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Definition of physical integrity of synaptosomes and myelosomes by enzyme osmometry

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Abstract. Isotonic requirements for synaptosomes were shown to vary with the concentration of sucrose or mannitol in the isolation medium, as well as with their differential permeability to polyols and ions. The technique of enzyme osmometry, which permits quantitation of the osmotic integrity in a heterogeneous population, was used to defined the osmotic requirements for synaptosomes and myelosomes in a variety of ionic and non-electrolyte media. Important differences, observed in the rank order of permeability of synaptosomal and myelosomal membranes to electrolyte media, were consistent with the known channel density/electrical activity of the corresponding plasma membranes.

Keywords. Enzymes osmometry; turbidimetry; synaptosomes; myelosomes; lactate dehydrogenase; osmolytes.

Introduction

Physical integrity of synaptosomes is an essential prerequisite for transport studies *in vitro* and awaits a clear definition. Basically, 3 types of definitive studies were reported in literature that question the inherent assumption of controlled physical integrity of synaptosomes and therefore the validity of such uptake studies in synaptosomes: (i) Sperk and Baldessarini (1977) showed that synaptosomes exhibit pronounced leakage of internal lactate dehydrogenase, when incubated in Krebs-Ringer medium, unless stabilized by sucrose; (ii) Adamvizi and Marchbanks (1983) claimed that osmotic 'disruption is never complete and although resealing does occur, the contribution of residual synaptosomes is difficulty to distinguish' in the experimental protocols for uptake studies *(cc.* Kanner and Sharon, 1978; Kanner, 1978; Roskoski *et al.,* 1981); (iii) dramatic changes in the permeability characteristics of biological membranes such as synaptosomes, mitochondria, peroxisomes (Sitaramam and Sarma, 1981a,b; Sambasivarao and Sitaramam, 1983), myelosomes (Sarma and Sitaramam, 1982), lysosomes (Reijngoud and Tager, 1977), hepatocytes (Sainsbury *et al.,* l979) were shown to occur during centrifugation, leading to equilibration of small molecular weight substances such as sucrose, with consequent altered isotonic requirements. Thus, a methodological reappraisal of the integrity of synaptosomes has become truly complex, not only in relation to preparative methods, but also in terms of osmotic requirements for studies on transport.

These considerations have prompted us to investigate and delineate the physical criteria of integrity of synaptosomes, which is long over due in neurochemical research. The still novel technique of enzyme osmometry is derived from a quantitative interpretation of the activity profiles of occluded enzymes, based on the robust physical theory of osmosis. In this paper, we document briefly the methodology and

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Abbreviations used: LDHase, Lactate dehydrogenase; B.P., break point

applications of enzyme osmometry as relevant to synaptosomal integrity, for the first time. This technique also aided in the elucidation of the structure of osmotically active myelin particles, the myelosomes (Sarma and Sitaramam, 1982) which was independently confirmed subsequently (Pereyra and Braun, 1983). The technique of enzyme osmometry was used also to demonstrate the different osmotic requirements for the integrity of synaptosomes and myelsomes.

Materials and methods

Materials

Sucrose, sodium pyruvate, mannitol,*ß*-NADH, Tris and Triton X-100 were Obtained from Sigma Chemicals Co., St. Louis, Missouri, USA. All other reagents were of analytical grade.

Isolation of synaptosomes

Synaptosomes from rat neocortex as well as motor cortex of Rhesus monkeys (*Macaca mullata*) were isolated by method of deRobertis *et al.* (1962). The crude mitochondrial fraction (P2) was resolved into 5 fractions labelled as A-E., corresponding to the interface of the discontinuous sucrose gradient: A (0·32–0·8 M), B(0·8–10 M). C(1·0–1·2 M), D(1·2–1·4 M) and E (as a pellet in 1·4 M). Aliquots of fractions C and D were diluted slowly, with ice-cold, glass distilled water under constant stirring, to a final sucrose concentration of 0·32 M sucrose. The particles were washed twice with 0·32 M sucrose at 0·4°C by centrifugation at 10,000 *g* to yield fractions C_L and D_L respectively (*cf.* Sitaramam and Sarma, 1981a).

Preparation of 'mannitol' synaptosomes

Whole brains from 4 rats were homogenized in 0·3 M mannitol medium (without EDTA or buffer to avoid undue clumping of particles) and were processed according to the method of Kanner and Sharon (1978) in a discontinuous ficoll gradient with 0·3 M mannitol throughout. The 8–12% ficoll interface were collected and diluted with 3 volumes of 0·3 M mannitol and centrifuged again to obtain 'mannitol' synaptosomes, free of ficoll, finally suspended in 0·3 M mannitol.

Isolation of myelosomes

Osmotically active myelin particles were obtained from primate cortical white matter as described earlier (Sarma and Sitaramam, 1982). Crude mitochondrial fraction (P_2 (M)) and purified myelin (fraction A(M) at 0·32–0.8 M sucrose interface) were harvested by an isolation technique identical to that of deRobertis *et al.* (1962).

Isolation of erythrocytes

Erythrocytes from unanaesthetized rats were obtained by washing heparinized blood

thrice with 15 vols of 0·9% (w/v) NaCl at room temperature. Erythrocyte osmolysis curves were obtained by measuring release of hemoglobin in NaCl media of varying tonicity as described earlier (Sambasivarao and Sitaramam, 1983).

Identification of density gradient fractions by marker enzymes

Relative specific activity of lactate dehydrogenase (LDHase, L-lactate:NAD^+-oxidoreductase, EC 1·1·1·27), monoamine oxidase, acetyl and butyryl-thiocholine-sterases, β-galactosidase, 2'3'-cyclic nucleotide 3'-phosphohydrolase were determined in each fraction to confirm that the isolation procedures match those reported in literature (*cf.* deRobertis *et al.,* 1962; Jones, 1974). The enriched fractions of the rat neocortex consisted of: (i), Myelin; (ii), nonspecific membranes; (iii), synaptosomes; (iv), synaptosomes with contamination of mitochondria and lysosomes; (v), mito-chondria and lysosomes. In the subfractionation of motor cortex of primate brain, results were similar with minor quantitative differences (data not given).

