Vol. 93, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 13, 1980 Pages 326-332

MEASUREMENT OF TRANSMEMBRANE PROTON DIFFUSION USING TWO LIPOSOME-SEQUESTERED pH INDICATOR DYES

William Stillwell and Keith Doram

Purdue University, School of Science

Biology Department

1201 East 38th Street, Indianapolis, Indiana 46205

Received February 1,1980

A new method is described for measuring the rate of transmembrane proton diffusion in liposomes. Two sequential pH indicator dyes (bromcresol green, pH 3.8 to pH 5.4 and bromthymol blue, pH 6.0 to pH 7.6) of similar color transitions were cosequestered inside liposomes and their rate of bleaching in the presence of imposed pH gradients was followed spectrophotometrically. This simple, novel method was used to directly measure the increase in membrane permeability to protons caused by the classic uncouplers of oxidative phosphorylation; 2,4 dinitrophenol, dicoumarol and carbonylcyanide metachlorophenylhydrazone. Increase in proton permeability caused by the uncouplers was measured from pH 4.0 to pH 8.0 and was found to correspond well with the uncoupler's pKa. Membrane permeability was also shown to decrease as a function of mole-percent cholesterol incorporated in the liposome and increase as a function of the membrane disruptive agent, n-butanol, in the aqueous phase. This method can be used to quickly test for transmembrane proton conductors as well as for agents causing general disruption of the membrane lipid bilayer and so can be used as a simple screening method for the detection of potential uncouplers of oxidative phosphorylation.

The last decade has greatly increased awareness of the importance of transmembrane pH gradients to biological systems (1), reaching its pinnacle recently with the awarding of a Nobel Prize to Peter Mitchell for his chemiosmotic hypothesis (2). pH gradients are now thought to drive ATP synthesis in plants, animals and bacteria as well as ion and metabolite transport in several membrane systems (3). pH gradients have been established across artificially constituted model membranes (liposomes and planar bimolecular lipid membranes) in an attempt to better understand the role of proton gradients and the nature of their energetically important discharge on simple, easily studied membranes (4).

A family of compounds have been described which stimulate electron transport while inhibiting ATP synthesis in respiring mitochondria (5). Known as uncouplers, these compounds often have the ability to discharge transmembrane pH gradients by serving as lipid-soluble proton conductors (3,6). Included in this series of compounds are the classic

Abbreviations. DNP, 2,4 dinitrophenol; CCCP, carbonylcyanide metachlorophenylhydrazone.

0006-291X/80/050326-07$01.00/0

uncouplers 2,4 dinitrophenol (DNP), carbonylcyanide metachlorophenylhydrazone (CCCP) and dicoumarol. Employing electrical measurements, Lehninger first observed that DNP could dissipate pH gradients across artificial planar bimolecular lipid membranes (7). Later CCCP and dicoumarol were shown to behave in a similar fashion (8). These compounds were found to conduct protons across membranes with well defined pH maxima often corresponding to their pKa's (6,9,10).

Observing pH inside cells or model liposome systems has not been a simple process. The small internal volume precludes direct potentiometric measurements. As a result internal pH has been determined by a number of indirect methods including fluorescent pH indicators such as umbilliferone (11), 9-aminoacridine (12) and carboxyfluorscein (13), entrappment of radioactive weak acids (14) and weak bases (15) and ammonium uptake followed with an external ammonium sensitive electrode (16).

Here we report the rate of proton diffusion across a bimolecular lipid membrane by spectrophotometrically measuring the titration of pH indicator dyes sequestered inside liposomes. To successfully follow proton diffusion over a wide range of pH's, two indicator dyes were cosequestered and the rate of bleaching followed as a function of transmembrane pH gradient magnitude and initial pH. Effect of the known uncouplers DNP, CCCP and dicoumarol on proton diffusion with this model system is reported here. This system can serve as an alternate method for the measurement of transmembrane proton diffusion and employes simple, inexpensive and readily available laboratory equipment.

