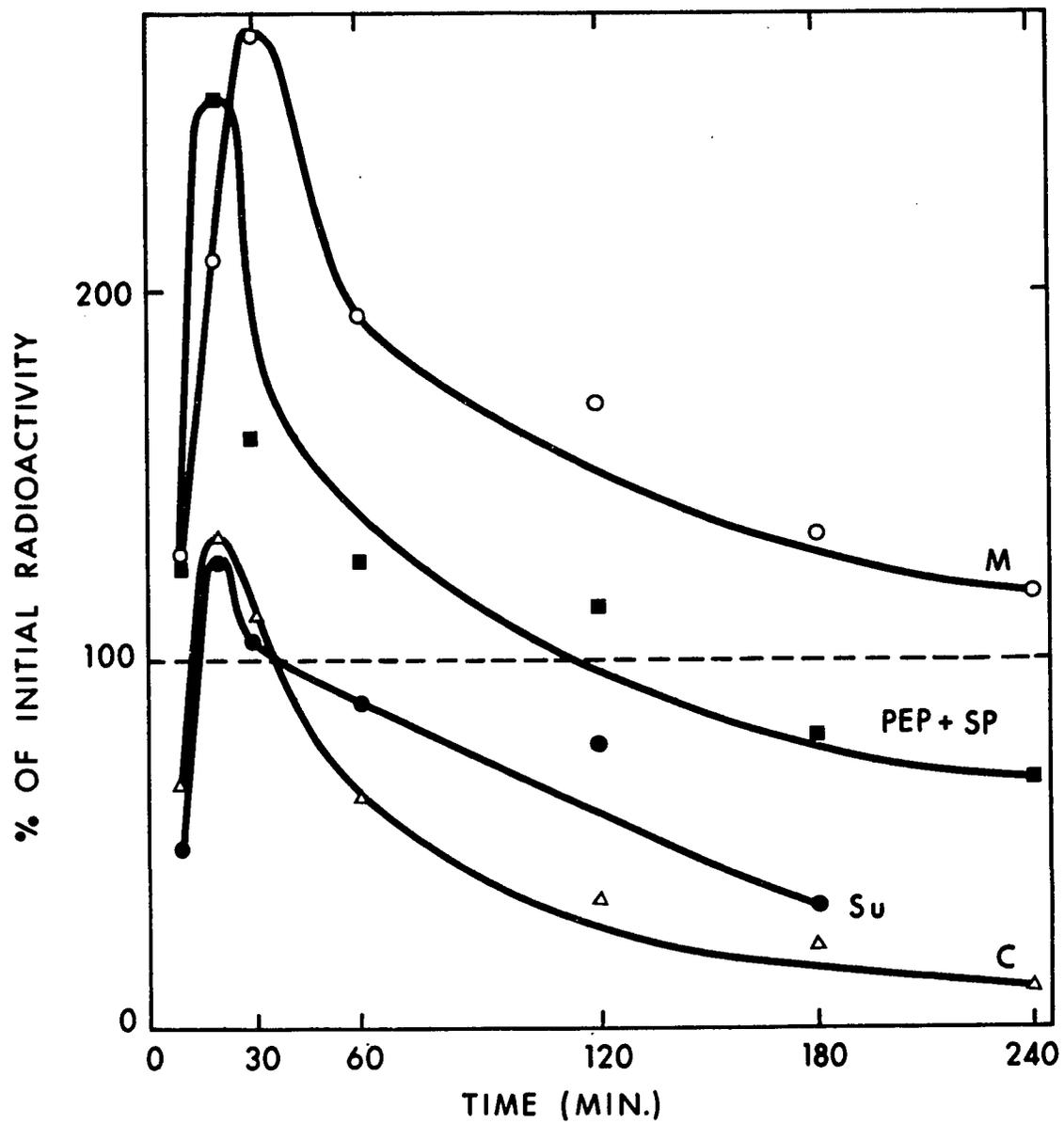
*Incorporation of  $C^{14}$  from glycine-2- $C^{14}$  into the components of the organic acid fraction. Changes in their radioactivities during post-pulse.*

The data presented are obtained from Table XI. Only the major radioactive components of Table XI are plotted.

The data are expressed as the percentages of the radioactivities incorporated at the end of the 50 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

C: citrate; M: malate; PEP + SP: phosphoenolpyruvate, phosphoglycerate and sugar phosphates, Su: succinate.

FIGURE 11



and labelled asparagine was not detected. After the pulse, the sugar-phosphates, phosphoenolpyruvate and 3-phosphoglycerate together, contained 39% of the activity recovered in the organic acid fraction, followed by malate and citrate each with about 20% of the total (Table XI, Fig. 11). During the post-pulse period these organic acids and the sugar-phosphates gave trends indicative of rapid turnover. The metabolic fate of serine was not easily determined in these experiments so an attempt was made to determine the degree of utilization of this amino acid under similar experimental conditions.

*Utilization of serine-3-C<sup>14</sup> by illuminated disks of radish cotyledons. Preliminary experiment.*

Serine-3-C<sup>14</sup> was administered to disks of radish cotyledons under the experimental conditions used in the previous glyoxylate-1,2-C<sup>14</sup> and glycine-1-C<sup>14</sup> experiments. The aqueous solution of serine-3-C<sup>14</sup> had a specific activity of 2  $\mu\text{C}/1\mu\text{mole}/0.1\text{ ml}$ . Aliquots of 0.1 ml. were supplied to each sample.

At the end of the 60 minute incubation, only 16% of the supplied amino acid had been utilized by the tissue; 7% of this was present in the sugar fraction, and 5% was in the organic acid fraction (Table XII, Fig. 12). A comparatively large amount (3.6%) of label was incorporated into lipids while the remainder was distributed in very small amounts among the other fractions.

In general, it is impossible to say whether the tissue

TABLE XII

*The utilization of serine-3-C<sup>14</sup> by illuminated disks of radish cotyledons as a function of time.*

Samples of disks (100 mgm) from eleven-day old radish cotyledons were incubated at room temperature in Warburg flasks containing 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of serine-3-C<sup>14</sup> (2  $\mu$ c/ $\mu$ mole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and fractionated as described in the text.

Note: Dash-lines in the table represent percentages lower than 0.01 which were considered not significant.

TABLE XII

Fraction	5 min.	15 min.	30 min.	60 min.
CO <sub>2</sub> as BaCO <sub>3</sub>	8 ---	32 (0.01)	40 (0.01)	152 (0.02)
		12 ---	50 (0.01)	196 (0.03)
Lipids	15,900 (2.23)	19,800 (2.96)	23,150 (3.48)	25,890 (3.57)
	4,950 (0.75)	9,020 (1.35)	19,240 (3.00)	49,325 (6.81)
Sugars	5,360 (0.75)	10,000 (1.54)	23,940 (3.42)	53,655 (7.36)
Organic acids	14,160 (1.99)	19,600 (2.94)	21,540 (3.24)	39,325 (5.43)
	688,900 (94.80)	618,840 (92.70)	600,000 (90.30)	609,250 (84.07)
Neutral and basic amino acids	675,220 (94.97)	599,390 (92.29)	625,400 (89.35)	612,010 (83.89)
Acidic amino acids	350 (0.06)	250 (0.04)	390 (0.06)	600 (0.08)
Amides	---	60 (0.01)	58 (0.01)	125 (0.02)
Total C <sup>14</sup> recovered	710,998	667,602	664,418	724,667
	726,662	649,485	699,945	729,470

Data expressed as cpm.

Figures in brackets indicate percent of total C<sup>14</sup> recovered.

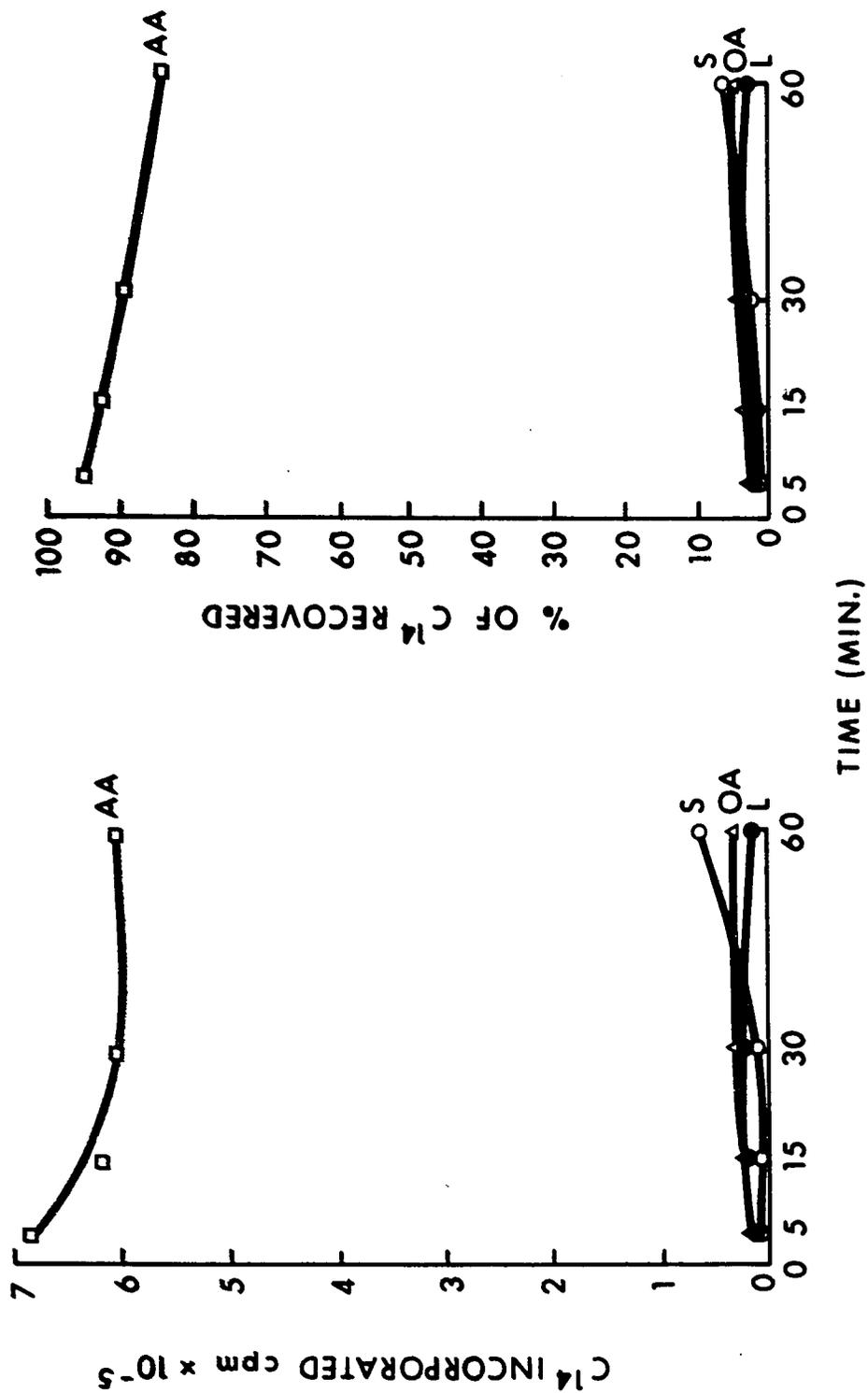
FIGURE 12

*The utilization of  $C^{14}$  from serine-3- $C^{14}$  by illuminated disks of radish cotyledons as a function of time.*

The data presented are obtained from Table XII.

