Studies on the Mechanism of Action of 2-Aminobutane

J. A. Bartz and J. W. Eckert

Former Research Fellow, Environmental Sciences Program, University of California, Riverside, now Assistant Professor of Plant Pathology, University of Florida, Gainesville 32601; and Professor of Plant Pathology, University of California, Riverside 92502, respectively. The authors gratefully acknowledge the assistance of Mrs. Soo Porter and M. L. Raum in the respiration experiments, and M. Matsumura who helped with K^+ analysis.

Supported in part by NIH Training Grant No. ES00084 from the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina.

Accepted for publication 8 September 1971.

ABSTRACT

Concentrations of the 2-aminobutane cation (2AB) that reduced the growth rate of *Penicillium digitatum* by 50% or more had no direct effect upon respiration or ability of treated cells to retain lower molecular weight metabolites. The level of K^+ in germinating conidia was reduced 50% by 1 mM 2AB, but even lower levels of K^+ were sufficient for growth in the absence of 2AB. 2-Aminobutane inhibited the transport of ^{14}C-labeled proline, lysine, glutamic acid, and leucine by 50-70% of the control value, and the incorporation of the label into protein to about the same extent. Uptake and incorporation of ^{33}P-phosphate into ribonucleic acid was also inhibited in the presence of 2AB. Cultures grown in an amino acid-free medium showed a greater increase in dry weight than 2AB-treated cultures growing in a complete medium. This indicated that inhibited transport of amino acids was not limiting the rate of growth.

Phytopathology 62:239-246.

Additional key words: RNA concentration, DNA concentration, amino acid pools.

2-Aminobutane (*sec*-butylamine, 2AB) is the only simple aliphatic amine of low molecular weight reported fungistatic in its cationic form. The antifungal spectrum of 2AB is relatively narrow, and no activity against bacteria has been noted (13). The major practical application of 2AB has been to control species of *Penicillium* on fruits after harvest (11, 13, 14).

An investigation of the relationships between chemical structure and fungistatic activity revealed that 2AB is a structurally specific inhibitor, and that most closely related amines are inactive (16). In fact, the (−) enantiomer of 2AB was found to be 60 times more active than the (+) enantiomer.

This investigation was undertaken to help elucidate the mechanism whereby a simple aliphatic amine, 2AB, could inhibit growth of certain fungi. An understanding of the biochemical processes involved could provide an insight into the unique facets of metabolism which render certain fungi vulnerable to inhibition by the cation of 2AB. Portions of these investigations have been reported (1).

MATERIALS AND METHODS.—To initiate germination, dry conidia of *Penicillium digitatum* Sacc. were suspended at 0.5 mg/ml in a medium of the following composition (per liter): 125 ml Valencia orange juice, 15 g sucrose, 1.25 g DL-asparagine, 1 g KH_2PO_4, and 0.15 g $MgSO_4 \cdot 7H_2O$ adjusted to pH 4.5 with KOH before autoclaving. Approximately 60% of the conidia had germ tubes after incubation in this medium at 27 C for 8 hr on a rotary shaker. After an appropriate period of incubation in the orange juice medium, conidia were transferred to simple salt solutions or to a synthetic medium before treatment with 2AB. The synthetic medium consisted, per liter, of 15 g sucrose, 0.25 g DL-asparagine, 1 g KH_2PO_4, 0.05 g KCl, 0.2 g $Ca(NO_3)_2$, 0.2 inositol, a trace of $Fe(NO_3)_3$, 0.2 ml of a vitamin solution (65 mg thiamin HCl, 0.5 mg biotin, 250 mg niacin, 150 mg Ca pantothenate, and 30 mg riboflavin in 50 ml 20% v/v acetone/water), and 0.2 ml of a trace element solution (100 mg $ZnSO_4 \cdot 7H_2O$, 70 mg $MnSO_4$, 80 mg $CaSO_4 \cdot 5H_2O$, 90 mg $CoCl_2 \cdot 6H_2O$, 35 mg MoO_3, 125 mg H_3BO_3 in 50 ml H_2O). The medium was adjusted to pH 4.5 and autoclaved, and the vitamins were added.

Standard manometric techniques using a Warburg apparatus were employed to study respiration. Neutral solutions of 2AB were added at various times after the conidia were suspended in the orange juice germination medium in Warburg flasks. Before being added to the Warburg flasks, the conidia were sometimes allowed to germinate for 8 hr, then incubated in a solution containing 0.1 g KH_2PO_4 and 0.03 g $MgSO_4 \cdot 7H_2O$/100 ml distilled water for 16 hr to reduce endogenous respiration. Exogenous respiration was measured after addition of 2AB and either glucose or citric acid (equivalent to 12 mg carbon/ml).

Absorption and retention of K^+ was investigated by transferring conidia from the orange juice medium after 4-hr incubation to the synthetic medium, with or without K^+, containing 2AB. After treatment with 2AB for 4 hr in the presence and absence of K^+, the conidia were washed by centrifugation and extracted with 70% (v/v) ethanol:water at 60 C for 30 min. The alcohol extract and rinses of additional 70% ethanol were combined and evaporated to dryness, and the residue was redissolved in distilled water. Potassium was determined with a Perkin-Elmer atomic absorption spectrophotometer.

The effect of 2AB on phosphorous metabolism was assessed by following the uptake of ^{33}P - $H_2PO_4^-$ and determining the distribution of ^{33}P in fungal tissue in the presence and absence of 2AB. Cells were also labeled before treatment with 2AB in order to

evaluate leakage of ^{33}P or transfer of ^{33}P to RNA during exposure to 2AB. The influence of 2AB on the uptake of $H_2PO_4^-$ and synthesis of nucleic acids was studied in a synthetic medium buffered with potassium citrate. Radioactive phosphorous was introduced as KH_2PO_4, 0.5 or 5 mM, with a specific activity of 2 μc/mM. Tissues were allowed to equilibrate in the synthetic medium for 1 hr before the 2AB and ^{33}P were added.

