THE UNIVERSITY OF MANITOBA

GLYCINE METABOLISM AND STUDIES OF SOME AMINOTRANSFERASES IN WHEAT LEAVES

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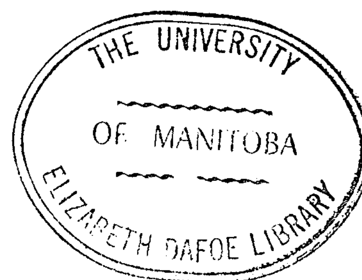

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ABSTRACT

Quantitative studies of the derangement in glycine-2-C^{14} metabolism to sugars in wheat leaves were carried out with the aid of benzimidazole. Specific activities of sucrose, glucose and fructose from immediately detached, water floated and benzimidazole treated leaves suggested that derangement of glycine-2-C^{14} metabolism after prolonged detachment was caused largely by an increase of free sugars from a non-radioactive source associated with photosynthesis. Chromatographic studies have been made on the following aminotransferases in crude dialysed extracts of wheat leaves:serine:glyoxylate (SGT); alanine:glyoxylate (AGT); glutamate: glyoxylate (CGT); aspartate:glyoxylate (AsGT); glutamate:pyruvate (GPT); glutamate:oxalacetate (GOT). A more detailed study of SGT showed that this and other glyoxylate aminotransferases have some properties apparently not shared by aminotransferases in general. Various aspects of glycine and carbohydrate metabolism have been discussed in relation to this and other work recently reported using wheat blades.

TABLE OF CONTENTS

PAGE

I. INTRODUCTION -- 1

II. LITERATURE REVIEW ---------------------------------- 3

1. The Glyoxylate-Serine Pathway ---------------- 3

2. Enzymes of the Glyoxylate-Serine Pathway------ 10

3. Aminotransferase Reactions Involving Glyoxylic Acid ---------------------------------- 12

(a) Non-enzymatic reactions ---------------- 12
(b) Enzymatic reactions -------------------- 12

4. Conclusion ---------------------------------- 14

III. MATERIALS AND METHODS ------------------------------ 17

1. General -- 17

2. Cultivation and Light Treatment of Wheat Leaves -- 17

3. Incubation of Wheat Leaves with Glycine-2-C^{14} and Extraction of Alcohol-Soluble Substances- 18

4. Ion-Exchange Fractionation -------------------- 18

5. The Separation and Quantitative Estimation of Sugars ------------------------------------ 19

(a) Quantitative estimation of radioactivity in each sugar -------------------------- 19
(b) Quantitative estimation of sugars using the anthrone sugar test ----------------- 20

6. Isolation and Assay of Invertase ------------ 21

7. Extraction of Some Aminotransferases from Wheat Leaves--------------------------------- 22

(a) Extraction of crude enzyme--------------- 22
(b) Fractionation with ammonium sulphate----- 23
(c) Fractionation on DEAE-cellulose columns-- 23

TABLE OF CONTENTS CONT'D

	PAGE
8. Assay Systems	24
(a) Qualitative estimation of amino acids	24
(b) Quantitative estimation of amino acids	25
(c) Quantitative estimation of β-hydroxypyruvic acid	26
9. Protein Estimations	28
10. Qualitative Tests of Keto Acids by Paper Chromatography	29
(a) Extraction of keto acids	29
(b) Assay of keto acids	30
11. Detections and Measuring Devices	30
IV. EXPERIMENTAL RESULTS	31
1. The Effect of Benzimidazole on the Metabolism of Glycine-2-C^{14} in Detached Khapli Wheat Leaves	31
(a) Preliminary extraction of alcohol-soluble substances	31
(b) Fractionation of water soluble substances on Dowex resin columns	33
(c) Final separation and quantitative measurement of sugars	49
(d) Invertase activity	51
2. Chromatographic Studies of Some Glyoxylate: Aminotransferases in Wheat Leaves	51
(a) Preliminary investigations of each aminotransferase	51
(b) Reaction products with single amino donors and multi-amino acceptors	56
3. Kinetic Studies of a Serine:Glyoxylate Aminotransferase in Wheat Leaves	59

TABLE OF CONTENTS CONT'D

PAGE

(a) General properties of the enzyme ———— 59
(b) Effect of metal-chelating compounds ———— 73
(c) Effect of pyridoxal-5-phosphate ———— 75
(d) Effect of pyridoxal-5-phosphate antagonists—— 75
(e) Serine:glyoxylate aminotransferase activity in etiolated leaves ———— 76
(f) An unidentified ninhydrin-reacting substance in in the serine:glyoxylate aminotransferase—— 77
(g) Initial velocity studies of serine:glyoxylate aminotransferase ———— 79

V. DISCUSSION ———— 87

1. Glycine-2-C^{14} Metabolism ———— 87

2. Aminotransferases ———— 96

3. Conclusion ———— 113

VI SUMMARY ———— 114

VII BIBLIOGRAPHY ———— 116

LIST OF TABLES

TABLE		PAGE
I	Fresh weights (g) of Khapli wheat leaf tissue used in feeding experiments with glycine-2-C^{14}	32
II	The incorporation of glycine-2-C^{14} into leaves of Khapli wheat	34
III	Total radioactivity recovered from Dowex resin columns	36
IV	Formation of sugars, amino acids and organic acids from glycine-2-C^{14} in immediately detached Khapli wheat leaves	38
V	The effect of benzimidazole on the transformation of glycine-2-C^{14} to sugars in detached leaves of Khapli wheat	40
VI	The effect of benzimidazole on the percentage of of glycine-2-C^{14} remaining in the free amino acid fractions of detached Khapli wheat leaves	43
VII	The effect of benzimidazole on the transformation of glycine-2-C^{14} to organic acids in detached leaves of Khapli wheat	44
VIII	Quantitative estimations of the components of the sugar fractions isolated from Khapli wheat leaves fed glycine-2-C^{14}	45
IX	Invertase activity in crude extracts of Khapli wheat leaves	52
X	R_f values of 2,4-dinitrophenylhydrazones of some keto acids	61
XI	Reaction between various amino acid donors and glyoxylate or glycolaldehyde	62
XII	Hydroxypyruvate disappearance in the presence of glycine	66
XIII	Stoichiometry of the forward reaction	67
XIV	Inhibition of serine:glyoxylate aminotransferase	73

LIST OF TABLES CONT'D

TABLE		PAGE
XV	Activation of serine:glyoxylate aminotransferase by Cu^{2+} ions	74
XVI	Effect of pyridoxal-5-phosphate on serine:glyoxylate aminotransferase activity	75
XVII	Purification of serine:glyoxylate aminotransferase	79

LIST OF FIGURES

FIGURE		PAGE
1	Radioautographs of chromatograms of free sugars from detached Khapli wheat leaves fed glycine-2-C^{14}	41
2	Plan of a chromatogram showing glycine formation from alanine:glyoxylate and serine:glyoxylate aminotransferases	54
3	Plan of a chromatogram showing glycine formation and irreversibility of glutamate:glyoxylate aminotransferase	55
4	Requirement for active enzyme protein for a serine: glyoxylate aminotransferase reaction in wheat leaves	60
5	A progress-time curve of serine:glyoxylate aminotransferase activity in the forward direction	64
6	Effect of increasing enzyme concentration on serine: glyoxylate aminotransferase activity in the forward direction	65
7	Effect of increasing glyoxylate concentration on serine:glyoxylate aminotransferase activity in the forward direction	68
8	Effect of increasing serine concentration on serine:glyoxylate aminotransferase activity	69
9	Effect of increasing pH on serine:glyoxylate aminotransferase activity	70
10	Effect of increasing temperature on serine: glyoxylate aminotransferase activity	71
11	Effect of phosphate ions on serine:glyoxylate aminotransferase activity	72
12	Effect of light on serine:glyoxylate aminotransferase activity in etiolated wheat leaves	78
13	Effect of changing substrate concentration on the initial velocity of serine:glyoxylate aminotransferase at high concentrations of the other substrate	80

LIST OF FIGURES CONT'D

FIGURE		PAGE
14	Fraction of a partially purified extract of serine: glyoxylate aminotransferase using a DEAE-cellulose column	81
15	Initial velocity pattern for serine:glyoxylate aminotransferase with serine as varied substrate	83
16	Initial velocity pattern for serine:glyoxylate aminotransferase with glyoxylate as varied substrate	84
17	Replots of maximum velocity data from Figures 15 and 16	85
18	Replots of slopes of lines from Figures 15 and 16	86
19	Pathways of amino group transfer from the aspartate, glutamate and serine metabolic pools to the glycine pool in wheat leaves	98

I. INTRODUCTION

Wang and Waygood (98) postulated a serine-glyoxylate cycle as being an important pathway for glycine synthesis in wheat leaves. Other results by Rabson et al (71) indicated that a similar pathway was operating in a wide variety of plant species. Further studies by Wang and Burris (96) and recently by McConnell (44) support earlier findings. In other experiments Wang and Hao (97) and Wang (95) showed that detachment of wheat leaves caused a number of metabolic derangements and among which the derangement of glycine metabolism was most pronounced. In Khapli wheat leaves floated on water the synthesis of sucrose from glycine-2-C^{14} was greatly decreased whilst on the other hand in benzimidazole treated leaves the metabolism of glycine-2-C^{14} and the synthesis of sucrose were found to be comparable to those in immediately detached leaves (95). In a continuation of Wang's (95) studies a more quantitative measurement of the effects of detachment and benzimidazole treatment on glycine-2-C^{14} metabolism in Khapli wheat leaves was undertaken. Quantitative estimates of sucrose, glucose and fructose confirmed Wang's (95) earlier work and also threw light on the mechanism of the decreased incorporation of glycine-2-C^{14} into free sugars of wheat leaves. This work was also a continuation of the studies being carried out in this laboratory on the effect of benzimidazole treatment on metabolism in wheat leaves recently reviewed by Kapoor (32) and Mishra (54).

Other investigations were undertaken primarily to study serine: glyoxylate aminotransferase, an enzyme believed to be involved in

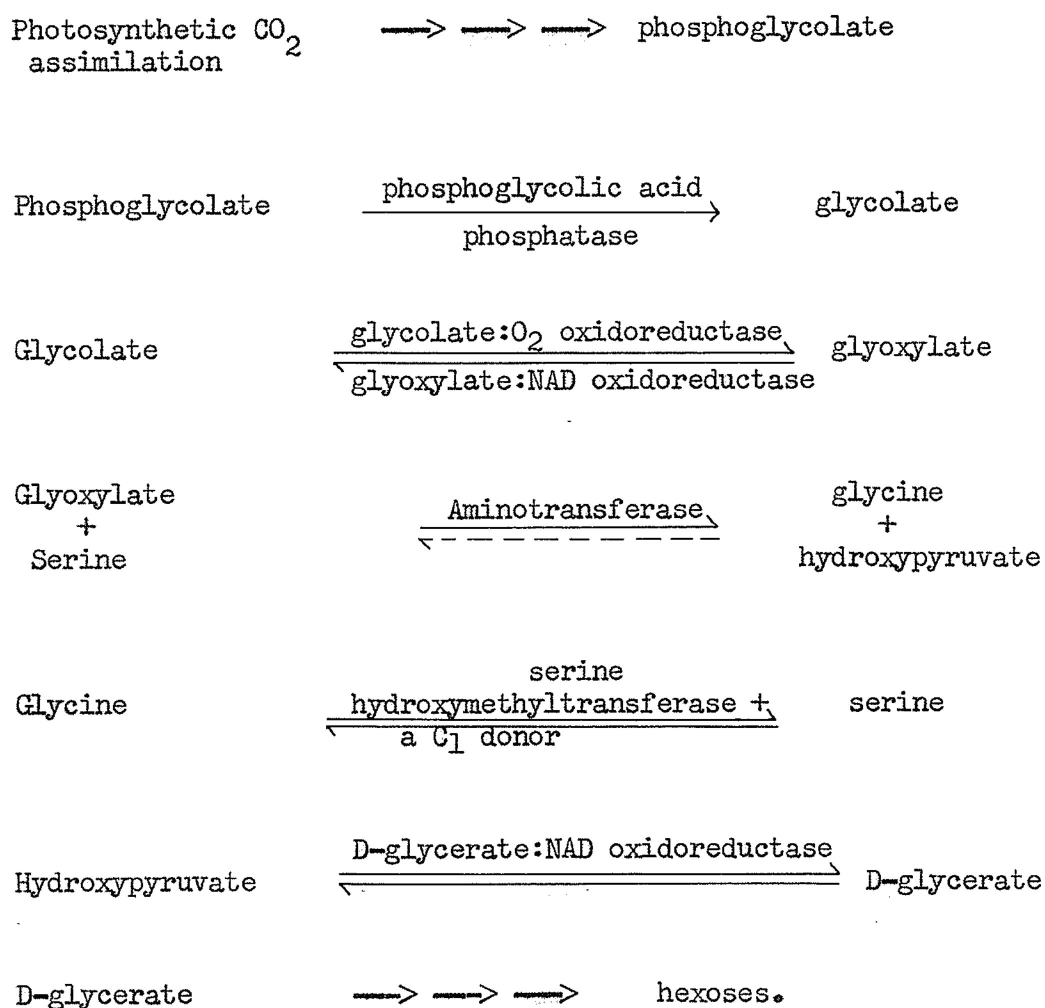
glycine:serine interconversion in wheat leaves (98), but also to assess the role of other aminotransferases in glycine metabolism. Glyoxylate:aminotransferases and particularly serine:glyoxylate aminotransferase, were shown to have some unusual properties apparently not shared by other aminotransferases.

II. LITERATURE REVIEW

1. THE GLYOXYLATE-SERINE PATHWAY

In 1962 Wang and Waygood (98), after investigating the conversion of glycine to sugars in wheat leaves, proposed a scheme for the conversion of photosynthetic CO_2 assimilation products to sugars which they called the "glyoxylate-serine pathway". They noticed the conversion of glyoxylic-1, 2-C^{14} acid to sugars in wheat leaves was drastically curtailed by the presence of non-radioactive glycine or serine. Non-radioactive serine also lowered the synthesis of sugars from glycine-2-C^{14} but the reverse was not so. They showed the effect of non-radioactive glycolate and glyoxylate on the formation of sugars from glycine-2-C^{14}. The presence of either of these compounds should have caused a reduction of sugar formation from glycine if the latter was metabolised to carbohydrates by a reversal of the reaction scheme of Weissbach and Horecker (98,102). On the contrary, both enhanced the rate of flow of isotope. In addition, the intramolecular distribution of isotope in the glucose moiety of sucrose after wheat leaves had been fed with glycine-1-C^{14} and -2-C^{14} was consistent with the idea that glucose was synthesised by the condensation of two three-carbon compounds derived from serine. In the same year, a similar pathway was reported by Rabson et al (71) to be operating in a wide variety of plant species. These workers determined the distribution of C^{14} in phosphoglyceric and glyceric acids, alanine and serine from five different plant species photosynthesising for 20 seconds in a $C^{14}O_2$ atmosphere. In each experiment the first three compounds above were predominantly carboxyl-labelled but serine was uniformally-labelled. They concluded

that serine biosynthesis proceeded by a different route than that for the other C_3 compounds. Results of additional experiments with other C^{14}-labelled isotopes were consistent with what Rabson et al (71) called a "glycolate pathway", essentially similar to Wang and Waygood's (98) "glyoxylate serine pathway" which may be summarised as follows:



More recent work by Wang and Burris (96) and Jimenez et al (31) has confirmed the existence of this pathway. Wang and Burris

(96) found that when glyoxylate-2-C^{14} was fed to wheat leaves it was converted rapidly to glycine and serine. Serine was formed rapidly from glycine and possessed 39% of the total radioactivity in the amino acid fraction after 10 minutes whereas glycine contained only 25%. In contrast, little glycine was formed from serine-1-C^{14} and possessed only 1.3%, 0.5% or 0.7% of the total radioactivity in amino acids after 10, 25 or 45 minutes, respectively. These data indicated a flow of plant products from glyoxylate to serine rather than in the reverse direction thus agreeing with the previously proposed pathway of glyoxylate-serine metabolism (98). Jimenez et al (31) followed the incorporation of C^{14} into sucrose from glycolate-C^{14} and serine-3-C^{14}. The latter compound was converted by 10 day-old wheat leaves in the light to sucrose labelled predominantly in C_1 and C_6. Glycolate-2-C^{14} gave rise to sucrose with C^{14} in C_1, C_2, C_5 and C_6 and after glycolate-1-C^{14} feeding to soybean leaves, sucrose was labelled in C_3 and C_4.

The formulation of the glyoxylate-serine pathway was based not only on reports from the laboratories already mentioned but also on information in numerous publications since 1950. Schou et al (77) found that when Scenedesmus cells were allowed to assimilate glycolate-2-C^{14} the major products of 6-10 minute dark anaerobic metabolism were glycine and serine. Conversion to serine in the light was much increased as was the ratio between serine and glycine. Other early experiments with C^{14}-labelled glycolate also showed serine and glycine to be intermediates in a glyoxylate-serine pathway. When α-labelled glycolate was

fed to Pawnee wheat leaves in the light, the glycine formed was also labelled in the α-carbon (86).

By 1952 this apparent connection between glycolate and its products and photosynthesis was confirmed (4,5). More recently Nelson and Krotkov (62) reported glycine as one of the earliest compounds to become radioactive when $C^{14}O_2$ was fed to leaves in the light. In rather different experiments Tolbert and Zill (87) demonstrated 3-10% of the total $C^{14}O_2$ fixed by Chlorella cells in 2-30 minutes of steady-state photosynthesis as glycolate excreted into the algal medium. Similarly, Warburg and Krippahl (99) reported that Chlorella during a ten minute period of photosynthesis, converted 92% of fixed CO_2 into glycolate. Extending the excretion experiments to whole spinach chloroplasts Kearney and Tolbert (33) allowed chloroplasts to fix $C^{14}O_2$ for 10 minutes in the light into products of the photosynthetic carbon cycle and found a small amount of C^{14} incorporated into glycolate. In 1962 Pritchard et al (69,104) published evidence showing glycolate production by Chlorella cells to be at a maximum at more natural (below 0.4%) CO_2 concentrations.

Mortimer (56) fed cyanide to photosynthesising sugar beet leaves and reasoned that if phosphoglyceric acid was reduced to glyceraldehyde-3-phosphate as postulated by Calvin in his photosynthesis carbon cycle, then cyanide should form a glyceraldehyde cyanhydrin and prevent hexose formation. Mortimer found no evidence

of cyanhydrin formation in the presence of cyanide and sucrose was still formed. These and other experiments, in which he followed the distribution of C^{14} amongst the products of 30 second and 5 minute photosynthesis in sugar beet leaves and found substantial amounts of C^{14} in serine, glycine and glyceric acid (57), led Mortimer to suspect an alternative pathway for carbon assimilation in some plant tissues.

McConnell and Finlayson (45) concluded that serine was an excellent precursor of carbohydrate in wheat plants since more than one half of the total radioactivity derived from L-serine-3-C^{14} in wheat kernels was found in starch.

When Nicotiana rustica (26) leaves were infiltrated with D-ribose-1-C^{14}, C^{14} was incorporated into glycolate, glycine, serine and alanine. Illumination of the leaves during the metabolising period increased the total amount of C^{14} incorporated into these compounds. The carbon isotope was found principally in the α-carbon of glycolate and glycine and in the α- and β-carbons of serine which suggested that the 1- and 2-carbons of ribose gave rise to glycolate. Other laboratories (84,110) also report ribose to be a precursor of glycine. All results are consistent with cleavage between C_2 and C_3 of a keto sugar phosphate so that carbon-2 of the sugar becomes the carboxyl group of glycolate. Phosphoglycolate could conceivably arise from such a keto sugar in this way and is known to be a precursor of glycolate (72,84). Experiments by Dickens and Williamson (20)

showed that the rat *in vivo* and rat liver tissue *in vitro* readily incorporated all three carbon atoms of β-hydroxypyruvate into the glucose molecule, the evidence suggesting that this occurred symmetrically by combination of two unbroken C_3 chains in a fairly direct route not involving L-serine or pyruvate as intermediates but possibly via D-glyceric acid and 3-phosphoglyceric acid.

A number of inhibition studies of the glyoxylate-serine pathway have added to the evidence for the pathway's existence. When either α-hydroxy sulfonates, which inhibit glycolate oxidase (109, 110) or isonicotinyl hydrazide (isoniazid), an inhibitor of aminotransferases (1,70) was added during *in vivo* photosynthesis experiments with $C^{14}O_2$, a more than 50% accumulation of C^{14} in glycolate products resulted in 10 minutes. According to Zelitch (109,110) and Pritchard *et al* (70) this accumulation occurred without a decrease in the rate of CO_2 fixation although Asada and Kasai (1) observed over 70% inhibition of photosynthesis by 0.01 M α-hydroxysulfonates in their experiments.

Although a good deal of evidence has accumulated for the existence of the glyoxylate-serine pathway there are some reports from some laboratories that must be considered and that may not be explained by the pathway as currently formulated. Mortimer's (58) results from iodoacetate inhibition studies of the transformation of phosphoglyceric acid to glyceraldehyde-3-phosphate were consistent

with the existence of an alternative pathway leading to sugars but did not agree with the proposed glyoxylate pathway. Mortimer concluded from his experiments (58) that glycine and serine biosyntheses were independent of one another. Furthermore, Towers and Mortimer (89) isolated keto acids from leaves of sugar beet and other plants exposed to $C^{14}O_2$ and found only pyruvate to be labelled in light periods up to 45 sec. Radioactive hydroxypyruvate was not detected. In contrast, glycine and serine were labelled after five seconds. The significance of the pathway found in animal tissues, notably by Ichihara and Greenberg (30), involving the production of serine from sugars via phosphohydroxypyruvate and phosphoserine is not understood in plant metabolism although Hanford and Davies (27) showed the formation of phosphoserine from D-3-phosphoglycerate with an enzyme from pea epicotyls and Wang and Burris (96) noticed a rapid labelling of phosphoserine when either glycolate-2-C^{14}, glycine-2-C^{14} or serine-1- or -2-C^{14} was fed to wheat leaves. Decisions regarding the possible participation of these compounds in the glyoxylate-serine pathway, as intermediates, must await further investigation. Finally, Milhaud et al (53) during experiments with algae found carboxyl-labelled glycolate produced copiously (85%) from hydroxypyruvate-2-C^{14} in accord with a decarboxylation by transketolase. The rather slow, observed formation of carbohydrates from hydroxypyruvate indicated that its aerobic metabolism by algae was largely oxidative and that it was unlikely to be a normal intermediate of carbohydrate biosynthesis (53).

2. ENZYMES OF THE GLYOXYLATE-SERINE PATHWAY

Richardson and Tolbert (72) isolated and partially purified a phosphoglycolate phosphatase from tobacco leaves. A similar enzyme was reported by Ullrich (90) to be active in spinach leaves. This enzyme has since been reported to be ubiquitous in the green parts of plants and apparently is not found in roots or etiolated leaves (84). Zelitch and Ochoa (112) isolated a glycolate:O_2 oxido-reductase from spinach leaves in a highly purified form and also a glyoxylate:NAD oxidoreductase from spinach leaves (113) an enzyme later crystallised by Zelitch (108) from tobacco leaves. Both of these glyoxylate oxidoreductases were NAD-dependent and a further NADP-dependent glyoxylate oxidoreductase has been isolated from both tobacco and spinach leaves by Zelitch and Gotto (111). For some time a D-glycerate:NAD oxidoreductase has been known from higher plant tissues capable of catalysing the reduction of hydroxypyruvate to D-glycerate. This enzyme was particularly active in green leaves (82).

The most puzzling step in the glyoxylate-serine pathway is glycine-serine interconversion. In mammalian tissues (78) and micro-organisms (9) glycine can be oxidised to CO_2 and formate through glyoxylate. However, there has been no proof that such a series of events takes place in plant tissues. On the contrary, Wang and Burris (96) showed clearly that glycine-2-C^{14} was converted rapidly to serine *in vivo* in wheat leaves and gave rise to highly radioactive glycerate but only slightly radioactive glycolate. The extent of conversion of

the α-carbon of glycine to the β-carbon of serine was reflected in the amount of C^{14} in carbons 1 and 6 of glucose formed from glycine-2-C^{14} (98). In animal tissues a direct cleavage of glycine to give CO_2 and formate was shown. Siekevitz and Greenberg (78) found rat liver slices when incubated with variously C^{14}-labelled glycine gave rise to serine. They reported the carboxyl-carbon of glycine to be metabolised probably to CO_2 whilst the α-carbon appeared as formate which then condensed with another glycine to give serine. The following year Kisliuk and Sakami (34) found the conversion of glycine-C^{14} to serine-C^{14} stimulated by the addition of tetrahydrofolate (THFA) in pigeon liver extracts, which evidence supported the hypothesis that the interconversion of serine and glycine was catalysed by a single enzyme, serine hydroxymethyltransferase. A similar active reversible THFA dependent enzyme was demonstrated by Wilkinson and Davies (105) in turnip tissue extracts. McConnell and Findlayson (45) indicated wheat plants also effected this conversion and more recently Cossins and Sinha (17) showed both carrot roots and sunflower cotyledons readily produced formate-C^{14} when incubated with glycine-2-C^{14}. Tolbert (83) found formate to be rapidly taken up into the β-carbon of serine in barley leaves. Wang and Burris (96) reported in their experiments with wheat leaves, little glycine to be formed from serine-1-C^{14} and suggested glycine was synthesised by one metabolic pathway and oxidised by another. It has been suggested (98) serine-glycine interconversion could also be effected by an aminotransferase with an equilibrium favoring glycine formation (see diagram of glyoxylate-

serine pathway in section 1 of this review.

3. AMINOTRANSFERASE REACTIONS INVOLVING GLYOXYLIC ACID

Aminotransferase reactions involving glyoxylate have been greatly neglected and only a few reports are available.