AA: amino acid fraction; L: lipid fraction; OA: organic acid fraction; S: sugar fraction.

FIGURE 12



did not take up the labelled compound, or whether after being taken up, serine was simply not metabolized. The latter possibility appears unlikely, as serine, produced endogenously from  $C^{14}O_2$ , showed rapid turnover during the post-pulse incubation. A possible explanation for these findings is that the metabolically active pools of serine within this tissue were not readily accessible to the exogenously supplied serine- $C^{14}$  under the conditions of the present experiment.

*Utilisation of glyoxylate- $C^{14}$  and glycine- $C^{14}$  by radish cotyledons. The effects of light and dark.*

While the preliminary experiments demonstrated that glyoxylate and glycine were extensively utilized by illuminated radish cotyledons, it was still not known whether this utilization was affected by light.

To elucidate these points a series of 30 minute pulse experiments were performed in dark and in light, with and without illumination. Glyoxylate-1- $C^{14}$ , glyoxylate-2- $C^{14}$  and glycine-1- $C^{14}$  were fed as aqueous solutions with specific activities of 2  $\mu$ c/ $\mu$ mole/0.1 ml of solution. Aliquots of 0.1 ml were fed to samples of tissue in each experiment.

a. *Utilisation of glyoxylate-1- $C^{14}$  in light and dark.*

*(Table XIII).*

Ninety percent or more of the activity recovered was found distributed between the organic and amino acid fractions. This distribution was essentially unaltered

when the tissues were illuminated. Similarly, incorporation of  $C^{14}$  into proteins and sugars was not affected by illumination. However, the lipid fraction showed a ten-fold increase in  $C^{14}$  when the tissues were in the light.

*b. Utilization of glyoxylate-2- $C^{14}$  in light and dark.*

*(Table XIV).*

In these experiments, up to 82% of the label was again present in the organic and amino acid fractions. In fact, the pattern of incorporation in all fractions was very similar to that of the previous experiment with glyoxylate-1- $C^{14}$ . However, the sugar and lipid fractions in this experiment did show a rather marked difference depending on whether they had been produced in dark or light. In the dark, incorporation of the label into lipids from glyoxylate-2- $C^{14}$  was less than 1%, while in the light this incorporation was increased by more than five fold. The activity found in the sugar fraction of the illuminated tests was one third lower than that produced in the dark.

*c. Utilization of glycine-1- $C^{14}$  in light and dark.*

*(Table XV).*

Similar to the above experiments, up to 83% of the total activity recovered was present in the organic and amino acid fractions. Here, however, the amino acids accounted for 70% of such activity as the organic acids. Also, incorporation of  $C^{14}$  into lipids was doubled with illumination. An interesting difference from the earlier experiments with glyoxylate-1- $C^{14}$  and glyoxylate-2- $C^{14}$  experiments was the increased incorporation of glycine-1- $C^{14}$  into  $CO_2$  in

darkness. When comparing the results obtained in these three 30 minute pulse experiments, it is clear that organic and amino acids are in all three cases the major products; the greatest activity being found in whichever compound was fed, since the media in these experiments were not separated or discarded, but included and processed with the tissues. It is evident, however, that in all three cases, there is an active interconversion of organic acids to amino acids and vice versa, and that this interconversion is largely light independent, although the presence of light enhances it slightly.

*The effects of glycine on the utilization of glyoxylate-2-C<sup>14</sup> in the light and dark. (Table XVI).*

It is clear from the earlier experiments that glyoxylate is an effective precursor of glycine in these tissues. In addition to synthesis of glycine, glyoxylate carbon was extensively incorporated into the other fractions. If glycine is the major precursor of these other fractions additions of unlabelled glycine should alter the distribution of C<sup>14</sup> when glyoxylate-C<sup>14</sup> was fed. In order to examine this point, unlabelled glycine was supplied to radish cotyledons 10 minutes before addition of glyoxylate-2-C<sup>14</sup>. The experiments were carried out with and without illumination. The results of such experiments carried out in the light are summarized in Table XIV.

When glyoxylate-2-C<sup>14</sup> was fed in the presence of an excess of unlabelled glycine, 53% of the recovered activity

TABLE XIII

*The utilization of glyoxylate-1-C<sup>14</sup> by disks of radish cotyledons. The effects of light and dark.*

Samples of disks (100 mgm) of eleven-day old radish cotyledons were incubated at room temperature for 30 minutes in light or dark as indicated. The incubation medium consisted of 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of glyoxylate-1-C<sup>14</sup> (2 µc/µmole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and fractionated as indicated in the text.

TABLE XIII

Fraction	Light			Dark	
CO <sub>2</sub> as BaCO <sub>3</sub>	---	---	---	8,771 (1.57)	8,064 (1.30)
Solids	358 (0.07)	342 (0.06)	376 (0.07)	266 (0.05)	259 (0.04)
Hydrolyzates	16,625 (3.03)	18,425 (3.28)	15,822 (2.74)	14,400 (2.57)	17,800 (2.58)
Lipids	26,660 (4.87)	24,420 (4.34)	21,820 (3.78)	2,220 (0.40)	2,180 (0.35)
Sugars	22,695 (4.14)	19,912 (3.54)	18,925 (3.28)	14,412 (2.57)	13,350 (2.16)
Organic acids	255,100 (46.57)	275,425 (48.97)	288,900 (50.01)	286,500 (51.10)	306,525 (49.54)
Amino acids	226,336 (40.93)	223,886 (39.81)	231,879 (40.13)	234,050 (41.75)	270,600 (43.73)
Total C <sup>14</sup> recovered	547,774	562,410	577,722	560,619	618,778

Data expressed as cpm.

Figures in brackets represent percent of total C<sup>14</sup> recovered.

Note: CO<sub>2</sub> was not collected in the light experiment.

TABLE XIV

*The utilization of glyoxylate-2-C<sup>14</sup> by disks of radish cotyledons. The effects of light and dark.*

Samples of disks (100 mgm) of eleven-day old radish cotyledons were incubated at room temperature for 30 minutes in light or dark as indicated. The incubation medium consisted of 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of glyoxylate-2-C<sup>14</sup> (2 µc/µmole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and fractionated as indicated in the text.

TABLE XIV

Fraction	Light			Dark	
CO <sub>2</sub> as BaCO <sub>3</sub>	---	---	---	5,128 (0.82)	4,831 (0.75)
Solids	148 (0.03)	519 (0.09)	284 (0.05)	1,908 (0.30)	1,618 (0.25)
Hydrolyzates	21,225 (3.63)	18,350 (3.22)	19,300 (3.66)	14,800 (2.36)	17,320 (2.68)
Lipids	27,800 (4.76)	30,880 (5.42)	34,290 (6.51)	7,060 (1.12)	10,660 (1.65)
Sugars	47,218 (8.08)	44,900 (7.89)	49,250 (9.35)	84,315 (13.41)	80,550 (12.46)
Organic acids	250,650 (42.91)	232,931 (40.88)	230,000 (43.66)	272,752 (43.39)	280,320 (43.36)
Amino acids	237,050 (40.58)	242,192 (42.51)	193,685 (36.77)	242,600 (38.60)	251,225 (38.87)
Total C <sup>14</sup> recovered	584,096	569,772	526,809	628,563	646,524

Data are expressed as cpm.

Figures in brackets indicate percent of total C<sup>14</sup> recovered.

Note: CO<sub>2</sub> was not collected in the light experiment.

TABLE XV

*The utilization of glycine-1-C<sup>14</sup> by disks of radish cotyledons. The effects of light and dark.*

Samples of disks (100 mgm) of eleven-day old radish cotyledons were incubated at room temperature for 30 minutes in light or dark as indicated. The incubation medium consisted of 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer at pH 5.0, 0.1 ml of glycine-1-C<sup>14</sup> (2  $\mu$ c/ $\mu$ mole/0.1 ml) and 0.1 ml of distilled water in a total volume of 0.7 ml. Tissues were killed and fractionated as indicated in the text.

TABLE XV

Fraction	Light			Dark	
CO <sub>2</sub> as BaCO <sub>3</sub>	---	---	---	35,714 (6.36)	32,985 (5.81)
Solids	1,682 (0.28)	2,012 (0.36)	1,825 (0.32)	344 (0.06)	365 (0.06)
Hydrolyzates	28,675 (4.84)	22,075 (3.91)	23,950 (4.17)	19,700 (3.51)	23,350 (4.12)
Lipids	71,520 (12.07)	70,460 (12.48)	76,360 (13.31)	42,450 (7.56)	41,925 (7.39)
Sugars	26,981 (4.55)	26,875 (4.76)	27,925 (4.87)	13,125 (2.34)	13,700 (2.42)
Organic acids	156,375 (26.39)	149,612 (26.49)	161,025 (28.05)	152,575 (27.18)	159,250 (28.07)
Amino acids	307,266 (51.80)	293,746 (52.11)	282,915 (49.28)	297,350 (52.75)	295,820 (52.07)
Total C <sup>14</sup> recovered	592,499	564,780	574,000	561,258	567,395

Data are expressed as cpm.