General

Protein was estimated by a modified method of Lowry in the presence of sodium dodecyl sulphate (Markwell *et al.,* 1978). LDHase and turbidimetry assays were carried out using Gilford Spectrophotometer with automatic cuvette positioner and recorder (Sitaramam and Sarma, 1981a). Bufer and substrate concentrations in the assays were reduced so as to minimize their influence on the osmolality of the external medium. Initial velocities were determined for specific activity calculations reaction velocities were first order with regard to synaptosomal protein.

Enzyme osmometric methodology (Sitaramam and Sarma, 1981a,b, Sambasivarao and Sitaramam, 1983)

The first premise in the technique of enzyme osmometry is that the activity of an occluded enzyme, E, under initial velocity conditions, is

$$J_r = KA_o/(1 + K/P)$$
$$\text{and, } A_i = \text{Ao}/(1 + K/P)$$
$$\text{since, } J_r = KA_i, \qquad (1)$$

where K is the rate constant of the enzyme and P, the permeability to the external substrate, A_O*. The second premise is that the osmotic behaviour of a population of

*The equation implies an equality that $P(A_0 - A_i) = KA_i$ which is valid only for an occluded enzyme. An occluded enzyme, typified by LDHase, is defined as that whose activity is stimulated significantly, at constant A_o,when the membrane barrier is removed by means such as solubilization (detergents) or disruption (freeze-thaw etc.), which do not significantly interfere with the activity of the occluded enzyme. Presence of occluded activity, therefore, necessarily implies $A_o > A_i > A_c$, where A_c is the minimal substrate concentration corresponding to V_{max}.-δ, where δ is an immeasurably small decr4ement in activity (since V_{max} requires an infinite substrate concentration). This excludes the possibility of inequality of the kind $P(A_O - A_i) > KA_i$ due to $A_i > A_c$. Similarly, initial velocity conditions (which is a rigorous equilibrium assumption) itself excludes the inequality of the kind, $P(A_O - A_i) < KA_i$ since $J_r = KAi$.

In principle, one may argue that non-osmotic leakage of enzyme, cofactors or inhibitors could affect

cells or organelles would obey the linear Boyle-van't Hof relationship only within critical limits of external osmolality as depicted in figure 1. The osmotic phenomena broadly fall into two categories. In one, the measurements are associated with lysis,

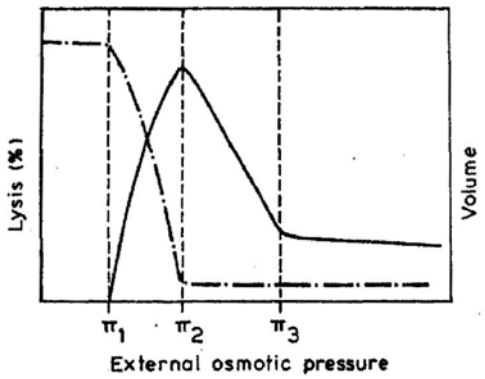

Figure 1. Osmotic profiles in membranous particles. The volume of a population of particles varies with external osmotic pressure linearly within two critical limits, π_2 and π_3, such that the isotonicity corresponds to $(\pi_2 + \pi_3)/2$. The onset of lysis corresponds to the maximal volume of the particles at π_2. Total lysis of the population is achieved at a critical external osmotic pressure, π_1, corresponding to 'zero' internal (i.e., occluded) volume of the particles. An evaluation of the points of inter-section of the individual regression lines yields the critical external osmotic pressures π_1–π_3. The Y-coordinate values corresponding to the break-points at π_2 and π_1 yield '0' and 100% lysis (or 100 and 0% integrity, respectively).

activity and even contribute to inequality. This is simply overcome by the fact that break-point is independent of activity, since it represents the critical external osmolality at which the activity exhibits a marked non-linearity. Break-point represents a true non-linearity *(i.e.,* discontinuity) because: (i) it is independent of transformations on either or both axes, and (ii) no single polynomial, that too of a high order, can account for all the experimentally observed profiles in a variety of systems and (iii) the break-point remains virtually the same even when a significant amount of the occluded enzyme is released and the particles resealed, as in fractions P_2, C_L and D_L. Non-osmotic mechanisms of leakage (of either enzyme or endogenous modulators of activity), if any, should exhibit only a monotonic profile as a function of osmolality. Any true non-linearity would arise only due to an osmotic mechanism and would only serve to reinforce the break-point measurement. It would be a truism to state that any or a set of linear operators would not impart non-linearity to a response. However, the signal-to-noise ratio (*K/P*) may be afected. Thus, absence of an osmometric profile need not be suficient evidence for the absence of a barrier, while its presence is the most conclusive evidence for osmotic behaviour.

Since there could always be some external contamination of the enzyme, a more complete expression would be,

$$J, = KA_a\,[\{E_b/(1 + K/P\} + E_f],$$
$$E_{total} = E_b + E_f$$

where E_b is the total occluded enzyme and E_f is the external free enzyme. Another kind of inequality can arise in the osmotic space, if either *K* or *P* vary with the volume of the particles. This is excluded by critically showing that break-point of osmometry is identical to that of osmolysis, as shown in the case of LDHase. Existence of a break-point in the non-lytic domain has interesting implications on the nature of transport (vide discussion). In any event, the kinetic and physical assumptions inherent to the osmometric analysis are eminently testable.

wherein the osmotic heterogeneity of the particles would result in two discontinueties, one corresponding to the onset and the other corresponding to the completion of lysis. The derivative of the lysis profile would be a direct measure of the variance associated with a population of particles, it being a cumulative integral of the osmotic heterogeneity of the entire population. The volume profile, the second category, which is the signal-averaged response of a population, does not yield readily information on the heterogeneity of the population and is also characterized by the presence of two discontinuities, corresponding to the limits of linear osmotic response of a population of particles. The discontinuities can be evaluated by fitting the individual slopes (i.e., $y = m_1x + c_1$ and $y = m_2x+c_2$) across each discontinuity (corresponding to π_1, π_2 and π_3 in figure 1) to regression lines, such that the point of intersection for any pair of slopes, may be obtained as the break-point concentration on x-axis; B.P. = $(c_2-c_1)/(m_1-m_2)$. Lysis can be monitored either by release of occluded enzymes into cell-free supernatants (osmolysis), or, on direct suspension of the particles in the assay medium adjusted to various tonicities by a chosen osmolyte and directly monitoring the activity (osmometry). Given the condition that $P \ll K$ (to ensure good signal-to-ratio), under initial velocity conditions, osmolysis and osmometry would yield identical break-points for the onset of lysis (*i.e.,* expression of occluded activity). If P is also a function of volume of the particle, a break-point to the right of lysis would be obtained in osmometric profiles. A break-point to the left of osmolytic profiles is forbidden. Since volume of the particles can also be monitored by rate of change in absorbance (turbidimetry) (Sambasivarao and Sitaramam, 1983; Sarma and Sitaramam, 1982), one can verify these assumptions in a turbid preparation such as myelosomes (though not readily in synaptosomes) that the break-point of osmometry of turbidimetric data (i.e., volume) always falls to the right of osmometry of occluded enzymes with impermeable substrates or osmolysis.