MATERIALS AND METHODS: Bromthymol blue and bromcresol green were cosequestered inside egg lecithin liposomes made via the ether-evaporation method (17). 70 mg of egg lecithin (Type IX-E, Sigma Chemical Co.) were dissolved in 42 ml of ethyl ether and this was slowly injected at 2.8 ml/min into 5.0 ml of aqueous buffer containing 0.1 mg/ml of each of the pH indicator dyes. The solution was kept under aspiration between 55° to 65°C during the procedure. The large, single-walled liposomes containing the trapped dyes were separated from the unsequestered dyes on a Sephadex G-50, medium, column (Pharmacia). (Use of the column was later shown to be optional as the external, unsequestered dyes are titrated instantaneously and so do not interfere with the slower spectrophotometric determination of the bleaching rate for the internal dyes). The highly colored liposomes were added to cuvettes where HCl or NaOH injections rapidly developed the required pH gradients. Transmembrane proton diffusion rates were determined by measuring the change in absorbance at 610 nm or 410 nm of the trapped indicator dyes on a Beckman DBGT Spectrophotometer. Identical liposome preparations, except not containing the dyes, were used as the reference. As a control, acid or base was simultaneously added to the reference liposomes as well as the sample cuvettes.

Rates of color change associated with the trapped indicator dyes were recorded on a Beckman Model 1005 Linear Recorder. The O.D. rate changes were followed for 5 minutes

and the first minute rates, averaged for at least 10 experiments, were reported. The uncouplers, DNP, CCCP and dicoumarol were added to the liposome-containing cuvettes as acetone solutions prior to establishment of the pH gradient. Uncoupler concentration in the cuvettes varied between 10^{-4}M and 10^{-9}M. The initial pH was established between about pH 3.5 to pH 9.0 and the pH of the solution external to the liposomes was rapidly adjusted with NaOH or HCl additions. Internal liposome pH's were monitored by comparing the 610 nm and 410 nm peak ratios of the dye-sequestered liposomes with a large volume of identical dye solution in which pH was accurately determined with a Beckman Model 3500 Digital pH Meter. Bleaching rates were followed continuously as a decrease at 610 nm.

RESULTS AND DISCUSSION: We were interested in developing a simple, rapid but inexpensive method for directly measuring transmembrane diffusion rates using common laboratory equipment. Previous methods to determine internal pH have involved fluoremetric measurements (11,12,13), radioactive labeling (14,15) or ammonium sensitive electrodes (16), all of which require specialized equipment and are subject to interpretional problems. We theorized that titration of sequestered, simple pH indicator dyes would directly yield results similar to those of the more complicated techniques. Therefore we chose to sequester pH indicator dyes into easily constructed, well defined liposomes and follow their titration spectrophotometrically as protons diffused across the membrane and entered the internal chamber.

Our original experiments used the single trapped dye, bromthymol blue. Although we could readily measure diffusion rates, the pH range over which the technique was sensitive was limited. Therefore we included a second dye, bromcresol green, which complimented the bromthymol blue and extended the pH range to about four pH units (from about pH 4 to pH 8). The novel, simultaneous use of two indicator dyes allowed us to compare the effect of a series of uncouplers as proton conductors over a wide range of pH's. The results are reported below.

By combining the two indicator dyes, bromcresol green with bromthymol blue, the pH range over which rapid color change occurred is greatly extended (Figure 1). These dyes were chosen because of their sequential indicator range (bromcresol green, pH 3.8 to pH 5.4 and bromthymol blue, pH 6.0 to pH 7.6), their water solubilities and the fact that their color transitions were very similar (yellow to blue). The ratio of blue peak to yellow peak (610 nm/410 nm) gave an accurate indication of the pH when compared to that determined potentiometrically. Therefore by monitoring the 610 nm/410 nm ratio of the dyes

