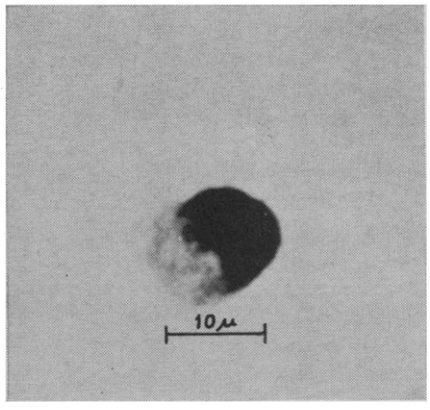

Fig. 1. Cytoplasmic alkaline phosphatase reaction of an isolated cell.

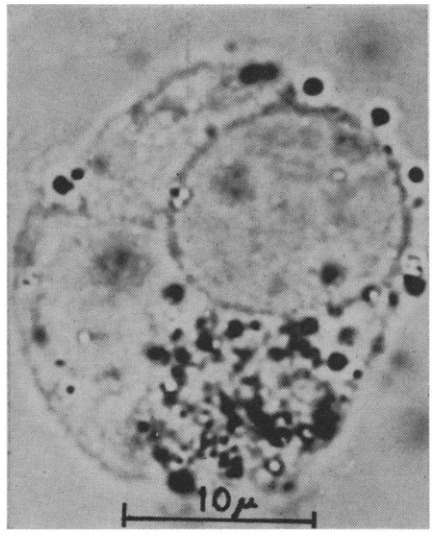

Fig. 2. Particles of neutral red within cytoplasm of an isolated cell.

penicillin-streptomycin at 50 units per milliliter, were incubated at 37°C in falcon plastic tissue culture vials. Cultures were examined daily and fed with newly prepared medium every 3 to 5 days. Subcultures were achieved by trypsinization of monolayers, and cell counts were done at each transfer. Tissue culture multiplication from cell isolates was apparent within 24 hours, and repeated subculturing was required as monolayers rapidly developed and separated from the bottom of the vial. Cell counts (after trypsinization) disclosed a threefold increase in cell population per week. No evidence of bone formation has been noted after one month in Eagle's medium. While round and stellate-shaped cells were noted in primary cultures, all cells assumed a stellate appearance after subculturing.

Isolated cells were characterized further by studies of glucose metabolism. Washed cells were suspended in Krebs-Ringer bicarbonate buffer, *p*H 7.4, with either 95 percent oxygen–5 percent carbon dioxide or air as the gas phase. An inoculum of known cell number was added to each 25-ml polyethylene flask containing buffer, glucose at 1 to 3 μmole/ml, and uniformly labeled glucose-C^{14}, glucose-1-C^{14}, or glucose-6-C^{14}, 0.5 to 1.0 mc/ml (*9*). After 2 to 4 hours' incubation with gentle shaking at 37°C, evolved $C^{14}O_2$ was trapped in hyamine hydroxide and counted in a liquid scintillation counter (*3*). After the addition of carrier lactic acid, radioactive lactic acid was isolated by ascending thin layer chromatography on cellulose plates (800 μ thick) developed with the upper phase of an ethyl acetate:water:formic acid (60:35:5) solvent system. Lactic acid spots were detected with bromcresol green spray and exposure to ammonium hydroxide vapor. Spots were then removed from the plates, placed in 10 ml of Bray's solution (*10*), and counted as above. Internal standards were added to account for quenching.

Inocula of 10^6 cells converted approximately 0.2 percent of medium glucose to carbon dioxide and 3.0 percent to lactic acid within two hours. While the release of $C^{14}O_2$ from glucose-1-C^{14} was three times that from glucose-6-C^{14}, there was 30 percent more lactic acid than $C^{14}O_2$ from glucose-6-C^{14} (Table 1). Substitution of air for 95 percent oxygen–5 percent carbon dioxide produced a 50 percent reduction in oxidation of glucose to carbon dioxide with a concomitant increase in lactic acid production. Oxidation was slightly less in Krebs phosphate buffer. Studies of glucose metabolism of cleaned, intact calvaria gave similar results, in agreement with the observations of Cohn and Forscher, which indicated that lactic acid is a principal end product of aerobic glucose metabolism by bone slices in vitro and qualitatively demonstrated significant hexose monophosphate shunt activity (*11*). Thus the impression gained by histologic examination, that cells isolated by collagenase treatment are representative of the entire cell population in the parent bone, is fortified by the similarity in the metabolism of glucose between whole bone segments and isolated cells.

Although we have demonstrated cell viability, it has not been possible to prevent dedifferentiation or overgrowth of fibroblasts in tissue culture, or both, and no bone formation has been noted. The presence of considerable alkaline phosphatase in the cytoplasm of many cells provides some evidence that these cells were osteoblasts or osteocytes (*12*).

