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EFFECT OF AMITROLE ON GROWTH AND ORGANIC NITROGEN METABOLISM IN SOYBEAN CELL CULTURE

Roger Shu Kong Suen

A Thesis

in

The Department

of

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ABSTRACT

EFFECT OF AMITROLE ON GROWTH AND ORGANIC NITROGEN METABOLISM IN SOYBEAN CELL CULTURE

Roger Shu Kong Suen

The effect of amitrole on growth and organic nitrogen metabolism has been studied in soybean cell culture. At a sublethal concentration (10^{-4}M), it caused a decrease in fresh weight, dry weight, cell number and both soluble and total proteins with a corresponding increase in free amino acids.

Amitrole inhibited the activities of both nitrate reductase (10^{-4} to 10^{-2}M) and glutamate dehydrogenase (10^{-3} to 10^{-1}M) of the cultured cells, though the nature of its noncompetitive inhibition remains to be determined. On the other hand, amitrole (10^{-4}M) stimulated the uptake of ^{14}C-leucine and its incorporation into TCA-precipitable protein of the treated cells. This stimulation has been shown to be due to de novo synthesis of protein which may not be a continuing process. There were slight quantitative, but no qualitative differences in the acrylamide gel protein patterns of control and 3AT-treated cells.

The results obtained in this investigation were discussed in relation to the effect of amitrole on nitrogen metabolism. To the author's knowledge, the effect of amitrole on the enzymes of organic nitrogen metabolism has not been reported before in either intact or in vitro cultured tissues.

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ABBREVIATIONS

3AT	Amitrole
3ATAL	Aminotriazolylalanine
CH	Cycloheximide
GAT	Amitrole-glucose adduct
GDH	Glutamate dehydrogenase
IAA	Indole-3-acetic acid
NR	Nitrate reductase
PPO	2,5-Diphenyloxazole
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography

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INTRODUCTION

The compound, 3-amino-1H-1,2,4-triazole or 'Amitrole' (3AT, Figure 1a) has been introduced in agriculture since 1954 by Amchem Products, Inc. (U.S. Patent No.2,670,282) as a plant growth inhibitor and defoliant (Herbicide handbook of the Weed Science Society of America, 1974).

Absorbed and easily translocated by both roots and leaves, 3AT has proved effective as a pre- and post-emergence spray and as a selective herbicide (Behrens, 1953). 3AT was originally introduced as a defoliant of cotton (Hall et al., 1953). In 1959 after the "Cranberry Incident" which led to the seizure and prohibition of sale of certain contaminated berries, 3AT was registered with the U.S. Department of Agriculture as an economic poison and its application on food crops was totally prohibited (DuShane, 1959).

3AT is considered to be positively carcinogenic (Dalgaard-Mikkelsen and Poulsen, 1962; NIOSH, 1977) causing thyroid adenoma and adenocarcinomas in rats (Hodge et al., 1966; Innes et al., 1969). However, ingestion of 3AT (20 mg/kg) by a 39-year-old woman caused no signs of intoxication and 3AT was excreted unchanged some hours later (Geldmacher — Mallinckrodt and Schmidt, 1970). Currently 3AT is being used for weed control in non-crop areas and is most efficient against weeds such as Canada thistle (Cirsium arvense) and quackgrass (Agropyron repens) and

some other perennial broadleaf weeds (Herrett and Linck, 1961; King, 1966; Crafts, 1975). 3AT cannot be legally used on crops or pasture land where food for man or animals may become contaminated.

The effect of 3AT on plant metabolism has drawn a large number of publications concerning mainly with proteins, nucleic acid and porphyrin metabolism. Its ultimate effect is the pronounced absence of chlorophyll in new growth, which was related to the blockage of the formation of 70S chloroplastic ribosomes and the 18S fraction I protein (Bartels et al., 1967). There are conflicting reports as to the effect of 3AT on protein synthesis in plants (Carter, 1969; Ashton and Crafts, 1973). Some workers reported the inhibition of glycine incorporation into proteins of wheat seedlings (Bartels and Wolf, 1965); Hilton (1966) reported that 3AT inhibited incorporation of ^{14}C from adenine-2-^{14}C into nucleic acids of yeast. Other workers, on the other hand, were unable to find a direct effect of 3AT on protein synthesis (Mann et al., 1965; Brown and Carter, 1968; Moreland et al., 1969).

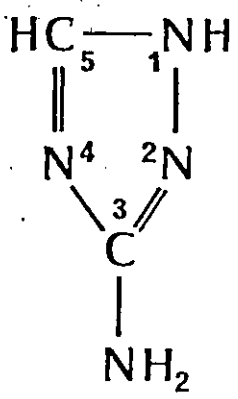
In view of these contradicting results and due to the lack of information on the effect of 3AT on the enzymes of organic nitrogen assimilation, it was considered important, therefore, to undertake this study using soybean suspension culture.

The objectives of the present study will be an attempt to determine:- a) the effect of 3AT on the growth of

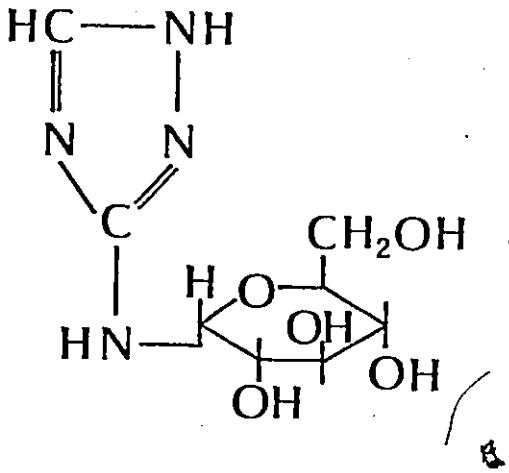
soybean cell suspension culture, b) the effect of 3AT on organic nitrogen metabolism, especially on the enzymes nitrate reductase (NR) and glutamate dehydrogenase (GDH), and c) the correlation between the herbicidal action of 3AT and protein synthesis.

Previous studies of the effects of 3AT have mostly employed intact plants or excised plant tissues which made it difficult to assess the herbicidal effects on the cellular level. Although plant cell suspension culture have been in existence for many years, they have not been widely used in metabolic studies of phytotoxicity. One may ask the question whether cell cultures actually represent the whole plant in terms of physiological and biochemical responses.

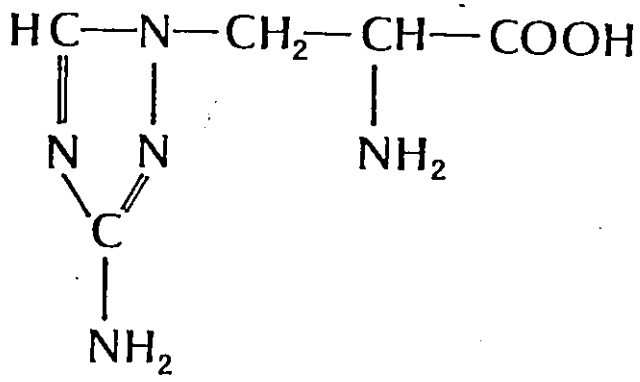
Cell cultures have many potential uses in the study of phytotoxic compounds:- a) they have the advantage of being a homogeneous cuticle-free systems, thus eliminating the problems of uptake, translocation and tissue differentiaion which are usually encountered with intact or excised plant tissues, b) they offer an axenic system where most of the cells are metabolically active and of uniform growth, c) all growth is on liquid medium, enabling easy addition and removal of compounds at all stages of growth, and d) growth cycle is relatively short (12-14 days) and offer ample cell yield.



(a) 3AT



(b) GAT



(c) 3ATAL

Figure 1. Structure of amitrole and its metabolites

Section B

LITERATURE REVIEW

B.1. Properties of Amitrole

3AT ($C_2H_4N_4$) is a heterocyclic nitrogen compound composed of a 5-membered ring having three nitrogen and two carbon atoms, as well as an amino group on the 3-position of the ring (Potts, 1961). The acute oral LD_{50} (albino rats) was found to be approximately 25 mg/kg (Sutherland, 1964).

3AT reacts with bases and acids to form salts; with aldehydes and ketones to form aldimines and ketimines; and with di- and trivalent metals such as iron, copper, nickel, magnesium, manganese and cobalt to form metal complexes (Naylor, 1964; Sutherland, 1964).

3AT was reported to form a number of conjugates with some endogenous plant constituents, which may be considered as a detoxification mechanism. Rogers (1957) first reported a D-glucose adduct of 3AT. This amine-glucoside was later identified as N-(1H-1,2,4-triazol-3-yl)-glucopyranosylamine (GAT, Figure 1b): GAT can act as a substrate for aldolase and also can be phosphorylated by hexokinase in the presence of ATP, forming phosphorylated-GAT (Fredrick and Gentile, 1962). The latter conjugate resembles AMP in structure and may interfere with adenine metabolism in plants (Fredrick, 1964). Further studies showed that the inhibition of phosphorylase by 3AT (Fredrick and Gentile, 1960) was not due to the chelating effect of 3AT on the Mn^{2+} of the enzyme, but rather to a competitive inhibition of phosphorylated-GAT with

AMP, in the conversion of active phosphorylase from its inactive form (Fredrick and Gentile, 1967). The blocking of the phosphorylating reaction would result in the accumulation of carbohydrates in the 3AT-treated tissue and thus affected carbohydrate metabolism in the treated plants (Fredrick and Gentile, 1967).

Another derivative of 3AT frequently reported is the 3-(3-amino-1,2,4-triazole-1-yl)-2-aminopropionic acid, or the aminotriazolylalanine (3ATAL, Figure 1c) (Massini, 1963). 3ATAL appears to be a complex between 3AT and serine or phosphoserine and is synthesized enzymatically in plants (Massini, 1959; Murakoshi et al., 1974). It is nontoxic to plants and, like β-pyrazol-1-ylalanine (Dunnill and Fowden, 1963), is considered to be an analogue of histidine. 3ATAL may be incorporated into cellular protein by E. coli (Williams et al., 1965), which implies the formation of nonfunctional proteins.

Kroller (1966) reported that 3AT also conjugates with tannins forming a 3AT-tannin complex.

B.2. Effects of Amitrole on Plant Metabolism

There have been numerous publications on the effect of herbicides on plant metabolism with a view to establish a mode of action of these compounds on the different metabolic processes (Hilton et al., 1963; Audus, 1976; Moreland, 1977). Despite the voluminous research that has been done, in no case has the mode of action of one particular compound been worked out. No single hypothesis may explain adequately the

action of one herbicide under all conditions (Ashton et al., 1977).

Based on current knowledge, 3AT seems to be involved in a wide spectrum of phytotoxic reactions in different organisms and 3AT has been classified as inhibitor of more than a single process (Andreae, 1963). On the basis of structural analogy, it is not surprising that 3AT has inhibitory actions and interferes with some of the natural biochemical compounds with an imidazole ring structure. 3AT acts as an aza-inhibitor to compounds such as histidine, 4-aminoimidazole, purines and pyrimidines (Balis, 1968).

The target site(s) of 3AT toxicity can be focussed on nitrogen metabolism, mainly amino acids metabolism; protein metabolism; enzyme inhibition and some other metabolisms such as that of purines and porphyrins.

B.3. Effects of Amitrole on Amino Acid and Protein Metabolism

3AT was reported to block histidine biosynthesis in bacteria (Bond and Akers, 1961; Hilton et al., 1965), yeast (Hilton, 1960; Klopotowski and Wiater, 1965), algae (Siegel and Gentile, 1966), fungi (Carsiotis et al., 1974) and some higher plants (Davies, 1971; Wiater et al., 1971), by inhibiting imidazole glycerol phosphate (IGP) dehydratase (E.C.4.2.1.19) competitively and resulting in the accumulation of IGP and imidazole glycerol. As a result of histidine deficiency, the biosynthesis of proteins and nucleic acids may be inhibited (Hilton and Kearney, 1965).

3AT may also affect glycine metabolism by depleting the endogenous serine-glycine pool as a result of its complexing with serine or phosphoserine (Carter and Naylor, 1961; McWhorter and Hilton, 1967; Burt and Muzik, 1970). Overbeek (1964) suggested that the interference with glycine metabolism may block the synthesis of purines and thereby alter the synthesis of RNA.

Treatment by 3AT usually induced a decrease in protein content with a corresponding increase in free amino acid content and free ammonia (Bartels and Wolf, 1965; McWhorter and Hilton, 1967). The increase in amino acid content, in 3AT-treated plants, appeared to be in stored form of nitrogen rather than that available for protein synthesis. This can be interpreted as a decrease in protein synthesis and/or an increase in protein hydrolysis, where the proteins may be the major source of respiratory energy of the 3AT-treated plants (McWhorter, 1963). Furthermore, the increase in free ammonia would enhance the toxic effect in the 3AT-treated plants (Baumann and Gunther, 1976).

There is a considerable controversy in the literature as to the effect of 3AT on protein synthesis. Bartels and Wolf (1965) reported that 3AT inhibited the incorporation of ^{14}C-glycine into proteins of wheat seedlings; while Mann et al. (1965) reported a stimulatory effect on the incorporation of ^{14}C-leucine into proteins of barley but an inhibitory effect with that of Sesbania sp. Hilton (1966) reported that 3AT inhibited ^{14}C-adenine incorporation into the nucleic acids of

yeast. Brown and Carter (1968), on the other hand, reported an increase in the incorporation of ^{14}C-serine into the soluble proteins of bean hypocotyl sections, whereas the incorporation of ^{14}C-alanine and ^{14}C-histidine was not affected. Similarly, Moreland et al. (1969) found that 3AT had a stimulatory effect on ^{14}C-leucine incorporation into proteins of soybean hypocotyl sections.

Herbicides are believed to affect also the proteolytic enzymes (Ashton et al., 1968). 3AT was found to inhibit both the dipeptidase and proteinase activities of squash cotyledons (Tsay and Ashton, 1971). These authors concluded that 3AT did not inhibit protein synthesis.

In view of these conflicting reports, the results seem to indicate that 3AT did not inhibit the protein synthesis reaction per se, but rather inhibited the formation of a critical component necessary for protein synthesis (Ashton and Crafts, 1973). Hence the reduced protein content in 3AT-treated plants may have been derived from a disruption in the amino acid or nucleic acid metabolism.

B.4. Effects of Amitrole on Enzyme Activity

In addition to phosphorylase and IGP dehydratase, 3AT has been found to inhibit many enzymes, especially the metallo-protein enzymes and those with a porphyrin prosthetic group (Wort, 1964).

Enzymes such as tryptophan peroxidase-oxidase (Auerbach et al., 1959); ascorbic acid oxidase, catalase, peroxidase

and catecholase (Palmer and Porter, 1959); fructoaldolase (McWhorter and Porter, 1960); fatty acid peroxidase, tyrosinase and DOPA-oxidase (Castelfranco, 1960); xanthine oxidase (Castelfranco et al., 1963) and isocitric dehydrogenase (DeKock et al., 1966) were all found to be inhibited by 3AT. These observations led Castelfranco and Brown (1963) to suggest that 3AT may undergo a one-electron oxidation to form free radical which attacked various enzymes causing irreversible inhibition. Other workers thought that 3AT may act primarily as a metal chelator which, by locking up essential micro-nutrients, might lead to a reduction in the activity of many different enzymes (Wort, 1964; Luckwill, 1968).

The effect of 3AT on catalase has been extensively investigated as early as 1955 (Heim et al., 1955; Pyfrom et al., 1957; Price, 1962). All the results indicated that the catalase activity was irreversibly inhibited by 3AT and the inhibition reaction was between catalase-H_2O_2-complex I and 3AT, and that the essential binding of 3AT was with the protein part of catalase (Margoliash et al., 1960). Close examination of the amino acid sequence of catalase revealed that 3AT bound covalently to a specific histidyl residue, the hist$^{\#}$74, which is implicated in the catalytic mechanism of the enzyme (Agrawal et al., 1970). Similar results were obtained by Chang and Schroeder (1972) with catalase and lactoperoxidase (Chang and Schroeder, 1973). A reduced catalase activity would mean a high H_2O_2 level in plant cells and since H_2O_2 is involved in chromosome breakage and IAA destruction, 3AT may indirectly

affect normal plant growth (Russell, 1957; Racusen, 1958).

B.5. Effects of Amitrole on Purine Metabolism

The release from 3AT toxicity by purines in treated plants may suggest that 3AT had an inhibitory effect on purine biosynthesis (Carter, 1969). Rabinowitz and Pricer (1956) reported that 3AT affected purine catabolism by inhibiting the enzymatic (4-aminoimidazole hydrolase activity) conversion of 4-aminoimidazole to formiminoglycine. Hulanicka et al. (1969) reported that 3AT inhibited purine biosynthesis at the cyclization of formylglycine-amidine ribotide to aminoimidazole ribotide and had a direct effect on phosphoribosylaminoimidazole synthetase. All these evidences supported the idea that 3AT interferes with purine metabolism. The impairment of purine synthesis by 3AT-treatment may lead to the inhibition of biosynthesis of cytokinin and other purine derivatives (Ashraf et al., 1976). This implied that 3AT treatment may result in a change in the growth hormone balance in plants.