(a) Non-enzymatic reactions

Nakada and Weinhouse (61) observed a rapid non-enzymic conversion of glyoxylate to glycine in the presence of various amino acids and amides. Fleming and Crosbie (24,25) found a non-enzymic aminotransfer between glycine-C^{14} and glyoxylate proceeding at 37° and pH 7.0. This reaction was catalysed by Cu^{2+} ions and inhibited by EDTA and pyridoxal phosphate. Chromatographic evidence for β-hydroxyaspartate formation was obtained and in the presence of pyridoxal phosphate, hydroxypyruvate was also formed. Mix (55) reacted glyoxylate with a number of amino acids using cuprous ions and pyridine as catalysts. When serine and glyoxylate were reacted together about 27% of the serine was converted to glycine. Pyridoxamine (52) was shown to undergo rapid aminotransfer with glyoxylate in aqueous solutions at pH 4.5 and $79-100^{o}$ to yield pyridoxal and glycine.

(b) Enzymatic reactions

As reported in the previous sub-section, glyoxylate is capable of participating in non-enzymic aminotransfer reactions independently of pyridoxal phosphate although glyoxylate can accept an amino group

from pyridoxamine (52). Meister (48) considers it conceivable that aminotransferases involving glyoxylate are not dependent on the presence of pyridoxal phosphate. With respect to this point, reports in the literature are conflicting. Wilson et al (107) isolated a glutamic-glycine aminotransferase from tobacco leaves which showed activity only in the presence of pyridoxal phosphate. In his investigations with Pseudomonas extracts, Campbell (9) found an aminotransferase reaction between glyoxylate and a number of amino acids which seemed to require pyridoxal phosphate. Nakada (60) purified a glutamate-glycine enzyme from rat liver and although he could not confirm the presence of pyridoxal - or pyridoxamine phosphate in the enzyme preparation, he inferred from inhibition data that a vitamin B_6 co-enzyme was present. None of these reports have furnished unequivocal evidence for a pyridoxal phosphate requirement and indeed there are reports of more searching but unsuccessful attempts to isolate pyridoxal derivatives from glyoxylate aminotransferases.

Sastry and Ramakrishnan (75) isolated and purified a glutamic-glycine enzyme from 'green gram' (Phaseolus radiatus) which when inhibited by isonicotinyl hydrazide (isoniazid) or L-penicillamine, not only was reactived by their commercially obtained pyridoxal phosphate but also by Mn^{2+} ions alone. McCurdy and Cantino (46) studied an alanine-glycine aminotransfer in extracts from the fungus Blastocladiella emersonii. The purified enzyme had a pH optimum at 8.5, proceeded

in the forward direction to only one-third completion and yet did not exhibit true reversibility. No treatment could induce stimulation by pyridoxal phosphate except that hydroxylamine inhibition could be overcome by addition of that compound. In their experiments with pea leaves Cossins and Sinha (15) demonstrated a transamination involving glyoxylate-C^{14} and various amino acids. The enzyme had pH optima at 5.6 and 8.6 and was reversible but the equilibrium favored glycine formation. In a more recent communication these workers (16) concluded that the enzyme is cytoplasmic in origin and could be strongly inhibited by hydroxylamine, which inhibition could not be reversed by pyridoxal phosphate.

A number of other reports of glyoxylate aminotransferases do not mention pyridoxal phosphate. Metzler *et al* (52) observed a reaction between serine and glyoxylate to yield glycine and hydroxypyruvate. The equilibrium of this enzyme favoured glycine formation to such an extent that any reverse reaction was not observable. Two reports by Meisrer (47,50) recorded aspartate, glutamate- and ornithine-glyoxylate aminotransferases from rat liver, the last of these unique in that an aldehyde was both a reactant and a product.

4. CONCLUSION

Glycolate was shown to be rapidly metabolised to sugars (96,98) and because it was also found to be excreted from chloroplasts (33), it may be that the glyoxylate-serine pathway constitutes a link between

the photosynthetic carbon cycle of chloroplasts and the synthetic pathways of the cytoplasm (84). Moses and Calvin (59) followed the incorporation of tritium from tritium oxide into Chlorella cells in the light and in the dark. Glycolate was labelled after five seconds in the light and this suggested an early role for 2-carbon compounds in photosynthesis, possibly in hydrogen metabolism, i.e., carrying hydrogen from photochemically reduced pyridine nucleotides, possibly to phosphoglycerate. Glycolate may indeed be a carrier across membranes within the cell as well as being an intermediate in a pathway of sugar biosynthesis.

Glycolate synthesis during photosynthesis requires normal CO_2 (69,104) and O_2 (103) concentrations and under such conditions Whittingham et al (103) cite evidence consistent with the light production of 2-carbon compounds from glucose which may be subsequently metabolised via serine to give either alanine in the dark (6,106) or sucrose in the light (84,98,103). The production of glycolate seems to be from a sugar phosphate of the photosynthetic carbon cycle. Bassham et al (3) demonstrated that carbons 1 and 2 of ribulose diphosphate, hexose phosphate and sedoheptulose phosphate were uniformly labelled and could thus give rise to a uniformly labelled C_2 moiety. These results and those of Whittingham et al (103) could be modelled after a transketolase reaction where a free C_2 piece would be liberated rather than transferred. Orth and Tolbert (in 84) reported

fast labelling of glycolate in algae when photosynthesis was performed in the absence of orthophosphate or phosphoglycolate but encountered considerable inhibition of glycolate formation in the presence of either of these compounds, results which suggest some phosphorylated intermediate between photosynthetic assimilation products and glycolate. From the literature cited, however, it can be seen that many qualitative and quantitative aspects of the metabolism of this key compound, glycolic acid and hence also of the glyoxylate-serine pathway, are still unresolved.

III. MATERIALS AND METHODS

1. GENERAL

Serine, glycine and sodium glyoxylate were purchased from the Sigma Chemical Co., St. Louis, Missouri, pyridoxal-5-phosphate and Li-β-hydroxypyruvate from Nutritional Biochemicals Corporation, Cleveland, Ohio, glycine-2-C^{14} (0.1 mc/0.93 mg) from the United Kingdom Atomic Energy Authorities, Amersham, England and glyoxylate: NAD oxidoreductase from Calbiochem, Los Angeles, California. Hydroxy-aspartic acid was a gift from Dr. H. J. Sallach, Department of Physiological Chemistry, University of Wisconsin Medical School. Both labelled and unlabelled chemical compounds were used without further purification. No evidence of contamination by other compounds was found during experiments using the chemicals listed above.

2. CULTIVATION AND LIGHT TREATMENT OF WHEAT LEAVES

First leaves of Khapli (*Triticum dicoccum* Schubler) wheat were grown under greenhouse conditions for seven to ten days depending on the season, excised at their bases with a clean razor blade and their cut ends placed immediately into distilled water. Healthy unmarked leaves were floated on either distilled water or 100 mg per litre benzimidazole in glass trays for either three or five days. The trays were covered with "Saran Wrap" punctured to allow air circulation. The leaves were placed in a growth chamber (21°) and subjected to various periods of light (1000 ft-c) and dark. Four different photoperiods were used, namely (a) continuous darkness, (b) eight hours, (c) sixteen hours and

(d) continuous light. Leaves treated thus were compared with immediately detached leaves.

3. INCUBATION OF WHEAT LEAVES WITH GLYCINE-2-C^{14} AND EXTRACTION OF ALCOHOL-SOLUBLE SUBSTANCES

The primary leaves of Khapli wheat, either immediately detached or after having been floated on water or benzimidazole,were cut into equal four inch lengths and 10 leaves placed in each of two small glass vials along with 0.1 ml (1µc) of glycine-2-C^{14}. The vials were then transferred to a growth chamber (21°) and incubated in the light (1000 ft-c) for three hours during which time water was added to the liquid in the vials to replace that absorbed by the leaves. During the three hour incubation period all of the radioactivity was taken up by the leaves. After incubation the leaves were removed from the vials, surface dried and their fresh weights determined. The leaves were then cut into 1/4 inch lengths and killed in 95% ethanol. The tissue sections were further extracted twice with boiling 80% ethanol and once with 40% ethanol for thirty minutes each time under reflux condensation. All extracts were combined and evaporated to dryness to remove ethanol. The dried residue was then partitioned between chloroform and water. Care was taken to facilitate complete removal of chloroform-soluble pigments from the water-soluble substances. Radioactivity in both fractions was then quantiatively determined.

4. ION-EXCHANGE FRACTIONATION

The water-soluble substances were further fractionated, using

Dowex 50W-X8 (H^+) and Dowex-1-X10 ($HCOO^-$) resins (94) into neutral (sugars), basic (amino acids) and acidic (organic acids) fractions. All three fractions were evaporated to dryness and the residues were taken up in 1 ml of 40% ethanol. The radioactive content of each fraction was determined quantitatively. With some minor differences, all of these methods were used previously by Wang and Waygood (98).

5. THE SEPARATION AND QUANTITATIVE ESTIMATION OF SUGARS

(a) Quantitative estimation of radioactivity in each sugar

Aliquots (100-200 µl) of the sugar-containing neutral fractions from the Dowex columns were used for one-dimensional ascending chromatography. Mono- and disaccharides were separated from one another by one-dimensional ascending chromatography on unwashed Whatman No.1 paper strip (one inch wide) with n-butanol:acetic acid:water (4:1:5 v/v) (66) as the developing solvent. In order to obtain a good separation of the sugars under investigation, three developments were required each of eighteen hours duration, drying the chromatogram papers between developments (95). Radioautographs were prepared in the conventional manner by superimposing X-ray film upon each chromatogram and with an exposure period of one to two weeks. The individual sugars were located on the chromatograms by comparison with the radioautographs and each sugar was quantitatively eluted from the filter paper in the following manner.

Radioactive areas of the filter papers were carefully cut out and elution of substances carried out overnight in test-tubes with five ml of 40% ethanol at 4°. The eluate was removed and replaced by a further five ml aliquot of 40% ethanol. A second elution was then carried out at 40° for three hours. This second eluate was removed and the residual filter-paper washed thoroughly with 40% ethanol. All three alcohol eluates were combined, evaporated to dryness, and taken up in one ml of water and characterised by co-chromatography with known sugars in an ascending manner using n-butanol:pyridine:water (2:1:2 v/v) (95) as solvent. The majority of the fractions were found to consist of single sugars. Quantitative estimation of the radioactivity contained in these fractions was carried out.

(b) Quantitative estimation of sugars using the Anthrone Sugar Test

Five millilitres of anthrone reagent (0.2% anthrone in H_2SO_4) were pipetted into test-tubes kept at 10-15°. With extreme care this solution was overlaid by a 2.5 ml solution containing between 25 and 100 µg of a sugar. The two layers were then mixed quickly in the cold and the test tubes transferred to a water bath at 90° for 16 minutes. After incubation the tubes were cooled and the color determined at 625 mµ against a blank containing only the anthrone reagent treated in the same manner as the sample tube. The large number of samples made it necessary to carry out these estimations over an extended period of time. Accordingly the anthrone reagent was prepared each day and

standardized against known sugar solutions. The three sugars under investigation, namely fructose, glucose and sucrose, were each found to give an almost exactly similar color with the anthrone reagent, between 10 and 150 µg concentrations.

From the quantitative measurements carried out in (a) and (b) above specific radioactivities of each sugar were determined.

6. ISOLATION (100) AND ASSAY OF INVERTASE

Twenty grams of Khapli wheat leaves were macerated in a mortar with acid-washed quartz sand and 20 ml of 0.2 M Na_2HPO_4-citric acid buffer, pH 4.5. After centrifugation at 30,000 x g for 10 minutes the supernatant fluid was dialysed overnight against a 0.02 M Na_2HPO_4-citric acid buffer, pH 4.5. The protein precipitated during dialysis was removed by centrifugation and the supernatant fluid diluted to 50 ml with cold distilled water. The invertase activity in this solution was assayed as follows:

Twenty ml of buffer, containing 8.08 ml of 0.2 M Na_2HPO_4 and 11.92 ml of 0.01 M citric acid, to give a pH of 4.5; 5 ml of 10% sucrose and 5 ml of enzyme were incubated at room temperature (25°) for one hour. A one ml aliquot was removed and added to 1 ml of 4% $HClO_4$ and immediately 0.4 g of $KHCO_3$ was added to the mixture and the resulting potassium perchlorate removed by centrifugation. To a one ml aliquot of the supernatant fluid after centrifugation was added one ml of reagent

A + B (25:1 v/v) (63). After thoroughly mixing the reagent and sample were heated for 20 minutes in a boiling water bath and cooled. With rapid mixing, one ml of arsenomolybdate reagent (63) was added, the whole made to 25 ml with distilled water and the color determined at 660 mμ. The composition of the reagents used in this modified Somogyi test were as follows (63):

Copper reagent A:

25 g anhydrous Na_2CO_3

25 g Rochelle salt (sodium potassium tartrate)

200 g anhydrous Na_2SO_4

Dissolved in 800 ml of warm water, cooled, filtered and diluted to one litre.

Copper reagent B:

15% $CuSO_4 \cdot 5H_2O$ + 1-2 drops H_2SO_4 per 100 ml

Arsenomolybdate reagent:

To 25 g ammonium molybdate in 450 ml water was added 21 ml H_2SO_4 with mixing. Three grammes of $Na_2HAsO_4 \cdot 7H_2O$ dissolved in 25 ml water was then added and the mixture incubated at 55° for 25 minutes with stirring.

7. EXTRACTION OF SOME AMINOTRANSFERASES FROM WHEAT LEAVES

(a) Extraction of crude enzyme

About 10 g of nine-day old Selkirk (*Triticum aestivum*, L.) wheat leaves were ground in a chilled mortar with acid-washed quartz sand and

an equal volume (w/v) of 0.2 M KH_2PO_4-K_2HPO_4 buffer, pH 5.7 or pH 7.0 or pH 8.0 for 15 minutes. The brei was pressed through four layers of muslin and the filtrate centrifuged at 1,000 x g for five minutes to remove cell debris. The supernatant fluid was then dialysed against a large volume of distilled water overnight at 4°. This dialysed extract is referred to as "crude enzyme".

(b) Fractionation with ammonium sulphate

To the crude enzyme preparation, extracted at pH 7.0 and before final dialysis, solid ammonium sulphate was added to a concentration of 20% (w/v). After 30 minutes stirring at 4° the protein precipitate was removed by centrifugation for 10 minutes at 20,000 x g. The ammonium sulphate concentration in the supernatant fluid was raised to 40 (w/v) and after a further 30 minutes stirring the protein precipitate was collected by centrifugation at 20,000 x g, suspended in a small volume of 0.2 M phosphate buffer, pH 7.0, and dialysed overnight against 0.002 M phosphate buffer, pH 7.0 at 4°. The resulting protein solution is referred to as "partially purified preparation".

(c) Fractionation on DEAE-cellulose columns

A partially purified enzyme preparation, extracted as outlined above, but suspending the protein finally in 0.05 M phosphate buffer pH 7.5, was dialysed overnight against 0.002 M phosphate buffer, pH 7.5. The resulting dialysed solution was quickly frozen and thawed

twice, centrifuged at 30,000 x g for 15 minutes and freeze-dried. The residue was taken up in a small volume of 0.05 M phosphate buffer, pH 7.5, and added to a chromatography column (measuring 80 cm x 1 cm) containing DEAE-cellulose equilibrated with 0.1 M phosphate buffer, pH 8.2. The protein was removed from the column in 2.5 ml fractions with 1.0 M phosphate buffer, pH 8.2. Those fractions containing serine-glycine aminotransferase activity, the "purified enzyme preparation", were combined and could be stored in a frozen state for some weeks.

In most experiments either the crude enzyme or the partially purified preparation was used.

8. ASSAY SYSTEMS

(a) Qualitative estimation of amino acids

In early experiments when a number of aminotransferases were being isolated from wheat leaves, a "crude enzyme" preparation only was used in the following assay system: 0.5 ml (100 µmoles) KH_2PO_4-K_2HPO_4 buffer, pH 5.7, 7.0 or 8.0; 0.1 ml (5 µmoles) amino group donor (L-isomer); 0.1 ml (5 µmoles) amino group acceptor, 0.013-0.13 µmoles pyridoxal-5-phosphate and 0.5 ml of enzyme in a total volume of 1.3 ml.

The reaction mixtures, with or without pyridoxal phosphate and boiled enzyme controls were incubated for 30-60 minutes at 30° and the reaction stopped by the addition of 0.2 ml of 5.4, 6.2 or 7.2% trichloroacetic acid at pH 5.7, 7.0 and 8.0, respectively. The clear

supernatant fluids obtained after standing were subjected to chromatographic analysis.

Five microlitres of the assay mixtures were applied to Whatman No.1 filter papers (23.1 x 28.6 cm) and chromatographed in phenol: water (4:1 v/v) solvent containing 0.004% 8-hydroxyquinoline for 16 hours at 20-22°. Chromatograms were dried in air for 3 hours and each was sprayed with 10 ml of 0.2% ninhydrin in 95% ethanol and placed in an oven, saturated with ethanol vapor, for one hour at 39-40°. After the ninhydrin spots had developed maximum color (16-40 hr at room temperature) intensities of the various spots were compared qualitatively on the filter paper.

(b) Quantitative estimation of amino acids

Serine-glyoxylate aminotransferase was assayed by quantitatively measuring the appearance of the new amino acid on paper chromatograms (80). The reaction system usually contained 0.1 ml serine (15 µmoles), 0.1ml sodium glyoxylate (15 µmoles), 0.5 ml enzyme preparation, 0.5 ml phosphate buffer (100 µmoles), pH 7.0 or 8.2 and distilled water in a final volume of 1.5 ml. The reaction was normally carried out at 30° for 30 minutes and terminated by immersing the assay tubes in boiling water for 10 minutes. The precipitated proteins were removed by centrifugation and the amino acids, serine and glycine, in the supernatant fluid separated by one-dimensional ascending chromatography

on unwashed Whatman No.1 filter paper (23.0 x 28.0 cm) with phenol: water (4:1 v/v) containing 0.004% hydroxyquinoline as solvent. The amino acids were then quantitatively estimated according to the method of Smith and Waygood (80). The amino acids were located on chromatogram paper by spraying with 1% ninhydrin in 95% ethanol. The paper was then heated to 60° for 30 minutes in an ethanol saturated atmosphere and allowed to stand at room temperature for two hours. The individual colored spots were identified by their known R_f values and cut out from the chromatogram paper. Removal of the colored compounds from each spot was effected by elution with three ml of 50% ethanol containing 0.025 M phosphate buffer pH 6.5 in small test tubes. The tubes were sealed with parafilm and stored overnight at 3° to allow complete elution of the color. The tubes were removed to room temperature one hour before determination of the absorbancy of the eluted color in a spectrophotometer at 570 mμ. Background corrections were made by cutting a comparable section of the paper from an amino acid free area and eluting as before. The procedure was calibrated with authentic samples of serine and glycine.

(c) Quantitative estimation of β-hydroxypyruvate

Glycine is only one product of the forward reaction catalysed by serine-glyoxylate aminotransferase from wheat leaves. The other is hydroxypyruvate which can be assayed spectrophotometrically using D-glycerate:NAD oxidoreductase, found by Stafford *et al* to be very

active in green leaves (82). However, sodium glyoxylate is also present in the assay mixture and glyoxylate:NAD oxidoreductase has been found also in green leaves (108). Difficulties could then have arisen in any hydroxypyruvate assay but later results will show that any such difficulties were overcome (see Experimental Results).

The assay was carried out in two parts:

(i) A reaction system was set up as in (b) above and the reaction was terminated after 30 minutes by the addition of 0.2 ml of 4N $HClO_4$. After removal of the precipitated protein and $KClO_4$, a one ml aliquot of the reaction mixture was transferred to a two ml, volumetric flask and the pH of the solution raised to 7.0 with the addition of ca 0.4 ml of 2N KOH. The neutralised solution was then made up to two ml with distilled water.

(ii) The level of hydroxypyruvate in this neutralised solution was determined as follows: Into a three ml silica cuvette was pipetted 0.5 ml of the unknown solution; 0.02 ml partially purified enzyme preparation (having D-glycerate:NAD oxidoreductase activity), 0.5 ml phosphate buffer (150 µmoles), pH 7.0; 0.04 ml NADH (a solution containing ca 8 mg NADH/ml) and distilled water in a final volume of three ml. The reference cell contained all components excepting NADH. The oxidation of NADH was followed at 340 mµ until equilibrium was attained. From the total drop in absorbancy at 340 mµ the amount of hydroxypyruvate produced in the aminotransferase reaction was estimated

assuming that one mole of hydroxypyruvate oxidises 1 mole of NADH and knowing that the equilibrium of the enzyme D-glycerate:NAD oxido-reductase favors hydroxypyruvate reduction (82). The molar extinction coefficient of NADH was taken as 6.22×10^3 (7).

9. PROTEIN ESTIMATIONS

In preliminary experiments with crude enzyme preparations, protein concentration were calculated (N x 6.25) from determinations of total nitrogen by a micro-Kjeldahl procedure (36) using peroxide digestion. Ammonia was distilled over by 13N NaOH into the boric acid reagent containing a modified Tashiro's mixed indicator (14) followed by titration with 0.01N HCl.

During investigations of a serine-glyoxylate aminotransferase the method of protein estimation used was similar to that described by Lowry _et al_ (43). Five or ten microlitres of enzyme solution were added to a Bausch and Lomb 'Spectronic 20' cuvette along with 0.5 ml of 0.1N NaOH followed by 2.5 ml of a reagent containing sodium carbonate, copper sulphate and sodium tartrate. The latter reagent was prepared by mixing 50 ml of a 2% Na_2CO_3 solution with one ml of a solution containing 0.5% $CuSO_4 \cdot 5H_2O$ in one percent sodium tartrate. After 10 minutes 0.5 ml of a phenol reagent, containing commercial phenol reagent (Fisher Scientific Co.,) diluted 1:1 with distilled water was rapidly added to the cuvette. Mixing was effected within five seconds. After 30 minutes the color formed was read at 500 mμ using a Bausch and Lomb 'Spectronic 20' colori-

meter. Abosrbancy levels were compared with curves of known amounts of a pure egg albumin solution.

10. QUALITATIVE TESTS OF KETO ACIDS BY PAPER CHROMATOGRAPHY

(a) Extraction of keto acids

Keto acids were separated and identified by paper chromatography of their 2,4-dinitrophenylhydrazones according to the method of Towers and Mortimer (89) as adopted by Wang (93). The aminotransferase assay (system b, section 8 above) was stopped by the addition of one ml of 0.14% 2,4-dinitrophenylhydrazone (dissolved in 2N HCl) to convert keto acids to the more stable phenylhydrazones. The protein precipitate was removed by centrifugation and discarded. The supernatant fluid was diluted with approximately 10 ml of water and extracted twice in a separatory funnel with 25 ml ethylacetate. These extracts were combined and washed with water and the alkali soluble 2,4-dinitrophenylhydrazones then extracted twice with 25 ml of one percent sodium carbonate. The alkaline extracts (lower phase) were combined and immediately adjusted to approximately pH 2.0 with one ml HCl, washed with water and re-extracted twice with 25 ml ethylacetate. After washing with water the ethylacetate extracts were evaporated to dryness at room temperature and the residue redissolved in one ml of 95% ethanol.

(b) Assay of keto acids

An aliquot (10 μlitre) of the extract was spotted together with known keto acid derivatives on Whatman No.1 filter paper and chromatographed overnight in an ascending manner using tert-amyl alcohol: ethanol: water (9:1:4 v/v) as solvent. A small beaker containing NH_4OH was kept in the chromatography chamber. The chromatograms were dried at room temperature and sprayed with 10% alcoholic sodium hydroxide The keto acid derivatives were identified by their characteristic colors and known R_f values.

11. DETECTION AND MEASURING DEVICES

Radioactivity in solution was quantitatively detected using a Phillips Electronic Counter, Model PW4035, fitted with an Amperex 200NB end-window counter tube. Radioactivity patterns on filter paper were permanently recorded on Kodak, No-screen X-ray film, 14x17 inches.

Colored compounds produced in the reaction between ninhydrin and amino acids, in the anthrone sugar test and in the measurement of invertase activity, were quantitatively determined using a Zeiss PMQII spectrophotometer set at 570 mμ, 625 mμ and 660 mμ respectively. Oxidation of NADH by hydroxypyruvate was followed quantitatively also using a Zeiss PMQII spectrophotometer, set at 340 mμ.

IV. EXPERIMENTAL RESULTS

The experiments carried out fall naturally into two sections and are presented under two overall headings. On the one hand was the investigation of glycine-2-C^{14} utilisation by wheat leaves and on the other the occurrence of aminotransferases in the same tissue. Whilst these two phenomena are in part presumably linked in cellular metabolism, no effort was made to assess the strength of the relationship experimentally.

1. THE EFFECT OF BENZIMIDAZOLE ON THE METABOLISM OF GLYCINE-2-C^{14} IN DETACHED KHAPLI WHEAT LEAVES

Wang and Hao (97) and Wang (95) found that detachment of Khapli wheat leaves caused a derangement in the metabolism of glycine-2-C^{14}. In leaves floated on water the rate of sugar formation from this amino acid was enhanced considerably and the synthesis of sucrose from glycine-2-C^{14} was greatly decreased. On the other hand, in leaves treated with benzimidazole the metabolism of glycine-2-C^{14} and the synthesis of sucrose were found to be comparable to those in immediately detached leaves. The work reported in this section was a continuation of these studies.

(a) Preliminary extraction of alcohol soluble substances

Exogenously supplied glycine-2-C^{14} was readily incorporated by detached Khapli wheat leaves (Table I). The preliminary results in

TABLE I. Fresh weights (g) of Khapli wheat leaf tissue used in feeding experiments with glycine-2-C^{14}

	Experiment No.							
	1	2	3	4	5	6	7	8
Immediately detached	1.26	1.25	1.11	1.05	1.19			
Continuous darkness	1.13	1.08	1.2	1.06	1.03	1.08	1.05	1.09
Eight hours light Sixteen hours darkness	1.18	1.25	1.45	1.34	1.25	1.26	1.3	1.38
Sixteen hours light Eight hours darkness	0.99	0.96	1.26	1.21	1.01	0.95	1.51	1.41
Continuous light	0.94	1.04	1.02	0.92	1.06	1.01	1.27	1.28

The numbers (1-8) correspond to those in Table II.