Figures in brackets indicate percent of total C<sup>14</sup> recovered.

Note: CO<sub>2</sub> was not collected in the light experiment.

TABLE XVI

*The effects of glycine on the utilization of glyoxylate-2-C<sup>14</sup> by disks of radish cotyledons in the light and dark.*

Samples of disks (100 mgm) from eleven-day old radish cotyledons were preincubated at room temperature in light and dark as indicated in a medium consisting of 0.5 ml of 0.1 M KH<sub>2</sub>PO<sub>4</sub>, and 0.1 ml of glycine solution (10 μmoles/0.1 ml). At the end of 10 minutes, 0.1 ml of glyoxylate-2-C<sup>14</sup> (2 μc/μmole/0.1 ml) were added to the flasks and incubation resumed in light or dark for 30 minutes. Tissues were killed and fractionated as described in the text.

TABLE XVI

Fraction	Light		Dark	
CO <sub>2</sub> as BaCO <sub>3</sub>	---	---	22,600 (3.72)	24,139 (4.05)
Solids	2,114 (0.35)	2,056 (0.35)	2,201 (0.36)	1,969 (0.33)
Hydrolyzates	19,310 (3.21)	11,400 (1.94)	15,220 (2.51)	20,420 (3.42)
Lipids	43,460 (7.23)	42,180 (7.19)	18,450 (3.04)	17,995 (3.02)
Sugars	145,875 (24.27)	142,162 (24.22)	157,200 (25.88)	154,525 (25.91)
Organic acids	316,887 (52.71)	314,720 (53.61)	260,405 (42.88)	249,152 (41.78)
Amino acids	73,532 (12.26)	74,517 (12.70)	131,261 (21.61)	128,129 (21.45)
Total C <sup>14</sup> recovered	601,178	587,035	607,338	596,329

Data are expressed as cpm.

Figures in brackets indicate percent of total C<sup>14</sup> recovered.

Note: CO<sub>2</sub> was not collected in the light experiment.

was found in the organic acids. In the control the organic acids contained 42% of the radioactivity supplied. Additions of glycine resulted in considerable reduction in the levels of  $C^{14}$  in the amino acids. However, this treatment greatly increased the incorporation of glyoxylate into sugars. Labelling of the lipid fraction was not appreciably altered by additions of glycine. When experiments were carried out in the dark, the results obtained were similar (Table XVI), thus supporting earlier conclusions that the utilization of glyoxylate was not appreciably affected by light. One exception to this, however, was a marked increase in the levels of  $C^{14}$  in the amino acid fraction of glycine treated tissues in the dark.

*Utilization of acetate-2- $C^{14}$  by illuminated disks of radish cotyledons. Pulse-chase experiment.*

In certain of the previous experiments, malic acid is an important labelled product. Furthermore, this acid is in a state of turnover in these tissues. Malic acid could conceivably become labelled from the substrates supplied via malate synthetase and by carboxylation of phosphoenolpyruvate. If malate were formed from glyoxylate via the malate synthetase reaction, then acetate- $C^{14}$  should share similar fates to glyoxylate when supplied under similar experimental conditions. On the other hand, if glyoxylate labels malate via three carbon intermediates, then the metabolic fates of acetate and glyoxylate would be significantly different. To examine these possibilities, further feeding experiments

were conducted using acetate-2-C<sup>14</sup>. The radioactive compound (as the sodium salt) was provided in 0.1 ml. aliquots of a solution having a specific activity of 2  $\mu\text{c}/\mu\text{mole}/0.1$  ml. of solution. The tissues were incubated with acetate-2-C<sup>14</sup> for 30 minutes in the light followed by transfer to equimolar unlabelled sodium acetate and the illumination continued for periods up to 4 hours. The results of these experiments are summarized in Tables XVII, XVIII and XIX; Figs. 13, 14 and 15.

At the end of the pulse period the bulk of the C<sup>14</sup> recovered was present in the medium. This radioactivity was highly volatile under acidic conditions and was therefore presumed to be mainly unused acetate-C<sup>14</sup>. In addition, C<sup>14</sup> was distributed among the various fractions isolated showing that this compound was metabolized (Table XVII).

After the 30 minute pulse period acetate carbon was located predominantly in the amino acid fraction. Chromatography of this fraction revealed the presence of labelled glutamate, glutamine and  $\gamma$ -aminobutyrate. Smaller levels of C<sup>14</sup> were found in aspartate, serine and some glutamate derivatives (Table XVIII). The organic acid fraction and the lipids together accounted for the bulk of the remaining C<sup>14</sup> incorporated. Analysis of the organic acid fraction (Table XIX) revealed heavy labelling of succinate with smaller levels of activity in citrate,  $\alpha$ -ketoglutarate and malate. Small amounts of acetate carbon were also present in the insoluble residue.

During the post-pulse period, radioactivity appeared in the medium, the amount being relatively constant after 10 minutes (Table XVII). The examination of the contents of these media gave results similar to that obtained for the medium of the pulse incubation, and was, therefore, assumed to be mainly acetate. This assumption is borne out by the observation that increasing levels of  $C^{14}$  were incorporated into the major fractions throughout the post-pulse period. For example, radioactivity in the lipids and organic acids was more than doubled during that period. In contrast to this trend, the amino acids showed evidence for turnover as observed in the earlier experiments.

When the levels of  $C^{14}$  in the various organic acids were examined (Table XIX) it was clear that the pools of succinate and  $\alpha$ -ketoglutarate were turning over. This turnover was most obvious for  $\alpha$ -ketoglutarate. The pools of the other acids continued to accumulate  $C^{14}$  during the post-pulse incubation.

Consideration of the amino acid fraction (Table XVIII) showed that glutamate, the major labelled component, was rapidly turning over. This turnover was accompanied by accumulations of  $C^{14}$  in glutamine and  $\gamma$ -aminobutyrate. Other amino acids showed a similar though smaller increase in  $C^{14}$ . In comparison with the data obtained in the previous experiments, acetate contributed little carbon to the sugar fraction.

From the results of the acetate feeding experiment

TABLE XVII

*The incorporation of  $C^{14}$  from acetate-2- $C^{14}$  and turnover of labelled compounds during a post-pulse incubation.*

Samples of disks (100 mgm) of eleven-day old radish cotyledons were incubated for 30 minutes with illumination in Warburg flasks containing 0.5 ml of 0.1 M  $KH_2PO_4$  buffer at pH 5.0, 0.1 ml of acetate-2- $C^{14}$  (1  $\mu c/\mu mole/0.1$  ml) and 0.1 ml of distilled water in a total volume of 0.7 ml (pulse). The tissues were subsequently transferred to Warburg flasks containing 0.5 ml of 0.1 M  $KH_2PO_4$  buffer pH 5.0 and 0.2 ml of sodium acetate solution (0.5  $\mu mole/0.1$  ml) and incubated for periods of time as indicated (post-pulse). The tissues were killed and fractionated as described in the text.

TABLE XVII

Incubation Time (min.)	Medium	Lipids	Amino acids	Organic acids	Sugars	Solids	Hydrolyzates
<i>Pulse</i>							
30	2,245,460	16,600	27,400	17,800	1,700	224	2,800
<i>Post-Pulse</i>							
10	17,952	11,200	16,000	17,200	2,200	195	2,600
20	21,756	13,800	22,600	18,500	2,100	177	3,200
30	20,976	15,400	24,900	22,800	2,000	187	3,600
60	21,103	19,600	26,100	28,000	1,800	299	5,300
120	22,231	30,200	21,800	29,200	1,600	343	6,200
180	23,079	38,300	20,600	34,700	1,500	315	7,300
240	22,834	39,200	18,900	37,900	1,500	512	7,700

Data was expressed in cpm.

### FIGURE 13

*The incorporation of  $C^{14}$  from acetate-2- $C^{14}$  and the turnover of labelled compounds during a post-pulse incubation.*

The data presented are obtained from Table XVII and are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

AA: amino acid fraction; H: hydrolyzate fraction; L: lipid fraction; OA: organic acid fraction; S: sugar fraction.