After introduction of 2AB, samples (2 or 3 ml) were withdrawn periodically from the culture and filtered through glass fiber filters (Whatman GF/A 2.4 cm). The fungal tissue in the filter was quickly rinsed with unlabeled phosphate and plunged into 10% trichloroacetic acid (TCA) at 0 C. Cell phosphorus was fractionated by the procedure of Kennell (20). Analysis for ribonucleic acid (RNA) (9) and deoxyribonucleic acid (DNA) (5) in the fractions revealed that these fractions were not significantly cross-contaminated. Leakage of lower molecular weight phosphorous compounds was evaluated by suspending young hyphae, which had been grown in a medium with ^{33}P-KH_2PO_4, in a medium with citrate buffer containing 2AB. Samples were withdrawn periodically and filtered. The level of radioactivity in the filtrates was determined.

The uptake and incorporation into protein of ^{14}C-L-glutamic acid, ^{14}C-L-leucine, ^{14}C-L-proline, and ^{14}C-L-lysine by 12-hr hyphae was determined in the presence of 2AB by three general procedures. 2-Aminobutane and the labeled amino acids were simultaneously added to 12-hr hyphae. The latter had been allowed to equilibrate for 1 hr in the synthetic medium. When ^{14}C-leucine and ^{14}C-lysine were used, the hyphae were suspended in the synthetic medium modified to contain 0.25 g asparagine and 0.3 g proline/liter. The ^{14}C-leucine or ^{14}C-lysine (used at 163 μc/mM and 167 μc/mM final specific activity, respectively) was dissolved in a solution 0.2 mM in nonradioactive leucine, lysine, and glutamic acid. Experiments with ^{14}C-proline were conducted in a similar fashion, except that the synthetic medium suspending the hyphae was modified to contain 0.5 g asparagine and 0.05 g proline. ^{14}C-proline (used as 2 μc/30 ml culture) was added to a solution containing 0.2 mM leucine, lysine, and glutamic acid. ^{14}C-glutamic acid (used at 1 or 2 mM and 25 μc/mM) was added alone to hyphae suspended in an amino acid-free synthetic medium.

In the second procedure, the 12-hr hyphae were fed ^{14}C-lysine for 2 hr, rinsed, and suspended in an amino acid-free medium. After 10-min equilibration, 2AB was added to the medium, and samples were removed after various intervals. The 12-hr hyphae were suspended in a synthetic medium modified to contain 1 g asparagine/liter. ^{14}C-lysine (167 μc/mM) was added to the culture in a 0.2-mM mixture of glutamic acid, leucine, proline, and lysine. In the third procedure, the ^{14}C-amino acids were "pulse" fed to 10-hr hyphae suspended in synthetic medium modified to contain 1.0 g asparagine/liter. At various intervals after addition of 2AB, portions of the culture were transferred for 5 min to Erlenmeyer flasks containing the labeled amino acids, then filtered. ^{14}C-leucine and ^{14}C-proline were fed in a 0.1-mM solution of leucine, proline, glutamic acid, and lysine. Final concentration of radioactivity during pulse feeding was 1 μc/20 ml. ^{14}C-lysine and ^{14}C-glutamic acid were fed similarly, but the concentration of the four amino acids was 0.2 mM, and the final radioactivity was 0.1 μc/20 ml in the pulsing medium.

Twelve-hr hyphae fed labeled amino acids were extracted with 5% TCA at 80 C for 30 min, rinsed twice with cold 10% TCA, and finally rinsed with cold 70% ethanol/H_2O (20). The extract and rinses were combined, and are hereafter referred to as the TCA-soluble fraction. The major portion of ^{14}C in this fraction is in the form of free amino acids ("amino acid pool"). The hyphae were further extracted with lipid solvents (20) and dried at 90 C for 1 hr. The extracted hyphae are hereafter referred to as the TCA-insoluble fraction, and the radioactivity in this fraction is a measure of the incorporation of ^{14}C-amino acids into protein.

Radioactivity in cell fractions was determined in a Beckman CPM liquid scintillation spectrometer. Radioactive carbon in aqueous extracts was counted in a mixture of toluene: Triton X-100 (2:1) containing 5 g 2,5-diphenyloxazole (PPO)/liter of toluene. Counting efficiency was determined by internal standardization and by channels ratio. Residual radioactivity in the hyphae (TCA-insoluble fraction) was determined by submerging the dried hyphal mat on the glass filter into scintillation solution comprised of 5 g PPO/liter toluene. Counting efficiency was determined by drying known quantities of ^{14}C-glutamic acid on hyphal mats, then counting in the same scintillation fluid. Radioactive phosphorus was counted in the same manner as the ^{14}C samples and reported as counts per minute (cpm).

^{33}P-KH_2PO_4 (50 mc/mM) was obtained from New England Nuclear Corp. The ^{14}C-amino acids obtained from Amersham/Searle Corp. were randomly labeled and had the following specific activities: L-leucine-^{14}C, 6.5 mc/mM; L-lysine-^{14}C, 12 mc/mM; L-proline-^{14}C, 7.5 mc/mM; and L-glutamic acid-^{14}C, 14.9 mc/mM. Solutions of 2 AB and *n*-butylamine were prepared from crystalline hydrochloride salts.