Table II indicate that a substantial amount of the glycine-2-C^{14} fed (1 µc) was recovered in water-soluble substances, the chloroform-soluble compounds accounting for only 5-15% of the total radioactivity extracted. Although the total amount of C^{14} activity recovered in the water-soluble fractions was greater in leaves floated on water or benzimidazole solution than in immediately detached leaves, there was no indication at this early stage whether benzimidazole was having any effect on the levels of substances in the various fractions. In both three and five day experiments the levels of radioactivity in water-soluble compounds were essentially similar in both benzimidazole treated and leaves floated on water. The activity in chloroform-soluble substances did fall more rapidly in leaves floated on water but not sharply.

(b) Fractionation of water soluble substances on Dowex resin columns

In all experiments and under any light conditions the greatest percentage of radioactivity was recovered in amino acid fractions (Table III). The amino acids were not analysed further. The average radioactivity in sugars in immediately detached leaves was approximately 33% of the total activity in the alcohol-soluble extracts (Table IV). When detached leaves were floated on water the rate of transformation of the carbon skeleton of glycine to free sugars decreased with time and appeared to reach a constant level after

TABLE II. The incorporation of glycine-2-C^{14} into leaves of Khapli wheat.

Treatment	Expt. No.	Water Soluble (cpm/fraction)		Chloroform soluble (cpm/fraction)
	1	148,000	(117,000)	17,000
	2	146,000	(115,000)	16,000
A	3	101,000	(91,000)	21,000
	4	100,000	(95,000)	21,000
	5	139,000	(117,000)	29,000
	1[1]	191,000	(169,000)	16,000
	2[1]	184,000	(170,000)	15,000
	3[2]	142,000	(120,000)	14,000
	4[2]	150,000	(120,000)	19,000
B	5[3]	153,000	(154,000)	7,000
	6[3]	163,000	(170,000)	7,000
	7[4]	144,000	(151,000)	10,000
	8[4]	163,000	(156,000)	12,000
	1[1]	181,000	(151,000)	14,000
	2[1]	184,000	(174,000)	16,000
	3[2]	170,000	(117,000)	14,000
C	4[2]	170,000	(127,000)	16,000
	5[3]	110,000	(87,000)	6,000
	6[3]	115,000	(95,000)	5,000
	7[4]	187,000	(184,000)	7,000
	8[4]	199,000	(216,000)	8,000

continued

TABLE II CONTINUED

Treatment	Expt. No.	Water Soluble (cpm/fraction)		Chloroform soluble (cpm/fraction)
D	1^1	175,000	(170,000)	10,000
	2^1	171,000	(158,000)	9,000
	3^2	170,000	(136,000)	11,000
	4^2	174,000	(138,000)	14,000
	5^3	191,000	(190,000)	17,000
	6^3	184,000	(194,000)	17,000
	7^4	131,000	(124,000)	6,000
	8^4	151,000	(150,000)	7,000
E	1^1	137,000	(130,000)	9,000
	2^1	143,000	(131,000)	10,000
	3^2	173,000	(133,000)	6,000
	4^2	173,000	(125,000)	6,000
	5^3	107,000	(71,000)	4,000
	6^3	146,000	(104,000)	3,000
	7^4	157,000	(124,000)	3,000
	8^4	157,000	(123,000)	3,000

Superscripts refer to experiments where leaves were floated on benzimidazole solution or water for twenty four hour cycles using conditions of continuous darkness (1), eight hours light and sixteen hours darkness (2); sixteen hours light and eight hours darkness (3); continuous light (4). Figures in brackets are cpm/fraction/g fr.wt. tissue.

Treatments A-E refer to experiments with wheat leaves immediately detached; floated for three or five days on benzimidazole solution and floated for three or five days on water, respectively.

TABLE III. Total radioactivity recovered from Dowex resin columns.

Treatment	Expt. No.	cpm/fraction Sugar fraction	Amino acid fraction	Organic acid fraction	% recovery of radioactivity
A	1	35,000	81,000	7,000	83
	2	51,000	66,000	8,000	86
	3	31,000	35,000	13,000	79
	4	27,000	34,000	12,000	73
	5	63,000	51,000	9,000	88
B	1[1]	32,000	114,000	9,000	81
	2[1]	25,000	115,000	9,000	81
	3[2]	11,000	90,000	11,000	79
	4[2]	13,000	121,000	12,000	97
	5[3]	8,000	109,000	11,000	84
	6[3]	14,000	99,000	12,000	77
	7[4]	27,000	93,000	13,000	92
	8[4]	28,000	80,000	14,000	75
C	1[1]	18,000	132,000	11,000	89
	2[1]	23,000	127,000	13,000	89
	3[2]	42,000	91,000	13,000	86
	4[2]	48,000	97,000	14,000	94
	5[3]	16,000	88,000	12,000	105
	6[3]	16,000	85,000	13,000	99
	7[4]	23,000	148,000	14,000	99
	8[4]	25,000	129,000	16,000	86
	1[1]	15,000	123,000	8,000	84
	2[1]	19,000	148,000	8,000	102
	3[2]	39,000	106,000	9,000	91

continued

TABLE III. CONTINUED

Treatment	Expt. No.	cpm/fraction Sugar fraction	Amino acid fraction	Organic acid fraction	% recovery of radioactivity
D	4[2]	47,000	106,000	8,000	93
	5[3]	14,000	157,000	11,000	95
	6[3]	18,000	131,000	10,000	87
	7[4]	20,000	100,000	6,000	96
	8[4]	26,000	107,000	7,000	93
	1[1]	16,000	99,000	7,000	89
	2[1]	18,000	106,000	8,000	92
	3[2]	32,000	110,000	8,000	87
E	4[2]	37,000	111,000	6,000	89
	5[3]	5,000	91,000	8,000	97
	6[3]	7,000	125,000	8,000	96
	7[4]	19,000	110,000	7,000	87
	8[4]	25,000	116,000	8,000	95

For an explanation of superscripts and the symbols A-E, see Table II.

TABLE IV. Formation of sugars, amino acids and organic acids from glycine-2-C^{14} in immediately detached Khapli wheat leaves.

Expt. No.	Radioactivity (cpm g fr wt tissue)					
	Sugars	%#	Amino Acids	%#	Organic Acids	%#
1	28,000	24	65,000	55	6,000	5.1
2	41,000	36	53,000	46	6,000	5.2
3	28,000	31.0	32,000	35	12,000	14
4	27,000	27	34,000	34	12,000	12
5	53,000	45	43,000	37	8,000	7

These figures represent the percentage of total radioactivity/ g fr wt tissue of the alcohol-soluble fraction (less chloroform-soluble substances).

three days (Tables III, IV and V). With a diurnal cycle of 16 hours light and 8 hours dark and to a lesser extent in an 8 hour light and 16 hour dark sequence, the level of radioactivity in sugars from leaves floated on water continued to decline up to the end of the five day experiment. In those leaves treated with benzimidazole the decrease in incorporation of glycine-2-C^{14} into sugars at the three day stage was greater even than the decreased incorporation in leaves floated on water in experiments carried out under alternating light and dark periods (Table V). After five days a reverse trend was observed, i.e., the control/benzimidazole ratio favored the latter. Under conditions of continuous light or darkness no significant fluctuations in this ratio occurred throughout the five day exposure period.

The metabolic derangement in detached leaves was not confined to the rate of transformation of glycine, the type of sugars labelled was also altered. In immediately detached leaves the carbon skeleton of glycine gave rise essentially to sucrose and to a much lesser extent to glucose and fructose especially in leaves grown under high light, summer, conditions in the greenhouse (Table VIII, treatment A, experiment 5 discussed more thoroughly later) (Fig.1). Leaves floated on water showed, qualitatively, a pronounced shift in isotopic distribution among the free sugars and the major part of the radioactivity was eventually found to reside in fructose and glucose rather than sucrose. On the other hand, leaves treated with benzimidazole gave

TABLE V. The effect of benzimidazole on the transformation of glycine-2-C^{14} to sugars in detached leaves of Khapli wheat.

Treatment	Expt. No.	Radioactivity (cpm/g fr wt tissue) Water Control*	%#	Benzimidazole	%#	Control*/ Benz.
Three days	1[1]	15,000	8.8	28,000	16.6	0.54
	2[1]	18,000	11.4	23,000	13.5	0.78
	3[2]	31,000	23.0	9,000	7.5	3.5
	4[2]	37,000	27.0	10,000	8.3	3.7
	5[3]	14,000	7.4	8,000	5.2	1.76
	6[3]	19,000	9.8	15,000	8.8	1.26
	7[4]	19,000	15.4	29,000	19.0	0.66
	8[4]	26,000	17.4	27,000	17.3	0.96
Five days	1[1]	15,000	11.5	15,000	9.9	1.0
	2[1]	17,000	13.0	22,000	12.6	0.77
	3[2]	30,000	23.0	29,000	25.0	1.0
	4[2]	27,000	24.0	35,000	28.0	0.77
	5[3]	3,000	4.3	13,000	15.0	0.23
	6[3]	5,000	4.8	13,000	13.7	0.39
	7[4]	15,000	13.0	23,000	12.8	0.65
	8[4]	21,000	17.4	27,000	8.1	0.78

For explanation of superscripts see Table II.

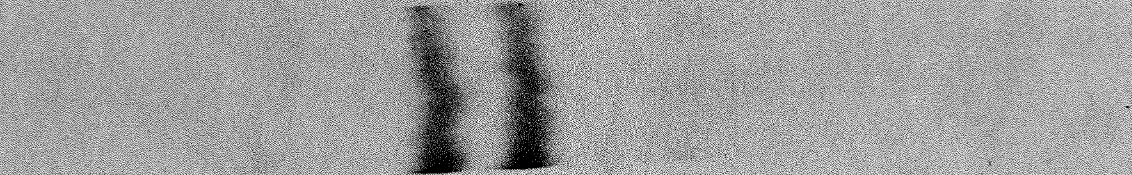
see Table IV.

* 'Control' refers to those experiments where leaves were floated on water.

Figure 1. Radioautographs of chromatograms of free sugars from detached Khapli wheat leaves fed glycine-2-C^{14}.

I, immediately detached leaves; 2 and 4, three and five days benzimidazole treated leaves respectively; 3 and 5, leaves floated on water for three or five days respectively.

F,G, S and O represent fructose, glucose, sucrose and origin, respectively. Developing solvent was n-butanol:acetic acid: water (4:1:5 v/v). Chromatograms were developed three times, ascending.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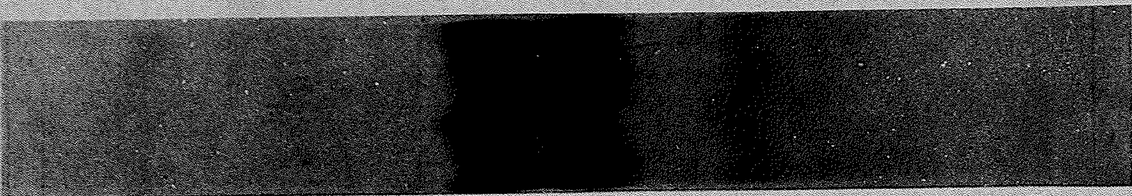
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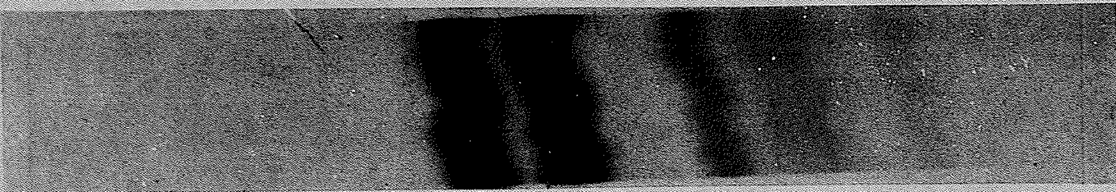
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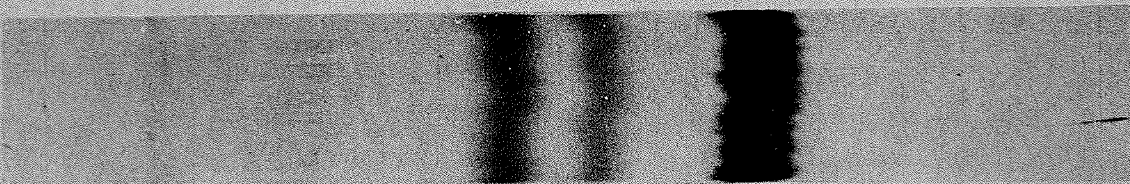
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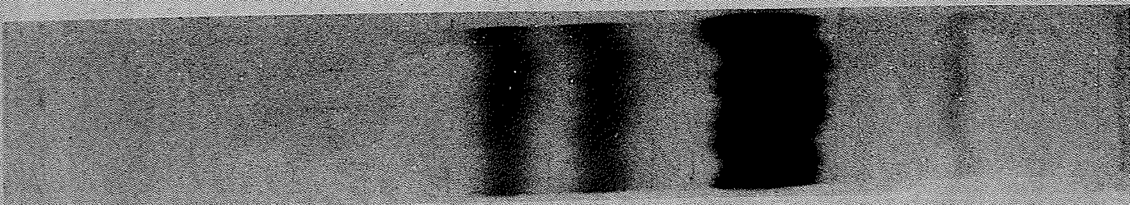
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a pattern of isotopic distribution more nearly like that found in immediately detached leaves. After five days in both leaves floated on water and those treated with benzimidazole a marked shift to glucose and fructose was observed. Throughout all experiments more than 90% of the radioactivity in sugars could be accounted for in glucose, fructose and sucrose.

Fluctuations in the rate of flow of isotope to sugars was accompanied by a concomitant variation in radioactivity in amino acids (Tables III, V and VI), but with little or no change of radioactivity of the organic acids (Table VII). This implied that variations in formation of sugars from glycine-2-C^{14} was at the expense of other amino acids.

The percentage recovery of radioactivity from Dowex columns varied between 73% and 100%. This variability seemed to be paralleled by a fluctuation of amino acid recovery from Dowex 50 resin beds. The reasons for this are obscure but may be due in part to the retention on these columns of red pigments, presumed to be anthocyanins, which were present in large quantities, especially in floated leaves. After five days treatment with benzimidazole or in leaves floated on water anthocyanins were formed to such an extent that the green color of chlorophyll was almost completely obscured by a deep purple pigmentation. This coloration persisted in water-soluble fractions and was substantially retained by Dowex 50 although a little of the color was removed during elution with HCl. It may not be unreasonable to specu-

TABLE VI. The effect of benzimidazole on the percentage of glycine-2-C^{14} remaining in the free amino acid fraction of detached leaves of Khapli wheat.

Treatment	Expt. No.	Radioactivity (cpm/g fr wt tissue) Water Control*	%#	Benzimidazole	%#	Control*/ Benz.
Three days	1[1]	121,000	71	101,000	60	1.19
	2[1]	137,000	87	106,000	61	1.29
	3[2]	85,000	62	76,000	63	1.1
	4[2]	84,000	61	97,000	81	0.75
	5[3]	156,000	82	110,000	71	1.42
	6[3]	138,000	71	103,000	61	1.34
	7[4]	94,000	76	90,000	59	1.04
	8[4]	106,000	71	77,000	48	1.38
Five days	1[1]	94,000	72	110,000	73	0.85
	2[1]	97,000	74	120,000	69	0.81
	3[2]	85,000	64	63,000	54	1.2
	4[2]	81,000	65	72,000	57	1.1
	5[3]	60,000	85	70,000	80	0.86
	6[3]	89,000	86	70,000	74	1.27
	7[4]	87,000	56	146,000	79	0.6
	8[4]	91,000	58	140,000	65	0.65

For explanation of superscripts see Table II

\# see Table IV.

* see Table V.

TABLE VII. The effect of benzimidazole on the transformation of glycine-2-C^{14} to organic acids in detached leaves of Khapli wheat.

Treatment	Expt No.	Radioactivity (cpm/g fr wt tissue) Water Control*	%#	Benzimidazole	%#	Control*/ Benz.
Three days	1[1]	8,000	4.7	8,000	4.7	1.0
	2[1]	7,000	4.4	8,000	4.7	0.88
	3[2]	7,000	5.1	9,000	7.5	0.78
	4[2]	6,000	4.4	10,000	8.3	0.6
	5[3]	11,000	5.8	11,000	7.1	1.0
	6[3]	11,000	5.7	12,000	7.1	0.92
	7[4]	6,000	4.8	14,000	9.2	0.43
	8[4]	7,000	4.7	13,000	8.1	0.58
Five days	1[1]	7,000	5.4	9,000	6.0	0.78
	2[1]	7,000	5.4	12,000	6.9	0.58
	3[2]	6,000	4.5	9,000	7.7	0.66
	4[2]	4,000	3.2	10,000	7.9	0.4
	5[3]	5,000	7.1	10,000	11.5	0.5
	6[3]	6,000	5.8	11,000	11.6	0.55
	7[4]	6,000	3.8	14,000	7.6	0.43
	8[4]	6,000	3.8	17,000	6.5	0.35

For explanation of superscripts see Table II.

see Table IV.

* see Table V.

TABLE VIII. Quantitative estimations of the components of the sugar fractions isolated from Khapli wheat leaves fed glycine-2-C^{14}.

Treat-ment	Expt. No.	Fraction	cpm fraction from paper eluates	% Recovery	Total Sugar (mg)/ fraction)	% Total Sugar/ Expt.	Total Sugar (mg/g fr wt leaf tissue	Specific activity cpm mg sugar
	1	F	6,000		0.98	17.4	0.78	6,180
		G	7,000	91	1.66	29.5	1.32	4,603
		S	19,000		2.98	53.0	2.37	7,177
	2	F	13,000		1.3	21.9	1.0	10,417
		G	14,000	90	2.32	39.1	1.86	6,651
		S	19,000		2.31	39.0	1.85	9,351
	3	F	4,000		0.44	14.2	0.4	10,750
A		G	4,000	90	0.84	27.1	0.76	5,658
		S	20,000		1.82	58.7	1.64	12,195
	4	F	3,000		0.3	12.0	0.29	11,852
		G	3,000	89	0.7	28.1	0.664	5,161
		S	18,000		1.49	59.8	1.42	13,233
	5	F	3,000		0.56	3.5	0.47	5,660
		G	4,000	94	1.99	12.5	1.67	1,872
		S	52,000		13.4	84.0	11.3	4,127
	1	F	3,000		0.16	7.1	0.14	23,076
		G	2,000	81	0.38	17.0	0.34	7,742
		S	21,000		1.7	75.9	1.5	15,220
	2	F	3,000		0.13	7.7	0.12	25,692
		G	2,000	100	0.27	16.0	0.25	8,630
B		S	20,000		1.29	76.3	1.19	15,194
	3	F	2,000		2.71	24.5	2.3	1,081
		G	3,000	82	4.31	38.9	3.65	708
		S	4,000		4.06	36.6	3.44	1,306

continued

TABLE VIII CONTINUED

B cont'd	4	F	6,000		3.38	33.1	2.7	1,760
		G	6,000	100	4.62	45.2	3.7	1,290
		S	4,000		2.22	21.7	1.78	1,982
	5	F	1,000		4.19	18.5	4.23	310
		G	1,000	100	6.48	28.6	6.55	201
		S	5,000		12.0	52.9	12.12	458
	6	F	2,000		3.48	14.5	3.63	555
		G	2,000	93	6.97	29.1	7.26	278
		S	9,000		13.5	56.4	14.1	724
	7	F	4,000		2.04	14.9	2.17	1,863
		G	3,000	100	4.36	31.8	4.64	768
		S	17,000		7.33	53.4	7.8	2,278
	8	F	5,000		2.58	18.3	2.48	1,977
		G	5,000	100	4.62	32.8	4.44	1,147
		S	19,000		6.89	48.9	6.63	2,823
C	1	F	9,000		0.71	44.4	0.59	12,958
		G	9,000	105	0.8	50.0	0.67	11,375
		S	1,000		0.09	5.6	0.08	13,778
	2	F	9,000		0.41	35.0	0.39	25,833
		G	8,000	87	0.61	52.1	0.58	14,717
		S	3,000		0.15	12.8	0.14	23,077
	3	F	15,000		8.8	39.1	6.1	1,716
		G	19,000	102	11.2	49.8	7.7	1,720
		S	5,000		2.49	11.1	1.72	2,048
	4	F	14,000		8.78	31.7	6.55	1,837
		G	21,000	88	15.4	55.5	11.46	1,562
		S	3,000		3.53	12.8	2.63	932
	5	a.	4,000		39.41	41.5	38.54	127
		b.	8,000	75	55.6	58.5	44.1	190
	6	a.	4,000		48.56	44.9	40.13	106
		b.	9,000	81	59.67	55.1	49.31	176

continued

	7	a.	17,000		31.7	78.0	31.1	658
C cont'd		b.	2,000	82	8.94	22.0	8.76	311
	8	F	2,000		4.34	18.6	4.72	686
		G	3,000	80	8.0	34.3	8.7	425
		S	14,000		11.0	47.1	12.0	1,602
	1	F	8,000		0.8	50.0	0.78	9,500
		G	8,000	100	0.73	45.6	0.71	10,411
		S	1,000		0.07	4.38	0.07	19,042
	2	F	9,000		0.67	38.5	0.62	13,134
		G	9,000	105	0.87	50.0	0.81	10,632
		S	2,000		0.2	11.5	0.19	10,800
	3	F	15,000		4.84	29.6	3.87	3,017
		G	18,000	100	9.8	59.9	7.8	1,832
		S	6,000		1.73	10.6	1.38	3,653
	4	F	14,000		4.95	27.1	3.93	3,248
D		G	16,000	87	10.2	55.8	8.1	1,775
		S	11,000		3.12	17.1	2.48	3,948
	5	F	6,000		0.71	36.4	0.7	7,746
		G	5,000	100	0.93	47.7	0.92	5,699
		S	3,000		0.31	15.9	0.31	8,226
	6	F	6,000		0.95	38.3	1.0	7,775
		G	7,000	84	1.27	51.2	1.34	6,589
		S	2,000		0.26	10.5	0.27	9,318
	7	F	3,000		6.55	19.7	6.18	553
		G	4,000	80	16.34	49.1	15.42	268
		S	8,000		10.39	15.9	9.8	939
	8	F	4,000		5.05	19.3	5.0	941
		G	4,000	81	12.62	48.2	12.5	396
		S	12,000		8.51	32.5	8.43	1,742
	1	F	4,000		0.31	29.0	0.3	15,370
E		G	4,000	88	0.31	29.0	0.3	14,814
		S	6,000		0.45	42.1	0.43	14,150

continued

E cont'd	2	F	7,000		0.6	44.4	0.55	12,453
		G	7,000	89	0.67	49.6	0.62	12,333
		S	2,000		0.08	5.9	0.07	21,488
	3	F	9,000		7.11	22.3	5.47	1,238
		G	19,000	103	22.04	69.2	16.95	853
		S	4,000		2.71	8.6	2.08	1,476
	4	F	10,000		7.77	22.9	5.63	1,416
		G	20,000	95	23.58	69.3	17.09	888
		S	3,000		2.66	7.82	1.93	1,225
	5	a.	2,000		19.24	30.8	12.74	124
		b.	4,000	100	43.33	69.3	28.7	88
	6	a.	3,000		19.62	35.5	13.91	158
		b.	5,000	100	35.71	64.5	25.33	146
	7	F	4,000		13.89	23.7	10.94	318
		G	7,000	95	31.58	53.9	24.87	228
		S	7,000		13.13	22.4	10.34	529
	8	F	5,000		15.31	24.2	11.96	364
		G	9,000	88	34.47	54.6	26.93	280
		S	8,000		13.41	21.2	10.48	636

The symbols F,G and S refer to fructose, glucose and sucrose, respectively. Only these three sugars were isolated and measured quantitatively. However, the "% recovery" column in the table also includes one or more other radioactive sugar components.

In some experiments the sugars were only partially separated from one another by paper chromatography. In these cases the fractions have been labelled "a" and "b" because of their uncertain identity.

The "experiment numbers" (1-8) coincide with those found in Table II.

For explanation of sybmols A-E see Table II.

late that some radioactivity was lost to anthocyanins but no attempt was made to assess this loss quantitatively or to associate it with pigment formation.

(c) Final separation and quantitative measurement of sugars

Quantitative measurements of fructose, glucose and sucrose (Table VIII) confirmed earlier qualitative data (Fig.1). Determinations of counts per minute per fraction showed that high levels of activity in sucrose were maintained in leaves treated with benzimidazole for three days whilst in leaves floated on water there was a major re-distribution of activity to the two monosaccharides. In addition, in all three day treatments a marked drop in total activity in sugars was observed but the shift of isotopic labelling from sucrose to glucose and fructose was less marked in those experiments carried out in continuous light. After five days the trend towards isotopic distribution largely in monosaccharides was clear in both benzimidazole treated and leaves floated on water excepting again under a continuous light regime. Total sugar determinations also reflected the shift from sucrose to monosaccharides and also a marked rise in levels of all sugars was observed the longer the floating period except in those leaves kept in continuous darkness.

In immediately detached leaves the total amounts of all sugars were below 4 mg per fraction but after five days these levels had increased many fold. For example, the average level of fructose in

leaves floated on water for five days, excluding those floated in the dark, was 15-fold higher than that in immediately detached leaves. Similarly glucose, in the same time period, increased 19-fold and sucrose approximately doubled its level. Whilst sucrose did not maintain its preponderance over the monosaccharides in long term experiments the ratio between glucose and fructose was always of the same order of magnitude. In almost all experiments there was more glucose present than fructose, a trend not reflected in total radioactivity levels.

In all experiments with immediately detached leaves, the specific activity ratio between the three sugars remained essentially constant. Fructose and sucrose had similar specific activities whilst glucose was always lower. After three days treatment with benzimidazole a two- to three-fold increase of specific activities for all three sugars was observed under a continuous darkness regime whilst under any light conditions a four- or more-fold decrease occurred. In leaves floated on water for three days similar trends were apparent. These high specific activities in dark treated leaves persisted throughout the five day floating period as did the sharp fall in specific activities amongst those leaves treated in the light. The high specific activities were due to a drop in total radioactivity incorporated into the sugars coupled with an even greater drop in all sugar levels. In contrast, whilst there is also a fall in total radioactivity incorporated into leaves under various light conditions this is

accompanied by a substantial rise in all sugars.

