Effects of the Mycotoxin Penicillic Acid on Electrogenic Sodium Transport and Associated Adenosine Triphosphatase

by

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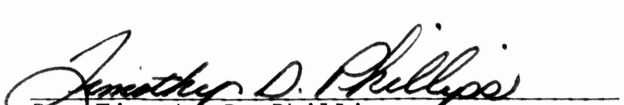

Dr. Timothy D. Phillips

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ABSTRACT

The effects of the mycotoxin penicillic acid on short circuited intact toad bladder and on Na^+-K^+, activated ATPase were examined to determine the relationship between toxin, the Na^+-K^+ ATPase enzyme system and associated active sodium transport. Penicillic acid inhibited transbladder short circuit current and Na^+-K^+ ATPase from isolated bladder preparations. The effect was time and dose dependent. The results are compatible with the assumption that Na^+-K^+ ATPase is associated with the pump mechanism since penicillic acid inhibited enzyme activity and reduced the rate of electrogenic Na^+ transport.

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DEDICATION

To Bif Marie Wilczynski.

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INTRODUCTION

Mycotoxins are toxic mold metabolites that can occur as contaminants of foodstuffs and feeds. Penicillic acid (PA), a tautomeric mycotoxin ($\Delta\alpha\beta$-γ-hydroxylactone or open ring substituted γ-keto hexenoic acid) was first isolated from corn contaminated with the mold Penicillium puberulum (1). The two forms of penicillic acid are shown in Figure 1. Penicillic acid is produced as a metabolite of a variety of Penicillium and Aspergillus species. PA possesses both antimicrobial and anti-tumor properties (2,3) but has been proven too toxic for therapeutic use. The toxin exhibits a digitalis-like effect when injected in rats and a cytotoxic effect in cultured cells from a variety of organs (4). It has also been shown to be a potent and specific inhibitor of Na^+-K^+ adenosinetriphosphatase (ATPase) in brain in vitro and in vivo (5,6). The toxin is selective in that mitochondrial Mg^{2+} ATPase activity is not affected at concentrations which significantly inhibit Na^+-K^+ ATPase.

It is theorized that the varied toxicity resulting from penicillic acid may be elicited either directly or indirectly through toxin interaction at membrane, intracellular, and/or subcellular active transport sites. The mode of action of penicillic acid may be linked to the reaction it exhibits with sulfhydryl groups of glutathione and cysteine and the amine groups of lysine, histidine, and arginine. Because of its simplicity of structure and its capacity to transport sodium electrogenically, the toad urinary bladder is a convenient preparation to study epithelial transport. Also Na^+-K^+ ATPase can be isolated from NaI-treated bladder preparations. Since inhibition of

ATPase may disrupt active membrane transport processes and alter a variety of physiological functions, the effects of penicillic acid on the Na^+-K^+ ATPase system and on active sodium transport by the toad bladder were studied to examine the relationship between penicillic acid, Na^+ transport and the transport enzyme.

METHODS

Penicillic acid was obtained from Makor Chemicals, Ltd., Jerusalem, Israel. Purity (99.8%) was confirmed by melting point, thin layer chromatography and infrared and mass spectra. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri). Dominican toads (Bufo marinus) were purchased from Bill Chase Co. Toads were housed in an aquarium with water ad libitum. Animals were fed fresh ground beef weekly.

Short-Circuit Current Analysis

A modification of the standard short-circuit current (SCC) apparatus (7) was used to measure electrogenic Na^+ transport across the toad hemibladder using a method previously described for frog skin. Toads were pithed and the bilobed bladder removed. The exposed bladder was placed between half cell anodic and cathodic chambers.

The exposed serosal and mucosal surfaces of urinary bladders were bathed with oxygenated Locke's solution in both chambers. The area of the exposed bladder was 1.13 cm^2.

A short circuit current of 1 μAmp was calculated to be equivalent to a Na^+ transport rate of 0.0330 μmol $Na^+/h/cm^2$. Active transport of

Na^+ across the bladder generates a potential difference (PD) between serosal and mucosal sides. Experiments were not initiated until stable, reproducible potentials were achieved. Bladders were in the open-circuited state except for intervals when short-circuit current (SCC) was measured.

Changes in the rate of Na^+ transport were determined from the post treatment peak rate and were expressed as a percentage of this rate. PA was added to either the mucosal or serosal bathing solutions and the time course of inhibition of SCC and PD was followed for at least 30 minutes.