RESULTS.—*Effect of 2-aminobutane on growth.*—The ED_{50} for 2AB against germination of *P. digitatum* conidia (10^6/ml) was indicated to be about 0.3 μmoles/ml (0.3 mM) (12). The response was fungistatic rather than fungicidal. As 0.5-1.0 mg fungus tissue/ml of culture were required for biochemical experiments, an evaluation of the concentration of 2AB required to suppress growth of this tissue mass was investigated. Introduction of 1 mM 2AB into the orange juice medium immediately after the conidia (0.5 mg/ml) were suspended reduced germination and dry weight to 8% and 26%, respectively, of the control value after 24-hr incubation. When the addition of the same dosage was delayed 4 hr, the corresponding values were 25%

and 37%, and finally, when the 2AB was added at 8 hr (60% germination after 8-hr incubation), dry weight of the treated culture was 57% of the control at 24-hr total incubation.

Although conidia in various stages of germination were used in early studies on respiration and K^+ retention, hyphae from 12-hr cultures were used in all later experiments. These hyphal suspensions were quite uniform in consistency, and could be filtered with greater ease than the conidial suspensions. The effect of several concentrations of 2AB on the growth of 12-hr hyphae is shown in Fig. 1.

Respiration.—The RQ_{O_2} and RQ_{CO_2} of conidia in the orange juice medium were not affected by 1 mM 2AB applied at 0, 4, or 8 hr after suspension of the conidia in the medium.

Oxygen uptake of young hyphae starved for 16 hr before suspension in a medium containing glucose or citric acid (equivalent to 12 mg carbon/ml) was not affected over a 3-hr period by the presence of 20 mM 2AB. The starved tissue rapidly respired glucose, but O_2 uptake by hyphae on the citric acid medium was not significantly greater than controls respiring endogenously. Respiration of rapidly growing young (12 hr) hyphae was reduced significantly in the presence of 1 mM 2AB. The rate of respiration in these cells appears to be controlled by growth; therefore, all growth inhibitors tested, including 2AB, brought about a reduced rate of respiration.

Cell membrane permeability.—2-Aminobutane (1 mM), applied after conidia had been in the process of germination for 4 hr, slightly reduced the K^+ content of the conidia treated for 4 hr in medium containing 8 mM K^+. This slight reduction in tissue K^+, however, could not be responsible for inhibition of germination. Conidia cultured in the absence of 2AB and K^+ showed almost normal germination, but had only one-half the cellular K^+ found in conidia cultured in the presence of both 2AB and K^+ (Table 1, see treatments 2 and 3). Furthermore, 2AB did not cause measurable leakage of cellular K^+ in low K^+ cultures. Further evidence that 2AB did not cause gross changes in cell permeability was provided by the fact that hyphal tissue uniformly labeled with ^{33}P or with ^{14}C-lysine in the soluble pool did not leak radioactivity during subsequent incubation in medium containing either 1 or 5 mM 2AB.

Effect of 2AB on the uptake of ^{14}C-amino acids and their incorporation into the protein fraction.—2-Aminobutane at 1 mM inhibited the uptake and incorporation of the four ^{14}C-amino acids tested (Fig. 2). When 2AB and the ^{14}C-amino acids were added simultaneously, inhibition of transport of glutamic acid, leucine, and lysine into the TCA-soluble pool was evident after 30-40 min; transport of proline was inhibited after 60 min. Inhibition in the rate of incorporation of ^{14}C-glutamic acid, ^{14}C-lysine, and ^{14}C-leucine into the protein fraction over the 60- to 80-min interval after the treatment was initiated was 56, 50, and 50%, respectively. The rate of incorporation of proline over the same time period was inhibited 70%. The ^{14}C in the TCA-soluble fraction resulting from the uptake of different amino acids cannot be compared because (i) glutamic acid and proline were metabolized, and (ii) for different amino acids there appeared to be different rates of transfer from the amino acid pool to protein. Nevertheless, the rate of transport of all the ^{14}C-amino acids into the cell is substantially inhibited by the presence of 1 mM 2AB.

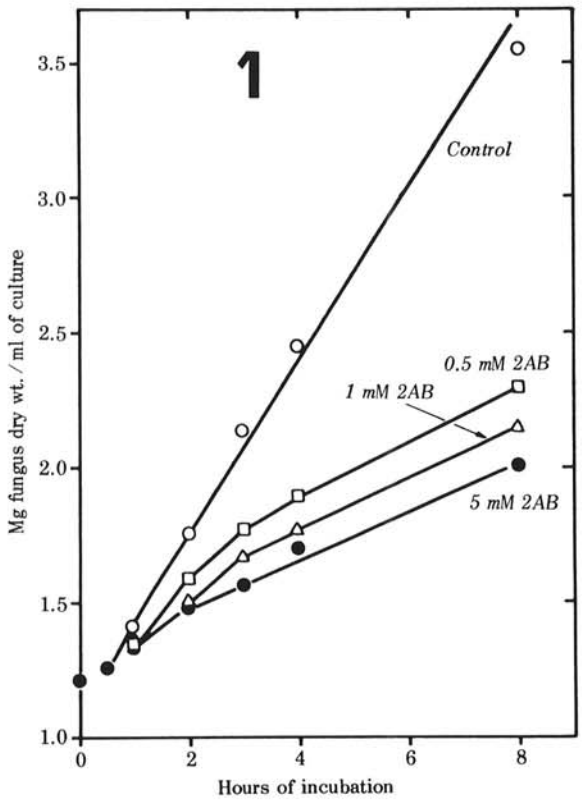
Experiments were conducted to determine whether 2AB was acting as a competitive inhibitor for one or more amino acid transport systems, or had to accumulate at some site of action before inhibition began. In those experiments, the hyphae were exposed to 2AB prior to the addition of a ^{14}C-amino acid. The results of an experiment in which 12-hr hyphae were exposed to 1 mM 2AB for 2 hr prior to ^{14}C-glutamic acid are illustrated in Fig. 3. The inhibition of transport of ^{14}C-glutamic acid into the amino acid pool was evident 30 sec after addition; the inhibition in the incorporation into protein was 10-15 min earlier than when the ^{14}C-amino acids were added simultaneously with the 2AB. Furthermore, the rate of incorporation of ^{14}C into the protein fraction over the interval 30-60 min after introduction of the label was inhibited 83%, as compared to 56% when both 2AB and ^{14}C-amino acid were added simultaneously. The inhibitory effect of 2AB on the rate of transport and incorporation of amino acids into protein increased during exposure to 2AB prior to pulse feeding of ^{14}C-amino acids (Table 2). These results indicate that the mechanism of action of 2AB is not exclusively due to competitive inhibition of an amino acid transport system. The delay in onset of inhibition of amino acid uptake suggests either that the site of action is not freely