Figure 2 illustrates these principles using erythrocyte osmolysis profiles. Addition of as little as 50 mM Tris HCl (82 mOs/kg) was seen to cause a measurable shift in the break-point of onset of lysis by ~62 mOs/kg. Similarly, loss of internal K^+ on addition of valinomycin should lower the instantaneous osmotic pressure exerted by internal K^+ and therefore ofer protection against hypotonic lysis by a left-ward shift in the break-point, while enhanced permeability to ions (particularly external Na^+) by addition of gramicidin S should make the cells more susceptible to lysis, as indicated by a marked right-ward shift of the break-point (figure 2C and D) (*cf.* Sambasivarao and Sitaramam, 1985; Sambasivarao *et al.,* 1986).

These results demonstrate the unique capability of the osmometric technique to measure the reflection coefficients to external and internal osmolytes and variations hereof. The instantaneous osmotic pressure required for constant tonicity by a permeable external solute, at time, $t = 1$ is,

$$\pi_{s_{(t=1)}} = \pi_{s_{(t=0)}} + RT.ds \int_{t=0}^{t=1} dt, \tag{2}$$

Such that the shift in break-point for an erythrocyte population was,

$$\Delta\text{B.P.} = RT.J_{Na} + \int_0^t dt - RT.J_K + \int_0^t dt, \tag{3}$$

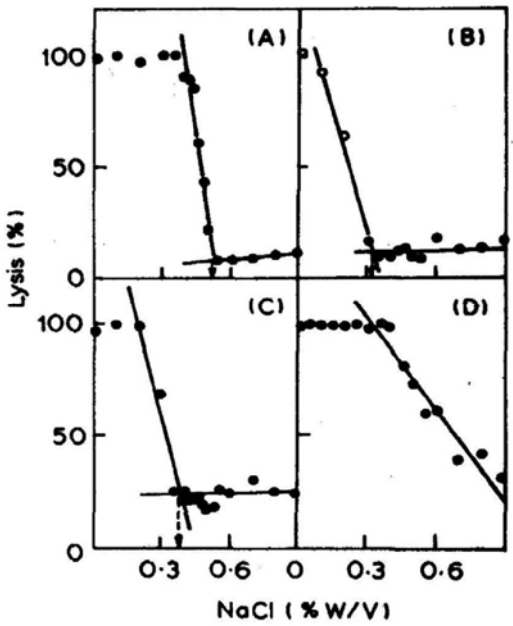

Figure 2. Erythrocyte osmolysis curves. (A), Control osmolytic profile in varying NaCl solutions with 3·3 mM Tris-HCl, pH 7·4; (B), osmolytic profile in the presence of 50 mM Tris-HCI, pH 7·4; (C), osmolytic profile as in A, in the presence of 10^{-6} M vaiinomycin in the osmolytic media; (D), osmolytic profiles as in A, in the presence of 10^{-5} M gramicidin S.

Break-point analysis was carried out as described in the text for both the onset and the completion of lysis, wherever measurable. The figures illustrate only the evaluation of the break-point of the onset of lysis. The break-points (B.P.) (expressed as % NaCl) for the onset of lysis were: (A), 0·53%, (B), 0·33%, (C), 0·387% and (D), not measurable (>0·9%) B.P. Indicated by a dashed arrow.

where J_{Na}^{+} and J_K+ represent the influx and efflux Na^+ and K^+ respectively* (Sambasivarao *et al.,* 1986).

The second unique feature of the technique osmometry of is that the confidence inter-- val associated with the break-point obeys an F distribution (Rao, 1967) such that the

*More generally,

$$\Delta B.P. = \frac{RT}{V_c}\left\{ J_p \underset{1\to2}{} - J_q \underset{2\to1}{} \right\}$$

where V_c is the critical, constant volume corresponding to the break-point (Os/kg), J, the fluxes, i.e., the influx (1→2) of the external (p) and the efflux (2→1) of the internal(q) osmolytes, in time, t, since π = RTC, where C is the concentration. Since,

$$J = L\Delta\mu + J_{act}$$

where L is the Onsager matrix of phenomenological coefficients (including permeability/diffusion coeffi–cints, tranport capacity etc.),- $\Delta\mu$ the passive forces (concentration, electrical forces) and $J_{act,}$ the contri-bution of active mechanisms, one can experimentally derive the relationship between the break-point and the net permeation of any given species of osmolyte, by appropriate design. For a more detailed discussion on channel properties, see Sambasivarao *et al.* (1986).

observed shifts in figure 2 were significant at $P<0.01$. The methodological precautions for the technique of osmometry have been published earlier (Sitaramam and Sarma, 1981b; Sambasivarao and Sitaramam, 1983). A detailed statistical theory (since no ready methodology exists) will be published elsewhere. Briefly, the negative slope is referred to as the determinant slope, whose coefficients of correlation is a measure of the fit of the data to a rectangular distribution, i.e., equal probability of osmotic susceptibility for the entire population, determined by its range, the break-points. The high sensitivity of break-point analysis to systematic error requires that the data on the same day be compared with adequate internal controls rather than data from different preparations on different days.

