Plant Physiol. (1970) 45, 169-172

Adenosine Triphosphatase Activities in Leaves of the Mangrove *Avicennia nitida* Jacq.

INFLUENCE OF SODIUM TO POTASSIUM RATIOS AND SALT CONCENTRATIONS[1]

Received for publication August 12, 1969

A. KYLIN[2] AND R. GEE
Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92037

ABSTRACT

Homogenates from the salt-excreting leaves of the mangrove *Avicennia nitida* were subjected to differential centrifugation and investigated for adenosine triphosphatase activities. At pH 6.75 a salt stimulation with peaks at three different sodium to potassium ratios could be demonstrated above the activity due to Mg^{2+} ions. The stimulation by sodium and potassium depends on the ionic strength of the test medium, higher salt concentrations being inhibitory. The plant system seems thus more complicated than the animal activities. Technically, this means that a search for $(Na^+ + K^+)$-activated ATPases in plants should be performed with a close spacing of Na:K ratios at several constant levels of salt. Literature data on the transport of Na^+ and K^+ indicate that the physiological situation is rather complex in plants.

The $(Na^+ + K^+)$-stimulated activity of membrane-bound ATPase can be considered as a biochemical expression of the active transport of Na^+ and K^+ across that membrane (15). Salt-stimulated ATPases from plant tissues have been reported (1, 3-6), but so far only sugar beets have been shown to contain an ATPase with an activity specifically related to the ratio of Na:K in the assay medium (7).

Saline habitats are generally dominated by Na^+. Plants and other organisms selectively absorb K^+ and tend to exclude Na^+. In a comparative study, Norkrans and Kylin (11) recently observed that the K^+-Na^+ exchange processes are better developed in the halotolerant yeast *Debaryomyces* than in the nonhalotolerant *Saccharomyces*, which shows more of a K^+-H^+ exchange. The occurrence of $(Na^+ + K^+)$-activated ATPases might be particularly correlated with halotolerant and halophilic species. As a matter of fact, it was postulated by Jennings (8) that such enzymes are important for an understanding of salt resistance.

The genus *Avicennia* is adapted to high salinity habitats. As other mangroves, *Avicennia* species are found in the upper tidewater zone of tropical shores. In the mangrove vegetation around La Paz and Magdalena Bay in Baja California, Mexico, the genera mostly occur in the order *Rhizophora-Laguncularia-Avicennia*, so that *Avicennia* grows in the most elevated and adverse part of the habitat, where the surface is often dry and very high in salts for extended periods.

The leaves of *Avicennia* excrete salts from special glands. Uptake of potassium in leaf pieces of the species *Avicennia marina* has been investigated (13). The optimum absorption of K^+ is a function of the concentration of Na^+ in the medium. We are inclined to interpret this dependence as potassium uptake coupled to sodium extrusion.

With the above considerations in mind, we started a search for ATPases dependent upon the Na:K ratio in leaves of *Avicennia*. This paper presents evidence that such enzyme activities are present and are dependent upon the ionic strength of the assay medium. Difficulties were encountered in developing a preparation procedure, as well as in cultivating the plants under laboratory conditions. At this stage of development it may be useful if such technical difficulties are discussed, since others have sought evidence for the occurrence of $(Na^+ + K^+)$-dependent ATPases without success (1, 5, 6).

MATERIALS AND METHODS

Plants. Small trees of *Avicennia nitida* Jacq. were cultivated together with other mangroves in a growth chamber with 14 hr of light and 10 hr of darkness. The light was from mixed cool white and warm white fluorescent tubes, giving between 20,000 and 30,000 lux at the level of the branches used. The day temperature was regulated at 24° and the night temperature at 16°.

The medium was fundamentally Johnson's solution and contained 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $(NH_4)H_2PO_4$, 1 mM $MgSO_4$, 0.04 mM $FeSO_4$ in 0.08 mM Na_3-citrate, and trace amounts of B, Mn, Zn, Cu, Mo, and Co. The medium was made up in filtered sea water and deionized water to give the required range of total salinity.

The media were originally made up with 50% sea water. In order to increase growth, the plants were then transferred to a medium with only the nutrient salts in deionized water. After a month, a group of plants of the genus *Aegialithis* were attacked by root fungi. In order to control this infection and keep it from spreading, the plants were returned to 50% sea water, which stopped the growth of the fungus. After the pest had been controlled, the main group of *Avicennia* plants were kept with a salinity of 25% sea water. A few plants were grown with only nutrients in deionized water. It should be noted that the *Avicennia* plants were never actually attacked by the fungus.