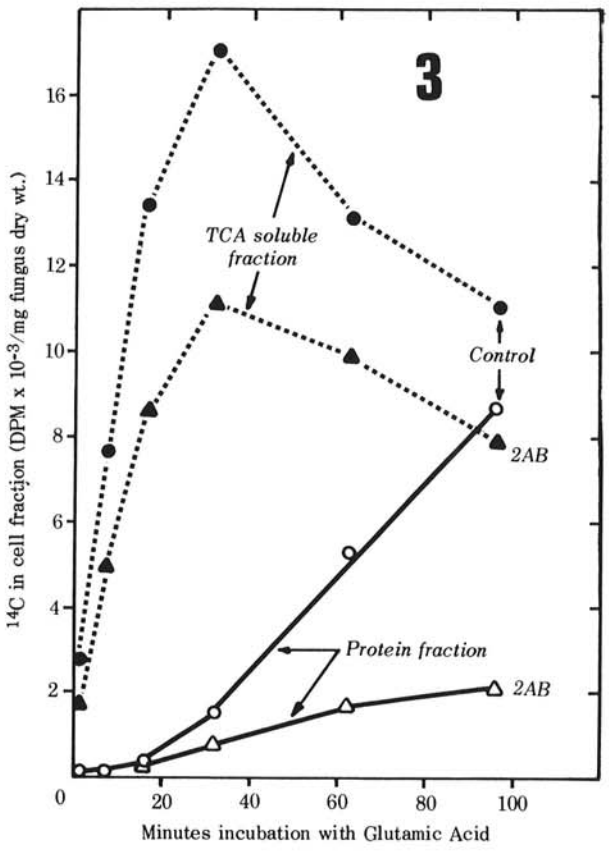
TABLE 1. Potassium content of conidia of *Penicillium digitatum* germinating in the presence of 1 mM 2-aminobutane (2AB)

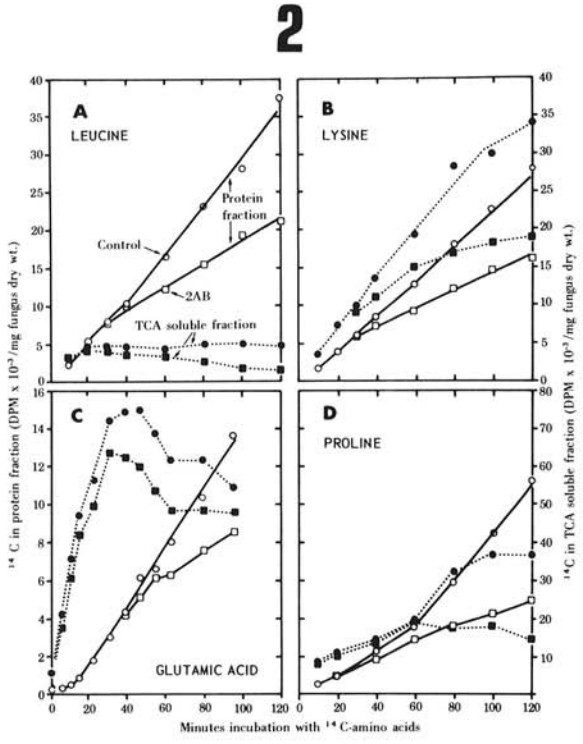
Treatment[a]	K^+ in synthetic medium	% Germination[b]	μgK^+/mg fungus dry wt
1) 1mM NaCl	8 mM	58	36
2) 1mM NaCl		49	12
3) 1 mM 2AB	8 mM	2	27
4) 1 mM 2AB		2	13

[a] Conidia incubated 4 hr in germination medium before transfer to synthetic medium containing 2AB or equivalent concentrations of NaCl.

[b] Determined after 4 hr on medium containing 2AB or NaCl. Average of two replications which were repeated twice.


1
Control
0.5 mM 2AB
1 mM 2AB
5 mM 2AB
Mg fungus dry wt. / ml of culture
Hours of incubation


3
TCA soluble fraction
Control
2AB
Protein fraction
2AB
14C in cell fraction (DPM x 10-3/mg fungus dry wt.)
Minutes incubation with Glutamic Acid


2
A
LEUCINE
Protein fraction
Control
2AB
TCA soluble fraction
B
LYSINE
C
GLUTAMIC ACID
D
PROLINE
14 C in protein fraction (DPM x 10-3/mg fungus dry wt.)
14C in TCA soluble fraction (DPM x 10-3/mg fungus dry wt.)
Minutes incubation with 14 C-amino acids

