MODE OF ACTION OF N^6-BENZYLADENINE IN THE INHIBITION OF RESPIRATION IN HIGHER PLANTS WITH SPECIAL REFERENCE TO BROCCOLI (*BRASSICA OLERACEA* VAR *ITALICA* CV. SPARTAN EARLY)

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Virendra Tuli

1964

THESIS

This is to certify that the

thesis entitled

Mode of Action of N^6-benzyladenine in the Inhibition of Respiration in Higher Plants with Special Reference to Broccoli (Brassica Oleracea var Italica cv Spartan Early).

presented by

Virendra Tuli

has been accepted towards fulfillment
of the requirements for

PhD degree in Horticulture

S. H. Wittwer

Major professor
S.H. Wittwer

Date July 29, 1964

O-169

ABSTRACT

MODE OF ACTION OF N^6-BENZYLADENINE IN THE INHIBITION OF RESPIRATION IN HIGHER PLANTS WITH SPECIAL REFERENCE TO BROCCOLI (*BRASSICA OLERACEA* VAR *ITALICA* CV. SPARTAN EARLY)

by Virendra Tuli

The mode whereby N^6-benzyladenine (BA), an active kinin, inhibits respiration of higher plants was studied with broccoli and cauliflower explants. Post harvest applications of BA (5×10^{-5} M) delayed chlorophyll and carotene breakdown of broccoli heads and leaves. This was associated with a delay in the onset of senescence and with respiration inhibition. Effects of BA on respiration were studied using broccoli and cauliflower mitochondria, isolated by fractional centrifugation. Adenosine triphosphate (ATP) was found essential for mitochondrial activity of both species when succinate was provided as the substrate. The presence of 3.3×10^{-5} M BA markedly inhibited the oxidation of succinate by the isolated mitochondria. The greater retention of chlorophyll and carotene resulted in higher photosynthetic $^{14}CO_2$ fixation by treated broccoli leaves. Distribution of ^{14}C among the various photosynthetic products was determined by autoradio-chromatography. Results suggested that the activity of certain phosphorylating enzymes (kinases) was impeded by BA.

An *in vitro* system involving hexokinase, ATP, Mg^{2+}, cysteine, glucose, glucose-6-phosphate dehydrogenase and

nicotinamide-adenine dinucleotide phosphate ($NADP^+$) indicated that the reduction of $NADP^+$ was greatly slowed by $3.3x10^{-5}$ M BA. This resulted from an inhibition of hexokinase activity. It is suggested that structural similarities between ATP and BA resulted in a competitive inhibition for an active site on hexokinase. The nature of inhibition was established by the use of classic enzyme kinetics. ATP also participates in the enzymic synthesis of glutamine, catalyzed by glutamine synthetase. The ineffectiveness of BA on a 10-fold purification of the enzyme from cauliflower suggested, however, that the inhibition was specific for the kinases. Specificity of BA for the kinases was further established by studying its effects on another in vitro system involving pyruvic kinase, adenosine diphosphate (ADP), K^+, Mg^{2+}, phospho(enol)pyruvic acid, lactic dehydrogenase and reduced nicotinamide-adenine dinucleotide (NADH). Here the rate of disappearance of NADH at 340 mμ was greatly reduced by the presence of $3.3x10^{-5}$ M BA. This was from an inhibition of pyruvic kinase activity. It was further established by enzyme kinetics that BA competed with ADP for an active site on pyruvic kinase.

It is proposed that post harvest applications of BA at the above mentioned concentrations delay senescence in broccoli by inhibiting respiration via a possible interference with the activity of the kinases. The structural similarity between ATP, ADP and BA suggests a competitive

inhibition of the nucleotides by BA for an active site on the enzyme surface. Since most kinases in respiration are involved in glycolysis, this is probably where the inhibition occurs.

MODE OF ACTION OF N^6-BENZYLADENINE IN THE INHIBITION OF RESPIRATION IN HIGHER PLANTS WITH SPECIAL REFERENCE TO BROCCOLI (*BRASSICA OLERACEA* VAR *ITALICA* CV. SPARTAN EARLY)

By

Virendra Tuli

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1964

Dedication

To my parents Ram and Mohini Tuli

ACKNOWLEDGMENTS

I am greatly indebted to Dr. Sylvan H. Wittwer for the opportunities afforded, and whose guidance, encouragement and concern made the presentation of this work possible. To Drs. R. R. Dedolph, D. R. Dilley and C. J. Pollard, who extended their counsel beyond the scope of these investigations and so generously gave of their time, I am indeed grateful.

I would also like to express my appreciation to Drs. M. J. Bukovac, I. W. Knobloch and H. M. Sell for their academic guidance and invaluable discussions whenever needed.

Finally, I would like to thank my wife, Patricia A. Tuli, who endured without complaint and was a source of inspiration throughout this work.

TABLE OF CONTENTS

Page

INTRODUCTION. 1

REVIEW OF LITERATURE. 3

Discovery and isolation of kinetin. 3

Effects of kinetin on plant growth and development. 4

Cell division and enlargement 4

Lateral bud growth. 5

Pigmentation. 5

Kinetin and light effects 6

Kinetin induced transport and mobilization of metabolites. 7

Kinetin and heat resistance 8

Effects of kinetin on plant metabolism. 8

Protein and nucleic acid metabolism 8

Enzyme activity and respiration 12

Effects of N^6-benzyladenine on plant metabolism . . 13

STATEMENT OF PROBLEM. 15

GENERAL METHODS 16

Plant materials and culture 16

Harvest of experimental materials 16

Treatment of plant materials. 17

Preparation of N^6-benzyladenine 17

Preparation of phosphate buffer 17

Preparation of Tris buffer. 17

Statistical methods 18

Page

RESULTS 19

Chlorophyll and carotene degradation. 19

Mitochondrial respiration 22

Broccoli mitochondria 22

Cauliflower mitochondria. 23

Photosynthetic ($^{14}CO_2$ fixation) studies 27

N^6-benzyladenine (BA) and hexokinase activity . . . 33

N^6-benzyladenine (BA) and glutamine synthetase activity 40

N^6-benzyladenine (BA) and pyruvic kinase activity . 48

DISCUSSION. 53

Chlorophyll and carotene metabolism 53

Photosynthesis. 54

Mitochondrial respiration 57

Mode of action of (BA) in respiration inhibition. . 59

SUMMARY 64

LITERATURE CITED. 65

INTRODUCTION

The science of plant growth substances began with Darwin (18). Early research in "tropisms" paved the way to practical applications of growth substances in agriculture. Well established practices in agriculture include the use of α-naphthaleneacetic acid as a stop drop and thinning agent for apple fruit, and 2,4-dichlorophenoxyacetic acid as an herbicide. Numerous other examples may be cited where growth substances play an important role in the production and marketing of farm products.

A continuing increase in the knowledge of uses and functions of plant growth substances has necessitated classification. Thus, they have been grouped as auxins, gibberellins, and kinins, based upon chemical structure and biological specificity. Most auxins are chemically characterized by an indole, naphthalene, or benzene nucleus; the gibberellins by a tetracyclic lactone, whereas the kinins have in common a purine nucleus.

N^6-benzyladenine (BA) is biologically one of the most active kinins. Applied as a pre-harvest spray or post-harvest dip to certain leafy or green vegetables, it produces remarkably beneficial results. The fresh, green, turgid appearance is maintained for much longer than normal periods (10, 96). It has been suggested (20, 21, 22, 94) that this results from a marked inhibition of

respiration subsequent to treatment.

This dissertation will describe some effects of post-harvest applications of BA on the respiratory metabolism of the leaves, heads, and cellular constituents thereof, of broccoli (Brassica oleracea var. italica, cultivar Spartan Early), and the curd of cauliflower (Brassica oleracea var. botrytis). Evidence of a possible mechanism involved in respiratory inhibition, at the enzymic and molecular level, will be presented.

REVIEW OF LITERATURE

Discovery and isolation of kinetin

A primary effect of kinetin appears to be the stimulation of cell division in a variety of plant materials. The presence of a kinetin-like substance in higher plants was first reported by Haberlandt (32). He observed that a diffusible substance originating in the phloem of potato tubers was capable of causing cell division in the adjacent parenchyma. Crushed cells also induced cell division. Haberlandt concluded that a "wound hormone" was responsible for the response. Several years later, Shantz et al. (79, 80) isolated from coconut milk concentrates a crystalline substance which stimulated cell division in carrot root tissue. They showed that the material was a heat stable, water soluble organic compound. However, their isolation procedure did not lend itself satisfactorily for chemical characterization, so that the exact nature and structure of the active compound remained unknown. Finally, in 1955, Miller et al. (62, 63, 64) isolated from autoclaved herring sperm deoxyribonucleic acid (DNA), a crystalline substance which markedly promoted cell division in various plant tissue cultures at concentrations as low as one microgram/liter. The chemical structure was shown by degradation and synthesis to be 6-furfurylaminopurine. The specific name kinetin was given to this compound since it promoted

cytokinesis. The generic name kinin was further suggested by the authors, for any substance which similarly stimulated cytokinesis. The natural occurrence of a kinetin-like compound in corn was reported by Miller (61). He observed that water or alcohol extracts of immature corn kernels substituted very effectively for kinetin in the growth of soybean callus tissue. Further purification of the factor, however, revealed that it was chemically different from kinetin. Letham purified a similar factor from plum fruitlets (50) and immature corn kernels (51). Melting point and spectral characteristics indicated that the biologically active factor was an N^6-substituted adenine. The possibility of its being kinetin was ruled out since the two substances could be distinguished chromatographically. It is possible that the factors purified by Miller and Letham were identical. So far, all attempts to isolate naturally occurring kinetin from plant material have been unsuccessful.

Effects of kinetin on plant growth and development

(a) Cell division and enlargement

Das et al. (19) showed that kinetin in combination with indoleacetic acid (IAA) induced mitosis and cell division in excised tobacco pith tissue, virtually all of which was followed by cytokinesis. Their data suggested that kinetin or a similar substance was required for mitosis and cytokinesis, and possibly for DNA duplication. Kinetin

also stimulated cell division in carrot root tissues (80) and pea root callus (87). Besides inducing cell division, kinetin can also cause cell enlargement in leaf tissue of Phaseolus vulgaris (59) and Jerusalem artichoke (1). Thus kinetin is both a cell enlargement and cell division factor.

(b) Lateral bud growth

It was reported by Wickson and Thimann (90) that kinetin antagonized the apical dominance of IAA. They showed that the growth of the lateral buds of isolated pea stem segments was inhibited by IAA; however, when kinetin was applied simultaneously with IAA, the inhibition was overcome. Later (91), the workers showed that kinetin hindered the transport of IAA, thereby negating the apical dominance of IAA applied to the stem segments.

(c) Pigmentation

The synthesis of certain plant pigments is enhanced by kinetin. Bamberger and Mayer (4) reported the increase of an unidentified red pigment in seedlings of Amaranthus retroflexus, as a result of treatment with kinetin. The possibility of this red pigment being a "nitrogenous anthocyanin" was indicated by the authors. Kinetin also facilitated the formation of anthocyanin in cultured petals of Impatiens balsima (42). The delay of chlorophyll and carotene degradation by N^6-benzyladenine, also a kinin, is described elsewhere in this dissertation. It is of interest to note that the breakdown of the cytoplasmic pigments (anthocyanins) and the particulate pigments (chlorophyll

and carotene) are both delayed by the kinins. Probably, these phenomena are mediated by an identical reaction.

(d) Kinetin and light effects

Several plant responses conditioned by red light and far red radiation appear to be subject to the same control mechanism. These include the germination of lettuce seed (12), the expansion of bean leaf disks (23), and the flowering of certain plants (13). An identical photoreaction apparently controls all of these responses.

Miller (59) erroneously reported that kinetin could replace the red light effect. He observed that kinetin promoted the expansion of bean leaf disks and germination of lettuce seeds in the dark. This led him to conclude that kinetin could substitute for red light. The work of Scott and Liverman (82) and Hillman (37) supported Miller's view, and the general concept was that kinetin could replace red light. Later, Miller (50) revised his earlier conclusions, when he realized that the stimulation of germination by kinetin was, in fact, due to an accidental exposure to light. Recently, Leff (49) has reported that the high percentage of germination of lettuce seeds is due to a synergistic interaction between kinetin and light.

It is apparent that light and kinetin work in the same direction. Both promote lettuce seed germination (59, 49), bud formation (90), and both antagonize apical dominance. Yet, they cannot generally substitute for one another and are not identical. The effect of kinetin is not

reversed by far red light as is that of red light. Evanari (27) proposed that kinetin favored the formation of the red light sensitive pigment Phytochrome. However, since Leff (49) has shown that kinetin is equally effective given before or after a red light exposure, it appears that kinetin does not promote the synthesis of this pigment but protects it against degradation.

(e) Kinetin induced transport and mobilization of metabolites

Kinetin also directs the transport of metabolites. Mothes and Engelbrecht (68), working with excised tobacco leaves, showed that numerous substances from non-treated parts migrated to the "kinetin locus," which was characterized by a limited area sprayed with the chemical. The phenomenon was demonstrated by using glycine-1-^{14}C, which was applied through the petiole or to the leaf surface. The kinetin-induced transport occurred in the dark, and was greatly promoted by light. In leaves, which were kept in the dark for a long period of time, the phenomenon could hardly be demonstrated. It was suggested that a factor other than kinetin, possibly ATP, derived from photophosphorylation, was necessary to make migration possible. Amino acids, which are not incorporated into proteins, such as α-aminoisobutyric acid, are also subjected to the kinetin-directed transport and accumulation (69).

Kinetin may also inhibit the outward migration of metabolites. Engelbrecht and Mothes (25) showed that

applications of kinetin to leaves of tobacco seedlings inhibited rooting and regeneration of roots. The chemical apparently arrested the downward migration of amino acids and other metabolites from the leaf blade. Thus kinetin appears to promote the process of attraction and accumulation.

(f) Kinetin and heat resistance

The heat resistance of certain plant tissues is increased by kinetin. Engelbrecht and Mothes (26) reported that immersion of tobacco leaves in water at 50° C for 1 minute induced premature withering owing to the loss of amino acids to neighboring sound tissue. Kinetin applied as a spray before or after heating fully counteracted the damage by stimulating the accumulation of amino acids. Application before heating increased the heat resistance of the tissue. Again, the kinetin effect was limited strictly to the area to which it was applied.

Effects of kinetin on plant metabolism

(a) Protein and nucleic acid metabolism

The aforementioned different, often contradictory, effects of kinetin on plant metabolism make it difficult to postulate a common mechanism of action underlying all of them. Yet, it is logical to assume that kinetin manifests itself similarly in all systems; the effect being relayed differently in the different systems.

Some enlightening effects of kinetin on plant

metabolism have been obtained. Guttman (30, 31) conclusively demonstrated that onion roots growing in the presence of kinetin markedly increased their ribonucleic acid (RNA) content within a few hours. Jensen and Pollock (39) confirmed this observation. Employing histochemical procedures, they showed that onion root tips treated with 1 ppm kinetin greatly increased their RNA and DNA contents, on a per cell basis. The protein content, however, was unaffected. On the other hand, Richmond and Lang (78) reported that 5 ppm kinetin greatly inhibited the decline of protein in detached Xanthium leaves. The magnitude of inhibition, in some cases, approached 45%. Protein degradation in tobacco leaves (67) was similarly arrested by kinetin.

Several reports indicate that protein synthesis is enhanced by kinetin. As shown by Parthier (72), the incorporation of ^{14}C-glycine into the protein fraction of Nicotiana tabacum leaves was greatly enhanced by kinetin. Optimal concentrations were from 10^{-7} to 10^{-5} M. Again, the effect was more pronounced in the light than in the dark, suggesting a possible interaction between kinetin and light. Thimann and Laloraya (85) cultured pea stem segments and buds, in media containing 1 per cent sucrose, in combination with IAA (1 ppm) and kinetin (4 ppm). They found that the dry weight of buds grown in the presence of sucrose, IAA and kinetin was much lower than that of the controls; however, the former segments contained twice

as much protein as the control segments. Localized application of kinetin to the buds increased the protein content therein. The internode below showed no response. The localization of the kinetin effect and its promotion of protein synthesis suggested to these workers that kinetin was rapidly incorporated into RNA, which was responsible for the increased synthesis of protein.

