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MEMBRANE-BOUND ATPase OF INTACT VACUOLES AND TONOPLASTS ISOLATED FROM MATURE PLANT TISSUE

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Summary

Intact vacuoles were isolated from petals of *Hippeastrum* and *Tulipa* (Wagner G.J. and Siegelman, H.W. (1975) Science 190, 1298—1299). The ATPase activity of fresh vacuole suspensions was found to be 2—3 times that of protoplasts from the same tissue. 70—80% of the ATPase activity of intact vacuoles was recovered in tonoplast preparations. The antibiotic Dio-9 at 6 $\mu g/10^6$ vacuoles or protoplasts causes 40% inhibition. However, only the protoplast ATPase is sensitive to oligomycin. *N,N'*-dicyclohexylcarbodiimide (DCCD) slightly stimulates ATPase activity in both vacuole and protoplast suspensions, whereas ethyl-3-(3-dimethylaminopropyl carbodiimide) (EDAC) strongly inhibits.

Spectrophotometric studies show that in the petal the vacuolar contents have a pH of 4.0 for *Tulipa* and 4.3 for *Hippeastrum*, whereas the intact isolated vacuole has an internal pH of 7.0 (in pH 8.0 buffer) for *Tulipa* and about 7.3 for *Hippeastrum*. Internal ion concentrations of 150, 46, 30, 30 and 6 mM were found for K^+, Na^+, Mg^{2+}, Cl^-, and Ca^{2+} respectively, which are about the same as those in protoplasts.

Introduction

Plant cells have long been thought to possess a tonoplast (vacuole membrane) ATPase [1]. However, the role of the vacuole in plant cell ion transport remains unclear since direct measurements on intact isolated vacuoles or pure tonoplast preparations have not been possible. The development of a large-scale isolation technique for intact vacuoles [2] enables us to explore the properties of tonoplast ATPase and the possible role of the vacuole in regulating cellular ion balance.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDAC, ethyl-3-(3-dimethylaminopropyl carbodiimide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CCCP, carbonylanide *m*-chlorophenylhydrazone.

The technique of Wagner and Siegelman [2] was used to isolate and purify vacuoles from petals and leaves of *Tulipa* cultivar Red Shine and *Hippeastrum* cultivar Red Christmas Amaryllis. In brief: petals or leaves were sliced into 2-mm strips and incubated in 2% thoroughly dialyzed Cellulysin (Calbiochem) and 0.6 M mannitol pH 5.8 at 25° C for 17—24 h with gentle shaking to obtain protoplasts. Vacuoles were then prepared by rapidly adding to the protoplasts, during 15 s, about 50 ml of 0.2 M K_2- or Na_2-HPO_4/HCl, pH 8.0 plus 0.5 mM dithiothreitol per $2 \cdot 10^6$ protoplasts. Vacuoles were washed by resuspension in 0.7 M mannitol, 0.5 mM dithiothreitol, 1.0 mM HEPES/NaOH or HEPES/KOH buffer at pH 8.0. Vacuoles and protoplasts were counted in a well-type slide having a depth of 0.2 mm. The washed vacuole suspension was layered on a step gradient of 15% sucrose (w/w) and 35% sucrose (w/w) containing 5 mM HEPES/NaOH pH 8.0, 1 mM $MgSO_4$ and 0.5 mM dithiothreitol, which was then centrifuged for 2 h at 100 000 $\times g$ and 4° C. Tonoplast membranes were collected at the 15/35% sucrose interface. The supernatant containing the vacuolar sap (as indicated by the presence of virtually all the anthocyanin) was transferred to dialysis tubing and concentrated by ultrafiltration against crystalline sucrose.

A modified Hodges and Leonard [3] method was used to assay ATPase. Enzymes were assayed at 38° C in 1.0 ml containing 0.9 ml of the appropriate substrate solution (see Tables) and 0.1 ml of organelle suspension (10^5—10^6 vacuoles, protoplasts or equivalent tonoplasts). Vacuoles and protoplasts undergo osmotic rupture in the assay media. After 30 min the reaction was terminated by addition of 1 ml of cold 5% trichloroacetic acid (w/v). The phosphate released was determined by the Martin and Doty [4] procedure. Substrate and organelle suspension blanks were determined and subtracted to calculate all enzyme activities.