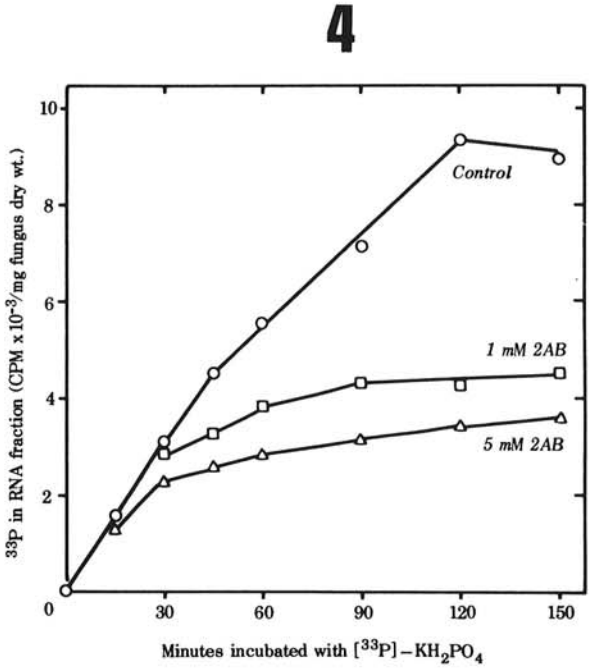

4
Control
1 mM 2AB
5 mM 2AB
33P in RNA fraction (CPM x10-3/mg fungus dry wt.)
Minutes incubated with [33P]-KH2PO4

TABLE 2. Absorption of amino acids over a 5-min interval by hyphae of *Penicillium digitatum* which were being exposed for varying lengths of time to 2-aminobutane (2AB)[a]

	1 mM 2AB				5 mM 2AB			
	^{14}C-leucine[b,c]		^{14}C-proline		^{14}C-leucine		^{14}C-proline	
Min exposure to 2AB	Pool	Protein	Pool	Protein	Pool	Protein	Pool	Protein
5	89	88	147	158	79	74	100	153
10	103	101	118	148	93	82	105	144
30	67	85	90	71	66	79	66	50
45	80	80	95	74	57	65	100	73
60	76	83	62	50	63	66	48	37
120	30	37	81	49	28	34	79	47
180	26	42	91	51	20	35	81	46
240	32	48	84	61	26	42	62	54

[a] Ten-hr hyphae were treated with 2AB for indicated time periods, and "pulse fed" the ^{14}C-amino acids for 5 min.
[b] ^{14}C in hyphal samples was fractionated into 5% TCA-soluble fraction ("amino acid pool") and 5% trichloroacetic acid (TCA)-insoluble ("protein fraction").
[c] Relative rate of appearance of ^{14}C in indicated fraction, dpm/mg dry wt treated ÷ dpm/mg dry wt control X 100.

accessible or that the lesion requires time to develop under the influence of 2AB.

Growth inhibition in nitrogen-free medium.—Growth utilizing endogenous nitrogen sources should not be inhibited by 2AB if the primary site of action involves an interference of transport of amino acids into the cell. This hypothesis was tested by comparing the growth inhibition brought about by 1 mM 2AB in a medium with or without glutamic acid. The standard synthetic medium modified to contain only trace amounts of N as $Ca(NO_3)_2$ was used. 2-Aminobutane inhibited weight gain of the cultures with or without glutamic acid over a 10-hr period by 47% and 42%, respectively. The minus amino acid culture without 2AB was inhibited only 27% (due to N deficiency), indicating that the prevention of uptake of amino acids could not be the primary site of action of 2AB.

Synthesis of protein from endogenous ^{14}C-lysine.—The observation that hyphae of *P. digitatum* developed a comparatively large pool of ^{14}C-lysine (see Fig. 2-B) suggested a means for measuring protein synthesis free from the complication of transport inhibition The latter is inherent in all experiments in which the ^{14}C-amino acid is supplied to the medium. Hyphal cultures were supplied ^{14}C-lysine for 2 hr, rinsed, and placed in reduced nitrogen-free medium containing 1 mM 2AB. Samples were withdrawn periodically, and radioactivity that remained in the soluble pool and incorporated into protein was measured. Ancillary experiments showed that the level of ^{14}C in the hyphae was constant over the period of the experiment. Measurement of loss of ^{14}C from the soluble pool or gain of ^{14}C in the protein fraction could, therefore, be used as a measure of protein synthesis from endogenous precursors (8). Depletion of pool lysine was followed because preliminary experiments revealed that the protein fraction was heavily labeled at the end of the period of exposure to ^{14}C-lysine. After 90 min in the absence of an exogenous N source, ca. 38% of the initial ^{14}C was transferred from the soluble pool of the control culture; whereas identical cultures treated with 5 mM 2AB lost only 15% of the radioactivity from the pool. These data suggested that 5 mM 2AB reduced protein synthesis from endogenous precursors to about 50% of the control value, and supported the conclusion that prevention of uptake of amino acids was not the primary site of action of 2AB.

Comparison of inhibition of 2-aminobutane and n-butylamine.—The effect of *n*-butylamine on growth and protein synthesis was determined in anticipation that this "nonfungistatic" amine (12) also might not inhibit incorporation of exogenous ^{14}C-leucine into protein, thereby indicating a unique physiological effect for 2AB. This, however, did not prove to be the case. The inhibition of dry weight increase over a 7-hr period for hyphae in cultures containing 5 mM and 20 mM *n*-butylamine, and those containing 5 mM 2AB, were 20%, 24%, and 66%, respectively. Comparable values for inhibition of incorporation of