FIGURE 13

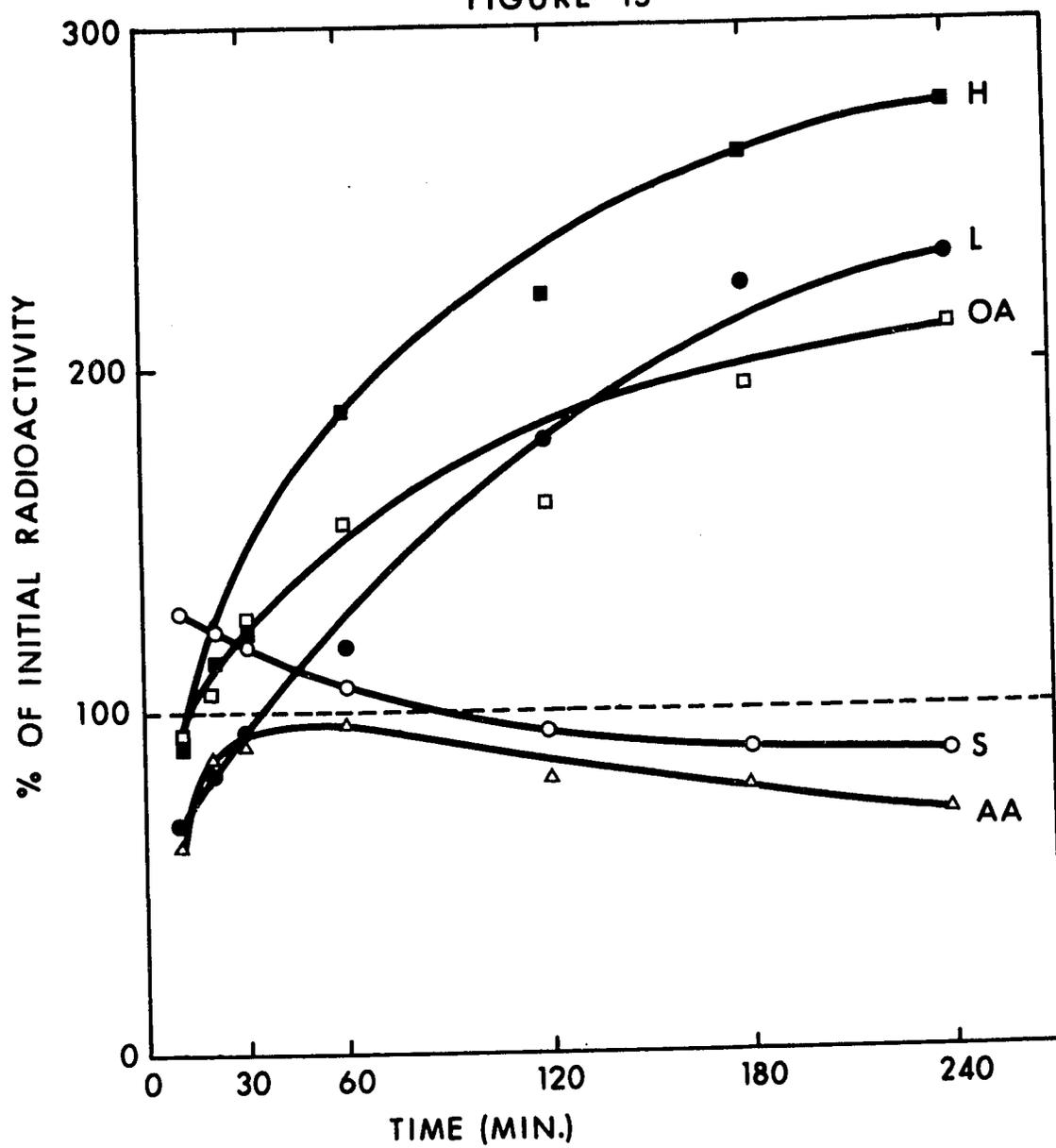


TABLE XVIII

*Incorporation of  $C^{14}$  from acetate-2- $C^{14}$  into the major radioactive components of the amino acid fraction and changes in their radioactivities during a post-pulse incubation.*

Separation of the component amino acids was obtained by thin-layer chromatography as described in the text.

TABLE XVIII

Incubation Time (min.)	Glutamate	Glutamine	γ-Amino butyrate	Aspartate	Serine	Unidentified	
						1	2
<i>Pulse</i>							
30	19,200	1,100	1,100	530	450	430	480
<i>Post-Pulse</i>							
10	8,200	800	700	280	420	410	450
20	12,600	1,200	800	370	560	540	570
30	17,300	1,300	900	420	650	700	690
60	17,600	1,600	900	530	---	520	580
120	14,200	1,900	1,200	550	800	---	430
180	11,700	2,300	1,500	650	820	360	400
240	7,500	2,500	1,700	700	1,030	660	450

Data are expressed as cpm.

Note: Dash-lines in the table stand for "not detected".

FIGURE 14

*Incorporation of  $C^{14}$  from acetate-2- $C^{14}$  into some of the soluble amino acids. Changes in their radioactivities during a post-pulse incubation.*

The data presented are obtained from Table XVIII. Only the major radioactive amino acids from Table XVIII are plotted. The data are expressed as percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period.

Asp: aspartate; Glu: glutamate; Gln: glutamine;  $\gamma$ -NH<sub>2</sub>:  $\gamma$ -aminobutyrate.

FIGURE 14

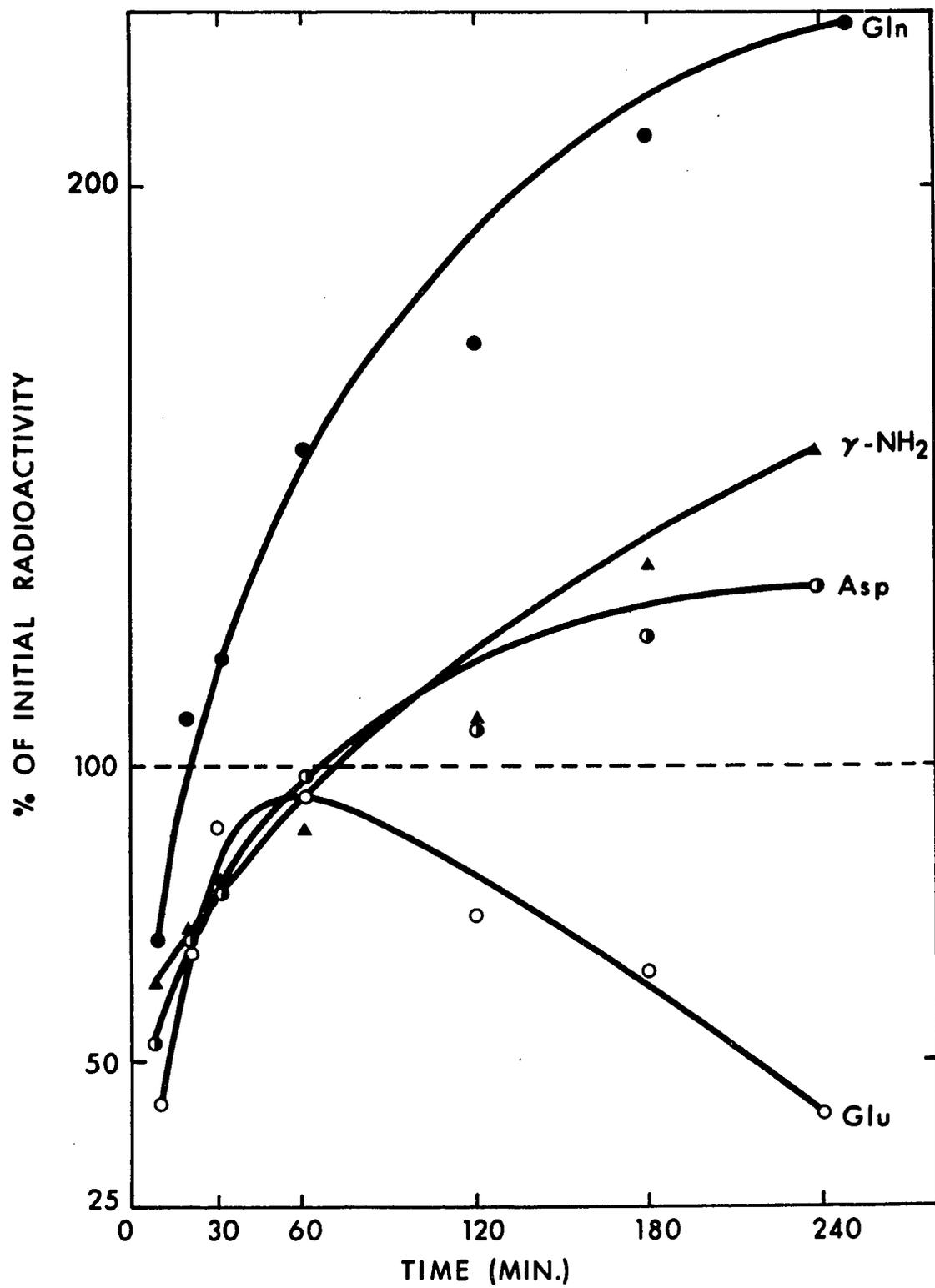


TABLE XIX

*Incorporation of C<sup>14</sup> from acetate-2-C<sup>14</sup> into some  
of the components of the organic acid fraction  
and changes in their radioactivities  
during a post-pulse incubation.*

Separation of the component organic acids was obtained by thin-layer chromatography and high-voltage electrophoresis as described in the text.

TABLE XIX

Incubation Time (min.)	Citrate & α-Keto-		Phospho- glycerate	Phosphoenol pyruvate
	Succinate Malate	Isocitrate glutarate		
<i>Pulse</i>				
30	8,690	1,050	1,500	1,890
				--
				400
				--
<i>Post-Pulse</i>				
10	4,890	980	1,040	1,280
20	8,760	2,820	1,570	1,470
30	9,960	4,680	1,920	1,370
60	12,900	5,800	2,390	1,200
120	13,670	6,780	2,900	770
180	12,770	9,900	3,850	500
240	9,820	14,330	4,850	--
				1,020
				2,380
				--
				400
				--
				700
				750
				800
				860
				920
				1,080
				--

Data are expressed as cpm.