The degradation of protein, nucleic acids and chlorophyll is symptomatic of senescence. Osborne (71) demonstrated that kinetin enhanced the synthesis of protein and nucleic acids, and inhibited the breakdown of chlorophyll in Xanthium leaves. Consequently, Osborne suggested that kinetin could control senescence, by substituting in some way for an essential factor necessary for the continued synthesis of RNA. Osborne (71) showed that incorporation of ^{14}C-leucine into protein, and ^{14}C-orotic acid into RNA, was markedly enhanced in Xanthium leaf disks pretreated with 40 ppm kinetin. Based upon specific activities, the data revealed that there was a net synthesis of protein and RNA, although the protein/RNA ratio remained constant in the control and kinetin-treated leaf disks. Osborne suggested that this was due to an increase in the synthesis of protein which reflected a stimulation of RNA synthesis. The synthesis of DNA was not enhanced by kinetin, and virtually no radioactivity was detected in the DNA fraction isolated from the leaf disks. Kinetin did, however, prevent the breakdown of DNA. Osborne indicated that the

inability of kinetin to promote DNA synthesis was probably related to the absence of cell division in the mature leaves used for the experiments. Patau et al. (75), using actively dividing tobacco pith tissue, showed that DNA synthesis was indeed stimulated by kinetin.

The synthesis of protein appears to be the primary effect of kinetin on plant metabolism. This is probably brought about by an increased synthesis of RNA. Thimann's view (85), that kinetin is incorporated into RNA, at best, remains largely speculative.

The direct effect of kinetin on protein synthesis was demonstrated by Kulayeva and Vorobyeva (46, 47). Barley leaves sprayed with 20 ppm kinetin enhanced protein synthesis, whereas 250 ppm chloramphenicol, a specific inhibitor of protein synthesis in bacterial systems, retarded protein synthesis. A combined spray of the two chemicals indicated that kinetin had overcome the inhibitory effect of choramphenicol.

Since kinetin promotes protein synthesis, it has a rejuvenating effect. Mothes (67) found that detached leaves and leaves on a plant remained alive longer following a spray of kinetin. Enhancement of protein synthesis by kinetin may be related to amino acid synthesis. Mothes et al. (69) reported that kinetin induced an accumulation of amino acids against a concentration gradient, in non-dividing leaf cells of Nicotiana tabacum. Thus an increase in the amino acid pool may facilitate protein synthesis.

(b) Enzyme activity and respiration

Most biological effects are best explained on an enzymic level. Little is known of kinetin in this respect. Henderson (35) and Henderson *et al.* (36) observed that the activity of xanthine oxidase (xanthine $\xrightarrow{\text{xanthine oxidase}}$ uric acid) was inhibited by kinetin. It was earlier reported by Bergman *et al.* (3) and Wyngaarden (95) that kinetin was oxidized to the 2,-8-dihydroxy derivative by xanthine oxidase, suggesting a non-specific nature of the enzyme towards its substrate. Henderson *et al.* (36) found that kinetin and its enzymatic oxidation product were potent inhibitors of xanthine oxidase, when xanthine was used as the substrate. The formation of uric acid, which is inhibited in this reaction, cannot be significant for it is not known to occur as an intermediary metabolite in plants. However, the accumulated xanthine may probably be converted to other purines, thus increasing the synthesis of RNA.

Starch degradation in wheat endosperm is also accelerated by kinetin (11). Increased activity of amylase, the enzyme which is responsible for the degradation of starch, was induced.

Maciejewska-Potapszyk (56) found that kinetin stimulated the *in vitro* activity of RNAase and DNAase of bean hypocotyl extracts. Kinetin at a concentration of 1 microgram/liter doubled the RNAase activity, whereas 1 mg/liter of kinetin was required for maximum DNAase activity. Since there is overwhelming evidence to the contrary, it is

apparent that *in vivo*, kinetin does not behave in this manner.

Bergmann (9) reported that 10-20 ppm kinetin markedly inhibited the respiration of cell suspensions from *Nicotiana tabacum* tissue cultures, in media containing glucose. The reduction in oxygen consumption was restored by the addition of pyruvate, succinate, and α-ketoglutarate. This was accompanied by an increased respiratory quotient. Kinetin apparently did not inhibit the Kreb's cycle, but inhibited the glycoytic pathway, the site of action being some enzymes involved in that pathway.

Effects of N^6-benzyladenine on plant metabolism

The kinin, N^6-benzyladenine (BA) is metabolized to a number of low molecular weight compounds by *Xanthium* leaves, as reported by McCalla *et al.* (58). The authors found that a major product was the riboside, benzyladenosine. The ribotide, benzyladenylic acid, was also produced. Addition of 8-^{14}C-BA to *Xanthium* leaves resulted in labeled adenylic, guanylic and inosinic acids, and small amounts of adenine and guanine. Substantial radioactivity was also found in urea and ureide. Labeled adenylic and guanylic acids derived from BA were likewise found incorporated in the RNA of the leaf. Benzyladenylic acid itself was not thus incorporated. Since kinetin and BA induce similar biological effects, it may be that they are similarly metabolized. It may also be that one or more of the

degradation products is biologically active.

BA is biologically more active than kinetin (48, 70, 84), hence much interest has recently been diverted to this compound. Bessey (10) and Zink (96) both reported that spray applications (5-10 ppm) of BA to leafy or green vegetables greatly delayed the onset of senescence. The delay in senescence was accompanied by a slower rate of chlorophyll breakdown in chlorophyllous tissue (88). Applications of BA (10 ppm) to nonchlorophyllous tissue, e.g., cauliflower, prevented decay and extended marketability. It was demonstrated that BA inhibited the overall respiratory process in broccoli (22), asparagus (21), and celery (94). Respiration rates as indexed by carbon dioxide evolution and oxygen uptake were greatly inhibited when BA was applied as a post-harvest dip or a pre-harvest spray, at a concentration of 10 ppm. Recently, Dedolph (20) reported that spray applications of BA to sugar beet plants resulted in a net accumulation of sucrose. This was attributed to an inhibition of respiration. Chlorophyll retention and the lesser protein degradation may also be a consequence of respiratory inhibition.

STATEMENT OF PROBLEM

Post-harvest applications of N^6-benzyladenine to leafy green vegetables cause a retention of freshness, and delay chlorophyll and protein degradation. The delay in senescence is apparently caused by an inhibition of respiration. This thesis is concerned with the respiratory metabolism of higher plants, as affected by N^6-benzyladenine at a sub-cellular level, and determination of the mode of action of the chemical as an inhibitor of respiration.

GENERAL METHODS

Plant materials and culture

Broccoli (*Brassica oleracea* var. *italica* cv. Spartan Early) leaves and heads, or snowball type cauliflower (*Brassica oleracea* var. *botrytis*) heads were exclusively used for the experiments. Broccoli plants were cultured in experimental greenhouses and cauliflower heads were purchased from a local supermarket.

The broccoli seed were germinated in flats of vermiculite placed in a greenhouse and maintained at day and night temperatures of 75 and 65° F, respectively. Water was applied as needed. Three-week-old seedlings were transplanted to two-inch peat pots, maintained at the above mentioned temperatures, and watered periodically. After four weeks they were transplanted to and retained in 6-inch clay pots.

Harvest of experimental materials

Leaves of similar physiological age were obtained by harvesting the fifth leaf from the top of the plant. Broccoli heads were medium-sized, firm, and those in which anthesis had not occurred. The detached leaves and heads were transported to the laboratory in wet paper towels to prevent wilting.

Treatment of plant materials

For treatment with N^6-benzyladenine, the leaves and heads were dipped in a 5×10^{-5} M solution of the chemical prepared from a stock solution. One tenth of one per cent Tween 20* was used as the wetting agent. Control samples were dipped only in 0.1 per cent Tween 20. After treatment the samples were wrapped in waxed paper and stored at 70° F until used.

Preparation of N^6-benzyladenine (BA)

A 10^{-4} M stock solution was prepared by boiling 22.5 mg. crystalline BA (Shell Development Co.) in 100 ml. of 0.05 M phosphate buffer (pH 7.6).

Preparation of stock solutions for phosphate buffer (ca. 0.05 M)

A: 0.2 M solution of monobasic sodium phosphate. Dissolved 27.8 grams of $NaHPO_4$ in 1 liter of glass distilled water.

B: 0.2 M solution of dibasic sodium phosphate. Dissolved 53.65 gm. $Na_2HPO_4.7H_2O$ in 1 liter of glass distilled water.

13 ml. of A + 87 ml. of B + 100 ml. of water = pH 7.6.

Preparation of stock solutions for Tris (hydroxymethyl) aminomethane (Tris) buffer (0.05 M)

A: 0.2 M solution of Tris. Dissolved 24.2 gm. Tris in 1 liter of glass distilled water.

---

*Polyoxyethylene (20) Sorbitan monolaurate (Atlas Powder Co.).

B: 0.2 M HCl. Dissolved 16.7 ml. of concentrated HCl in 1 liter of glass distilled water.

50 ml. of A + 38.4 ml. of B + 111.6 ml. of distilled water = pH 7.6.

Statistical methods

Each experiment was conducted at least three times to establish reproducibility. The standard deviation was never greater than 0.25 for any experiment. The points used for the plotting of lines in the various graphs were averages of discrete experiments.

Optimum concentrations of BA ($3\text{-}5\times10^{-5}$ M), based on preliminary experiments were used throughout these investigations.

RESULTS

Chlorophyll and carotene degradation

Chlorophyll and carotene contents were used for indexing the freshness of broccoli heads, subsequent to treatment with BA. Broccoli heads were dipped in a 5×10^{-5} M solution of BA. The heads were then divided into equal halves for estimating the chlorophyll and carotene content at 0, 24, 48 and 72 hours after treatment.

Chlorophyll was extracted according to Withrow et al. (93). Values were expressed as µgm. $\text{chlorophyll}_{(a+b)}$/gm. fresh weight, using the following equation:

$$C_{a+b} = (7.9\ A_{665} + 17\ A_{645} - 0.56\ A_{625})\ V/Wb$$

where,

C_{a+b} = $\text{Chlorophyll}_{(a+b)}$ in µgm./gm. fresh weight.

A = Absorbancy at indicated wavelength in millimicrons.

V = Volume of solution in milliliters.

W = Fresh weight of tissue in grams.

b = Length of light path in centimeters.

Chlorophyll values for treated and non-treated heads showing a delay in breakdown, are summarized in Fig. 1-A.

Carotene was extracted by a modification of the methods of Wiseman et al. (92) and Lime et al. (52). The entire procedure was carried out at 4° C. Weighed samples of fresh broccoli were placed in a Waring blendor to which

was added a 50% acetone-hexane mixture, at the rate of 5 ml./gm. tissue. The blendor was brought up to one-fourth its maximum speed for 30 seconds, by means of a variable transformer, and then turned on to full voltage for 2 minutes. The brei was filtered by suction through a Büchner funnel and washed with 20 ml. of the extraction medium. The filtrate was transferred to a 250 ml. separatory funnel; 50 ml. of water were added and the aqueous acetone layer was removed and re-extracted with hexane until the hexane extract was colorless.

The combined hexane extracts were washed three times with 50 ml. portions of water, to which were added a few drops of methanol to prevent foaming. After washing, the hexane extract was filtered through a pad of anhydrous sodium sulfate on a medium porosity fritted glass funnel and volume of the hexane extract noted.

Total carotene was determined spectrophotometrically, using hexane as the blank. Readings were made at 450 mμ with a Beckman Model DU spectrophotometer, in a 1 cm. cell. The following equation was used:

$$\mu\text{gm. carotene/gm. fresh tissue} = \frac{(A)\ (\text{total volume})}{(a)\ (\text{weight of sample})}$$

where,

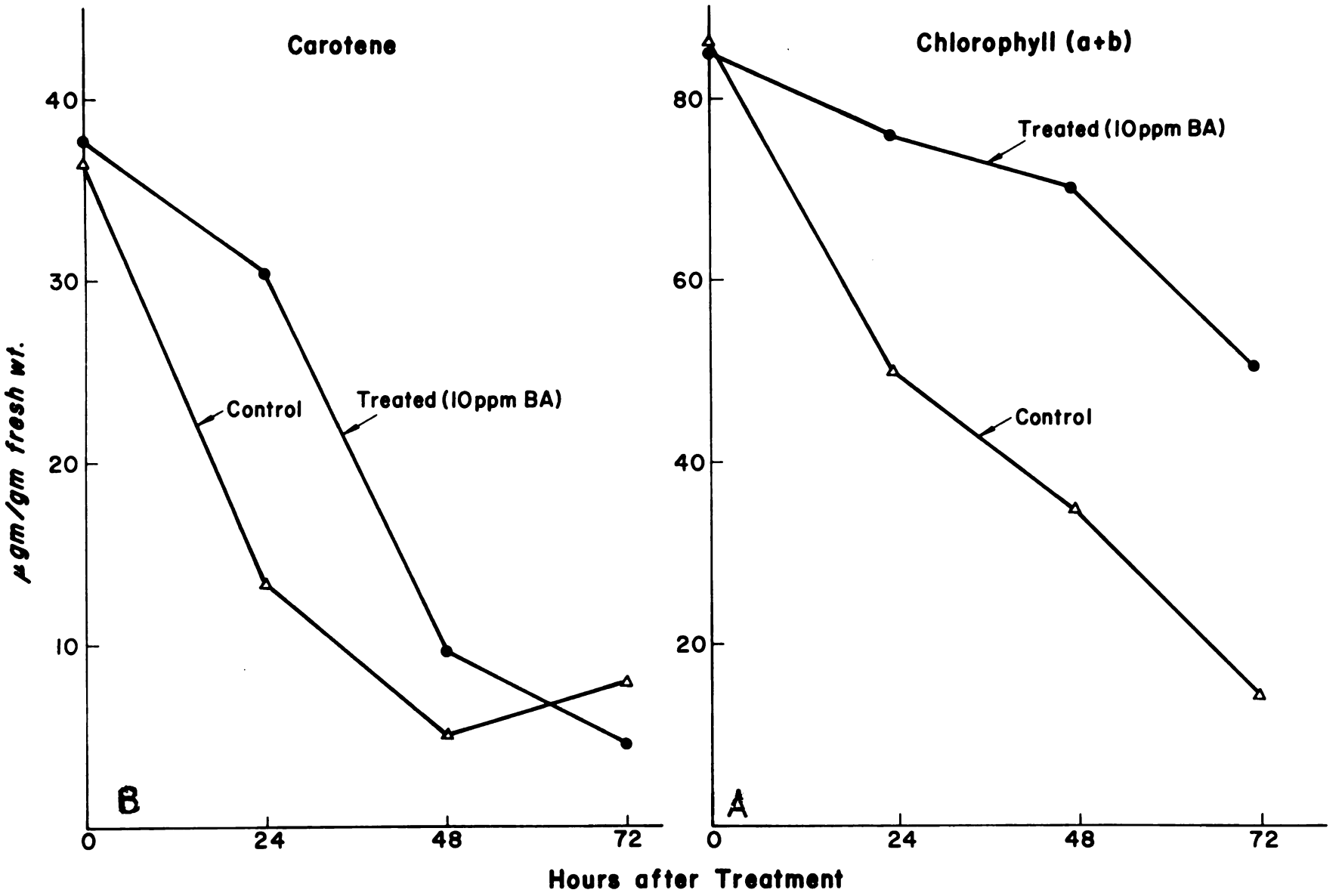
A = Optical density.

a = Extinction coefficient of carotene.

A summary of the effects of BA, showing a delay in carotene degradation of broccoli heads, is presented in Fig. 1-B.

Figure 1

Delay of chlorophyll and carotene degradation in fresh broccoli following treatment with 10 ppm BA. Each value is an average of three discrete experiments.


Carotene
Chlorophyll (a+b)
μgm/gm fresh wt.
40
30
20
10
80
60
40
20
Control
Treated (10ppm BA)
Control
Treated (10ppm BA)
B
A
0
24
48
72
0
24
48
72
Hours after Treatment

Mitochondrial respiration

It was mentioned earlier that BA markedly inhibited the respiration of explants (22, 21, 94). The following experiments were conducted to determine the effects of BA on mitochondrial respiration. Broccoli leaves and cauliflower heads were sources of mitochondria. Isolation procedures closely paralleled those of Millerd *et al.* (65). All solutions were prechilled and operations carried out at 4° C.

