Glycoside-Sensitive ATPase from Arachis hypogaea

by

Harry Darrow Brown[1] and Aaron M. Altschul
Seed Protein Pioneering Research Laboratory[2]
New Orleans, Louisiana

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In the last several years there has been a considerable renaissance of interest in ATPase catalyzed reactions. This has followed reports of demonstrable relationships between the enzyme's activity and membrane transport (particularly of sodium and potassium). (See for examples, Skou, J. C. 1957; 1960; Post, R. L., et al., 1960; Klein, R. L. 1963; Ulrich, F. 1963.) In crab nerve and human red-blood-cell ghosts it has been demonstrated that transport and ATPase activity were inhibited by the cardiac glycoside ouabain, stimulated by magnesium and by sodium and potassium together. The facts suggest that these same criteria may be applied to systems in which transport itself cannot be measured. We have, assuming that such an activity may approach universality, sought an enzyme with these characteristics in a plant system.

Arachis hypogaea seeds were washed in .5% sodium hypochlorite solutions, blotted dry, and dusted with Spergon[3], thence germinated at room temperature (25C) upon moistened filter paper; harvested after 72 hours; washed; blotted dry; and weighed. The seedlings, in toto, were ground for 6 minutes in a Servall Omnimixer together with a volume of .2M Tris/HCl buffer pH 7.2 equal

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1/ Postdoctoral Research Associate.

2/ One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

3/ Trade names are used in describing exact experimental conditions; their mention does not imply recommendation by the Department over others equally suitable.

to five times their weight. The homogenate was filtered through glass wool and then centrifuged (5000 x g, 10 min.). This supernatant was again filtered through glass wool and centrifuged (20,000 x g, 20 min.). After collection of the sediment, the filtration and centrifugation were repeated and the supernatant collected as "the supernatant enzyme," the sediments pooled and resuspended in Tris as "the pellet enzyme." Grinding and all subsequent handling was carried out at 0-4C.

Assay was accomplished by incubating the enzyme (1cc), together with ATP solution (1cc; 1.5 mg/cc disodium ATP, Sigma, or in experiments involving metals, Tris ATP, Sigma, in Tris buffer) brought to 3cc with buffer, pH 7.2, at 38C for 1 hour or, in some experiments, 2 hours. Protein was then precipitated with .2cc 50% w/v trichloroacetic acid; centrifuged 5000 x g for 10 minutes and the supernatant assayed for inorganic phosphate (Technicon modification of Fisk-Subbarow method) (Technicon Manual, 1958). Activity is reported as the increase in inorganic phosphate per 100ml of test solution. Alkaline-earth metals were measured with a Gilford modified Beckman DU with flame attachment and with a Hilger-Watts flame absorption spectrometer.

Both the supernatant and the pellet enzyme were sensitive to the presence of crystalline ouabain, but while the supernatant enzyme was inhibited in much the manner as had been reported (Skou, J. C.; Post, R. L. *et al.*, *op. cit.*), the pellet enzyme was stimulated to the same extent. Maximum effectiveness in either instance was at concentrations of 1.5mM ouabain (max. stimulation at 52.4%; inhibition 51.2%), though in other experiments maxima were at lower concentrations. Addition of metals did not affect the rate of activity of either enzyme preparation.

After these early experiments it appeared that: 1) the supernatant and the pellet enzyme might not be the same though an alternative hypothesis which is presently being tested is that the activity difference is a reflection not of different enzymes but rather of conditions which result in differing conformation; 2) the enzyme preparations must contain metals in sufficient quantity, or even in excess, to obscure any effect of added salts. The pellet

enzyme was not further studied and, after confirmation that alkali metals did indeed remain in the supernatant enzyme preparation (9 ppm Na), methods were sought to lower the level of the metals. Dialysis against Tris buffer for 48 hours was ineffective (Table 1). Dialysis against Tris for 48 hours (4C) with Amberlite GC50 (previously converted to the Tris form) suspended in the dialyzing medium reduced the sodium content of the preparation to 6 ppm. In this case, the preparation not containing added salts showed 14.5 percent more activity than with Na-K-Mg added. Apparently the point where metals were limiting had not been reached. When EDTA was added to the system to reduce further the metal concentration (30 mg EDTA/3cc reaction mixture was optimum), a stimulation of 28.3 percent was observed. And moreover, when the salts and EDTA were added together an additional stimulation of 12.1% was measured. The salts of Mg and K were stimulatory; addition of sodium still inhibited (Table 1).