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13. We thank Dr. Leon Sokoloff for the histology and Drs. Martin Rodbell, John Bader, Peter Goldman, and Gerald Aurbach for their advice.

14 September 1964

N^6-Benzyladenine: Inhibitor of Respiratory Kinases

Abstract. N^6-*benzyladenine, an active phytokinin, inhibits the respiration of many explants. The chemical also delays senescence and extends the postharvest life of many green or leafy vegetables. Evidence is presented that these phenomena may be linked to a competitive inhibition of the glycolytic kinases by* N^6-*benzyladenine.*

Treatment with N^6-benzyladenine delays the breakdown of certain leafy or green vegetables after they have been harvested (*1*). There is a greater retention of chlorophyll and carotene, less loss in dry and fresh weights, and an apparent delay in the onset of senescence. This has been attributed to an inhibition of the overall respiratory rates of the explants as measured by the uptake of oxygen and the evolution of carbon dioxide (*2*). Phosphorylation of hexoses in excised leaves and heads of broccoli is also greatly in-

hibited in the presence of N^6-benzyladenine (*3*). This suggested that the respiratory kinases were inactivated after treatment with the chemical. Our report describes the effects, in vitro, of N^6-benzyladenine on hexokinase, pyruvic kinase, and glutamine synthetase. The specificity of N^6-benzyladenine as an inhibitor of respiration appears to be associated with the kinases.

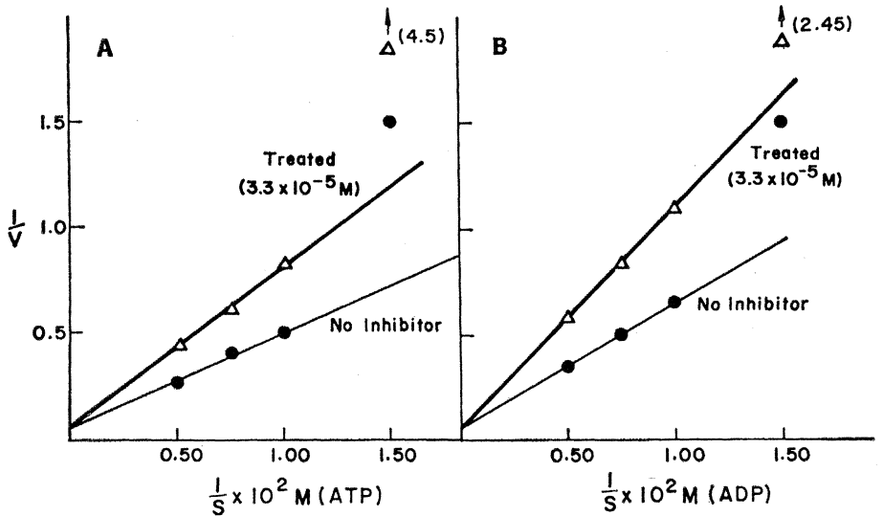
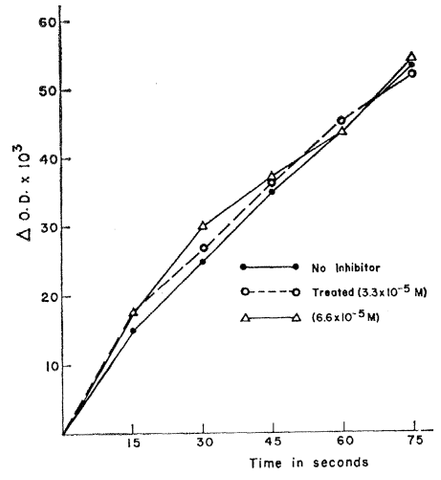


Fig. 1. The reciprocals of the reaction velocities ($1/v$) and of the concentrations ($1/S$) of ATP and ADP for hexokinase (*A*) and pyruvic kinase (*B*), respectively. Points are mean values for three experiments. The reaction mixtures contained the following: *A*, Tris buffer, *p*H 7.6, 50 μmole; glucose, 40 μmole; $MgCl_2$, 10.2 μmole; ATP, 20 to 60 μmole; NADP, 0.30 μmole; cysteine, 28 μmole; hexokinase, 100 μg; glucose-6-phosphate dehydrogenase, 300 μg in a total volume of 3.0 ml. Treated samples contained 0.1 μmole N^6-benzyladenine. *B*, Tris and $MgCl_2$, as in *A*; KCl, 5 μmole; ADP, 20 to 60 μmole; phosphoenolpyruvate, 20 μmole; $NADH_2$, 0.30 μmole; pyruvic kinase, 85 μg; and lactic dehydrogenase, 350 μg in a total volume of 3.0 ml. Treated samples contained 0.1 μmole N^6-benzyladenine.