In higher plants, processes requiring purine precursors such as histidine, riboflavin and nucleic acid synthesis, appeared to be most sensitive to 3AT treatment (Hilton and Kearney, 1965; Henderson, 1972). 3AT is known to block riboflavin production (Sund and Little, 1960; Suzuki et al., 1978), and moreover 3AT elicited disruption in nucleic acid metabolism as shown by the inhibition of the incorporation of labelled precursors into RNA, DNA and acid soluble

nucleotides (Schweizer and Rogers, 1964).

B.6. Other Effects of Amitrole Treatment

3AT disrupted porphyrin metabolism by interfering with the conversion of pyrroles into porphyrins (Aaronson, 1960); inhibition of δ-aminolevulinic acid dehydrase activity (Tschudy and Collins, 1957); accumulation of coproporphyrin (Dorfling et al., 1970) and the production of a modified form of chlorophyll a (Inoue et al., 1972).

Perhaps the most obvious effect of 3AT upon higher plants is the pronounced absence of chlorophyll in new growth (Bruinsma, 1965). Extensive research indicated that 3AT affected chloroplast structure quite differently from the Hill-reaction inhibitors (Hilton et al., 1969; Vivekanandhan and Gnanam, 1972; Anderson and Thomson, 1973). The most convincing evidence was that 3AT blocked the formation of 70S chloroplastic ribosome and 18S fraction I protein (Bartels et al., 1967). The study by Ayanian and Block (Special study, 1974) showed that 3AT exhibited a direct effect on the 16S chloroplastic ribosomal RNA, whereas the cytoplasmic ribosomal RNA and the 23S chloroplastic ribosomal RNA were not affected. The lack of the 16S RNA would undoubtedly result in the inhibition of the assembly of the 70S ribosomes.

Ultrastructural examination revealed that 3AT affected plastid development and resulted in the formation of abnormal grana-fret membrane system and chloroplast ribosomes (Bartels and Weier, 1969). On the other hand, the mitochondria, golgi

apparatus and endoplasmic reticulum were morphologically unaffected (Bartels and Weier, 1965). Similarly, Vivekanandhan and Gnanam (1975) showed that the formation of chloroplast membrane was specifically inhibited.

Burns et al. (1971) reported that 3AT inhibited normal carotenogenesis. Other workers believed that the interference with carotenoid synthesis alone would be sufficient to explain the phytotoxicity of 3AT; since in the absence of carotenoids, the chlorophyll is susceptible to photodestruction resulting in the oxidation and disruption of 70S ribosomes and internal membranes (Bartels and Hyde, 1970; Corbett, 1974).

Other secondary herbicidal effects of 3AT were also reported such as :- chromosomal aberrations (Epstein and Legator, 1971; Mohandas and Grant, 1972); accumulation of starch (DeKock and Innes, 1970) and anthocyanins (Bartels and Wolf, 1967); and inhibition of the development of microbodies (Feierabend and Beevers, 1972).

Section C

MATERIALS AND METHODS

C.1. Soybean Cell Suspension Culture

Soybean culture (Glycine max, variety Kanrich), previously initiated from root callus by Dr. D.T. Nash (Lady Davis Institue, Montreal), was brought into cell suspension in our laboratory. This culture had the advantage of the lack of chlorophyll synthesis, even when grown under light condition. Its growth was maintained on MS salt nutrient medium (Murashige and Skoog , 1962; Appendix 1) that was supplemented with 3% sucrose, 1 g/l casein hydrolysate (Sigma Chemical Company), 2 ppm 2,4-dichlorophenoxyacetic acid and 0.5 ppm kinetin.

The pH of the medium was adjusted to 5.7 and 50-ml aliquots of the liquid medium were transferred to 250-ml Erlenmeyer flasks before being autoclaved at 15 p.s.i. for 20 min. Batch cultures were agitated on a gyrotary shaker (Model G-10, New Brunswick Scientific, N.J.) at 150 r.p.m. and 24±1°C in the dark. Subculturing was done at bi-weekly intervals using a 10% inoculum. Except where stated otherwise, 3AT was added as an aqueous solution to the culture medium before autoclaving and in a final concentration of 10^{-4}M.

C.2. Determination of Growth Parameters

Growth of the culture was determined by measuring the fresh weight, dry weight and cell number at regular intervals during the growth cycle.

C.2.1. Fresh and dry weight

For fresh weight determination, the cell cultures were filtered through fritted glass funnel (coarse porosity) using suction and air was allowed to pass through the filter for 30 seconds before the fresh weight of the cells was determined. The cells were then dried in an oven at 60°C for 24 hr or until constant weight. Both the fresh and dry weight values were determined on triplicate samples and were expressed in g/l of the culture medium.

C.2.2. Cell number

Cell numbers were determined on freshly filtered cells. A known weight of cells was incubated with 5 ml of a mixture of 10% HCl and 10% chromic acid (1:1, v/v) for 2 hr. Cell aggregates were mechanically separated by drawing them into a syringe through a #21-gauge hypodermic needle, several times, until a homogeneous suspension of free cells was obtained. The cells were counted on a Fuchs-Rosenthal ruled hemocytometer and the counts were expressed in 10^6/l of the culture medium.

C.3. Determination of Soluble Protein Content

Soluble protein was extracted by homogenizing weighed samples of fresh cells with ice-cold, 0.2 M Tris-HCl buffer, pH 7.5 in a 1:10 (w/v) ratio. The homogenate was centrifuged at 15,000 g for 15 min and the supernatant was used for protein assay using the method of Lowry et al. (1951).

One ml of the sample solution was mixed with 5 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium citrate:2% Na_2CO_3 in 0.1 N NaOH

(1:50, v/v) and was allowed to stand for 10 min at room temperature. Then 0.5 ml of 1 N Folin-Ciocalteu reagent was added and the mixture was allowed to stand for 30 min. The absorbance of the color developed was read at 750 nm in a spectrophotometer (Spectronic 700, Bausch and Lomb). Bovine serum albumin (BSA) was used as a standard for the preparation of a calibration curve and the results were expressed in mg/g fresh weight of cells.

C.4. Determination of Total Nitrogen Content

Total nitrogen content of the cells was determined on freeze-dried tissue using the micro-Kjeldahl digestion-distillation method (Bailey, 1967). A weighed sample of dry cells (ca. 2 g) was mixed with 1 g of a catalyst mixture consisting of K_2SO_4:$CuSO_4 \cdot 5H_2O$:sodium selenate (40:10:0.17, w/w/w) and was digested in the presence of 2 ml of concentrated H_2SO_4 overnight (micro-Kjeldahl digestor, Labconco, Kansas City, Missouri). The mixture was allowed to cool and was diluted to a constant volume (ca. 10 ml).

One-ml samples of the hydrolysate was added to 5 ml of 45% NaOH in the micro-Kjeldahl distillation unit (Labconco, Kansas City, Missouri) and the distillate was collected in 10 ml of 2% boric acid containing few drops of the indicator. The latter consisted of a mixture of 0.2% methyl red and 0.1% methylene blue (1:1, v/v) in absolute ethanol. About 20 ml of the distillate was collected and titrated against standardized 0.01 N HCl to a neutral grey end point. Analyses were

carried out on duplicate samples and the results were expressed in mg nitrogen/g dry weight of cells, on the basis that 1 ml of 0.01 N HCl was equivalent to 0.14 mg of nitrogen.

C.5. Determination of Free Amino Acid Content

The determination of free amino acids was carried out on the ethanol-soluble extracts of fresh cells using the method of Moore and Stein (1954). Cells were ground with hot 80% ethanol (1:5, w/v) and the homogenate was centrifuged. Measured aliquots of the ethanol extract were mixed with the borate-KOH reagent, at a ratio of 2:1 (v/v) and 2 ml of the ninhydrin reagent. The mixture was incubated for 15 min in a boiling water bath. After cooling, the reaction mixture was diluted with absolute ethanol (1:5, v/v) and the absorbance of the color developed was measured at 560 nm. A calibration curve was prepared using α-alanine as standard and the amount of free amino acids was calculated in mmol/g fresh weight of cells.

C.6. Thin-Layer Chromatography of Alcohol-Soluble Amino Acids

Six days old control and 3AT-treated cells were ground with hot 80% ethanol (1:5, w/v). The ethanol extract was centrifuged and reduced to a small volume by flash evaporation under reduced pressure. Two-directional, ascending, thin-layer chromatogrqphy (TLC) on cellulose plates was carried out for each sample. The chromatographic plates were coated with 250 μm-thick layer of cellulose MN300G (Macherey and Nagel Company). The solvent systems used for developing the

plates consisted of n-butanol:acetone:NH_4OH:H_2O (10:10:5:2, v/v/v/v) for the first direction and isopropanol:formic acid:H_2O (20:1:5, v/v/v) for the second (Pillay and Mehdi, 1970). The developed chromatograms were evenly sprayed with 0.5% ninhydrin in 95% ethanol and heated in the oven at 100°C for 10 min. The amino acid spots were identified by comparing their Rf values and their colors with those of reference compounds. 3AT was identified using the H-acid reagent of Racusen (1958) as shown in Appendix 2. 3AT appeared as a bright red spot with Rf values of 0.65 and 0.56 in both solvents, respectively.

C.7. Extraction and Assay of Nitrate Reductase (NR) and Glutamate Dehydrogenase (GDH)

The activities of both NR (E.C.1.6.6.1) and GDH (E.C.1.4.1.2) were determined during the 2-week growth cycle of the cell culture. This was done by preparing a buffer extract of washed, filtered cells at 48 hr intervals according to the following procedure.

C.7.1. Determination of NR activity

A weighed amount of fresh cells (ca. 2 g) grown in the presence or absence of 3AT (10^{-4}M) was homogenized in a chilled mortar with 5 volumes of ice-cold, 0.1 M Tris-HCl buffer, pH 7.5 (Sanderson and Cocking, 1964). The homogenate was centrifuged at 15,000 g for 15 min and the supernatant was used directly as the enzyme source. The assay condition for NR was similar to that reported by Filner (1966),

consisted of:-

0.5 ml of 0.1 M KH_2PO_4, pH 7.5,

0.1 ml of 0.1 M KNO_3,

0.1 ml of 1 mM NADH and

0.3 ml of crude enzyme extract in a total volume of 1.0 ml. The reaction was started by the addition of the enzyme protein and the mixture was incubated at 30°C for 30 min in a constant temperature water bath. At the end of the incubation period, 1 ml of 1% sulfanilamide in 3 M HCl and 1 ml of 0.02% N-naphthylethylene-diamine diHCl were added and allowed to stand at room temperature for 10 min. The optical density of the color developed was measured at 540 nm against a blank solution containing all of the above mentioned reagents except the NADH. The concentration of nitrite produced was calculated from a calibration curve which was prepared by using known amounts of sodium nitrite. Assays were carried out in duplicates. One unit of NR activity was defined as the amount required to produce 1 µmol of NO_2^-/g fresh weight/hr under the assay conditions.

Specific activity was calculated after determining the soluble protein content using the Lowry method, and was defined as mUnits/mg protein/hr.

C.7.2. Determination of GDH activity

A known amount of fresh cells, grown in the presence or absence of 3AT (10^{-4}M), were ground with ice-cold 50 mM Tricine (N-Tris-hydroxy methyl-methylglycine) buffer, pH 7.5 containing 1 mM β-mercaptoethanol. The homogenate was

centrifuged at 15,000 g for 15 min and the supernatant was used directly as the enzyme source. The enzyme assay mixture was similar to that of Gamborg and Shyluk (1970), consisted of:-

1 ml of 20 mM ammonium sulfate,

1 ml of 10 mM α-ketoglutarate,

0.1 ml of 0.1 mM NADH and

1 ml of crude enzyme extract.

The reaction mixture was incubated at 30°C in a water bath and the decrease in the absorbance of the pyridine nucleotide was measured every min for 10 min at 340 nm during which the reaction rate was linear with respect to time. One unit of GDH activity was defined as the amount required to produce 1 mmol NAD^+/g fresh weight/hr under the assay conditions. The specific activity was defined as mUnits/mg protein/hr.

C.8. Partial Purification of Nitrate Reductase (NR) and Glutamate Dehydrogenase (GDH)

The kinetics of NR and GDH were studied using partially purified enzymes. All purification procedures were carried out at 0-4°C.

C.8.1. Nitrate reductase

Control cells, six days old, were homogenized with 5 volumes of ice-cold 0.1 M phosphate buffer, pH 7.8 containing 0.5 M EDTA and 1 mM β-mercaptoethanol (Schloemer and Garrett, 1973). The homogenate was centrifuged at

15,000 g for 15 min. The supernatant was fractionated by the gradual addition of solid ammonium sulfate and the protein which precipitated between 10 and 40% saturation was collected by centrifugation. This fraction which was found to contain approximately 54% of the total enzyme (NR) activity was dissolved in the minimal amount of 0.1 M phosphate buffer, pH 7.8 and was desalted on Sephadex G-25 (medium grade) column using the same buffer. The protein eluate was continuously monitored at 280 nm using a UV-absorbance monitor (Model 100, Pharmacia Fine Chemicals, Uppsala, Sweden). This treatment yielded a 3-fold increase in specific activity of the enzyme as compared with that of the crude extract.

According to Hageman and Hucklesby (1971), the assay mixture of the partially purified NR consisted of:-

1 ml of 0.1 M KNO_3 (33 mM),

0.5 ml of 1 mM NADH (0.166 mM),

0.5 ml of H_2O/3AT (1.66×10^{-4} to 1.66×10^{-2} M) and

1 ml of enzyme (0.6 mg protein/ml) in a total volume of 3.0 ml. The assay conditions and the determination of enzyme activity were similar to those described previously in section C.7.1.

C.8.2. Glutamate dehydrogenase

Control cells, eight days old, were homogenized with 5 volumes of ice-cold 0.2 M Tris-HCl buffer, pH 8.0 containing 1 mM β-mercaptoethanol (King and Wu, 1971).

The homogenate was centrifuged and protein fractionation was carried out using solid ammonium sulfate. The protein which precipitated between 40 and 60% saturation was collected by centrifugation. This fraction was found to contain approximately 65% of the total enzyme activity. The protein pellet was dissolved in a small volume of Tris-HCl buffer and desalted on a Sephadex G-25 column using the same buffer. This treatment yielded a 2-fold increase in the specific activity of the enzyme as compared with that of the crude extract.

According to Bulen (1956), the reaction mixture for the assay of GDH consisted of:-

0.2 ml of 1.5 M ammonium sulfate, pH 8.0 (100 mM),

0.2 ml of 0.2 M ∝-ketoglutarate (13.33 mM),

0.5 ml of 1 mM NADH (0.166 mM),

0.5 ml of H_2O/3AT (1.66×10^{-3} to 1.66×10^{-1} M),

1.5 ml of 0.2 M Tris-HCl buffer, pH 8.0 and

0.1 ml of enzyme (1.6 mg protein/ml) in a total volume of 3.0 ml. The assay conditions and the determination of enzyme activity were similar to those described previously in section C.7.2.

C.9. Incorporation of ^{14}C-Leucine into Protein

Four days old soybean cells growing in the presence or absence of 3AT (10^{-4}M), were administered 0.2 µCi of L-leucine [^{14}C(U)] (325 mCi/mmol, New England Nuclear, Boston, Mass.) for different periods of time. This was carried out in 50-

ml Erlenmeyer flasks containing 1:3 (w/v) of cells in culture medium under aspectic conditions. At the end of the labelling period, the cells were thoroughly rinsed with distilled water, filtered and then homogenized with 0.2 M ice-cold Tris-HCl buffer, pH 7.5. The homogenate was centrifuged (15,000 g, 15 min) and the supernatant was made up to a constant volume (ca. 5 ml) with the same buffer.

Measured aliquots (in duplicate) were withdrawn from the buffer extract for the determination of radioactivity by liquid scintillation. The radioactivity of these samples was used as a measure of amino acid uptake, referred to as 'activity of buffer-extract fraction'. The amount of soluble proteins in the supernatant was also determined using the Lowry method. The remaining supernatant was mixed with 1:1 (v/v) ice-cold 10% trichloroacetic acid (TCA) and was left to stand for 20 min at 0-4°C. The protein which precipitated was collected by centrifugation, then washed twice with 5% TCA and twice with ethanol-ether-chloroform (2:2:1, v/v/v). The protein pellet was solubilized with 0.2 ml of Protosol (New England Nuclear, Boston, Mass.) at 50°C over a period of 1 hr and then counted for radioactivity by liquid scintillation, referred to as 'activity of TCA-precipitated fraction'.

Radioactivity was determined by adding 10 ml of a scintillation liquid consisting of 0.4% 2,5-diphenyloxazole (PPO) in toluene, and counting in a Unilux II liquid scintillation spectrometer (Nuclear Chicago, Chicago,

Illinois). Counts per min (cpm) were transformed to disintegrations per min (dpm) using the channel-ratio method and a quench correction curve. The radioactivity was defined as dpm/mg protein.

In another set of similar experiment, the cultured cells were first incubated for 3 hr in a culture medium containing 10^{-5}M non-labelled L-leucine, in order to saturate their endogenous leucine pool. The cells were then administered 0.5 µCi of ^{14}C-leucine and processed as described above.

