GLUTATHIONE X. HUMAN ERYTHROCYTE MEMBRANE DIFFUSION COEFFICIENTS FOR DIAZENE DERIVATIVES OF THE DIP SERIES VIA INTRACELLULAR THIOL OXIDATION

Nechama S. Kosower[a], Edward M. Kosower[b,c], Gilda Saltoun[b] and Lilly Levi[a]

[a]Dept. of Human Genetics, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel

[b]Dept. of Chemistry, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel

[c]Dept. of Chemistry, State University of New York, Stony Brook, New York, USA 11794

Received October 10, 1974

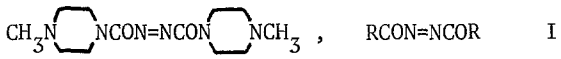
The thiol-oxidizing agent, DIP(diazenedicarboxylic acid bis (N'-methylpiperazide), I) and its homologs with different N'alkyl groups can be utilized to obtain membrane diffusion coefficients for human erythrocytes at 1°. From the initial rates of intracellular glutathione (GSH) oxidation and the 2-octanol partition coefficients for the specific DIP homolog, diffusion coefficients of ca. 1 x 10^{-8} cm^2/sec are obtained. The method (irreversible loss of permeant through well-defined intracellular reaction) can be used to evaluate membrane properties and possibly for the study of substituent effects on drug entry into cells.

Methods for measuring the rates at which various molecules ("permeants") enter cells, vesicles or liposomes, may be divided into two general classes: (a) particle size perturbation methods and (b) quasi-steady-state measurements of internal or external permeant concentrations. The first class involves an alteration in the size of the particle through a response to a change in external permeant concentration, and is followed by changes in light scattering or absorption (e.g., for hemolysis). Although adaptable to high rates, uncertainties in the extrapolations to zero perturbation time make the rate constants inaccurate. (for an excellent summary, see Sha'afi and Gary-Bobo (1)) Furthermore, influences such as an increase in the resistance of the particle membrane to expansion would be interpreted as a lower permeability.

Quasi-steady-state methods can be carried through under well-specified conditions, but suffer from the necessity to separate the

particle, a process which can damage the membrane through the excessive pressure associated with a high speed of separation. Thus, quasi-steady-state methods are most useful for rates with half-lives of ten seconds or more.

We noted that a glutathione(GSH) oxidizing agent, (DIP diazenedicarboxylic acid bis(N' -methylpiperazide), I) oxidized the GSH in red blood cells more slowly than might have been expected on the basis of independently measured rate constants for the reaction shown in eq. 1(2,3) and the rate constant for the reaction of GSH with the thiol-oxidizing agent, diamide. (4,5)



, RCON=NCOR I

(1) RCON=NCOR + 2GSH → RCONHNHCOR + GSSG

We have therefore synthesized a series of homologs of DIP and investigated their usefulness for the measurement of membrane diffusion coefficients through following the initial rates of GSH oxidation within red blood cells.

Piperazines: N-methylpiperazine, commercially available. Others: After refluxing an equivalent of alkyl bromide of iodide with piperazine monohydrochloride in ethanol, filtering off bis-salt, neutralizing, extracting with chloroform, and distilling, mono-N-alkyl-piperazines were obtained in 40-75% yield. B.p., ethyl-, 58°/23 mm; n-propyl-, 71°/20mm; isopropyl-, 66°/19mm; allyl-, 68-70°19mm; n-butyl-, 93-4°/23mm; sec-butyl-, 78°/17mm; isobutyl-, 72°/17mm; pentyl-, 110-112°/25mm. Structures were confirmed by nmr and purity established by tlc.

DIP homologs: (cf. Bock and Kroner (6)) Mono-N-alkylpiperazines were added to diethyl diazenecarboxylate ($EtO_2CN=NCO_2Et$) in petroleum ether: ether (3:1) at 0°, the precipitate filtered off and crystallized from either hexane or benzene-hexane. Compounds were purified if necessary by column chromatography on silica gel. Structures were confirmed by nmr and purity

by tlc. M·p(°C): M, 141; E, 141-3; n-Pr, 100-102; i-Pr, 139-141; A, 109-110; n-Bu, 84-6; S-Bu, 62-64; i-Bu, 111-113; n-Pen, 76-7.

Membrane diffusion coefficients: Freshly prepared solutions (4-6 x 10^{-4}M) of DIP homologs (see Table 1) in NaCl-phosphate buffer (pH 7.2) were cooled to 0-1°C, and mixed with an equal volume of 40-60% human red blood cell suspensions previously cooled to 1°C. Samples were removed at frequent intervals, added to an equal volume of ice-cold GSH solution (4-6 x 10^{-4}M), further diluted by 10 volumes of cold buffer, centrifuged, deproteinized and analyzed for GSH (7). The initial rate of GSH disappearance (k_1) was determined from the initial slope of a plot of ln [GSH] versus time. The initial intracellular DIP homolog concentration was calculated from the relation $k_1 = k_2[DIP]$, using the independently determined rate constant for the reaction of DIP and GSH at pH 7.2 . (3) Setting the flux of DIP through the membrane equal to the initial rate of GSH disappearance leads to an equation for the apparent membrane diffusion coefficient. (Eq. 2)

$$D_a = \frac{1000\ k \cdot \Delta x \cdot r \cdot [DIP]_{12} \cdot [GSH]}{3\ (\ [DIP]_{01} - [DIP]_{12})}$$

Symbols: k(k_2 for DIP + GSH), 2.5 x $10^4 M^{-1}sec^{-1}$; Δx, membrane thickness, 75A; r, cell radius, 2.75 x 10^{-4}cm; $[DIP]_{12}$, initial intracellular DIP concentration; $[DIP]_{01}$, initial extracellular DIP concentration, (GSH], initial intracellular GSH concentration.