From studies of oat root homogenates, Leonard et al. [5] suggested that tonoplast or plasmalemma ATPases show more activity at pH 6.0 than pH 9.0 and are more sensitive to activation by KCl. Similarly, our pH effect studies (Table I) show that vacuolar ATPase has about twofold more activity at pH 6.0 than at pH 9.0, though in protoplast suspensions the pH dependence is less marked. This could arise from the contribution of mitochondrial ATPases having high (pH 9.0) optima [5,6] to the overall ATPase activity of protoplasts.

Table I indicates that the ATPase activity of intact vacuoles is reproducibly

TABLE I

EFFECT OF pH ON PETAL ATPase ACTIVITY

Reaction mixture contained 3 mM ATP (sodium salt), 1.5 mM $MgSO_4$, 50 mM KCl, and 33 mM Tris/2-(*N*-morpholino)ethanesulfonic acid. pH adjusted with 5 M HCl or NaOH.

	Vacuole		Protoplast	
	Hippeastrum	*Tulipa*	*Hippeastrum*	*Tulipa*
pH 6	3.52 *	2.75	1.12 **	1.05
pH 9	1.98	1.24	1.01	0.86

* pmol P_i/(vacuole · h).

** pmol P_i/(protoplast · h).

TABLE II

EFFECT OF VARIOUS COMBINATIONS OF Mg^{2+} AND K^+ ON THE PETAL ATPase ACTIVITY

Reaction mixture contained 3.3 mM ATP (Tris salt), 33 mM Tris/2-(*N*-morpholino)ethanesulfonic acid, ± 1.5 mM $MgSO_4$, and ± 50 mM KCl, pH 6.0.

	+Mg, +K	+Mg, —K	—Mg, +K	—Mg, —K
Vacuole				
Hippeastrum	2.48 *	1.06	0	0
Tulipa	1.52	0.90	0.16	0.08
Protoplast				
Hippeastrum	1.17 **	0.62	—	—
Tulipa	1.28	0.95	—	—

* pmol P_i/(vacuole · h).
** pmol P_i/(protoplast · h).

higher than that of protoplasts from the same tissue. This is unexpected since, as noted above, protoplasts rupture in the assay medium and release about an equal number of vacuoles, which in turn break down to tonoplast vesicles. Approximately equal enzyme activities would be expected on a protoplast, vacuole or equivalent tonoplast basis. Further experiments are needed to clarify the findings in Table I, but two explanations under consideration are (1) that a natural or exogenous inhibitor is present in protoplasts or protoplast suspensions, which is removed during vacuole isolation; (2) that the process of membrane annealing following rupture of protoplasts results in vesicles of mixed membrane origin with imperfectly oriented or exposed ATPase.

Vacuolar ATPase shows clear dependence on Mg^{2+} and stimulation by KCl (Table II). ATPase activity in (ruptured) protoplasts is also increased by KCl. The location of KCl-stimulated ATPases in membrane fractions from roots is

TABLE III

EFFECT OF VARIOUS INHIBITORS ON THE PETAL ATPase ACTIVITY

Reaction mixture contained 3 mM ATP (sodium salt), 33 mM Tris/2-(*N*-morpholino)ethanesulfonic acid, 1.5 mM $MgSO_4$, and 50 mM KCl, pH 6.0. The method of Hirs [25] was used for protein determination, with bovine serum albumin as standard.

	Vacuole		Tonoplast		Protoplast	
	Hippeastrum	*Tulipa*	*Hippeastrum*	*Tulipa*	*Hippeastrum*	*Tulipa*
Control	2.76 *	1.72	1.94 **	1.39	0.99 ***	1.06
Oligomycin, 5 μg/ml	2.69	1.73	—	—	0.65	0.58
Dio-9, 5 μg/ml	1.79	1.18	1.18	0.66	0.60	0.57
CCCP, 10 μM	2.90	1.85	—	—	1.04	1.17
DCCD, 25 μM	3.10	2.01	—	—	1.11	1.25
EDAC, 5 mM	0	0	—	—	0.23	0.51
Tartarate, 15 mM	1.50	0.75	0	0.41	—	—

* pmol P_i/(vacuole · h) or 15.9 μmol P_i/(mg protein · h)
** pmol P_i/(tonoplast · h) or 216 μmol P_i/(mg protein · h)
*** pmol P_i/(protoplast · h) or 1.65 μmol P_i/(mg protein · h). Average protein content per tonoplast, vacuole or protoplast was respectively 0.009, 0.174 and 0.604 ng. A unit tonoplast is the amount of membrane recovered from the lysis of one vacuole.

disputed [7,8]: our data do not exclude the possible existence of KCl-sensitive ATPases in the plasmalemma or other membranes in addition to the tonoplast.