Samples from the buffer-extract and TCA-precipitated fractions were hydrolyzed with 10 N HCl at 120°C for 18 hr and the hydrolysis products were chromatographed on thin-layer plates as described in section C.6. The developed chromatograms were autoradiographed using Kodak no-screen X-ray film in order to study the distribution of label of the administered ^{14}C-leucine.

C.10. Discontinuous Polyacrylamide Gel Electrophoresis of Soluble Proteins

Polyacrylamide gel electrophoresis of the buffer-soluble proteins was carried out at 0-4°C according to the method of Davis (1964), using a 7.5% (w/v) acrylamide, but without a sample gel.

All essential stock solutions were stored in brown glass bottles at 2-4°C, except for the ammonium persulfate which was freshly prepared before use. The glass tubes

were 7 cm long with an inner diameter of 6 mm. Prior to use they were soaked in cleaning solution, rinsed with 0.5% Photo-flo (Kodak) solution and allowed to dry.

For a run of 12 gels, 18 ml of the separating gel solution was used. Each tube was filled with 1.5 ml of the solution. Onto the gel solution, a layer of distilled water was placed carefully by means of a micropipet. The gels were allowed to polymerize under a day-light fluorescent tube for about 20 min. After the gels had solidified, 200 µl of the stacking gel (3.1%) was added and allowed to polymerize on top of the separating gel. The gel tubes were placed in the electrophoretic chamber (Buchler Instruments, N.J.) and the electrode buffer reservoirs were filled with Tris-glycine buffer, pH 8.3.

Cells grown in the presence or absence of 3AT (10^{-4}M) were harvested at 4, 8 and 12-day period and were ground with ice-cold 0.01 M Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 15,000 g for 15 min and the supernatant was concentrated by freeze-drying. The protein content of the sample was determined by the Lowry method.

Samples containing approximately 2.0 mg protein were mixed with 40% sucrose (4:1, v/v) and layered directly onto the stacking gel. A few drops of 0.001% bromophenol blue was added to the sample protein and served as tracking dye. A constant current of 2 mA per gel was applied initially and then increased to 5 mA per gel with the cathode (-) being connected to the upper reservoir and the

anode (+) to the lower one. Immediately at the end of electrophoresis, the gels were removed from the glass tubes by using a gentle stream of water. The gels were stained for 1 hr in 1% (w/v) naphthol blue black (Amido-black) in 7% glacial acetic acid. The gels were destained by electrophoresis in 7% acetic acid and stored in the same solution. Gel scanning was performed using an ISCO gel scanner (Model 659) with a model UA-4 absorbance monitor. The protein profiles of the gels were also recorded by photography.

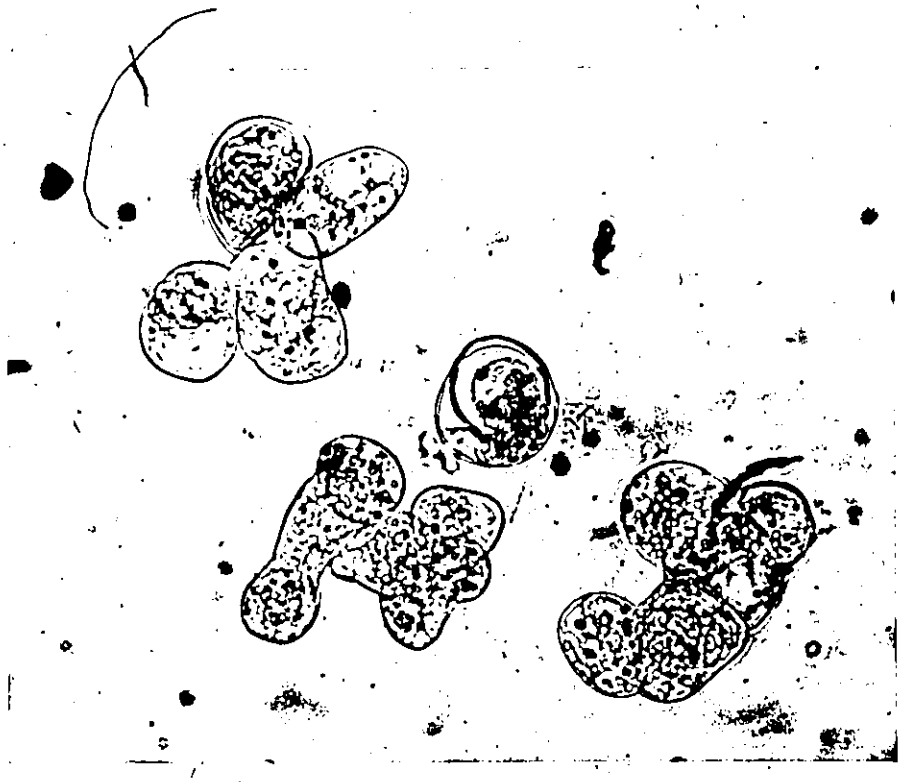
RESULTS

D.1. Tolerance of Soybean Cells to Different Amitrole Concentrations

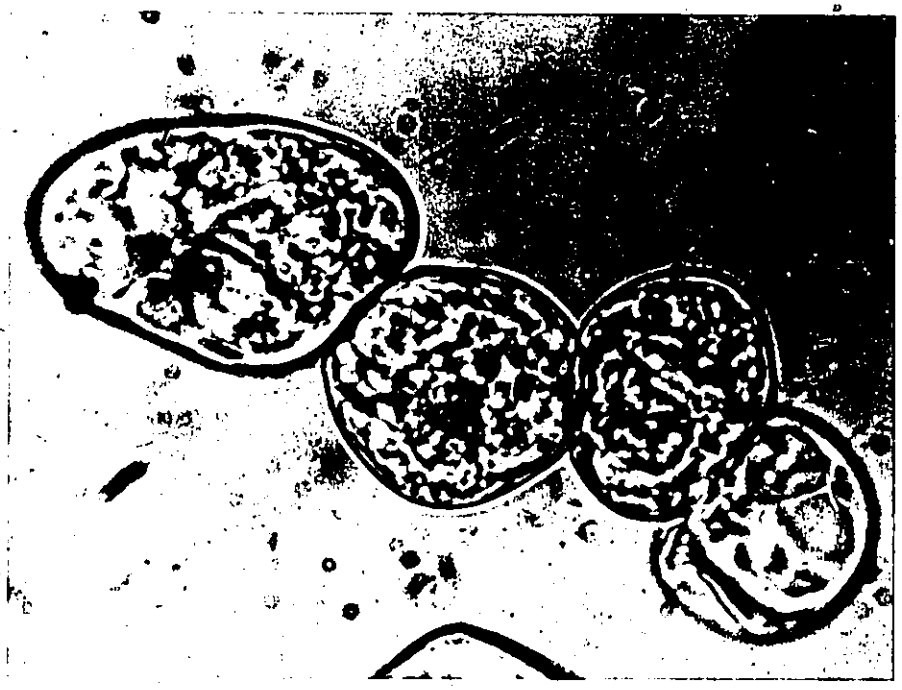
Table I shows the results of the effect of different concentrations of 3AT on growth parameters of soybean culture. Low concentrations of 3AT treatment (10^{-5} to 10^{-7} M) resulted in an increase in fresh weight and a corresponding decrease in both dry weight and soluble protein content. This apparent increase in fresh weight was probably due to high water content which does not really lead to increased growth. This response misled some workers to report on the stimulatory effect of low 3AT concentration on plant growth (Homes, 1966; Brown and Carter, 1968; Metcalf, 1971).

At a sublethal concentration (10^{-4}M), 3AT treatment resulted in a 20-25% drop in fresh weight, dry weight and soluble protein as compared with the controls. In contrast, cells grown in 10^{-3}M of 3AT exhibited no visible growth and turned brown after a few days from subculture of the inoculum. Therefore, 3AT at a final concentration of 10^{-4}M was considered to be the optimum concentration for growth inhibition and unless otherwise stated, this concentration was used in the study.

D.2. Effects of Amitrole on Growth Parameters of Soybean Cell Culture



(150X)



(600X)

Figure 2. Soybean cells cultured in MS medium

Table I

Effect of different amitrole concentrations on growth parameters of soybean cells

Growth parameter*	Amitrole concentration (M) 0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Fresh weight (g/l)	240±4% (100%)	292 (122%)	282 (117%)	268 (112%)	194±5% (81%)	50 (21%)
Dry weight (g/l)	16.1±3% (100%)	15.2 (94%)	14.7 (91%)	13.8 (86%)	13.1±6% (81%)	2.5 (16%)
Soluble protein (mg/g fresh weight)	16.4±1% (100%)	15.7 (96%)	14.2 (87%)	13.0 (79%)	12.1±3% (74%)	- -

* Values in between parentheses are percentage of controls. For actual data see Appendix 3.

D.2.1. Fresh weight, dry weight and cell number

Soybean culture, grown under control conditions, appeared as a homogenous suspension of mostly free cells with some cell aggregates (Figure 2).

The growth patterns of soybean cultures grown in the presence or absence of 3AT (10^{-4}M) are illustrated in Figure 3. Both fresh and dry weight values exhibited normal S-shaped growth curves with a short lag period, which are characteristic of most in vitro grown cultures. The mid-exponential phase was reached at about 6 days of culture growth. The response of cultured cells to 3AT was characterized by a decline of about 20% in both fresh and dry cell weights and a 40% decrease in cell number as compared with the controls. Regardless of the inhibiting effect of 3AT on culture growth, however, there was no change in the pattern of the growth curves.

D.2.2. Soluble protein

3AT-treated cells also exhibited lower levels of soluble protein than those of the control over the entire growth cycle (Figure 4). This drop was more pronounced during early and mid-exponential phases (about 30%) than at later growth stage (about 20%). However, the maximum yield of soluble protein was reached during the early exponential phase—about 4 days from subculture—for both treated and control cultures.

D.2.3. Total nitrogen and free amino acids

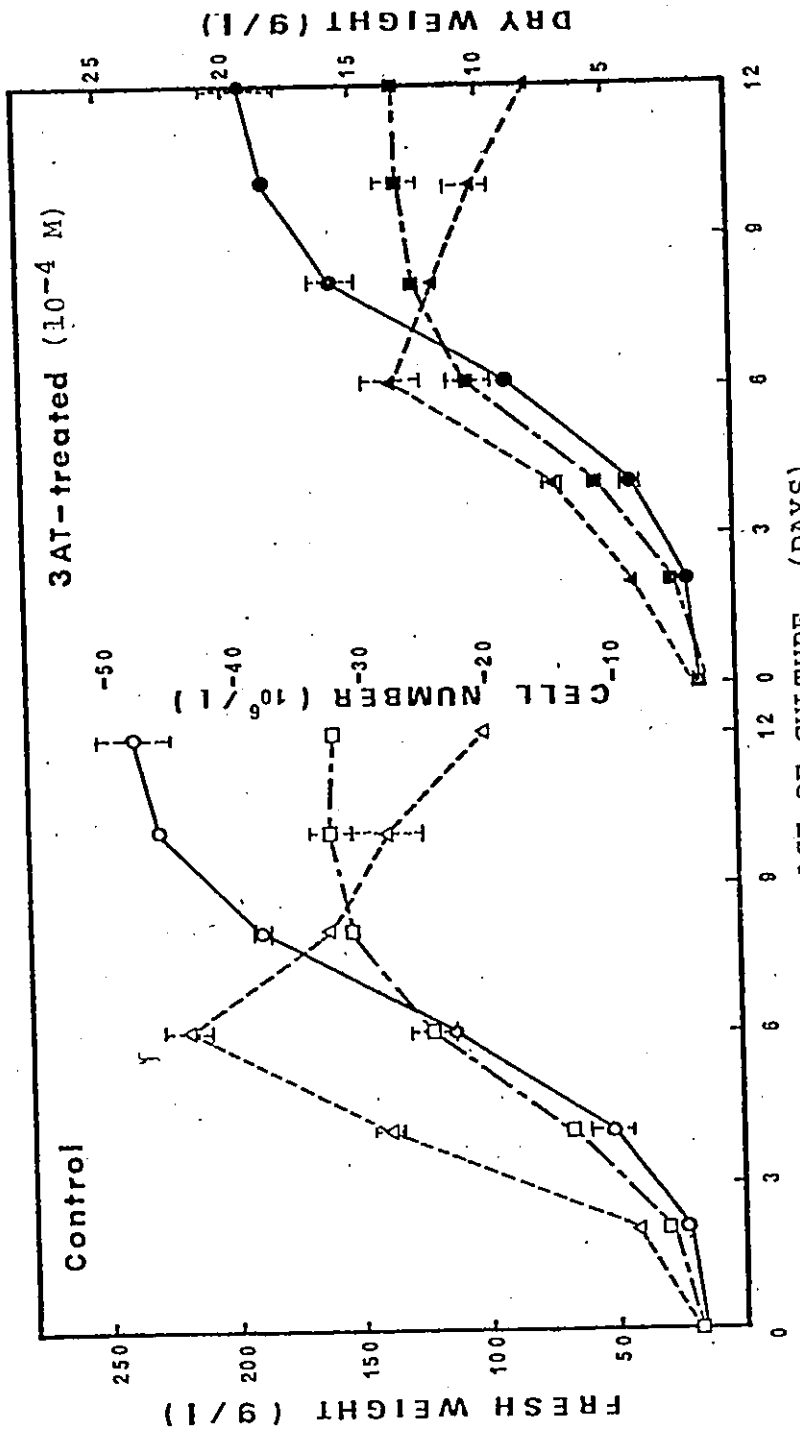


Figure 3. Growth parameters of control and 3AT-treated soybean cells

——— fresh weight

— — — dry weight

---------- cell number

For actual data see Appendix 3.

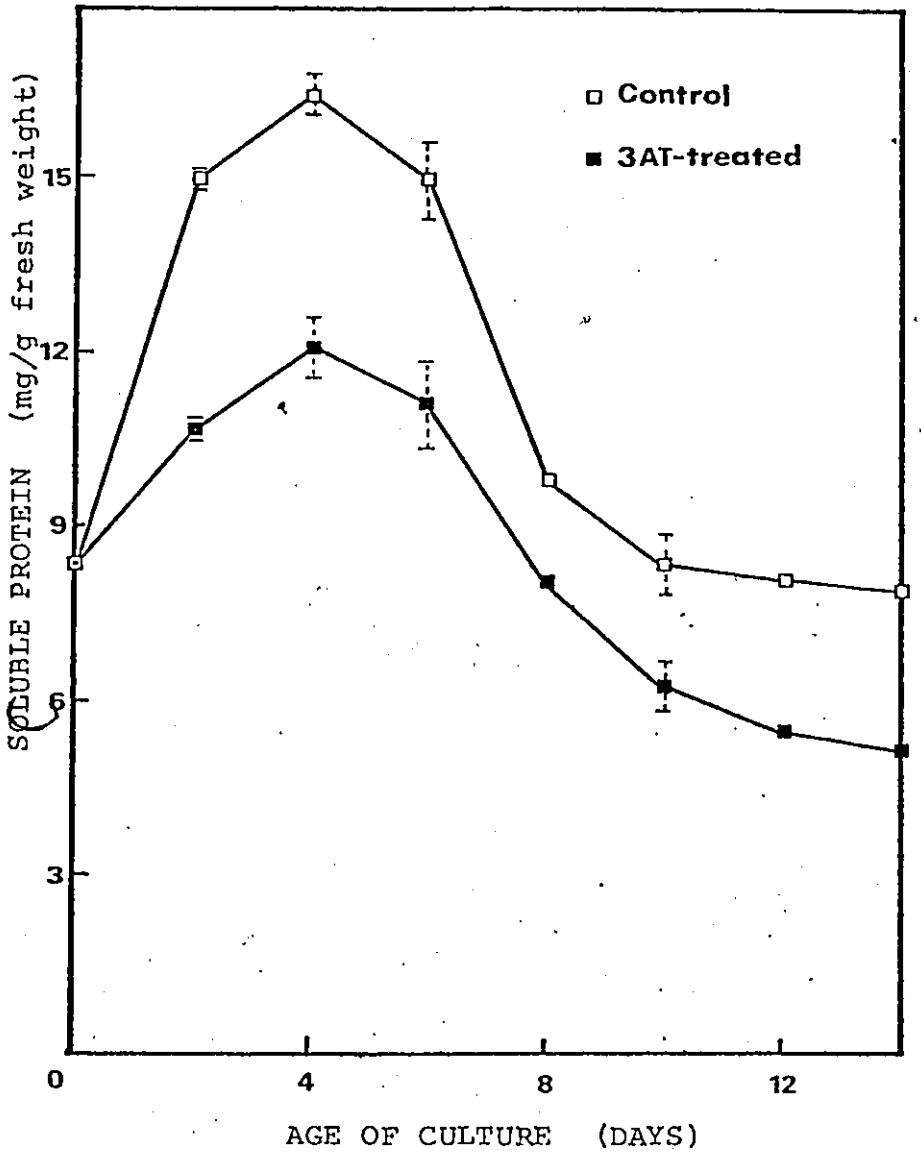


Figure 4. Soluble protein content of control and 3AT-treated soybean cells

For actual data see Appendix 3.