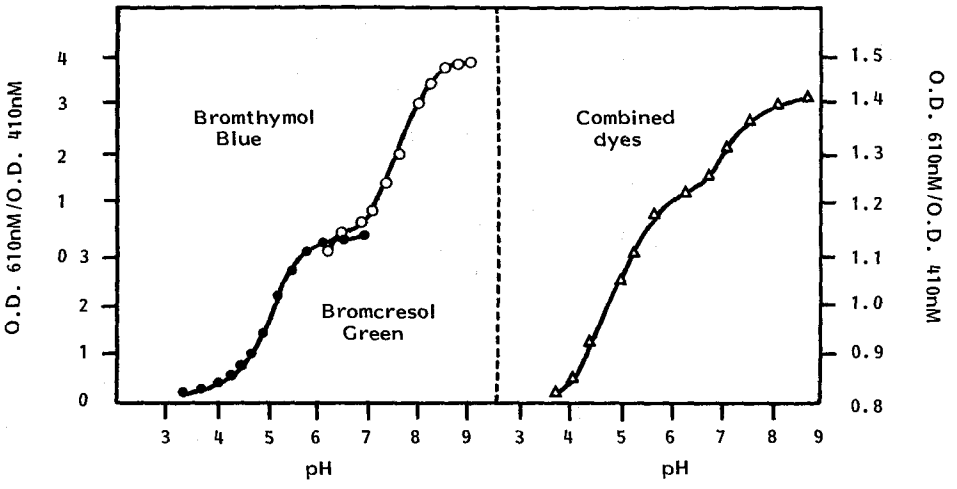

Figure 1. Titration of the pH indicator dyes bromcresol green (-●-) and bromthymol blue (-○-) independently (left and combined (right) followed spectrophotometrically and reported as the O.D. 610 nm/O.D. 410 nm.

sequestered in liposomes, the internal pH could be accurately determined. The initial external pH could be rapidly measured with a pH electrode.

Proton diffusion rates across the liposome membranes were measured by establishing at time zero a known pH gradient with either HCl or NaOH. Absorbance change of the internal indicator dyes associated with the subsequent changing internal pH was followed spectrophotometrically. Reproducible rates of proton diffusion could be determined with identically prepared sets of liposomes. Titrations in both directions was successfully attempted with liposomes initially at pH 4.0 and titrated with NaOH or at pH 8.0 and titrated with HCl. Successive titrations and back titrations could be performed on the same liposome preparations (Figure 2).

Chemical agents known to effect membrane structure were added to the system and their effect on proton permeability measured. Cholesterol is known to tighten membrane structure and so should cause a decrease in proton permeability (18). Butanol on the other hand is often used to disrupt membranes (19) and so would be expected to increase proton diffusion. This is confirmed experimentally in Figure 3. Using this technique, cholesterol greatly decreased proton diffusion across the liposomal membrane while butanol was shown to increase diffusion.

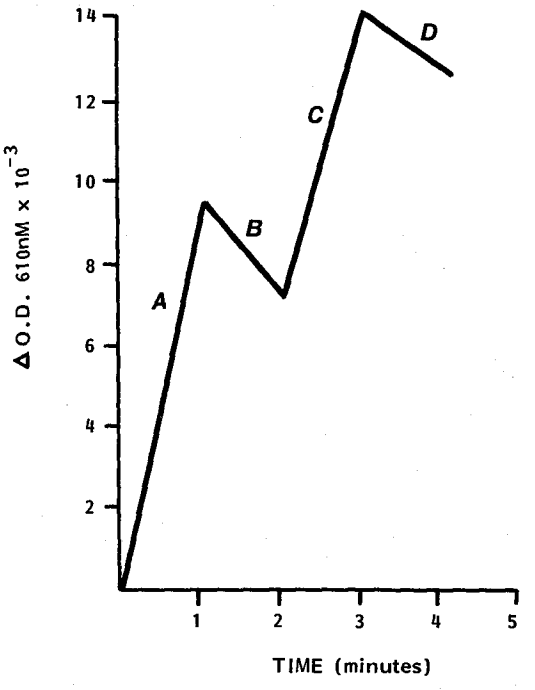

Figure 2. Reversible acid-base titrations with dye-sequestered liposomes. Reference and sample cuvettes each contained initially 1.9 ml of egg lecithin liposomes in 0.02M sodium phosphate buffered at pH 6.5. To both cuvettes were sequentially added: a) 50 µl of 1.25N NaOH, B) 50µ l of 0.625M HCl, C) 50 µl of 1.25N NaOH and D) 100 µl of 1.25N NaOH.