70—80% of the intact vacuole ATPase activity was recovered in tonoplast preparations (Table III), but no ATPase activity was found in the vacuolar sap. Oligomycin at 5 μg/ml was found to have no effect on vacuole ATPases but inhibits protoplast ATPase activity by 35—40% (Table III). Oligomycin is known to inhibit the ($Na^+ + K^+$)-ATPase of animal cell membranes [9] and the uncoupler-stimulated ATPase of plant mitochondria [10]. However, Leonard and Hodges [11] found the plasmalemma ATPase of oat root to be insensitive to oligomycin. The inhibitory effect of oligomycin on mitochondrial ATPase may account for the observed inhibition of protoplast ATPase. The oligomycin insensitivity of vacuole suspensions provides evidence for the homogeneity of the preparation.

In chloroplasts, Dio-9 was found to inhibit photophosphorylation, ATP formation in the acid-bath phosphorylation system [12] and Ca^{2+}-ATPase activity of the purified coupling factor [13]. Dio-9 at 5 μg/ml inhibited vacuolar, tonoplast and protoplast ATPase to the same extent, approx. 40%, suggesting that Dio-9 is a general inhibitor for ATPases.

Collapse of trans-membrane proton gradients by uncoupling agents is a well known phenomenon. When energy for creating the proton gradient arises from ATP hydrolysis, uncouplers should increase the ATPase activity [14]. At 10 μM, carbonyl cyanide m-chlorophenylhydrazone (CCCP) facilitated ATP hydrolysis in both vacuole and protoplast suspensions (Table III), in keeping with a role for the ATPase in ion pumping.

DCCD is a powerful inhibitor of plasmalemma ATPase in oat root [11]. It appears to bind covalently to the membrane-bound ATPase system from *Streptococcus* [15]. However, in spinach chloroplasts Douce et al. [16] found the envelope associated Mg^{2+}-dependent ATPase to be insensitive to DCCD. At low concentration DCCD stimulates photophosphorylation in EDTA-treated spinach chloroplasts [17] and it also stimulates oxidative phosphorylation in submitochondrial particles [18]. Our results show that at 25 μM, DCCD stimulates the rate of ATP hydrolysis in both vacuole and protoplast suspensions by about 10%.

The water soluble carbodiimide, EDAC, completely blocks vacuole ATPase activity and strongly inhibits protoplast ATPase activity (Table III). An inhibitory effect of EDAC can be expected if the ATPase is exposed to the outer surface of the membrane. McCarty [19] found that EDAC inhibited photophosphorylation but not acid-bath phosphorylation in chloroplasts and suggested that the electron flow rather than the phosphorylative reaction was blocked. However, in the vacuole system where there is no electron flow coupled to ATPase function, EDAC is still effective.

(+)-Tartarate is known to be a very potent inhibitor of certain phosphatases [20]. However, its effect on ATPases has not been carefully studied. As shown in Table III, (+)-tartarate at 15 mM, is a very powerful inhibitor of tonoplast ATPase. Porcine cerebral cortex microsomal ($Na^+ + K^+$)-activated ATPase (Sigma) was completely inhibited by the same concentration of (+)-tartarate.

Nucleotide specificity studies showed that the vacuole ATPase is specific for

TABLE IV

EFFECT OF VARIOUS SALTS ON THE ATPase ACTIVITY OF *TULIPIA* VACUOLES

Reaction mixture contained 3 mM ATP (Tris salt), 50 mM Tris, 33 mM 2-(*N*-morpholino)ethanesulfonic acid, 1.5 mM $MgSO_4$, and 50 mM salt, pH 8.0.