The effect of 3AT on the total nitrogen content of soybean cells (Figure 5) showed a small drop (10%) at mid-exponential phase, as compared with a significant decrease (44%) at the end of the exponential growth. The levels of free amino acids, on the other hand, exhibited a different pattern (Figure 5). 3AT-treated cells showed increases of approximately 82% and 71% above the control during early and mid-exponential phases and another increase (30%) during later growth stage. It may be difficult to suggest, at this point, whether the increase in free amino acid content was the result of protein hydrolysis or a slowdown in protein synthesis in the 3AT-treated cells.

Comparsion of the free amino acid patterns by TLC (Figure 6), showed no qualitative difference between the control and 3AT-treated cells.

D.3. Effects of Amitrole on the Activities of Nitrate Reductase and Glutamate Dehydrogenase During Growth

The specific activities of NR and GDH of both control and 3AT-treated cultures are shown in Figure 7. This pattern, which was reproducible, indicated that maximum activity of NR was reached at mid-exponential phase and then declined sharply thereafter, possibly due to the depletion of nitrate ion in the culture medium. The activity of GDH, on the other hand, continued to increase steadily from early exponential until stationary phase. 3AT-treated cells exhibited the same patterns of enzyme

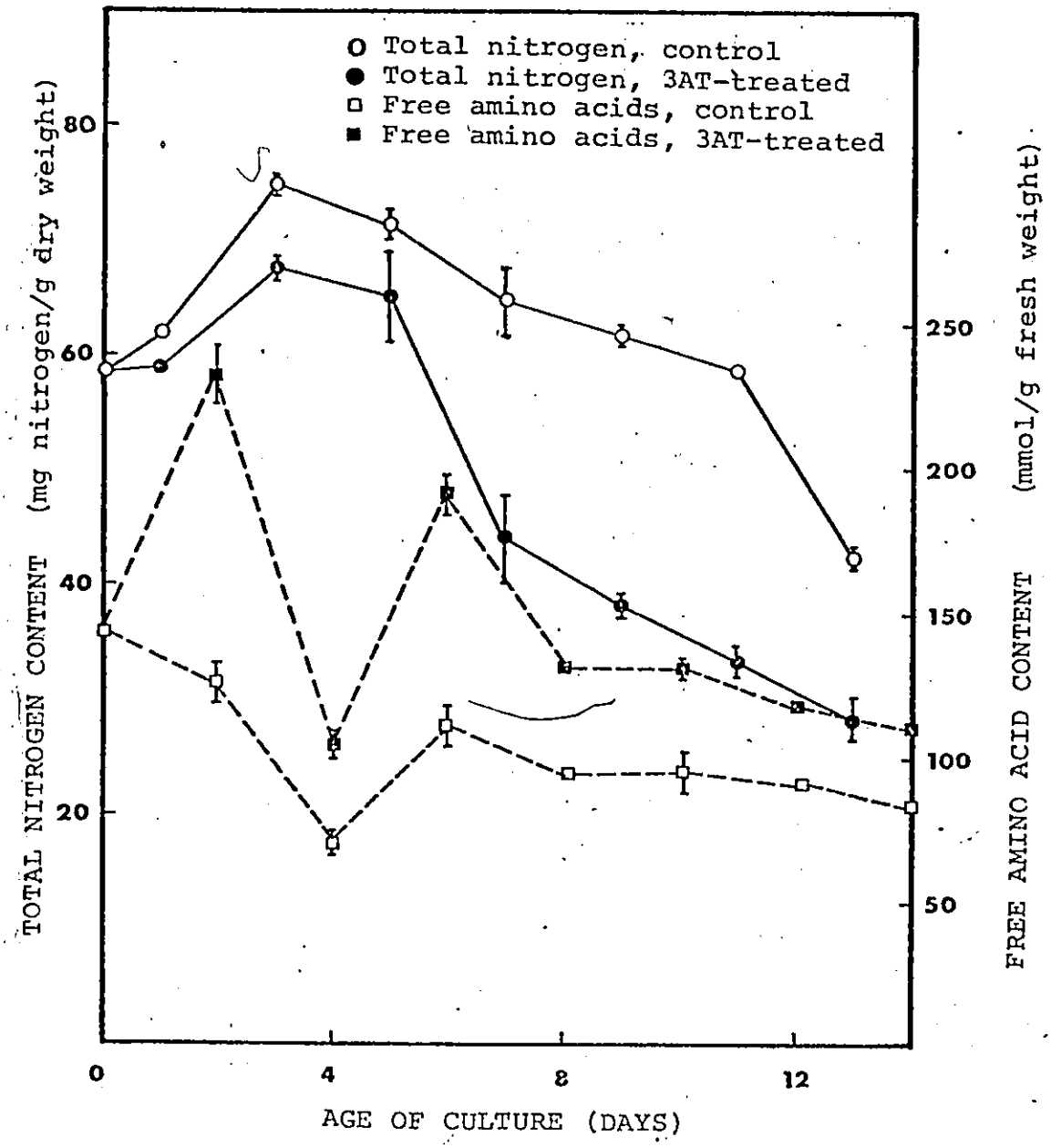


Figure 5. Total nitrogen and free amino acid contents of control and 3AT-treated soybean cells

For actual data see Appendix 3.

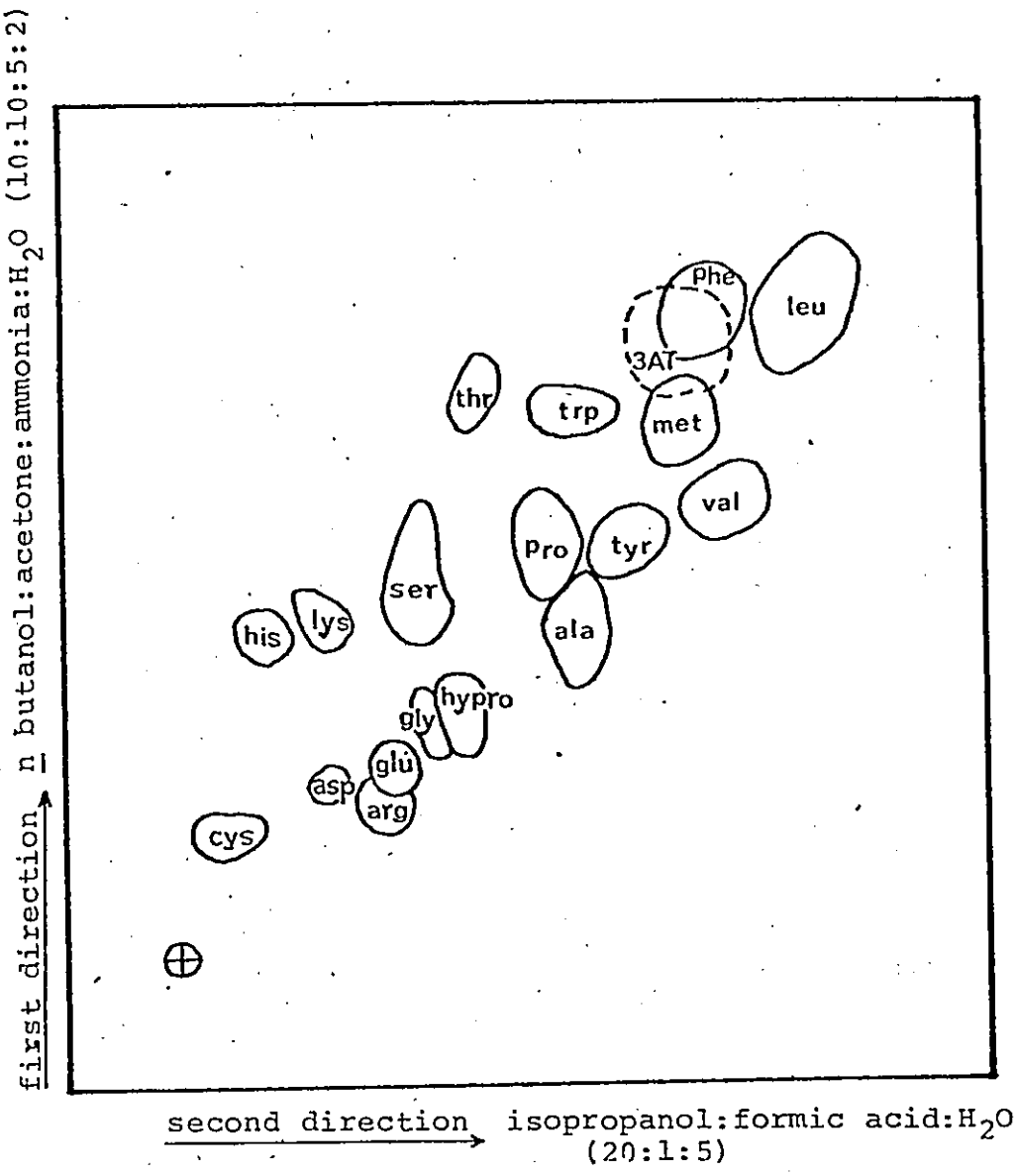


Figure 6. Diagram of thin-layer chromatogram of free amino acids of soybean cells

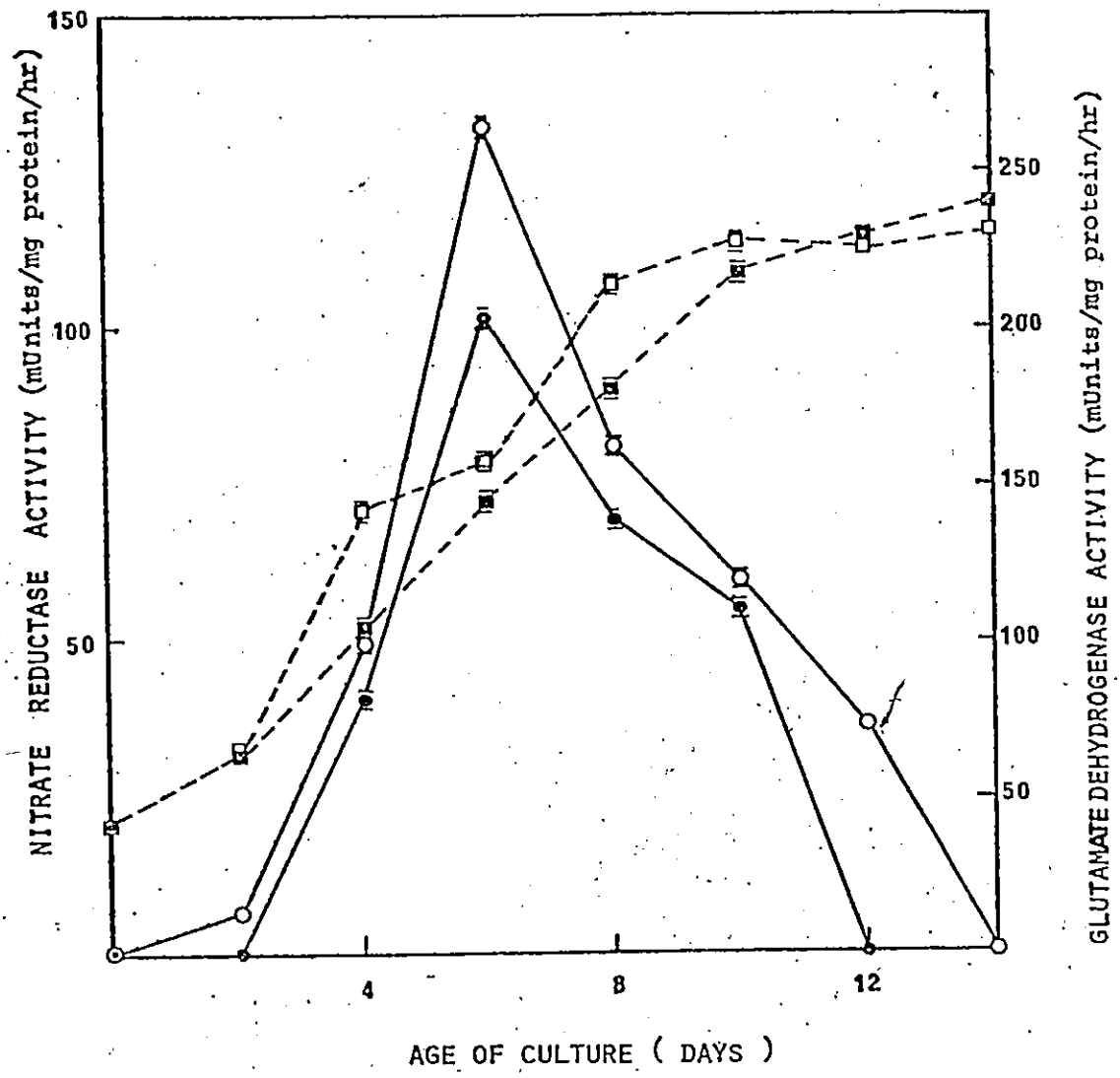


Figure 7. Nitrate reductase and glutamate dehydrogenase activities of control and 3AT-treated cells during growth

——— nitrate reductase activity

- - - - - - glutamate dehydrogenase activity

open symbols : control

solid symbols: 3AT-treated

activities as those of the controls, except for a marked drop of about 20% for both NR and GDH activities especially during the exponential phase.

D.4. Effects of Amitrole on the Kinetics of Nitrate Reductase and Glutamate Dehydrogenase

D.4.1. Nitrate reductase

The effect of different concentrations of 3AT on the kinetics of partially purified NR (Figure 8) was studied at different substrate (NO_3^- and NADH) concentrations. The apparent Km values for NO_3^- and NADH, using the Lineweaver-Burk double reciprocal plots (Lineweaver and Burk, 1934), were found to be 66.6 and 11.7 µM, respectively. These values are within the same order of magnitude as those reported for soybean leaf tissue (Beevers et al., 1964; Campbell, 1976). Increasing concentration of 3AT resulted in a corresponding increased inhibition of the specific activity of NR against different substrate concentrations (NO_3^- and NADH) as shown in Figures 9 and 10, respectively. These results indicated a noncompetitive inhibition of NR by 3AT.

K_I values shown in Table II were calculated according to the following equation:-

$$Vm_i = \frac{\frac{V_{max}}{K_I + I}}{K_I}$$

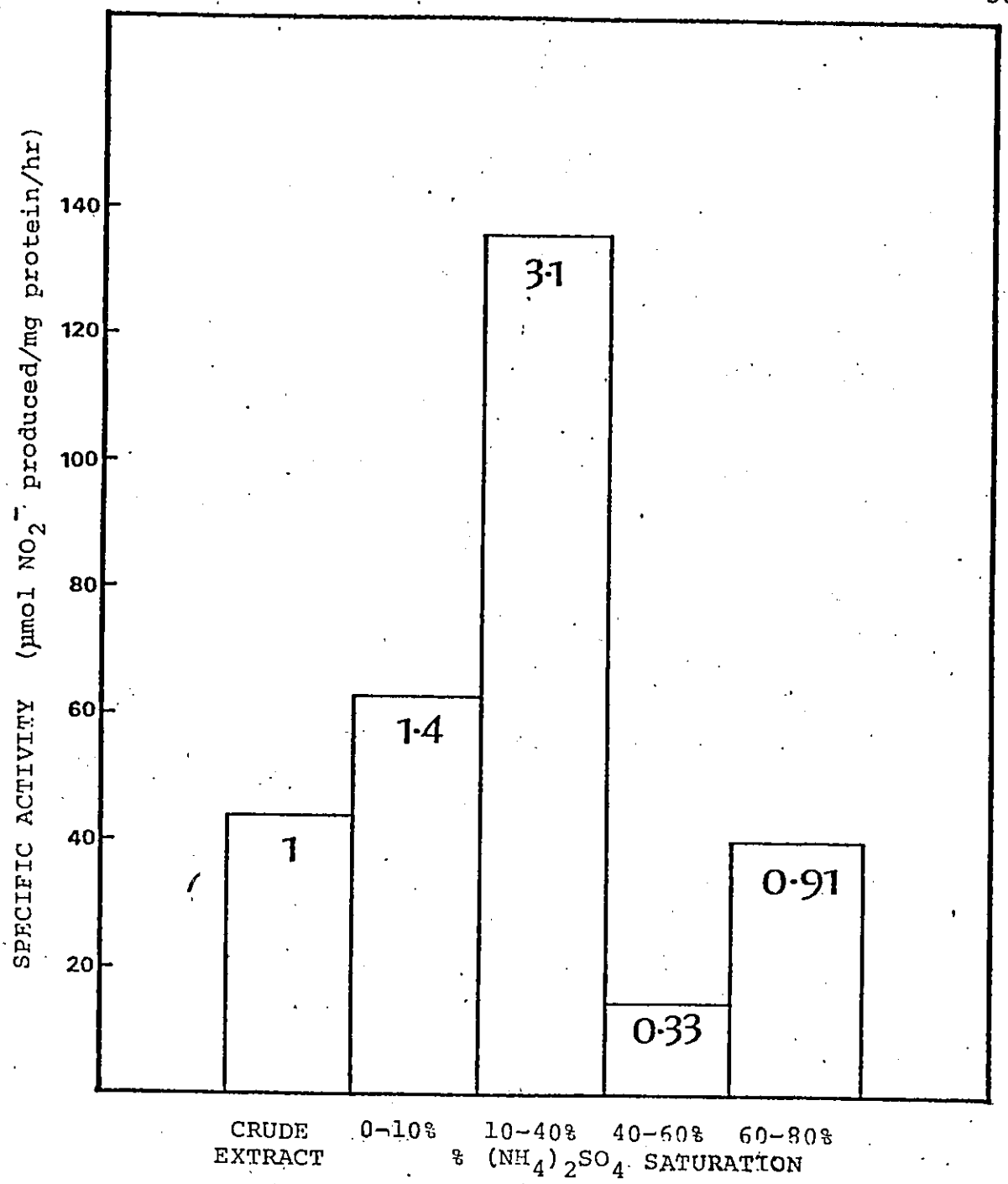
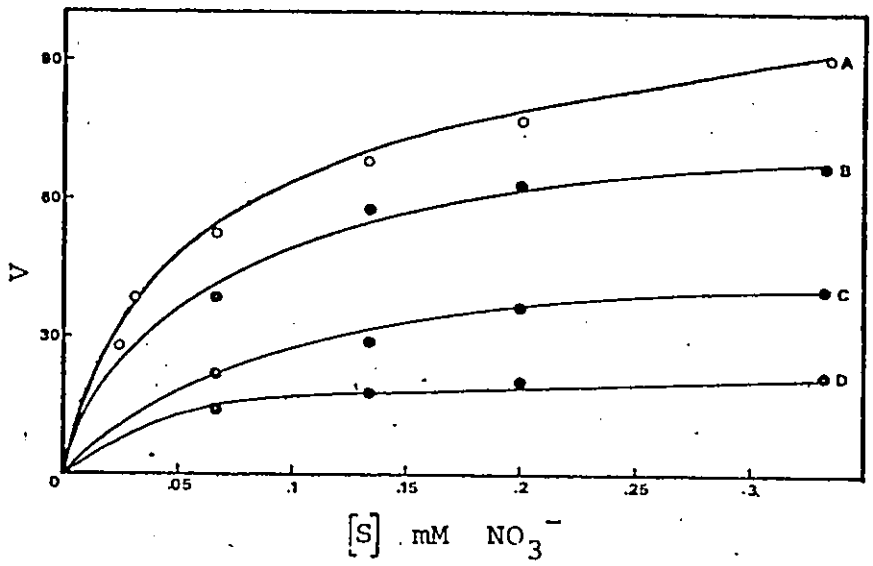