One could assume that an enzyme similar to that described by other authors was present in the seed extract, but that consistent with the thesis of Skou (op. cit. 1960) metal ions were bound in the system. The methods employed to remove them had sufficed to bring the amount of Mg and K below the optimum for activity, but Na was still present in sufficient amount so that its addition inhibited the reaction. One might suggest that the excess of any of the metals results in some occupation of alternate sites with resultant diminution in activity.

An additional experiment has introduced a complication of some weight. Ouabain in an EDTA stimulated system is not, as it had been in the original system, inhibitory but stimulatory and in a most dramatic fashion. Ouabain stimulates activity 289 percent and has its maximum effect at a concentration of .3mM (Figure 1). Thus in a system which resembles the transport-ATPase, when activity is stimulated by the presence of EDTA, the cardiac glycoside, ouabain, stimulates rather than inhibits, with a nearly 3-fold effect.

While it may be that the pellet enzyme (very likely associated with the plasma membrane which adheres to the cell walls) and the supernatant enzyme

Preparation	Sample Treatment	Addition	Δ P/100cc	Inhib. or Stim.%		Incubation at 38C
115*	Dialysis	none	6.11	-------		1 hr.
115*	Dialysis	Na + K + Mg	5.96	2.5-		1
1122*	Dialysis and amberlite	none	5.39	-------		2
1122*	"	Na + K + Mg	4.61	14.5-		2
1218	"	none	2.19	----	Reference	2
1218	and EDTA	none	2.81	Reference	28.3+	2
1218	"	Na + K + Mg	3.15	12.1+	43.8+	2
1218	"	Na	1.91	29.5-	9.6-	2
1218	"	K	2.97	6.0+	36.0+	2
1218	"	Mg	3.21	14.0+	46.6+	2

Table 1. Effect of metals upon ATP hydrolysis(1.5mgATP/3cc sample;*15mg/3cc). NaCl .12g; KCl 1.4g; $MgCl_2$ .4g, all per 1000cc; EDTA 30mg/3cc.

are different, the ouabain activity difference upon which this supposition was based ceases to exist in the presence of EDTA.

We suggest that a "transport-ATPase" is present in extracts of germinating seed. The enzyme is similar to that described by Skou and by Post et al. It is Mg activated and sensitive to Na and K. It is inhibited by ouabain at concentration levels reported by these workers. The fact that conditions which could be expected to lower the Mg level result in stimulation by ouabain is being further investigated. The results of Klein in which only statistical evidence of ouabain inhibition could be developed with frequent

examples of stimulation also suggest the possibility of conditions (perhaps those determining conformation) under which ouabain stimulates.

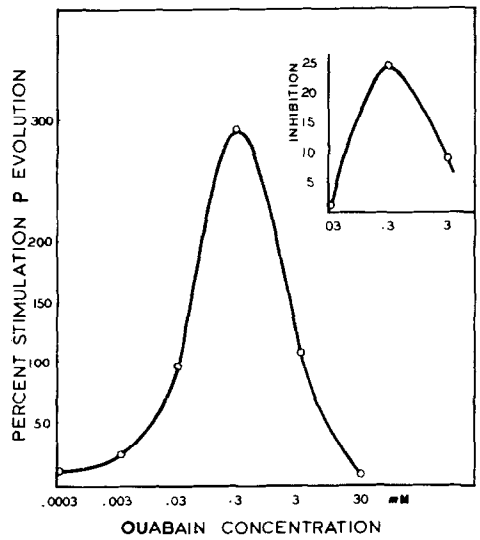

Figure 1. Stimulation of P-evolution by ouabain in the presence of EDTA. Inset: inhibition of P-evolution by ouabain in the absence of EDTA.

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