Preparation of ATPase from Toad Bladder

A fraction high in Na^+-K^+ activated ATPase was isolated from the toad bladder by a modification of the procedure of Cortas and Walser (9). Toads were pithed and the bilobed bladder surgically removed, emptied and placed in ice-cold 1 M NaI/Tris buffer (pH 7.3) for 10 minutes to loosen the mucosal layer (9,10). Bladders were then washed 2 times in 15 mM tris buffer containing 5 mM EDTA (pH 7.0) and 3 times in 50 mM tris buffer (pH 7.0). Tissues were homogenized gently in a 0.32 M sucrose solution containing .1M imidazole in a ground glass homogenizer. Aliquots of the resulting homogenates were immediately analyzed for ATPase activity.

ATPase Analysis

ATPase activity was measured using endpoint phosphate analysis as shown in Fig. 2 (11,12,13) and continuous monitoring of NADH depletion as illustrated in Fig. 3 (14). In each of the two assays, treated and

control preparations of the enzyme were preincubated simultaneously at 37°C with PA for 15 minutes. For the endpoint assay, a 1 ml reaction mixture contained in final concentration: 5.0 mM ATP (vanadium free), 5.0 mM Mg^{2+}, 100 mM Na^{+}, 20 mM K^{+}, 135 mM imidazole HCl buffer (pH 7.5) and enzyme protein. Total ATPase activity was measured with Na^{+}, K^{+} and Mg^{2+} present in the reaction mixture. Mg^{2+} ATPase activity was measured by omitting both Na^{+} and K^{+}; thus the Na^{+}-K^{+} activated component was obtained by the difference between total ATPase and Mg^{2+} ATPase activity. The incubation reaction was initiated with ATP and stopped after 10 minutes by the addition of trichloroacetic acid at a final concentration of 5% (w/v). The coupled continuous ATPase assay contained the same molarities of Mg^{++}, Na^{+}, and K^{+} as in the endpoint method. Total ATPase activity was measured with Na^{+}, K^{+} and Mg^{++} present in the reaction mixture. Mg^{++} ATPase activity was measured by adding ouabain (1 mM) to inhibit Na^{+}-K^{+} ATPase; thus the Na^{+}-K^{+} activated component was obtained by the difference between total ATPase and Mg^{++} ATPase activity. The change in NADH content was measured every 60 seconds for 20-30 minutes on a Cary 219 recording uv-visible spectrophotometer. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard.

RESULTS

Figure 4 shows a representative experimental trial using a high concentration of PA (10^{-2}M). When a bladder is first removed, it has undergone quite a bit of trauma and at this point the potential is

quite low. It is allowed to stabilize in an oxygenated Lock's solution for 20 to 30 minutes. Toxin is then added and its effect is monitored. In this trial we see a slight stimulatory response and then a marked dropoff in SCC. Sodium transport was significantly inhibited by PA in a concentration-dependent manner when applied to the mucosal side of the bladder (Fig. 5). Inhibition of SCC was time dependent. Thirty minutes after the addition of toxin, 70% inhibition was observed. Complete inhibition of the SCC was not obtained. Serosal addition, in contrast, had no measurable effect.

Bladder ATPase preparations yielded various levels of Na^+-K^+ sensitive activity ranging from 1%-10%. The protein concentration was tested using the Lowry method and averaged between 80 and 85 μg protein per 100 μl preparation. Of the tested concentrations, Na^+-K^+ activated ATPase activity from bladder preparation was significantly inhibited by only high concentrations of PA (Table 1). PA did not significantly affect Mg^{++} ATPase activities at the same high concentrations.

DISCUSSION

Penicillic acid is toxic to the toad urinary bladder sodium transport system in a time and dose responsive manner as demonstrated by the inhibition of transbladder SCC. It is generally recognized that the Na^+-K^+ ATPase system is intimately related to the Na^+ pump at the serosal side of the bladder (16). It is interesting to note that penicillic acid's effects were one-sided (mucosa only). A number of chemicals exert their effects at either the serosal or mucosal surfaces

while others affect transport equally on both sides of the bladder. The finding that penicillic acid exerted its effect mainly on the mucosal side may indicate that the serosal surface presents accessible sites for non-specific binding or restricts toxic permeation.