Broccoli mitochondria--Medium-sized leaves were thoroughly washed with cold tap water. Fifty grams of interveinal tissue were ground in a prechilled mortar with 5 gm. of acid-washed sand. The grinding medium (150 ml.) consisted of 0.25 *M* sucrose, and 0.01 *M* neutralized ethylenediaminetetraacetate (EDTA) in a 0.05 *M* phosphate buffer at pH 7.6. The homogenate was strained through eight layers of cheesecloth, into four centrifuge tubes, and centrifuged at 1,500x *g*. for 10 minutes. The mitochondrial fraction was sedimented from the supernatant at 14,000x *g*. for 20 minutes. The particles were washed by re-suspending in a 0.25 *M* sucrose-0.05 *M* phosphate buffer (pH 7.6), and re-centrifuged at 14,000x *g*. for 20 minutes. The washing was repeated and the mitochondrial pellet suspended in 5 ml. of the washing medium. The contents of two tubes (10 ml.) were combined for measuring oxygen uptake, and the two remaining tubes, representing 25 gm. leaf tissue, were used for determining total nitrogen.

One ml. of the enzyme (mitochondrial) suspension was pipetted into each of eight Warburg flasks placed in cracked ice, containing 0.2 ml. KOH placed in the center well. Sodium succinate, cytochrome c, and adenosine triphosphate (ATP) were added to the suspension, so that the final concentration in 3 ml. was $2x10^{-2}$ M, $2x10^{-5}$ M and 10^{-3} M respectively. BA (final concentration $3.3x10^{-5}$ M) was added to the side arms of four flasks. The medium was equilibrated for 10 minutes at 30° C in the Warburg bath, after which the system was closed and oxygen uptake measured by conventional manometric techniques (89). At the end of 30 minutes (Fig. 2), BA was tipped in and the measurements continued. After the addition of BA, the resulting pH was 7.2-7.4. Values were expressed as µl O_2 uptake/mg. nitrogen. Nitrogen was determined according to Folin and Farmer (28).

Inhibitory effects of BA on the oxidation of succinate by broccoli leaf mitochondria are shown in Fig. 2.

Cauliflower mitochondria--A medium-sized head was rinsed with cold tap water, and 50 gm. of the curd grated with a plastic disk. Isolation procedures were similar to the aforementioned for broccoli, except that the grinding medium consisted of 0.25 M sucrose in distilled water. The mitochondrial fraction was washed twice with 0.25 M sucrose-0.05 M phosphate (pH 7.6), and finally suspended in 5 ml. of phosphate buffer. The incubation medium (3 ml.) consisted of $2x10^{-2}$ M sodium succinate and 10^{-3} M ATP.

BA (3.3×10^{-5} M) was added to the side arm and oxygen uptake measured as previously described. The oxidation of succinate by cauliflower mitochondria was greatly inhibited by BA (Fig. 3).

Figure 2

Inhibition of oxygen uptake by broccoli leaf mitochondria upon the addition of 3.3×10^{-5} M BA. Mitochondria were fortified with ATP and cytochrome c. Succinate was provided as the substrate. Points presented are the averages of three experiments.

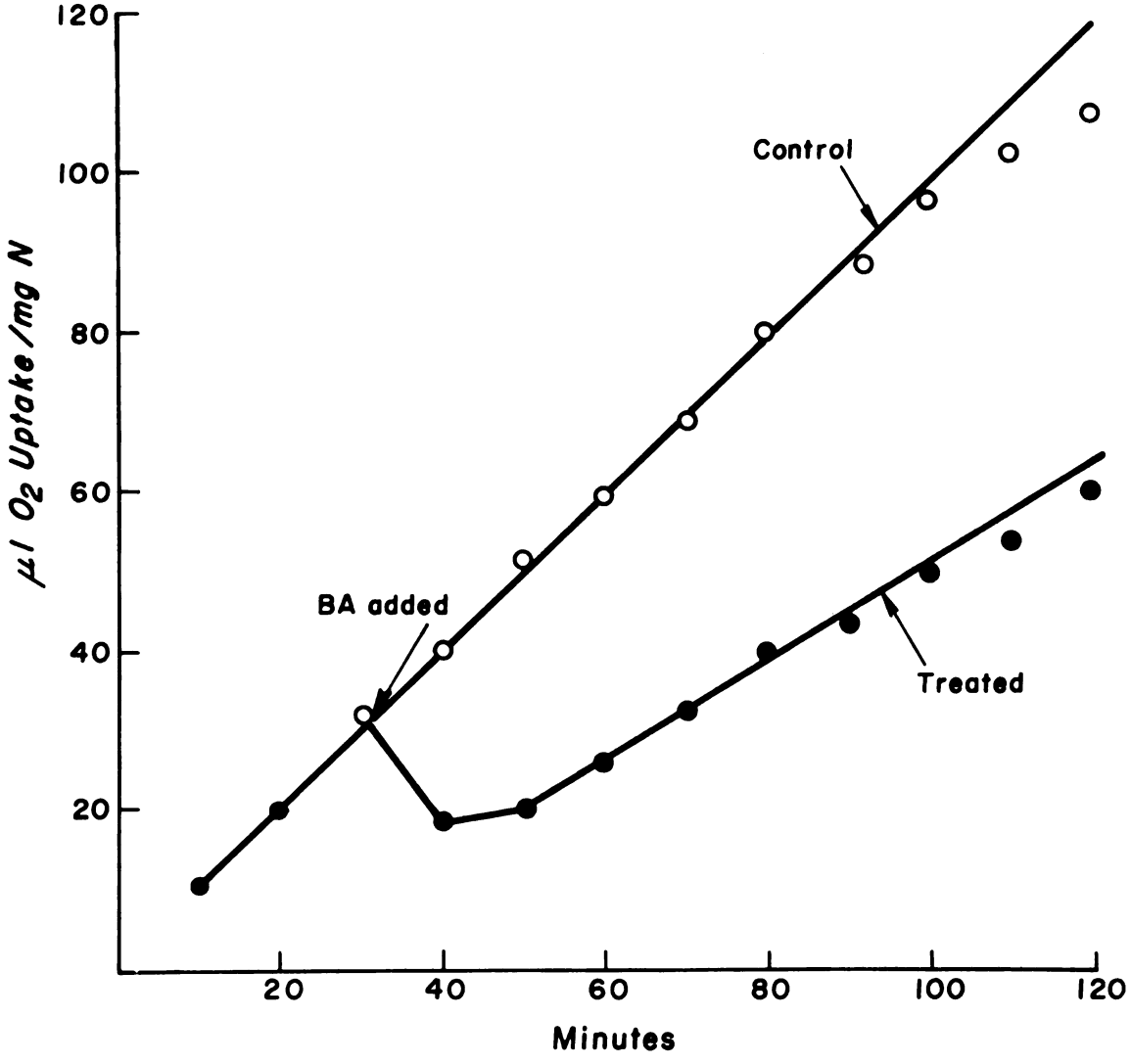
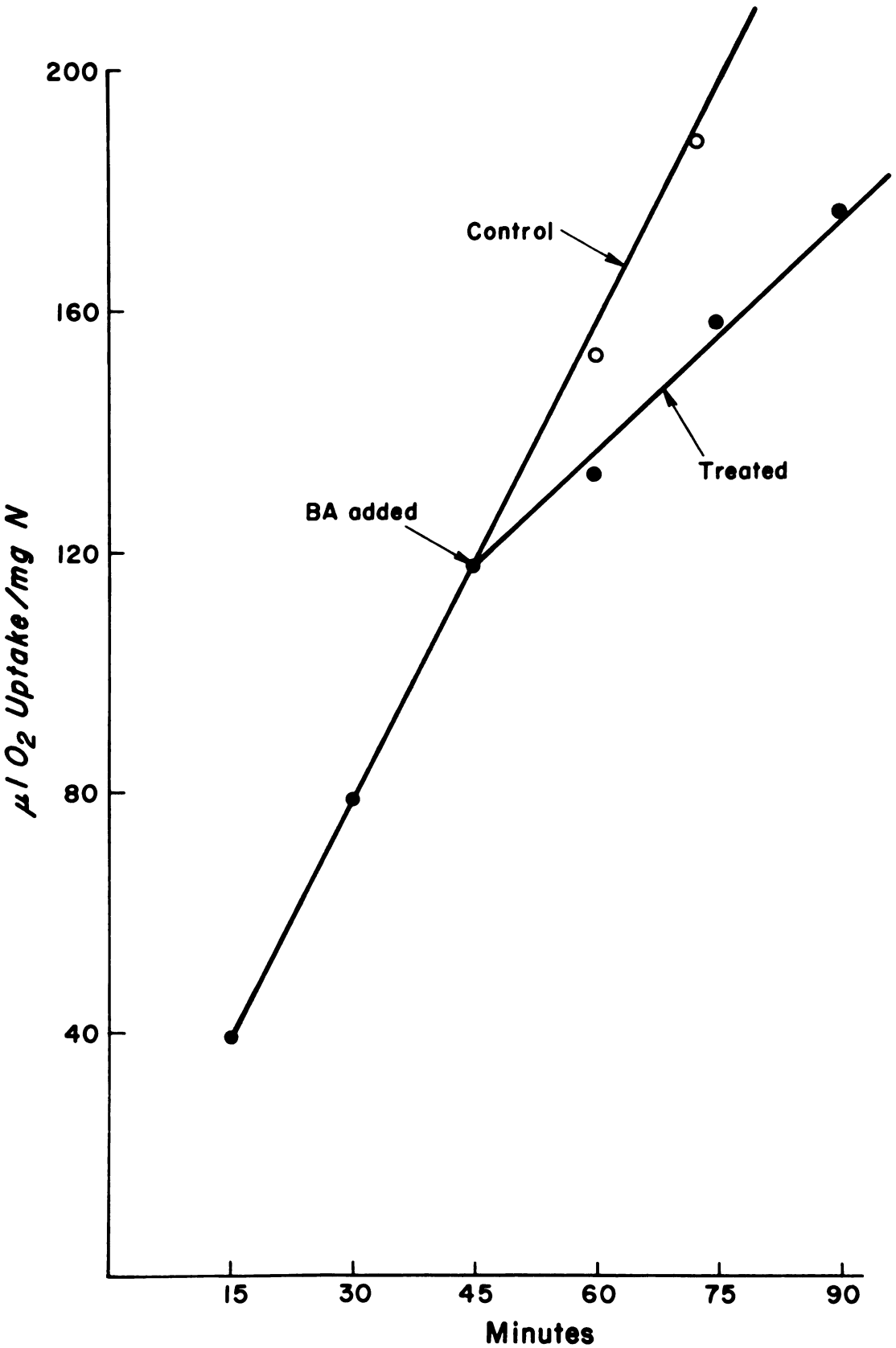

120
100
80
60
40
20
µl O2 Uptake/mg N
Control
BA added
Treated
20
40
60
80
100
120
Minutes

Figure 3

Inhibition of oxygen uptake by cauliflower mitochondria upon the addition of 3.3×10^{-5} M BA. The mitochondrial suspension was fortified with ATP and succinate was provided as the substrate. Points presented are the averages of three experiments.


200
160
120
80
40
μl O2 Uptake/mg N
Control
Treated
BA added
15
30
45
60
75
90
Minutes

Photosynthetic ($^{14}CO_2$ fixation) studies

Fixation of ^{14}C by treated and non-treated broccoli leaves provided an estimate of the photosynthetic rates subsequent to treatment with BA. Measurements were recorded at 0, 24, 48 and 72 hours after treatment.

Weighed and comparable leaves for treated and control were selected for each experiment and enclosed together in a glass photosynthesis chamber (Fig. 4). The petioles were immersed in water to prevent wilting. $NaH^{14}CO_3$ representing 5 microcuries ^{14}C was placed in a porcelain boat and kept in the side arm. The lights were turned on and, with the stop cock open, the system was equilibrated at 25° C in a constant temperature bath for 10 minutes. After equilibration, the system was closed, and $^{14}CO_2$ released inside the sealed chamber by injecting 5 N lactic acid through a serum cap. The leaves were exposed to $^{14}CO_2$ for 10 minutes. The lights were then turned off and the leaves dropped in boiling 80% ethanol for 3 minutes, and re-extracted two more times with boiling water. The ethanol and water extracts were combined and brought to a common volume with water. One ml. aliquots were then drawn into aluminum planchets and evaporated under a bank of 250-watt infrared heat lamps. The samples were counted with a mica end-window Geiger-Müller tube using a Nuclear Chicago Model 161A scaler. Data were expressed as counts per minute (cpm/gm.) fresh weight. The total amounts of ^{14}C fixed as recovered in the various ethanol and water soluble

fractions of treated and control leaves, at various intervals after treatment, are shown in Fig. 5.

The combined extracts were evaporated to about 1 ml. on a hot plate and chromatographed two-dimensionally, to determine the distribution of ^{14}C among the various photosynthetic products. Ten microliters of the concentrate were spotted on an 18 $\frac{1}{4}$" x 22$\frac{1}{2}$" Whatman No. 1 filter paper, and developed first in 80% phenol and then in 125 ml. butanol, 87 ml. distilled water, and 62 ml. redistilled propionic acid. After two-dimensional development, the chromatograms were exposed to sheets of 14" x 17" Kodak blue brand x-ray film for 4 weeks. The exposed films were processed using Kodak x-ray developer and fixer. The spots depicted in Fig. 6 were identified by co-chromatography and color specific reagents (5, 7, 34, 66, 74). The chromatograms were scanned for radioactivity with a large Geiger tube covered by a mylar film. The tube was gassed by helium flowing through cold ethanol. Results were expressed as percentage distribution of ^{14}C among the various compounds (Table I).

Figure 4

Design of the photosynthesis chamber. Experiments conducted at 8,000 foot candles and 25° C.

A - 150W projector bulb

B - 5 liter bacteria culture bottle

C - 2 liter beaker

D - photosynthesis chamber

E - side arm

F - porcelain boat

G - serum cap

H - thermometer

I - treated and control leaf

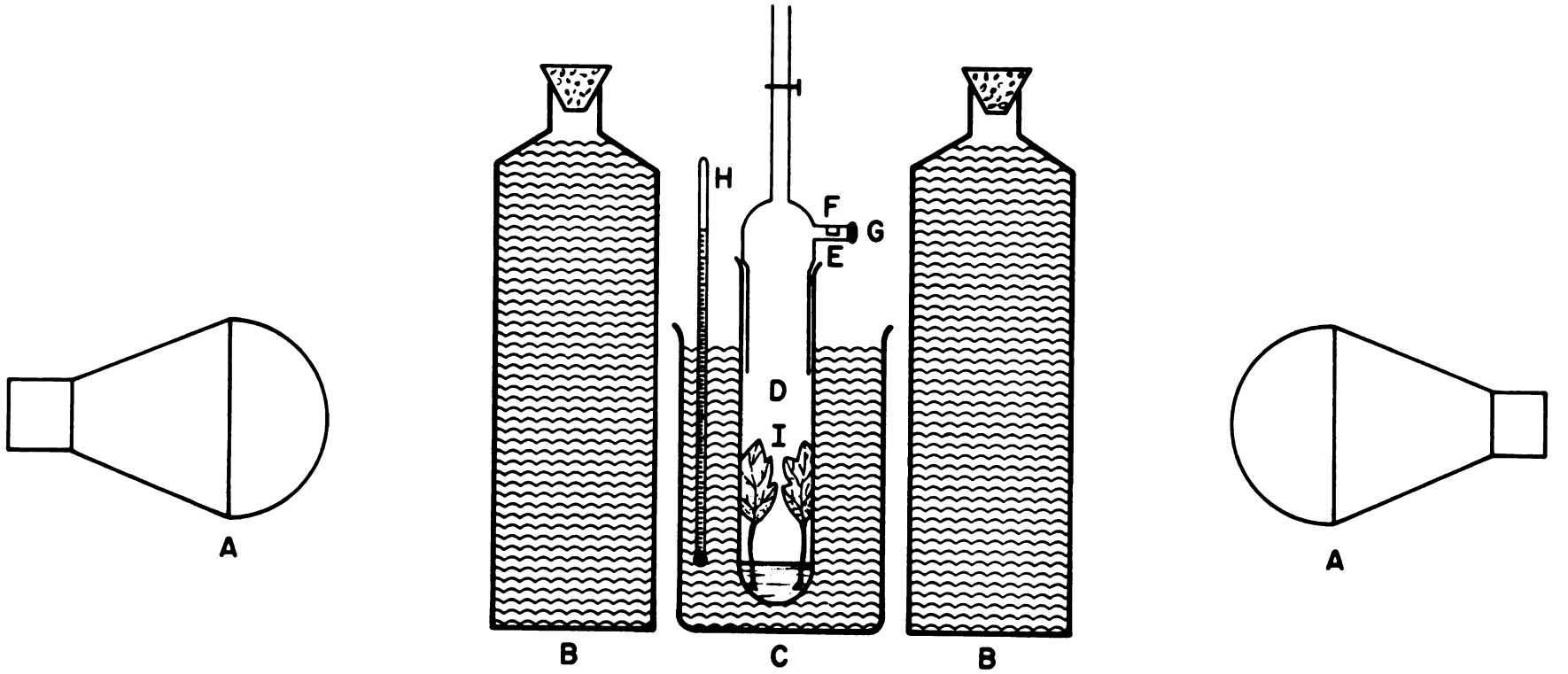

A
H
F
G
E
D
I
B
C
B
A

Figure 5

Photosynthetic fixation of $^{14}CO_2$ by BA treated ($5x10^{-5}$ M) and control broccoli leaves. Radioactivity expressed as counts per minute per gram (cpm/gm.) fresh weight.

