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Determination of Catecholamine Permeability Coefficients for Passive Diffusion Across Phospholipid Vesicle Membranes

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Summary. A convenient catecholamine transport assay has been developed which permits continuous, instantaneous monitoring of transmembrane flux. Epinephrine transport has been examined by spectrophotometrically monitoring adrenochrome formation resulting from the passive diffusion of catecholamine into unilamellar phospholipid vesicles containing entrapped potassium ferricyanide. Ferricyanide oxidation of epinephrine under the conditions employed is fast compared to membrane transport, which obviates the need for intravesicular concentration or volume determinations. Epinephrine transport data over a pH 6 to 7 range have been fitted to an integrated rate equation from which a permeability coefficient for neutral epinephrine of $2.7 \pm 1.5 \times 10^{-6}$ cm/sec has been obtained.

The physiologically active catecholamines epinephrine, norepinephrine, and dopamine are hormones and neurotransmitters which are synthetically derived from the amino acid tyrosine in mammals. These substituted beta-hydroxyphenylamines are stored in remarkably high concentrations within specialized membrane-bound granules or vesicles. Epinephrine, for example, is stored within chromaffin granules in the adrenal medulla at a concentration of 0.5 M; these secretory granules also contain 0.125 M ATP and a soluble, acidic protein, chromogranin A [1, 11]. The accumulation and storage of epinephrine within chromaffin granules has been actively investigated and has been suggested as a model for accumulation and storage of biogenic amines in other secretory granules and in neuronal synaptic vesicles [9, 11].

The ability of chromaffin granules to create and maintain high internal concentrations of epinephrine in opposition to passive efflux has been regarded as a composite function of several features of the granule. Epinephrine accumulation has been proposed to occur by a carrier-mediated process involving a membrane-bound, ATP-driven, proton-translocating ATPase which pumps protons into the chromaffin granule [1, 3, 5, 6, 9]. The resulting proton electrochemical gradient drives epinephrine transport by lowering the chemical potential of internal neutral (unprotonated) catecholamine, thereby inducing an internally directed flux of permeable external neutral amine. Furthermore, it has been proposed that internal ATP and chromogranin A bind epinephrine to effectively trap the amine through the formation of impermeable osmotically inactive aggregates [2, 10].

In order to more fully assess and evaluate the mechanisms which have been proposed to explain the chromaffin granule's ability to oppose passive efflux of high levels of internal catecholamine, a systematic determination of the membrane passive permeabilities of these biogenic amines is required. We report here a convenient and novel procedure for determining catecholamine permeability coefficients for passive transport across unilamellar phospholipid vesicles. The procedure to be described is based upon spectrophotometric monitoring of the epinephrine oxidation product formed when epinephrine passively diffuses into vesicles containing impermeable potassium ferricyanide [4]. In contrast to many commonly used transport assay procedures, the method permits instantaneous and continuous monitoring of catecholamine flux from which permeation coefficients can be calculated in a straightforward manner.

Materials and Methods

The following describes a typical epinephrine transport experiment. Unilamellar 24 nm diameter phospholipid vesicles contain-

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ing potassium ferricyanide were prepared by sonicating a 10% (wt/vol) egg yolk phosphatidylcholine suspension in an aqueous pH 7 solution containing 0.10 M $K_3Fe(CN)_6$ and 0.06 M maleate buffer. The phospholipid used in these experiments was obtained from Sigma (St. Louis, Mo.) and purified by preparative liquid chromatography using a Waters (Milford, Mass.) Prep LC-500 chromatograph. The exterior ferricyanide solution of the vesicle preparation was replaced with an equiosmolar pH 7 solution of maleate buffer (0.06 M) and K_2SO_4 (0.13 M). This exchange was accomplished by eluting the vesicles with the above maleate-K_2SO_4 solution from a 1 × 20 cm Sephadex G-50 (Pharmacia, Piscataway, N.J.) column. The filtered vesicles containing impermeable ferricyanide were placed in the sample and reference beams of a Varian (Palo Alto, Calif.) Superscan 3 ultraviolet-visible spectrophotometer. Aqueous epinephrine (8 mM) was mixed with the vesicle preparation in the sample beam to produce an 0.08 mM extravesicular epinephrine solution. The absorbance of adrenochrome produced by ferricyanide oxidation of permeating epinephrine was monitored at 510 nm at ambient temperature with the spectrophotometer operating in the time mode.