Preparation of ATPase. In their studies of the ATPase of sugar

[1] Supported by United States Public Health Service Grants GM-12310 and AM 12386-03. Traveling expenses for Dr. Kylin were provided by a Fulbright Scholarship of the United States Government, by the Hahn Scholarship of the Royal Swedish Academy of Sciences, and by a Scholarship from the Wallenberg Foundation 50th Anniversary Donation to the University of Stockholm.

[2] Permanent address: Institute of Botany, University of Stockholm, Lilla Frescati, S-104 05 Stockholm, Sweden.

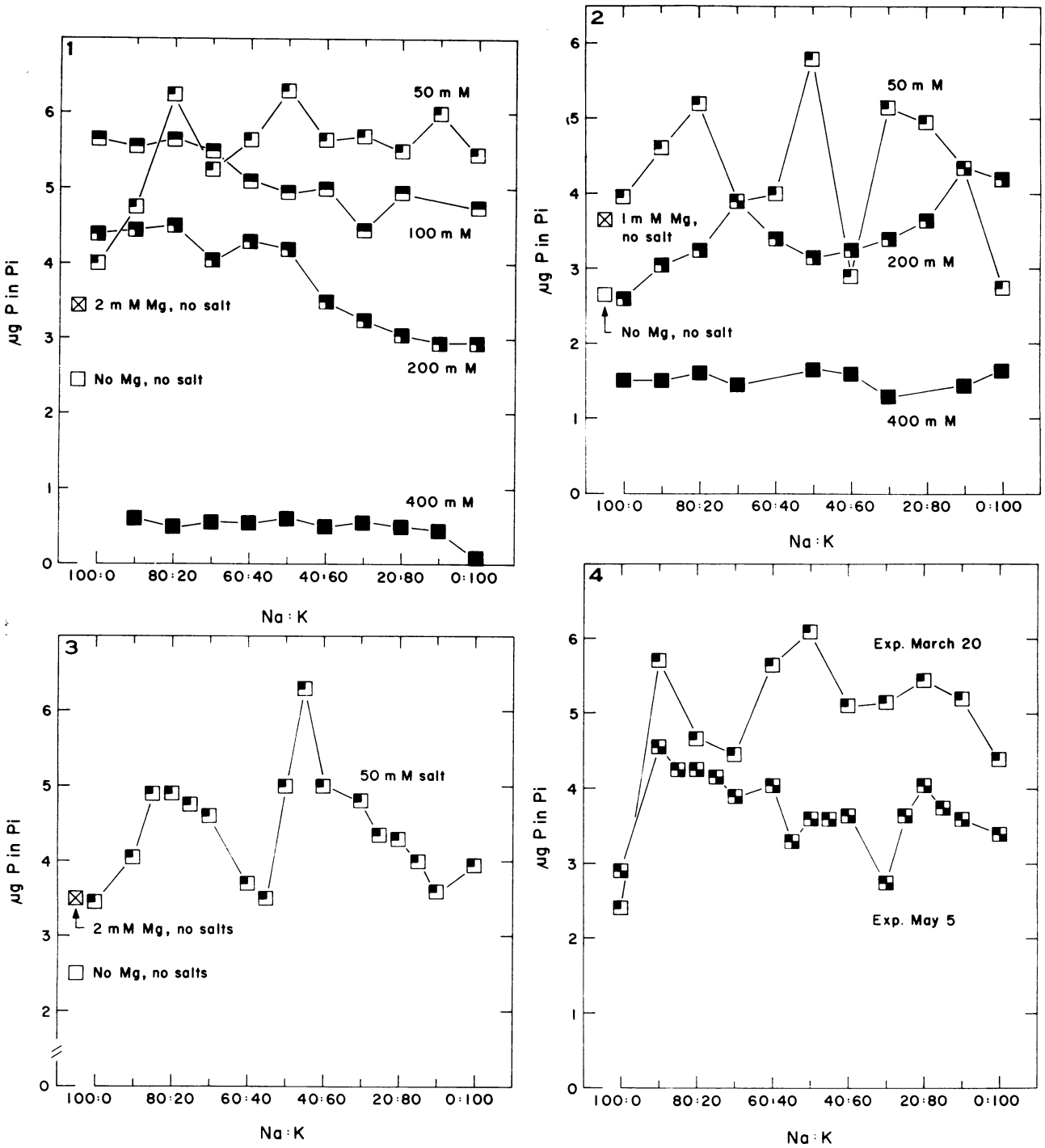

FIG. 1. ATPase activity of *Avicennia* leaves at different salt levels and different Na:K proportions. Fraction obtained between 7,000*g* and 20,000*g*. After several months with nutrient solution made up in 50% sea water, the plants had been grown a week in nutrient salts alone. Preparation made January 17, test run January 23.