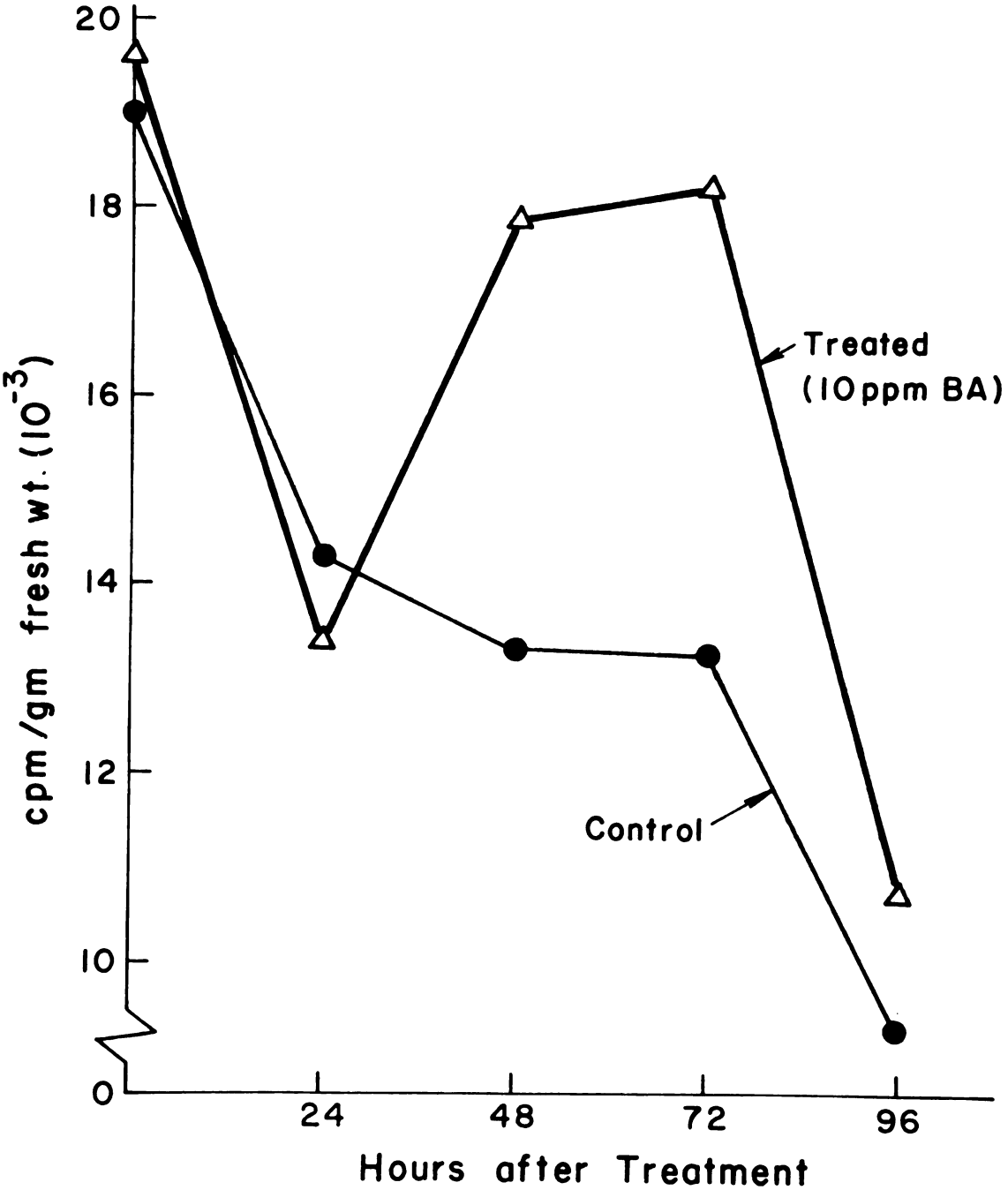

20
18
16
14
12
10
0
cpm/gm fresh wt. (10^{-3})
Treated
(10ppm BA)
Control
24
48
72
96
Hours after Treatment

Figure 6

Paper chromatographic distribution of ^{14}C in treated ($5x10^{-5}$ M) and control broccoli leaves. Autoradiograms prepared of chromatographed extracts 48 hours after treatment.

A - Uridine diphosphate glucose (UDPG)

B - phosphate esters

C - sucrose

D - glucose

E - glycine

F - serine

G - aspartate + glutamine

H - citrate

I - fructose

J - alanine

K - malate

L - glycolate

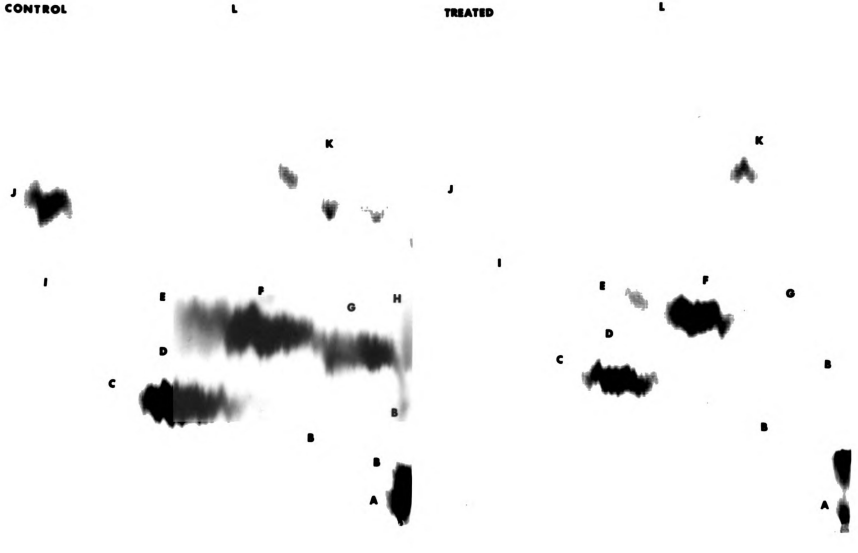

CONTROL
L
TREATED
L
K
J
I
E
F
G
H
D
C
B
A

Table I

Effects of BA on the percentage distribution of ^{14}C among the various organic constituents of broccoli leaves at 0, 24, 48 and 72 hours after photosynthetic $^{14}CO_2$ fixation

	0 hr		24 hr		48 hr		72 hr	
Compound	C*	T**	C	T	C	T	C	T
Sucrose	32	26	28.6	26	21.4	27.6	20.9	37
UDPG	12.4	10	8.6	8.7	7.8	9.1	9	9
Glucose	-	-	0.8	0.3	1.4	0.6	3	1.3
Fructose	-	-	0.8	0.3	1.4	0.6	3	1.3
Serine	16.5	17.6	19.2	25.2	23.1	30.5	26.5	18.5
Glycine	2.9	3.0	3.6	3.5	3.6	5.3	3.0	10
Glycolate	1.1	1.4	0.8	1.3	0.4	1.2	-	0.6
Citrate	0.7	0.7	0.9	0.1	1.3	0.3	8.0	-
Malate	17.4	19.1	15.6	18.5	11.5	12.5	5.4	7.9
Alanine	4.9	5.3	6.5	4.5	8.7	4.2	3.4	4.9
Glutamic	0.2	1.0	0.5	0.3	0.8	-	1.2	-
Aspartate	8.0	11.1	9.4	7.7	12.8	3.2	3.0	1.4
P-esters	3.7	5.0	5	4.2	7.2	3.1	11.3	4.2

* - Control

** - Treated

N^6-benzyladenine (BA) and hexokinase activity

The reduction in the formation of phosphate esters in treated broccoli leaves (Table I) suggested that the activity of certain phosphorylating enzymes was impeded by BA. An *in vitro* system was thus devised to study the effects of BA on hexokinase activity. Hexokinase catalyzes the phosphorylation of glucose by ATP as follows:

(1) Glucose + ATP $\xrightarrow{\text{Hexokinase, } Mg^{2+}}$ Glucose-6-phosphate + Adenosine diphosphate (ADP).

Glucose-6-phosphate is oxidized in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$), by glucose-6-phosphate dehydrogenase (GDH):

(2) Glucose-6-phosphate + $NADP^+$ $\xrightarrow{\text{GDH}}$ 6-phosphoglucono-δ-lactone + NADPH + H^+.

The two above enzymic reactions were coupled to proceed stoichiometrically and quantitatively. The reduction of $NADP^+$ in the second equation was measured spectrophotometrically at 340 mμ and served as a measure of hexokinase activity.

Solutions were prepared as follows, after which they were stored at 4° C, and held in ice during use.

Tris buffer (0.05 M; pH 7.6)--Solutions were made up each day as described under general methods.

Glucose (0.1 M)--Three hundred sixty mg. of glucose were dissolved in distilled water and the volume made up to 20 ml.

Magnesium chloride (0.1 M)--One-half gm. $MgCl_2.6H_2O$ was dissolved in distilled water and the volume made up to 25 ml.

Adenosine triphosphate (0.1 M ATP)--Approximately 600 mg. ATP-$Na_2H_2.3H_2O$ were dissolved in distilled water and the volume made up to 10 ml.

Serum albumin--Twenty mg. crystalline serum albumin were dissolved in 5 ml. distilled water. The solution served as a protective protein for hexokinase.

Nicotinamide-adenine dinucleotide phosphate (1.5×10^{-3} M $NADP^+$)--Approximately 13 mg. NADP-NaH_2 were dissolved in distilled water and brought up to 10 ml.

Cysteine (ca. 0.28 M)--Thirty mg. of cysteine.HCl were dissolved in 0.8 ml. distilled water and neutralized with 0.2 ml. N NaOH.

Hexokinase (140 units)--One-half gm. of crystalline yeast hexokinase (Nutritional Biochemicals Co.) was diluted with 0.5 ml. serum albumin solution. One enzyme unit was defined as that amount of protein which caused the phosphorylation of one micromole of glucose per minute, at 25° C and pH 7.6.

Glucose-6-phosphate dehydrogenase (140 units)--An ammonium sulfate suspension of the enzyme (Sigma Chemical Co.) was reconstituted by the addition of 0.1 ml. cold distilled water for each 50 units. One enzyme unit caused the reduction of 1.0 micromole of $NADP^+$ per minute at 25° C and pH 7.6.

N^6-benzyladenine (10^{-3} M)--Solutions were prepared as described under general methods.

Preliminary experiments indicated that the magnitude of inhibition caused by BA was dependent upon the ATP concentration; hence, kinetic studies with the ATP concentration as a variable were conducted.

Optical density measurements, relating to the reduction of $NADP^+$, were made with a Beckman DU spectrophotometer at 340 mμ, a light path of 1 cm., and a final volume of 3.0 ml. made up with distilled water. The following solutions were pipetted into silica cuvettes in the order listed.

1	Tris	1.0 ml.
2	glucose	0.4 ml.
3	ATP	varied
4	$MgCl_2$	0.1 ml.
5	cysteine	0.1 ml.
6	water	to make up 3.0 ml.
7	Hexokinase	0.1 ml. (28 units)
8	GDH	0.3 ml. (42 units)
9	BA	-
10	$NADP^+$	-

One treatment consisted of a blank with no addition of BA or $NADP^+$. The control received only $NADP^+$ (0.2 ml.) and the treated BA (0.1 ml.) plus $NADP^+$ (0.2 ml.).

After the addition of BA in the treatment, the contents were stirred and equilibrated at 25° C, after

which initial optical densities (O.D.) were read against the blank; 0.2 ml. of $NADP^+$ were then added and change in O.D. measured each 15 seconds.

Reaction 2 (page 33) was used as a basis for determining the effect of BA on GDH activity. Components of the in vitro system were the same as those previously described for hexokinase.

Reaction velocities in the presence of BA and varying concentrations of ATP are shown in Fig. 7. A double reciprocal plot of the results of kinetic studies indicating competitive inhibition of hexokinase by BA is shown in Fig. 9. GDH activity was not affected by BA at two different concentrations (Fig. 8).

Figure 7

Effect of BA on hexokinase activity. Changes in reaction velocity (Δ O.D./min.) correspond with the reduction of $NADP^+$ in *in vitro* systems containing various concentrations of ATP and 3.3×10^{-5} M BA.

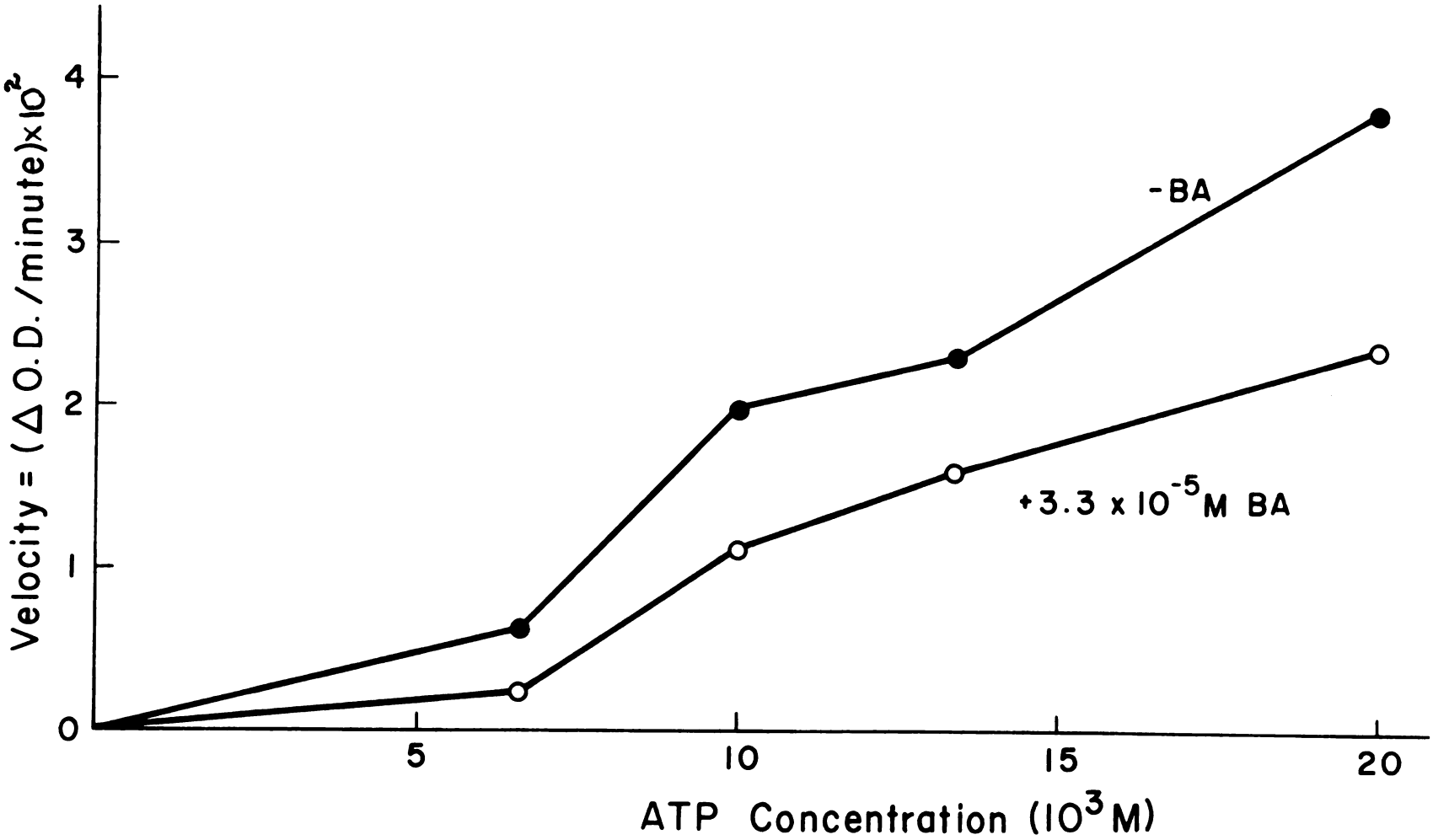

Velocity = (Δ O.D./minute)x10²
4
3
2
1
0
5
10
15
20
ATP Concentration (10³ M)
-BA
+3.3 x 10⁻⁵M BA

Figure 8

Effect of BA (3.3 and 6.6×10^{-5} M) on glucose-6-phosphate dehydrogenase activity. Changes in optical density correspond with the reduction of $NADP^+$ in in vitro systems.