It should be noted that the variance associated with the break-point (*i.e.,* intrinsic to each profile) results in a coefficients of variation of magnitude less than 5%. However, the profiles, when assayed under identical conditions on the same preparation yield break-points which are almost indistinguishable (i.e., they differ within 1%). As the profiles are susceptible to systematic errors likely to occur over different days, it is natural to expect larger variation in the data base and hence, also the break-points, which indeed was the case. For instance, when the break-points of 6 different $P2$ fractions assayed on different days were compared, the break-points varied within 10% of coefficients of variation. Using a single day preparation, we could show that the variations in the specific activity of occluded LDHase activity (due to its variable removal by lysis) as in P_2, C_L and D_L fractions, did not contribute significantly to a change in break-points. Therefore, one should not pool the data collected over different days to arrive at estimates of reliability or robustness of the break-points by mere calculation of standard deviation for the break-points.

Osmolality vs concentration

The osmolality of various assay media were routinely determined using a Wescor vapour pressure osmometer. In osmometric as well as osmolytic experiments, break-point analysis was routinely performed, using concentration of external osmolyte rather than osmolality, since the break-points represent a robust measurement of material properties, relatively independent of transformations (*i.e.*, molarity, molality, osmotic pressure (bar)) on the primary variable, *viz.* the osmotic pressure of the external medium.

Results

The experimental results reported here have 4 specific objectives, *i.e.*, (i) to illustrate the efficacy of the technique of osmometry to achieve a clear definition of the physical integrity synaptosomes and myelosomes; (ii) to resolve the controversy whether 100% lysis of synaptosomes can indeed be achieved, as prelude to studies on transport with defined internal milieu; (iii) to identify standardized protocols in the investigation of integrity of synaptosomes; and lastly, (iv) to investigate the differences between the osmotic behaviour of synaptosomes and myelosomes. The definition of integrity was throughout restricted to physical integrity in terms of matrix space enclosed by a limiting membrane (which alone is measurable and is applicable to all cells and organelles), avoiding more qualitative notions such as metabolic integrity.

Enzyme osmometry of LDHase in synaptosomal fractions

Phenomenological homology (i.e., identical break-points) of LDHase osmometry (*i.e.,* direct measurement of activity of LDHase in particulate suspensions without phase separation) and osmolysis (i.e., release of the enzyme into supernatants, as in the case of hemoglobin) was documented by us earlier (Sitaramam and Sarma, 1981a). This equivalence emerges as a necessary consequence of the impermeability of the exogenous NADH to the cell membrane. LDHase osmometry in various synaptosomal fractions isolated in a constant 0·32 M sucrose media was consistent with the theoretical predictions (Sitaramam and Sarma, 1981a,b) thus: (i) a marked negative slope bounded by a discontinuity (break-point) was seen in the osmometric profiles of various fractions (also in figure 3 A–C); (ii) the physical basis of LDHase osmometry was identical to that of osmolysis, since the break-points were identical; (iii) the break-point of LDHase osmometry matched not only the sucrose content within the organelle, but also the ambient sucrose concentration of each fraction

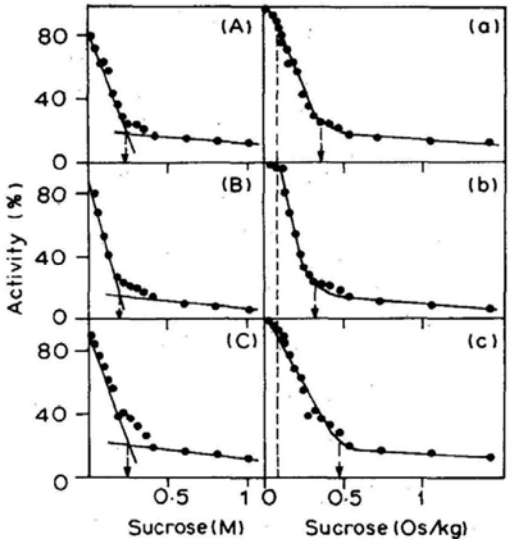

Figure 3. LDHase osmometry of synaptosomal fractions, P_2(A,a), C_L(B,b) and D_L(C,c), isolated in 0·32 M sucrose. The osmometric profiles in A–C represent the activity of the enzyme plotted as a function of external sucrose concentration, assayed in the presence of 50 mM Tris-HCl. The osmometric profiles in a–c represent the activity plotted as a function of osmolality of the assay medium; the osmolality was lowered further by decreasing Tris HCl concentration from 50–10 mM (i.e., below zero sucrose concentration indicated by a vertical dashed lines). Break-point analysis was carried out in all cases as described in the text. B.P. (indicated by a dashed arrow) values were: (A), 026 M; (B), 0·27 M; (C), 0·27 M, external sucrose; and (a), 0·35; (b), 0·3; (c), 0·37 Os/kg. The regression lines of break-point analysis were depicted in A–C; a-c represent visual fit of the sigmoidal osmometric profiles to facilitate comparison with figures 1 and 2. 100% activity was taken as that obtained in 10 mM Tris-HCl, for reasons discussed in the text. Specific activities of total LDHase in these fractions were comparable to those reported by Sitaramam and Sarma (1981a), for rat neocortical synaptosomal fractions.

isolated by density gradient centrifugation at 0–4° C; (iv) the break-point was Sensitive to the permeability to the external solute, such that the osmometric profiles yielded the rank order of permeability, i.e., sucrose < Na^+ < K^+.

Methodological investigations in a wide variety of systems led to the following rules in obtaining osmometric profiles of good statistical quality. (i) Osmotic pressure of the external medium should be predominantly contributed by a single osmolyte of choice. (ii) The substrate should have very low permeability and the occluded enzyme high activity, to ensure good signal-to-noise ratio in break-point analysis. (iii) Initial velocity conditions should be strictly adhered to. (iv) Velocity should be first order with regard to protein in the assay medium at all osmolalities, (v) The external osmolyte should exhibit only a monotonic profile of inhibition of the solubilized enzyme with a slope much smaller than that obtained in enzyme osmometry.