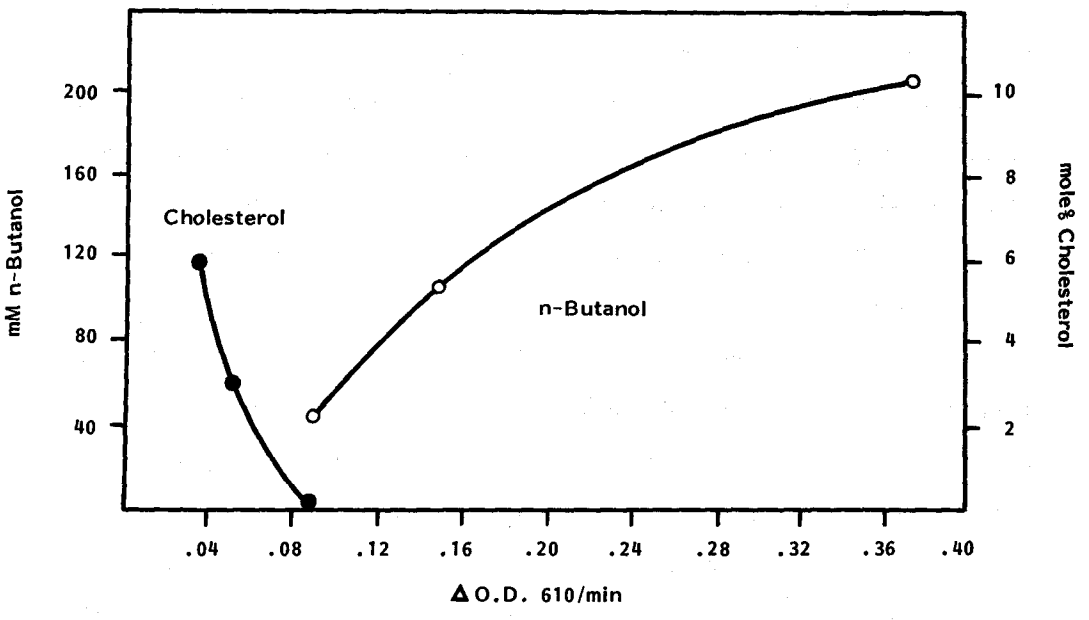

Figure 3. Effect of cholesterol (-●-) and n-butanol (-o-) on the permeability rates for protons. The initial membrane pH gradient is 4.7 units (pH 9.2 external, pH 4.5 internal).

This new model system was developed primarily to measure proton conducting abilities of potential uncouplers of oxidative phosphorylation. Three classic uncouplers, DNP, CCCP and dicoumarol was tested. All showed a stimulation in proton conduction from 10^{-4} to 10^{-9}M with the relative efficacies being CCCP > dicoumarol > DNP. Additionally, DNP displayed a large decrease in proton conducting ability in the highest (millimolar) ranges tested. This is consistant with the sharp decrease in electrical conductivity Lehninger noted for millimolar DNP on planar bimolecular lipid membranes (7). All three uncouplers showed a strong pH dependence on their ability to conduct protons. The liposome-sequestered dual pH indicator method reported here showed maximum proton conducting rates at; DNP, pH 4.1, dicoumarol, pH 5.7 and CCCP, pH 7.3 (Figure 4). This is in good agreement with Lehninger's electrical measurements on planar bimolecular lipid membranes which showed pH maxima for conductivity in the presence of the uncouplers of; DNP, pH 4.0, dicoumarol, pH 5.5 and CCCP, pH 7.2 (8). Subsequent experiments from other labs confirmed these maxima. Hopfer measured a maximum for DNP at pH 3.9 to 4.2 (6) and Lea and Croghan (20) reported