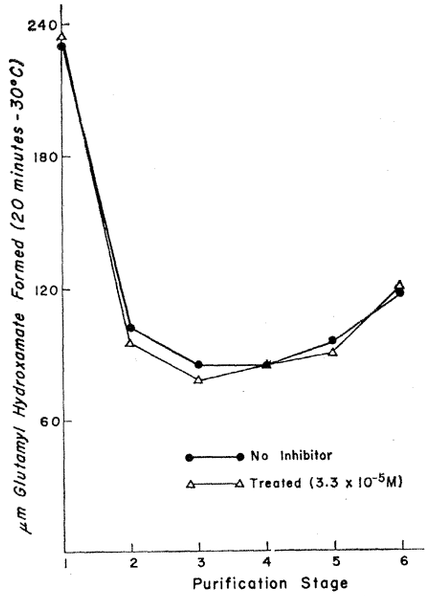


Fig. 2 (left). Effects of N^6-benzyladenine on the activity of glucose-6-phosphate dehydrogenase. The reaction mixture contained: tris buffer, $MgCl_2$, cysteine, glucose-6-phosphate dehydrogenase, and NADP as in Fig. 1*A*; N^6-benzyladenine, 0.1 or 0.2 μmole; and glucose-6-phosphate, 40 μmole in a total volume of 3.0 ml. Fig. 3 (right). N^6-benzyladenine and glutamine synthetase activity. The incubation mixture contained: tris buffer, *p*H 7.6, 26 μmole; sodium glutamate, 250 μmole; ATP, 35 μmole; $MgSO_4$, 120 μmole; cysteine, 28 μmole; NH_2OH, *p*H 7.6, 110 μmole; enzyme preparations, 0.3 ml in a total volume of 2.25 ml. Treated samples contained 0.1 μmole N^6-benzyladenine. Enzyme activity was determined by the conversion of glutamine to glutamylhydroxamate and measured colorimetrically at 540 mμ (*4*).

Hexokinase and pyruvic kinase activities were determined spectrophotometrically by coupling them to glucose-6-phosphate dehydrogenase and lactic dehydrogenase (*3*), respectively. The rates of reduction of nicotinamide adenine dinucleotide phosphate (NADP) by glucose-6-phosphate dehydrogenase and the oxidation of the reduced form of nicotinamide adenine dinucleotide ($NADH_2$) by lactic dehydrogenase served to measure the respective enzyme activities. Spectrophotometric measurements were made with a Beckman DU spectrophotometer at 340 mμ and 25°C, in 1-cm silica cuvettes.

Hexokinase catalyzes the phosphorylation of glucose by adenosine triphosphate (ATP); pyruvic kinase catalyzes the phosphorylation of adenosine diphosphate (ADP) by phosphoenolpyruvate. Preliminary experiments revealed that the magnitude of inhibition induced by N^6-benzyladenine was dependent on the ATP and ADP concentrations, hence kinetic studies with varying concentrations of the nucleotides were conducted.

The graph of the reciprocals (Fig. 1*A*) of the ATP concentration ($1/S$) and the velocity of the reaction ($1/v$) in the presence or absence of inhibitor indicates that hexokinase activity was competitively inhibited by N^6-benzyladenine. Nonspecific inhibition of respiratory kinases was suggested from the observation that N^6-benzyladenine also competitively inhibited the phosphorylation of ADP by pyruvic kinase (Fig. 1*B*). N^6-benzyladenine did not affect the activity of glucose-6-phosphate dehydrogenase (Fig. 2) nor that of lactic dehydrogenase used to assay the kinases.

The activity of glutamine synthetase was not affected by N^6-benzyladenine (Fig. 3). This enzyme requires ATP for the conversion of glutamic acid to glutamine, and in the reverse reaction it catalyzes the formation of ATP from inorganic phosphate and glutamine. The glutamine synthetase employed was purified tenfold from cauliflower (*3*) according to the procedure described by Elliott (*4*) and assayed for inhibition by N^6-benzyladenine at each stage of purification.

The effect of N^6-benzyladenine on other reactions involving ATP or ADP was not determined. However, similari-

ty in the nature of inhibition of the two kinase reactions investigated suggests that this phytokinin may inhibit similar phosphorylation reactions involving ATP.

Our results are not proposed as the explanation for all the varied effects of the phytokinins on plant metabolism (cell division and enlargement, germination, protein synthesis, directed transport, delay of senescence, and others). Nevertheless, these experiments suggest that respiratory inhibition induced by N^6-benzyladenine, which is also related to the delay in senescence of explants, is a consequence of inhibition of the glycolytic kinases. The structural similarity of ATP, ADP, and N^6-benzyladenine offers a possible explanation for the competition between the nucleotides and N^6-benzyladenine.