Because higher concentrations of penicillic acid were required to significantly inhibit the Na^+-K^+ ATPase than were required for significant inhibition of SCC, an availability of membrane nonspecific binding sites in the homogenate preparation vs. the intact bladder or a reduced number of non-specific sites may be indicated.

Our results suggest that PA directly inhibits the sodium pump in toad bladder, possibly via critical sulfhydryl moieties of the Na^+-K^+ ATPase. This inhibition could be a common mechanism in many types of cells and might thus play a significant role in PA toxicity since inhibition of ion transport across bioelectrically active membranes may disrupt a variety of physiological functions.

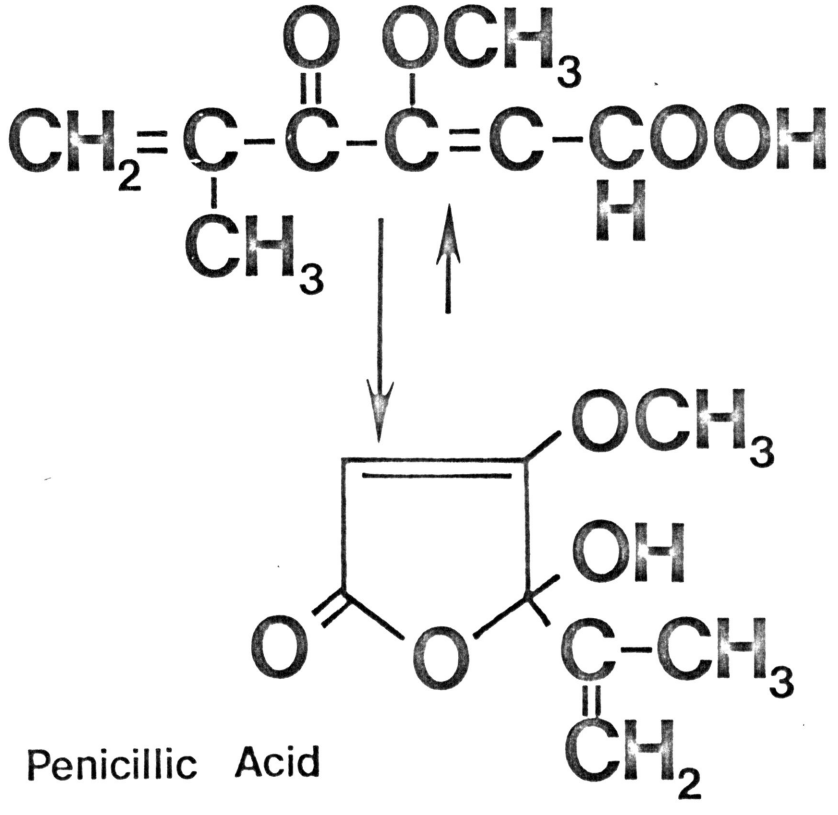


Fig. 1. Tautomeric structures of penicillic acid.