Figure 8. Partial purification of soybean nitrate reductase by ammonium sulfate precipitation

(Values at top of columns represent the purification-fold. The protein which precipitated between 10-40% saturation was used.)



3AT conc. (M)

A= 0
B= $1.66X10^{-4}$
C= $1.66X10^{-3}$
D= $1.66X10^{-2}$

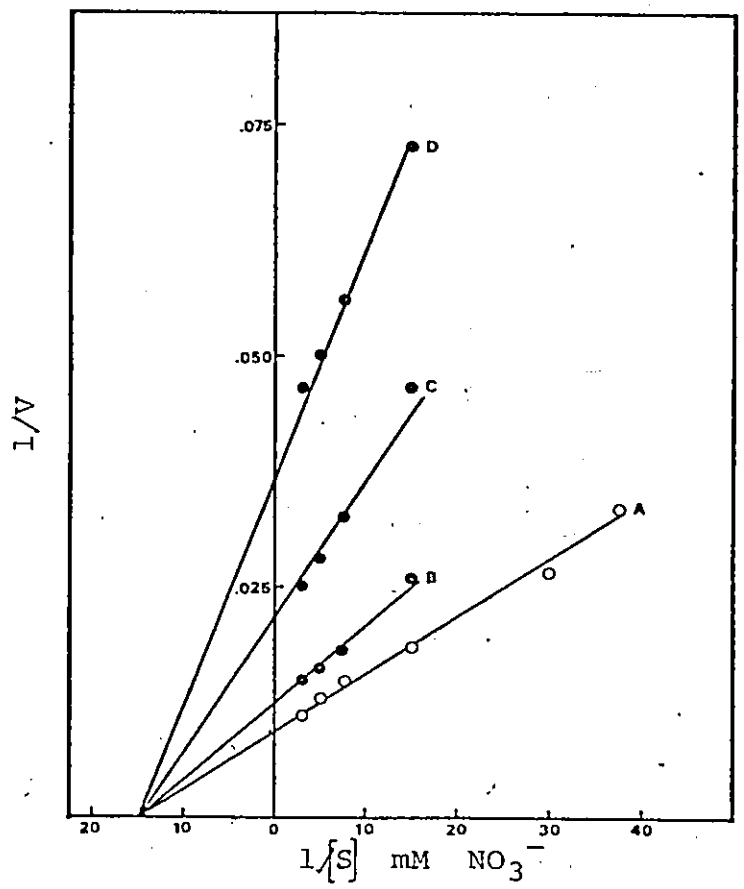
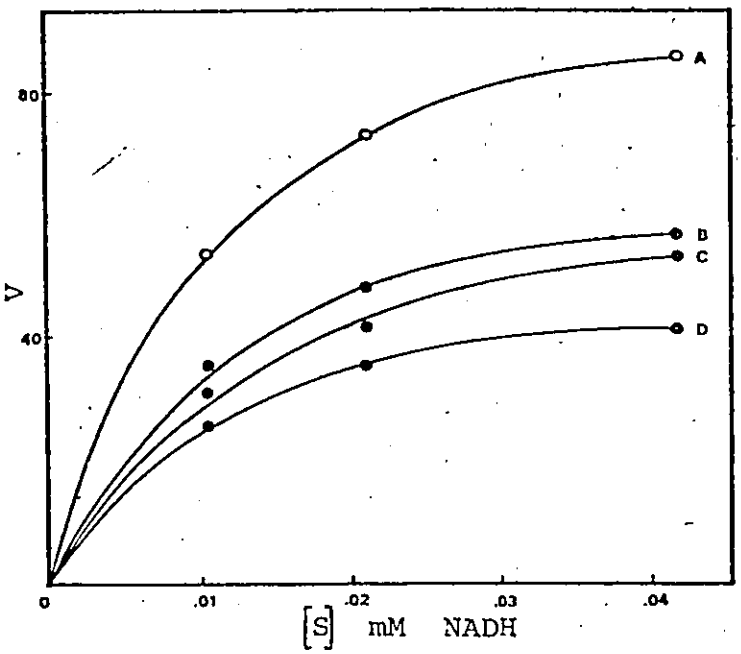


Figure 9. Lineweaver-Burk double reciprocal plot of nitrate reductase for different concentrations of 3AT using NO_3^- as substrate



3AT conc. (M)

A= 0
B= $1.66X10^{-4}$
C= $1.66X10^{-3}$
D= $1.66X10^{-2}$

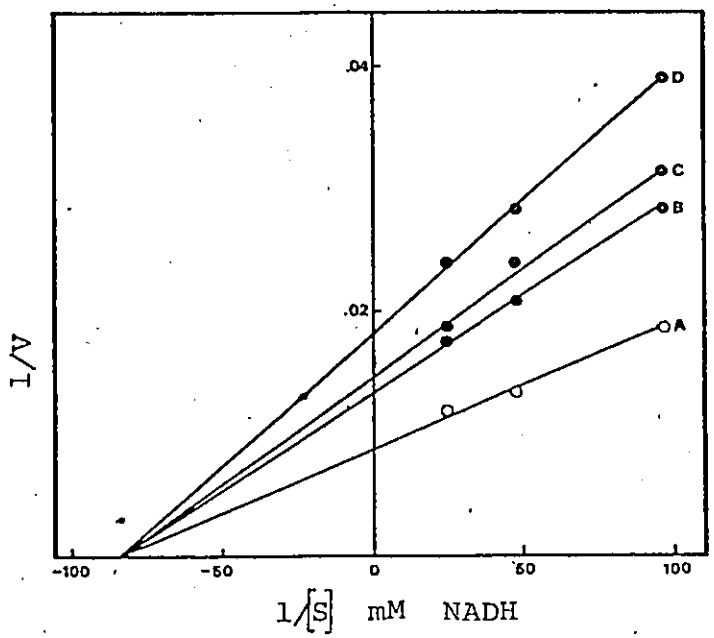


Figure 10. Lineweaver-Burk double reciprocal plot of nitrate reductase for different concentrations of 3AT using NADH as substrate

Table II.

Inhibition of soybean nitrate reductase by different concentrations of amitrole

Substrate	3AT concentration (M)	Relative activity[a]	K_I (M)
NO_3^-[b]	0	100	-
	1.66×10^{-4}	81	0.85
	1.66×10^{-3}	46	0.64
	1.66×10^{-2}	26	0.49
NADH[c]	0	100	-
	1.66×10^{-4}	66	0.81
	1.66×10^{-3}	62	0.76
	1.66×10^{-2}	48	0.68

(a) % of control (in the absence of 3AT), calculated on the basis of specific activity.

(b) NO_3^- concentration ranged between 0.066-0.33 mM, while [NADH] remained constant at 0.166 mM.

(c) NADH concentration ranged between 0.01-0.04 mM, while $[NO_3^-]$ remained constant at 33 mM.

where,

V_{max} = maximum velocity

Vm_i = maximum velocity in the presence of inhibitor

K_I = dissociation constant of the enzyme-inhibitor complex

I = concentration of inhibitor

This data indicates that the extent of inhibition of NR by different concentrations of 3AT was almost comparable with both substrates.

D.4.2. Glutamate dehydrogenase

Kinetic studies of partially purified GDH (Figure 11) were carried out using different substrate concentrations of α-ketoglutarate and NADH. The Km values for both substrates were found to be 1.667 mM and 25.0 μM, respectively. These values are quite similar to those reported for several intact tissues (Fawole and Boulter, 1977). Increasing concentration of 3AT also resulted in a corresponding increased inhibition of the specific activities of GDH against different substrate concentrations, as shown in Figures 12 and 13, respectively. These results also indicated a noncompetitive inhibition of GDH by 3AT.

K_I values were calculated using the same equation given in section D.4.1. and are shown in Table III. The data also indicated that the extent of inhibition of GDH by different concentrations of 3AT was comparable for both

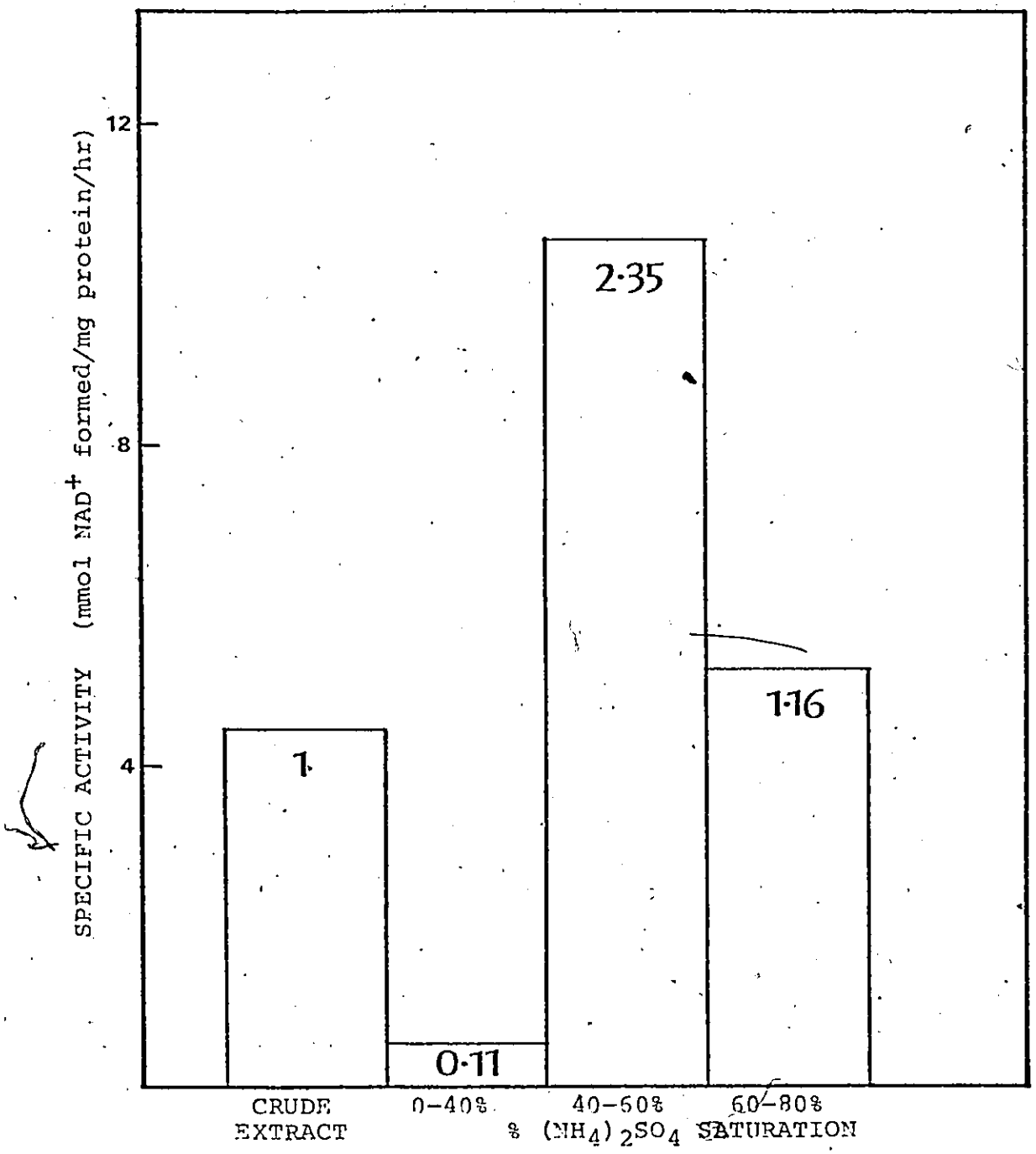
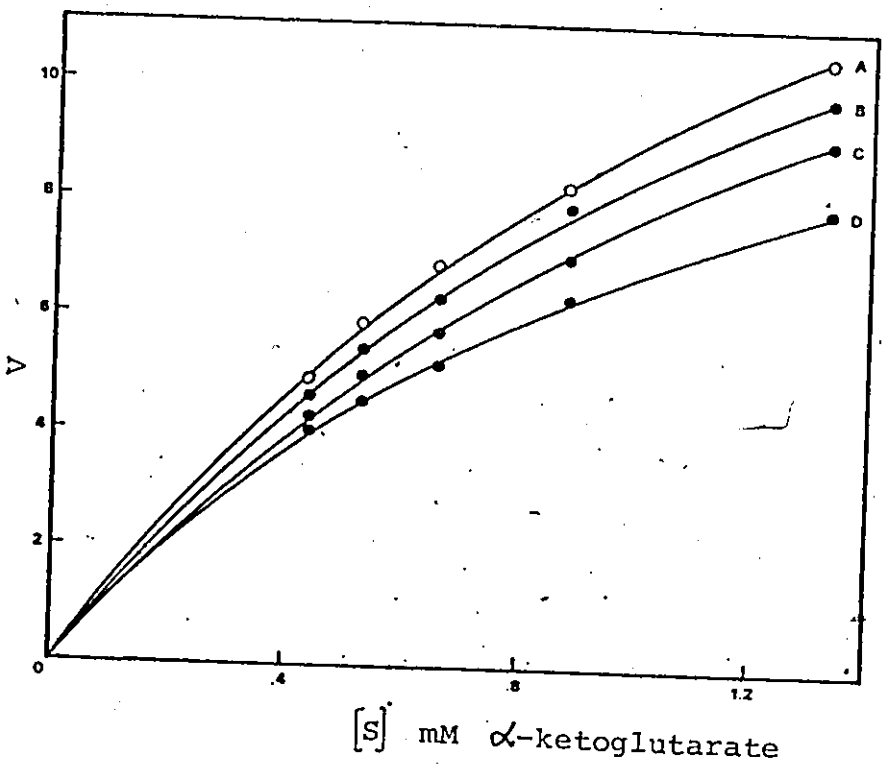


Figure 11. Partial purification of soybean glutamate dehydrogenase by ammonium sulfate precipitation

(Values at top of columns represent the purification-fold. The protein which precipitated between 40-60% saturation was used.)