(d) Invertase activity

The increase of total sugars in detached leaves could have been due, at least in part, to increased invertase activity. However when levels of this enzyme were measured in variously treated Khapli wheat leaves a slight decrease in activity was observed in all leaves tested over a five day exposure period (Table IX), results which may be compared to those obtained by Roberts (73) who found a decrease in invertase activity in first leaves of Khapli wheat beyond seven days after germination.

2. CHROMATOGRAPHIC STUDIES OF SOME GLYOXYLATE:AMINOTRANSFERASES IN WHEAT LEAVES

(a) Preliminary investigations of each aminotransferase

Studies were initiated in this laboratory on the following aminotransferases in crude extracts of wheat leaves:serine:glyoxylate (SGT); alanine:glyoxylate (AGT); glutamate:glyoxylate (GGT); aspartate:glyoxylate (AsGT); glutamate:pyruvate (GPT); glutamate: oxalacetate (GOT). Following these preliminary investigations a more thorough analysis of SGT was carried out.

In crude dialysed enzyme preparations extracted at pH 7.0 and

TABLE IX. Invertase activity in crude extracts of Khapli wheat leaves.

Treatment	Enzyme Activity (OD) Replicates 1	2	3	Boiled enzyme control OD	Enzyme (mg protein per ml)
A	0.49	0.49	0.37	0.049	0.58
B	0.37	0.38	-	0.045	0.59
C	0.4	0.32	0.33	0.14	1.28
D	0.37	0.43	0.46	0.047	0.46
E	0.27	0.26	0.33	0.11	0.84

For an explanation of the symbols A-E see Table II.

Enzyme activity is expressed as the optical density (OD) of the colored compound formed in the Somogyi test for sugars measured at 660 mμ (see p.21 for details of invertase assay).

tested at pH 7.0 and pH 8.0, activity of SGT was demonstrable in the forward direction only (Fig.2) but at pH 5.7 no activity was detected. The greatest activity was demonstrated, qualitatively, at pH 8.0.

In wheat leaf preparations extracted at pH 7.0 and 8.0 and tested at pH 8.0, AGT similarly was active only in the forward direction whereas GOT showed activity in both directions (Fig.2). In a thirty minute incubation period five µmoles of alanine were almost completely utilised in the production of glycine in a reaction mixture containing three mg of protein. When tested at pH 7.0 similar results were obtained but neither AGT nor GOT showed as much activity as at pH 8.0. At pH 5.7, GOT was slightly active but AGT was completely inactive.

GGT was readily demonstrated when extracted at pH 7.0 and tested at pH 7.0 and 8.0 (Fig.3) but AsGT had only slight activity under similar conditions of pH. Both reactions proceeded in the forward direction only. At pH 5.7 GGT was only slightly active and AsGT completely inactive.

Chromatographic evidence for a reversible GPT in these extracts was also obtained but not for a glutamate:hydroxypyruvate aminotransferase. In fact β-hydroxypyruvate at one-tenth or the same concentration as glutamate had an inhibitory effect on GGT, but α-ketoglutarate had no reciprocal effect on SGT. Further studies of the effect of

Figure 2. Plan of a chromatogram showing glycine formation from alanine:glyoxylate and serine:glyoxylate aminotransferases.

Without Pal. P* (1,1'); with 0.07 μmoles of Pal.P (2,2'); boiled enzyme controls (3,3'). A, B: glycine and alanine, and serine markers respectively.

* Pal.P: pyridoxal-5-phosphate.

N.B. ninhydrin-positive spot at $R_f = 0.24$ in 1' and 2'.

Developing solvent:phenol:water (80:20 v/v) containing 0.004% 8-hydroxyquinoline. Ascending for 14 to 16 hours.

solvent front

0

3 2 1 A B 1' 2' 3'

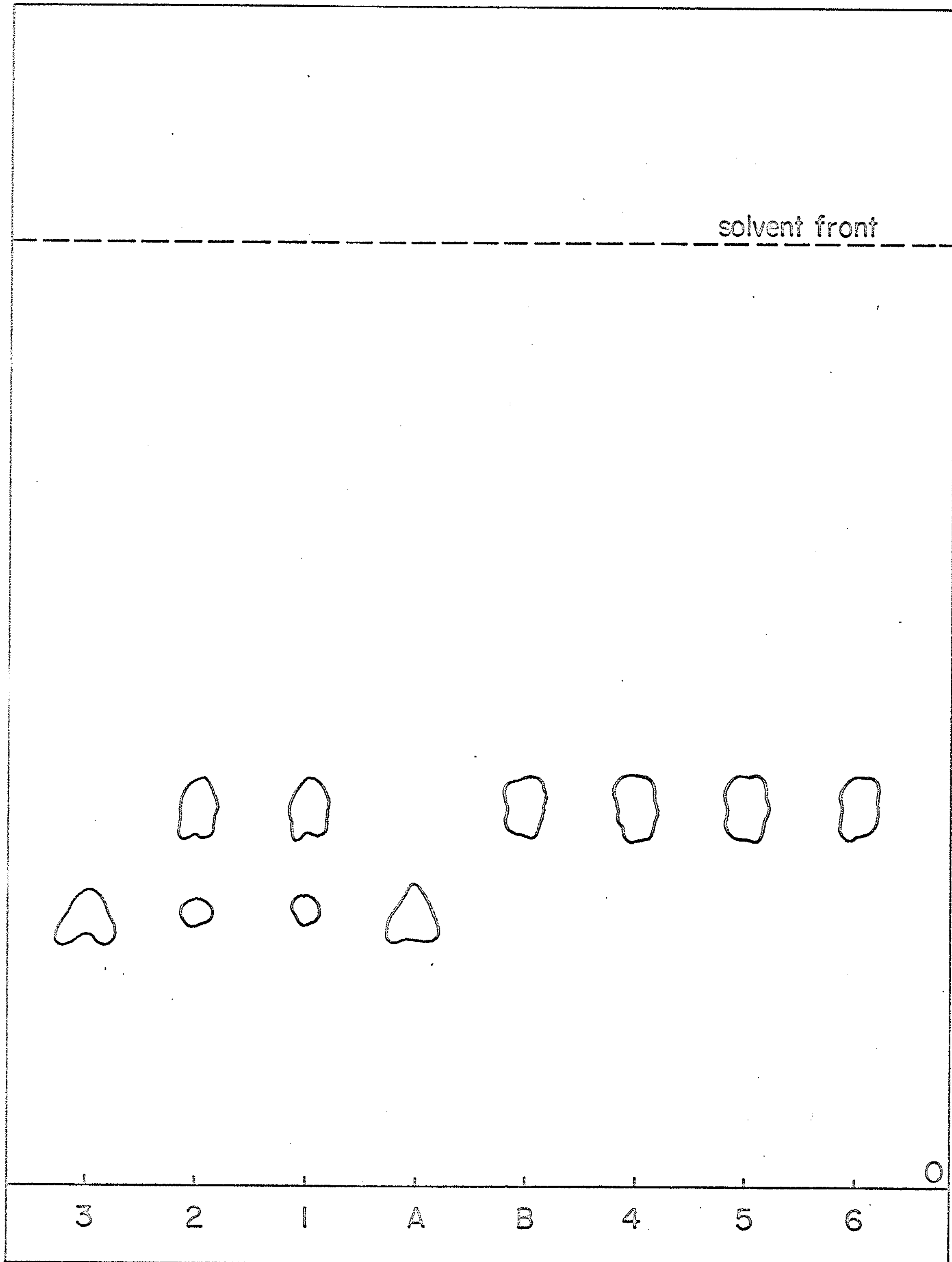
Figure 3. Plan of a chromatogram showing glycine formation from and irreversibility of glutamate:glyoxylate aminotransferase.

A, 1, 2 and 3: forward reaction.

B, 4, 5 and 6: backward reaction.

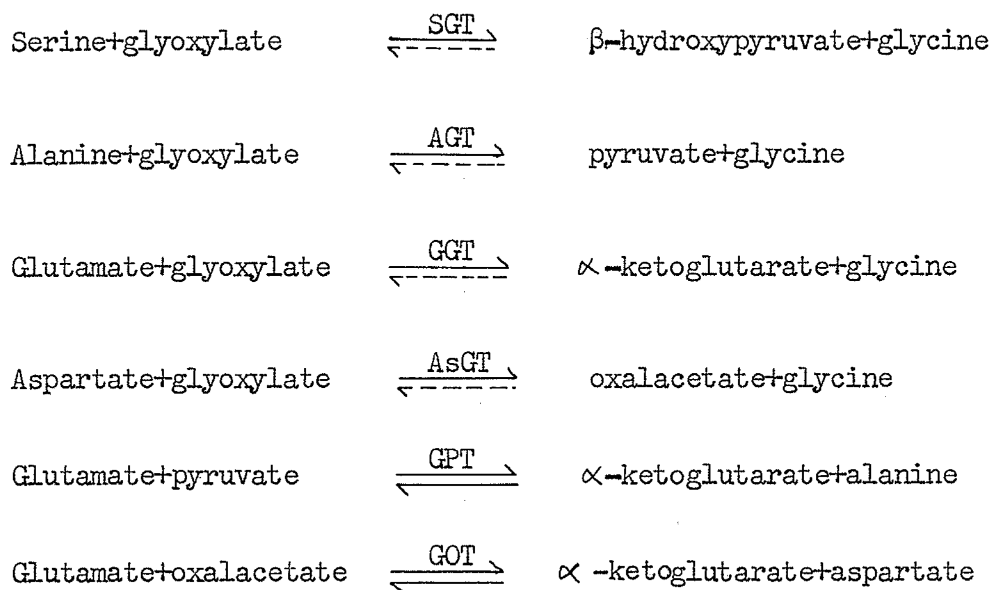
Without Pal.P* (1,4); with 0.07 μmoles of Pal.P (2,5); boiled enzyme controls (3,6); glutamate without glyoxylate and glycine without α-keto glutarate (A and B, respectively).

* See Fig.2. developing solvent: see Fig.2.


solvent front
O
3
2
1
A
B
4
5
6

β-hydroxypyruvate (0.5 or 5.0 µmoles) showed that it had no effect on SGT, AsGT or GOT (in either direction) whereas it inhibited GGT, AGT and GPT (in both directions). Inhibition was more pronounced with higher β-hydroxypyruvate concentrations judging from the intensity of ninhydrin-positive spots.

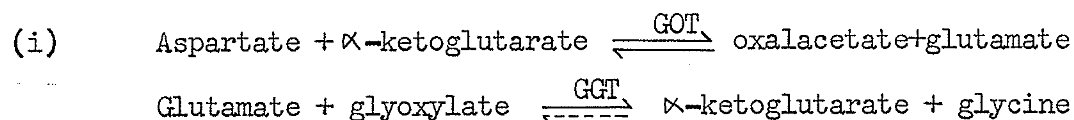
These results can be summarised as follows:



(b) Reaction products with single amino donors and multi-amino acceptors

The evidence obtained thus far indicated that the synthesis of glycine could be catalysed by any of four glyoxylate:aminotransferases present in crude dialysed extracts of wheat, none of which were readily reversible, and that the contribution of AsGT would be small in comparison to the other three. However, the fact that the extracts also

contain both an active reversible GOT and an irreversible GGT should make possible a substantial synthesis of glycine from aspartate by providing the substrates, α-ketoglutarate and glyoxylate, to couple these aminotransferases as follows:


(i) Aspartate + α-ketoglutarate ⇌(GOT) oxalacetate+glutamate
Glutamate + glyoxylate ⇌(GGT) α-ketoglutarate + glycine

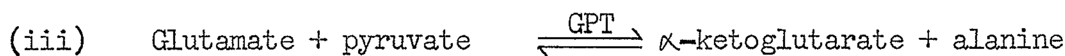
This reaction would involve glutamate as an intermediate and be independent of the weakly active AsGT. In experiments to test this supposition, dialysed enzyme containing three or 3.4 mg protein was incubated for 30 minutes with aspartate and equimolar amounts of α-ketoglutarate and glyoxylate. Substantial amounts of glycine were formed, glutamate was detected an chromatogram paper and some aspartate remained unmetabolised. Controls showed that GGT functioning alone yielded a similar amount of glycine whereas with AsGT the yield of glycine was only slight. The equilibrium of GOT functioning singly in comparable experiments appeared to favor aspartate formation, a fact established for the enzyme extracted from other plants (18,80) and animal tissues (38). Reaction (i) represents a mechanism where the equilibrium of a reaction, in this case GOT, is disturbed by coupling with an irreversible reaction. The fact that glutamate was detected on chromatogram paper indicated that the rate of GOT exceeds that of GGT. That this is dependent on the α-ketoglutarate concentration was shown in experiments in which

only one-tenth of the concentration of this keto acid was employed. The formation of glycine was markedly decreased and a much smaller amount of the intermediate glutamate was detected.

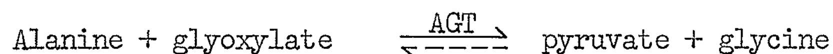
In comparable experiments using glutamate as the amino donor together with equimolar amounts of either (ii) oxalacetate and glyoxylate, or (iii) pyruvate and glyoxylate the following reactions were co-ordinated:

(ii) Glutamate + oxalacetate $\underset{}{\overset{GOT}{\rightleftharpoons}}$ α-ketoglutarate + aspartate

Glutamate + glyoxylate $\underset{}{\overset{GGT}{\rightleftharpoons}}$ α-ketoglutarate + glycine


(iii) Glutamate + pyruvate $\underset{}{\overset{GPT}{\rightleftharpoons}}$ α-ketoglutarate + alanine

Glutamate + glyoxylate $\underset{}{\overset{GGT}{\rightleftharpoons}}$ α-ketoglutarate + glycine


Alanine + glyoxylate $\underset{}{\overset{AGT}{\rightleftharpoons}}$ pyruvate + glycine

in which (ii) represents a competitive system and (iii) a mixed competitive, coupled system. In both, there was a predominant formation of glycine, as compared to aspartate in system (ii) and alanine in system (iii). Glycine formation was about the same when one-tenth the concentration of either oxalacetate or pyruvate was used but aspartate could hardly be detected and alanine was only just detectable on chromatogram paper.

These results indicate that GGT preferentially utilises

glutamate as compared to GOT even though the rate of the GOT reaction, despite the unfavorable equilibrium, exceeded that of GGT in system (i). It can be concluded that GGT has a greater affinity for glutamate than does GOT, which can only be confirmed by a study of the kinetics of the individual enzymes.

3. KINETIC STUDIES OF A SERINE:GLYOXYLATE AMINOTRANSFERASE IN WHEAT LEAVES

(a) General properties of the enzyme

The enzyme was routinely stored at -10° in KH_2PO_4-K_2HPO_4 buffer, pH 7.0 and under these conditions it remained stable for several weeks. However, fresh preparations were made before each experiment and used within 24 hours of extraction. Early experiments showed that the reaction in the presence of active enzyme was 10 to 15 times that of control experiments with boiled enzyme or in the absence of protein (Fig.4). In the majority of experiments with L-serine and glyoxylate as substrates the only compounds present after incubation with enzyme protein were serine, glyoxylate, glycine and β-hydroxypyruvate. Serine and glycine were located on paper chromatograms and the keto acids identified by formation of their 2,4-dinitrophenylhydrazones, following the method of Towers and Mortimer (89) as modified by Wang (93). The enzyme is specific for L-serine as neither D-serine nor phospho-L-serine could replace the L-isomer (Table XI). Glycolaldehyde could not replace glyoxylate but when five µmoles of γ-hydroxyglutamate were

Figure 4. Requirement for active enzyme protein for a serine:glyoxylate aminotransferase reaction in wheat leaves.

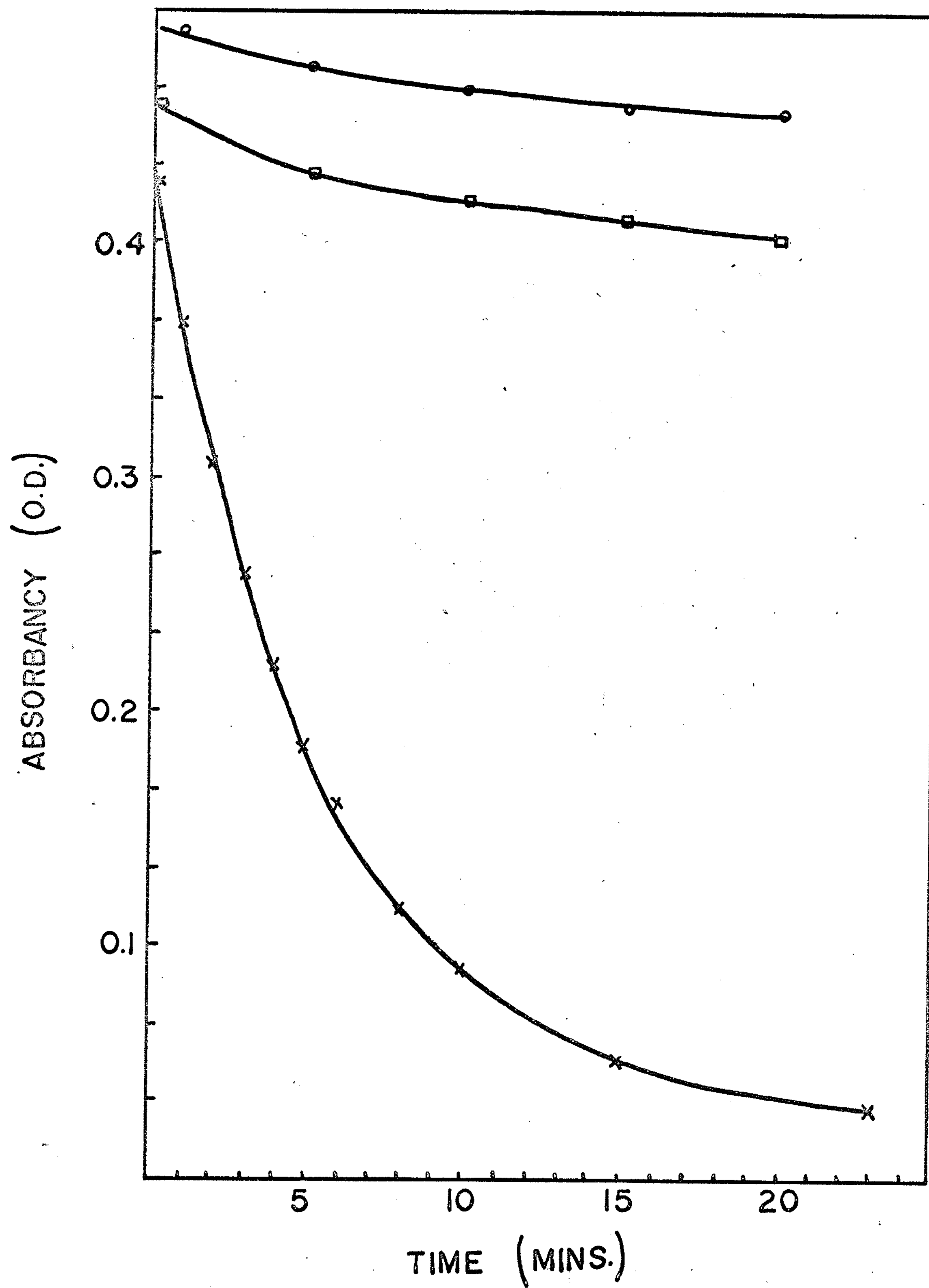
O - boiled enzyme control

□ - without enzyme

X - active enzyme

Assay system: phosphate buffer, 100 µmoles (pH 7.0); serine, 45 µmoles; sodium glyoxylate, 6 µmoles; enzyme, 1.34 mg protein; water.
Final volume: 1.5 ml. Incubation: 30 min. at 30°

Disappearance of NADH at 340 mµ in the D-glycerate: NAD oxidoreductase reaction used as a measure of hydroxypyruvate formation.



ABSORBANCY (O.D.)
0.4
0.3
0.2
0.1
5
10
15
20
TIME (MINS.)

TABLE X. R_f values of 2,4-dinitrophenylhydrazones of some keto acids

Keto acid	R_f values	
	Reaction mixture	Known compounds
Glyoxylate	0.41, 0.63	0.41, 0.65
β-hydroxypyruvate	0.46, 0.55	0.47, 0.55
Pyruvate	-	0.2, 0.57, 0.76

Assay system: 100 µmoles phosphate buffer, pH 8.2; 15 µmoles serine and glyoxylate; enzyme protein (1.52 mg); water. Final volume: 1.5 ml. Hydrazones separated by ascending chromatography in vessels containing tert. amyl alcohol:ethanol:water (9:1:4 v/v) and a beaker of 4% NH_4OH (89).

TABLE XI. Reaction between various amino acid donors and glyoxylate or glycolaldehyde.

Substrates	Hydroxypyruvate (μmoles) formed (hr/mg protein) with: Active enzyme	Boiled enzyme control
L-serine + glyoxylate	2.35	0.12
D-serine + glyoxylate	0.15	0.12
Phospho-L-serine + glyoxylate	0.13	0.11
γ-hydroxyglutamate + glyoxylate	0.12	0.07
L-serine + glycolaldehyde	0	0
γ-hydroxyglutamate + L-serine + glyoxylate	0.81	0.14

Assay system: 100 μmoles phosphate buffer, pH 8.2; 15 μmoles L-serine or 5 μmoles D-serine, phospho-L-serine or γ-hydroxyglutamate; 15 μmoles glyoxylate or glycolaldehyde; 1.3 mg enzyme protein; water. Final volume: 1.5 ml. Incubation time: 20 min at 30°.

added to a reaction system containing serine and glyoxylate a three-fold retardation of the forward reaction was observed. This was probably due to a transamination between γ-hydroxylglutamate and glyoxylate rather than an inhibition of SGT by the former compound. The partially purified enzyme shows a linear relationship with time for the first 10 minutes (Fig.5) and on all occasions the forward reaction proceeded to no more than ca one-third to one-half completion (Fig.5). Neither increasing time nor increasing enzyme concentration (Fig.6) could improve on this conversion despite the fact that a reverse reaction could not be detected. On various occasions attempts were made to induce a reverse reaction using equimolar amounts of hydroxypyruvate and glycine as high as 45 µmoles for incubation periods up to 140 minutes at pH values between 5.5 and 10.0. No serine could be detected on chromatogram paper, only the 2,4-dinitrophenylhydrazone of hydroxypyruvate was found and no glyoxylate could be detected when incubation mixtures were assayed with a commercially purified glyoxylate oxidoreductase. However in a further series of experiments glycine and hydroxypyruvate were incubated together at pH 8.2 for 30 minutes at 30° in the presence of 1.98 mg protein. In the presence of glycine and active enzyme some hydroxypyruvate could not be accounted for (Table XII). This result was not due to an inhibition of D-glycerate: NAD oxidoreductase activity, used to measure hydroxypyruvate levels, by glycine as the loss of hydroxypyruvate occurred only in the

Figure 5. A progress-time curve of serine:glyoxylate aminotransferase activity in the forward direction.

Assay system: phosphate buffer, 100 μmoles (pH 8.2); L-serine, 9 μmoles; sodium glyoxylate 9 μmoles; enzyme, 1.74 mg protein; water. Final volume: 1.5 ml.

Incubation times varied at 30°.