DIAGRAMATIC ILLUSTRATION OF CHEMICAL ENDPOINT
PHOSPHATE ANALYSIS OF ATPase ACTIVITY

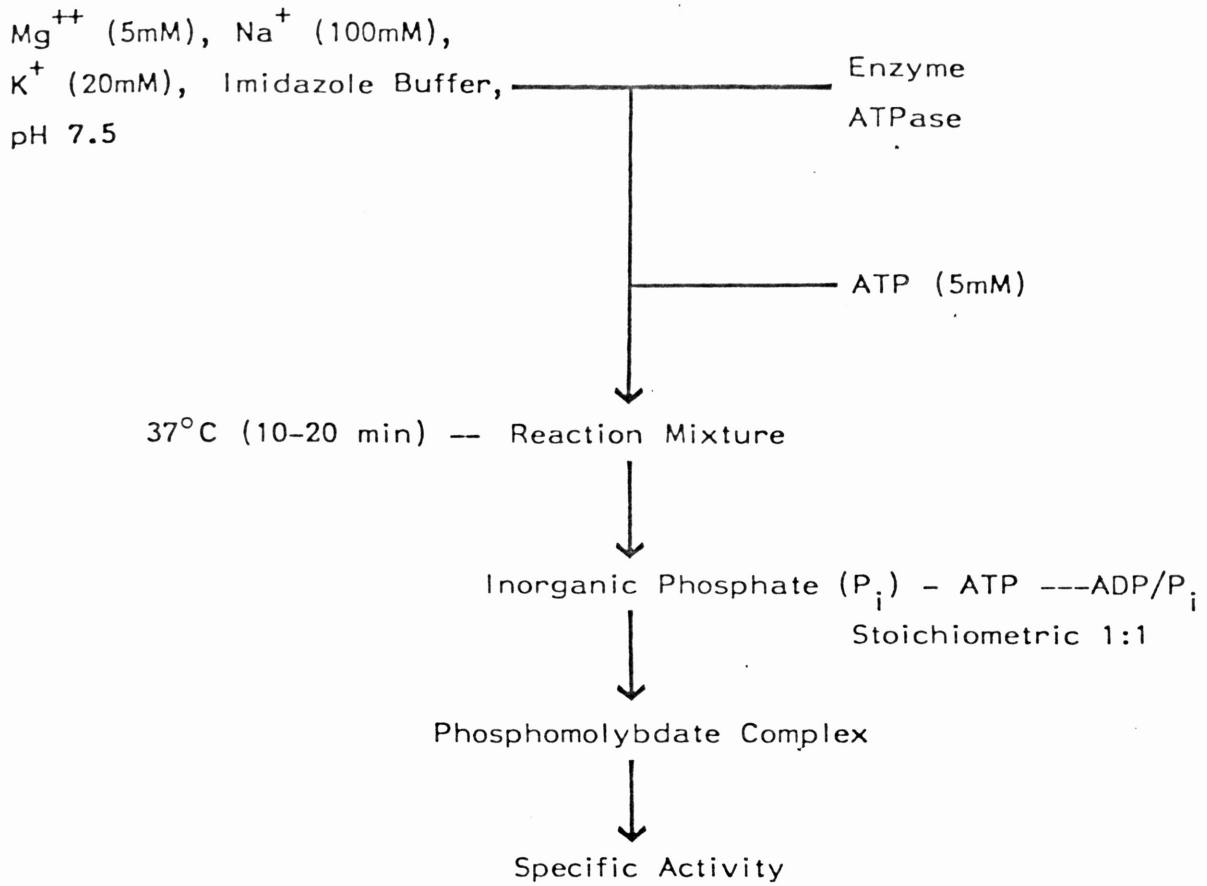
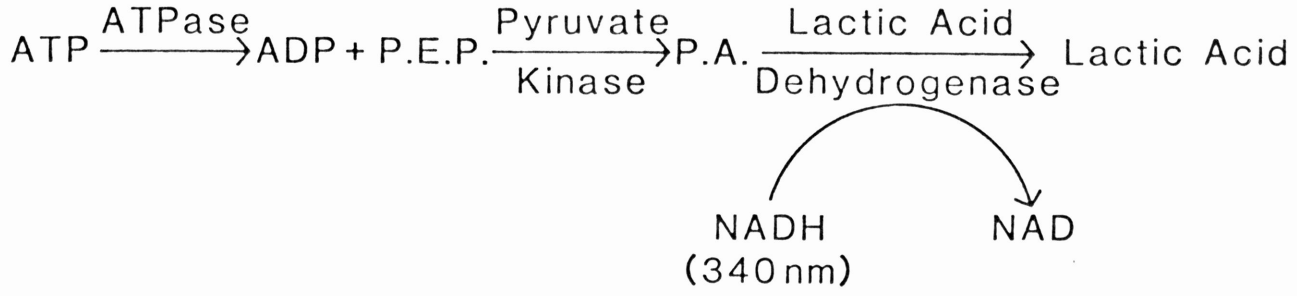


Fig. 2. The phosphate analysis of ATPase activity involves combining the release inorganic phosphate with a molybdate to form a phosphomolybdate complex that can be measured using a colorimetric reaction (Absorbance at 750 nm).



Sp. Act. - μMoles P_i/mg Protein/hr

Fig. 3. The enzyme coupled continuous ATPase assay utilizes the ADP released from the ATPase action. Phosphoenol pyruvate, in the presence of ADP and pyruvate kinase will form pyruvic acid. When lactic acid dehydrogenase acts on pyruvic acid, lactic acid is formed. During this step, NADH is converted to NAD. By spectrophotometrically measuring the amount of NADH (340 nm) in the reaction mixture, one can tell how much ADP is released from the ATPase reaction because ADP has a 1 to 1 stochiometric relation to NADH.