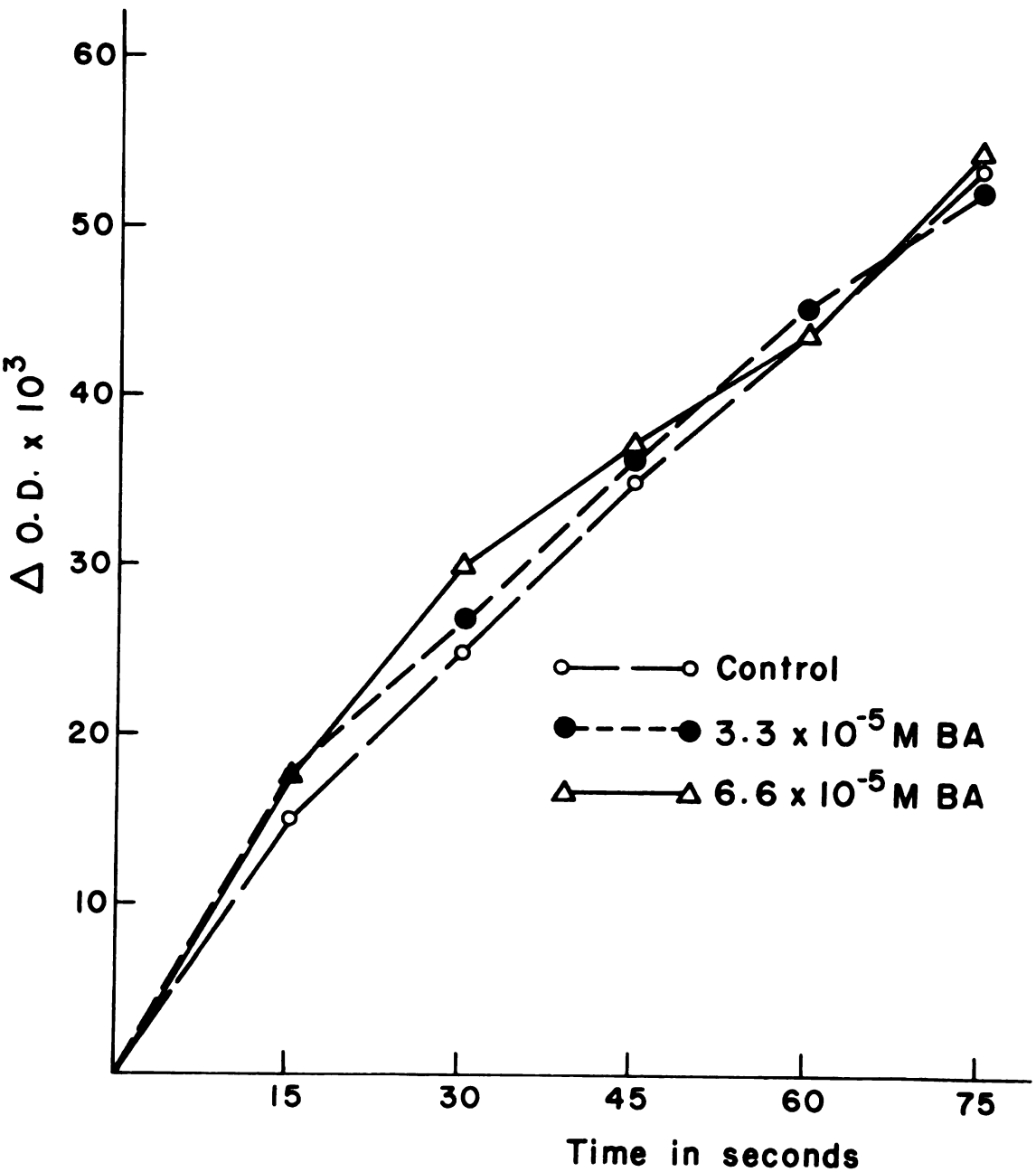
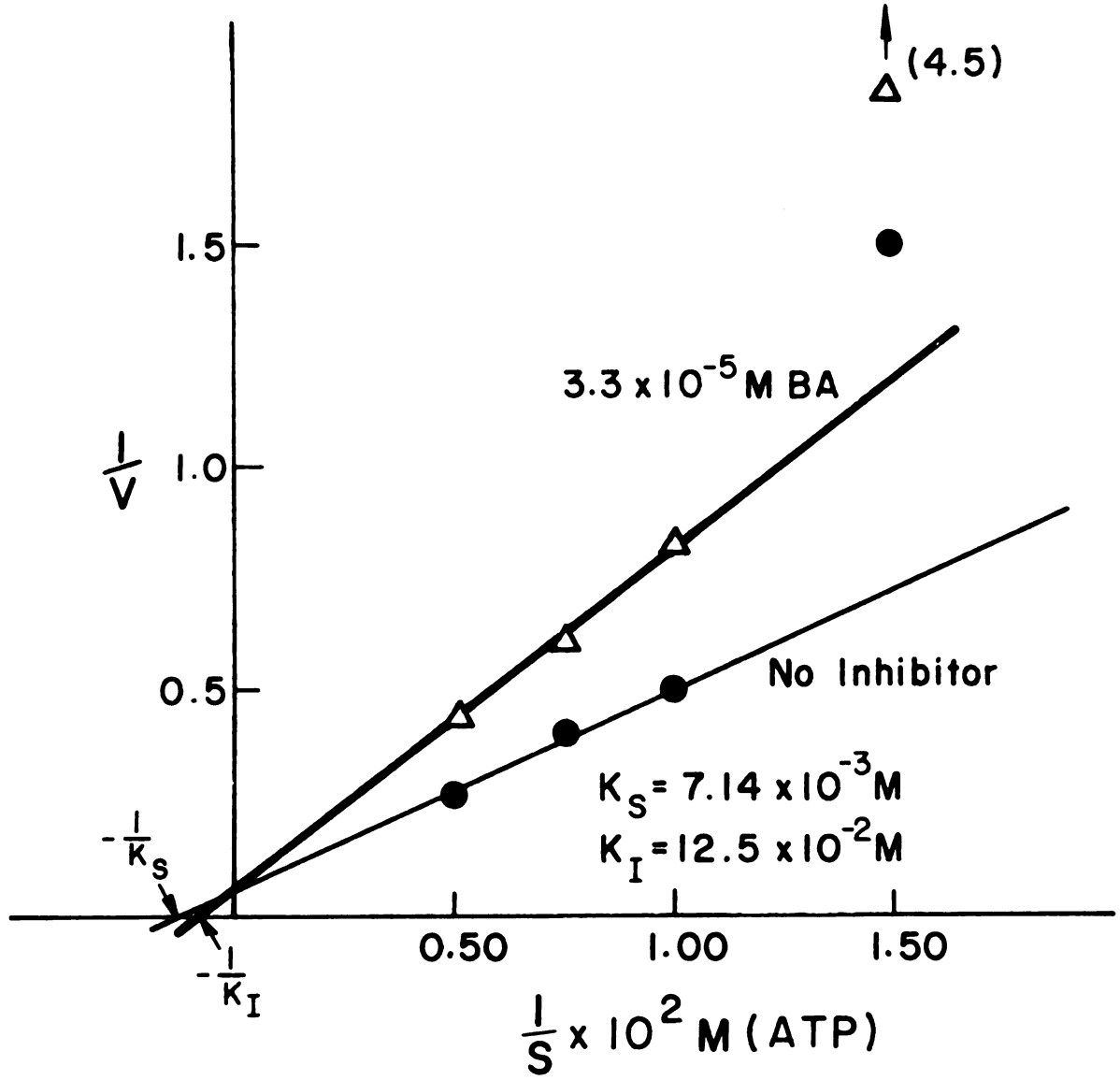

60
50
40
30
20
10
Δ O.D. x 10³
15
30
45
60
75
Time in seconds
Control
3.3 x 10⁻⁵ M BA
6.6 x 10⁻⁵ M BA

Figure 9

Inhibition of hexokinase by 3.3×10^{-5} M BA. The velocities and substrate concentrations (ATP) are plotted as the reciprocal of their values. The common intercept of the treated and control curves on the $\frac{1}{V}$* axis confirms competitive inhibition. K_S and K_I are the apparent substrate and inhibitor constants respectively.

---

*$\frac{1}{V}$ axis should read $\frac{1}{V} \times 10^{-2}$.


(4.5)
1.5
1.0
0.5
1/V
3.3 x 10^-5 M BA
No Inhibitor
K_S = 7.14 x 10^-3 M
K_I = 12.5 x 10^-2 M
-1/K_S
-1/K_I
0.50
1.00
1.50
1/S x 10^2 M (ATP)

N^6-benzyladenine (BA) and glutamine synthetase activity

The competitive inhibition of hexokinase by BA suggested that BA might inhibit other enzymic reactions where molecular ATP was involved; hence, the activity of glutamine synthetase, in the presence of BA, was tested.

The enzymatic synthesis of glutamine occurs as follows:

glutamic acid + ammonia + ATP $\xrightarrow{\text{Glutamine synthetase}}$ glutamine + ADP + phosphate.

Ammonia in the above equation can be replaced by hydrazine or hydroxylamine. All three bases react at the same rate (24). With hydroxylamine, a hydroxamic acid (glutamyl-hydroxamic acid) may be produced, the estimation of which provides a convenient colorimetric test (54).

Preparation of solutions and reagents

Sodium glutamate (0.5 M)--Four hundred forty mg. of sodium glutamate (MW 176) were dissolved in distilled water and made up to 50 ml.

ATP (0.05 M)--Six hundred mg. of ATP-$Na_2H_2.3H_2O$ were dissolved in distilled water and made up to 20 ml.

Tris buffer, BA, and Cysteine--Prepared as in the hexokinase experiments.

Magnesium sulfate (1 M)--Twelve gm. anhydrous $MgSO_4$ were dissolved in distilled water and made up to 100 ml.

Hydroxylamine (4 M; pH 6.4)--Prepared by nearly neutralizing $NH_2OH.HCl$ (28%) with an equal volume of 14%

NaOH (3.5 M), so that the resulting solution remained slightly acidic. This labile solution was prepared daily.

Hydroxylamine (1 M; pH 7.6)--Prepared by dissolving 0.33 gm. $NH_2OH.HCl$ in Tris (pH 7.6) and brought up to a volume of 10 ml.

Acetate buffer (0.1 M; pH 5.4)--One-tenth molar solutions of acetic acid and sodium acetate were mixed in 1:4 proportions.

Ferric chloride (5%)--A solution of $FeCl_3.6H_2O$ was made in 0.1 N HCl.

Trichloracetic acid (12% w/v)--This solution was prepared with distilled water.

Glutamyl hydroxamic acid standard curve--One-half gm. of L-glutamine was dissolved in 4 ml. of 2 M hydroxylamine, in a 10 ml. volumetric flask. Ten minutes were allowed for the conversion of glutamine to glutamyl-hydroxamic acid. The flask was then filled to the 10 ml. mark with distilled water. Aliquots were pipetted into standardized colorimetric tubes containing 1 ml. acetate buffer (pH 5.4) and the volume adjusted to 3 ml. at room temperature. Thereafter, 1 ml. each of HCl, TCA, and ferric chloride solution was added in the indicated order. Absorption of the purple iron complex formed was measured at 540 mμ on a Bausch and Lomb colorimeter (54).

Protein standard curve--Constructed after the method of Lowry et al. (55).

Glutamine synthetase activity was determined according

to Elliott (24). Incubations were carried out at 30° C for 20 minutes. The system consisted of the following components for the control. Each treatment was replicated three times.

Tris	0.5 ml.
Na-glutamate	0.5 ml.
ATP	0.5 ml.
$MgSO_4$	0.1 ml.
Cysteine	0.1 ml.
NH_2OH (pH 7.6)	0.1 ml.
Enzyme	0.3 ml.
Water	0.15 ml.
BA	-
	2.25 ml.

Treated samples were provided with 0.1 ml. BA (final concentration ca. 4.4×10^{-5} M) and only 0.05 ml. of water.

Immediately following incubation, 1 ml. aliquots were drawn into centrifuge tubes containing 1 ml. of 4 M hydroxylamine and 1 ml. acetate buffer. After 10 minutes, when the glutamine formed during incubation had been converted to glutamyl-hydroxamic acid, 1 ml. each of HCl, TCA, and $FeCl_2$ was added respectively. The precipitated protein was removed by centrifugation and the supernatant decanted into colorimetric tubes. Glutamyl-hydroxamic acid was measured quantitatively using an appropriate standard. One enzyme unit was arbitrarily defined as the production of

1.50 micromoles of glutamyl-hydroxamic acid in 20 minutes at 30° C.

The procedure used in the purification of glutamine synthetase was a modification of that proposed by Elliott (24). Temperatures were maintained at 0°-4° C. Centrifugation was carried out at 10,000x g. for 15 minutes at 0° C.

Stage 1 - Extraction--Washed cauliflower curd (100 gm.) was homogenized in a Servall omni-mixer with 200 ml. of 0.1 M $NaHCO_3$ for 1 minute. Twelve gm. of $MgSO_4$ were then stirred in and the precipitate allowed to settle overnight at 0° C. The supernatant fluid was poured off and the remaining suspension centrifuged. The two supernatant fluids were combined. Aliquots were used for estimating protein content and glutamine synthetase activity, in the presence and absence of BA.

Stage 2 - Fractionation with ammonium sulfate--The extract was adjusted to pH 6.5 by the addition of 2 M KH_2PO_4 and 0.33 gm. of solid ammonium sulfate per ml. The precipitate was allowed to settle overnight at 0° C and the supernatant discarded. The precipitate was suspended in 220 ml. of cold distilled water and brought to pH 7.2 by the addition of N NaOH. The thick suspension was put into cellophane tubes, and dialyzed with stirring against two changes of 7 liters of cold distilled water, for about 40 hours. A small sample of the cloudy dialysate was centrifuged. This product was used for protein determination and enzyme assay.

Stage 3 - Treatment with Protamine--The dialyzed extract was treated with a 2 per cent solution of protamine sulfate, until a small sample, after centrifugation, gave no further precipitate on the addition of a drop of protamine solution. About 200 ml. were required. The inactive precipitate was centrifuged and the supernatant retained for protein and enzyme assay.

Stage 4 - Second ammonium sulfate fractionation--The above supernatant was adjusted to pH 7.6 by the addition of phosphate buffer (prepared as described under general methods) and 300 ml. of cold, saturated ammonium sulfate were added. The bulky inactive precipitate was removed by centrifugation and 240 ml. of the cold, saturated ammonium sulfate solution were added to the supernatant. The resulting precipitate was centrifuged and redissolved in cold water, with the addition of M K_2HPO_4 to a pH of 7.3. A small sample of redissolved precipitate was used for protein and enzyme assay.

Stage 5 - Dialysis--The remainder of the above suspension was dialyzed with stirring against three changes of 7 liters of distilled water for about 30 hours. The precipitate was discarded after centrifugation and the supernatant retained for protein and enzyme assay.

Stage 6 - Third ammonium sulfate fractionation--The above supernatant was adjusted to pH 7.4 by the addition of phosphate buffer and 50 ml. of cold, saturated ammonium sulfate were then added. The precipitate was discarded

and a further 50 ml. of the saturated ammonium sulfate were added to the supernatant and left overnight at 0° C. The precipitate was centrifuged and redissolved in cold distilled water. $\underline{M}$ K_2HPO_4 was added to bring the pH of the redissolved precipitate to 7.3. This was tested for protein and glutamine synthetase activity.