These studies posed specific cautions with regard to the criteria of integrity of synaptosomes. Release of macromolecules such as LDHase or hemoglobin is the only reliable measure of the integrity of membranes, but not that of ions such as K^+ because: (i) large changes in break-point in K^+ media (compared to sucrose media) indicating rapid permeation of K^+; (ii) the reported prelytic increase in K^+ permeability in erythrocytes (Seeman *et al.,* 1969); (iii) osmotic dependence of K^+ exchangers (Garlid, 1980); (iv) enhanced permeability to K^+ in synaptosomes (Sitaramam and Sarma, 1981a) and erythrocytes (Davson and Danielli, 1938) during centrifugation and (v) mediation of K^+ influx depending on the metabolic state *via* Na^+, K^+ ATPase. Attempts to quantify the integrity of synaptosomes in terms of K^+ content (Adam Vizi and Marchbanks, 1983) would be unfructuous, since marked changes in K^+ content could occur without membrane disruption, hypotonic or otherwise. Even the release of LDHase should be interpreted Width caution, as shown below.

The specific question with regard to release of LDHase was whether it yields evidence for 100% lysis of synaptosomes. 100% lysis would be indicated by (i) ability to assay all the occluded activity in hypotonic media, the total activity judged from triton X-100 solubilized synaptosomes and (ii) the true sigmoidal shape of the osmolytic/osmometric profile as in the case of erythrocytes, such that maximal lysis corresponds to 100% lysis. Osmometric profiles in figure 3A–C, of fractions P_2, C_L and D_L (all of which were isolated in 0·32 M sucrose) show that even at zero sucrose concentration, 10–15% synaptosomes remain intact, as also pointed out by AdamVizi and Marchbanks (1983). Since the usually recommended bufer concentration, *i. e.,* 50 mM Tris-HCl (Marchbanks, 1967; Whittaker and Barker, 1972), alone would contribute to a finite (measured) osmotic pressure of 82 mOsm/kg, we varied Tris-HCl from 10–50 mM in the absence of sucrose, as an extension of the hypotonic domain and measured the LDHase activity as a function of true osmolality. Indeed the osmometric profiles yielded not only a sigmoidal profile as in the case of erythrocytes but also further release of LDHase. The activity of LDHase on solubilization by Triton X-100 was actually lower by 5–10% than that obtained in 10 mM Tris-HCl buffer. Thus, the activity of LDHase in these fractions in 10 mM Tris-HCl buffer approximated the total activity rather than that with Triton, which was marginally inhibitory. The break-points in the osmolality scale (figure 3a–c) were larger than those in the molarity scale (figure 3A–C), as expected of the contribution of the buffer to osmolality. It should also be noted that such a conversion of the ordinate amounts to a transformation of the independent variable, leading to

finite, though relatively minor, variations in the true break-point concentration. In figure 3, plots in both scales were given specifically to illustrate the similarity regardless of the scale chosen in osmometric studies. Plotting in the osmolality scale also revealed the close proximity of the break-point to the isotonic medium of 0·32 M sucrose (350 mOs/kg), showing that the nearly spherical synaptosomal populations exhibit very little capacity for volume increase. Even the biconcave erythrocytes cannot withstand the surface area expansion beyond 3% without lysis (Linderkamp and Meiselman, 1982). The YYintercept at the break-point corresponds to the osmotically insensitive basal activity, the external LDHase contamination, by 8–10% . It may be recalled that solubilized LDHase exhibits only a monotonic inhibition in the presence of sucrose (Sitaramam and Sarma, 1981a).

Extent of lysis in synaptosomes prepared in mannitol media

Kanner and Sharon (1978) prepared synaptosomes in 0·3 M mannitol, lysed in 5 mM Tris-HCl and resealed in 0.1 M potassium phosphate buffer, after storage and freeze-thaw in 0·32 M sucrose. Adam-Vizi and Marchbanks (1983) questioned the wisdom of such a complex procedure since no attempt was made by Kanner to quantitate the contribution of non-lysed synaptosomes.

In order to investigate the extent of lysis in 'mannitol'-synaptosomes, synaptosomes were prepared by the method of Kanner and Sharon (1978) and the osmometry of LDHase was carried out with sucrose as an external osmolyte (figure 4). The break-point shifted to left by 25% compared to 0·32 M sucrose synaptosomes (correcting for the differences in the osmolality of the different isolation media), suggesting that the internal mannitol was more permeable than the external sucrose.

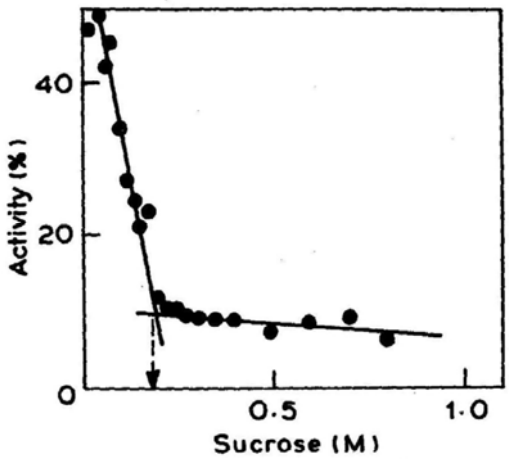

Figure 4. LDHase osmometry for mannitol synaptosomes. Synaptosomes were prepared in 0·3 M mannitol, as described in the text. Total activity, assayed in the presence of Triton X-100 was taken as 100%; the activity assayed in the presence of 50 mM Tris-HCl bufer, pH 7·4, was plotted as a function of the external sucrose concentration. B.P. represented by a dashed arrow, was 0·19 M external sucrose.

A corresponding shift in the negative slope to left resulted in a lysis of only 70-75% of the synaptosomes even in water, or ~50% lysis in 50 mM Tris-HCl. The criticism levied by Adam Vizi and Marchbanks (1983) was therefore valid in so far as the lysis step in 5 mM Tris-HCl was concerned. However, Kanner employed a further step of freeze-thaw which would be expected to disrupt all the synaptosomes. Doubt persists since possible lysis during transport assays was not monitored and the absence of a critical control study of uptake at 0°C at all time points tested was conspicuous.