FIG. 2. ATPase activity of microsomal fraction of *Avicennia* leaves at different salt levels and different Na:K proportions. After several weeks in nutrient solution made up with 50% sea water, the plants were grown 10 days in nutrient solution made up with 25% sea water. Preparation from March 17, test performed March 20. In the interest of legibility the curve for 100 mM salts has not been drawn. It was intermediate between the curve for 50 and the curve for 200 mM salts.

FIG. 3. Effect of freeze-storage at −20° on the ATPase activity in *Avicennia* leaves. Same microsomal preparation as in Figure 2, but test performed May 5. Total monovalent salts, 50 mM.

FIG. 4. Effect of freeze-storage on the ATPase activity of the microsomal fraction of *Avicennia* leaves. These plants had been grown 10 days in nutrients made up in deionized water. Previously they had been cultured several weeks in nutrients made up with 50% sea water. Preparations from March 17, tests from March 20 and May 5. Conditions as in Figures 2 and 3. ($MgCl_2$, no salt) controls at 3.1 and 3.4 µg of P, respectively.

beets, Hansson and Kylin (7) developed a grinding medium with 0.2 M sucrose and 0.03 M histidine buffered at 7.2 with HCl. In our first attempts to homogenize leaves of *Avicennia* in this medium, we met two main difficulties, namely, bursting of chloroplasts and peroxidation. Bursting of chloroplasts leads to release of Mg^{2+}, so that the stimulation due to added Mg^{2+} becomes difficult to demonstrate; and peroxidation is generally destructive for the system.

Excessive bursting of chloroplasts could be avoided by increasing sucrose to 1 M. Peroxidation could largely be prevented by 0.025 M ascorbic acid. To maintain suitable pH in the presence of ascorbic acid, histidine was increased to 0.1 M. A minimal addition of HCl then gave a pH of 6.5, which gave active preparations for the assays.

The leaves were collected and washed with deionized water to remove excreted salts, the midribs were removed, and the tissue was precooled in a plastic bag. All subsequent operations were carried out at 0 to 5°. Homogenization was accomplished in 30 sec in a Waring Blendor at full speed, with 10 ml of cooled medium added per g of tissue. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 20 min at 2500*g*, and the pellet was discarded. This removed debris and chloroplasts.

The mitochondria were then sedimented by centrifugation at 7,000*g* for 20 min and removed. A solution of 1% sodium deoxycholate was added while the supernatant was stirred, until the concentration was 0.1%. The stirring was continued for 20 min, and the mixture was left for another 40 min. Particulate fractions were then collected by differential centrifugation for 1 hr at 20,000*g*, followed by 1 hr at 100,000*g*. After three washings in (1 M sucrose + 0.04 M histidine, pH 6.5), the sediments were individually suspended in small amounts of the washing medium, about 1 ml/g of initial tissue for the pellet obtained at 20,000*g* and 3 ml/g of initial tissue for the fraction obtained at 100,000*g*. The preparations were stored at −20° until used.

Analytical. The ATPase activity was assayed in a total volume of 1 ml, containing 0.75 mM ATP (disodium salt; sodium was removed by treatment with the H^+ form of Dowex-50 resin, filtering directly into the histidine), 40 mM histidine-HCl, and other additions as indicated. The reaction was started by the addition of enzyme corresponding to 75 μg of protein, and stopped after 60 min by the addition of 0.25 ml of 20% trichloroacetic acid, which was neutralized by 4 ml of 0.1 M Na-acetate. After centrifuging, the supernatant was decanted, and 2.5 ml were used for a spectrophotometric determination of P_i by the modified Fiske-SubbaRow method worked out by Bertram and van Herk and published by Lindeman (10). All figures are the means of duplicates; the variation between the two tubes was 10% or less.

Less than 30% of the ATP was hydrolyzed during an assay. Special tests showed that with Mg:ATP 1:1, the ATP addition was sufficient to give maximal velocity of the reaction.

Protein determinations were made according to the standard biuret method.

RESULTS

All preparations were first tested with a series of Mg^{2+} concentrations over a range of pH. In the active preparations, the greatest stimulation due to Mg^{2+} was found at pH 5.5 and 6.75. This corresponds to the results reported for sugar beets (7).

The preparations were then tested with combinations of (NaCl + KCl) at different total ionic strengths as shown in the figures. At 50 mM total concentration of (NaCl + KCl) activity peaks were obtained in Na:K ratios between 9:1 and 8:2 and around Na:K 5:5 (Figs. 1–4). A similar peak seems to exist around Na:K 2:8 (Fig. 2), although it is in most cases less noticeable (Figs. 1, 4) and sometimes occurs as a shoulder only (Fig. 3).

At higher salinities, the curves tend to flatten out. At 100 mM (NaCl + KCl) one would tend to think of them as showing a general salt stimulation (Fig. 1). At 200 mM (NaCl + KCl) the salt additions produce no consistent activation as compared with the no salt control, and at 400 mM the salt addition inhibits the activity to levels below those obtained with the enzyme in the absence of any salts (Figs. 1, 2).