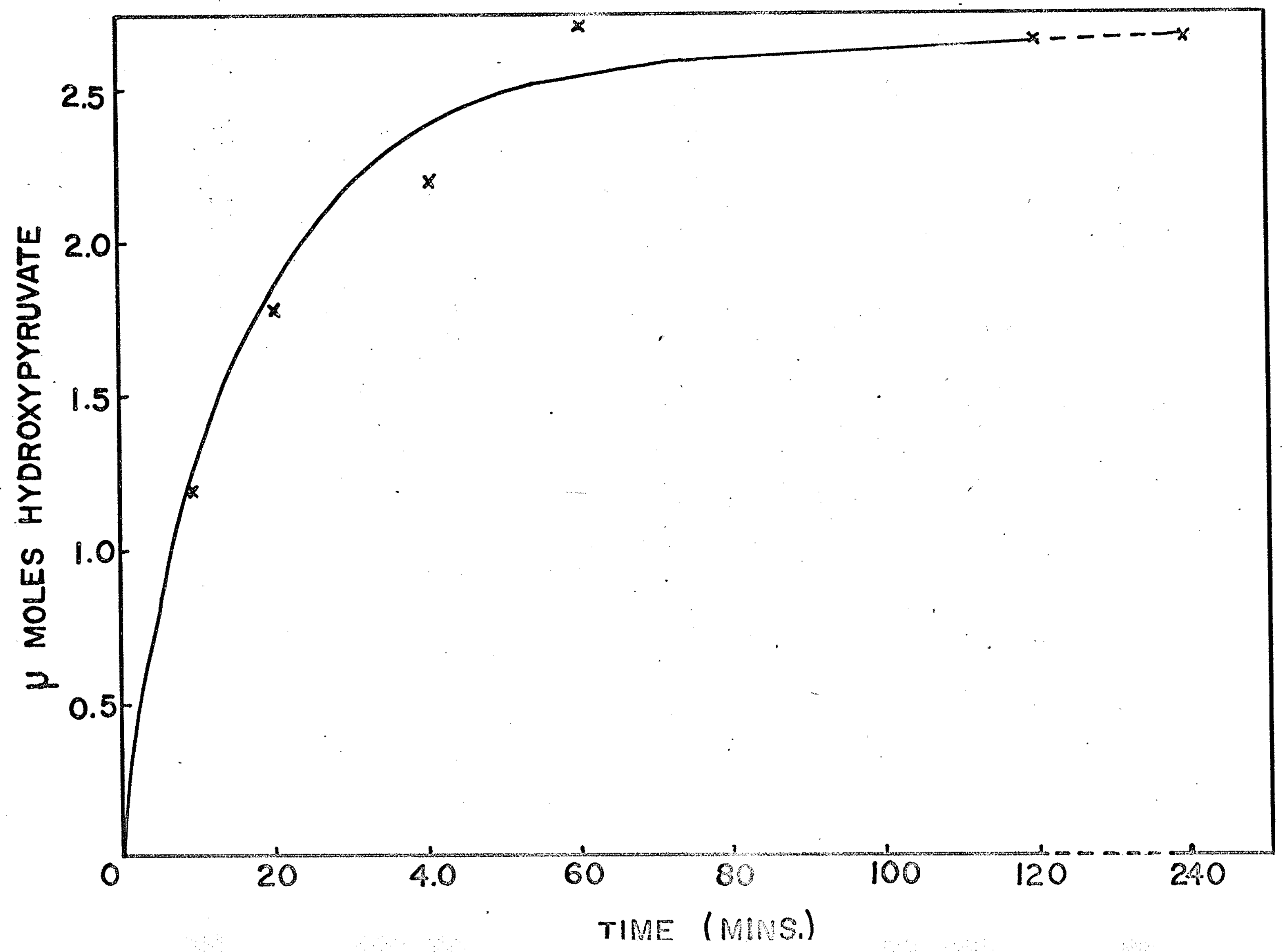
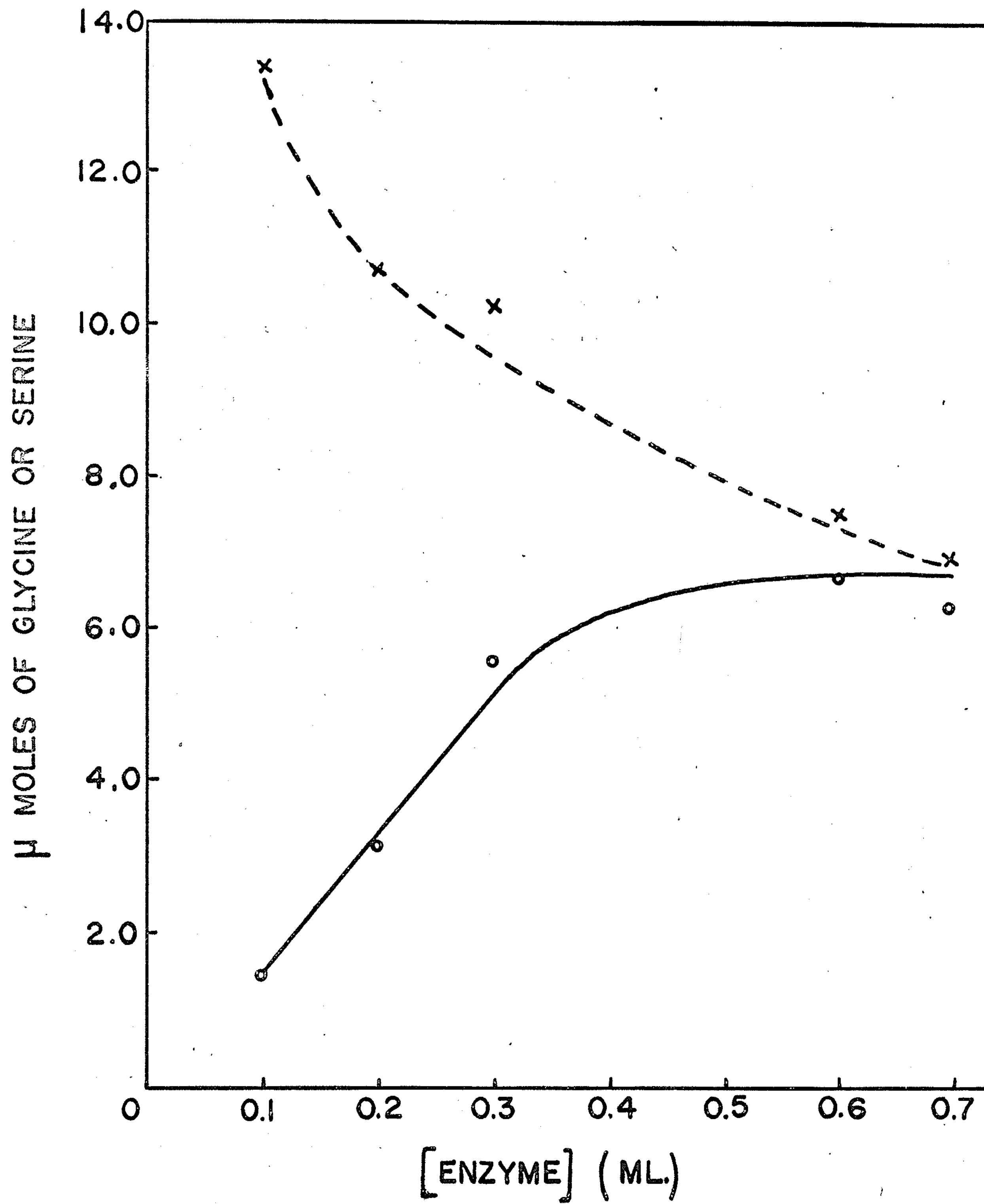

μ MOLES HYDROXYPYRUVATE
2.5
2.0
1.5
1.0
0.5
0
20
4.0
60
80
100
120
240
TIME (MINS.)

Figure 6. Effect of increasing enzyme concentration on serine:glyoxylate aminotransferase activity in the forward direction.

X - serine disappearance

O - glycine formation

<u>Assay system:</u> phosphate buffer, 100 µmoles (pH 7.0); L-serine, 15 µmoles; sodium glyoxylate, 15 µmoles; enzyme, containing 6.3 mg protein/ml; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.



14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
μ MOLES OF GLYCINE OR SERINE
[ENZYME] (ML.)

presence of active enzyme and in other experiments the oxidoreductase was shown to be unaffected by 15 µmoles of added glycine.

TABLE XII. Hydroxypyruvate disappearance in the presence of glycine.

Substrates	Hydroxypyruvate (µmoles/hr/mg protein)
Hydroxypyruvate alone	3.06
Hydroxypyruvate + glycine with enzyme	2.45
Hydroxypyruvate + glycine without enzyme	3.06

Stoichiometry of the forward reaction confirmed that it proceeded to only one-third to one-half completion at pH 8.2 and in the presence of 1.8 mg enzyme protein (Table XIII). Serine and glycine were estimated by elution from chromatogram paper, hydroxypyruvate by D-glycerate:NAD oxidoreductase activity and glyoxylate by using a commercial glyoxylate oxidoreductase.

No evidence was obtained that either glycine (up to 30 µmoles) or hydroxypyruvate (30 µmoles) inhibited the forward reaction but in a number of experiments using partially purified enzyme an inhibition of the forward reaction by high levels of glyoxylate

was observed (Fig.7). Inhibition occurred at a glyoxylate level of 21 µmoles when serine was being held at 45 µmoles. Serine had no such inhibition effect (Fig.8). The maximum activity of SGT was attained at pH 8.2 (Fig.9) when tested in phosphate buffer over the pH range 6.0 to 10.5 and at a temperature of 38° (Fig.10).

TABLE XIII. Stiochiometry of the forward reaction.

Incubation (Time Min)	Total concentration (µmoles) in reaction system			
	Serine	Glyoxylate	Glycine	Hydroxypyruvate
0	15	15	0	0
30	10.3	9.9	4.0	4.4

When attempts were made to isolate the enzyme in 0.2 M Tris-HCl buffer, pH 7.0, no activity was found. Nisonoff and Barnes (64,65) reported that a glutamate:aspartate aminotransferase reaction in hog heart extracts was enhanced by phosphate ions. They suggested that phosphate may act as a catalyst in some transaminations. Two enzyme preparations from wheat leaves, one extracted with 0.2 M Tris-HCl buffer, pH 7.0 and the other with distilled water were reactivated by the addition of sodium or potassium phosphate buffer. Maximum effect of the phosphate ion seemed to be, in either case, at a concentration of between 0.03 and 0.04 M (Fig.11). In other experiments with a Tris-extracted enzyme five µmoles of ATP

Figure 7. Effect of increasing glyoxylate concentration on serine:glyoxylate aminotransferase activity in the forward direction.

Assay system: phosphate buffer, 100 µmoles (pH 8.2); L-serine, 45 µmoles; sodium glyoxylate, varied; enzyme, 1.22 mg protein; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.

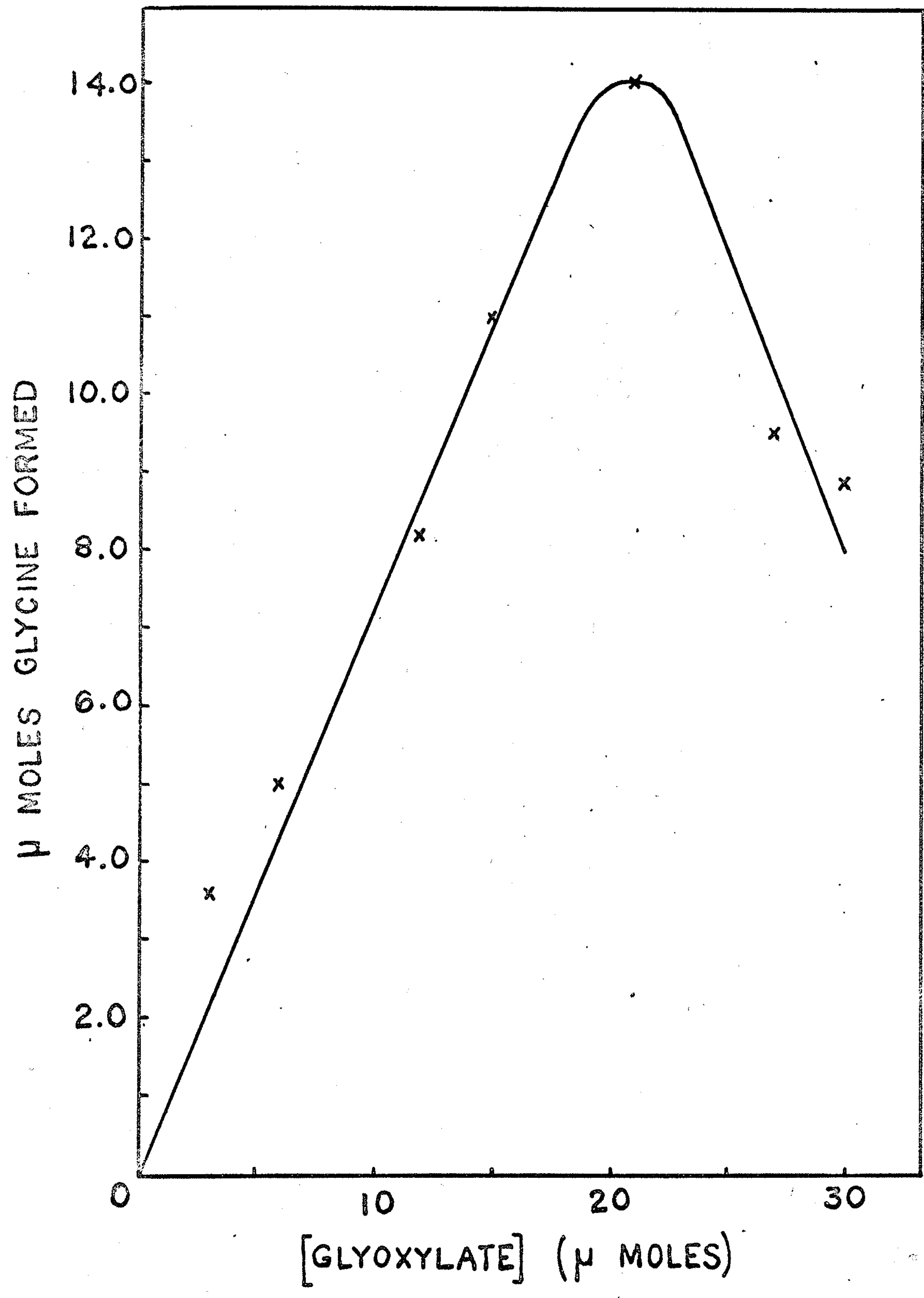

μ MOLES GLYCINE FORMED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
10
20
30
[GLYOXYLATE] (μ MOLES)

Figure 8. Effect of increasing serine concentration on serine:glyoxylate aminotransferase activity from wheat leaves.

Assay system: As in Fig.7 but varying serine.

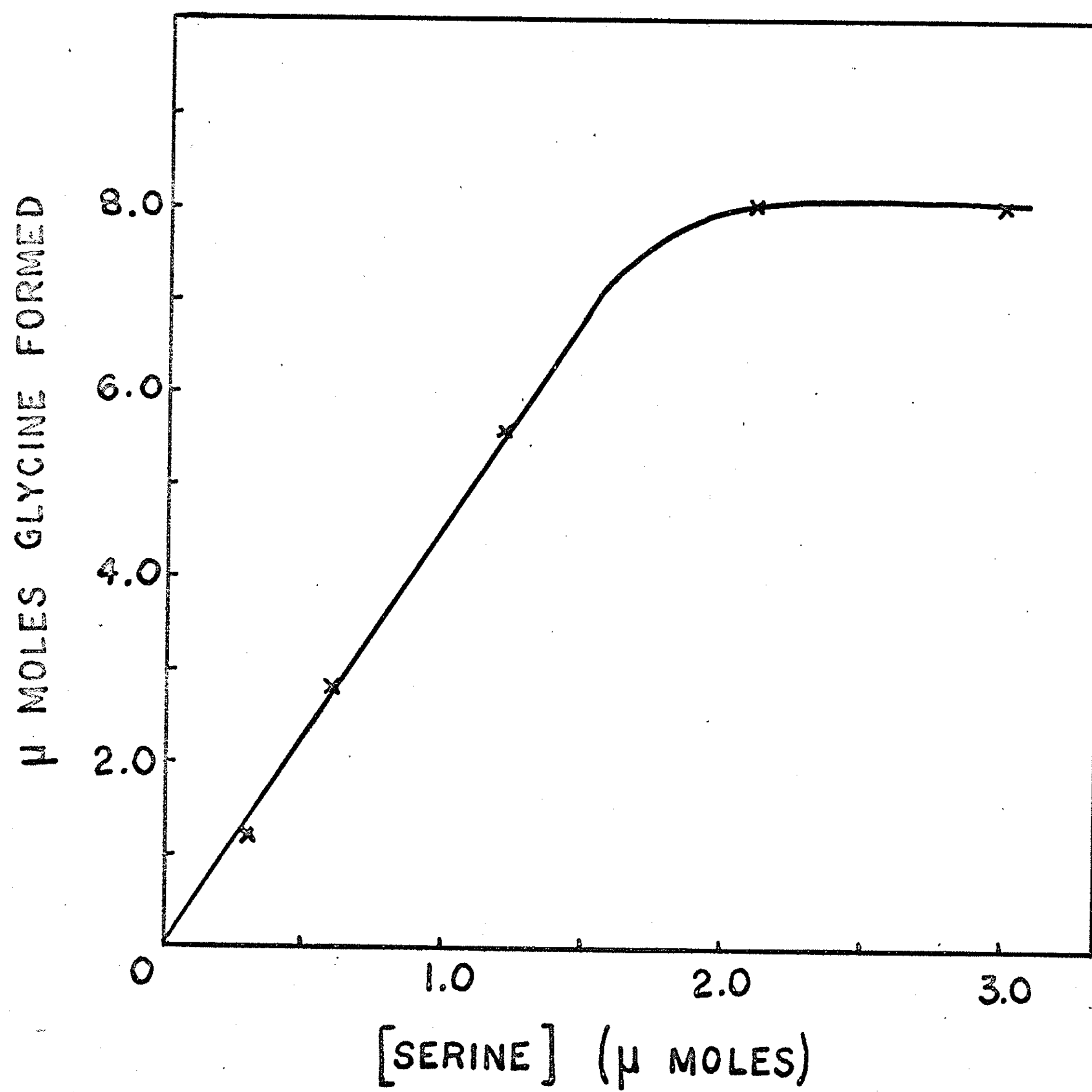
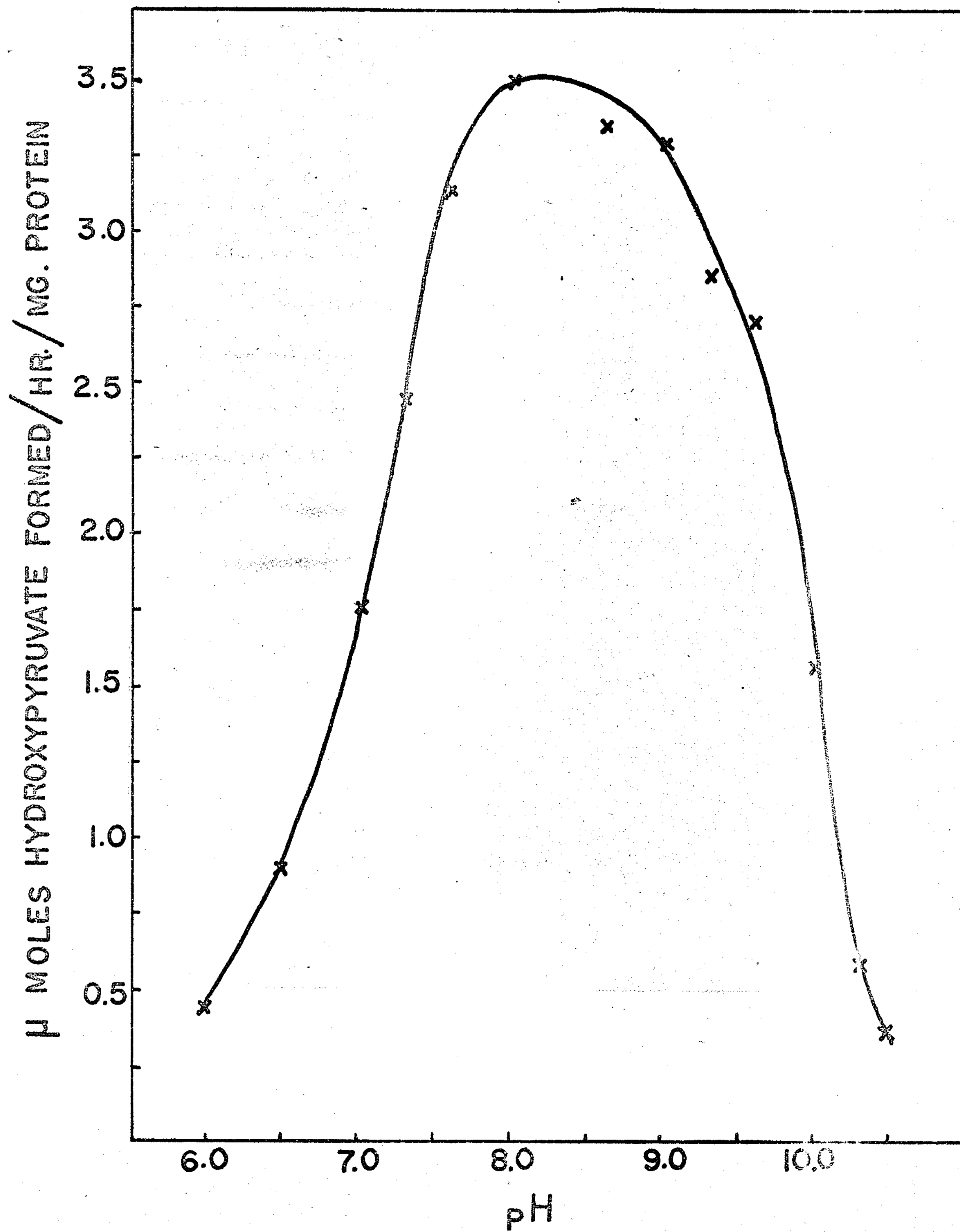

µ MOLES GLYCINE FORMED
8.0
6.0
4.0
2.0
0
1.0
2.0
3.0
[SERINE] (µ MOLES)

Figure 9. Effect of increasing pH on serine:glyoxylate aminotransferase activity.

Assay system: phosphate buffer, 100 µmoles (pH varied); L-serine, 9 µmoles; sodium glyoxylate, 9 µmoles; enzyme, 1.68 mg protein; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.



μ MOLES HYDROXYPYRUVATE FORMED/HR./MG. PROTEIN
3.5
3.0
2.5
2.0
1.5
1.0
0.5
6.0
7.0
8.0
9.0
10.0
pH

Figure 10. Effect of increasing temperature on serine: glyoxylate aminotransferase activity.

Assay system: phosphate buffer, 100 μmoles (pH 8.2); L-serine, 9 μmoles; sodium glyoxylate, 9 μmoles; enzyme, 1.74 mg protein; water. Final volume: 1.5 ml. Incubation time: 30 min at various temperatures.

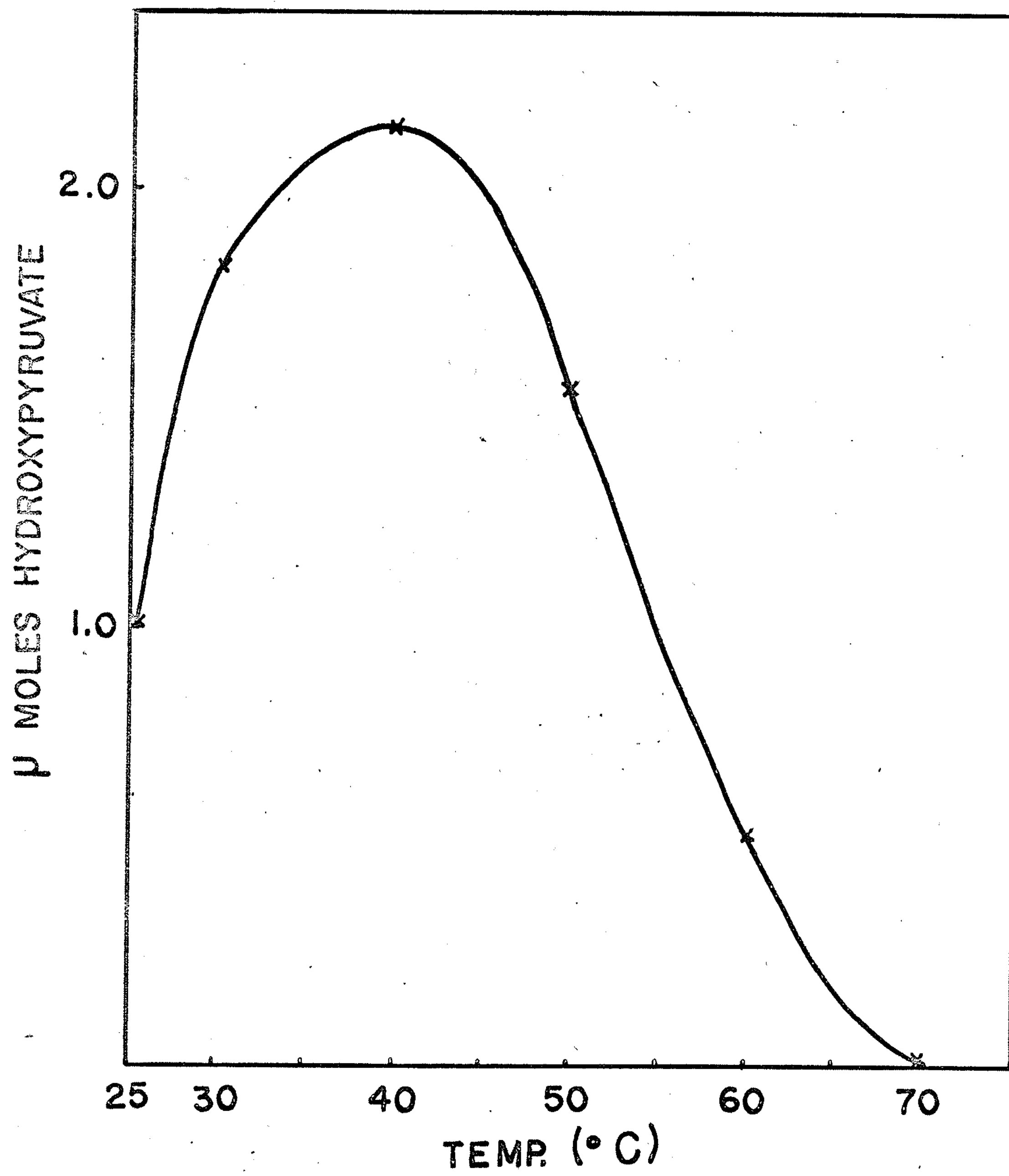

2.0
1.0
μ MOLES HYDROXYPYRUVATE
25
30
40
50
60
70
TEMP. (° C)

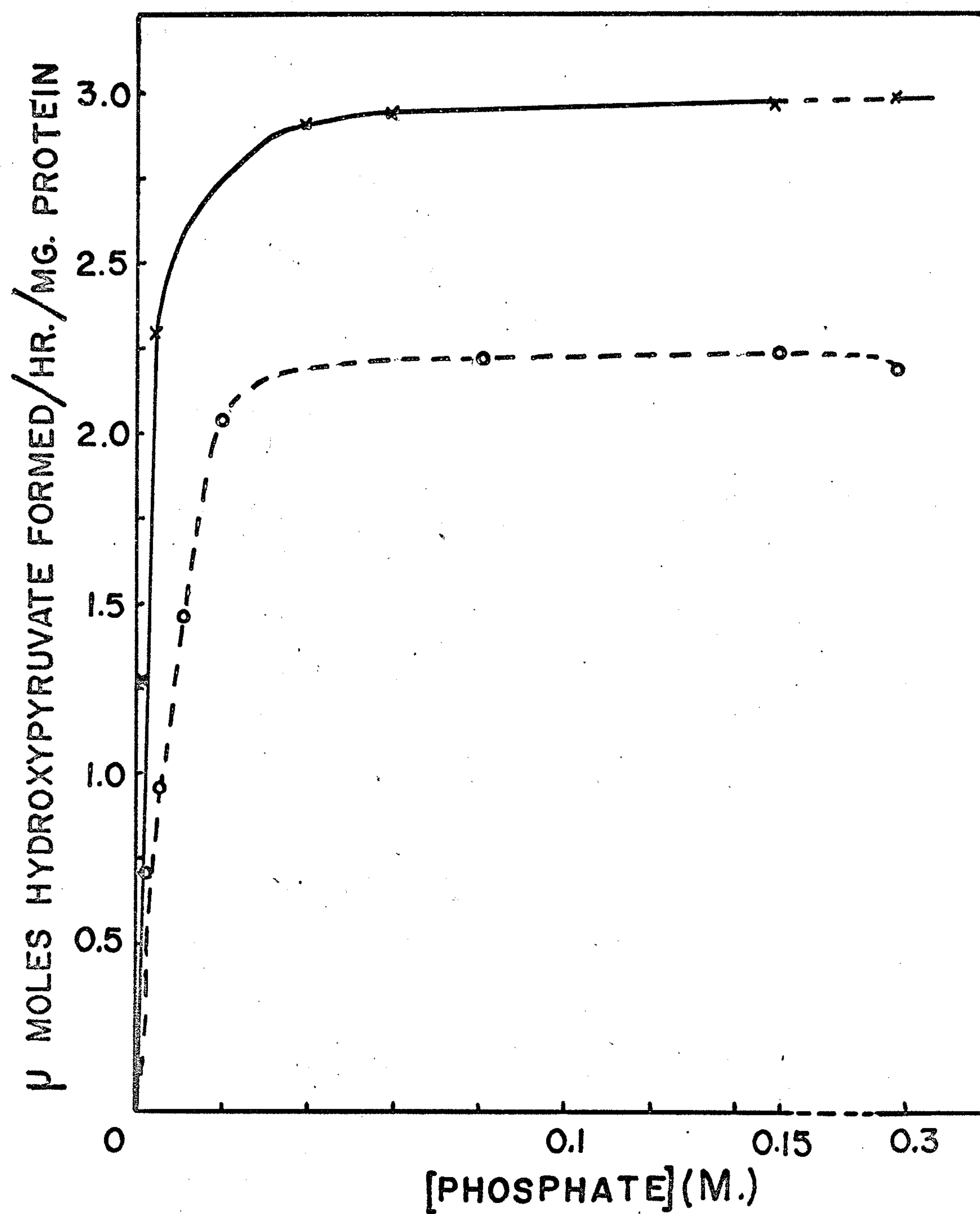
Figure 11. Effect of phosphate ion on serine:glyoxylate aminotransferase activity.

