A new intracellular medium for prolonged viability of noradrenergic storage vesicles from rat brain[1]

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Summary. The biochemical lifetime of noradrenergic storage vesicles from rat brain is greatly extended in a medium based upon the membrane impermeable anion D-tartrate. Examination of the MgATP-dependent accumulation of (−)-^{3}H-norepinephrine suggests that this medium may more closely approximate the intracellular environment of the storage vesicle, and may better support electrochemical gradients existing across the vesicle membrane.

We have recently described an intracellular medium based upon the potassium salt of polyacrylic acid[3]. This medium greatly extended the period of biochemical viability of rat brain storage vesicles as measured by the MgATP-stimulated accumulation of (−)-^{3}H-norepinephrine (NE). The utility of this medium was ascribed to the membrane impermeability of polyacrylic acid and the corresponding potassium salt. The osmotic integrity of the vesicles would thus be preserved in spite of the substantial electrochemical gradients established across the vesicle membrane[4–6]. In conjunction with this work, it was considered advantageous to develop a low molecular weight equivalent of polyacrylate. The following study details the biochemical behavior of noradrenergic storage vesicles from rat brain in a medium prepared from D-tartaric acid, a monomeric equivalent of polyacrylic acid.

Materials and methods. Tartrate buffer was prepared from neutral potassium tartrate (110 mM; pH 7.4). Ascorbic acid (10^{-5} M) and iproniazid (10^{-5} M) were included. The crude vesicle fraction was prepared from rat brain by the method of Seidler et al.[7]. Briefly, Sprague-Dawley rats (150–200 g, either sex) were decapitated, and the brains (less cerebellum) were quickly removed. The tissue was homogenized (Duall glass/glass) in 4 vols of cold 0.32 M sucrose, and sequentially centrifuged (3000 × g, 20 min; 20,000 × g, 30 min; 100,000 × g, 30 min) to obtain a final crude vesicle pellet. The pellet was gently resuspended (teflon/glass homogenization) in 500 µl of tartrate buffer. The suspension was brought to a total of 2.0 ml containing (−)NE (10^{-5} M; 20 µCi) and MgATP (10^{-3} M). Uptake studies were performed by warming this suspension to 37 °C. Tissue suspensions reached 37 °C within 10 sec with gentle swirling. At desired intervals 100 µl aliquots were removed, filtered on Whatman GF/A glass fiber filters, and washed with 5 ml of warm (37 °C) buffer. The tritium content of each filter was determined by liquid scintillation counting (minimum efficiency 35%). All samples were corrected for quenching. Uptake values at 4 °C were subtracted. For kinetic analysis, various concentrations of NE were employed, and uptake was terminated at 2.0 min of incubation.

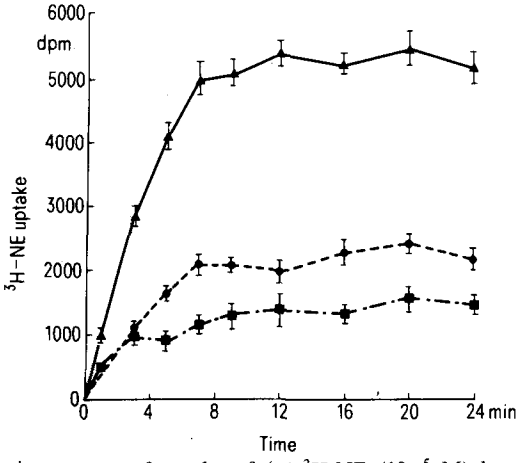
When employed, reserpine pretreatment consisted of a single dose of reserpine (Ciba-Serpasil, 2.5 mg/kg, i.p.) administered 18 h prior to sacrifice. Osmotic strength determinations were made with a Model 3 Osmometer (Advanced Instruments, Inc.). MgATP and (−)-NE bitartrate were obtained from Sigma Chemical Co. (−)-^{3}H-NE (44 Ci/mmole) was obtained from New England Nuclear Corp.