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22 October 1964

Conjunctiva Contains Factor Inhibiting Growth of Candida albicans

Abstract. *Intact conjunctiva and conjunctival extracts have a strong inhibitory effect upon* Candida albicans. *The fungistatic effect is not attributable to antimetabolic action and is not caused by lysozyme or tears, neither of which inhibit growth and viability of* Candida albicans.

We have found in the normal conjunctiva a factor which inhibits the growth of *Candida albicans*. This factor may account for the rarity (*1*) of conjunctivitis caused by *C. albicans* in contrast to the relative frequency of invasion of other mucous membranes by this organism (*2*).

The normal conjunctiva has a sparse and limited microflora. In a study of 520 eyes (*3*) free of mycotic disease, cultures for fungi yielded 38 isolates, of which only two were *C. albicans*. Tears have been shown to inhibit the growth of some bacteria (*4*) by a mechanism distinct from lysozyme action, but their effect on fungi has not been reported. In our investigations pooled human tears did not affect the viability of *C. albicans*.

For our study calf palpebral conjunctiva were brought to the laboratory in chilled containers within 2 hours after slaughter. The conjunctivae were dissected, away from the Maibomian glands and down to the tarsus plate, dipped in a mixed antibiotic solution which did not inhibit *C. albicans* (*5*), and freed of excess moisture with sterile filter paper. The mucosa was tested for inhibitory activity by culturing the organism in the presence of the tissue. Cultures were prepared by applying a thin layer of Sabouraud's agar to sterile microscope slides and inoculating the agar evenly with a suspension of an 18-hour culture of *C. albicans* containing 2×10^4 spores per milliliter. The mucosal surface of the excised conjunctivae was placed on the seeded agar and incubated for 18 hours at 37°C in sterile petri dishes.

When the conjunctivae were removed, no growth was visible on the slide where the agar was in contact with the tissue. No growth could be detected microscopically in the central portions of the areas of contact. Stunted colonies were occasionally present in the periphery. A thick, viscid exudate was present in the covered areas. Growth of the yeast was normal in control areas of the slide not covered by the conjunctivae (Fig. 1).

The inhibition was not caused by anoxia, since *C. albicans* appears to grow well under anaerobic conditions.

The inhibitory effect of the intact conjunctivae toward *C. albicans* remained unimpaired after exposure of the tissues to temperatures of 4°, 22°, and 37°C for 24 and 48 hours, and after warming to 42°C for 2 hours.

An excess of nitrogenous and mineral metabolites, supplied by increas-

Table 1. Decrease in viable plate count of *Candida albicans* after 2½ hours' incubation in conjunctival extracts. The figures reported are averages of duplicates which were plated in duplicate.

Initial	2½ hours	Decrease (%)
	Conjunctival extract	
1,155,000	128,000	89
870,000	10,000	99
1,305,000	304,000	77
	Water diluent	
1,290,000	990,000	23
	50% alcohol evaporated to half-volume	
1,050,000	1,115,000	0

Table 2. Decrease in counts (units/ml) of *Candida albicans* exposed to conjunctival extracts (three preparations) in rotator at 37°C. The counts were made in a white-blood-cell chamber; budding spores were counted as one unit; a group of three spores were counted as one unit.

0 hour	2½ hours	Decrease (%)
885,000	295,000	67
1,100,000	170,000	85
1,195,000	440,000	64

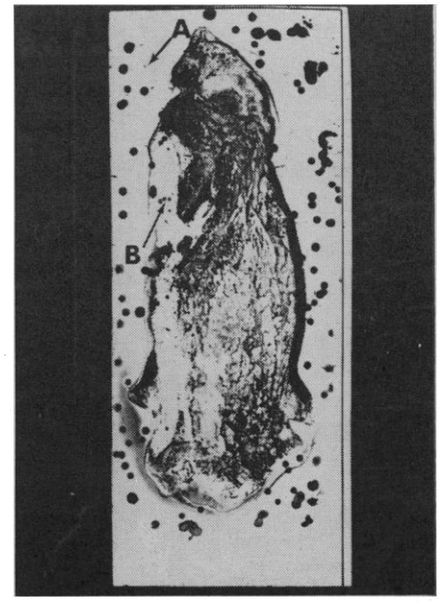

Fig. 1. Agar culture on microscope slide of *C. albicans* in presence of fresh palpebral conjunctiva (calf). A viscid exudate marks the area of contact. *A*, Normal colonies at a distance from the tissue. *B*, Stunted colonies at periphery of the tissue.