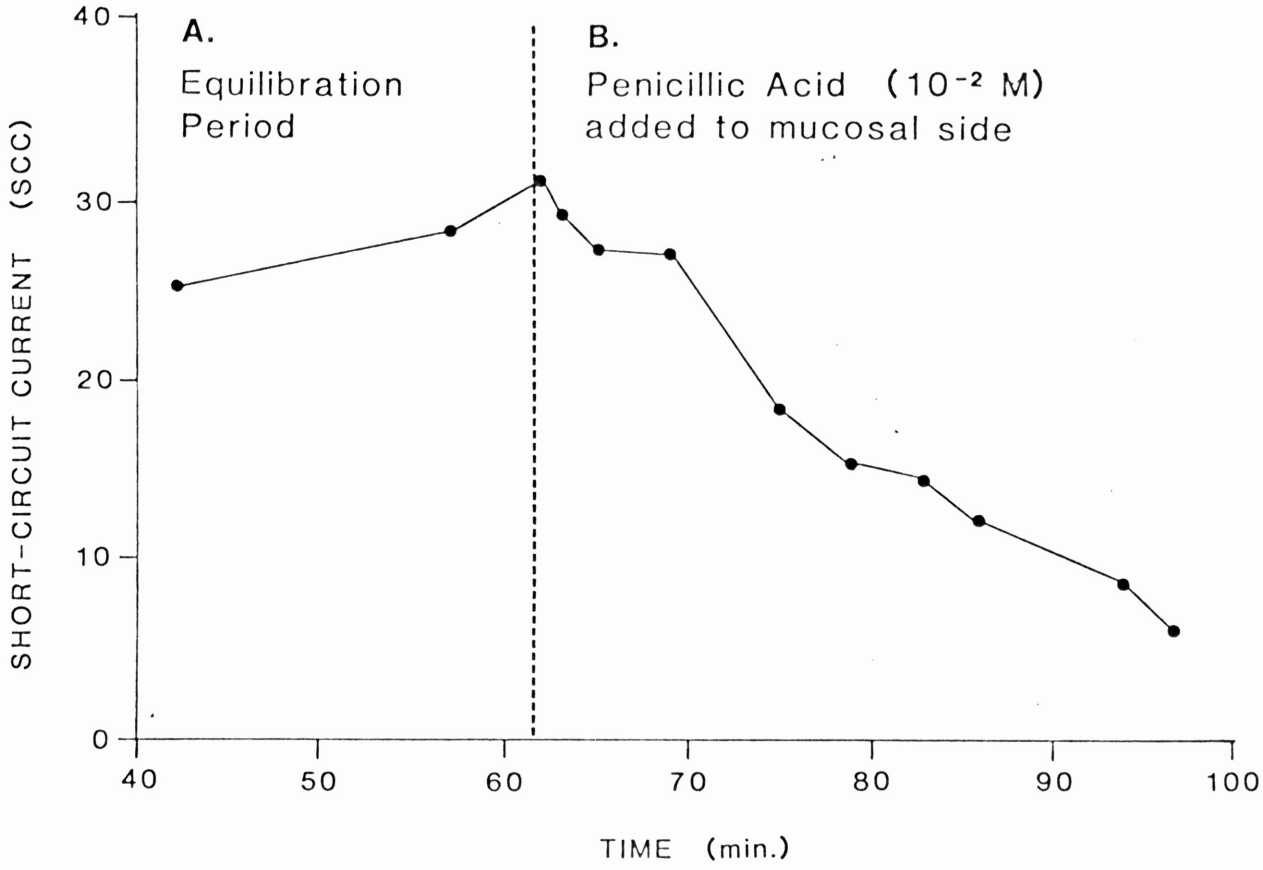


Fig. 4. Typical short-circuit current experiment using a penicillic acid concentration of 10^{-2}M.