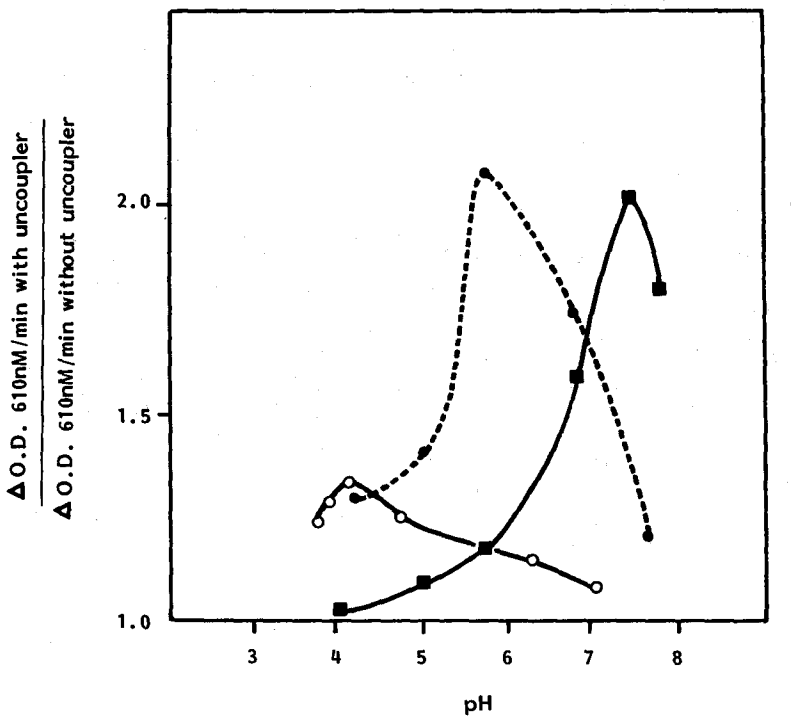

Figure 4. Effect of pH on uncouplers ability to increase proton conductivity across the liposomes membrane: (-o-), 2,4 dinitro phenol, 10^{-5} M; (---•---), dicoumarol, 10^{-6} M; (-■-), carbonylcyanide metachlorophenylhydrazone, 10^{-7} M.

a similar maximum at pH 4.2. LeBlanc (21) demonstrated a broad conductivity maximum for CCCP at pH 7.0 to 8.5. The sequestered dual indicator dye technique reported here yields results in good accord with those obtained by other unrelated techniques.

This novel approach of sequestering two sequential pH indicator dyes inside liposomes can serve as a simple but sensitive method for determining rates of proton movement across bimolecular lipid membranes. This method has been successfully employed for DNP, CCCP and dicoumarol and will be further used to test for other potential proton conducting uncouplers of oxidative phosphorylation. By this technique it should also be possible to observe small membrane perturbations which result in proton leakage. Such experiments are currently underway.

REFERENCES

1. Mitchell, P. (1979) Science 206, 1148-1159.
2. Harold, F. M. (1978) Science 202, 1174-1177.
3. Harold, F. M. (1972) Bact. Rev. 36, 172-230.
4. Tien, H. T. (1974) Bilayer Lipid Membranes (BLM) Theory and Practice, Marcel Dekker, Inc., New York.
5. Hanstein, W. G. (1976) Biochim. Biophys. Acta 456, 129-148.
6. Hopfer, U., Lehninger, A. L. and Lennarz, W. J. (1970) J. Memb. Biol. 3, 142-155.
7. Bielawski, J., Thompson, T. E. and Lehninger, A. L. (1966) Biochem. Biophys. Res. Comm. 24, 948-954.
8. Hopfer, U., Lehninger, A. L. and Thompson, T. E. (1968) Proc. Natl. Acad. Sci. U.S. 59, 484-490.
9. Liberman, E. A. and Topaly, V. P. (1968) Biofizika 13, 1025-1035.
10. Finkelstein, A. (1970) Biochim. Biophys. Acta 205, 1-6.
11. Gruenhagen, H. H. and Witt, H. T. (1970) Z. Naturforsch B 25, 373-386.
12. Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64-70.
13. Szoka, F. C., Jacobson, K. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 551, 295-303.
14. Kashket, E. R. and Wong, P. T. S. (1969) Biochim. Biophys. Acta 193, 212-214.
15. Rottenberg, H., Grunwald, T. and Avron, M. (1971) FEBS Lett. 13, 41-44.
16. Rottenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71-74.
17. Deamer, D. and Bangham, A. D. (1966) Biochim. Biophys. Acta 443, 629-634.
18. Harrison, R. and Lunt, G. G. (1975) Biological Membranes, Their Structure and Function pp. 79-80, Halsted Press, New York.
19. Johnson, S. M. and Bangham, A. D. (1969) Biochim. Biophys. Acta 193, 92-104.
20. Lea, E. J. A. and Croghan, P. C. (1969) J. Memb. Biol. 1, 225-237.
21. LeBlanc, O. H. (1971) J. Memb. Biol. 4, 227-251.