3AT conc. (M)

A= 0
B= $1.66X10^{-3}$
C= $1.66X10^{-2}$
D= $1.66X10^{-1}$

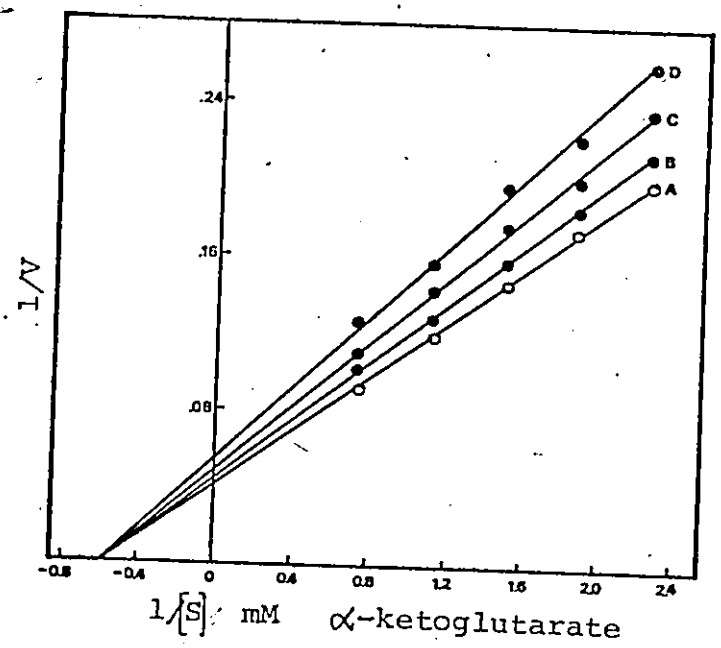
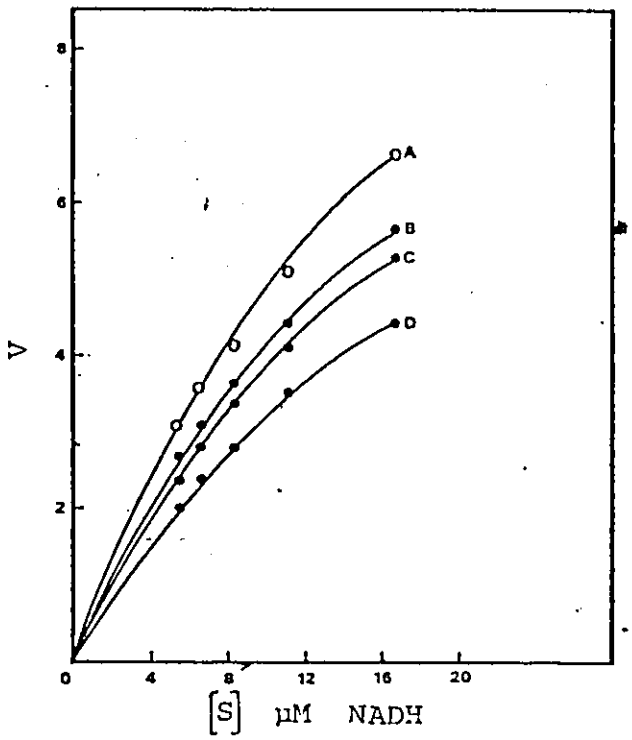


Figure 12. Lineweaver-Burk double reciprocal plot of glutamate dehydrogenase for different concentrations of 3AT using α-ketoglutarate as substrate



3AT conc. (M)

A= 0
B= $1.66X10^{-3}$
C= $1.66X10^{-2}$
D= $1.66X10^{-1}$

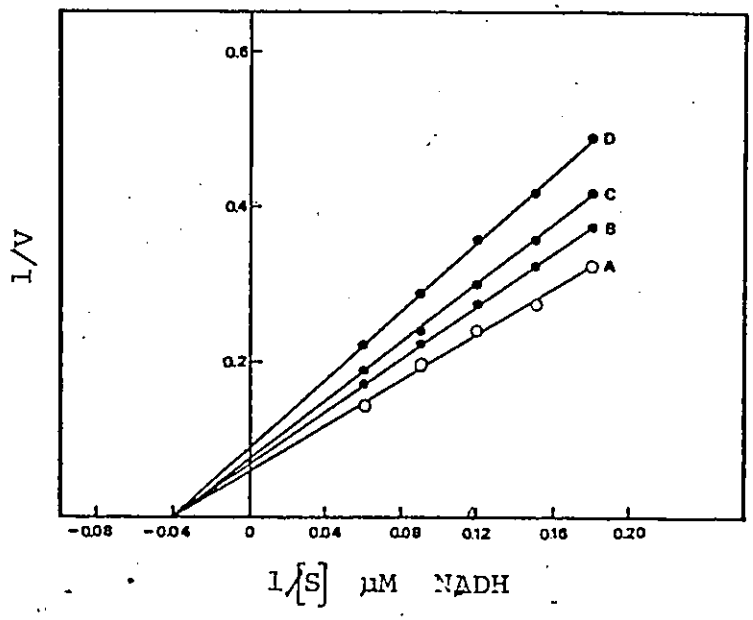


Figure 13. Lineweaver-Burk double reciprocal plot of glutamate dehydrogenase for different concentrations of 3AT using NADH as substrate

Table III

Inhibition of soybean glutamate dehydrogenase by different concentrations of amitrole

Substrate	3AT concentration (M)	Relative activity[a]	K_I (M)
α-keto-glutarate[b]	0	100	-
	1.66×10^{-3}	93	0.98
	1.66×10^{-2}	86	0.93
	1.66×10^{-1}	75	0.78
NADH[c]	0	100	-
	1.66×10^{-3}	85	0.93
	1.66×10^{-2}	79	0.86
	1.66×10^{-1}	66	0.73

(a) % of control (in the absence of 3AT), calculated on the basis of specific activity.

(b) α-ketoglutarate concentration ranged between 0.44-1.33 mM, while [NADH] remained constant at 0.166 mM.

(c) NADH concentration ranged between 5.55-16.66 μM, while [α-ketoglutarate] remained constant at 13.33 mM.

substrates.

D.5. Effects of Amitrole on the Uptake of ^{14}C-Leucine and its Incorporation into Soybean Proteins

The effect of 3AT on protein synthesis was measured by following the uptake and incorporation of ^{14}C-leucine into soluble proteins, as described in section C.9.

D.5.1. ^{14}C-Leucine uptake

^{14}C-Leucine uptake was measured by determining the radioactivity of the buffer extract of the cultured cells after incubation for 1-6 hr. The results (Figure 14) show that, in both the control and 3AT-treated cells, the uptake of ^{14}C-leucine was proportional to the time of incubation. However, it is remarkable to note that the label uptake by 3AT-treated cells was 30-90% higher than that of the control (Table IV).

The addition of cycloheximide (CH) (50 µg/ml) to the incubation medium resulted in a significant drop in label uptake, which amounted to approximately 30-50% in both control and 3AT-treated cells (Figure 14). This seems to indicate that the label uptake by the cultured cells was directed towards protein synthesis (see following section).

D.5.2. ^{14}C-Leucine incorporation into TCA-precipitable protein

The results given in Figure 15 show that there was a significantly higher incorporation of label into proteins

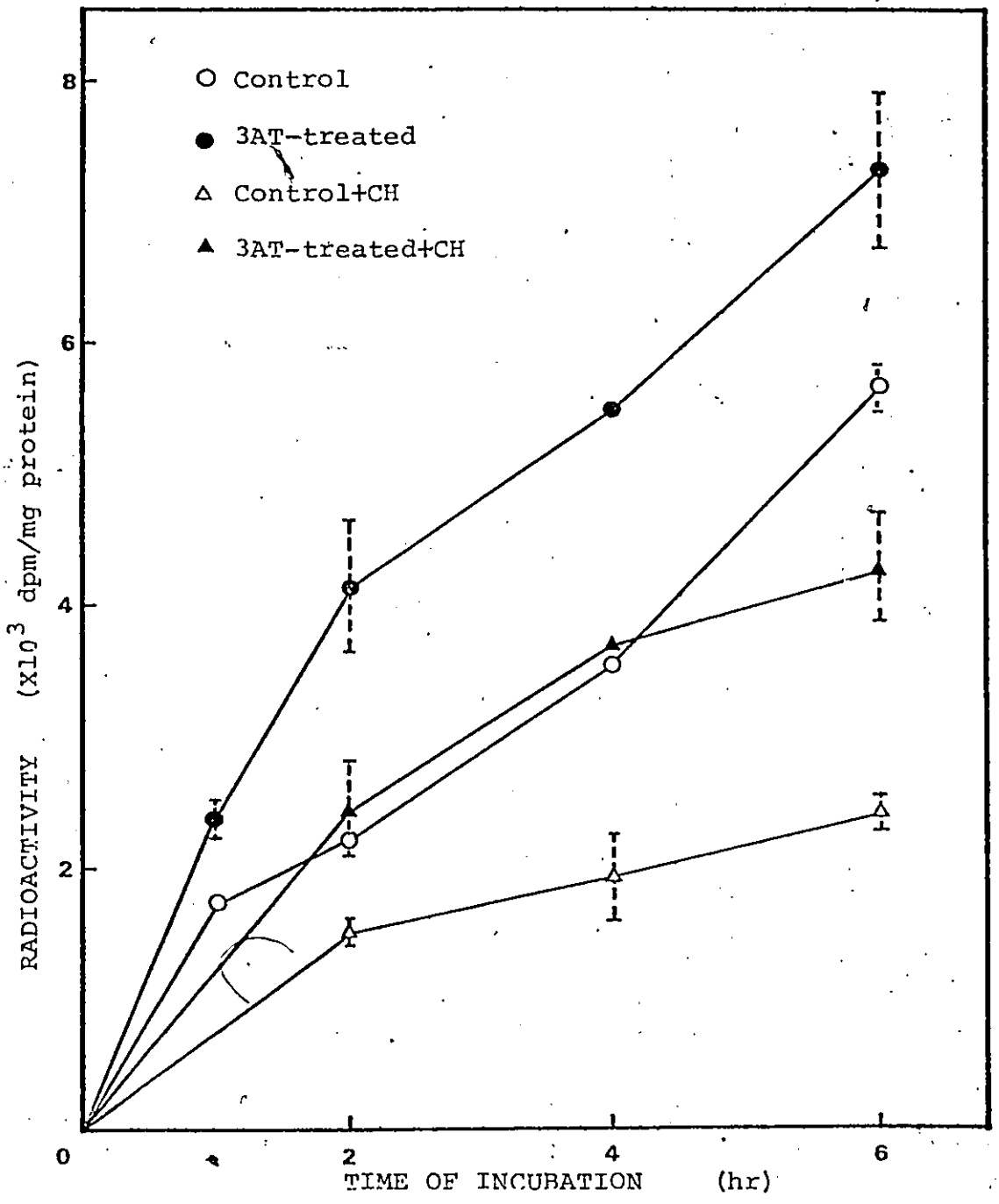


Figure 14. Uptake of ^{14}C-leucine by control and 3AT-treated cells

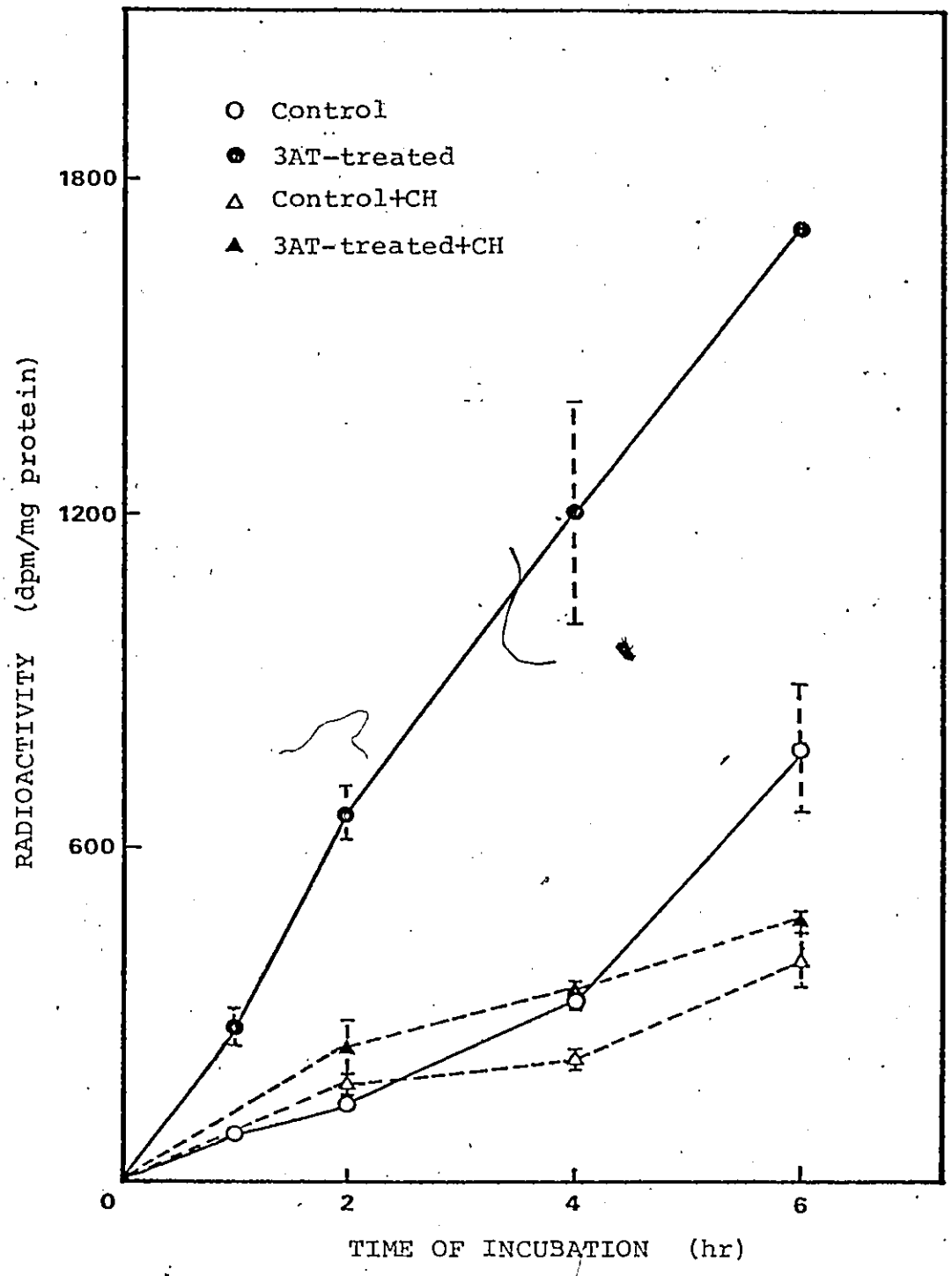


Figure 15. Incorporation of ^{14}C-leucine into TCA-precipitable protein of control and 3AT-treated soybean cells

Table IV

Effect of amitrole on ^{14}C-leucine uptake and its incorporation into TCA-precipitated protein of cultured soybean cells

Time of incubation (hr)	^{14}C-Leucine uptake (% above control) (a)	Activity in TCA-pptd protein (% above control) (b)
1	+39%	+224%
2	+89.8%	+384%
3	+54.7%	+273%
4	+29.6%	+121%

(a) values correspond to points of 3AT treatment in Figure 14.

(b) values correspond to points of 3AT treatment in Figure 15.

of the 3AT-treated cells than that of the control. This increase was highest during the first 4 hr of incubation and amounted to 2-4 times that of the control. Furthermore, the addition of cycloheximide resulted in a 30-50% drop in label incorporation of the control cells as compared with 60-70% in 3AT-treated cells.

D.5.3. The effect of saturating the endogenous leucine pool

In another experiment, the leucine pool of soybean cells was saturated by incubation in 10^{-5}M of non-labelled (cold) leucine, prior to label administration. The uptake of label was followed and its incorporation into protein was determined during a period of 1-6 hr. The results given in Figures 16 and 17 were similar to those observed in the absence of cold leucine (Figures 14 and 15), i.e., there was a significant increase in label uptake and its incorporation into protein by the 3AT-treated cells. However, saturation of the leucine pool of the cultured cells resulted in a 37-55% drop in both label uptake and its incorporation into protein of both control and 3AT-treated cells.

D.5.4. Label distribution into protein hydrolysate

Chromatography of the acid hydrolysates of the labelled TCA-precipitable proteins, followed by autoradiography, indicated that the label was totally confined to the amino acid, leucine (Figure 18).