The flux of DIP across the membrane is related to the concentration of agent within the membrane rather than the extracellular concentration, $[DIP]_{01}$ used in Eq. 2. To approximate the partition coefficient for the distribution of DIP between the external milieu and the exterior portion of the membrane, we utilized the partition coefficients between buffer and 2-octanol, an approach which has been extensively used by Hansch and co-workers (8). From the definition of $K = [DIP]_{2\text{-octanol}}/[DIP]_{buffer}$ and the knowledge that the initial intracellular DIP concentration, $[DIP]_{12}$, is

TABLE 1

MEMBRANE DIFFUSION CONSTANTS FOR DIP PERMEANTS THROUGH HUMAN RED BLOOD CELL MEMBRANE AT 1°.

DIP Homologs	Apparent Membrane Diffusion Coeff., D_a $cm^2/sec. \times 10^9$	Partition Coefficient[a] K	Membrane Diffusion Coeff., D $cm^2/sec. \times 10^8$
CH_3	1.2 ± 3[b]	0.1	1.2
CH_3CH_2	8.6 ± 1.9[c]	0.75	1.2
$CH_3CH_2CH_2$	48[d]	8.7	.55
$(CH_3)_2CH$	7.1 ± 1.1[e]	3.3	.21
$CH_2=CHCH_2$	40 ± 9[e]	6.1	.61
$CH_3CH_2CH_2CH_2$			
$CH_3CH_2CHCH_3$	too fast		
$(CH_3)_2CHCH_2$	to measure		
$CH_3CH_2CH_2CH_2CH_2$			

[a] defined by the relationship, $K = [DIP]_{2\text{-octanol}}/[DIP]_{buffer}$. Values were determined by spectrophotometric analysis at 25°.

[b] average of 10 determinations. Variations outside the indicated range may arise from (a) the procedure, (b) real differences between membranes of red blood cells from different individuals, (c) variations in membranes of a single individual.

[c] average of 6 determinations. See footnote b.

[d] average of 2 determinations which gave almost identical results.

[e] average of 3 determinations. See footnote b.

extremely small with respect to the external DIP concentration, the membrane diffusion coefficient may be obtained from the relation, $D = D_a/K$.

The apparent membrane diffusion coefficients, the 2-octanol/water partition coefficients and the "true" membrane diffusion coefficients are listed in Table 1 for a series of DIP homologs.

A report on the derivations, assumptions and temperature effects

must be deferred to a full paper. However, we may note that the diffusion coefficients obtained in this work are in good accord with those obtained by Devaux and McConnell (9) for phosphatidylcholine in lipid bilayers (1.8×10^{-8} cm^2/sec at 25°) after correcting for the difference in molecular weights, following the approach of Lieb and Stein (10) (see also Cohen and Bangham (11)), and additional results at 25°

The present approach (irreversible loss of permeant through a well defined intracellular reaction) offers a new quasi-steady-state method for the determination of membrane diffusion coefficients. Effects of substituent groups on the entry of molecules into cells may also be probed in view of the relative ease with which the necessary molecules may be synthesized. Further improvement would be possible through the development of a suitable quenching technique for the extracellular agent, perhaps by a modification of the approach used by Kanaoka and coworkers (12) in which a fluorescent compound is generated from a non-fluorescent reagent and a thiol.

Acknowledgement: H. Kenety-Londner is thanked for advice and materials. Support from the Chief Scientist's Office, Ministry of Health, Government of Israel, is acknowledged.

REFERENCES

1. Sha'afi, R.I. and Gary-Bobo, C.M., Progr.Biophys.Mol.Biol. 26, 103-146 (1973)
2. Kosower, E.M., Kosower, N.S., Kenety-Londner, H. and Levy, L., Biochem. Biophys.Res.Commun. 59, 347-351 (1974)
3. Kosower, E.M. and Kenety-Londner, H., in preparation for publication.
4. Kosower, N.S., Kosower, E.M., Wertheim, B. and Correa, W., Biochem.Biophys. Res.Commun. 37, 593-596 (1969).
5. Kosower, E.M., Correa, W., Kinon, B.J. and Kosower, N.S., Biochim.Biophys. Acta 264, 39-44 (1972).
6. Bock, H. and Kroner, J., Chem.Ber. 99, 2039-2051 (1966).
7. Kosower, N.S., Song, K.R. and Kosower, E.M., Biochim.Biophys.Acta 192, 1-7 (1969).
8. Leo, A., Hansch, C. and Elkins, D., Chem.Revs. 71, 525-616 (1971).
9. Devaux, P. and McConnell, H., J.Am.Chem.Soc. 94, 4475-4481 (1972).
10. Lieb, W.R. and Stein, W.D., Nature New Biology 234, 220-222 (1971).
11. Cohen, B.E. and Bangham, A.D., Nature 236, 173-174 (1972).
12. Kanaoka, Y., Machida, M., Sekime, T., Biochim.Biophys.Acta 317, 563-9 (1973).