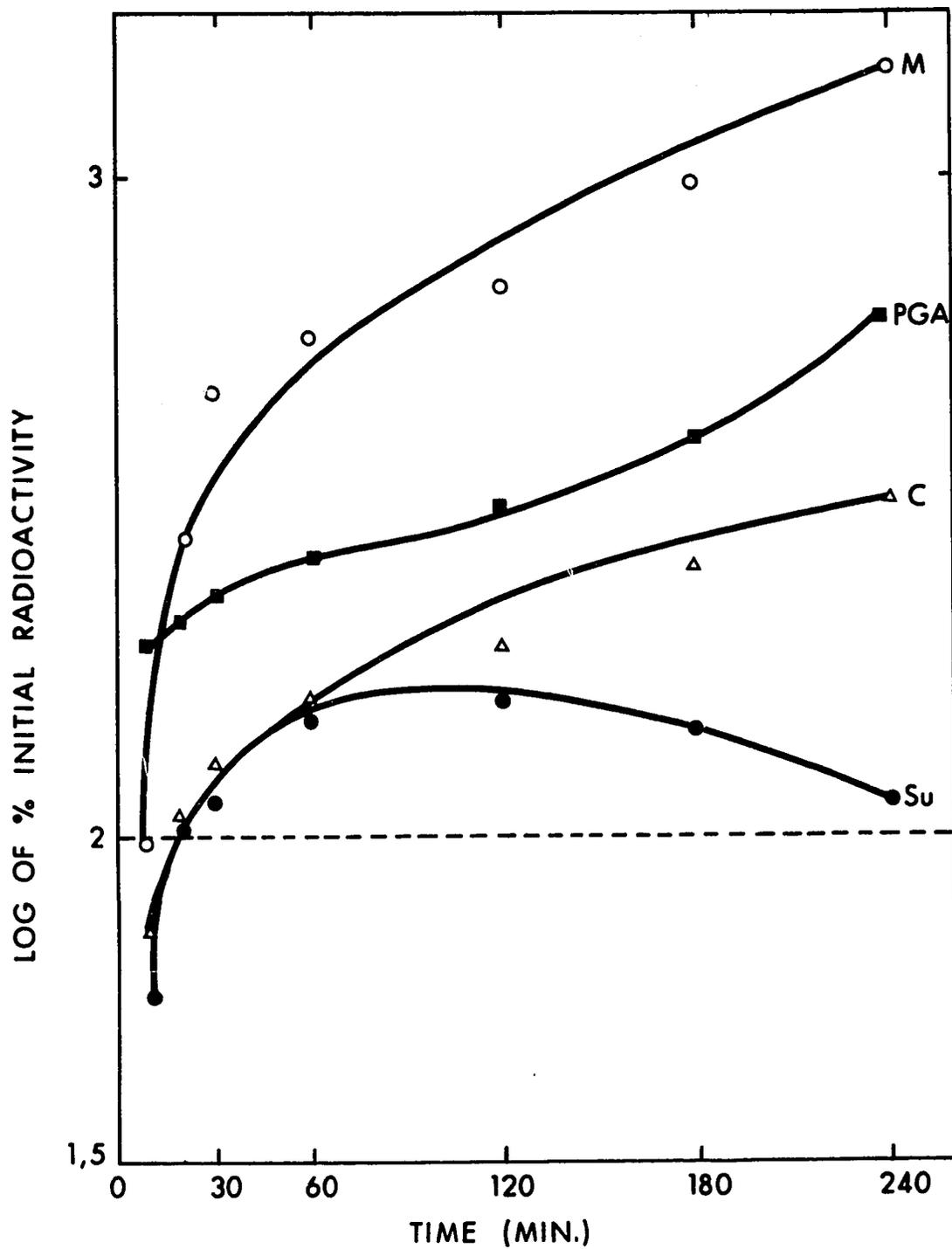
Note: Dash-lines in the table stand for "not detected".

## FIGURE 15

*Incorporation of  $C^{14}$  from acetate-2- $C^{14}$  into some of the components of the organic acid fraction. Changes in their radioactivities during post-pulse.*

The data presented are obtained from Table XIX. Only the major radioactive components of Table XIX are plotted. The data are expressed as the logarithms of the percentages of the radioactivities incorporated at the end of the 30 minute pulse period (initial radioactivities) and plotted as a function of time during the post-pulse period. C: citrate; M: malate; PGA: phosphoglycerate; Su: Succinate.

FIGURE 15



it is apparent that this compound is utilized by the cotyledons by pathways which are essentially different from those of glyoxylate metabolism.

## DISCUSSION

The significance of certain observations related to each of the present experiments has been discussed in the appropriate sections. However, the implications of these experimental findings deserve more detailed attention.

A further examination of these findings under the following separate headings will be presented in this section of the thesis.

*Carbon dioxide fixation and turnover of photosynthetic products.*

The experimental results in Tables I and II and Figures 1 and 2 clearly show that carbon dioxide is actively utilized by illuminated radish cotyledons. This photosynthetic ability is particularly evident by incorporation of the label into the sugar fraction, which shows a linear increase in radioactivity with time (Fig. 1) and an appreciable turnover rate (Fig. 2) during a post-pulse incubation.

In general terms, the findings presented in Table IV regarding incorporation of  $C^{14}O_2$  into the amino acid fraction are those usually observed in studies of photosynthesis. The large amount of radioactivity incorporated into serine, however, might be due to the operation of more than one pathway for its formation. This possibility is supported by the presence of labelled glycollic, glyoxylic, phosphoglyceric and phosphohydroxypyruvic acids in the organic

acid fraction (Table V, Fig. 5) which may act as precursors of serine via the reactions of the glycolate pathway. Such serine would be uniformly labelled. In addition, the possibility exists that serine could be formed from 3-phosphoglyceric acid via a sequence of phosphohydroxypyruvic  $\rightarrow$  phosphoserine  $\rightarrow$  serine, and from 2-phosphoglyceric acid via hydroxypyruvate.

The activities found in certain volatile and keto-acids such as glycollic, glyoxylic, etc., are not intended to be interpreted as exact due to losses suffered during chromatography, although in most cases a pattern emerges. The fact that all the above named acids, including glycine, show a peak of incorporation immediately after the serine pool starts showing signs of being saturated cannot be considered purely coincidental (Tables IV and Va). The same trend was also observed in the sugar phosphates (Table Vb). Since serine has been shown to be rapidly incorporated into sugars via glycerate in the light, (Wang and Waygood, 1962) it is possible that turnover of serine is largely responsible for the changes in radioactivity of the sugar phosphates during the post-pulse incubation.

A rapid loss of radioactivity from any compound during a post-pulse incubation which is not associated by loss of the compound to the medium, is indicative of metabolic turnover. Using this criterion it is obvious from the experiment summarized in Table II that several of the photosynthetic products were in a state of metabolic turnover. Perhaps the most interesting trend was the continued increase in

radioactivity of 3-phosphoglycerate during the post-pulse incubation (Table Vb). As this compound is established as the primary product of  $\text{CO}_2$  fixation in illuminated tissues with a Calvin sequence, it is surprising that this acid was not saturated with  $\text{C}^{14}$  during the pulse period. In fact, maximal labelling did not occur until 30 minutes of the post-pulse incubation. This observation may be explained, in part, by assuming that more than one pool of 3-phosphoglycerate occurs in this tissue. Such compartmentation of 3-phosphoglyceric acid may involve the chloroplast and the cytoplasm.

Some interesting studies of the intracellular localization of  $\text{C}^{14}$  following exposure of tobacco leaves to  $\text{C}^{14}\text{O}_2$  have been reported by Ongun and Stocking (1965b). These workers have shown that various photosynthetic products exist in both chloroplastic and cytoplasmic pools. The relationships of these pools under various experimental conditions were examined in pulse-chase experiments. They found that  $\text{C}^{14}$  rapidly moved from the chloroplasts into the cytoplasm where further metabolism would conceivably take place. It is likely that a similar situation prevails in radish cotyledons. Exchange of photosynthetic products between the chloroplasts and the cytoplasm would lead to pronounced changes in labelling patterns when the  $\text{C}^{14}\text{O}_2$  was replaced by  $\text{C}^{12}\text{O}_2$ .

Considering the early products of  $\text{C}^{14}\text{O}_2$  fixation (Tables IV and V) there are indications that the label is incorporated into two and four carbon acids. The early

labelling of glycollate observed in the present work has been observed by many workers as reviewed in the Introduction. This synthesis was accompanied by heavy labelling of glycine and serine indicating that such a route of  $C^{14}O_2$  fixation was quantitatively significant in radish cotyledons.

The major four-carbon acid labelled from  $C^{14}O_2$  was found to be malate (Table V) and as suggested earlier may be formed by the action of phosphoenolpyruvate carboxylase. The importance of this route of  $C^{14}O_2$  incorporation in some plant species has been demonstrated recently by Hatch and Slack's group (Hatch *et al.*, 1967). Although plants with this pathway do not display photorespiration (Downton and Tregunna, 1968) the possibility exists that radish, which displays some photorespiration (Dr. C. D. Nelson, Personal Communication) may also fix some  $CO_2$  via this route.

The synthesis of organic acids from  $C^{14}O_2$  and the redistribution of the label into related amino acids during incubation in  $C^{14}O_2$  give further indications of the inter-relationships of photosynthesis and intermediary metabolism in this tissue. For example, the data presented in Table IV show that a considerable increase in the labelling of glutamate took place throughout the post-pulse period. This increase in  $C^{14}$  was not accounted for by the decreases in  $\alpha$ -ketoglutarate labelling (Table V). Clearly  $C^{14}$  was being withdrawn from the acids of the tricarboxylic acid via  $\alpha$ -ketoglutarate to support this labelling of glutamate. In contrast, aspartic acid reached maximal radioactivity after ten minutes of post-pulse incubation followed by a rapid

decrease in radioactivity (Table IV). At the end of 4 hours (Table IV) aspartate- $C^{14}$  was not detected in the tissues. While glutamine- $C^{14}$  was readily detected in the tissues (Table IV) asparagine- $C^{14}$  was not. However, examination of the medium in which the disks were incubated (Table VI) revealed considerable labelling of this amide which was greatly increased during the incubations in  $C^{12}O_2$ . The presence of this and other labelled compounds in the medium cannot be entirely due to physical phenomena such as diffusion. The predominance of labelling in compounds like sucrose, asparagine and glutamine, which are commonly translocated in plants (Crafts, 1961) argues for a similar removal from the disks in the present work. Vascular tissue is well developed in radish cotyledons as is evident from microscopic examination (Appendix, Plate II). It is very likely, therefore, that photosynthates, accumulating at the sites of synthesis, were readily translocated to adjoining vascular tissues and from there to the medium.

*The utilization of glyoxylate by illuminated radish cotyledons.*

It is clear from the experiments with  $C^{14}O_2$  that glyoxylate- $C^{14}$  was produced and utilized by the tissues under illumination (Table V, Fig. 5a). Glyoxylate, when provided exogenously was also quite rapidly utilized (Table VII, Fig. 7). Examination of this data points to a rapid transfer of the label from the organic acid fraction, allowing the assumption that conversion of glyoxylate

to glycine was rapidly taking place. The conversion of glycine to serine was also observed, and actual accumulation of radioactivity in serine took place. Since conversion of glycine to serine and glyoxylate to serine involve cleavage of these two 2-carbon compounds into  $C^{14}O_2$  and formate- $C^{14}$  and since no significant  $C^{14}O_2$  was recovered (Table VII), it can be assumed that this tissue possesses a very active metabolism involving 1-carbon compounds together with ability to refix  $CO_2$ . Some radioactivity was also present in glutamic and aspartic acids, which suggests that some carbon was entering tricarboxylic acid cycle intermediates.