X - extracted with 0.2 M Tris-HCl buffer (pH 7.0).

O - extracted with water.

Assay system: phosphate buffer, varied (pH 8.2); L-serine, 9 µmoles; sodium glyoxylate, 9 µmoles; enzyme, Tris-HCl extracted, 1.92 mg protein, water extracted, 0.78 mg protein; water to make 1.5 ml.

Enzyme , phosphate buffer and water pre-incubated for 30 min before assay performed for 30 min at 30°.



μ MOLES HYDROXYPYRUVATE FORMED/HR./MG. PROTEIN
3.0
2.5
2.0
1.5
1.0
0.5
0
0.1
0.15
0.3
[PHOSPHATE](M.)

or pyrophosphate or three µmoles of pyridoxal-5-phosphate failed to stimulate reactivation.

(b) Effect of metal-chelating compounds

The forward reaction of SGT was inhibited by 0.15 µmoles of 8-hydroxyquinoline and by 1.5 µmoles of EDTA (Table XIV) which suggested that a metal ion was involved in the transamination.

TABLE XIV. Inhibition of serine:glyoxylate aminotransferase.

Addition	Concentration (µmoles/0.1 ml)	Hydroxypyruvate formed (µmoles/hr/mg protein)
Sodium bisulphite	1.5	1.24
8-hydroxyquinoline	0.15	1.35
Isonicotinyl hydrazide	1.5	1.36
EDTA	1.5	1.24
Control	0	1.4

Assay system: 100 µmoles phosphate buffer, pH 8.2; 15 µmoles serine and glyoxylate; inhibitor (conc as above); 2.18 mg enzyme protein; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.

Since there was some suggestion of inhibition by metal-chelating compounds, a partially purified enzyme preparation was dialysed against 0.6% EDTA, pH 7.0, overnight and further dialysed for

12 hours against distilled water to remove excess EDTA. The enzyme was then assayed with the addition of a number of metal ions at concentrations between 0.4 and one µmole. It was observed that dialysis against EDTA had completely inhibited the reaction and that some activity could be restored by addition of notably Cu^{2+}, Fe^{3+} or Mn^{2+}. Co^{2+}, Mg^{2+} and Ni^{2+} had no observable effect. Both the enzymic and non-enzymic reactions were stimulated most markedly by Cu^{2+} ions (Table XV) at a concentration of one µmole (75).

TABLE XV. Activation of serine:glyoxylate aminotransferase by Cu^{2+} ions.

	E+ Cu^{2+}	E+PP + Cu^{2+}	EB+ Cu^{2+}	EB+PP + Cu^{2+}	EO+ Cu^{2+}	EO+PP + Cu^{2+}	E
Glycine formed (µmoles)	6.8	2.5	4.3	0	3.4	0	0

E = active enzyme; EB = boiled enzyme; EO = without enzyme; PP = pyridoxal-5-phosphate.

Assay system: 100 µmoles phosphate buffer, pH 8.2; 15 µmoles serine and glyoxylate; 1.0 µmoles $CuCl_2.2H_2O$; 3 µmoles pyridoxal-5-phosphate; 1.3 mg enzyme protein; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.

More glycine was formed in the presence of active enzyme and Cu^{2+} than with boiled enzyme and the metal ion. In all cases, when pyridoxal phosphate was added a retardation of activity was observed. It is suggested that pyridoxal phosphate retards the non-enzymic reaction catalysed by Cu^{2+} but these ions appear to

stimulate an enzymic reaction in addition. Mn^{2+} and Fe^{3+} also stimulated a reactivation but no attempt was made to assess the extent of this reactivation quantitatively.

(c) Effect of pyridoxal-5-phosphate

A number of attempts were made to stimulate enzyme activity by the addition of pyridoxal phosphate. No clear indication of a requirement for the coenzyme was obtained (Table XVI).

TABLE XVI. Effect of pyridoxal-5-phosphate on serine: glyoxylate aminotransferase activity.

	Hydroxypyruvate formed (µmoles/hr/mg protein)	
Enzyme	With pyridoxal phosphate	Without pyridoxal phosphate
1	3.27	2.96
2	3.12	3.67
3	4.42	4.07

Assay system: 100 µmoles phosphate buffer, pH 8.2; 9 µmoles serine and glyoxylate; 3 µmoles pyridoxal phosphate; 1.7 mg partially purified enzyme; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.

(d) Effect of pyridoxal phosphate antagonists

Isonicotinyl hydrazide (INH) has been reported (75) to inhibit

a glutamate:glycine aminotransferase and this inhibition has been attributed to the binding of this compound with the carbonyl group of pyridoxal phosphate (59). However, at a concentration of 1.5 µmoles INH did not inhibit the SGT from wheat leaves significantly (Table XIV). Since pyridoxal phosphate contains a reactive aldehyde group the effect of aldehyde-binding reagents on the activity of SGT was tested. Tris, which forms Schiff's bases (48) with aldehyde groups was found to retard the activity of SGT when the former compound was used as a buffer (see Fig.11) while phosphate ion could reverse this inhibition. Sodium bisulphite is also an aldehyde-binding agent and at a concentration of 1.5 µmoles caused slight retardation of this aminotransferase (Table XIV). The inhibition by Tris cannot however be reversed by addition of pyridoxal phosphate and inactivation of SGT by combination of Tris with the aldehyde group of pyridoxal phosphate does not explain why enzyme extracted in water alone is also inactive (see Subsection (a), Experimental Results).

(e) Serine:glyoxylate aminotransferase activity in etiolated leaves

Leaves were cultivated in a growth chamber in the dark at 21° for one week, after which time a sample of leaves was taken, the lights (1000 ft-c) switched on and further samples of leaves harvested at specified intervals up to 35.5 hr after termination of the dark period. Crude enzyme preparations were made of each

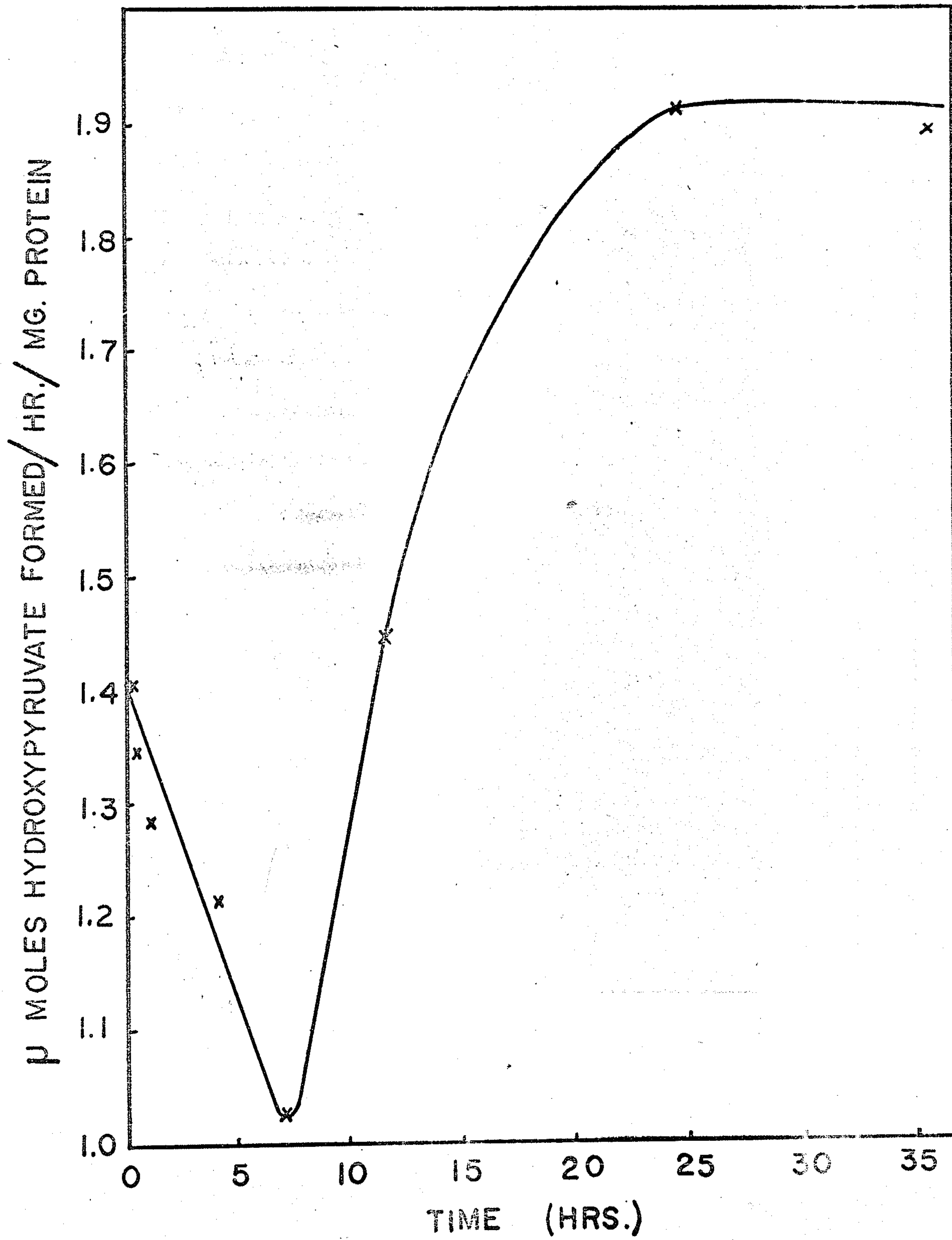
sample and enzyme activity tested (Fig. 12). Within the first seven hours of light treatment the activity per mg protein of SGT decreased to 91% that in etiolated leaves and then during a further 17 hour period increased to 140% of that value. After 24 hours the activity appeared to have reached a maximum.

(f) An unidentified ninhydrin-reacting substance in the serine:glyoxylate aminotrasferase reaction

During some experiments an unknown ninhydrin-positive spot appeared on chromatogram papers (Fig.2) when serine and glycine from an active assay system were being separated. The unknown compound had an R_f value of 0.24 in phenol:water solvent. Attempts were made to identify the compound but without success. It was thought to be β-hydroxyaspartate, a condensation product of glycine and glyoxylate, but when 45 µmoles of these latter two compounds were incubated together in the presence of SGT no ninhydrin spot with an R_f of 0.24 appeared on chromatogram paper. In addition, the R_f's of authentic β-hydroxyaspartate and γ-hydroxyglutamate, another possibility, in phenol:water (R_f 0.08 and 0.15 respectively) did not coincide with the unknown compound. In a further experiment involving 45 µmoles of glycine and β-hydroxypyruvic acid a similar spot with the same R_f value as the unknown was detected. The problem of the identity of the unknown compound remains unresolved.

Figure 12. Effect of light on serine:glyoxylate aminotransferase activity in etiolated wheat leaves.

Assay system: phosphate buffer, 100 µmoles (pH 8.2) L-serine, 9 µmoles; sodium glyoxylate, 9 µmoles; enzyme, 1.35, 1.44, 1.43, 1.8, 1.89, 1.95, 2.04, 1.91 and 2.1 mg protein after 0, 0.5, 1.0, 2.0, 4.0, 7.0, 11.5, 24.0 and 35.5 hr of exposure to light respectively; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°. Zero time in the figure is the point at which light treatment was begun.



μ MOLES HYDROXYPYRUVATE FORMED/ HR./ MG. PROTEIN
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
0
5
10
15
20
25
30
35
TIME (HRS.)

(g) Initial velocity studies of serine:glyoxylate aminotransferase

By employing the method of Lineweaver and Burk (41), the Michaelis constants of the enzyme for serine and glyoxylate were determined. Figure 13 gives the reciprocal plots of initial velocity against one or other of the substrates from which the K_M for serine was calculated as 9.0×10^{-5}M and for glyoxylate, 2.5×10^{-5}M.

For other kinetic studies the enzyme was further purified by addition to a DEAE-cellulose column (see Methods). Figure 14 illustrates that most SGT activity occurred in tubes 4-14 and protein from these tubes was combined and used in subsequent experiments. Table XVII shows that the enzyme was purified 35 times by this treatment.

TABLE XVII. Purification of serine-glyoxylate aminotransferase

Fraction	Volume (ml)	Protein (mg/ml)	Aminotransferase	
			Specific Activity (Enzyme units*/ mg protein)	Purification
Crude dialysed extract	50	8.8	3	1
Ammonium sulphate fraction (20-40%)	50	4.42	4	1.3
DEAE-cellulose fraction	2.5	1.2	105	35

* For definition of Enzyme Unit see Figure 14, p.81.

Figure 13. Effect of changing substrate concentration on the initial velocity of serine:glyoxylate aminotransferase at high concentrations of the other substrate.

A - serine concentration.

B - glyoxylate concentration

Assay system: phosphate buffer, 100 µmoles; varied substrate (as in the Fig.); fixed substrate, 45 µmoles; enzyme, 1.8 mg protein; water. Final volume: 1.5 ml. Incubation time: 30 min at 30°.

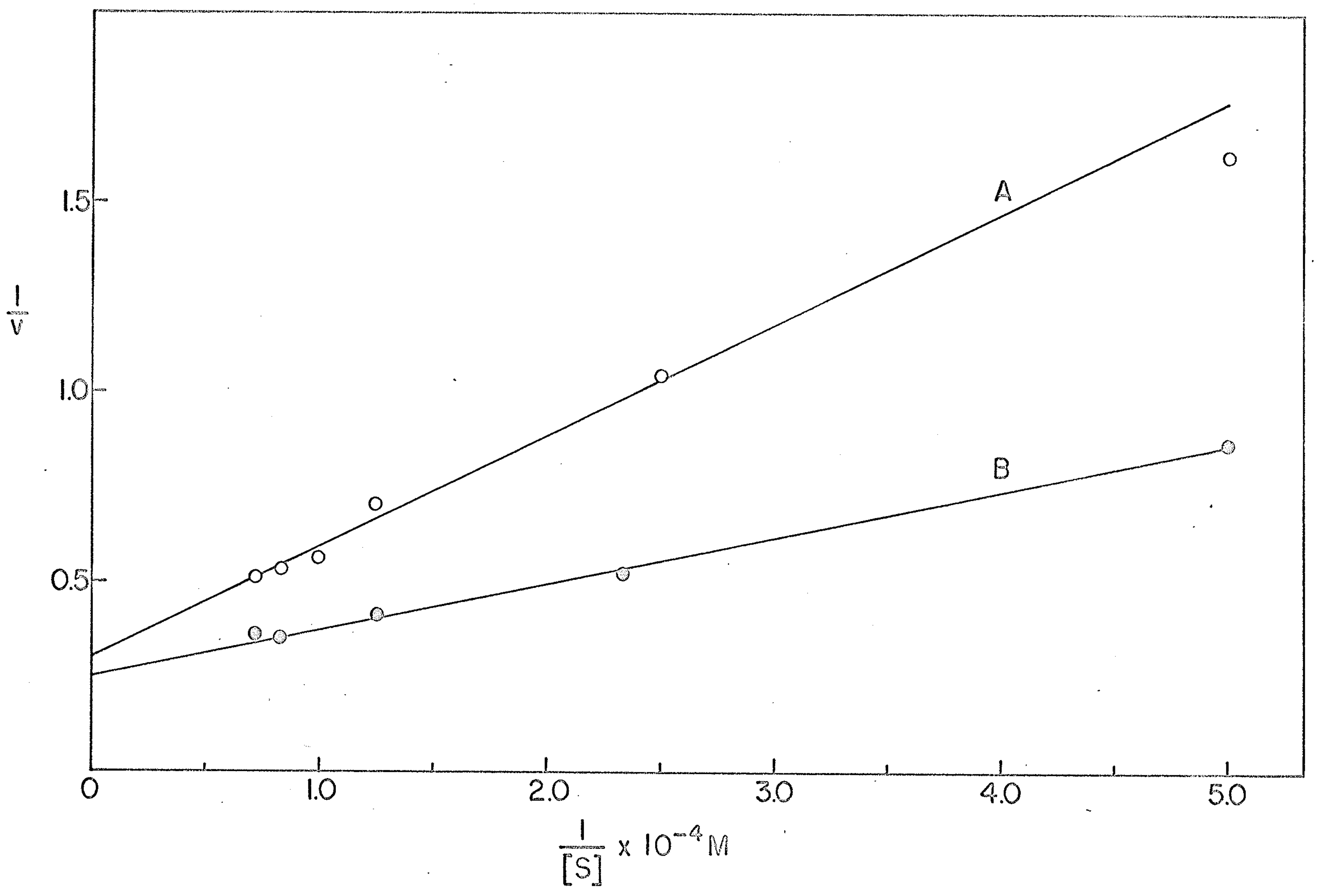
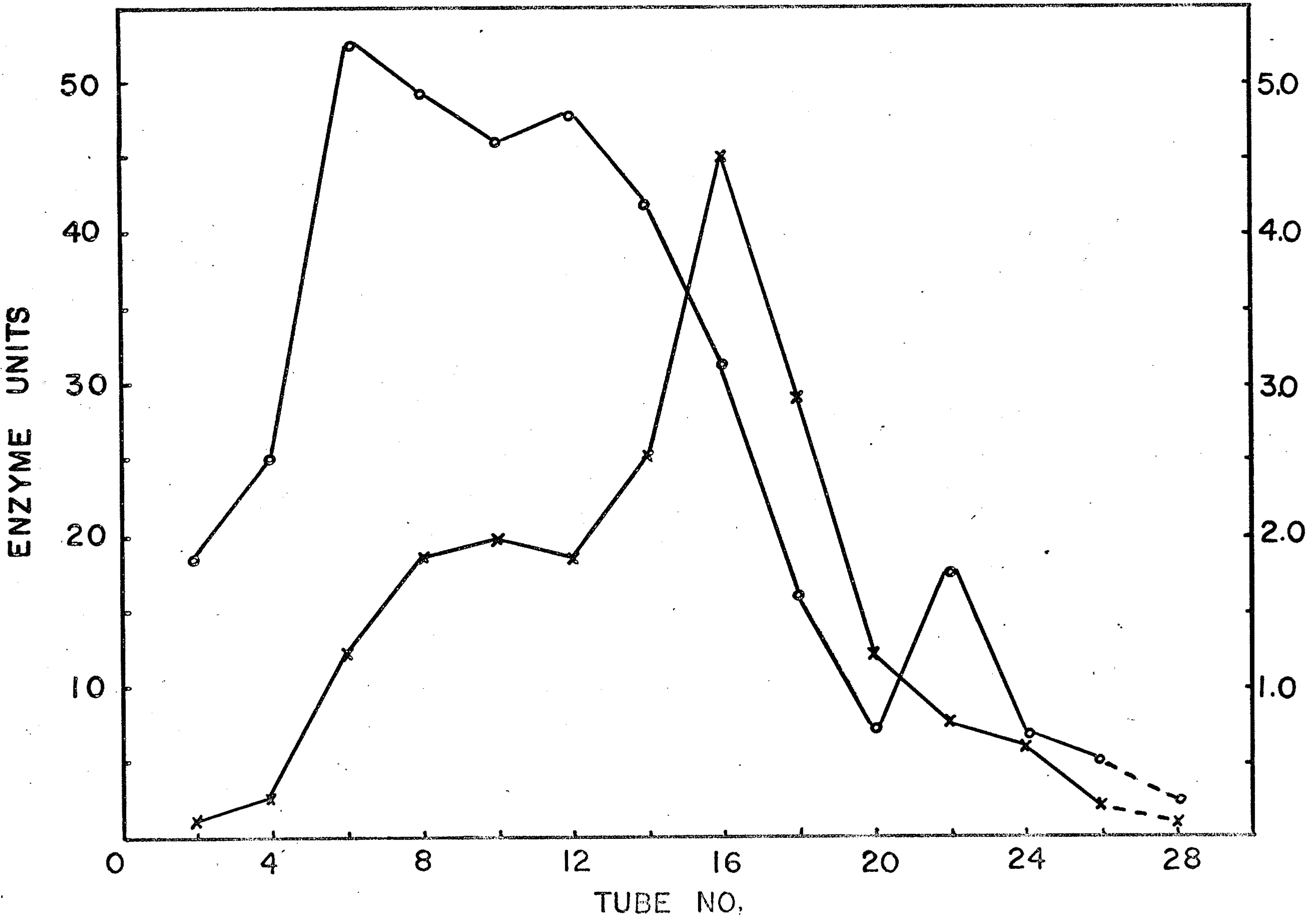

A
B
1.5
1.0
0.5
0
1.0
2.0
3.0
4.0
5.0
$\frac{1}{v}$
$\frac{1}{[S]} \times 10^{-4}$ M

Figure 14. Fractionation of a partially purified extract of serine:glyoxylate aminotransferase using a DEAE-cellulose column.

O - enzyme units

X - mg protein

One enzyme unit = drop in OD of 0.01/minute during the assay of β-hydroxypyruvate using D-glycerate:NAD oxidoreductase as assay enzyme.



ENZYME UNITS
50
40
30
20
10
0
4
8
12
16
20
24
28
TUBE NO.
MG. PROTEIN / ML. ELUATE
5.0
4.0
3.0
2.0
1.0

Using the purified enzyme a series of experiments involving the measurements of initial velocities of the forward reaction for several concentrations of one substrate at a series of constant concentrations of the other substrate were carried out. When reciprocal plots of velocity against serine or glyoxylate concentration were constructed a series of straight lines was obtained (Figs 15, 16) which intersected to the left of the ordinate and above the abscissa. The abscissa-coordinates of the two intersects gave the dissociation constants for serine (Kia) and glyoxylate (Kib) which were as follows:

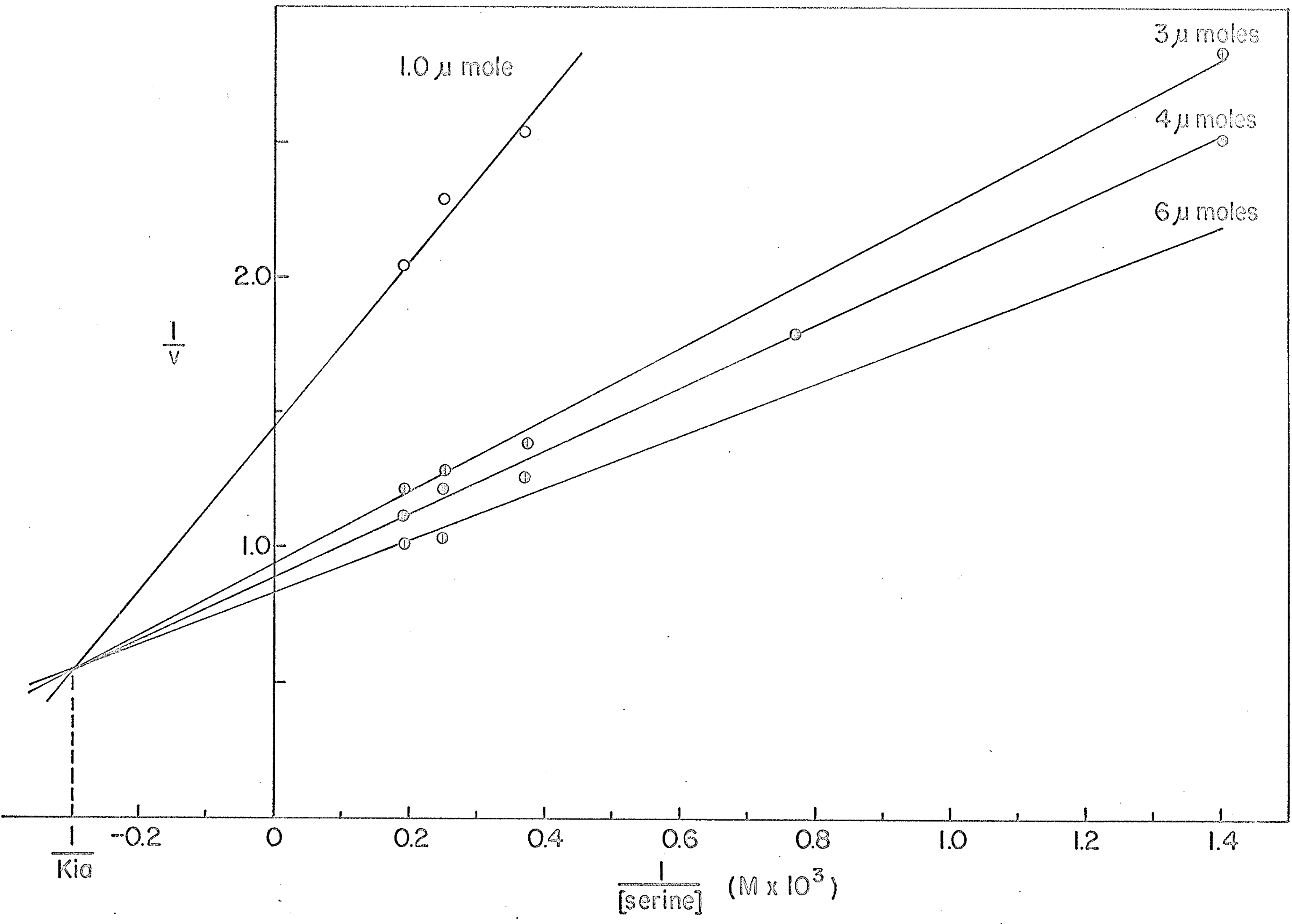
$$Kia = 3.35 \times 10^{-3} \text{ M}$$

$$Kib = 3.5 \times 10^{-3} \text{ M}$$

The kinetic data (Figs 17, 18) suggests that this reaction is of the Ordered Bi Bi type (11) and this is borne out by replots of the reciprocals of the intercepts on the ordinate ($1/V_{max}$) against reciprocals of substrate concentration (12). The replots gave a straight line relationship, a result consistent with an Ordered Bi Bi reaction (Fig. 17). In addition a straight line relationship between replots of the slopes of lines in Figures 15 and 16 against the reciprocal of substrate concentration would be expected from Ordered Bi Bi kinetic data (12). Figure 18 illustrates that such a relationship was obtained.