Results. The osmotic strength of 0.110 M potassium tartrate buffer was determined to be 276 mOsM by freezing point depression, from which a free potassium ion concentration of 165 mM is readily calculated.

The time-course of accumulation of (−)-^{3}H-NE at a saturating concentration (10^{-5} M) of NE is shown in the figure. At 37 °C, uptake saturated by 10 min and remained stable for 25 min. Maximal NE accumulation (13.9 ± 0.26 pmoles/mg protein) was reduced to 3.8 ± 0.12 pmoles/mg protein after reserpine pretreatment in vivo, and to 5.7 ± 0.11 pmoles/mg protein in the absence of MgATP. Accumulation at 4 °C was less than 10% of the 37 °C-value.

Kinetic analysis of vesicular ^{3}H-NE accumulation revealed an apparent K_m of 7.6×10^{-7} M.

Discussion. A membrane bound, proton translocating MgATPase maintains substantial electrochemical gradients across the membranes of chromaffin granules and brain storage vesicles[4–6]. Since the gradients are directly coupled to vesicular catecholamine accumulation, it is essential that membrane-impermeable species be employed in experimental media to insure biochemical viability of the organelle. We have previously reported data which suggest that during the process of ^{3}H-NE accumulation by brain vesicles, the vesicle membranes are permeable to phosphate and chloride anions[3]. In these media we have observed vesicular ^{3}H-NE accumulation for periods of 5–10 min, after which time accumulated NE is rapidly lost, presumably by vesicle lysis. This type of behavior has been observed by other investigators[4,7]. The D-tartrate appears to circumvent this behavior, and stable uptake is maintained for periods of 20–40 min. Vesicles can be preincubated in the medium for 20–40 min before the addition of ATP, and still display the same stable uptake properties. It was felt that a total osmotic strength of 276 mOsM with a free potassium ion concentration of 165 mM would represent reasonable approximations of the ionic parameters of the intracellular environment. These values are similar to those observed with potassium phosphate and potassium chloride buffers used by other investigators[4,7]. The characteristics of vesicular ^{3}H-NE accumulation in this medium support the validity of this assumption. ^{3}H-NE accumulation is saturable and stable for extended periods of time. The observed K_m of 7.6×10^{-7} M is within the range of reported values[4,7].



The time course of uptake of (−)-^{3}H-NE (10^{-5} M) by storage vesicles from whole rat brain. ▲, Uptake at 37 °C ●, uptake in the absence of MgATP; ■, uptake after reserpine pretreatment in vivo. (Mean ± SEM for 3–5 determinations).

It is interesting to note that reserpine sensitivity of NE accumulation (77%) is significantly greater than MgATP sensitivity (60%). These results suggest that some residual electrochemical gradient may be preserved in this medium, and may serve to drive some uptake of NE. It has been demonstrated by other investigators that the internal pH of 5.7 observed in chromaffin granules in the absence of ATP represents the zero-potential energy, equilibrium state of the granule[8]. A similar situation may be operative in the brain storage vesicle, and may serve to drive some ^{3}H-NE accumulation. The non-permeant nature of the anions used in this buffer would preserve any resting gradient of the vesicles, a situation which might not be observed with buffers comprised of phosphate or chloride anions. The obervation that reserpine completely inhibits ATP-dependent ^{3}H-NE accumulation, and inhibits half of the ATP-independent accumulation supports this possibility. A similar situation has been observed previously[3]. Alternatively, some exchange with endogenous NE may be occurring in an isoenergetic fashion. HPLC analysis (electrochemical detection) reveals 17.1 pmoles/mg protein of endogenous NE in the vesicle pellet after isolation (data not shown).

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A comparative study of membrane related phenomena in normal and crown gall tissues of red beet (*Beta vulgaris* L.)