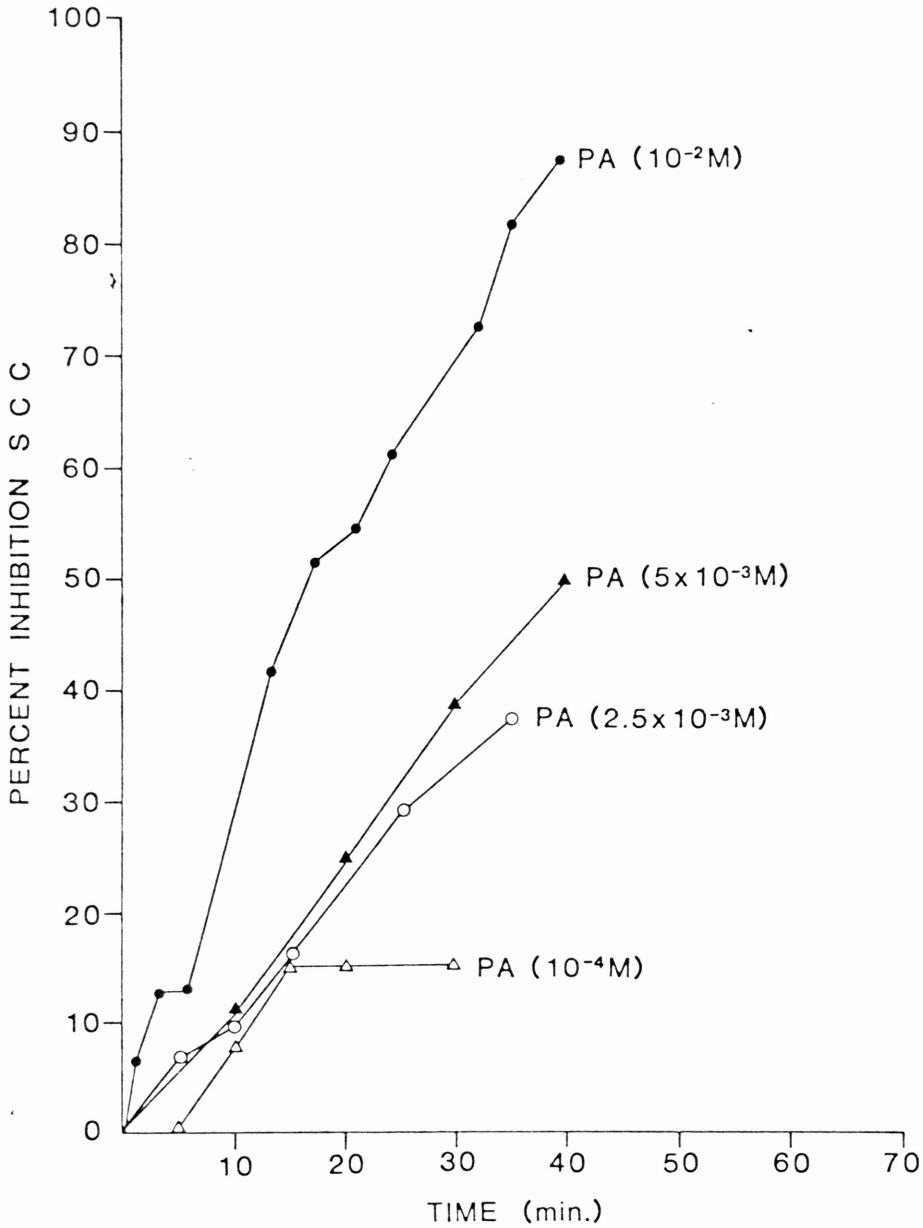


Fig. 5. Dose reponse effect of PA on short-circuit current. Concentrations of 10^{-2}M, 5 x 10^{-3}M, 2.5 x 10^{-3}M and 10^{-4}M penicillic acid assayed over a 30 min. period.

SCHEMATIC ILLUSTRATION OF PARTIAL REACTION
OF THE (Na^+-K^+) -ATPase SYSTEM

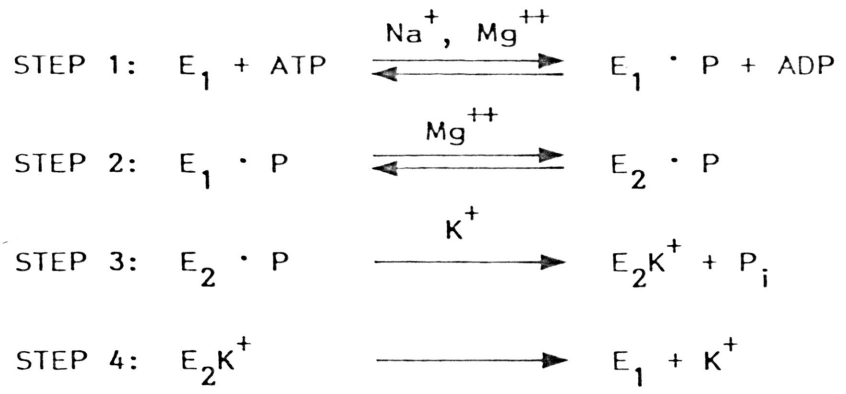


Fig. 6. Steps in ATPase reaction.