After stage 6, a 10-fold purification of the enzyme, glutamine synthetase had been obtained. A purification summary is presented in Table II. The comparative activity of glutamine synthetase at each purification stage, in the presence and absence of BA, is portrayed by Fig. 10.

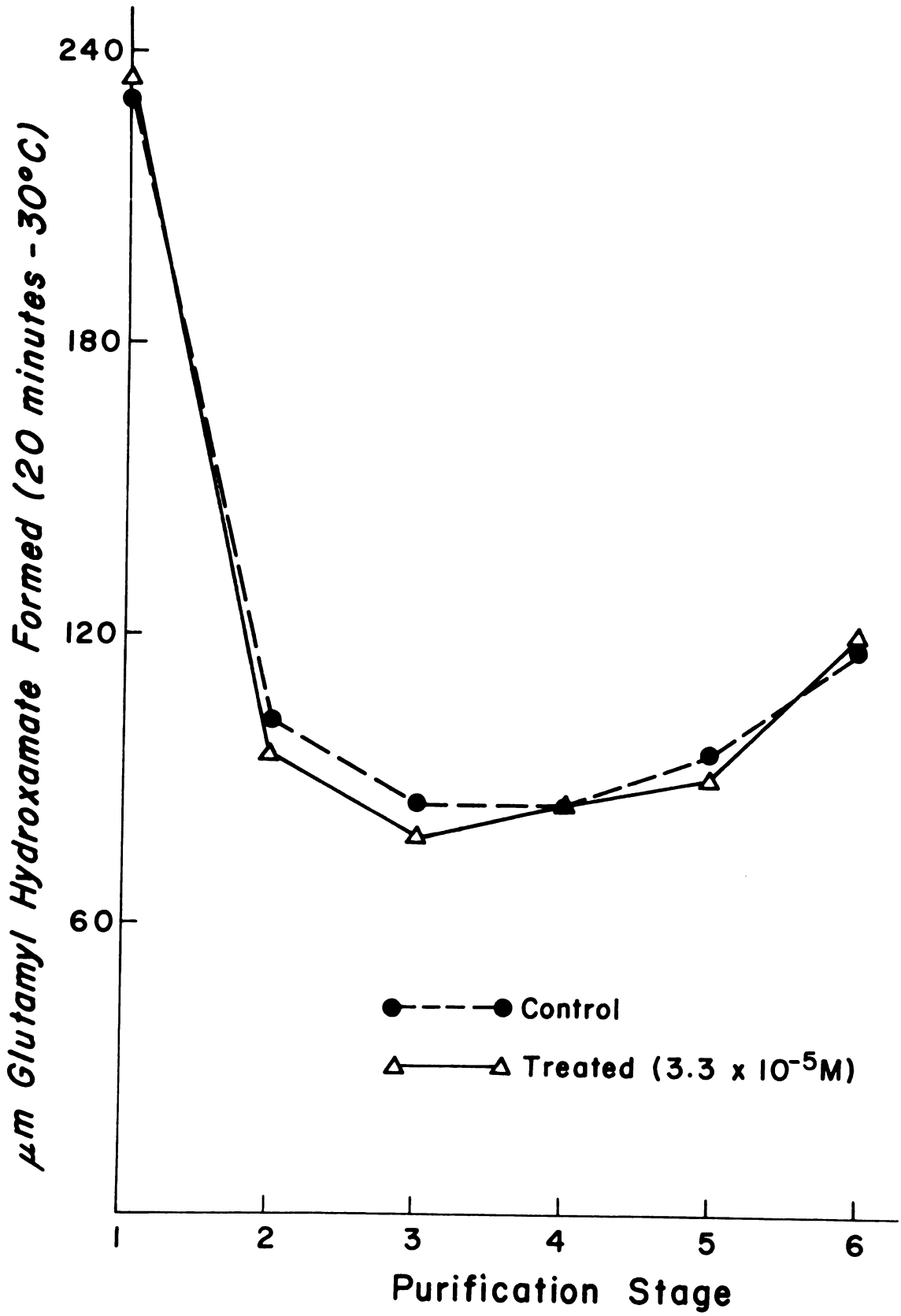
Table II

Steps in the purification of cauliflower glutamine synthetase and the effects of 4.4×10^{-5} M BA on the enzyme at each stage of purification

Stage	Treatment	Vol. (ml.)	Aliquot (ml.)	Units/ml.	Total Units (10^4)	% Yield	Protein (mg/ml.)	Specific Activity (10^2)	Fold Purified
1.	Control	193	0.2	1150	22.2	100	1.4	8.2	1
	Treated	193	0.2	1160	22.4		1.4	8.3	
2.	C	302	0.3	343.4	10.4	47	0.325	10.6	1.3
	T	302	0.3	317.2	9.6		0.325	9.8	
3.	C	342	0.3	284	9.7	44	0.300	9.5	1.2
	T	342	0.3	260	8.9		0.300	8.7	
4.	C	50	0.3	284	1.4	6.4	0.150	18.9	2.3
	T	50	0.3	284	1.4		0.150	18.9	
5.	C	56	0.3	317	1.8	8	0.105	30.2	4.0
	T	56	0.3	300	1.7		0.105	28.6	
6.	C	20	0.3	361	0.7	3	0.04	90.0	10
	T	20	0.3	364	0.7		0.04	90.0	

Figure 10

The effects of BA (4.4×10^{-5} M) on glutamine synthetase activity at various stages in the purification of the enzyme. Activity was determined colorimetrically by measuring the conversion of glutamine to glutamyl-hydroxamic acid.


240
180
120
60
μm Glutamyl Hydroxamate Formed (20 minutes - 30°C)
Control
Treated (3.3 x 10⁻⁵M)
1
2
3
4
5
6
Purification Stage

N^6-benzyladenine (BA) and pyruvic kinase activity

The ineffectiveness of BA on the ATP mediated glutamine synthetase activity suggested that inhibition was specific for the kinases. Accordingly, the activity of pyruvic kinase (PK), in the presence of BA, was studied.

PK catalyzes the following reaction in the presence of adenosine diphosphate (ADP).

Phospho(enol)pyruvate (PEP) + ADP $\underset{\rightleftharpoons}{PK,Mg^{2+}}$ Pyruvate + ATP.

The above equation was coupled with lactic dehydrogenase (LDH):

Pyruvate + NADH + H^+ $\underset{\rightleftharpoons}{LDH}$ Lactate + NAD^+.

The activity of PK was measured by the decrease in optical density at 340 mμ, from the oxidation of NADH (reduced nicotinamide-adenine dinucleotide). Quantitative conversion was assured because of the equilibria of the reactions catalyzed by PK and LDH.

The following solutions were prepared using deionized glass distilled water, and maintained as in the hexokinase experiments.

Tris buffer (0.05 M; pH 7.6) and BA (10^{-3} M)--See general methods.

Magnesium chloride (0.1 M)--Prepared by dissolving 0.19 gm. $MgCl_2$ in water and made up to a volume of 20 ml.

Potassium chloride (0.5 M)--The chemical (0.75 gm.) was dissolved in water and brought up to 20 ml.

Adenosine diphosphate (0.1 M ADP)--The solution

consisted of 0.511 gm. of ADP-Na_3 dissolved in 10 ml. of water.

Phospho(enol)pyruvate (0.1 M)--Solution prepared by dissolving 0.234 gm. PEP-Na_3 in 10 ml. of water.

Reduced nicotinamide-adenine dinucleotide (ca. 0.01 M NADH)--Prepared by dissolving 35 mg. NADH-Na_2 in 5 ml. Tris buffer (pH 7.6).

Pyruvic kinase (Sigma Chemical Co., 2,500 units)--A unit was defined as that amount of enzyme which catalyzed the conversion of 250 micromoles of PEP to pyruvate per minute at a pH of 7.6 and at 25° C. The enzyme suspension was reconstituted by the addition of 0.1 ml. Tris (pH 7.6) for each 50 units.

Lactic dehydrogenase (Sigma Chemical Co., 10,000 units)--Each unit was that amount required to catalyze the conversion of 400 micromoles of NADH per minute at a pH of 7.6 and at 25° C. The suspension was reconstituted by the addition of 0.1 ml. Tris (pH 7.6) per 200 units.

Optical density (O.D.) values utilized for kinetic measurements were obtained with a Beckman DU spectrophotometer at 340 mμ, a light path of 1 cm., and a final volume of 3 ml. made up with water. Measurements were made against a blank, using silica cuvettes. Components of the blank were pipetted into the cuvettes, in the indicated order.

Tris	1.0 ml.
KCl	0.1 ml.
$MgCl_2$	0.2 ml.

ADP	varied
PEP	0.2 ml.
NADH	-
*DGD water	to make up 3.0 ml.
BA	-
LDH	0.4 ml. (200 units)
PK	0.1 ml. (50 units)

The control samples received only NADH (0.2 ml.), and the treated received NADH (0.2 ml.), plus BA (0.1 ml.). The final concentration of BA was 3.3×10^{-5} M.

After the addition of lactic dehydrogenase (LDH), the contents were mixed by inversion and allowed to equilibrate for three minutes at 25° C. When the O.D. was constant, pyruvic kinase (PK) was added and O.D. measured every 30 seconds. Reaction velocities in the presence of BA and varying concentrations of ADP are shown in Fig. 11. Exploratory experiments indicated that LDH activity was not affected by BA. Double reciprocal plots of the velocities and substrate concentrations of ADP, portraying the nature of BA inhibition of pyruvic kinase activity, are presented in Fig. 12.

---

*Deionized glass distilled water.

Figure 11

Effect of BA on pyruvic kinase activity. Changes in velocity (Δ O.D./min.) correspond to the oxidation of NADH in *in vitro* systems containing various concentrations of ADP and 3.3×10^{-5} *M* BA.

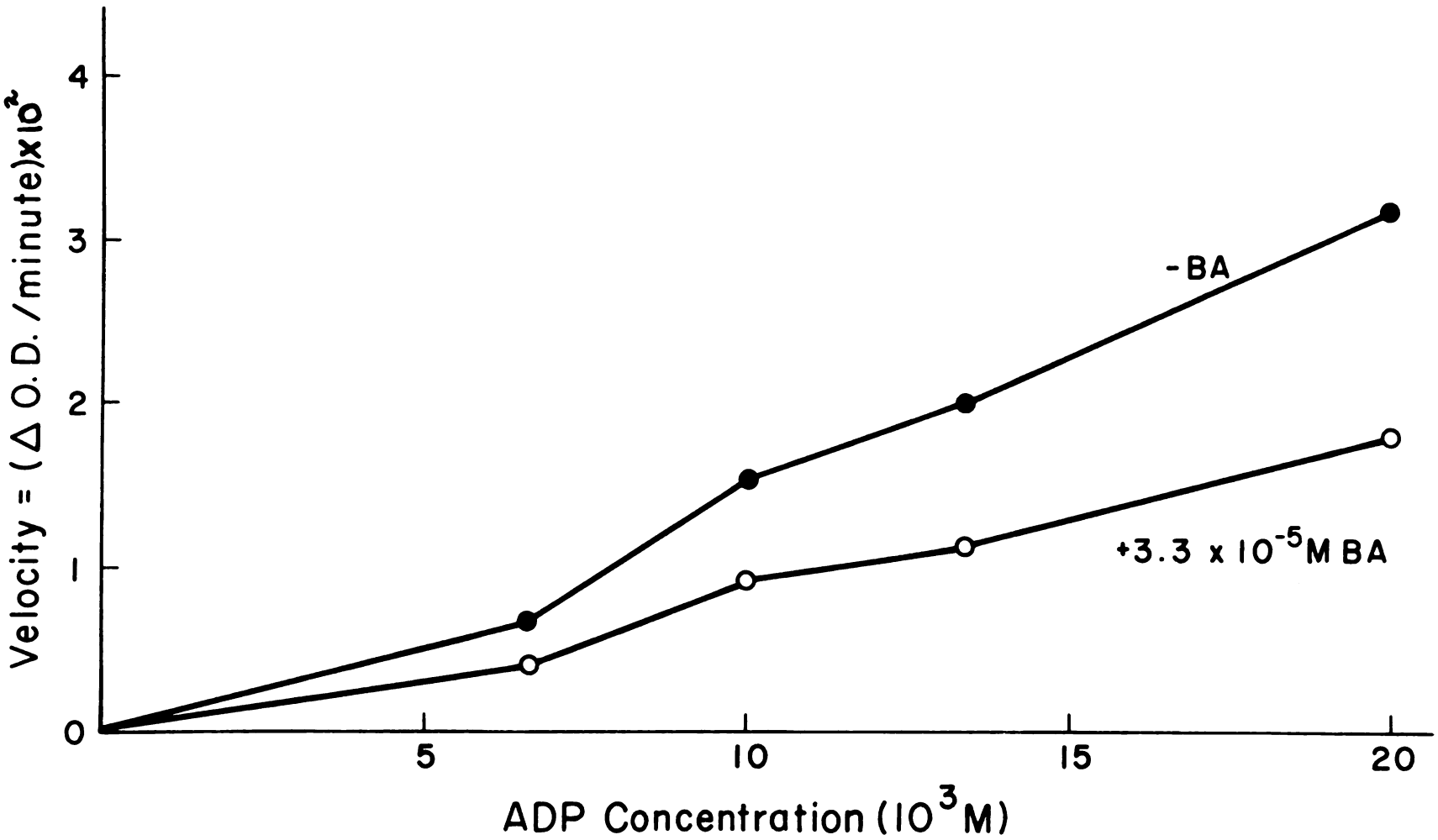
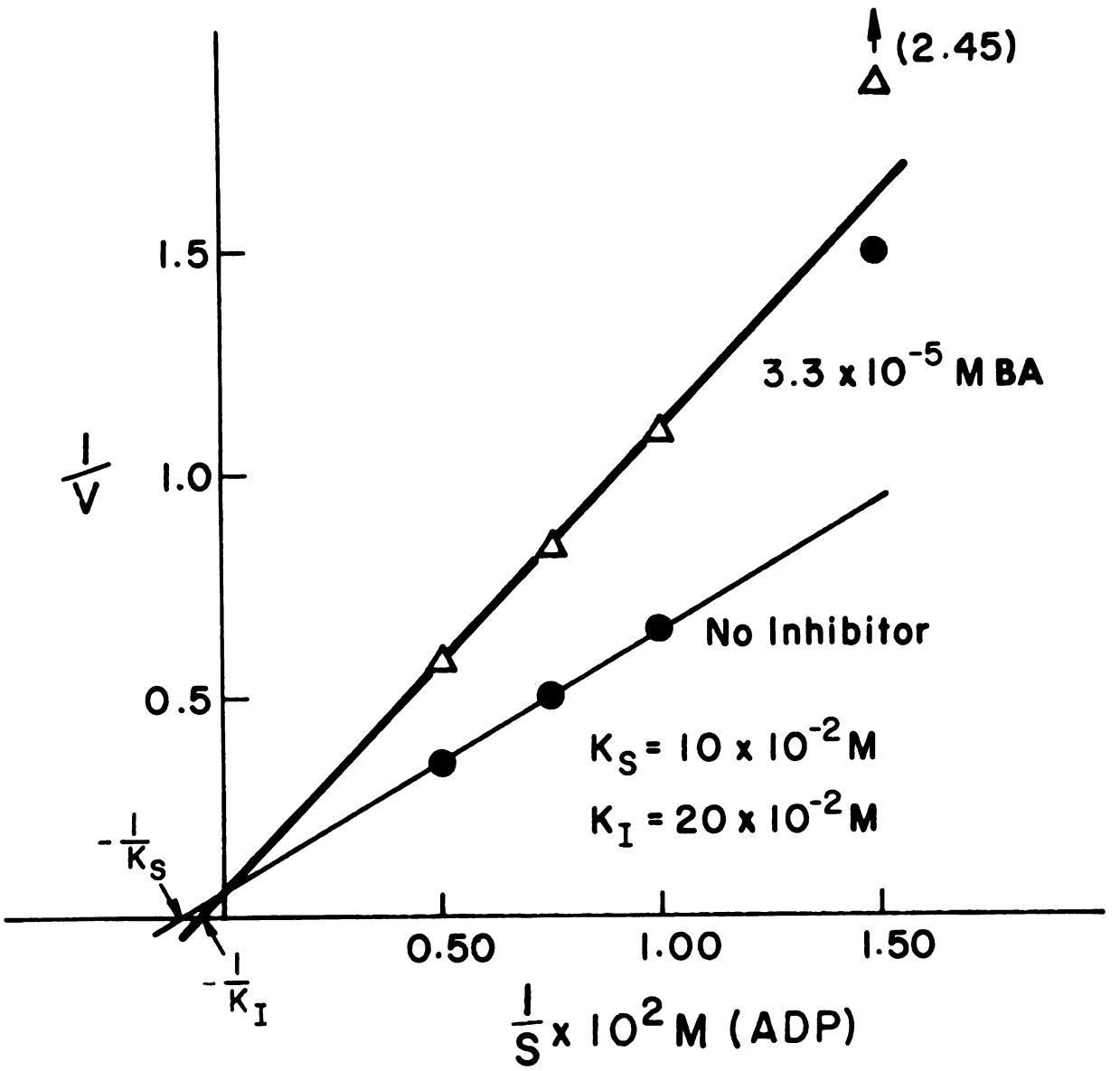

Velocity = (Δ O.D. /minute)x10²
4
3
2
1
0
5
10
15
20
ADP Concentration (10³M)
-BA
+3.3 x 10⁻⁵M BA

Figure 12

Double reciprocal plots of the velocities* and substrate concentrations (ADP) illustrate competitive inhibition of pyruvic kinase by 3.3×10^{-5} M BA. K_s and K_I are the apparent substrate and inhibitor dissociation constants respectively.