Isotonic requirements for synaptosomal fractions

The recommended procedure for an evaluation of the integrity of synaptosomes was to assay for LDHase in synaptosomes in 50 mM Tris-HCl buffer and to obtain fold stimulation of the activity on subsequent addition of Triton X-100 during the assay itself (Marchbanks, 1967; Whittaker and Barker, 1972). The magnitude of fold stimulation depends directly on (i) the integrity of synaptosomes, (ii) the amount of LDHase within, (iii) the relative impermeability of the substrate and inversely on (iv) the amount of external contamination of free LDHase, provided that adequate controls exist for absence of NADH oxidation by other enzymatic means and interference due to changes in turbidity on addition of Triton X-100. Since the assay of LDHase as in 50 mM Tris-HCl bufer results in ~ 90% lysis (with consequent reduction in fold stimulation by Triton), we investigated fold stimulation of LDHase activity by Triton, using different fractions of rat brain as well as primate cortical synaptosomes (which were subsequently needed for comparison with myelosomes) obtained by the method of deRobertis *et al.* (1962), as a preliminary step to arrive at their isotonic requirements. Bigure 5a shows that fold stimulation in 50 mM Tris-HCl bufer (i.e., without sucrose) was barely 2-5 fold in various fractions of synaptosomes from primate motor cortex. Though specific activity of LDHase was highest in

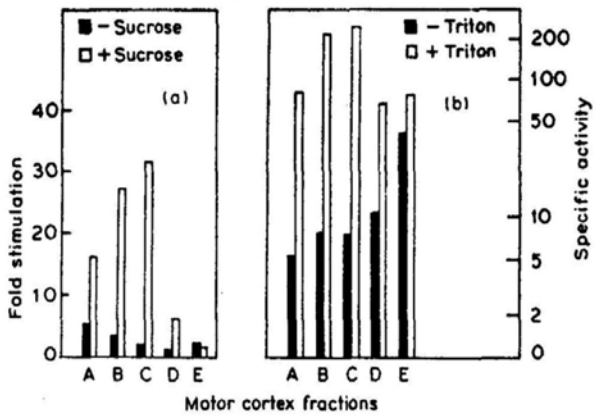

Figure 5. Occluded LDHase activity profiles in various deRobertts *et al.* (1962) fractions isolated from the P_2 fraction of primate motor cortex. LDHase activity was determined in the presence of 50 mM Tris-HCl, pH 7·4, with and without 0·32 M sucrose. Fold stimulation (a) was expressed as the activity in each fraction after solubilization with 0·1% (v/v) Triton X-100, divided by the activity in the particulate suspension. Specific activity in the presence of 0·32 M sucrose and 50 mM Tris-HCl ± Triton was plotted for each fraction in (b). Specific activity was expressed as μmol of NADH oxidized min^{-1} mg^{-1} of protein.

fraction C (figure 5b), fold stimulation by Triton was the least in synaptosomal fractions C and D (figure 5a). Contamination with external LDHase was difficult to visualize in these fractions, since density gradient centrifugation would remove much of the external contamination. On the other hand, inclusion of 0·32 M sucrose in the assay medium resulted in marked improvement of fold stimulation, particularly in fraction C, though not in fraction D. Clearly, fold stimulation would be an ideal marker for integrity and the choice of the assay medium, provided that the choice of the medium is applicable to all the fractions. Figure 5b shows the specific activity of all the fractions in 0·32 M sucrose with 50 mM Tris-HCI bufer with and without Triton. The total activity of LDHase recovered from P_2 was mainly in fractions C, D and A, in that order (data not given). The total specific activity (i.e., in the presence of Triton) was maximal in fraction C, but not in the absence of Triton. In fact, when assayed in 0·32 sucrose media, the basal activity of LDHase in these fractions progressively increased corresponding to sucrose concentration actually ambient during the density gradient isolation of these fractions. This anomalous behaviour could be reconciled with only if the isotonic requirements vary with the ambient sucrose concentration during centrifugal isolation, thereby affecting integrity of various fractions differentially, when assayed at a constant osmolality (Sitaramam and Sarma, 1981a).

Since equilibration of sucrose across biological membranes during centrifugal isolation was unequivocally established (Sitaramam and Sarma, 1981a,b; Sambasivarao and Sitaramam, 1983), we compared fold stimulation of various fractions by varying the sucrose concentration in the assay medium corresponding to that of the medium of isolation for each fraction. Data in table 1 compared LDHase activity (thereby, fold stimulation) in the deRobertis *et al.* (1962) fractions of rat neocortex. Clearly,

Table 1. Occluded LDHase activity in deRobertis *el al.* (1962) fractions obtained from rat neocortex.

Fraction	Isotonic[a] sucrose, M	LDHase specific[b] activity (total)	LDHase specific activity[c] 50 mM Tris-HCl buffer only	+ 0·32 M sucrose	+ isotonic sucrose
P_2	0·32	0·509	0·417 (82)	0·059 (11·6)	0·059 (11·6)
A	0·5	0·284	0·136 (48)	0·063 (22)	0·0457 (16)
B	0·9	0·44	0·387 (88)	0·156 (35)	0·080 (18)
C	1·1	1·04	0·758 (73)	0·066 (6·3)	0·030 (2·8)
D	1·3	0·401	0·340 (85)	0·151 (37·6)	0·037 (9·2)
E	1·4	0·601	0·674 (112)	0·34 (56·5)	0·053 (8·7)

[a]Isotonic sucrose corresponds to the average sucrose concentration of the density interface at which each fraction is isolated, since sucrose was known to equilibrate with the interior during centrifugal isolation of synaptosomes *(vide* text).

[b]Total activity was assayed on prior solubilization in 0·1 % (v/v) Triton X-100.

[c]Aliquots of each fraction were assayed in duplicate in media of different composition, as indicated. Numbers in parentheses represent % activity, taking total activity(b) as 100. Fold stimulation (i.e., b/c) can be computed from the activities given in the table.

fold stimulation was maximal, or basal activity least, at a sucrose concentration corresponding to that of the isolation medium. The phenomenon was valid regardless of whether the source of synaptosomes was rat or primate cortex, or even the primate retina (data not given).

The significance of these experiments is two fold: (i) Since an experimental protocol such as in table 1 permits rapid screening of media for the required tonicity, detailed LDHase osmometric profiles, otherwise tedious and time consuming, may be substituted by such a protocol, (ii) the practice of diluting subcellular fractions from sucrose density gradients directly into the so called 'isotonic' media (0·32 M sucrose for synaptosomes or 0·25 M sucrose for mitochondria) would be logically absurd and would result in uncontrolled bursting and resealing, however slowly diluted. Oxidative phosphorylation in mitochondria, a sensitive index of their integrity, cannot be demonstrated in mitochondria isolated in sucrose density gradients and diluted into 0·25 M sucrose (*cf.* Sambasivarao and Sitaramam, 1983).