Fig. 1-4. 1) Dry weight increase of 12-hr cultures of *Pencillium digitatum* in the presence of different concentrations of 2-aminobutane (2AB). **2)** Uptake of ^{14}C-amino acids into the amino acid pool (- - -) and their incorporation into the protein fraction (——) of 12-hr hyphae of *P. digitatum* exposed to 1 mM 2AB (□, ■); (A) leucine; (B) lysine; (C) glutamic acid; and (D) proline. The controls (○, ●) contained 1 mM NaCl. **3)** Uptake of ^{14}C-glutamic acid into the trichloroacetic acid (TCA)-soluble pool (- - -) and its incorporation into the protein fraction (——) of 12-hr hyphae of *P. digitatum* exposed to 2AB for 2 hr before the glutamic acid was added (□, ■). 1 mM NaCl was present in the control (○, ●). **4)** Incorporation of ^{33}P from ^{33}P-KH_2PO_4 into the RNA fraction of 12-hr hyphae of *P. digitatum* treated with 1 or 5 mM 2AB. 2-Aminobutane was added simultaneously with the ^{33}P. The control culture contained 5 mM NaCl.

leucine into the protein fraction over a 90-min period were 22%, 31%, and 49%, respectively. The correlation between inhibition of growth and inhibition of protein synthesis by 2AB and *n*-butylamine do not seem to be a cause and effect relationship because the incorporation rates are expressed on a dry weight basis. It is more likely that both of these aliphatic amines affect the same site to different degrees of intensity dependent upon their steric "fit" (16).

Effect on nucleic acid metabolism.—Twelve-hr hyphae were incubated for 4 hr in a synthetic medium containing 1 or 5 mM 2AB. Samples were removed periodically and fractionated by the procedure of Kennell (20), and RNA and DNA determined by the orcinol and indole methods, respectively. 2-Aminobutane had no quantitative effect upon either fraction (expressed on a dry wt basis) over a period of 4 hr during which growth was strongly inhibited.

Possible effects of 2AB on RNA metabolism were further evaluated by incubating hyphae with ^{33}P-KH_2PO_4 and determining radioactivity incorporated into the RNA fraction. Exposure of 12-hr hyphae to 1 or 5 mM 2AB and ^{33}P-$H_2PO_4^-$, simultaneously, resulted in a substantial reduction in the rate of incorporation of ^{33}P into the RNA fraction about 15 min after initiation of the treatment (Fig. 4). A similar depression in the rate of appearance of ^{33}P in RNA was observed when the hyphae were incubated in 2AB for 2 hr before introduction of ^{33}P-KH_2PO_4 into the culture. In the foregoing experiments, the level of ^{33}P in the low-molecular-weight phosphorous fraction (extracted with cold 10% TCA) was lower in the treated than in the control tissue when 2AB was applied simultaneously with ^{33}P, but not significantly different when the 2AB was applied 2 hr prior to the ^{33}P. To determine if the total amount of ^{33}P-RNA-phosphate present in the tissue was altered by 2AB, the tissue was uniformly labeled with ^{33}P prior to the addition of 2AB. Conidia were germinated in the orange juice germination medium with ^{33}P added to give the same specific activity as would be present subsequently in the synthetic citrate medium in which 2AB was introduced. The uniformly labeled hyphae were removed after 12-hr growth on the ^{33}P-containing germination medium, rinsed, and resuspended in a citrate buffered medium. The tissue was allowed to equilibrate for 1 hr, then 2AB and ^{33}P-$H_2PO_4^-$ were added simultaneously. Samples were withdrawn periodically for 2 hr and ^{33}P-RNA was determined. No obvious difference in the increase of ^{33}P-RNA was observed between 2AB treated and control cultures, although the large amount of ^{33}P-RNA present in the tissue at the time 2AB was applied reduced the sensitivity of measuring synthesis of RNA over the subsequent 2-hr period.

In a final experiment, hyphae were incubated in phosphorous-deficient medium for 9 hr to increase subsequent uptake of ^{33}P-$H_2PO_4^-$ in the presence of 2AB. 2-Aminobutane at 1 mM had no effect upon either ^{33}P uptake or incorporation into RNA by low-phosphorous hyphae.

DISCUSSION.—Tritium-labeled (-) 2AB is absorbed and concentrated, but not metabolized by hyphal cells of *P. digitatum* prior to the onset of inhibition (15). The present studies have demonstrated that the 2AB cation, unlike basic fungicides with long chain aliphatic components (4, 29), has no effect upon the ability of *P. digitatum* cells to retain small molecules or ions such as ^{14}C-lycine, K^+, or soluble ^{33}P compounds. The relative permeability of the cells to exogenous glucose and citrate was not altered as in the case of polyene antibiotics, which are known to alter the cytoplasmic membrane of sensitive fungi (3, 21). 2-Aminobutane at 20 mM (at least 20 times the ED_{50}) had no effect upon endogenous respiration or on O_2 uptake by starved cells fed glucose. 2-Aminobutane does not appear to have a direct effect upon the respiratory pathways of the cell.

The 2AB cation reduced the level of K^+ in germinating conidia to ca. 75% of that of controls. This phenomenon probably is a weak cation antagonism of the K^+ transport carrier as reported for the effect of other amines on K^+ concentration in yeast (6). The reduced K^+ level of 2AB-treated cells does not appear to have a significant effect on fungal growth, however, as conidia germinating on low K^+ medium germinated normally despite having a lower K^+ content than did the conidia inhibited by 2AB.

2-Aminobutane at 1 mM, which inhibited growth (dry weight increase) in 4 hr by ca. 50%, strongly inhibited the uptake of the four ^{14}C-labeled amino acids tested; it also inhibited incorporation of their label into the protein fraction of the cell. Both inhibitory effects were apparent within 1-2 hr after exposure to 2AB, and were quantitatively similar.