	Relative activity	
	Petals	Leaves
Control (- salt)	1.00 *	1.00 **
KCl	1.52	2.10
NaCl	1.22	2.04
LiCl	0.77	1.76
Choline Cl^-	0.88	1.76
NH_4Cl	1.31	2.22
$KHCO_3$	0.70	1.22
KNO_3	0.98	1.10
K^+ acetate	1.22	1.50
KI	0.48	0.52
KBr	0.82	1.23

* Absolute control value in petals is 1.24 pmol P_i/(vacuole · h).
** Absolute control value in leaves is 1.68 pmol P_i/(vacuole · h).

ATP. For *Tulipa* petal vacuoles, the relative activities were 1.0 : 0.3 : 0.5 : 0.5 for ATP : GTP : CTP : UTP respectively as substrate, and for leaf vacuoles, 1.0 : 0.35 : 0.37 : 0.45. No acid phosphatase activity was found when *p*-nitrophenyl phosphate was tested as a substrate under the same assay conditions. The enzyme activity determined in this study is clearly ATPase and not acid phosphatase.

Table IV shows that the vacuole ATPase in *Tulipa* petals is stimulated by cations only, whereas in *Tulipa* leaves it is stimulated by both cations and anions. An anion-sensitive ATPase, possibly released from the outer face of the tonoplast, has been found in a turnip root microsome fraction [21], and Cambraia et al. [22] have described a cation- and anion-stimulated ATPase from the plasmalemma of oat roots.

Cation contents of intact vacuoles and protoplasts were determined by atomic absorption spectrophotometry, after protein and other macromolecules were precipitated by addition of an equal volume of cold 10% trichloroacetic acid (w/v). For Cl^-, 2 ml of sample was mixed with 2 ml concentrated HNO_3 and

TABLE V

MILLIMOLAR ION CONCENTRATION IN INTACT VACUOLES AND PROTOPLASTS

Values were taken from the mean of six determination.

	Vacuoles		Protoplasts	
	Hippeastrum	*Tulipa*	*Hippeastrum*	*Tulipa*
K^+	150 ± 15	148 ± 18	135 ± 20	140 ± 10
Na^+	46 ± 8	50 ± 10	42 ± 5	40 ± 7
Mg^{2+}	30 ± 5	33 ± 4	32 ± 6	26 ± 7
Ca^{2+}	6 ± 2	8 ± 3	8 ± 3	10 ± 3
Cl^-	30 ± 7	32 ± 6	35 ± 8	34 ± 6

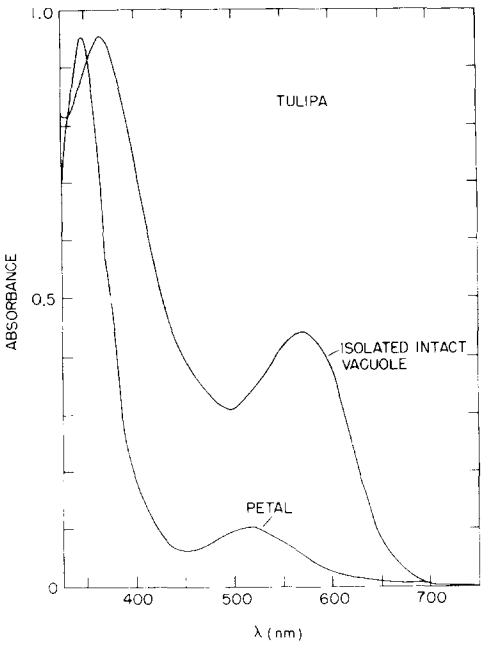

Fig. 1. Absorption spectra of isolated intact vacuole suspension and of epidermis peel from *Tulipa* petal. The traces were normalized at the blue absorbance maxima.

0.5 ml $AgNO_3$ (5000 μg/ml Ag). The mixture was then diluted with distilled water to 10 ml and centrifuged at 2500 × *g* for 10 min. Supernatant aliquots were then diluted 1 : 100 with H_2O for determination of silver concentration by atomic absorption.