Rank order of permeability to electrolytes and non-electrolytes in synaptosomes and myelosomes

Uncontrolled lysis and resealing of synaptosomes during transport assays can be preempted only if the rank order of permeability to electrolytes and non-electrolytes is critically assessed for a given preparation of synaptosomes. Data in figure 6 shows detailed osmometric profiles in fraction C_L of primate motor cortex. Since NaCl and

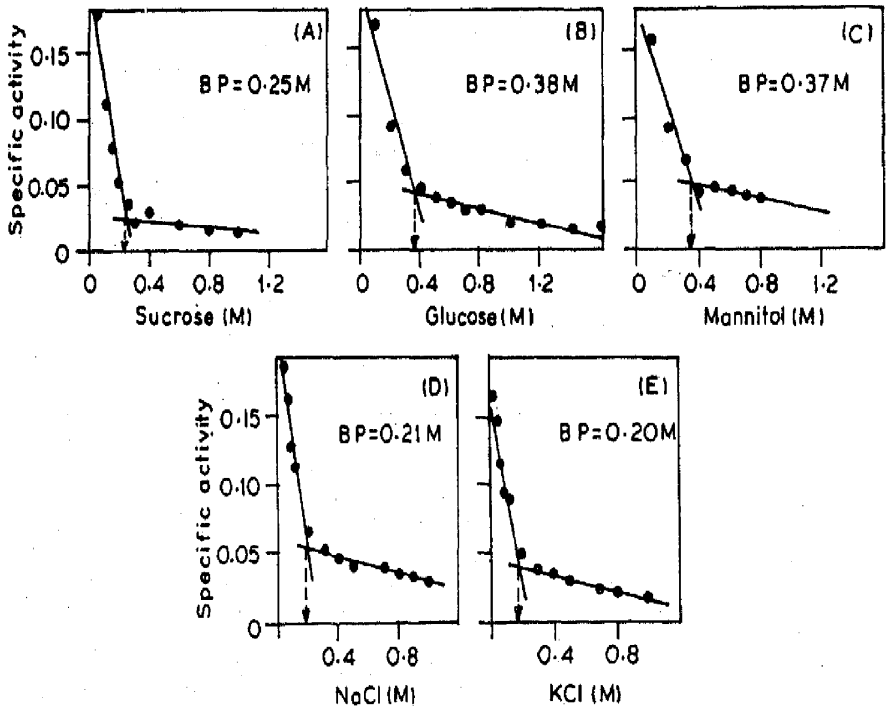

Figure 6. LDHase osmometry in synaptosomal fraction, C_L, from primate motor cortex, in sucrose (A), glucose (B), mannitol (C), NaCl (D), KCl (E), media in the presence of 50 mM Tris-HCl buffer. Break-point analysis was carried out as described in the text (B.P., indicated by a dashed arrow). The osmometric profiles were obtained in a single experiment. Specific activity was defined as in figure 5.

KCl ionize, the osmolality of these salt solutions is ~ 1·86 times its molarity. The data clearly shows that the rank order of permeability (=rank order of break-points) was sucrose < glucose ~ mannitol < NaCl ~ KCl. Thus, as predicted by eq. (2), the technique of osmometry appears to be a reliable method to assess the reflection coefficients to external solutes.

Onset of lysis should correspond to the maximal volume of the particles, *i. e.,* the break-point of volume changes should be larger than that of lysis (figure 1). Classical measurements of reflection coefficients rely on the monitoring of volume changes by light scatter methodology (Goldstein and Solomon, 1961). Carvalho and Carvalho (1979) and Kamino and Inouye (1975) attempted light scatter studies in synaptosomes. Experience with the application of light scatter measurements in erythrocytes showed that it would be hazardous to measure volume changes with concommitant lysis by light scatter (Seeman *et al.,* 1969; Goldstein and Solomon, 1961; Mlekoday *et al.,* 1983; Passow, 1964). The osmometric analysis of turbidimetry circumvents this limitation since it could be critically compared with osmolytic profiles and break-points. Assessment of permeability by the osmometric technique is less direct than the classical method of reflection coefficient measurements. It was therefore necessary to assess volume changes, atleast in a comparable particle, by osmometric methodology. The requirement for such a particle would be: (i) origin from the same tissue/cell, (ii) the presence of occluded LDHase activity and (iii) high capacity of the particle for light scatter. Myelosomes would be the obvious choice, since these particles, as we described earlier (Sarma and Sitaramam, 1982), would also be the choice control material for uptake studies on synaptosomes.

Data in table 2 summarizes the break-point analysis of detailed osmometric profiles of LDHase and turbidimetry in NaCl, KCl, sucrose, glucose and mannitol media in various fractions of synaptosomes from rat and primate cortex as well as myelosomes isolated from the white matter of primate brain. A major advantage in such a comparison was that, while marginal contamination of myelin material in synaptosomes is unavoidable, preparation of myelosomes from pure white matter guaranteed the lack of contamination by synaptosomes. The rapidity of changes in

Table 2. Rank order of permeability to polyols and electrolytes in synaptosomal and myelin (axonal) plasma membranes, as measured by LDHase osmometry and osmometry linked to turbidimetry

	Rat neocortex[a]		Cortical white matter (primate)			Motor cortex[b] (primate)
	C	D	P_2 (M)	A (M)		C_L
Osmolyte	LDHase	LDHase	Turbidimetry	Turbidimetry	LDHase	LDHase
Sucrose	0·49	0·86	0·36	0·61	0·39	0·245
Glucose	N.D.	N.D.	—	—	>0·40[c]	0·378
Mannitol	N.D.	N.D.	—	—	>0·40[c]	0·368
NaCl	0·46	0·59	0·133	0·24	0·12	0·207
KCl	0·51	0·8	0·115	0·1	0·102	0·202

[a]Data from Sitaramam and Sarma, 1981a.

[b]Data from figure 5. N.D. not done. –Data omitted since statistical criteria not satisfied.