Strong inhibition of transport of glutamic acid, leucine, and proline by 2AB is somewhat surprising, as previous reports have shown that amino acid uptake is not greatly inhibited by aliphatic amines, in particular those derived by decarboxylation of an amino acid (17, 19, 27, 33). Interference of 2AB with the metabolism of lysine is comprehensible, as the basic amino acids, lysine and arginine, are known to inhibit growth of mutants of *Neurospora crassa* which require an exogenous source of histidine, lysine, or arginine (10, 24). The mechanism of this inhibition is due to inhibition of transport of the amino acid required for growth. Mandelstam (22, 23) reported that 1,4-diaminobutane and certain other homologous diamines competitively inhibit transport of lysine, arginine, and/or ornithine, but not glutamic acid, into several species of bacteria. The diamines inhibited growth of bacterial strains which were auxotropic for lysine, but had no effect upon growth of the wild type.

The observation that the fungistatic effect of 2AB is antagonized by certain amino acids (J. W. Eckert, *unpublished data*) suggests the possibility that 2AB may bind with amino acid-transporting sites on the cell membrane. This is not necessarily the case, however, as amino acid transport in fungi is inhibited by polyene antibiotics (30), certain fungicides (19), and general cell poisons (2).

The relationship of inhibition of amino acid transport by 2AB to the primary cause of growth inhibition is questionable. First, hyphal growth in 10 hr was shown to be less in an adequate nitrogen supply with 1 mM 2AB than in a control culture without nitrogen or 2AB. Obviously, the primary mechanism of 2AB action cannot be inhibition of amino acid transport into the cell, although the nitrogen pool in the cell would eventually become depleted unless a reduction in protein synthesis offset the inhibited transport. The second line of evidence discrediting inhibition of amino acid transport as the primary mechanism of 2AB action is that hyphae incubated for 8 hr in a medium containing aspartic acid and 2AB had a pool of TCA-soluble amino acids which appeared to be quantitatively and qualitatively similar to control hyphae (J. A. Bartz, *unpublished data*). Some differences in ratios of amino acids were noted, but the significance of these findings is not yet clear.

The observed reduction in incorporation of amino acids into the protein fraction in the presence of 2AB could be a consequence of inhibition of amino acid transport into the pool that furnishes amino acids for protein synthesis (7, 33). Sterling & Henderson (32) proposed this mechanism to explain an apparent inhibition of protein synthesis in ascites tumor cells by the nonmetabolizable amino acid, 1-aminocyclopentane-1-carboxylic acid. The converse interrelationship, i.e., that inhibition of protein synthesis results in feedback control over the amino acid transport system, is very unlikely as it has been demonstrated that cycloheximide completely inhibits protein synthesis in *P. chrysogenum* but has no effect upon transport of methionine into this fungus (2). Jones (19) reported that the chloramphenicol, another inhibitor of protein synthesis, had no effect upon transport of amino acids in *Botrytis*.

The apparent coincidence of inhibition of transport and protein synthesis following exposure of hyphae to 2AB suggests that the latter phenomenon might be an artifact. However, the 40- to 60-min delay in the onset of transport inhibition after application of 2AB (Table 2) indicates that the inhibition phenomenon involves more than competition for a transport site on the outer surface of the cytoplasmic membrane. The observed decrease in rate of transfer of ^{14}C-lysine from the endogenous pool during protein synthesis also indicates a site of 2AB action in the cytoplasm. The correlation between growth inhibition and incorporation of ^{14}C-leucine into protein produced by 2AB and by *n*-butylamine implies a common site of action of all amine cations with intensity of inhibition governed by steric properties of the molecule (16).

In addition to competition for permeases (22, 23), at least two mechanisms are documented wherein simple amine cations interfere with enzymes involved in nitrogen metabolism. Metzler (26) demonstrated that 2AB complexed more strongly with pyridoxal under physiological conditions than did 12 amino acids tested, although not so strongly as did *n*-butylamine or *t*-butylamine. Matsuo (25) correlated stability constants of amino acids and amines with pyridoxal and inhibition of homoserine deaminase which utilizes pyridoxal as a prosthetic group. Tris buffer [tris (hydroxymethyl) amino methane] formed Schiff bases with more stability than did some of the amino acids. Pyridoxal phosphate did not activate homoserine deaminase when added to that enzyme in Tris buffer. Inagami (18) investigated the inhibition of trypsin by cations of alkylamines, and showed that the *n*-butylammonium ion was most active because of its similarity to the distal amino group of lysine which is the site of trypsin attack on proteins.

The inhibition of ^{33}P incorporation into RNA by 2AB conceivably could be due to a reduction in permeability of the cell to PO_4, but it is difficult to envision a mechanism for such an interaction and how it would be counteracted by phosphate starvation. One possibility is that a PO_4-permease which is not sensitive to 2AB develops under phosphorous starvation much as a methionine-specific transport develops during sulfur starvation of *P. chrysogenum* (2), or as a nonspecific amino acid transport system develops in carbon-starved mycelium of *N. crassa* (27). It is more likely that RNA synthesis is inhibited because of abnormalities in intermediary amino acid metabolism. In auxotrophic bacteria, deficiency of one amino acid repressed the rate of RNA synthesis (28, 31). This theory is further supported by the observed changes in the ratios of specific amino acids within the soluble amino acid pool of 2AB-treated hyphae (J. A. Bartz, *unpublished data*). The effect of 2AB upon growth could then be due to an indirect inhibition of protein synthesis which was caused by deficiency of a particular amino acid or acids within the amino acid pool of the treated fungus. A detailed investigation of the effect of 2AB on nitrogen and phosphorous metabolism by *P. digitatum* is under way.