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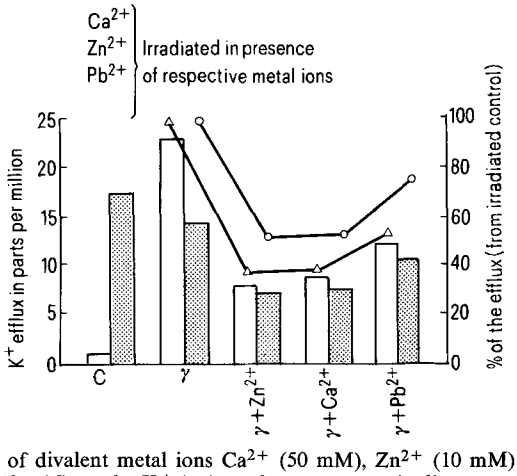
Summary. Divalent metal ions have been found to protect membranes of red beet crown gall tissue more than their adjacent normal regions from thermal or gamma radiation stress, suggesting the possibility of an alteration on the surface charge accompanying tumor formation in plants. Further, tumor tissue has been observed to possess enhanced membrane ATPase activity, a higher tissue sulfhydryl content and increased protein levels, thus suggesting a model of 'source' (normal tissue) and 'sink' (tumor tissue) relationship.

The transformation of a normal cell into a neoplastic cell in plants as well as in animals involves progressive changes in the properties of the membrane systems[3-6]. Altered membrane permeability reported in crown gall tissues (neoplastic tissues in plants formed due to the infection of *Agrobacterium tumefaciens*) by Braun[3] may be due to changes in the membrane lipid composition as has been reported for some crown gall tissues recently[4,5]. Alterations of the membrane surface charge have been frequently encountered with the transformation of cells in animal tissues[6]. There is little evidence to show any such change in the surface charge accompanying tumor formation in plants. In the present study, an attempt has been made to analyze the data obtained on the loss in membrane permeability of normal and crown gall beet root (*Beta vulgaris* L.) tissues. The tissues have been subjected to thermal and/or gamma-radiation stress in the presence of divalent metal ions to show that alteration of the membrane surface charge accompanying tumor formation is also possible in plants.

Besides changes in the membrane permeability, the membrane ($Na^+ + K^+$) ATPase activity and the tissue -SH content which are important factors in the altered function of membrane transport have been studied for both tumors and their adjoining normal regions.

Materials and methods. a) *Tumor induction.* Tumors were induced in beet roots with the help of *Agrobacterium tumefaciens* LBA201 (obtained through the courtesy of Professor S.C. Maheswari, Dept. of Botany, University of Delhi) as had been described earlier[7].

b) *Membrane permeability.* 2-month-old tumors and adjacent normal regions were harvested and cut into slices 4 mm long and 2 mm diameter and washed thoroughly.



Effect of divalent metal ions Ca^{2+} (50 mM), Zn^{2+} (10 mM) and Pb^{2+} (5 mM) on the K^+ leakage from tumor and adjacent normal irradiated tissues. ■, Adjacent normal; □, tumor. ○, %K^+ efflux in presence of metalions in normal tissues; △, %K^+ efflux in presence of metal ions in tumour tissues; C, control; γ, γ-irradiated.

Table 1. Effect of divalent cations on the heat-induced leakage of 260 and 280 nm absorbing materials from tumor and adjoining normal regions (10-12 discs weighing approximately 400 mg). Mean of 3 values. Incubation time - 60 min

Temperature °C	Incubating medium	Tumor		Adjacent normal	
		Optical density (nm)			
		260	280	260	280
20	Buffer (Tricine-NaOH)	0.050	0.070	0.022	0.033
30	-do-	0.069	0.079	0.062	0.077
45	-do-	0.394	0.324	0.090	0.085
45	-do + Ca^{2+} 50 mM	0.086	0.136	0.05	0.052
45	-do + Zn^{2+} 50 mM	0.142	0.155	0.052	0.057
45	-do + Pb^{2+} 5 mM	0.200	0.224	0.05	0.037