The almost equivalent amount of radioactivity found in the sugars and in the lipid fractions suggests the possibility that in both cases the flow of label was from a common three-carbon intermediate. The appreciable labelling of the sugar fraction may be due to the intermediary formation of a two-carbon-thiamine pyrophosphate adduct which enters the Calvin sequence (Bassham, 1964), or by metabolism of glyoxylate through the sequence of reactions suggested by Wang and Waygood (1962). The possible pathways for sugar biosynthesis from the two-carbon substrates used in the present work was examined in later experiments and will be discussed later.

The contribution of label to the lipid fraction (ether solubles) would include not only true fats but in addition, terpenoids which are readily derived from glyoxylate in photosynthetic tissues (Rogers *et al.*, 1968).

*Utilization of glycine-C<sup>14</sup> by illuminated radish cotyledons and turnover of labelled products in pulse-chase experiments.*

The considerable ability of this tissue to carry the conversion of glycine into serine became particularly evident in the experiments where glycine-1-C<sup>14</sup> and glycine-2-C<sup>14</sup> were supplied (Tables VIII and X, Figs. 8 and 10). This conversion would involve addition of a one-carbon fragment in a reaction involving serine hydroxymethyltransferase (Cossins and Sinha, 1966). Although the presence of this enzymes in radish cotyledons was not examined in the present work, there are several reports of its occurrence in higher plants (e.g. Mazelis and Liu, 1967). Regarding the transfer of one-carbon fragments in radish cotyledons it is of interest to note that recent work in this laboratory (Spronk, A. and Cossins, E.A., unpublished data) has shown an active biosynthesis of tetrahydrofolate derivatives in these tissues. This reaches a maximum in cotyledons of the age used in the present work.

The data obtained from the glycine feeding experiments (Tables VIII - XI, Figs. 8 - 11) when compared with similar data from the C<sup>14</sup>O<sub>2</sub> experiments, show that there exist significant differences in the utilization of this amino acid according to whether it is endogenously produced or externally supplied. For example, aspartic acid which showed a marked decline throughout the C<sup>14</sup>O<sub>2</sub> chase experiment (Table IV) presumably due to a rapid conversion to asparagine, in the glycine-2-C<sup>14</sup> experiment appears late

and increases throughout the post-pulse incubation (Table X). This result is to be expected if aspartate is derived from a pathway involving the carboxylation of phosphoenolpyruvate. Thus glycine carbon would be diluted by passage through the pools of several intermediates before appearing in aspartate.

The major labelled products of glycine-2-C<sup>14</sup> metabolism showed various degrees of turnover during the post-pulse incubation (Tables IX - XI). The only exception to this being the lipid fraction which showed a progressive increase in radioactivity. As carbon dioxide was not collected during the post-pulse incubation some of this metabolic turnover may result in loss of C<sup>14</sup> from the tissues as carbon dioxide. Furthermore, a considerable portion of the radioactivity present at the end of the pulse period in glycine and serine, appeared in the medium during the post-pulse period, in progressively increasing amounts.

Considering the data from the glycine-C<sup>14</sup> experiments, it is clear that this amino acid when supplied exogenously is metabolized by pathways which appear to be similar to those for the utilization of C<sup>14</sup>O<sub>2</sub> in the light. The minor differences between the metabolic fates of these two substrates could be attributed to the extent to which these compounds were metabolized under the present experimental conditions. Although considerably more radioactivity was supplied in the C<sup>14</sup>O<sub>2</sub> experiment, and therefore several of the products exhibited a higher radioactivity, data from the pulse chase incubation show that C<sup>14</sup>O<sub>2</sub> was more *extensively* metabolized than glycine. Therefore, the

exogenously supplied glycine may not have entered the same metabolic sites as the  $C^{14}O_2$ . Extensive metabolism of glycine outside the chloroplast could account for some of this difference. Regarding this point, similar conclusions were drawn by Miflin *et al.* (1966) who studied the metabolism of two-carbon compounds in pea leaves. These workers found that glycine and serine, formed in photosynthesis were rapidly converted to sucrose in the light when the tissues were incubated in  $C^{12}O_2$ . Although similar data were obtained when glycine was supplied exogenously, this transfer of radioactivity was not observed when higher partial pressures of  $C^{12}O_2$  were supplied. On the basis of these findings, Miflin *et al.* (1966) concluded that although the metabolic pathway was similar for the exogenous and endogenous substrates, the metabolism of the former may have taken place at a site spatially separated from the path of carbon in photosynthesis.

*The effects of light and dark on the utilization of glyoxylate and glycine.*

The detailed studies of Wang and Waygood (1962) with wheat leaves and Miflin *et al.* (1966) with pea leaves, have clearly shown that the synthesis of sugars from two-carbon compounds is stimulated in the light. Similar findings were obtained in the present studies. However, stimulations were only observed when the labelled substrate contained label in the carboxyl carbon (Tables XIII, XV). This would occur if upon cleavage of the glyoxylate or glycine molecule,

CO<sub>2</sub> arising from the carboxyl carbon was refixed in photosynthesis. When the 2-position was labelled (e.g. glyoxylate-2-C<sup>14</sup> experiment, Table XIV) the incorporation of activity into the sugar fraction was considerably higher in the dark.

In agreement with published work (e.g. Jimenez *et al.*, 1962; Wang and Waygood, 1962) the present experiments (Tables XIII and XIV) clearly show that the 2-position of glyoxylate contributes more C<sup>14</sup> to the sugar fraction than the 1-position. This observation is consistent with a utilization of glyoxylate-2-C<sup>14</sup> for the biosynthesis of serine which would be labelled in both the 2 and 3 positions. Metabolism of such serine through the glycollate pathway as suggested by Wang and Waygood (1962) would result in hexoses containing four labelled carbons per molecule. On the other hand, glyoxylate-1-C<sup>14</sup> would produce serine-1-C<sup>14</sup> and subsequently a hexose molecule labelled in the 3 and 4 positions.

Another effect of light on the metabolism of these compounds was the considerable stimulation in labelling of the lipid fraction. As this fraction includes pigments as well as true fats, the light dependent biosynthesis of pigments (Rogers *et al.*, 1968) could account for this stimulation.

*The effects of added glycine on the utilization of glyoxylate-2-C<sup>14</sup> by radish cotyledons in light and dark.*

In tissues containing an active glycollate pathway

the incorporation of intermediates like glyoxylate or glycollate is known to be drastically reduced by additions of glycine or serine (e.g. Mifflin *et al.*, 1966; Wang and Waygood, 1962). Such data are consistent with the incorporation of such 2-carbon compounds into sugars by the intermediary formation of glycine and serine. As considerable glyoxylate carbon was incorporated into the sugar fraction particularly when the label was in the carbon-2 position (Table XIV), it was of interest to determine whether such incorporation would be affected by the addition of unlabelled glycine. The data from such experiments are summarized in Tables XIV and XVI.

When comparisons are made between the results obtained in the glyoxylate-2-C<sup>14</sup> feeding experiments in light and dark (Table XIV) and those carried out in the presence of glycine (Table XVI) the most outstanding difference observed was the incorporation of label into sugars which was more than doubled in the presence of that amino acid.

The values obtained for the amino acid fractions can be attributed to the diluting action of the added glycine. The large decrease in the C<sup>14</sup> of the amino acid fraction is probably due to decreased synthesis of serine-C<sup>14</sup>. However this decrease in radioactivity in the amino acid fraction was not accompanied by decreases in the activity of the sugar fraction. Clearly, there must be another pathway for the rapid incorporation of the 2-position of glyoxylate into carbohydrates. This pathway does not appear to be light dependent.

*Utilization of acetate-C<sup>14</sup> by illuminated radish cotyledons and turnover of labelled products in pulse-chase experiments*

The results obtained in the previous experiments show considerable incorporation of radioactivity into malate. This is of particular interest in the glyoxylate and glycine experiments, since little evidence was shown for the operation of a glyoxylate cycle in this tissue. This lack of evidence could point to other pathways for the incorporation of C<sup>14</sup> into malate, such as carboxylation of phosphoenolpyruvate or via the tricarboxylic acid cycle.

These questions are partially answered by examination of the results obtained in the acetate-2-C<sup>14</sup> pulse-chase experiment (Tables XVII - XIX, Figures 13 - 15). These data show that this tissue has a limited permeability for the radioisotope fed. Only 3% of the radioactivity supplied was incorporated into the different fractions at the end of the pulse period (Table XVII). Furthermore, when compared with similar data from the C<sup>14</sup>O<sub>2</sub> (Tables II - V) and the glycine-2-C<sup>14</sup> pulse-chase experiments (Tables IX - XI) significant differences in the utilization of acetate-C<sup>14</sup> by radish cotyledons became apparent. For example, the C<sup>14</sup> incorporation into the sugar fraction at the end of the pulse period is considerably reduced in proportion to the other fractions of this experiment, including the protein fraction (Table XVII, Figure 13). In fact, only 3% of the total C<sup>14</sup> incorporated was found in sugars. In contrast,

in the  $C^{14}O_2$  and glycine- $C^{14}$  pulse-chase experiments, that fraction contains a proportionally much larger percentage of the total radioactivity incorporated, with an apparent rapid turnover of the pools involved (Tables II and IX, Figures 2 and 9).

On the other hand, the organic acid fraction shows a continuous increase in  $C^{14}$  uptake throughout the acetate- $C^{14}$  experiment (Table XVII, Figure 13).

$C^{14}$  incorporation into malate, however, is considerably lower than in the previous pulse-chase experiments (Tables Va, IX and XIX, Figures 5a, 9 and 15). The greatest incorporation within the components of the organic acid fraction at the end of the pulse period, is found in succinate. This high incorporation continues in this compound until after 3 hours of post-pulse incubation. At that time, saturation of the succinate pool occurred (Table XIX, Figure 15).