LITERATURE CITED

1. BARTZ, J. A., & J. W. ECKERT. 1969. Studies on the fungistatic action of 2-aminobutane against Penicillium digitatum. Phytopathology 59:1017 (Abstr.).
2. BENKO, P. V., TRUDY C. WOOD, & I. H. SEGEL. 1967. Specificity and regulation of methionine transport in filamentous fungi. Arch. Biochem. Biophys. 122:783-804.
3. BRADLEY, S. G., P. J. FARBER, & L. A. JONES. 1961. Action of polyene antibiotics of Candida, p. 558-569. Antimicrobial Agents Ann. 1960. Plenum Press, New York.
4. BROWN, I. F., & H. D. SISLER. 1960. Mechanisms of fungitoxic action of *n*-dodecylguanidine acetate. Phytopathology 50:830-839.
5. CERIOTTI, G. 1952. A microchemical determination of desoxyribonucleic acid. J. Biol. Chem. 198:297-303.
6. CONWAY, E. J., & F. DUGGAN. 1958. A cation carrier in the yeast cell wall. Biochem. J. 69:265-274.
7. COWIE, D. B., & F. F. MC CLURE. 1959. Metabolic pools and the synthesis of macromolecules. Biochim. Biophys. Acta 31:236-245.
8. COWIE, D. B., & BARBARA P. WALTON. 1956.

Kinetics of formation and utilization of metabolic pools in the biosynthesis of protein and nucleic acid. Biochim. Biophys. Acta 21:211-226.
9. DISCHE, Z., & ELLEN BORENFREUND. 1957. A new color reaction for the determination of aldopentose in presence of other saccharides. Biochim. Biophys. Acta 23:639-642.
10. DOERMAN, A. H. 1944. A lysineless mutant of Neurospora and its inhibition by arginine. Arch. Biochem. 5:373-384.
11. ECKERT, J. W. 1969. Chemical treatments for control of postharvest diseases. World Rev. Pest Control 8:116-137.
12. ECKERT, J. W., & M. J. KOLBEZEN. 1963. Control of Penicillium decay of oranges with certain volatile aliphatic amines. Phytopathology 53:1053-1059.
13. ECKERT, J. W., & M. J. KOLBEZEN. 1964. 2-Aminobutane salts for control of postharvest decay of citrus, apple, pear, peach and banana fruits. Phytopathology 54:978-986.
14. ECKERT, J. W., & M. J. KOLBEZEN. 1970. Fumigation of fruits with 2-aminobutane for control of certain postharvest diseases. Phytopathology 60:545-550.
15. ECKERT, J. W., M. L. RAHM, & BIRGITTE BRETSCHNEIDER. 1970. Accumulation and metabolism of aliphatic amines by hyphae of Penicillium digitatum in relation to growth inhibition. Phytopathology 60:1290 (Abstr.).
16. ECKERT, J. W., M. L. RAHM, & M. J. KOLBEZEN. 1972. Fungistatic activity of cations of non-aromatic amines. J. Agr. Food Chem. (in press).
17. HALVORSON, H. O., & G. N. COHEN. 1958. Incorporation des amino-acides endogènes et exogènes dans les proteines de la levure. Ann. Inst. Pasteur 95:73-87.
18. INAGAMI, T. 1964. The mechanism of specificity of trypsin catalysis. I. Inhibition by alkylammonium ions. J. Biol. Chem. 239:787-791.
19. JONES, O. T. G. 1963. The accumulation of amino acids by fungi, with particular reference to the plant parasitic fungus, Botrytis fabae. J. Exp. Bot. 14:399-411.
20. KENNELL, D. 1967. Use of filters to separate radioactivity in RNA, DNA, and protein, p. 686-693. *In* L. Grossman & K. Moldave [ed.]. Methods in enzymology, Vol. XII, Part A. Academic Press Inc., New York.
21. KOTTKE, MARGARET, & H. D. SISLER. 1962. Effect of fungicides on permeability of yeast cells to the pyruvate ion. Phytopathology 52:959-961.
22. MANDELSTAM, J. 1956. Factors affecting the passage of basic amino acids into coliform bacteria. Biochim. Biophys. Acta 22:313-323.
23. MANDELSTAM, J. 1956. Inhibition of bacterial growth by selective interference with the passage of basic amino acids into the cell. Biochim. Biophys. Acta 22:324-328.
24. MATHIESON, M. J., & D. C. CATCHESIDE. 1955. Inhibition of histidine uptake in Neurospora crassa. J. Gen. Microbiol. 13:72-83.
25. MATSUO, Y. 1956. Formation of Schiff bases of pyridoxal phosphate. Reaction with metal ions. J. Amer. Chem. Soc. 79:2011-2015.
26. METZLER, D. E. 1957. Equilibria between pyridoxal and amino acids and their amines. J. Amer. Chem. Soc. 79:485-490.
27. PALL, M. L. 1968. Amino acid transport in Neurospora crassa. Biochim. Biophys. Acta 173:113-127.
28. PARDEE, A. B., & LOUISE B. PRESTIDGE. 1955. The dependence of nucleic acid synthesis on the presence of amino acids in Escherichia coli. J. Bacteriol. 71:677-683.
29. SOMERS, E. 1963. The uptake of dodine acetate by Neurospora crassa. Mededel. Landbouwhogeschool Opzoekingssta. Gent. 28:580-589.
30. STACHIEWICZ, F., & J. H. QUASTEL. 1963. Amino acid transport in yeast and the effects of nystatin. Can. J. Biochem. Physiol. 41:397-407.
31. STENT, G. S., & S. BRENNER. 1961. A genetic locus for the regulation of ribonucleic acid synthesis. Nat. Acad. Sci. Proc. 47:2005-2014.
32. STERLING, W. R., & J. F. HENDERSON. 1963. Studies on the mechanism of action of 1-aminocyclopentane-1-carboxylic acid. Biochem. Pharm. 12:303-316.
33. WILEY, W. R., & W. H. MATCHETT. 1966. Tryptophane transport in Neurospora crassa. I. Specificity and kinetics. J. Bacteriol. 92:1698-1705.