Table V shows the ion concentrations within isolated vacuoles and protoplasts. No significant difference was found between the vacuole and protoplast. Since vacuoles usually occupy 90% or more of the volume of a mature plant

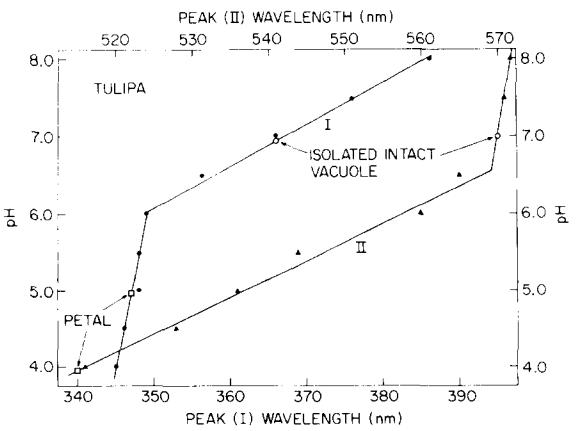

Fig. 2. Effect of pH on wavelengths of maximal absorbance of anthocyanin extracted from *Tulipa* petal. ●——●, shorter wavelength peak (peak I); ▲——▲, longer wavelength peak (peak II).

cell, this is to be expected. The internal P_i concentration cannot be detected owing to the use of P_i buffer in vacuole isolation, but from analysis of intact protoplasts we conclude it must be negligible. Large amounts of organic acid anions inside the vacuole presumably make up the balance of the charge.

Absorption spectra of isolated intact vacuoles in pH 8.0 buffer and of freshly peeled petal epidermis were recorded and compared with spectra of anthocyanin in buffer solution at different pH values. Absorption peak locations were used to determine the vacuole pH. Fig. 1 shows the absorption spectra of *Tulipa* petal peels and isolated intact vacuole suspensions. Two major peaks were found in both spectra, the blue peak being named peak I and the red, peak II.

In Fig. 2, showing wavelength maxima plotted against pH, biphasic curves were found for both peaks I and II. For the lower wavelength maximum (peak I), more peak position shift was found in the higher pH region, whereas for peak II more shift was found in the lower pH region. Both peak I and II show that the isolated intact *Tulipa* vacuole has an internal pH of 7.0, but that the vacuoles in petal epidermis peel have an internal pH of 4.0 when peak II position is used for the determination, or 5.0 when peak I is used. Because there is more peak position shift with pH change for peak II, we suggest that pH 4.0 is the more probable value.

Fig. 3 shows the same experiment as described in Fig. 1 using *Hippeastrum* petals and preparations. Again, two major peaks were found in both samples. By using the same analysis procedure used for *Tulipa*, it was found that *Hippeastrum* petal has an internal pH of about 4.3 while isolated intact vacuoles have an internal pH of about 7.3 (Fig. 4).

The loss of protons and organic acid anions from the vacuoles, during isolation could lead to this observed pH rise. Alternatively, vacuoles might rupture and reseal during isolation, thus allowing partial equilibration of pH

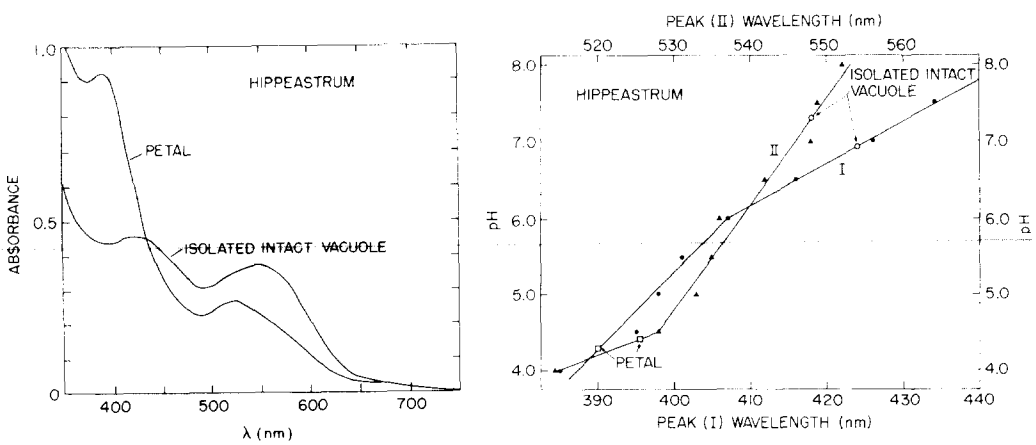

Fig. 3. Absorption spectra of isolated intact vacuole suspension and of epidermis peel from *Hippeastrum* petal. The vacuole spectrum was normalized at 430 nm to that of the petal.