These results, together with the presence of a saturated pool of  $\alpha$ -ketoglutarate at the end of the pulse period, support the findings of previous experiments regarding the possible lack of a working glyoxylate cycle in radish cotyledons under these experimental conditions. If such a cycle exists in this tissue, it is possible that its operation occurs in a cellular locale not accessible to the compounds supplied exogenously in the present experiments.

The high amount of radioactivity incorporated in succinate from acetate- $C^{14}$  feedings is supported by similar

results obtained when this radioisotope was fed in experiments with other plant tissues (Davies *et al.*, 1959; Goulding and Merrett, 1966; Merrett and Goulding, 1967). In fact, succinate was the primary product of photo-assimilation of acetate-2- $C^{14}$  by *Chlorella pyrenoidosa* (Merrett and Goulding, 1967).

The mechanism of succinate formation from acetate, however, is not readily apparent. Experiments using unlabelled acetate and  $C^{14}O_2$  with *Chlorella* in the light failed to demonstrate the formation of succinate (Goulding and Merrett, 1966). Similarly, the pulse-chase experiments carried out in these studies with  $C^{14}O_2$  do not show succinate as one of the most conspicuously labelled organic acids (Table Va). It seems unlikely, therefore, that succinate is formed by a carboxylation reaction. Furthermore, since succinate contains the highest percentage of the total  $C^{14}$  incorporated at practically all times as compared with citrate, it is also unlikely that succinate is formed exclusively via the tricarboxylic acid cycle (Table XIX).

Evidence has been obtained, however, which suggests that green leaves catalyze the Thunberg condensation, which introduces label into carbons 1 and 4 of succinate (Buhler *et al.*, 1956; Davies *et al.*, 1959) and that the acetate- $C^{14}$  produced succinate could be formed via such a reaction (Davies *et al.*, 1959).

On the other hand, some evidence exists for the operation of the tricarboxylic acid cycle in this tissue.

Considerable incorporation of  $C^{14}$  has been detected in some of the member acids of this cycle. Glutamic acid, the principal component of the amino acid fraction, shows a pattern of activity and turnover parallel to that of  $\alpha$ -ketoglutarate (Tables XVIII and XIX). It is conceivable that while a large portion of the  $\alpha$ -ketoglutarate- $C^{14}$  is incorporated into glutamate, this amino acid, in turn, passes on its activity to some of its derivatives, such as glutamine and  $\gamma$ -aminobutyrate. These two latter amino compounds consistently increase their  $C^{14}$  incorporation through the post-pulse period (Table XVIII, Figure 14).

Alternatively,  $\gamma$ -aminobutyrate might be produced in a transamination of succinic semialdehyde derived reductively from succinate with glutamate and glutamine serving as the immediate amino donors (Effer and Ranson, 1967).

There is a large and consistently increasing incorporation of  $C^{14}$  from acetate into the ether soluble fraction (Table XVII). This incorporation may represent a rapid formation of both cytoplasmic sterols and chloroplastic pigments, as well as incorporation into triglycerides in both locales. Rogers *et al.* (1968) pointed out the great permeability shown by chloroplasts to acetate as precursor of acetyl-CoA, which in turn is an obligatory intermediate in fatty acid biosynthesis. Also, terpenoid formation in young tissues is essential for further development of the seedling. Concurrently, Duperon (1968) demonstrated the large increase in total sterols in illuminated radish

cotyledons. It is assumed, therefore, that the acetate- $C^{14}$  supplied is efficiently used by this tissue in the light for the production of all these ether soluble components.

In general terms it may also be concluded that radish cotyledons metabolize acetate-2- $C^{14}$  by pathways which are essentially different from those used to metabolize  $C^{14}O_2$  and the other radioactive compounds supplied exogenously in the present experiments.

*Fluctuations in  $C^{14}$  levels during transfer of tissues from labelled to unlabelled media*

In all the previously described pulse-chase experiments, a sudden drop of activity was observed in many of the fractions at the end of ten minutes of post-pulse.

This phenomenon has been observed by several workers in light-dark transition experiments and it is closely related to the leaching of some compounds from the cells into the medium (Tolbert and Zill, 1956; Smith *et al.*, 1961; Whittingham *et al.*, 1963; Bassham *et al.*, 1968).

This initial drop in activity is known as the light-dark transient effect. It may be due to a sudden cessation of  $C^{14}O_2$  fixation in photosynthesis, or to a slow down in the metabolic processes that were taking place in light. Usually, this drop is compensated later by an increase in the production of the affected compounds (e.g. Tables II, IX and XVII) indicating that the metabolic processes have been resumed in the dark, either by the same or by other

pathways, or by renewed exposure of the tissues to the light.

In the present experiments, after the pulse period, the tissues were removed from the light source in order to wash and transfer them to a non-radioactive medium. This transfer, which took no more than 1 to 1½ minutes to complete, seems to have been enough to produce the transient drop. Smith *et al.* (1961) observed this drop when light had been shut off for 30 seconds, even when - as in the case of the present work - a steady-state of photosynthesis, or a pool saturation had been achieved.

As shown in the distribution of the activity in the different amino acids however, not all the components of that fraction showed this transient effect (Tables IV and X). Thus, while the total amount of C<sup>14</sup> in the amino acid fraction decreased at 10 minutes of the post-pulse period, the activity in glutamic acid and alanine for instance, (Tables IV and X) increased. This increase was probably initiated at the beginning of the post-pulse, when the tissues were removed from the light source and the washing and transfer done in relative darkness.

If we consider valid the assertion of Smith *et al.* (1961) that during the first 8 minutes of darkness the rate of ammonia uptake and utilization increases, then the increases shown in glutamate and alanine could thus be explained.



## CONCLUSIONS

On the basis of the present work with radish cotyledons, the following major conclusions can be drawn regarding the pathways of intermediary metabolism in this tissue. In addition, suggestions can be made regarding areas for future research with this tissue.

- I. Illuminated radish cotyledons are capable of an active photosynthetic fixation of  $\text{CO}_2$ .

Considering the nature of the products obtained in the experiments with  $\text{C}^{14}\text{O}_2$ , this fixation proceeds mainly through the Calvin photosynthetic carbon reduction cycle. Carbon dioxide is extensively metabolized and the tissue shows a large affinity for  $\text{CO}_2$ . This latter characteristic is demonstrated by the apparent re-fixation of  $\text{C}^{14}\text{O}_2$  liberated from the supplied substrates.

- II. Radish cotyledons can efficiently utilize some of the intermediates of the glycollate pathway, such as glyoxylate and glycine. The products obtained, particularly from pulse-chase experiments in which glycine-2- $\text{C}^{14}$  was fed, demonstrate that a complete glycollate pathway is operative in this tissue, in light and dark.

- III. There is evidence for an efficient conversion

of glycine to serine and glyoxylate to serine, which would implicate an active transfer of one-carbon fragments.

- IV. While the pulse-chase experiments using acetate-2-C<sup>14</sup> give some evidence for operation of the tricarboxylic acid cycle, this and the previous experiments do not give any definite evidence for an operative glyoxylate cycle in radish cotyledons.
- V. Radish cotyledons actively synthesize ether soluble compounds. This synthesis is light dependent. Since this is a young growing green tissue, it is assumed that these compounds are mainly chloroplastic pigments of pyrrolic and terpenoid nature. In addition, the acetate label might be incorporated into ether soluble compounds such as fatty acids via acetyl-CoA.

More detailed research is necessary to give more support to the conclusions drawn above, particularly within the topics of transfer of one-carbon fragments and possible operation of the glyoxylate cycle. More conclusive evidence could be obtained from: (a) investigations of the key enzymes catalyzing these metabolic pathways; (b) determination of the pool sizes of key intermediates and (c) the intramolecular distribution of label, within the products formed.

Recent work has been carried out on the intracellular localization of the enzymes responsible for operation of the glycollate pathway and glyoxylate cycle (Breidenbach and Beevers, 1967; Tolbert *et al.*, 1968; Tolbert *et al.*, 1969). In radish cotyledons the former pathway has been found to be active while evidence for the latter is still lacking. It would, therefore, be of interest to attempt an isolation of peroxisomes and, if existent, glyoxysomes from this tissue. A subsequent comparison of the component enzymes could be of considerable significance, as no work of this nature has been reported for green cotyledons to date.

Another area worthy of further investigation is a detailed study of glyoxylate metabolism in the presence of unlabelled glycine. Considering the results obtained from such experiments in the present work, it is possible that an alternate pathway(s) for the conversion of glyoxylate into sugars exists in both light and dark.