---

*$\frac{1}{V}$ should read $\frac{1}{V} \times 10^{-2}$.


(2.45)
1.5
1.0
0.5
$\frac{1}{V}$
3.3×10^{-5} M BA
No Inhibitor
$K_S = 10 \times 10^{-2}$ M
$K_I = 20 \times 10^{-2}$ M
$-\frac{1}{K_S}$
$-\frac{1}{K_I}$
0.50
1.00
1.50
$\frac{1}{S} \times 10^2$ M (ADP)

DISCUSSION

It is evident from the results herein presented that the primary effect of treatment of broccoli with N^6-benzyladenine (BA) at a concentration of $3.3\text{-}5 \times 10^{-5}$ M is to delay the onset of senescence (indexed by chlorophyll and carotene degradation), and inhibit respiration, phenomena which are closely related. In the following discussion, it is attempted to elucidate some of the possible mechanisms involved in the inhibition of respiration.

Chlorophyll and carotene metabolism

BA delays chlorophyll and carotene degradation (Fig. 1). The negative slopes of the curves show that BA neither enhances the synthesis of the pigments nor maintains them at a constant level. The rate of degradation is merely slowed down.

Degradation of chlorophyll is brought about by chlorophyllase as follows (3, 6):

$$\text{Chlorophyll} \xrightarrow{\text{Chlorophyllase}} \text{Chlorophyllide} + \text{Phytol}$$

In the presence of BA, degradation may be inhibited by a reaction product and/or accompanied by progressive enzyme inactivation. Inhibition of carotene degradation may likewise be explained, assuming that a similar system is operative.

Purine derivatives are known to prevent protein

degradation (78, 85), enhance protein and RNA synthesis (30, 31, 71), and delay chlorophyll and carotene breakdown (Fig. 1). In excised tissue, protein and pigment metabolism are closely related. The breakdown of one usually results in the breakdown of the other. A delay in such degradation suggests that BA maintains the physiological integrity of the cell for longer than normal periods.

Photosynthesis

Broccoli leaves were subjected to photosynthesis at 8,000-foot candles for ten minutes in an atmosphere of $^{14}CO_2$ (Fig. 4). The higher rate of ^{14}C fixation by treated broccoli leaves (Fig. 5) was probably associated with a higher chlorophyll and carotene content, since both pigments are active in photosynthesis. Furthermore, the photosynthetic rate and pigment content (Fig. 1) can be superimposed.

Distribution of ^{14}C among the various photosynthetic products revealed that certain metabolites accumulated to a greater extent in the treated leaves, whereas others were relatively less as compared to the controls (Table I).

Sucrose, which is generally the end product of photosynthesis, accumulated in the treated leaves. This could have been the result of greater synthesis, or the prevention of degradation of sucrose in the presence of BA. The work of Cardini et al. (15) has shown that extracts of plant tissues contain an enzyme ("UDPG-fructose trans-glycosylase") that catalyzes the transfer of the glucosyl residue of

uridine diphosphate glucose (UDPG) to fructose, thus forming sucrose and uridine diphosphate (UDP). The breakdown of sucrose, on the other hand, is enzymatically brought about by invertase, which hydrolyzes sucrose to glucose and fructose. The presence of approximately equal amounts of UDPG (Table I) in the treated and control leaves at successive time intervals, and the progressive increment of sucrose in the treated leaf samples suggested that sucrose breakdown was reduced by BA. This possibility was further strengthened in that both free glucose and fructose (breakdown products of sucrose) accumulated in the control samples.

Distribution of ^{14}C in serine followed a peculiar pattern. A greater accumulation occurred in the treated leaves up to 48 hours, after which it dramatically dropped and more was found thereafter in the controls.

The metabolism of serine and glycine is closely related in animals and plants. The principal source of glycine is serine (83). It is degraded to glycine and formate. The metabolic conversion of serine to glycine and a C_1 unit (serine $\longrightarrow$ glycine + C_1 unit) is reversible (41). The sudden accumulation of serine after 72 hours, and the concomitant decrease of glycine in the control leaves suggested that the leaf tissue was becoming senile. The continued production of glycine in treated leaves indicated that BA kept the system intact longer. The synthesis of glycine is not restricted, however, to the aforementioned reaction. Glycolic acid is also known

to be a precursor of glycine in higher plants (86), and microbial systems (14). The sequence is as follows:

Glycolate $\longrightarrow$ glyoxylate $\longrightarrow$ glycine.

The intermediate glyoxylate could not be detected in either the treated or control leaves; whereas glycolate accumulated more in the treated leaves. In animals and higher plants, the C_2 precursor of glycine could also arise from carbohydrates by transketolase catalyzed reactions.

Isotopic studies further revealed that, of the organic acids, citrate accumulated more in the control leaves, while greater quantities of malate accumulated in the treated leaves. The significance of this became clear when the net pool of aspartic and glutamic acids was examined. These acids are normally fed into the tricarboxylic acid (TCA) cycle (45). An increase in these acids led to more production of citric acid in the control leaves. The lesser amounts of glutamate and aspartate in treated leaves suggested that they were utilized instead for the synthesis of proteins, which may be an explanation for the classical concept that protein synthesis is enhanced by kinins.

Whenever the TCA cycle is set "off balance," as in the above case, an alternative pathway known as the glyoxylic acid cycle becomes operative (2, 43). In this pathway an enzyme system ("malate synthetase") effects the condensation of acetate with glyoxylic acid to form malic acid, a reaction formally analogous to the enzymic condensation of acetyl-CoA with oxaloacetic acid to form citric

acid. The presence of slightly greater amounts of malate in treated leaves was probably due to the operation of the glyoxylic acid cycle.

Striking differences were encountered in the distribution pattern of ^{14}C among the hexose and pentose phosphate esters of treated and control leaves. The rate of formation of these phosphate esters remained constant in the treated leaves, whereas it increased steadily in the control leaves. The formation of phosphate esters is generally mediated by a group of enzymes known as the kinases, e.g.,

Hexose $\xrightarrow{\text{Hexokinase}}$ hexose-phosphate.

Similar systems are operative for the conversion of pentoses to pentose-phosphates (77).

Phosphate esters are of signal importance in the metabolism of carbohydrates, fats, proteins, and vitamins. Intermediary metabolism essentially occurs via the phosphate esters (57). Thus differences observed in the amounts of phosphate esters fixed in the control and treated leaves first suggested that BA had an effect on the kinases.

A discrepancy in the data was noted, however. There was no apparent accumulation of free sugars in the treated leaves, even though phosphorylation was inhibited. It appeared, therefore, that enzymes other than the kinases were also inhibited subsequent to treatment with BA.

Mitochondrial respiration

Some reports (22, 21, 94) have indicated that

respiration of explants is inhibited by BA. Mitochondrial respiration is likewise inhibited (Figs. 2 and 3).

The broccoli mitochondrial fraction was highly contaminated with chloroplasts, and required fortification with cytochrome c and ATP before any oxidation of succinate could be detected. Citrate which is known to enhance the oxidation by chloroplast contaminated mitochondria (76) had no effect. Oxygen uptake was measured by covering the Warburg apparatus with a black cloth, so that no photosynthesis could occur. The cumulative oxygen uptake was linear with time (Fig. 2). The sudden drop in oxygen uptake by mitochondria treated with BA probably arose from exposure to light. The manometers had to be removed from the Warburg bath to tip in the BA. The exposure time was apparently long enough to cause adequate CO_2 fixation and O_2 evolution. Equilibrium was attained after about 20 minutes and oxygen uptake was again linear. The magnitude of inhibition caused was about 48 per cent.

Cauliflower mitochondria, also, had to be fortified with ATP before any oxidation of succinate could be detected. Oxygen uptake was linear (Fig. 3), and about a 20 per cent inhibition was induced by BA.

Preliminary experiments revealed that oxidation of pyruvate by mitochondria of both species (broccoli and cauliflower) was inhibited in the presence of BA. The inhibition could not be overcome by the addition of citrate, α-ketoglutarate, or succinate. This suggested that the

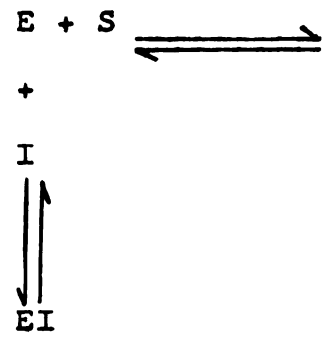
inhibition was not specific for the TCA cycle. It should be noted, however, that fortification of the mitochondria with ATP was necessary.

Mode of action of BA in respiration inhibition

It was mentioned earlier that phosphates did not accumulate in the treated leaves. Further, it was implied that BA impeded the activity of certain phosphorylating enzymes. This was indeed true in the case of hexokinase. The reduction of $NADP^+$ in an *in vitro* system was greatly inhibited in the presence of BA. The magnitude of inhibition was independent of the glucose concentration, but was greatly affected by the concentration of ATP. The reaction rates were measured by the rate of reduction of $NADP^+$ to NADPH at 340 mμ. It may be seen from Fig. 7 that the velocity of the reaction was accelerated by increasing the concentration of ATP and holding the concentration of BA constant. The lag noted at low concentrations of ATP was from product inhibition. ADP formed in the reaction likely interfered with the binding of ATP on the enzyme surface (29).

The tendency of high ATP concentrations to overcome the inhibitory effect of BA suggested that a competition existed between ATP and BA for an active site on hexokinase. Results of kinetic studies plotted after the method of Lineweaver and Burk (53) showed that the maximum velocities attained in the presence and absence of BA were the same

(Fig. 9). This is typical of a competitive inhibition and may be explained as follows:



where,

E = concentration of enzyme

S = concentration of substrate

I = concentration of inhibitor

ES = concentration of E-S complex

EI = concentration of E-I complex

The substrate (ATP) and inhibitor (BA) compete for the same site on hexokinase. At low concentrations of ATP, BA predominated, and the situation was reversed at high ATP concentrations. Thus the inhibitory effect of BA was overcome. The competitive inhibition may be explained on the basis of a similarity in chemical structure between ATP and BA. Both compounds have a common purine nucleus. Glucose-6-phosphate dehydrogenase, the other enzyme used in the assay, was not affected by BA at two different concentrations (Fig. 8). It is apparent that the activity of hexokinase, a phosphorylating enzyme was, indeed, specifically inhibited by BA.

ATP participates in several enzyme catalyzed reactions

including the synthesis of glutamine, catalyzed by glutamine synthetase. A 10-fold purification of the enzyme was prepared from cauliflower curd (Table II). The activity was tested at each purification step. Conversion of glutamine to glutamyl-hydroxamate served as an index of enzyme activity. It may be noted (Fig. 10) that BA had no effect on glutamine synthetase activity. The enzymic synthesis of glutamine is coupled to the cleavage of ATP. In contrast, however, to the reactions discussed for hexokinase, the nucleotide is cleaved to ADP and phosphate (24), without affecting any substrate phosphorylation, although phosphorylated enzymes appear to be formed as intermediates (44).

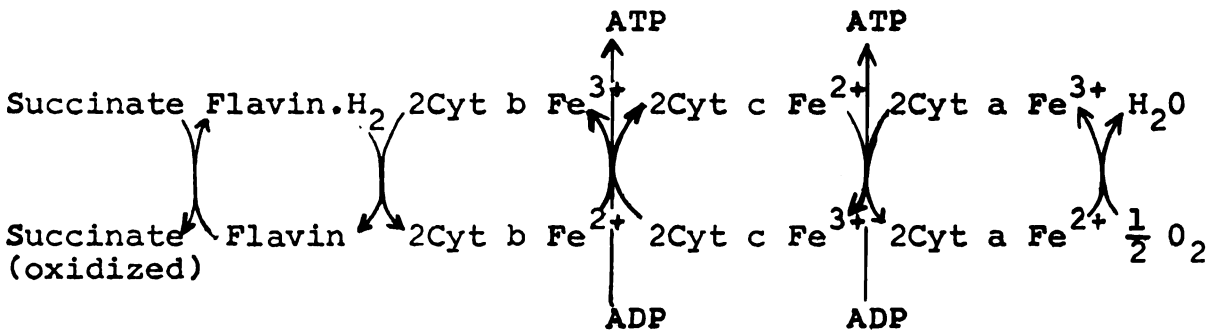
Specificity of BA for the kinases was established by studying its effects on pyruvic kinase. The enzyme catalyzes the conversion of phospho(enol)pyruvic acid (PEP) to pyruvate, which is further reduced to lactic acid by lactic dehydrogenase, in the presence of NADH. The coupled enzyme reaction was measured by noting the disappearance of NADH at 340 mμ. In preliminary experiments it was noted that high PEP concentrations inhibited the reaction rate, since the pyruvate formed caused a potent "substrate inhibition" of lactic dehydrogenase (33). Presence of BA enhanced the reaction rate, probably by arresting the formation of pyruvate. Evidently, in this case, BA was competing with ADP for the active site on pyruvic kinase.

By decreasing and increasing the concentrations

of PEP and lactic dehydrogenase respectively, the formation of pyruvate was slowed down, and consequently its turnover rate increased. Under such circumstances, a marked inhibition in the reaction rate was observed in the presence of BA. Fig. 11 shows that a situation analogous to hexokinase exists. By increasing the ADP concentrations, the inhibitory effect of BA was again overcome. In this case, the lag came from the production of ATP. The double reciprocal plot (Fig. 12) indicates that the nature of inhibition is competitive, again, from a similarity in chemical structure between ADP and BA. It is suggestive from the foregoing that BA is a specific inhibitor of the kinases. Since most kinases occur in glycolysis, it may be assumed that BA inhibits anaerobic respiration.

Reactions in the glycolytic pathway are mediated by cytoplasmic enzymes which are distinct from the mitochondrial enzymes involved in aerobic respiration. It will be recalled that oxidation of succinate by broccoli and cauliflower mitochondria was inhibited by BA. The complete catalytic system for the oxidation of succinate ("succinoxidase") is located in the mitochondria (38). Mitochondria thus provide an intact pathway for electrons from a metabolite to molecular oxygen.

Inhibition noted in the two distinct systems thus becomes difficult to correlate. However, the sequence of electron transfer to oxygen in the succinoxidase system appears to be as follows (16, 17):



It will be noted that certain sites are provided on the electron transport chain for the phosphorylation of ADP to yield ATP. It is possible that BA may intervene in these steps and thus cause an inhibition in the overall reaction. Another explanation may be that ATP is essential for mitochondrial activity. The role of ATP might possibly be that of maintaining the integrity of the system, and BA may interfere with this role.

SUMMARY

Mechanisms of the action of N^6-benzyladenine (BA) in inhibiting respiration of chlorophyllous and non-chlorophyllous tissue of broccoli and cauliflower are described.

Post-harvest dip applications of 5×10^{-5} M BA to broccoli heads delayed chlorophyll and carotene degradation which was ascribed to an inhibition of respiration.

Oxidation of succinate by broccoli and cauliflower mitochondria was markedly inhibited by 3.3×10^{-5} M BA.

Photosynthetic ($^{14}CO_2$ fixation) studies revealed that the per cent of ^{14}C fixed in the phosphate esters of BA treated broccoli leaves was much less as compared to the controls. This suggested that BA interfered with the activity of the phosphorylating enzymes (kinases).