[c]Exact break-point could not be determined *(vide* text), Various fractions were isolated at the interfaces of sucrose solutions. C, (1·0–1·2 M), D, (1·2-1·4 M) and C_L (0·32 M) represent synaptosomal fractions; P_2 (M) (0·32) and A (M) (0·32–0·8 M) represent myelosome fractions.

turbidity associated with larger permeability to glucose and mannitol resulted in a severe restriction on the number of reliable data points for osmometry; on the other hand, the high permeability to these polyols also resulted a shift in the break-point of LDHase osmometry to right, to the extent that the break-point could not be evaluated (*cf.* figure 2D). The data clearly showed that glucose and mannitol were more permeable than sucrose. Since neither mannitol nor sucrose would be expected to share the glucose transporter (they being foreign and inert) (Sitaramam and Sarma, 1981a; Sambasivarao and Sitaramam, 1983), the enhanced permeability to mannitol requires adequate explanation. In the case of electrolytes, synaptosomes exhibited greater permeability, while, myelosomes exhibited lesser permeability by osmometry of LDHase as well as turbidimetry. The data confirms the validity of LDHase osmometry, since turbidimetric profiles yielded consistently larger break-points than the corresponding profiles of LDHase. The differences in the rank order of permeability between myelosomes and synaptosomes for electrolytes supports our earlier contention that the myelosomes offer a unique control material to study what is specific to the plasma membrane of nerve endings.

Discussion

The experiments reported here demonstrate unequivocally the importance of the technique of osmometry in the quantitative delineation of the physical integrity of synaptosomes and myelosomes. The underlying statistical theory, based on an assumption of rectangular distribution of osmotic susceptibility of particles and its validation by least square regression fit of the determinant slope permit conclusions valid for the entire population. An evaluation of shifts in break-points offers a volume-independent method of evaluation of fluxes of osmolytes, overcoming the inherent limitation of centrifugal methodology for phase separation. The studies also confirm the results of Sperk and Baldessarini (1977) with regard to the interference due to coincident lysis during transport assays due to permeability to external Na^+ and K^+. Lysis of synaptosomes leading to 100% activity in 10 mM Tris-HCl indicated that the synaptosomes exhibit an osmolytic hole similar to that observed in erythrocytes (Lieber and Steck, 1982). Presence of 100% activity during LDHase osmometry indicated that the closure of the osmolytic hole was achieved by sub-sequent step of centrifugation for reisolation of synaptosomes after osmolysis, which again imparts osmotic behaviour to the LDHase remaining within. Thus 100% lysis can only be evaluated by direct osmometric methodology and not after attempts at phase separation in conventional lysis experiments.

Failure to observe good osmometric profiles in literature invariably resulted from complex assay conditions and the use of synaptosomes from sucrose density gradients, without reisolation in sucrose media of constant osmolarity. Enhanced heterogeneity (i.e., larger range of the determinant slope) could be shown to be a result of variable internal osmolyte content as well as presence of permeant osmolytes externally. On the other hand, strict observance of the methodological rules of osmometry invariably led to distinct break-points of high statistical quality, provided that, (i) synaptosomes were isolated in large diameter centrifuge tubes in swing-out buckets (to avoid wall effects), (ii) synaptosomes were repeatedly pelleted in the same sucrose medium and (iii) external osmolality was largely due to a single osmolyte of low permeability.

The differential osmotic behaviour of synaptosomes and myelosomes is of considerable importance for several reasons: (i) Enhanced permeability of the membranes of both these particles to mannitol, compared to sucrose, was in accordance with the observations of Villegas *et al.* (1966) on the enhanced permeability of the electrically active squid axonal plasma membrane to non-electrolytes. Since permeation of non-electrolytes, such as mannitol, would be thermodynamically forbidden (Sitaramam and Sarma, 1981a,b; Sambasivarao and Sitaramam, 1985), it is important to investigate the effect of membrane potential on non-electrolyte conductance to investigate possible alterations in the fine structure of these membranes due to electrical activity, (ii) Enhanced permeability of the synaptosomal membrane to Na^+ and K^+ as opposed to the myelosomal membrane would be consistent with the known electrical activity and channel density for these ions in these membranes ($25/\mu m^2$ for the axonal plasma membrane which is relatively inert, as opposed to a density of $10{,}000/\mu m^2$ for the comparable electrically active internodal membrane (Ritchie and Rogart, 1977). Thermal fluctuations in these channels would account for the observed fluxes of these ions, which are indeed large, considering the measurable variations in the instantaneous osmotic pressure exerted by these ions, as evidenced by osmometric studies.

Our studies, therefore, critically question certain assumptions inherent to transport studies. Boremost is the assumption that transport across the membrane is independent of the physical state of the membrane, *i. e.,* osmotic stretch. In a variety of enzyme systems, transporters and even channels, the activity, *A,* of the protein was shown to obey the relationship,

$$A = A_{max} - \tilde{K}\pi, \tag{4}$$

where $\tilde{K}$ is a newly described, empirical, elastic constant relating the activity of such hydrophobic proteins to the external osmotic pressure, π (Sambasivarao and Sitaramam, 1985; Sambasivarao *et al.,* 1986; Garlid, 1980; Kaiser, 1982; Rao and Sitaramam, 1984; Takanaka and O'Brien, 1975). Brom an osmometric point of view, existence of $\tilde{K}$ relates to a relaxation of the assumption inherent to present studies on LDHase that *P* (permeability to the substrate) is independent of the volume of the particle. The data of Kanner and Sharon (197D indeed suggests that L-glutamate transport activity in synaptosomes exhibits $\tilde{K}$. Another related assumption in transport studies relates to the thermodynamic interpretation of force-flux relationships, which assume a constant volume of the matrix space (Marchbanks, 1975), which would not be valid in the face of a permeable electrolyte*. Studies are in progress to delineate the importance of such physical interactions in transport across synaptosomal and myelosomal membranes.

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*The dimensionless quantity, K/P (eq. 1), is one form of Thiele modulus (see Bunow, 1978a.b for a more detailed exposition), used by chemical engineers to measure the strength of transport effects on chemical reactions. Existence of *K* for the rate constant of catalysis(K) and/or permeability or transport activity(P) shows very tight regulation between metabolism, transport and volume and the former cannot be studied in isolation without detailed osmometric analyses as indicated here.

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