Prolonged storage at −20° affects the shape of the curves and the activity. The greatest change in shape seems to occur in the high-K region (Fig. 2 compared with Fig. 3; Fig. 4).

As for the active fraction, our attention was first directed toward the ATPase of the mitochondria and of the fraction obtained between 7,000*g* and 20,000*g*, since together they correspond to the preparations from sugar beet by Hansson and Kylin (7). Little activity was found in the mitochondria. The first positive results were obtained with the 20,000*g* pellet (Fig. 1). When the salinity of the cultivation media had to be changed, as described in the methods section, the activity in the 20,000*g* pellet vanished. Instead, it was found in the microsomal fraction from the 100,000*g* centrifugation (Figs. 2–4).

Of the salt-stimulated plant ATPases reported in the literature, the one of Atkinson and Polya (1) has a more acid pH optimum, and the one of Fisher and Hodges (5) a more alkaline one than the enzyme described by Hansson and Kylin (7) and the one studied in the present investigation, which both show optimal Mg^{2+} responses at pH 6.75. We have checked our preparations for salt stimulation in the Mg^{2+} stimulation peak at pH 5.5. There was little or no stimulation at 50 mM (NaCl + KCl) and no evident synergism. At 400 mM (NaCl + KCl) the salts were inhibitory. Over the pH range 7.0 to 8.0 there was a continuous decrease in both background and Mg^{2+}-stimulated activity (investigated with tris buffers; duplicated with the histidine buffer of our main experiments).

Discussion

There are now at hand reports on salt-stimulated ATPases in the acid (1) as well as in the slightly alkaline (5) region, and also reports on synergistic effects of Na^+ and K^+ (7, present investigation), which are modified by the ionic strength of the assay medium (present investigation). The synergistic effects are interesting, because they do not represent a single peak at only one Na:K ratio as seen in animal systems (15). Instead, there are two (7) or three (present investigation) peaks, which can be interpreted either in terms of several enzymes in the membrane systems, or as structural changes which allow more than one ion to activate a transport site (7).

With the experience gained in the present investigation, some technical points must be stressed. First of all, the grinding media may have to be reinvestigated from species to species, as discussed in "Materials and Methods." Furthermore, the multiple peaks obtained at different Na:K ratios and the influence of total salt concentration necessitate a special caution. Working with only a few Na:K ratios, synergistic effects may easily escape detection. It is also necessary to work with closely spaced Na:K ratios at more than one level of total salt. The classical technique from animal systems, keeping one cation constant and varying the other, may lead to difficulties during the exploratory stage, since synergistic effects between Na and K may be easily obscured by the concomitant change in ionic strength. For these reasons it may be advisable to investigate further the acid and the alkaline salt-stimulated systems (1, 5), to make sure that there are no hidden synergisms between Na and K.

Disregarding these technical problems, it is still interesting to compare the properties of the enzyme with known physiological phenomena. Acidification of the medium of yeast (14) or lowering the salt content of barley roots (12) will induce a change

from Na^+-K^+ exchange to H^+-K^+ exchange. It would, therefore, be logical to expect less Na:K synergisms in the ATPases at lower pH, which is indicated so far (1, here); but the lowering of the Na:K synergism when the salt content of the test medium increases is surprising in a salt-tolerant plant. Damage to the membranes during the preparation procedure might be an explanation for this, since such damage is the most likely explanation for the losses induced by prolonged storage.

A change in the root culture medium induces a change in the composition of the salts excreted from the salt glands of the tamarac (2, 16). In *Scenedesmus*, the system for exchange of Na^+-K^+ will increase the exchange of K^+ (as measured with ^{86}Rb) when no sodium is present (9). Such findings could correspond to multiple Na:K interactions in an ATPase system. Another possibility is offered by the knowledge that Na^+-K^+ exchange occurs both at the plasmalemma and tonoplast (see 8 for references), and in the present case the salt excretion system might represent a third exchange system. In future experiments it is important to consider and to relate the properties of the salt-stimulated ATPases and their synergisms with transport phenomena on the physiological level.

Without being able to prove it for the moment, we have the feeling that the switching of the activity from the 20,000*g* pellet (Fig. 1) to the microsomal fraction (Figs. 2–4) was somehow correlated with the osmotic changes in the medium accounted for in "Materials and Methods." It might be pointed out that the microvesicles forming the anatomical basis for salt excretion in the tamarac (16) are of a size that can be expected to occur in the active fraction of Hansson and Kylin (7), and in the 20,000*g* pellet, where we first found the activity.

Acknowledgments—The continuous interest and hospitality of Professor A. A. Benson and of Professor Paul Saltman are gratefully acknowledged.

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