Internal volumes of gel-filtered vesicle preparations were determined by measuring the ferricyanide absorbance at 420 nm before and after filtration of the sonicated vesicles. Internal volumes of 1% were typically obtained under conditions employed in these studies. Total membrane surface area was determined by employing a colorimetric assay of phosphate formed by perchloric acid digestion of vesicle aliquots [12].

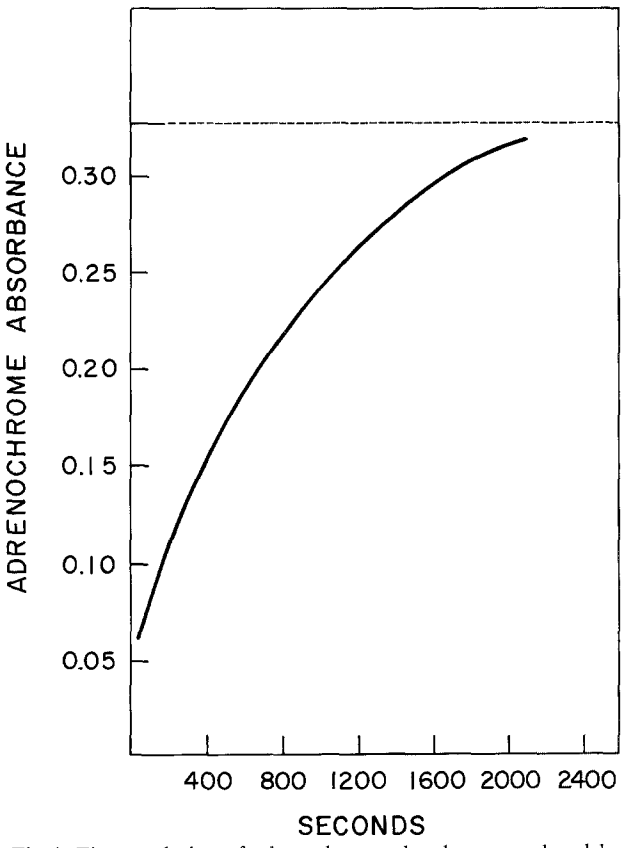

Fig. 1. Time evolution of adrenochrome absorbance produced by passive transport at pH 7 of epinephrine into sonicated phospholipid vesicles containing potassium ferricyanide. Dashed line represents extrapolation of absorbance trace produced by the direct addition of an equal quantity of epinephrine to potassium ferricyanide internal solution in the absence of vesicles

Results

Figure 1 represents a trace of adrenochrome absorbance *vs.* time after addition of epinephrine to an external concentration of 0.08 mM in a vesicle sample at pH 7. Figure 1 also indicates that direct addition of epinephrine to ferricyanide, in the absence of vesicles, produces full adrenochrome color development in less time than is required for mixing and positioning of the sample in the spectrophotometer (about 30 sec). Thus under the conditions employed in these studies, ferricyanide oxidation is fast compared to transmembrane catecholamine flux. Therefore, the rate of increase of adrenochrome absorbance can be directly related to epinephrine transport rates, provided the leakage of internal ferricyanide is slow on the time scale of epinephrine accumulation. This leakage was demonstrated to be suitably slow by monitoring intravesicular ferricyanide levels of a vesicle preparation initially containing 0.10 M entrapped ferricyanide. The entrapped-ferricyanide assay was performed at various time intervals by removing leaked extravesicular ferricyanide by gel filtration followed by spectrophotometric measurement of internal ferricyanide at 420 nm. No significant leakage was detected over a period of two hours; at 18 hours approximately 90% of the original internal ferricyanide still remained entrapped. In order to determine the dependence of catecholamine passive diffusion on pH, transport experiments were also performed at pH 6 and 6.5. In these experiments both intravesicular and extravesicular regions were buffered at the same pH. Curves similar to the one summarized in Fig. 1 were obtained; however, transport rates were observed to decrease with decreasing pH.

Since epinephrine oxidation is fast, the internal concentration of the catecholamine is always effectively zero. Thus transport should occur until all the exterior epinephrine has traversed the vesicle membrane. This prediction is supported by the fact that the observed limiting adrenochrome absorbance for the transport study depicted in Fig. 1 is the same as the absorbance observed by the direct addition of an equal quantity of epinephrine to ferricyanide.