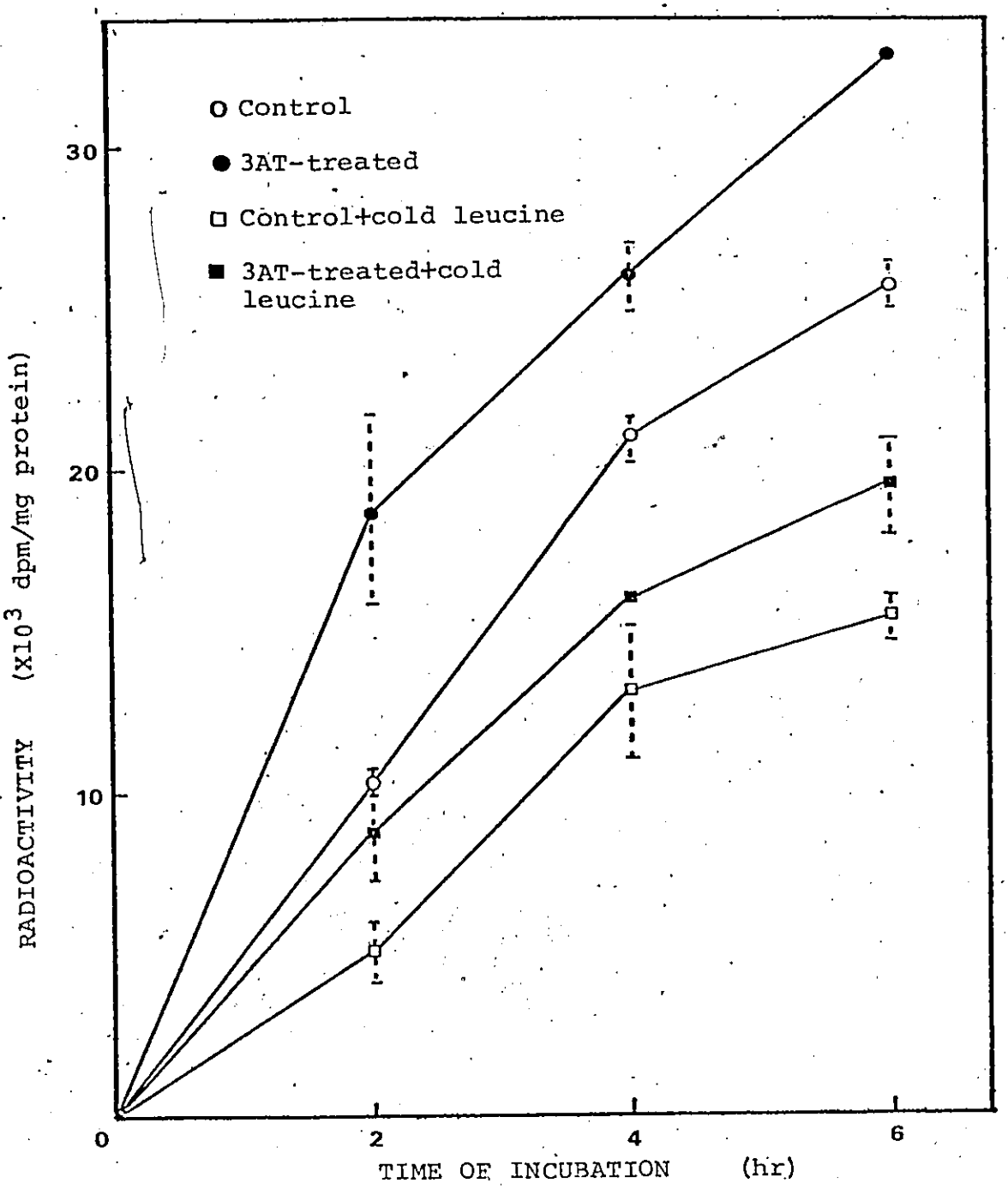


Figure 16. Effect of the saturation of leucine pool of the cells on label uptake

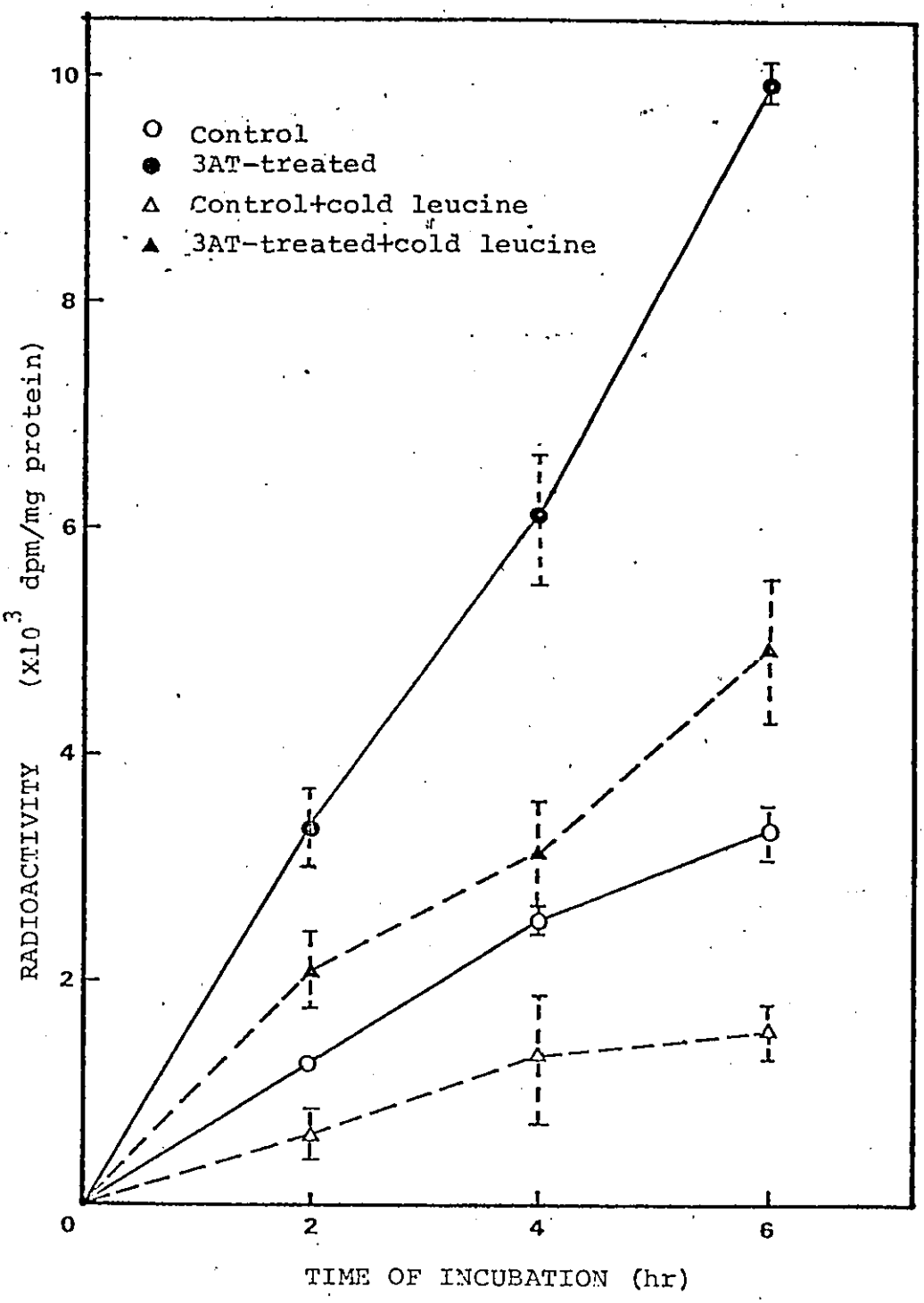


Figure 17. Effect of the saturation of leucine pool of the cells on label incorporation into protein

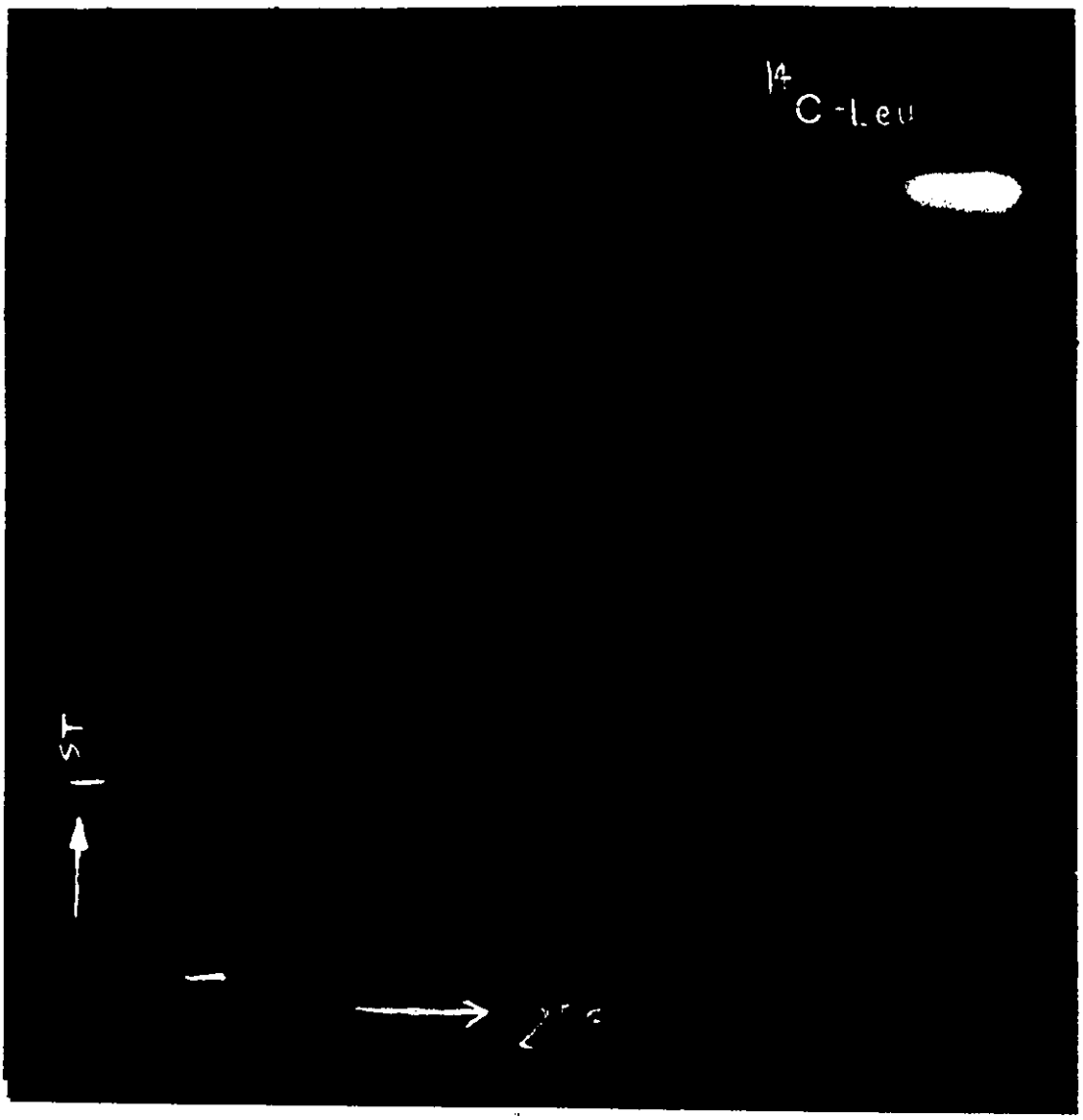


Figure 18. Print of an autoradiogram of the acid hydrolysate of TCA-precipitated protein of control or 3AT-treated cells

D.6. Electrophoretic Patterns of Soluble Proteins

The acrylamide gel protein patterns of the control and 3AT-treated soybean cells, cultured for 4, 8 and 12 days are shown in Figures 19-21, respectively. There was no significant change in the protein complements of 4- and 8-day-old cultures (Figures 19 and 20). However, few more protein bands were observed in the 12-day-old cultures (Figure 20). The latter may appear to be degradation products of some major proteins, which usually takes place in stationary-phase cells.

Examination of the recordings of gel profiles indicated slight quantitative, but no qualitative, differences between the control and 3AT-treated cells.

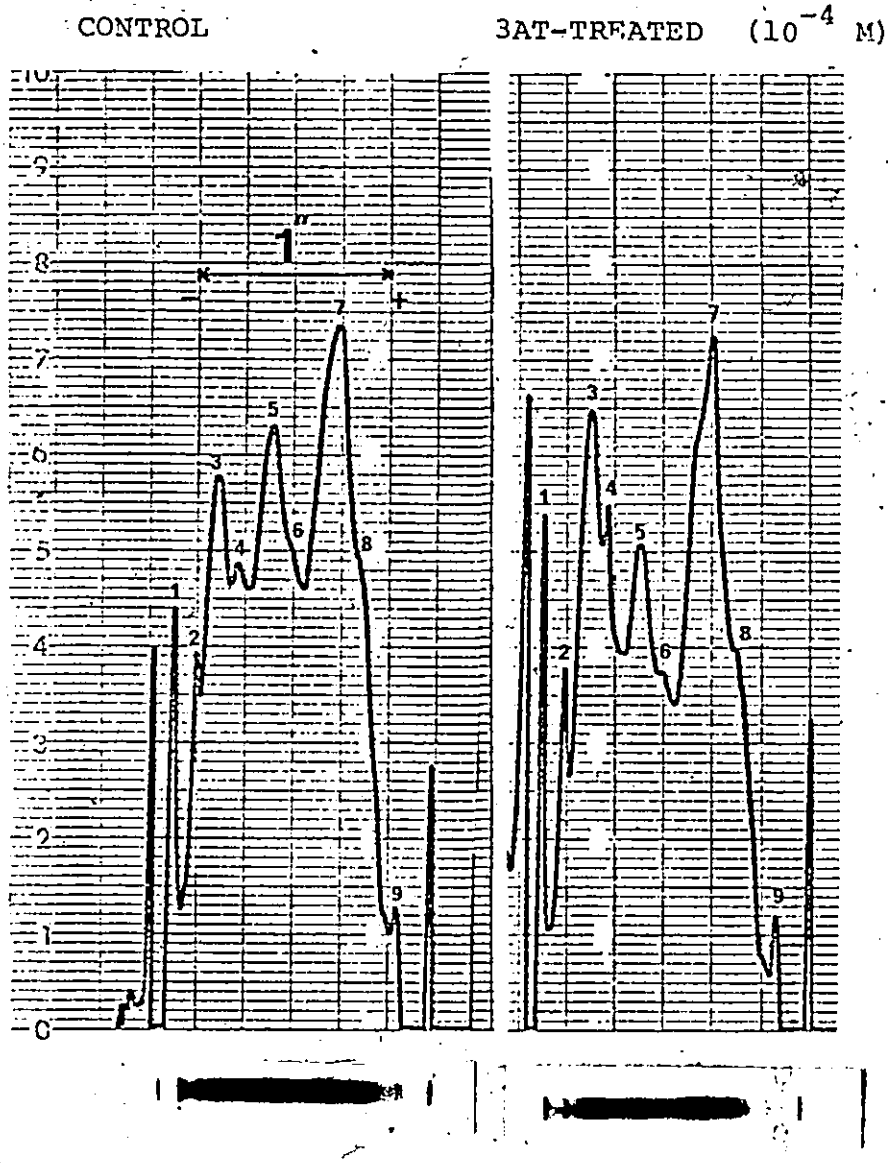


Figure 19. Acrylamide gel protein patterns of 4-day-old control and 3AT-treated cells

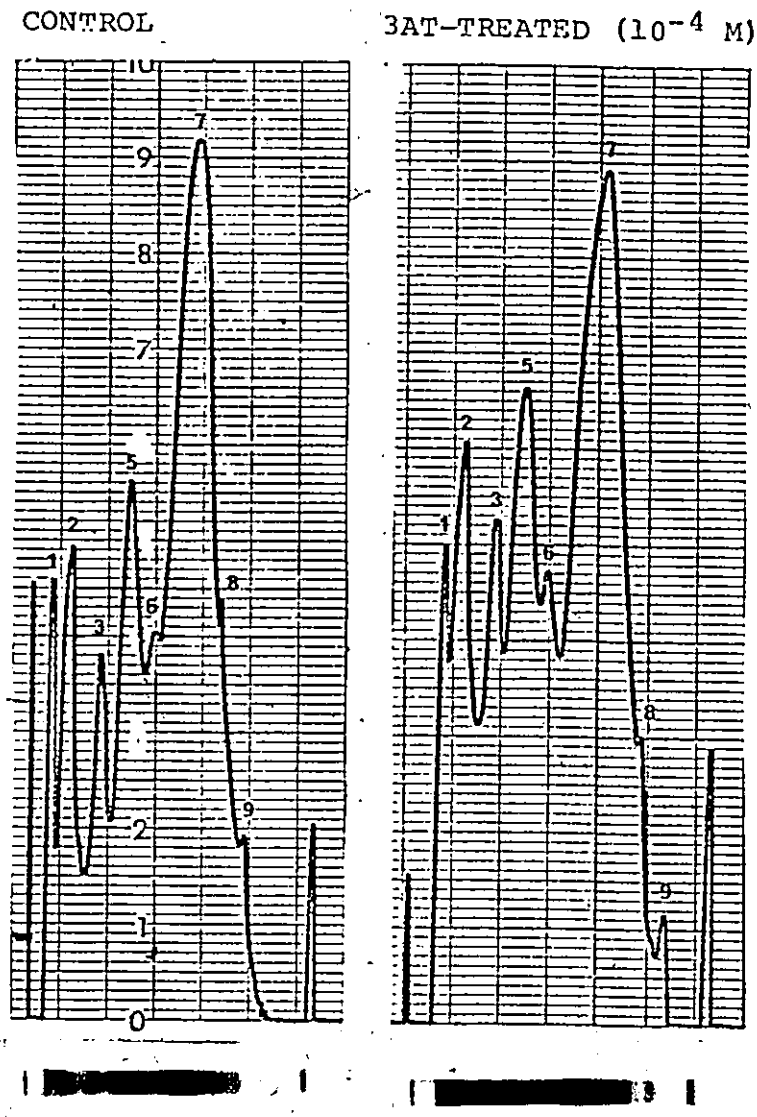


Figure 20. Acrylamide gel protein patterns of 8-day-old control and 3AT-treated cells