Figure 15. Initial velocity pattern for serine:glyoxylate aminotransferase with serine as varied substrate.

Assay system: phosphate buffer, 100 μmoles (pH 8.2); L-serine, 1.0, 2.0, 4.0, 6.0, and 8.0 μmoles; sodium glyoxylate, 1.0, 3.0, 4.0 and 6.0 μmoles; enzyme, 0.82 mg protein; water. Final volume: 1.5 ml. Incubation time: 7.5 min at 37°.



1.0 μ mole
3 μ moles
4 μ moles
6 μ moles
1/v
2.0
1.0
1/Kia
-0.2
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/[serine] (M x 10^3)

Figure 16. Initial velocity pattern for serine:glyoxylate aminotransferase with glyoxylate as varied substrate.

Assay system: phosphate buffer, 100 µmoles (pH 8.2); L-serine, 1.0, 3.0 and 4.0 µmoles; sodium glyoxylate, 1.0, 2.0, 4.0, 6.0 and 8.0 µmoles; enzyme, 0.82 mg protein; water. Final volume: 1.5 ml. Incubation time: 7.5 min at 37°.

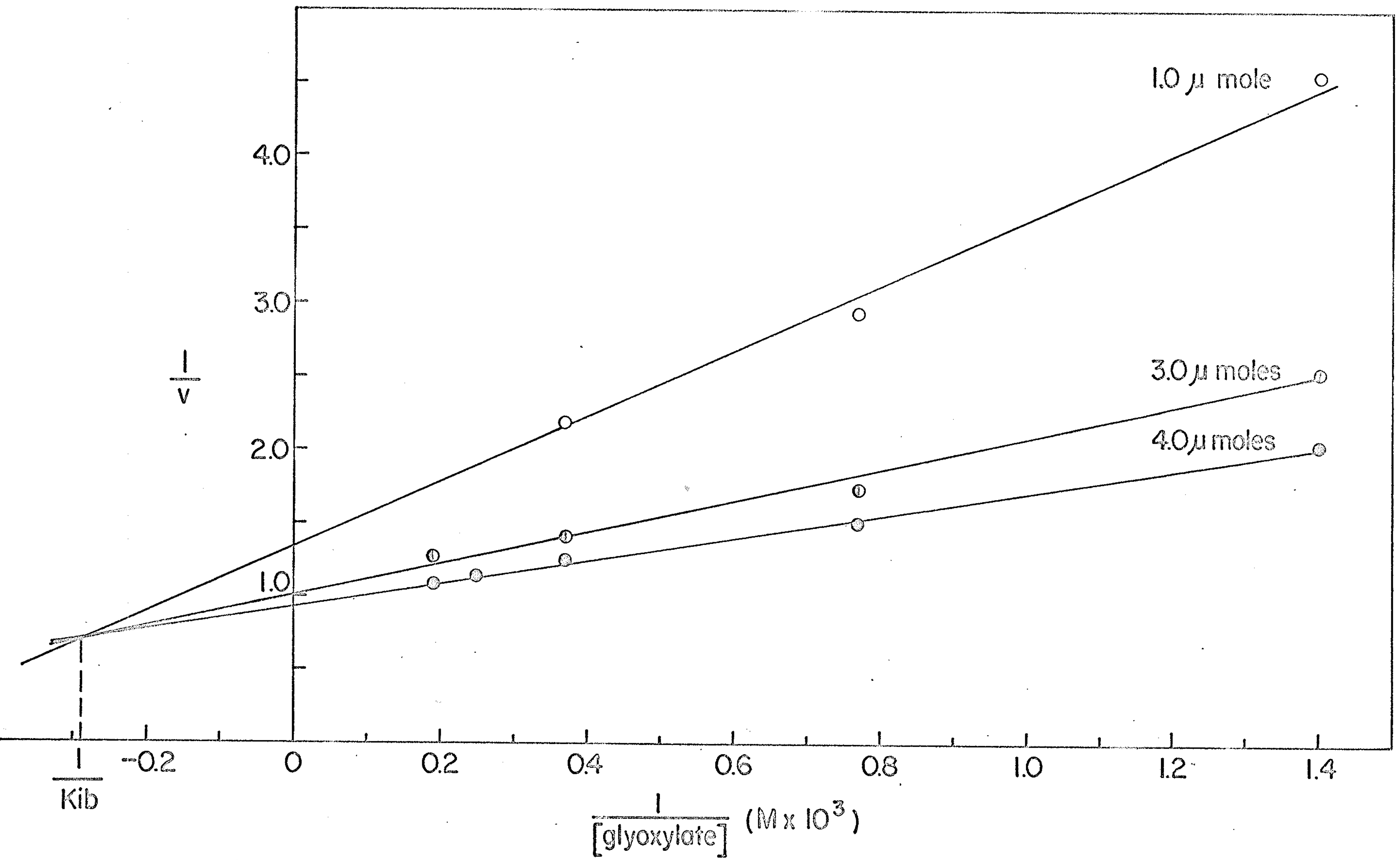
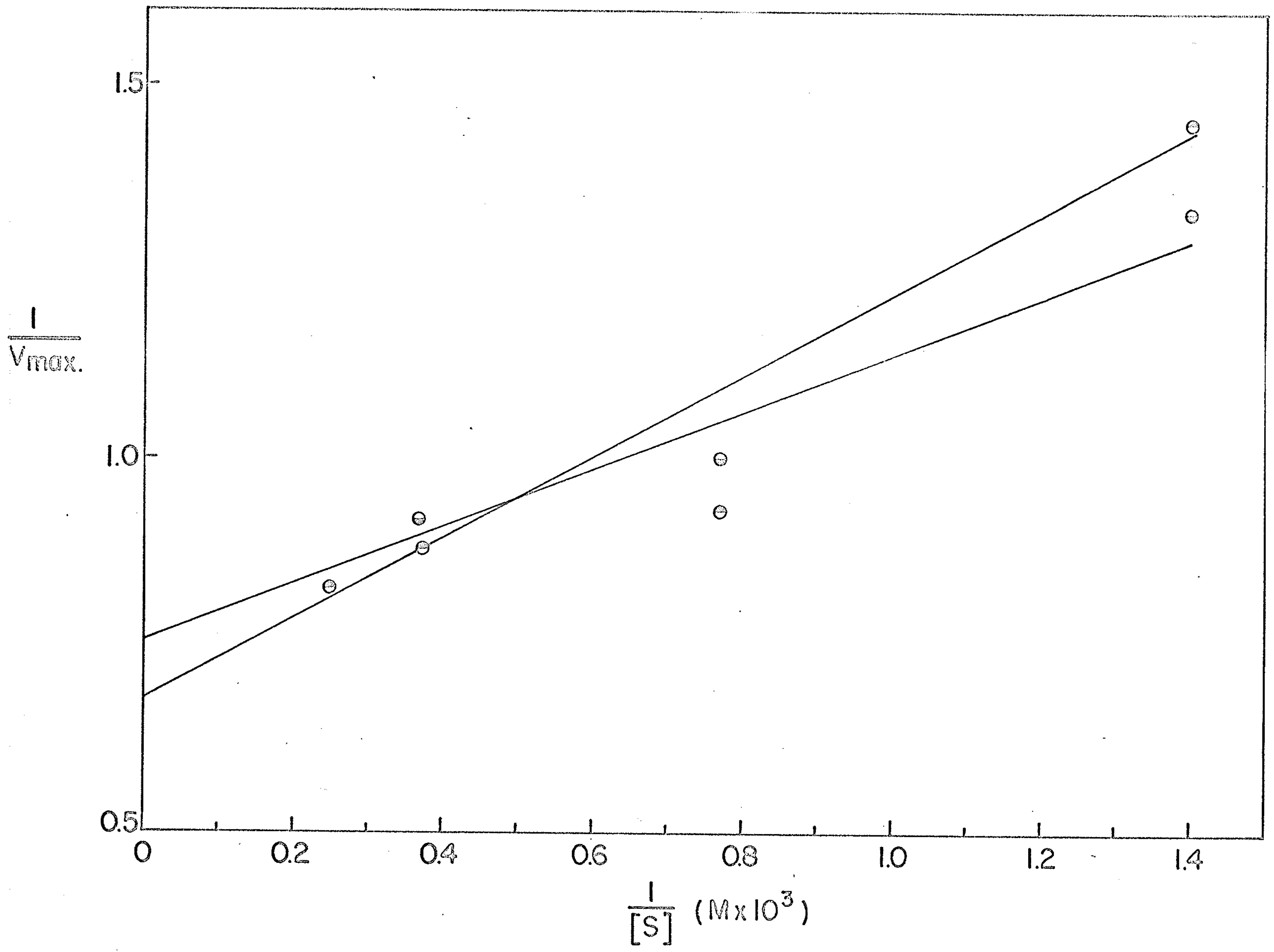

1.0 μ mole
3.0 μ moles
4.0 μ moles
1/v
4.0
3.0
2.0
1.0
1/Kib
-0.2
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/[glyoxylate] (M x 10³)

Figure 17. Replots of maximum velocity data from Figures 15 and 16.

⊖ - serine fixed variable substrate

⊖ - glyoxylate fixed variable substrate

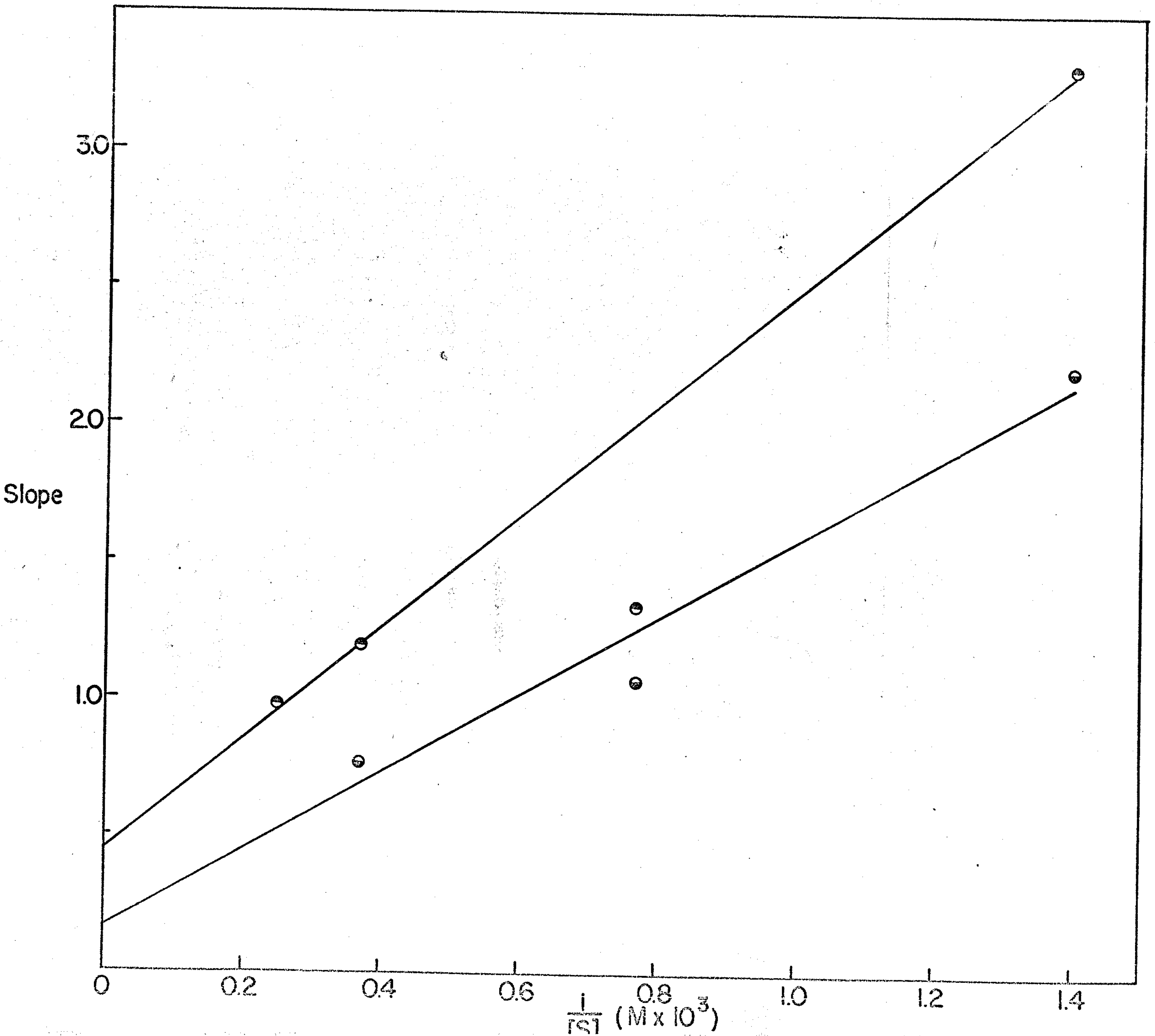


1/Vmax.
1.5
1.0
0.5
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/[S] (M x 10^3)

Figure 18. Replots of slopes of lines from Figures 15 and 16.

⊖ - serine fixed variable substrate

⊖ - glyoxylate fixed variable substrate



Slope
3.0
2.0
1.0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/[S] (M x 10^3)

V. DISCUSSION

1. GLYCINE-2-C^{14} METABOLISM

The experimental results confirmed earlier observations by Wang and Hao (97) and Wang (95) that exogenously supplied glycine-2-C^{14} is readily metabolised by detached Khapli wheat leaves and incorporated into free sugars. An average of 33 percent of the total radioactivity in water soluble substances from immediately detached leaves was located in free sugars and upon further fractionation of these sugars it was observed that the isotopic labelling was concentrated largely in sucrose and to a lesser extent in fructose and glucose confirming that sucrose is a major carbohydrate constituent in normal wheat leaves. In all experiments with detached wheat leaves over 90 percent of the total radioactivity incorporated into free sugars could be accounted for in sucrose, glucose and fructose.

When detached leaves were floated on water for three or five days under various light regimes a major derangement of glycine-2-C^{14} metabolism occurred. The incorporation of glycine-2-C^{14} into all sugars decreased, a marked shift in isotopic distribution from sucrose to fructose and glucose occurred and a 2-, 15- or 19-fold increase in sucrose, fructose and glucose levels, respectively, when compared to immediately detached leaves, was noted after five days of treatment. The isotopic shift was less pronounced in those

leaves treated under continuous illumination than in those with eight or sixteen hours light daily. In contrast, when detached leaves were floated on benzimidazole for three days under various light regimes the C^{14}-labelling pattern in free sugars closely resembled that in sugars from immediately detached leaves even though the rate of transformation of glycine-2-C^{14} to sugars often decreased more sharply in benzimidazole treated leaves than in leaves floated on water for a similar length of time and under similar light conditions, particularly in those experiments carried out in an eight or sixteen hour light regime daily. Benzimidazole treatment of detached leaves for three days also maintained more normal levels of all the sugars. Although some increase of total sugars occurred in those leaves treated with benzimidazole for three days the increase was much less than in leaves floated on water for the same length of time. It is apparent from these results that benzimidazole delays the onset of metabolic derangement in wheat leaves induced by detachement for at least three days. These observations are in agreement with the widely noted effect of some purine analogues, particularly benzimidazole and kinetin, on the metabolism of wheat leaves, namely a delaying of the onset of senescence (32,54). After five days benzimidazole appeared to be losing its influence on metabolic events and a glycine-2-C^{14} metabolism similar to that in leaves floated on water was evident in benzimidazole treated leaves. In his experiments,

Wang (95) found that when detached Khapli wheat leaves were floated on water the rate of transformation of glycine-2-C^{14} to sugars increased with time and attained a level two-fold higher than that in immediately detached leaves at approximately four days after detachment. In contrast this author found a decrease in the rate of glycine-2-C^{14} incorporation into sugars in leaves similarly treated for three and five days. The author's results also indicated a decrease in glycine-2-C^{14} transformation to sugars in leaves treated with benzimidazole whilst Wang (95) reported little or no increase in rate of transformation of glycine-2-C^{14} in similar experiments with benzimidazole. Critical examination of the techniques and results from both reports did not reveal the reasons for these quantitative differences but the differences maybe related to varying physiological states of the Khapli wheat leaves used by the two authors. However, qualitatively substantial agreement between the two reports was noted. Wang (95) found that in immediately detached leaves the carbon skeleton of glycine-2-C^{14} gave rise essentially to sucrose and to a much lesser extent to glucose and fructose whereas leaves floated on water showed a pronounced shift in isotopic distribution to glucose and fructose. Leaves floated on benzimidazole gave a similar pattern of isotopic distribution to that of the immediately detached leaves. The author's observations (Fig.1) closely paralleled those reported by Wang (95).

The increase in total sugar levels and the decrease in rate of transformation of glycine-2-C^{14} to free sugars in detached leaves gave rise to a four- or more-fold decrease in specific activities of sucrose, glucose and fructose after three days of treatment under any light regime. In contrast when leaves were treated with benzimidazole or floated on water in continuous darkness the specific activities of all sugars increased two- to three-fold. These wide divergences of specific activities amongst the sugars in benzimidazole treated leaves and leaves floated on water from those found in immediately detached leaves seemed to be due to variations in sugar levels under light or dark conditions rather than to variations in radioactivity incorporation into sugars from glycine-2-C^{14}. In continuous darkness free sugar pools were severely depleted in all experiments whilst under any light regime all sugar levels increased but in either light or dark experiments the rate of transformation of glycine-2-C^{14} did not parallel these massive changes of sugars. Indeed the rate of glycine-2-C^{14} transformation to sugars decreased whilst total sugar levels were increasing and decreasing. It appears that although specific activities varied widely the mechanism of transformation of glycine to sugars was not impaired either by detachment or by varying the light treatment. This observation was confirmed when it was noted that the ratio between the specific activities of fructose, glucose and sucrose in each experiment remained constant in all experiments under any

light or dark regime and with either immediately detached or variously treated leaves. Variations in the specific activities were due simply to production of free sugars from a source not involving any or at least very little of the radioactivity exogenously supplied as glycine-2-C^{14}.

An increase of invertase activity induced by detachment could not explain many of the observations already mentioned for the following reasons:

(i) Whilst fructose and glucose levels increased in detached leaves, sucrose was also observed to increase although to a lesser extent. It is unlikely that increase of invertase activity would give rise to an increase of sucrose levels.

(ii) The hydrolysis of radioactive sucrose to fructose and glucose should have maintained specific activities of all three sugars at a constant level and yet these activities were observed to vary markedly.

(iii) Measurements of invertase in leaves floated on water or benzimidazole for five days showed a decrease in activity of that enzyme rather than an increase.

A number of observations during other experiments in this laboratory have thrown some light on the problem of (a) the maintenance of normal metabolism by benzimidazole treatment of detached wheat leaves and (b) the rapid increase of free sugar levels and consequent decrease in specific activities of free sugars in varisouly treated,

detached wheat leaves fed glycine-2-C^{14}. Waygood (101) reported that in electron micrographs of Selkirk wheat leaves floated on water the chloroplasts changed shape, their lamellae deteriorated and electron dense particles were lost when compared to chloroplasts from immediately detached leaves. In contrast, chloroplasts from benzimidazole treated leaves had more closely spaced grana and intergrana lamellae and more electron dense particles than chloroplasts from immediately detached leaves. In parallel studies Waygood (101) found the rate of photophosphorylation to follow the improvement and deterioration of chloroplast structure with benzimidazole and water treatment. In chloroplast fragments isolated from leaves floated on water for 120 hours photophosphorylation had decreased to zero whereas in fragments from benzimidazole treated leaves the rate of photophosphorylation showed an initial rise followed by a decline to zero after 168 hours. In other experiments with Selkirk wheat leaves (S.M.Pathak, personal communication) glucose-6-phosphatase activity in chloroplasts from leaves floated on water increased to 150 percent that in immediately detached leaves after 72 hours and to 300 percent after 120 hours. In benzimidazole treated leaves after 72 hours glucose-6-phosphatase activity had decreased to 10 percent that in immediately detached leaves but then increased to 100 percent in the following 48 hours.

For purposes of speculation it is assumed that Khapli wheat

leaves react in a similar manner to the presence of benzimidazole as do Selkirk leaves, a contention supported by results from this laboratory, and (b) that other hexose phosphatases, notably fructose-6-phosphatase, follow a similar pattern of activity to glucose-6-phosphatase in detached wheat leaves. Increased photophosphorylation in leaves floated on benzimidazole for three days might be expected to give rise both to the formation of more hexose phosphates via normal photosynthetic pathways and to greater amounts of ATP. Higher levels of ATP might then cause an inhibition of phosphofructokinase (2,91) leading to an accumulation of hexose phosphates which in turn might be expected to cause an increase in free sugars, assuming hexose phosphate and free sugar pools to be in dynamic equilibrium in the cell. In support of this speculation Waygood (101) noted that in chloroplasts of Selkirk leaves treated with benzimidazole for 72 hours starch grains were present suggesting that a more active carbohydrate synthesising system was operating in these chloroplasts than in chloroplasts from immediately detached leaves. Starch is not normally found in wheat leaves. In leaves floated on water after three and five days and in benzimidazole treated leaves after five days increasing activity of hexose phosphatases could give rise to the formation of more free sugars. If it is assumed that C^{14} label from glycine-2-C^{14} is not quickly incorporated into hexose phosphates through normal photosynthetic channels but proceeds to sugars mainly by an alternative "serine-glyoxylate pathway" (98) then those sugars formed from photosynthetic

sources would be substantially non-radioactive. Increased levels of free sugars and hexose phosphates from a photosynthetic source might be expected (a) to retard the incorporation of glycine-2-C^{14} into free sugars along the "serine-gloxylate pathway" and give rise to a lowering of the specific activities of fructose and glucose and (b) enter into competition with sugars formed from glycine-2-C^{14} for the sucrose-forming mechanism causing both a lowering of transformation of labelled glucose and fructose to sucrose and an accumulation of radioactivity in these two monosaccharides, and a lowering of the specific activity of sucrose. The observation that glucose and fructose levels rose more rapidly than sucrose levels in detached leaves suggests that the anabolic pathway from fructose and glucose to sucrose is more carefully regulated than that leading to the production of these two monosaccharides possibly because of a dependence on the availability of ATP for uridine triphosphate (UTP) formation by the sucrose forming system (22,51). It is also conceivable that decreased transformation of glycine-2-C^{14} to sugars and increased formation of sugars from a non-radioactive hexose phosphate source in variously treated and detached wheat leaves in the light are two entirely independent unrelated processes in the intact cell which have an apparent connection only when compartments within the cell are broken down during the extraction of alcohol soluble substances from the wheat leaves. In one compartmentalised cellular process glycine-2-C^{14} transformation to sugars along the glyoxylate-serine

pathway in leaves floated on water declines with time and a breakdown of the sucrose forming mechanism occurs leading to an accumulation of fructose and glucose. Treatment of detached leaves with benzimidazole delays this derangement of glycine-2-C^{14} metabolism for at least three days. In other areas of the cells in leaves an improved photosynthetic process over that found in immediately detached leaves causes an increased photophosphorylation and an accumulation of massive pools of hexose phosphates and free sugars. In leaves floated on benzimidazole for five days and on water for three or five days increased hexose phosphatase activity gives rise to similar massive increases in free sugar pools. Upon extraction of alcohol soluble substances from the variously treated leaves intracellular barriers separating the two cellular processes outlined above are destroyed and radioactive sugars from glycine-2-C^{14} metabolism and sugars from non-radioactive sources are extracted and measured together. The immediate conclusion from quantitative estimations of the total sugars is that the decline of incorporation of glycine-2-C^{14} into sugars and the increase of sugars from non-radioactive sources are inversely related processes. The discussion above suggests that this may not be so. However compartmentalisation within the cell cannot explain all of the experimental results satisfactorily. It is difficult to explain on a compartmentalisation basis why the sucrose synthesising mechanism involved in the glyoxylate-serine pathway breaks down whilst the same mechanism associated with sucrose formation from

other sources is improving. The level of sucrose in leaves floated on water for five days increased two-fold whilst incorporation of glycine-2-C^{14} into sugars in the same leaves declined to near zero. It is more reasonable to assume that as fructose and glucose levels increased by phosphatase action on hexose phosphates in leaves floated on water for five days glycine-2-C^{14} incorporation into sucrose was inhibited either by feedback control at some point in the glyoxylate-serine pathway or by direct competition at sucrose forming sites between hexoses from the radioactive and non-radioactive sources. In addition Wang and Waygood (98) postulated a close connection between photosynthesis and the glyoxylate-serine pathway based on their results and those from many other laboratories. Therefore it is concluded that regulation of glycine-2-C^{14} incorporation into free sugars of detached wheat leaves is closely associated with variations in sugar levels from other sources.