Discussion

Permeability coefficients are proportionality constants relating membrane flux to transmembrane concentration gradients. They are of interest because they reflect intrinsic membrane permeabilities of molecules. In practice vesicular transmembrane concentration differences, necessary to determine permeability coefficients, are difficult to measure, since they require simultaneous monitoring of internal

and external concentrations [8]. The difficulty lies in discriminating inside and outside solutes as well as in determining internal volumes of small vesicular structures. Transport studies have typically been performed by sequentially removing aliquots and separating inside and outside contents by various techniques such as gel filtration, centrifugation, dialysis, and ultrafiltration [7]. A major problem with these indirect methods is that transport rates must be slow enough to avoid significant solute redistribution during the time required for separation of internal and external solute. This consideration is particularly crucial for transport studies involving small vesicular membranes, since these structures tend to have rapid transport rates due to their large surface area to volume ratios.

The experimental procedure described herein for the quantitative study of catecholamine transport utilizes sonicated unilamellar vesicular membranes which are well characterized and hence ideally suited for transport studies [7]. Determination of internal concentration or volume is not required in these experiments, since the catecholamine gradient is simply equal to the exterior concentration of the amine. This is particularly advantageous, since internal solutions of small vesicular structures have activities which are difficult to predict. If we assume that only the neutral form of the catecholamine permeates, an expression may be written for inward epinephrine flux as shown in Eq. (1), where E_o refers to external epinephrine, V_o is the external volume, S is the total membrane surface area, Ka is the acid dissociation constant of protonated amine, and P is the permeability coefficient for neutral epinephrine.

$$\frac{d[E_o]}{dt} \times \frac{V_o}{S} = -\frac{P \times [E_o] \times Ka}{[H^+]}. \quad (1)$$

In this expression the concentration of external neutral epinephrine has been approximated by $([E_o] \times Ka)/[H^+]$, since greater than 97% of the epinephrine exists in the protonated state over the pH range of these studies.

External epinephrine concentration in Eq. (1) was expressed as a function of adrenochrome absorbance to give the integrated rate expression shown in Eq. (2), where A is adrenochrome absorbance at time t and A_∞ is the infinity absorbance. This equation is based in part upon the observed

$$\ln[A_\infty - A] = -\frac{P \times S \times Ka \times t}{V_o \times [H^+]} + \ln A_\infty \quad (2)$$

linear Beer's law behavior of adrenochrome.

Equation (2) predicts that a plot of $\ln[A_\infty - A]$ *vs.* time should yield a straight line having a slope

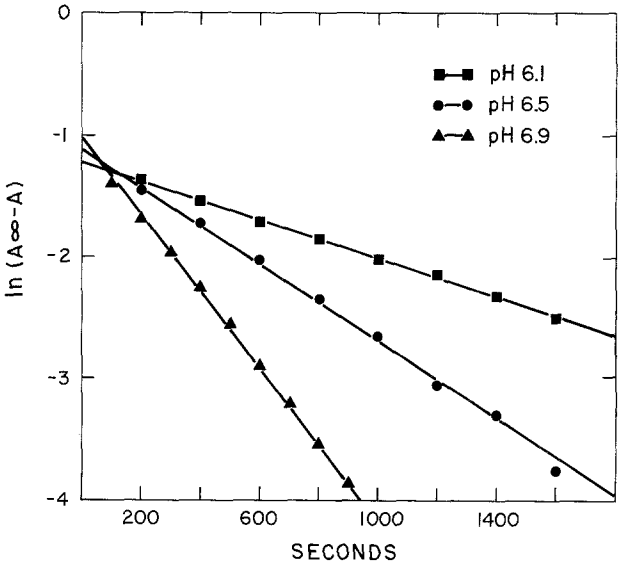

Fig. 2. Adrenochrome absorbance data, as in Fig. 1, plotted according to Eq. (2) for epinephrine transport experiments conducted at pH 6.1, 6.5, and 6.9

containing the neutral epinephrine permeability coefficient. Figure 2 presents three of these plots at pH 6, 6.5, and 7. The excellent linearity of these plots for experiments conducted at three different pH values offers compelling support for the validity of Eq. (2). The increase in transport rate observed with increasing pH confirms the supposition that transport occurs preferentially through permeation by the neutral form of the catecholamine.