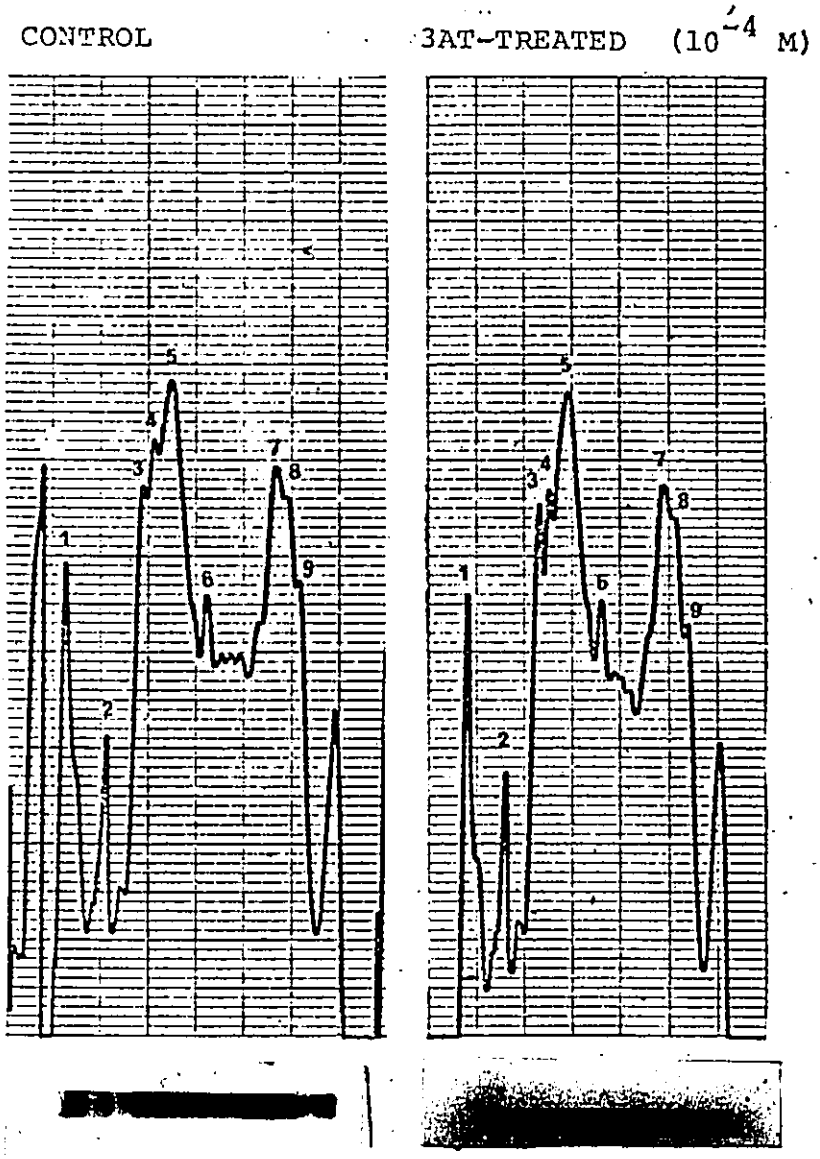


Figure 21. Acrylamide gel protein patterns of 12-day-old control and 3AT-treated cells

Section B

DISCUSSION

The results presented in this investigation show that the effects of 3AT on growth parameters of cultured soybean cells are of quantitative rather than qualitative nature. 3AT seems to affect growth by partially blocking cell division and consequently, resulted in a significant drop in cell number, fresh and dry weights and both total and soluble protein contents. The inhibitory effect of 3AT on multiplication of cells was observed in photosynthetic microorganisms (Aaronsqn and Scher, 1960), in Scenedesmus sp. (Castelfranco and Bisalputra, 1965) and in carrot (Homes, 1966) and tobacco (Barg and Umiel, 1977) callus cultures.

3AT was reported to induce chromosomal aberrations and impair the metabolic activities of cells (Sawamura, 1965; Wuu and Grant, 1967). The decrease in both soluble protein and total nitrogen and the corresponding increase in free amino acids, which were observed in 3AT-treated cells, appear to be similar to those reported for Zea mays and wheat seedlings (McWhorter, 1963; Bartels and Wolf, 1965). On the other hand, the increase in retention of water by cells treated with low concentrations of 3AT (10^{-7} to 10^{-5}M) may be the consequence of an increased osmotic pressure resulting from the hydrolysis of nitrogenous compounds and carbohydrates (Wort, 1964). This may provide an explanation for the increase in fresh weight and the decrease in dry

weight and protein content of cells treated with low concentrations of 3AT.

Nitrate is the principal source of nitrogen for most higher plants. NO_3^- is reduced by the enzyme NADH:nitrate reductase (E.C.1.6.6.1) to nitrite (Beevers and Hageman, 1969) and ultimately to NH_4^+ which, in turn, is utilized in the amination of organic acids by glutamate dehydrogenase (E.C.1.4.1.2) (Smith et al., 1975). These are considered two key enzymes in organic nitrogen metabolism. Amitrole inhibited the activities of both NR (10^{-4} to 10^{-2}M) and GDH (10^{-3} to 10^{-1}M) of cultured soybean cells, though the former was more sensitive to 3AT-inhibition than the latter enzyme. The noncompetitive inhibition observed for NR and GDH by 3AT (Figures 9, 10 and 12, 13 respectively) appears similar to the effect of chelating agents on both enzyme activities as was reported by Yamasaki and Suzuki (1969) and Garrett and Greenbaum (1973).

It was rather difficult to elucidate the nature of the noncompetitive inhibition of 3AT on enzyme activity since NADH, which is a required cofactor for both enzymes, exhibited no significant change in its spectral characteristics when incubated with different concentrations of 3AT (Figure 22). As far as the author is aware, there are no published reports on the effect of amitrole on the enzymes of organic nitrogen metabolism in either intact or cultured tissues. However, it is obvious that 3AT-inhibition of NR

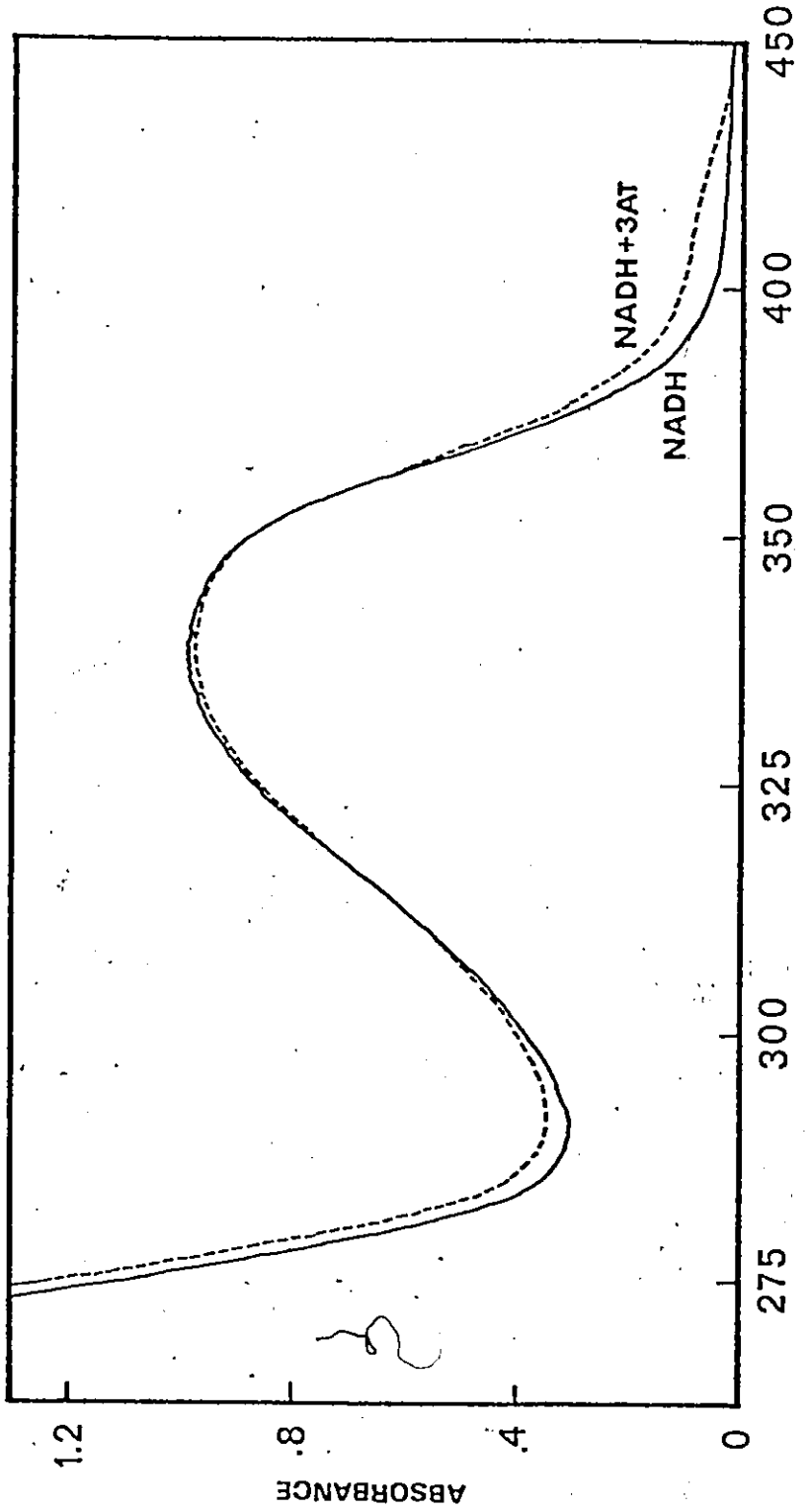


Figure 22. UV-Absorption spectra of NADH before and after incubation in 10^{-1} M 3AT for 30 min

activity may result in impairing the nitrogen metabolism of the treated cells. Furthermore, the inhibition of GDH activity may also prevent the conversion of ammonia to amino acids, thus resulting in its accumulation. Recently, Baumann and Gunther (1976) reported an enhanced 3AT-toxicity in oat seedlings as a consequence of protein breakdown and accumulation of ammonia. It is possible, therefore, that 3AT-inhibition of both NR and GDH activities may have resulted in the decrease in protein content and the corresponding increase in amino acids. Furthermore, the accumulation of a large pool of free amino acids may have caused the repression of various enzymes, including NR and GDH (Filner, 1966). The mechanisms by which 3AT inhibited enzyme activity may be diverse: it may be the result of covalent modification (direct binding) of the enzyme protein (Agrawal et al., 1970); locking up the metal prosthetic group, thus rendering the enzyme non-functional; or it may have a direct effect on enzyme synthesis. Our results seem to indicate that the effect of 3AT on enzyme inhibition may be due to inhibition of enzyme activity rather than on enzyme synthesis.

The effect of amitrole on increased uptake of ^{14}C-leucine and its incorporation into soybean protein is striking, in spite of the fact that saturation of the leucine pool of the cells resulted in a drop in both label uptake and its incorporation into proteins. The inhibition, by cycloheximide, of label incorporation into the protein

fraction further indicates de novo synthesis of protein and rules out the possibility of being an artifact.

A possible explanation may be that the decrease observed in NR and GDH activities and the significant drop in the protein content of 3AT-treated cells obviously put a stress on the tissue's metabolic system, causing an increase in respiration and energy output (McWhorter, 1963; Wort, 1964; Kirkwood, 1976). This energy output may be utilized in a repair mechanism that resulted in a stimulation of de novo protein synthesis, at least for a short period of time (ca. 4-6 hr). Whereas several workers reported a stimulatory effect of 3AT on protein synthesis (Mann et al., 1965; Brown and Carter, 1968; Moreland et al., 1969), however, Bartels and Wolf (1965) found that 3AT inhibited the incorporation of ^{14}C-glycine into wheat seedling proteins. However, the results presented here gave no indication that 3AT was an inhibitor of protein synthesis, it may have had an indirect effect on that process.

The effect of amitrole on green tissues has been reported to inhibit chlorophyll synthesis in new growth (Bruinsma, 1965), by blocking the formation of the 70S chloroplastic ribosome and the 18S fraction I protein (Bartels et al., 1967) and modifying the ultrastructure of chloroplast development (Bartels and Weier, 1969). Whereas the use of a non-chlorophyllous, in vitro cultured system was thought to eliminate the effects of 3AT on chlorophyll synthesis, however, it did not help establish the direct effect of

the herbicide on general metabolism. Cherry (1976) suggested that the direct effect of amitrole, as well as several other herbicides, might be on nucleic acid metabolism, particularly purines.

The mode of action of 3AT remains difficult to pinpoint. The primary site of action does not appear to be the same in all organisms. 3AT seems to be metabolically nonspecific; instead of having a single site of action, there may be several sites and mechanisms involved in the inhibition (Barg and Umiel, 1977). Different sites may vary in their sensitivity to the herbicide and while the primary site would be affected first, this initial response may be difficult to single out and identify. As the concentration of herbicide builds up inside the cells, additional sites may become involved and complicate the problem (Ashton et al., 1977). It was stated by Moreland (1967) that "no investigator has been able to name a single mechanism through which the phytotoxicity of amitrole to higher plants can be explained..."; unfortunately, the situation has not changed very much since. The author hopes, however, that the results presented in this investigation have added to our knowledge of the hitherto unreported effect of this herbicide on the enzymes of organic nitrogen metabolism in in vitro cultured cells.

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APPENDIX 1

Composition of MS medium (Murashige and Skoog, 1962), pH 5.7±0.1

		mg/l
Macro-elements	$CaCl_2 \cdot 2H_2O$	440
	$MgSO_4 \cdot 7H_2O$	370
	KNO_3	1900
	NH_4NO_3	1650
	KH_2PO_4	170
Iron	$FeSO_4 \cdot 7H_2O$	27.8
	Na_2EDTA	37.3
Micro-elements	$MnSO_4 \cdot 4H_2O$	22.3
	$ZnSO_4 \cdot 4H_2O$	8.6
	H_3BO_3	6.2
	$Na_2MoO_4 \cdot 2H_2O$	0.25
	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.025
	KI	0.83
Vitamins	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.1
	Glycine	2.0
Myo-inositol		100
Casein-hydrolysate		1,000
2,4-D		2
Kinetin		0.5
Sucrose		30,000

APPENDIX 2

Detection of amitrole by the H-acid reagent (Racusen, 1958)

The dry chromatogram was sprayed first with 1% sodium nitrite in 80% acetone-water and followed by 20% concentrated HCl in water. The plate was allowed to stand for 10 min, then sprayed with 0.25% monosodium salt of H-acid (8-amino-1-naphthol-3,6 disulfonic acid, Eastman-Kodak Company). Amitrole appeared immediately as a bright red spot.

Appendix 3

Growth parameters of soybean cell culture grown in the absence or presence of 3AT (10^{-4}M) as calculated on dry weight basis

Days of culture	Dry wt / Fresh wt [a]	Soluble protein [b] /fresh wt	Soluble protein [b] /dry wt	Free amino acid [c] /fresh wt	Free amino acid [c] /dry wt	Total N_2 [c] /dry wt
CONTROL						
0	$\frac{1.4}{14}=0.10$	8.3	83	144	1440.0	58.70 (DAY 0)
2	$\frac{2.8}{22}=0.1273$	15.0	117.83	126	989.79	62.22 (DAY 1)
4	$\frac{6.7}{52}=0.1288$	16.4	127.33	70	543.48	74.87 (DAY 3)
6	$\frac{12.3}{114}=0.1079$	15.0	139.02	112	1037.99	71.61 (DAY 5)
8	$\frac{15.4}{188}=0.0819$	9.8	119.66	94	1147.74	64.81 (DAY 7)
10	$\frac{16.2}{230}=0.0704$	8.4	119.32	95	1349.43	61.80 (DAY 9)
12	$\frac{16.1}{240}=0.0671$	8.2	122.21	92	1371.08	58.70 (DAY 11)
14	$\frac{15.8}{236}=0.0669$	8.0	119.58	84	1255.60	42.60 (DAY 13)
3AT-TREATED (10^{-4}M)						
0	$\frac{1.4}{14}=0.10$	8.3	83	144	1440.0	58.70 (DAY 0)
2	$\frac{2.3}{20}=0.115$	10.7	93.04	230	2000.0	58.715 (DAY 1)
4	$\frac{5.5}{42}=0.1309$	12.1	92.40	104	794.19	67.55 (DAY 3)
6	$\frac{10.5}{88}=0.1193$	11.2	93.88	192	1609.38	65.40 (DAY 5)
8	$\frac{12.4}{158}=0.0785$	8.1	103.21	132	1681.95	44.58 (DAY 7)
10	$\frac{13.1}{184}=0.0712$	6.4	89.90	130	1826.10	37.98 (DAY 9)
12	$\frac{13.1}{194}=0.0675$	5.6	82.96	120	1777.77	33.19 (DAY 11)
14	$\frac{13.0}{200}=0.065$	5.3	81.54	110	1692.30	28.62 (DAY 13)

(a) see Figure 3
(b) see Figure 4
(c) see Figure 5