TABLE 1: Effect of Penicillic Acid on ATPase[a]

Assay	% Inhibition
Endpoint[b]	20
Continuous[c]	27.5

[a]Concentration 10^{-2}M PA

[b]Method described by Lowry, O. H. and J. A. Lopez. J. Biol. Chem. 162 (1946) 421.

[c]Method described by P. J. Fritz and M. E. Hamrick. Enzymologia, 30:57, (1965).

APPENDIX

Review of the Literature

Penicillic Acid

CHEMISTRY

Penicillic acid, referred to as PA, is a tautomeric mycotoxin existing as a gamma hydroxy lactone or an open ring substituted gamma keto hexanoic acid (17). PA is a white powder. It has a molecular weight of 170.2 and it is soluble in water, alcohol, ether, benzene, chloroform and ethyl acetate. The melting point is 84-87°C (9,5). It has been noted that the ammoniated derivative of PA is fluorescent (18). PA absorbs Br_2, becomes yellow in alkaline solution, reduces Fehlings solution when heated, turns deep red when exposed to ammonia and causes ferric chloride solution to become brown-red (1,17,19), but most important is the reaction it undergoes with sulfhydryl groups of amino acids such as cysteine, glutathione, lysine, arginine and histidine. After it has reacted with the sulfhydryl groups the resulting product is not toxic (20).

OCCURRENCE

Fungi occur in two distinct groups, field fungi and storage fungi. Penicillic acid is produced primarily by storage fungi. PA was first isolated by Alsberg and Black in 1913 (1). Since then PA has been documented in 18 other Penicillium species, 6 Aspergillus species and one Paecilomyce specie (21,22,23). PA is detected primarily in starch rich commodities such as rice and sorghum but not in high protein commodities such as peanuts, soybeans, or cottonseed (24).

PA has been isolated from commercial corn at concentrations ranging from 5 to 231 ug/kg (25), and tobacco at concentrations ranging from 100 to 230 ug/kg (26). PA produced by P. martensii on high-moisture yellow dent corn is stable for only 45-90 days when stored at temperatures of 15°C and 20°C respectively (27). When PA production on mold-fermented sausage was studied, 88 of the 422 cultures (21%) were toxin producing species. Approximately half of the isolates capable of producing toxin actually did produce PA (28). PA was examined in flour and fruit juices and found to be quite unstable in high protein commodities such as flour presumably due to the toxin reaction with the sulfhydryl groups available in these products. Examples of commodities in which PA is relatively stable are apple juice and grape juice (29).

BIOSYNTHESIS

According to studies carried out by Bentley and Keil, PA is formed by the condensation of one acetyl coenzyme A unit with three malonyl Co A units to form orsellinic acid. The orsellinic acid is cleaved and CO_2 is lost giving the open-ring structure of PA (30,31).

METABOLISM

Oral ingestion of PA by rats results in the absorption of approximately 98% of the administered dose within 48 hours with the maximum blood levels reached within 2 to 4 hours. Within the next 7 days, 82% of the PA was recovered in the urine and 13% in the feces (32). The main distribution areas in the body after 24 hours are the heart, lung, and liver (33).

MEDICAL USE

Although penicillic acid possesses antimicrobial (S. aureus, E. coli, and A. melleus), antiviral (Newcastle Disease) and anti-tumoral (mouse Ehrlich ascites carcinoma and mouse leukemia SN-36) properties (2,3) it has been proven too toxic for medicinal use. The LD_{50} (lethal dose, 50%) values for intraperitoneal injection range from 70-100 mg/kg (34,35). The oral LD_{50} is approximately 600 mg/kg (4). The main effect of Penicillic acid is its digitalis-like action on frog heart, rabbit auricle, perfused cat heart and canine heart-lung preparation and a vasodilator effect on coronary and pulmonary vessels therefore creating a systemic rise in blood pressure when injected. In rats, rabbits, and cats, penicillic acid exhibits an antidiuretic effect at sublethal doses.