Table 1 lists epinephrine permeability coefficients calculated from transport experiments conducted at pH 6, 6.5 and 7, assuming a membrane surface area of 6.7×10^4 cm^2/cm^3 based upon a representative lipid analysis, an external volume of 99%, and a value of 2.8×10^{-9} for Ka. These coefficients, calculated from data spanning a wide range of neutral amine gradients, are quite constant, suggesting that neglect of protonated amine permeation is valid. In natural systems large differences in neutral and protonated amine permeability coefficients would allow

Table 1. Permeability coefficients calculated for neutral epinephrine transport across unilamellar phospholipid vesicles at pH 6, 6.5 and 7

pH	P (cm/sec)[a]
6	$3.5 \pm 0.5 \times 10^{-6}$
6.5	$3.0 \pm 1.1 \times 10^{-6}$
7	$1.9 \pm 0.4 \times 10^{-6}$

[a] Indicated uncertainties are estimated standard deviations involving three or more measurements.

pH gradients to mediate transport essentially by raising or lowering the transmembrane gradient of the permeating species.

In an initial investigation, norepinephrine appears to exhibit a lower permeability than epinephrine. This result is consistent with an expected lower lipophilicity for norepinephrine, a primary amine, compared to that for epinephrine which is a secondary amine. There are a number of other obvious extensions of this study. By a suitable adjustment of pH with provision for releasing transmembrane electrical gradients, the permeability coefficients for protonated catecholamines might be determined. The suggested role of ATP and acidic proteins in retarding biogenic amine efflux might be quantitatively investigated, and permeation as a function of membrane composition might be examined. Furthermore, since Eq. (2) is independent of vesicle geometry, the effect of vesicle radius of curvature on membrane permeability might be studied, permitting an assessment of the fidelity of small sonicated vesicles as model systems for larger vesicular structures.

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References

1. Bashford, C.L., Casey, R.P., Radda, G.K., Ritchie, G.A. 1976. Energy-coupling in adrenal chromaffin granules. *Neuroscience* **1**:399
2. Berneis, K.H., Da Prada, M., Pletscher, A. 1974. Catecholamine transport through a lipid barrier. *Nature (London)* **248**:604
3. Johnson, R.G., Carlson, N.J., Scarpa, A. 1978. pH and catecholamine distribution in isolated chromaffin granules. *J. Biol. Chem.* **253**:1512
4. Johnson, R.G., Scarpa, A. 1976. Ion permeability of isolated chromaffin granules. *J. Gen. Physiol.* **68**:601
5. Kanner, B.I., Fishkes, H., Maron, R., Sharon, I., Schuldiner, S. 1979. Reserpine as a competitive and reversible inhibitor of the catecholamine transporter of bovine chromaffin granules. *FEBS Lett.* **100**:175
6. Njus, D., Radda, G.K. 1978. Bioenergetic processes in chromaffin granules a new perspective on some old problems. *Biochim. Biophys. Acta* **436**:219
7. Poste, G., Papahadjopoulos, D., Vail, W.J. 1976. Lipid vesicles as carriers for introducing biologically active materials into cells. *Methods Cell Biol.* **14**:33
8. Prestegard, J.H., Cramer, J.A., Viscio, D.B. 1979. Nuclear magnetic resonance determinations of permeation coefficients for maleic acid in phospholipid vesicles. *Biophys. J.* **26**:575
9. Schuldiner, S., Fishkes, H., Kanner, B.I. 1978. Role of a transmembrane pH gradient in epinephrine transport by chromaffin granule membrane vesicles. *Proc. Natl. Acad. Sci. USA* **75**:3713
10. Uvnäs, B., Åborg, C.-H. 1977. The ability of ATP-free granule material from bovine adrenal medulla to bind inorganic cations and biogenic amines. *Acta Physiol. Scand.* **99**:476
11. Winkler, H. 1976. The composition of adrenal chromaffin granules: An assessent of controversial results. *Neuroscience* **1**:65
12. Yang, S.F., Freer, S., Benson, A.A. 1967. Transphosphatidylation by phospholipase D. *J. Biol. Chem.* **242**:477

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