2. AMINOTRANSFERASES

The preliminary chromatographic study provided evidence for the presence of five aminotransferases and a weakly active AsGT in extracts from wheat leaves. All of these have already been demonstrated in preparations from other plants as well as animal and microbial sources. Extraction of these enzymes from wheat leaves can be accomplished with phosphate buffer at either pH 5.7,7.0 or 8.0 but their activity was significant

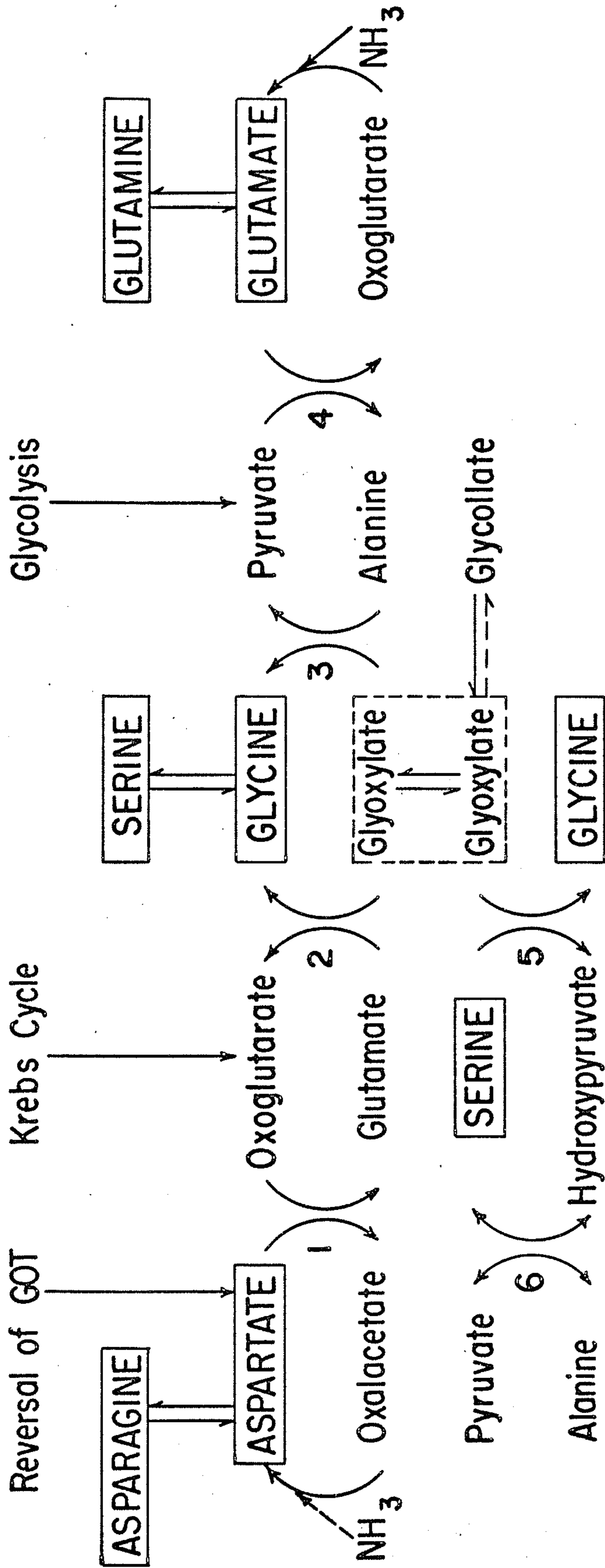
only at higher pH values. Non-enzymic formation of glycine could not be detected in the pH range tested.

The synthesis of glycine in wheat leaves can be accomplished by any one of the four glyoxylate transaminases demonstrated in this study, but to a much lesser extent by the weakly active AsGT. However, aspartate could be an important donor of the amino group of glycine by a coupled reaction involving GOT and GGT (reactions 1 and 2, Fig.19). Glutamate can also donate its amino group to glycine directly through GGT or indirectly in an analogous coupled reaction involving AGT and GPT (reactions 3 and 4, Fig.19). A source of α-ketoglutarate from the Krebs cycle is probably essential for the aspartate amino transfer but pyruvate is not necessarily required for the glutamate aminotransfer. The reaction involving serine:glyoxylate amino transfer operates in competition with AGT and GGT for the available glyoxylate. Figure 19 summarises these reactions schematically and shows the central position occupied by glyoxylate which provides the carbon skeleton of glycine and serine.

The synthesis of glyoxylate is still something of a puzzle. It is thought to arise as a fragment from ribulose diphosphate in the photosynthetic carbon cycle (2,84,103). Large quantities of labelled glyoxylate appear in wheat seedlings when fed allantoin, a product of purine catabolism (40). Synthesis of glyoxylate via isocitrate lyase is probably excluded (40) since this enzyme is

Figure 19. Pathways of amino group transfer from the aspartate glutamate and serine metabolic pools to the glycine pool in wheat leaves mediated by the following amino transferases (1) glutamate: oxalacetate, (2) glutamate:glyoxylate, (3) alanine: glyoxylate, (4) glutamate:pyruvate, (5) serine: glyoxylate, (6) alanine:hydroxypyruvate.

N.B. Glutamate can transfer its amino group directly to glycine by enzyme (2) and therefore a source of pyruvate is not essential.


Reversal of GOT
Krebs Cycle
Glycolysis
ASPARAGINE
ASPARTATE
SERINE
GLYCINE
GLUTAMINE
GLUTAMATE
NH_3
Oxalacetate
1
Oxoglutarate
Glutamate
2
3
Pyruvate
Alanine
4
Oxoglutarate
NH_3
Glyoxylate
Glyoxylate
Glycollate
Pyruvate
Alanine
6
SERINE
Hydroxypyruvate
5
GLYCINE

associated with organs having a large fat-carbohydrate turnover (10). It has never been demonstrated in leaves and in this laboratory it has not been found in wheat leaves (Waygood - unpublished data).

Wang and Waygood (98) suggested a pathway of serine-glyoxylate metabolism involving serine:glyoxylate aminotransferase which they demonstrated as a key enzyme in this cycle. This reaction is discussed more thoroughly later but one of the products, β-hydroxypyruvate, has been shown in these initial studies to inhibit GPT, GGT and AGT which are essential for the transfer of amino groups from aspartate and glutamate to glycine. While these experiments indicate inhibition of AGT this may be more apparent than real since a reversible alanine:hydroxypyruvate aminotransferase has been observed in wheat leaves by Willis and Sallach (106) and clearly demonstrated by Wang (personal communication). This enzyme would compete for the alanine of the AGT system and although there was no evidence of serine formation when β-hydroxypyruvate was added, this possibility cannot be excluded and the enzyme system has been included in the scheme of Figure 19. The same considerations may also apply to the inhibition of GPT and GGT by β-hydroxypyruvate. It is unlikely that β-hydroxypyruvate would normally accumulate to any extent in wheat leaves with alanine:hydroxypyruvate aminotransferase and an active glycerate:

NAD oxidoreductase (82), the equilibrium of which favors the utilisation of β-hydroxypyruvate and which has been suggested as an enzyme involved in carbohydrate synthesis in wheat leaves (98). However, this would not necessarily be the case with senescent wheat leaves which accumulate relatively large quantities of glyoxylate (39) and which subsequently synthesise carbohydrate from β-hydroxypyruvate by the serine:glyoxylate pathway.

It is suggested that normally glycine and serine, the latter synthesised by 'hydroxymethyltransfer' (17,34,45,83,105), arise from the major pools of aspartate and glutamate by transamination with glyoxylate, reactions mediated by both pyridoxal phosphate dependent and independent aminotransferases (Fig.19). Small amounts of β-hydroxypyruvate would be converted to serine by the pyridoxal phosphate dependent alanine:hydroxypyruvate aminotransferase. Any increased production of glyoxylate, during senescence, with probably an accompanying loss of pyridoxal phosphate, would be aminated preferentially by the pyridoxal phosphate independent serine: glyoxylate aminotransferase resulting in an increased production of β-hydroxypyruvate, part of which is channeled to carbohydrates via glycerate:NAD oxidoreductase. The remainder may be sufficient to "inhibit" the transfer of amino groups from aspartate and glutamate to glyoxylate via GGT thus conserving these metabolic pools. Also a glutamate:hydroxypyruvate aminotransferase, if present, may be inhibited by lack of coenzyme.

That some aminotransfer of this kind can occur in plant extracts was shown when γ-hydroxyglutamate inhibited SGT in the forward direction. The competition by serine:glyoxylate aminotransferase for glyoxylate is illustrated in Figure 19 and also represents further evidence for an alternative pathway for carbohydrate synthesis via β-hydroxypyruvate (98).

Figure 19 also includes the relationship of these amino-transfer systems to the asparagine and glutamine pools and also the reactions involved in the de novo synthesis of glutamate and aspartate from NH_3 and their respective carbon skeletons. While L-glutamate:NAD oxidoreductase (deaminating), which catalyses the synthesis of glutamate from α-ketoglutarate and NH_3 is well known in plants (80). There is no evidence as yet for the existence in plants of its counterpart with aspartate as the end-product (hence the dotted line from NH_3, Fig. 19). However, there is no doubt that GOT with an equilibrium in favor of aspartate formation represents a major pathway for its synthesis in plants. The aspartate pool may be built up rapidly prior to any utilisation by GOT in glycine synthesis or the enzyme may be spatially separated into different compartments of the cell (79,107).

The absence of AGT and lower activity of SGT from etiolated seedlings and leaves respectively indicates some relationship to glycollate:O_2 oxidoreductase which is confined to green leaves (84, 85).

A further investigation of serine:glyoxylate aminotransferase from wheat leaves showed that the enzyme had some unusual properties not shared by any other aminotransfer reaction so far recorded. Whilst the forward reaction proceeded to only one-third to one-half completion no reverse reaction could be detected. The irreversibility seemed to be real as the assay methods used were sufficiently accurate to detect any significant degree of serine or glyoxylate formation. Other results from this laboratory indicated that 0.08 µg of glycine and 0.12 µg of serine can just be detected on chromatography paper which in experiments by the author using 45 µmoles of glycine and β-hydroxypyruvate would be a 3×10^{-5} percent conversion of glycine to serine. No serine was detected in these experiments. The 2,4-dinitrophenylhydrazone method of Towers and Mortimer (89) for the detection of keto acids is also of considerable accuracy and yet again in SGT assays involving 45 µmoles of glycine and hydroxypyruvate no gloxylate was detected when 2,4-dinitrophenylhydrazones were prepared. These results indicate that if SGT is a reversible reaction the equilibrium must be greatly towards glycine formation. Reversibility of glyoxylate aminotransferases, as reported in experimental results recorded here and from other laboratories, is by no means certain. McCurdy and Cantino (46) with an alanine:glyoxylate aminotransferase from _Blastocladiella emersonii_ could not demonstrate true reversibility. Pitts _et al_ (68) have shown that extracts of _Escherichia coli_ possess an irreversible glutamate:

glyoxylate aminotransferase and in this laboratory Fegol (23) studied alanine: glyoxylate aminotransferase in wheat leaves which was not easily reversible. Recently, however, Cossins and Sinha (16) observed some reversal of an aminotransferase involving glyoxylate and several amino acids and Willis and Sallach (106) reported the formation of serine from glycine and β-hydroxypyruvate in their investigation of an alanine:β-hydroxypyruvate aminotransferase from plant tissue. Whilst reports are conflicting, all indicate the equilibria of glyoxylate:aminotransfer reactions to be in favor of glycine formation.

The SGT from wheat leaves was activated by phosphate ions with a maximum activation between 0.03 and 0.04 M phosphate concentration. The mechanism of phosphate activation is obscure but Nisonoff and Barnes (64) observed a similar activation of a glutamate:aspartate aminotransferase from hog heart by 0.033 M sodium or potassium phosphate and they suggested that phosphate ions may act as a catalyst in some aminotransfer reactions. This may be true of SGT from wheat leaves.

All of the glyoxylate aminotransferases (AGT, SGT, GGT, AsGT) studied were not activated by pyridoxal phosphate. Neither were the reversible enzymes GOT and GPT activated by pyridoxal phosphate in the dialysed preparations although these enzymes from wheat germ (18) and sunflower cotyledons (80) and other plant and animal sources have been shown to have a vitamin B_6 requirement. Pyridoxal

phosphate is firmly bound to these enzymes and although Smith and Waygood (80) found GOT unsaturated with respect to the coenzyme in healthy sunflower cotyledons, the reverse is true of wheat leaves (23). Other reports (9,16,46,60,75,107) give conflicting evidence of the participation of pyridoxal phosphate in glyoxylate aminotransferases. In its present state of purity SGT from wheat leaves is not activated by exogenously added pyridoxal phosphate. Experiments with aldehyde-binding agents such as sodium bisulfite suggest that the coenzyme may be involved but the bisulfite may also be causing inhibition by binding with the aldehyde group of one of the substrates, glyoxylate. The fact that isonicotinylhydrazide which binds to the carbonyl group of pyridoxal phosphate (75), did not have any inhibitory effect adds support to this view. Tris-hydroxymethylamino methane (Tris) completely inhibited the enzyme and this too is an aldehyde-binding agent but this inhibition could not be reversed by addition of pyridoxal phosphate, only by phosphate ions. The fact that the enzyme was similarly inactive when extracted in distilled water does not support the contention that Tris is acting by binding the free aldehyde group of pyridoxal phosphate. Further purification of SGT is necessary to clarify the role of a coenzyme in this reaction and it may be pertinent to recall once again the statement by Meister (48) that, "it is conceivable that transaminase reactions involving aldehydes (e.g., glyoxylic acid) may not involve vitamin

B_6". Although the results in this report do not unequivocally indicate that all glyoxylate:aminotransferases are independent of pyridoxal phosphate this is considered possible, especially in the case of AGT, GGT and SGT in plants.

SGT was inhibited slightly by 8-hydroxyquinoline and completely by dialysis against 0.6 percent EDTA. Since these compounds are metal chelators it is possible that a metal ion is involved in this aminotransferase reaction. The participation of metals in transaminase activity has not been well established. That Cu^{2+}, Al^{3+} and Fe^{3+} activate non-enzymatic transamination is known (24,25,29,55,61) but the role of metals in enzymatic transamination is still a controversial one. Cammarata and Cohen (8) and Cohen (13) were of the opinion that purified glutamic:aspartic aminotransferase could be activated by a metal ion, possibly Fe^{2+} or Mg^{2+}, but they did not demonstrate metal participation in their preparations unequivocally. Patwardhan (67) found that glutamic: aspartic aminotransferase from Dolichos lablab was activated by Fe^{2+} and that the metal ion had no effect on non-enzymatic aminotransfer. In the case of Phaseolus radiatus glutamic:glycine aminotransferase, activation by Mn^{2+}, Mg^{2+} and Fe^{2+} appears to be well established (75). Some activity of SGT from wheat leaves could be restored after EDTA dialysis by addition of notably Cu^{2+}, Fe^{3+} and Mn^{2+} and both the enzymic and non-enzymic reactions were stimulated most markedly by Cu^{2+} ions. Pyridoxal phosphate completely

inhibited the non-enzymic reaction stimulated by Cu^{2+} but in the presence of active enzyme considerable aminotransferase activity remained even though pyridoxal phosphate concentration was greater than that of the metal ion. This suggested that some Cu^{2+} was bound to the enzyme and could not be removed by added pyridoxal phosphate and was acting as a cofactor in a truly enzymic reaction. However, unequivocal evidence for metal ion participation in this reaction was not shown. An enzyme cannot be classified as metal ion activated simply by showing that its activity is increased by metal ions. As pointed out by Malmstrom and Rosenberg (43) this is a necessary but insufficient criterion. The metal can influence the reaction rate without playing an intrinsic part in the mechanism. A thorough study must be made of the kinetics and stability of all possible complexes especially since the most active ion in these studies was Cu^{2+} which also stimulates the non-enzymatic reaction of this and other amino-transfer reactions (24,25,55), markedly. The metal ion may for instance, be removing an enzyme inhibitor. Three main roles have been visualised for a metal ion coenzyme (35) namely:

(i) It may be necessary to induce or maintain the active conformational state of the protein molecule.

(ii) It may have a purely co-ordinative role by acting as a bridge in the formation of a ternary complex between substrates and enzyme protein surface.

(iii) It may be directly involved in changing the molecular structure of the substrate molecule. Snell (81) points out that metal ions may be involved in some enzymatic reactions involving pyridoxal phosphate. Scardi et al (76) found that when an aspartate aminotransferase from pig heart was partially separated from its coenzyme pyridoxamine phosphate the residual activity of the apoenzyme when the latter was incubated with its substrates for 30 minutes was directly related to phosphate ion concentration in the assay medium. As phosphate ion concentration increased, residual activity of the apoenzyme decreased. This was ascribed to a competition between phosphate ions and the phosphate group of pyridoxamine phosphate for the cationic site of the apoenzyme. In SGT from wheat leaves where pyridoxal phosphate involvement is doubtful and where phosphate ions are known to activate the enzyme, it is conceivable that phosphate ions occupy the cationic site of the apoenzyme and in association with a metal ion simulate the role played by pyridoxalphosphate in other aminotransferases. The SGT reaction involves a compound, glyoxylate, with a highly reactive aldehyde group and a phosphate ion-metal ion complex acting as a bridge to bind the substrate glyoxylate to the enzyme surface may be sufficient to bring about aminotransfer reaction between glyoxylate and serine. Hydroxypyruvate is also a highly reactive compound and it is not clear why this aminotransferase reaction cannot be reversed. More information about active sites

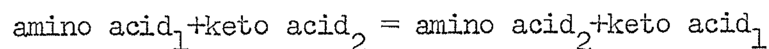
on the enzyme protein are required before questions about the mechanism of the reaction can be answered.

Kinetic data of a more pure SGT from wheat leaves revealed an enzyme mechanism differing from that normally ascribed to aminotransferases. According to Cleland (11) aminotransferase reactions are of the Ping Pong Bi Bi type (Cleland's nomenclature) and this was borne out after further work by the same author (28) using a glutamic:oxalacetate aminotransferase from pig heart. Characteristics of the Ping Pong Bi Bi mechanism are (a) that the enzyme oscillates between two stable forms (designated E and F (11)) and (b) that graphic plots of the reciprocals of the initial velocities against the reciprocals of one substrate at a series of fixed concentrations of the other substrate gives a family of parallel straight lines (28). Such a pattern indicates that the substrates are not reversibly connected because of their addition to two stable forms of the enzyme. During kinetic studies of SGT from wheat leaves it was found that plots of reciprocals of initial velocity data against reciprocals of one substrate concentration at a series of fixed concentrations of the other substrate gave a family of lines intersecting to the left of the ordinate and above the abscissa (Figs. 15, 16). Such data is characteristic of either an Ordered or Random Bi Bi reaction (11) in which the two substrates (serine and glyoxylate) react at two points in the sequence connected by reversible reactions.

Intersecting plots do not give any information as to the order of addition of the two substrates because both random and sequential mechanisms show the same effect. However replots of the reciprocals of V_{max} (Fig. 17) or slopes (Fig.18) of lines from Figures 15 and 16 against the reciprocals of the fixed, variable substrates should be linear (12) according to an Ordered Bi Bi mechanism. Examination of the rate equation for Ordered (11) and Random (108) Bi Bi reactions shows that only in the case of an ordered mechanism would there be linear replots as found in Figure 18 and Figure 19. The rate equation for the Random Bi Bi reaction is as follows (21):

$$v = \frac{(c_1ab+c_2a^2b+c_3ab^2-c_4z-c_5az-c_6bz)e}{c_7+c_8a+c_9b+c_{10}ab+c_{11}a^2+c_{13}a^2b+c_{14}ab^2+c_{15}z+c_{16}az+c_{17}bz+c_{18}abz}$$

where v is initial velocity; ab are substrate concentrations; z is product concentration; and c, rate constants. The presence of terms involving squares of substrate concentrations would have given rise to non-linear replots of data in Figures 15 and 16. The fact that linear relationships were established suggests that the mechanism is sequential not random. The implications of this data are that this reaction whilst falling into the group of reactions known as transferases (21) does not have the characteristics of true aminotransferases. It may still be termed an aminotransferase since the reaction follows the pattern:



$$\text{amino acid}_1+\text{keto acid}_2 = \text{amino acid}_2+\text{keto acid}_1$$

characteristic of transaminations. The kinetic data and irreversible nature of SGT point to an aminotransferase reaction differing fundamentally from other reported transaminations. Final decisions regarding the mechanism of this reaction must however await further purification of the enzyme, and possibly other glyoxylate: aminotransferases, and the elaboration of a more rapid and reliable assay system. The two stage assay method used in these kinetic studies did not lend itself to an unequivocal determination of the reaction mechanism because of the number of possible areas where errors in measurement of β-hydroxypyruvate could have occurred and also where the reactions involving one or other of the substrates or products could conceivably be proceeding at the same time. Stoichometric measurements of the forward reaction showed that not all substrates and products could be fully accounted for. The failure to recover theoretical amounts of the substrates and products could have been due to experimental error or to disappearance of substances in alternative reactions. A possible source of loss was in the formation of the ninhydrin-positive compound (Fig.2) whose identity is still unresolved. Mix (55) found an aldo-like condensation between glycine and glyoxylate to give β-hydroxyaspartate in the presence of Cu^{2+}. Sallach and Peterson (74) showed the formation of β-hydroxyaspartate from dihydroxyfumarate and L-glutamate in acetone powder extracts of sheep tissues and the acid has been synthesized chemically by Kornguth and

Sallach (37). Since both glycine and glyoxylate are involved in SGT it was believed that β-hydroxyaspartate was being formed during the prolonged aminotransferase assay. However, chromatography of an authentic sample of this acid in phenol:water gave an R_f of 0.08, very different from that of the unknown compound with an R_f of 0.24 in the same solvent. On the other hand, Tominaga et al (88) report the isolation of β-hydroxyasparagine from normal human urine which in phenol:water solvent had an R_f, of 0.23 and which upon acid hydrolysis gave a new ninhydrin-positive compound with an R_f of 0.07. The R_f of β-hydroxyasparagine agrees closely with that of the unknown but gave a characteristic orange ninhydrin-positive reaction whereas the unknown compound gave a clear purple spot. The possibility that it is β-hydroxyasparagine cannot, however, be finally ruled out. In a further search for clues to the identity of this unknown compound it was noticed that γ-hydroxyglutamic acid resembles a condensation product of glycine and β-hydroxypyruvate in that both would have five carbon chains, two carboxyls and one hydroxyl group. In experiments with the reverse reaction of SGT the ninhydrin-positive spot at R_f 0.24 did appear and in addition although no reverse reaction could be detected in the presence of active enzyme some β-hydroxypyruvate was disappearing. Chromatography of an authentic sample of γ-hydroxyglutamate in phenol:water produced a ninhydrin-positive spot with an R_f of 0.15 agreeing closely with that of Virtanen and Hietala (92) whose γ-hydroxyglu-

tamate from <u>Phlox decussata</u> had an R_f of 0.17 in phenol:NH_3. Whilst this R_f value agreed more closely with that of the unknown compound than did that of β-hydroxyaspartate the values were not sufficiently close to warrant a constructive comparison. Although the identity of this compound remains unsolved it may be associated with the inhibition of the forward reaction of SGT from wheat leaves. The reason for the forward reaction proceeding to only ca one-third to one-half completion is also unsolved but may be due to the formation of a complex between the products of the reaction on the enzyme surface at critical concentrations of the two products thus blocking any further reaction. As mentioned already hydroxypyruvate is a highly reactive compound and may form a complex with glycine which cannot be broken down by the enzyme and which may block active sites on the enzyme protein surface. However by increasing enzyme concentration the reaction might be expected to proceed further towards completion. Examination of enzyme concentration data did not reveal a linear relationship between the degree of product formation and the amount of enzyme protein present in the assay mixture, beyond a certain point, and the inhibition is assumed to be for some more complex reason e.g. an interaction between the transaminase and other proteins in the relatively crude enzyme preparations (64).

3. CONCLUSION

These experimental results further confirm the operation of a

pathway leading from glycine-2-C^{14} to sugars in wheat leaves (98) and a key enzyme in this pathway, serine:glyoxylate aminotransferase, was clearly demonstrated. That this enzyme has an equilibrium favoring glycine formation exclusively suggests that Wang and Burris (96) were correct in assuming that glycine is synthesised through one metabolic reaction and oxidised by another in this pathway. Studies with other aminotransferases show how the glyoxylate-serine pathway might be integrated with amino acid and keto acid pools in the cell.

VI SUMMARY

1. Exogenously supplied glycine-2-C^{14} was readily incorporated and metabolised to free sugars in immediately detached, water floated and benzimidazole treated detached Khapli wheat leaves under various light regimes over a period of five days.

2. The free sugars were isolated by resin column chromatography, separated by paper chromatography and the specific activities of the most abundant of them, namely sucrose, fructose and glucose, determined.

3. The rate of transformation of glycine-2-C^{14} to and isotopic distribution among the sugars was affected by five-day detachment but those leaves treated with benzimidazole in the light for three days had a metabolism more nearly like that in immediately detached leaves. In immediately detached leaves glycine-2-C^{14} was incorporated almost exclusively into sucrose whilst after five days of detachment under any light regime after either water or benzimidazole treatment a shift of isotopic distribution to fructose and glucose was observed. In all experiments, except those carried out in complete darkness, specific activities of sucrose, glucose and fructose decreased markedly after three days of detachment and remained low for the following two days, of treatment.

4. The metabolic derangement in glycine-2-C^{14} metabolism caused

by prolonged detachment was associated with increased formation of sugars from a non-radioactive source in the light, leading to a retardation of glycine-2-C^{14} incorporation into sugars.

5. Speculations were made on the mechanism of this impediment of glycine-2-C^{14} incorporation into free sugars.

6. Chromatographic studies were made on a number of aminotransferases in crude extracts of wheat leaves and discussed in relation to glycine metabolism.

7. A more thorough investigation of the partially purified serine:glyoxylate aminotransferase in Selkirk wheat leaves was undertaken.

8. The SGT reaction was found to be irreversible, no requirement for the coenzyme pyridoxal phosphate could be shown, some evidence of metal and phosphate ion involvement was obtained and kinetic data suggested that the enzyme mechanism was not typical of aminotransferase reactions in general.

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