In vitro systems involving (a) hexokinase and adenosine triphosphate (ATP), and (b) pyruvic kinase and adenosine diphosphate (ADP) revealed that the activity of the kinases was, indeed, inhibited by the presence of 3.3×10^{-5} M BA. Alternatively, the ineffectiveness of BA on other enzyme systems involving molecular ATP, namely, glutamine synthetase, was demonstrated.

Classic enzyme kinetics indicated that the similarity in structure between the nucleotides and BA was the origin of a competitive inhibition for an active site on the kinases along the glycolytic pathway.

LITERATURE CITED

1. Adamson, H. 1959. The physiology of auxin action. Doctoral thesis, Sydney University, Sydney, Australia.
2. Ajl, S. J. 1956. Conversion of acetate and glyoxalate to malate. Jour. Amer. Chem. Soc. 78:3230-3231.
3. Ardao, C., and B. Vennesland. 1960. Chlorophyllase activity of spinach chloroplastin. Plant Physiol. 35:368-371.
4. Bamberger, E., and A. M. Mayer. 1960. Effect of kinetin on formation of a red pigment in seedlings of Amaranthus retroflexus. Science. 131:1094-1095.
5. Bandurski, R. S., and B. Axelrod. 1951. The chromatographic identification of some biologically important compounds. Jour. Biol. Chem. 193:405-410.
6. Barrett, J., and S. W. Jeffrey. 1964. Chlorophyllase and formation of an atypical chlorophyllide in marine algae. Plant Physiol. 39:44-47.
7. Benson, A. A., J. A. Bassham and M. Calvin. 1950. The path of carbon in photosynthesis. V. Paper chromatography and autoradiography of the products. Jour. Amer. Chem. Soc. 72:1710-1716.
8. Bergman, F., and H. Kweitny. 1960. Oxidation of kinetin by mammalian xanthine oxidase. Biochim. Biophys. Acta. 28:100-106.

9. Bergmann, L. 1963. Action of 6-furfurylamino purine on the respiration of cell suspensions of Nicotiana tabacum. Naturforsch. 11:942-946.

10. Bessey, P. M. 1960. Effects of a new senescence inhibitor on lettuce storage. Univ. Ariz. Exp. Sta. Rep. 189:5-8.

11. Boothby, D., and S. T. C. Wright. 1962. Effects of kinetin and plant growth regulators on starch degradation. Nature. 196:389-390.

12. Borthwick, H. A., S. B. Hendricks and M. W. Parker. 1952. A reversible photoreaction controlling germination. Proc. Natl. Acad. Sci. U.S. 38:662-666.

13. Borthwick, H. A., S. B. Hendricks and M. W. Parker. 1952. The reaction controlling floral initiation. Proc. Natl. Acad. Sci. U.S. 38:929-934.

14. Campbell, L. L., Jr. 1956. Transamination of amino acids with glyoxylic acid in bacterial extracts. Jour. Bact. 71:81-83.

15. Cardini, C. E., L. F. Leloir and J. Chariboga. 1955. The biosynthesis of sucrose. Jour. Biol. Chem. 214: 149-155.

16. Chance, B., and G. R. Williams. 1955. Respiratory enzymes in oxidative phosphorylation. III. The steady state. Jour. Biol. Chem. 217:409-427.

17. Copenhaver, J. H., and H. A. Lardy. 1952. Oxidative phosphorylations: Pathways and yield in mitochondrial preparations. Jour. Biol. Chem. 195:225-238.

18. Darwin, Charles. 1880. The Power of Movement in Plants. J. Murray, London.

19. Das, N. K., K. Patau and F. Skoog. 1956. Initiation of mitosis and cell division by kinetin and IAA in excised tobacco pith tissue. Physiol. Plantarum. 9:640-651.

20. Dedolph, R. R. 1964. Phytokinins and net sugar accumulation under field conditions. Bioscience. 14: 53-54.

21. Dedolph, R. R., S. H. Wittwer and V. Tuli. 1961. Senescence inhibition and respiration. Science. 134: 1075.

22. Dedolph, R. R., S. H. Wittwer, V. Tuli and D. Gilbart. 1962. Effect of N^6-benzyladenine on the respiration and storage behavior of broccoli (Brassica oleracea var. italica). Plant Physiol. 37:509-512.

23. Downs, R. J. 1955. Photoreversibility of leaf and hypocotyl elongation of dark grown red kidney bean seedlings. Plant Physiol. 30:468-473.

24. Elliott, W. H. 1953. Isolation of glutamine synthetase and glutamotransferase from green peas. Jour. Biol. Chem. 201:661-672.

25. Engelbrecht, L., and K. Mothes. 1961. The effect of kinetin on the development of roots. Plant and Cell Physiol. 2:271-276.

26. Engelbrecht, L., and K. Mothes. 1961. Kinetin as a heat resistance factor. Ber. Deut. Bot. Ges. 73:246-257.

27. Evanari, M. 1961. A survey of the work done in seed physiology by the Department of Botany, Hebrew Univ., Jerusalem. Proc. Internatl. Seed Testing Association. 26:597-658.

28. Folin, O., and C. J. Farmer. 1912. A new method for the determination of total nitrogen in urine. Jour. Biol. Chem. 11:493-506.

29. Gamble, J. L., and V. A. Najjar. 1955. Studies of the kinetics of the reverse reaction of hexokinase. Jour. Biol. Chem. 217:595-601.

30. Guttman, R. 1956. Effects of kinetin on cell division, with special reference to initiation and duration of meiosis. Chromosoma. 8:341-350.

31. Guttman, R. 1957. Alterations in nuclear RNA metabolism induced by kinetin. Jour. Biophys. Biochem. Cytol. 3:129-131.

32. Haberlandt, G. 1922. Uber Zellteilungshormone und ihre beziehungen zur Wundheilung, Befruchtung, Parthenogenesis und Adventivembryonie. Biol. Zentr. 42:145-149.

33. Hakala, M. T., A. J. Glaid and G. W. Schwert. 1956. Lactic dehydrogenase. II. Variation of kinetic and equilibrium constants with temperature. Jour. Biol. Chem. 221:191-209.

34. Hanes, C. S., and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature. 164:1107-1112.

35. Henderson, T. R. 1960. Inhibition of xanthine oxidase by kinetin and kinetin analogues. Federation Proc. 19:314.

36. Henderson, T. R., C. G. Skinner and R. E. Eakin. 1962. Kinetin and kinetin analogues as substrate and inhibitors of xanthine oxidase. Plant Physiol. 37:552-555.

37. Hillman, W. S. 1957. Nonphotosynthetic light requirements in Lemna minor and its partial satisfaction by kinetin. Science. 126:165-166.

38. Hogeboom, G. H., W. C. Schneider and G. E. Pallade. 1948. Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. Jour. Biol. Chem. 172:619-635.

39. Jensen, W. A., and E. G. Pollock. 1958. Effect of kinetin on the protein and nucleic acid content of root tip cells. Plant Physiol. 33:suppl., xv.

40. Kaufman, J., and S. M. Ringel. 1961. Tests of growth regulators to retard yellowing and abscission of cauliflower. Proc. Amer. Soc. Hort. Sci. 78:349-352.

41. Kislink, R. C., and W. Sakami. 1955. A study of the mechanism of serine biosynthesis. Jour. Biol. Chem. 214:47-51.

42. Klein, A. O. 1959. Biochemical and physiological aspects of anthocyanin synthesis in detached petals of Impatiens balsima L. Doctoral thesis, Indiana University, Bloomington, Indiana.

43. Kornberg, H. L., and H. A. Krebs. 1957. Synthesis of cell constituents from C_2 units by a modified tricarboxylic acid cycle. Nature. 179:988-991.

44. Kowalsky, A., C. Wyttenbach, L. Langer and D. E. Koshland, Jr. 1955. Transfer of oxygen in the glutamine synthetase reaction. Jour. Biol. Chem. 219:719-724.

45. Krebs, H. A. 1953. The equilibrium constants of the fumarase and aconitase systems. Biochem. Jour. 54: 78-82.

46. Kulayeva, O. N., and I. P. Vorobyeva. 1961. The mode of action of kinetin on protein synthesis. Fiziol. Rast. 9:77-79.

47. Kulayeva, O. N., and I. P. Vorobyeva. 1961. Mechanism of the action of kinetin on protein synthesis. Fiziol. Rast. 9:106-108.

48. Kuraishi, S. 1959. Effect of kinetin analogues on leaf growth. Sci. Papers Coll. Gen. Educ. Univ. Tokyo. 9:67-104.

49. Leff, J. 1964. Interaction between kinetin and light on germination of Grand Rapids lettuce seed. Plant Physiol. 39:299-303.

50. Letham, D. S. 1963. Purification of factors inducing cell division extracted from plum fruitlets. Life Sciences No. 3. 152-157.

51. _____. 1963. Zeatin: A factor inducing cell division isolated from Zea mays. Life Sciences No. 8. 569-573.

52. Lime, B. J., F. P. Griffiths, R. J. O'Connor, D. C. Heinzelman and E. P. McCall. 1957. Spectrophotometric methods for determining pigmentation--beta carotene and lycopene--in ruby red grapefruit. Jour. Agr. Food Chem. 5:941-944.

53. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56:658-663.

54. Lipmann, F., and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl phosphates. Jour. Biol. Chem. 159:21-28.

55. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. Jour. Biol. Chem. 193:265-275.

56. Maciejewska-Potapczyk, W. 1959. Influence of kinetin, IAA, and gibberellic acid on nuclease activity of bean hypocotyls. Nature. 184:557-558.

57. McElroy, W. D., and B. Glass. 1951-1952. Phosphorus metabolism. Vols. I and II. Johns Hopkins Press, Baltimore.

58. McCalla, D. R., J. Moore and D. Osborne. 1962. The metabolism of a kinin, benzyladenine. Biochem. Biophys. Acta. 55:522-528.

59. Miller, C. O. 1956. Similarity of some kinetin and red light effects. Plant Physiol. 31:318-319.

60. Miller, C. O. 1958. The relationship of the kinetin and red light promotions of lettuce seed germination. Plant Physiol. 33:115-117.

61. _____. 1961. A kinetin like compound in maize. Proc. Natl. Acad. Sci. U.S. 47:170-174.

62. Miller, C. O., F. Skoog, H. M. Von Saltza and F. M. Strong. 1955. Kinetin, a cell division factor from DNA. Jour. Amer. Chem. Soc. 77:1392-1397.

63. Miller, C. O., F. Skoog, F. S. Okumara, M. H. Von Saltza and F. M. Strong. 1955. Structure and synthesis of kinetin. Jour. Amer. Chem. Soc. 77:2662-2666.

64. Miller, C. O., F. Skoog, F. S. Okumara, M. H. Von Saltza and F. M. Strong. 1956. Isolation, structure and synthesis of kinetin, substance promoting cell division. Jour. Amer. Chem. Soc. 78:1375-1381.

65. Millerd, A., J. Bonner, B. Axelrod and R. S. Bandurski. 1951. Oxidative and phosphorylative activity of plant mitochondria. Proc. Natl. Acad. Sci. U.S. 37:855-862.

66. Moffat, E. D., and R. I. Lytle. 1959. Polychromatic technique for the identification of amino acids in paper chromatography. Anal. Chem. 31:926-928.

67. Mothes, K. 1960. Uber das Altern der Blatter und die moglichksit ihrer Weiderjungung. Naturwissenschaften. 47:337-351.

68. _____., and L. Engelbrecht. 1961. Kinetin induced directed transport of substances in excised leaves in the dark. Phytochemistry. 1:58-62.

69. _____., et al. 1961. Uber die Akumulation von x-Aminoisobuttersaure im Blattgewebe unter dem Einfluss von Kinetin. Physiol. Plantarum. 14:72-75.

70. Osborne, D. J., and D. R. McCalla. 1961. Rapid bioassay for kinetin and kinins using senescing leaf tissue. Plant Physiol. 36:219-221.

71. Osborne, D. J. 1962. Effect of kinetin on protein and nucleic acid metabolism in Xanthium leaves during senescence. Plant Physiol. 37:595-602.

72. Parthier, B. 1961. Und Wolligiehn uber den Einflub des Kinetins auf den Eiweif und Nuklamsaurestoffwechsel in Isolierten Tabakblattern. Berichte der Deutschen Botanischem Gessel Schaft. LXXLV(sondernummer):47-51.

73. Partridge, S. M. 1948. Filter paper partition chromatography of sugars. Biochem. Jour. 42:238-249.

74. Paskova, J., and V. Munk. 1960. A combined detecting reagent for the identification of organic acids on paper chromatograms. Jour. Chromatography. 4: 241-243.

75. Patau, K., N. K. Das and F. Skoog. 1957. Induction of DNA synthesis by kinetin and IAA in excised tobacco pith tissue. Physiol. Plantarum. 10:949-966.

76. Pierpont, W. S. 1959. Mitochondrial preparations from tobacco leaves. Biochem. Jour. 71:518-528.

77. Quayle, J. R., R. C. Fuller, A. A. Benson and M. Calvin. 1954. Enzymatic carboxylation of ribulose diphosphate. Jour. Amer. Chem. Soc. 76:3610-3611.

78. Richmond, A. E., and A. Lang. 1957. Effect of kinetin on protein content and survival of detached Xanthium leaves. Science. 125:650-651.

79. Schantz, E. M., and F. C. Steward. 1952. Coconut milk factor: The growth promoting substances in coconut milk. Jour. Amer. Chem. Soc. 74:6133-6135.

80. Schantz, E. M., K. Mears and F. C. Steward. 1958. Comparison between the growth promoting effects on carrot tissue of coconut milk and of kinetin and of certain of its analogues. Plant Physiol. 33:suppl., xvi.

81. Schneider, W. C., and G. H. Hogeboom. 1950. Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome c in rat liver homogenates. Jour. Biol. Chem. 183:123-128.

82. Scott, R. A., and J. L. Liverman. 1956. Promotion of leaf expansion by kinetin and benzylaminopurine. Plant Physiol. 31:321-322.

83. Shemin, D. 1946. The biological conversion of l-serine to glycine. Jour. Biol. Chem. 162:297-307.

84. Strong, F. M. 1958. Kinetin and kinins, in topics in microbial chemistry. John Wiley and Sons, Inc., N.Y.

85. Thimann, K. V., and M. M. Laloraya. 1960. Changes in nitrogen in pea stem sections under the action of kinetin. Physiol. Plantarum. 13:165-178.

86. Tolbert, N. E., and M. S. Cohn. 1953. Products formed from glycolic acid in plants. Jour. Biol. Chem. 204: 649-654.

87. Torrey, J. G. 1958. Differential mitotic response of diploid and polyploid nuclei to auxin and kinetin treatment. Science. 128:1148-1149.

88. Tuli, V., R. R. Dedolph and S. H. Wittwer. 1962. Effects of N^6-benzyladenine and dehydroacetic acid on the storage behavior of cherries and strawberries. Mich. Agr. Exp. Sta. Quart. Bull. 45:223-226.

89. Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1964. Manometric Techniques. Fourth edition. Burgess Publishing Co.

90. Wickson, M., and K. V. Thimann. 1958. The antagonism of auxin and kinetin in apical dominance. Physiol. Plantarum. 11:62-74.

91. Wickson, M., and K. V. Thimann. 1960. The antagonism of auxin and kinetin in apical dominance. II. The transport of IAA in pea stems in relation to apical dominance. Physiol. Plantarum. 13:539-554.

92. Wiseman, H. G., H. M. Irwin and L. A. Moore. 1957. Determination of carotene in silages and forages. Jour. Agr. Food Chem. 5:134-137.

93. Withrow, R. B., W. H. Klein, L. Price and V. Elstad. 1953. Influence of visible and near infrared radiation energy on organ development and pigment synthesis in bean and corn. Plant Physiol. 28:1-14.

94. Wittwer, S. H., R. R. Dedolph, V. Tuli and D. Gilbart. 1962. Respiration and storage deterioration of celery (Apium graveolens) as affected by postharvest treatments with N^6-benzyladenine. Proc. Amer. Soc. Hort. Sci. 80:408-416.

95. Wyngaarden, J. C. 1957. 2,6-Diaminopurine as substrate and inhibitor of xanthine oxidase. Jour. Biol. Chem. 224:453-458.

96. Zink, F. W. 1961. A senescence inhibitor for vegetables. Jour. Agr. Food Chem. 9:304-307.