BIOCHEMICAL EFFECTS

Noncompetitive inhibition of muscle aldolase in vitro by penicillic acid was demonstrated in 1973. The concentration required to inhibit activity by 50% was reported to be 9.5×10^{-6} M. The proposed mode of action was toxin covalent binding with $-NH_2$ and -SH groups (36). PA also inhibits alcohol dehydrogenase and lactic dehydrogenase (37). Other penicillium toxins including luteoskyrin, rugulosin, and patulin, as well as penicillic acid, were shown to inhibit DNA-dependent RNA polymerase from rat liver nuclei (38). Penicillic acid also inhibits brain and kidney Na^{+}-K^{+} adenosinetriphosphatase in the mouse at I_{50} levels of 2.5 and 3.5×10^{-5} M PA respectively. Moreover, the Mg^{++}-ATPase was not affected (5).

Another study indicated that penicillic acid inhibits swine brain Na^+-K^+ ATPase at an I_{50} level of 1.8×10^{-8} M PA. The inhibition of ATPase was both time and pH dependent and complete after a 20-30 minute preincubation with the toxin. Also, PA was active only in a very narrow range of physiological pH. The mode of action was suggested to involve nucleophilic addition of reactive enzyme sulfhydryl groups with penicillic acid thus altering the conformation and activity (6).

TOXICITY

Penicillic acid toxicosis has not yet been reported in humans, but it has been associated with disease in animals (39). Alsberg and Black demonstrated convulsions and paralysis in mice injected subcutaneously with PA (1). Significant weight loss and pulmonary hemorrhages have been reported in mice after intraperitoneal injection of PA (5). Pentobarbital, acting as a mixed function oxidase inducer, increases the toxicity of PA (40). Ciegler recorded toxicity of PA expressed as generalized cell necrosis in mouse liver and severe edema in rabbit skin (23). When combined with either ochratoxin A or citrinin, a synergistic lethal response is noted (34). Moreover, PA is cytotoxic (41) and carcinogenic in rats, with doses as low as 0.1 mg initiating tumor development (42).

CONTROL

Mold toxin production peaks in a low moisture environment at a temperature between 1°C and 10°C; therefore, cold storage of susceptible products represent a public health hazard (43). One way to reduce such a hazard is to add CO_2 to the environment at a level of 60%. Drying is

also an effective method of control, if combined with temperatures above 25°C (27,43,44).

Sodium-Potassium Adenosinetriphosphatase:

CHEMISTRY

Adenosinetriphosphatases (ATPases) are structure-bound enzymes that hydrolyze the terminal phosphate in ATP releasing ADP and orthophosphate. These enzymes act as machanochemical enzymes translocating other molecules across themselves as part of their specific roles in cellular activity.

The Na^+-K^+ activated ATPase has a molecular weight of about 250,000. It is made up of four polypeptide chains; two identical chains with a molecular weight of 50,000 and two with a molecular weight of 100,000. The two 100,000 molecular weight polypeptide chains may or may not be identical. One or both of these heavier subunits contains the ATP binding site and the specific binding site for ouabain. The 50,000 molecular weight chain has ionophoric properties for certain ions. In decreasing order of specificity they are Na^+, NH^4+, Cs^+, Rb^+, and Li^+ (45).

OCCURRENCE

Adenosinetriphosphatases are found in the cells of animals, plants, and bacteria. The highest level of activity has been reported in excitatory and secretory tissues, especially of the brain cortex (45), kidney outer medulla, electroplax of the electric eel, the rectal gland of the dog fish (46), and the isolated urinary bladder of the toad (47).

ACTIVITY

Na^+-K^+ ATPase acts to transfer 3 Na^+ ions from outside the cell and 2 K^+ inside the cell during the hydrolysis of one ATP keeping a low Na^+ and high K^+ concentration outside (45). This action is commonly referred to as the sodium pump. Steps in the ATPase reaction are shown in Figure 6. Free enzyme, in the presence of Na^+, Mg^{++} and ATP undergoes transphosphorylation resulting in the formation of a phosphorylated enzyme. This phosphoenzyme complex in the presence of Mg^{++} undergoes a change in conformation to form the second phosphoenzyme complex. This second complex reacts with K^+ to form a potassium-enzyme complex and to release inorganic phosphate. The potassium-enzyme complex reacts with ATP to restore and recycle the enzyme and to release the potassium inside the cell (48).

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