Fig. 4. Effect of pH on wavelengths of maximal absorbance of anthocyanin extracted from *Hippeastrum* petal. ●——●, shorter wavelength peak (peak I); ▲——▲, longer wavelength peak (peak II).

with the more alkaline buffer phase. For the following reasons we tend to rule out this latter explanation: (a) the ionic contents of the isolated vacuoles are markedly different from the buffers used and are similar to the contents of *Nitella* vacuoles [23] and higher plant root cells [24]; (b) no anthocyanin leakage was detected during the vacuole isolation [2]; (c) when *Tulipa* leaf vacuoles, which are colorless, were isolated in phosphate buffer saturated with Evan's Blue, no blue vacuoles can be seen in the preparation; and (d) several hydrolytic enzymes which are very active in the cytoplasm are not present in the vacuole sap (Butcher, Wagner and Siegelman, in preparation).

The finding of ATPases in the tonoplast implies that the vacuole has an important role in regulating the ion balance of plant cells, and experiments are in progress to determine ion flux rates, especially for H^+, in the intact vacuole. The direct measurements of ATPase activity, ion fluxes, and the membrane potential of the vacuole will improve our understanding of its function in plant cells.

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References

1 Hodges, T.K. (1973) Adv. Agronom. 25, 163—207
2 Wagner, G.J. and Siegelman, H.W. (1975) Science 190, 1298—1299
3 Hodges, T.K. and Leonard, R.T. (1974) Methods Enzymol. 32, 392—406
4 Martin, J.B. and Doty, D.M. (1949) Anal. Chem. 21, 965—967
5 Leonard, R.T., Hansen, D. and Hodges, T.K. (1973) Plant Physiol. 51, 749—754
6 Fisher, J. and Hodges, T.K. (1969) Plant. Physiol. 44, 385—395
7 Leigh, R.A., Williamson, F.A. and Wyn Jones, R.G. (1975) Plant Physiol. 55, 678—685
8 Leonard, R.T. and VanDerWoude, W.J. (1976) Plant Physiol. 57, 105—114
9 Whittman, R., Wheeler, K.P. and Blake, A. (1964) Nature 203, 720—724
10 Stoner, C.D., Hodges, T.K. and Hanson, J.B. (1964) Nature 203, 258—261
11 Leonard, R.T. and Hodges, T.K. (1973) Plant Physiol. 52, 6—12
12 Jagendorf, A.T. and Uribe, E. (1966) Brookhaven Symp. Biol. 19, 215—245
13 McCarty, R.E., Guillory, R.J. and Racker, E. (1965) J. Biol. Chem. 240, PC 4822—4823
14 Lin, W. and Hanson, J.B. (1974) Plant Physiol. 54, 250—256
15 Harold, F.M., Baarda, J.R., Baron, C. and Abrams, A. (1969) J. Biol. Chem. 244, 2261—2268
16 Douce, R., Holtz, R.B. and Benson, A.A. (1973) J. Biol. Chem. 248, 7215—7222
17 McCarty, R.E. and Racker, E. (1967) J. Biol. Chem. 242, 3435—3439
18 Racker, E. and Horstman, L.L. (1967) J. Biol. Chem. 242, 2547—2551
19 McCarty, R.E. (1974) Arch. Biochem. Biophys. 161, 93—99
20 Webb, J.L. (1966) Enzyme Metabol. Inhibitors II, 440—442
21 Rungie, J.M. and Wiskich, J.T. (1973) Plant Physiol. 51, 1064—1068
22 Cambraia, J., Balke, N.E. and Hodges, T.K. (1976) Plant Physiol. 57, 84 suppl.
23 Dainty, J. (1962) Annu. Rev. Plant Physiol. 13, 379—402
24 Higinbotham, N., Etherton, B. and Foster, R.J. (1967) Plant Physiol. 42, 37—46
25 Hirs, C.H.W. (1967) Methods Enzymol. 11, 325—329