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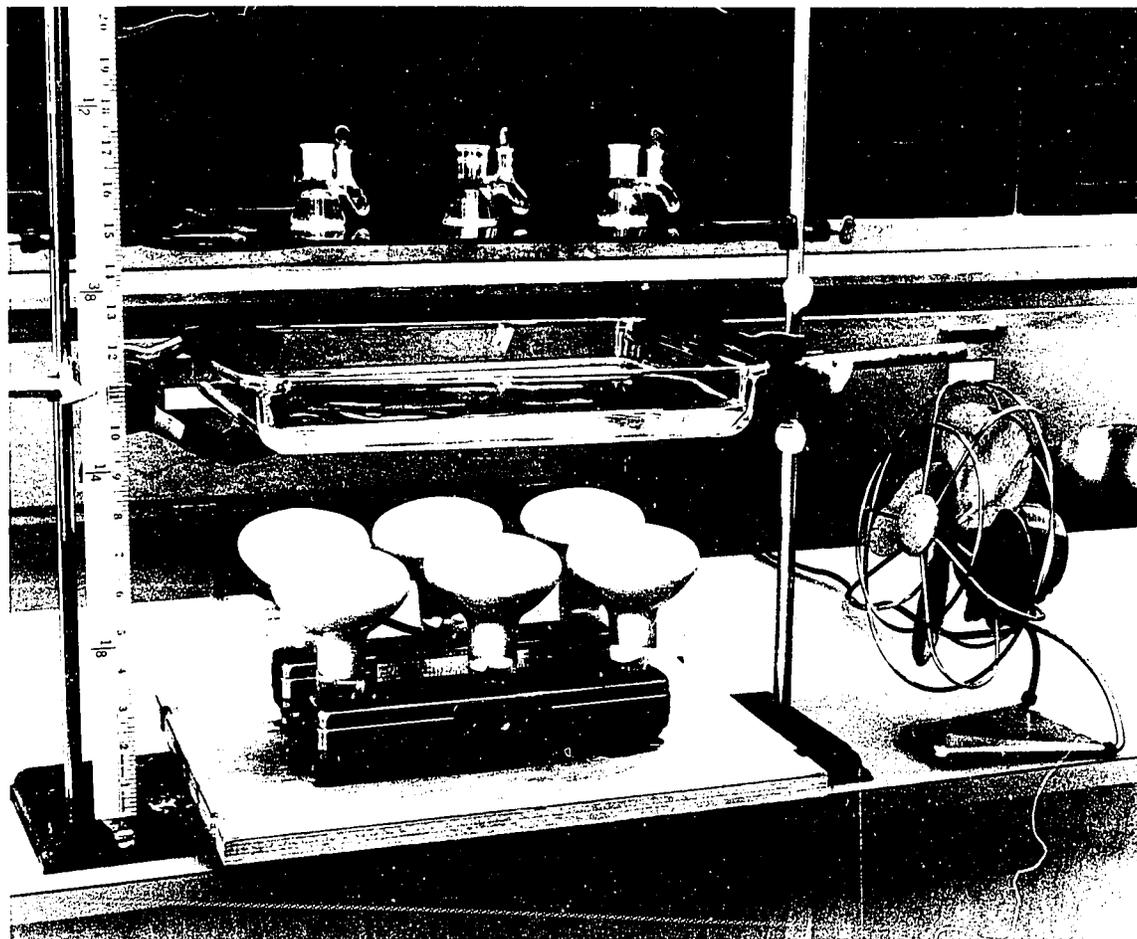
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APPENDIX

PLATE 1

Apparatus used to provide illumination  
to tissues in all the experiments  
carried out in the light.



*Anatomical description of transverse sections of radish cotyledons*

Transverse sections 8  $\mu$  thick were cut from eleven-day old radish cotyledons. The sections were stained with haematoxylin-eosin, mounted and observed microscopically.

In general, the tissue has an appearance characteristic of rapidly expanding and collapsible tissues. Such structure could be placed between an aerenchyma and a spongy parenchyma. The cells are non-differentiated and very large with large vacuoles as expected in tissues capable of rapid collapse. There is no significant difference between epidermal and mesophyll cells. The cuticle is absent and stomata are present on both surfaces of the organ. A palisade parenchyma as such, is absent; however, the mesophyll cells contain large numbers of chloroplasts.

The vascular tissues are represented by well developed primary phloem and xylem.

PLATE 2

*Illustration of the plant material used  
in all the experiments*

The upper photograph shows the stage of growth and the relative size of the seedlings.

The lower photograph shows a thin transverse section ( $8\mu$ ) of the tissue used.

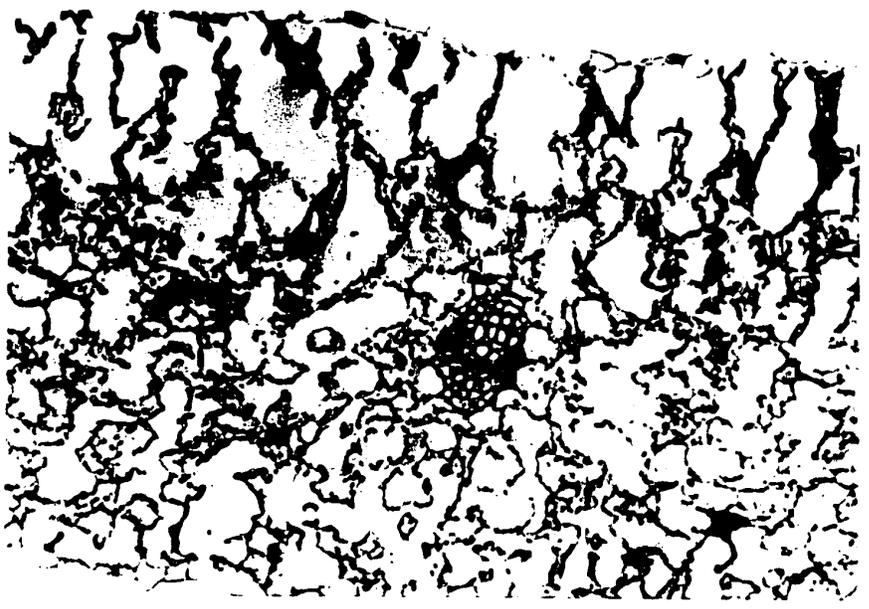


PLATE 3

*Separation of amino acids by thin-layer  
chromatography*

A mixture of known amino acids was placed at the origin and two directional ascending chromatography was carried out as described in the text. The developed plate was sprayed with ninhydrin and later the thin-layer was peeled off the plate for preservation, as described.

1-aspartate; 2-glutamate; 3-serine; 4-glycine;  
5-alanine; 6-asparagine; 7-glutamine; 8-cysteic  
acid; 9-lysine; 10-histidine; 11-proline; 12-  
methionine; 13-valine; 14-phenylalanine; 15-  
leucine and isoleucine; 16-threonine.

o - origin.

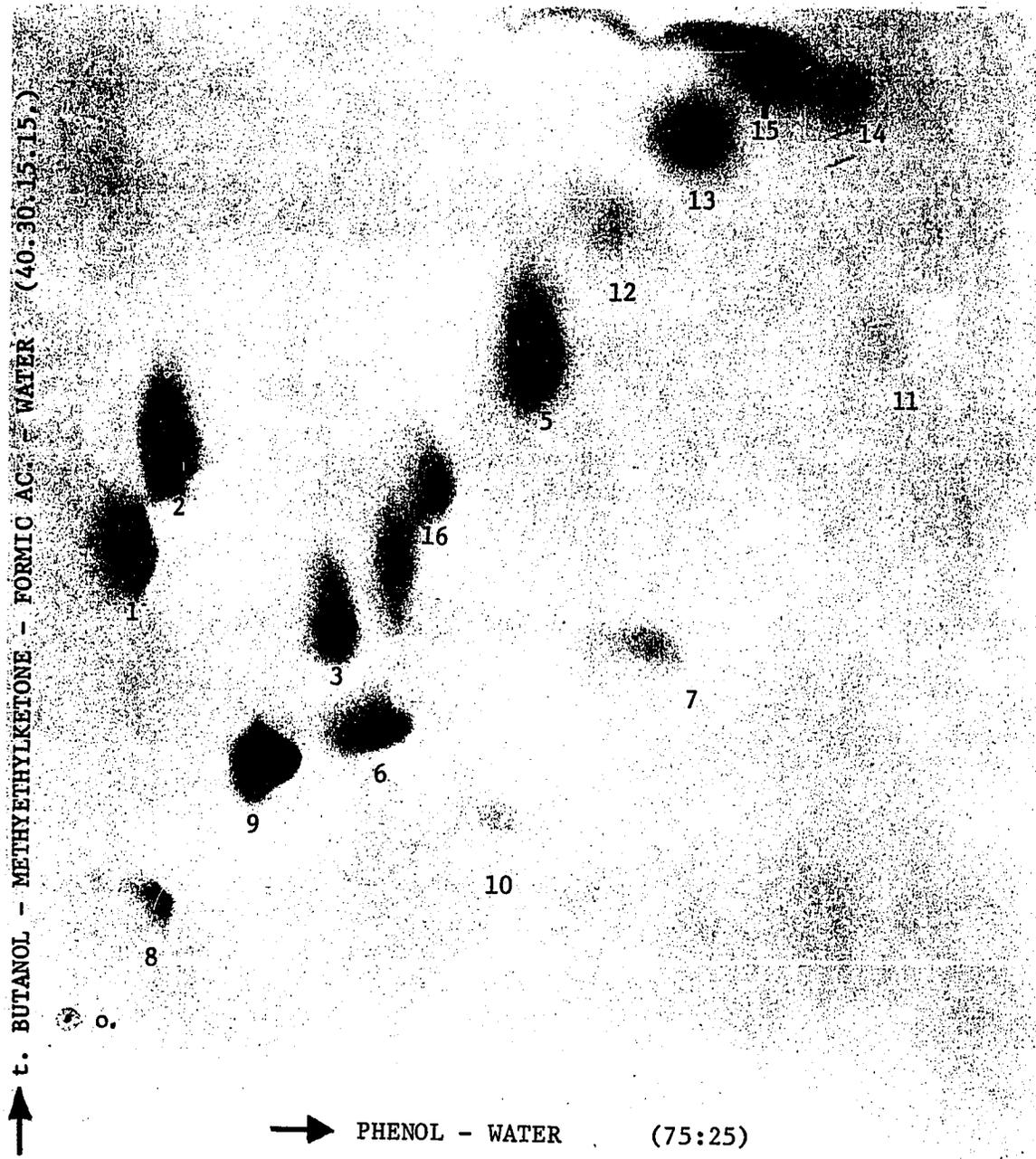


PLATE 4

*Separation of organic acids by thin-layer  
electrophoresis and chromatography*

As an example of separation of organic acids by the technique described in the text, a radioautograph of an organic acid fraction as obtained in one of the experiments is shown (Organic acid fraction, Pulse-chase experiment, 30 minutes incubation with  $C^{14}O_2$ ).

1-succinate; 2-glycollate; 3-pyruvate; 4-malate;  
5-citrate; 6-unidentified; 7-phosphoenolpyruvate;  
8-3-phosphoglycerate; 9- $\alpha$ -ketoglutarate; 10-gly-  
oxylate; 11, 12 and 13-unidentified sugar phos-  
phates; 14-fumarate.

o - origin.

