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Black lipid membranes as a model for intestinal absorption of drugs

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Black lipid membranes were generated in isotonic buffer (pH 4·5 and pH 6·5) from egg phosphatidylcholine and intestinal lipid, and the permeability to salicylamide, salicylic acid, *p*-aminobenzoic acid and tryptophan of these membranes was studied. Electrical resistance of intestinal lipid membranes was higher than that of phosphatidylcholine membranes. The presence of cholesterol produced an increase in the electrical resistance of black lipid membranes and a small decrease in the permeability of membranes to drugs. The permeability coefficient of salicylamide, an uncharged drug, was much larger than the coefficients of the charged drugs examined. The values for salicylic acid and *p*-aminobenzoic acid were much larger than comparable values predicted from their partition coefficients. Intestinal lipid membranes were more permeable to acidic drugs than phosphatidylcholine membranes. It is suggested that phospholipids and other lipid components of the small intestine may play an important role in the membrane permeability to acidic drugs. This method may be of interest in studying the complex processes of drug absorption from intestine.

The gastrointestinal mucosa and other biological membranes are generally considered to be lipid in nature and to possess aqueous filled pores. Although intestinal absorption of most weak organic acids and bases can be explained in terms of simple diffusion of uncharged molecules across a lipid-like boundary, the mechanism by which organic ions are absorbed remains unsolved (Schanker, Tocco & others, 1958; Hogben, Tocco & others, 1959; Kakemi, Arita & others, 1969). Advances in the understanding of membrane structure and function have led to the construction of *in vitro* membrane models which have been employed to simulate the *in vivo* absorption process (Doluisio & Swintosky, 1965; Perrin, 1967). Levy & Mroszczak (1968) reported on the use of three compartment transport model, consisting of a lipid-impregnated Millipore membrane separating two aqueous compartments.

On the other hand, artificial lipid bilayer membranes have been used in the past as models for biological membranes. These model membranes have been made mainly using the technique developed by Mueller, Rudin & others (1962) which allows the formation of optically black films from a large variety of lipids. Several researches have been performed on the electrical properties of black lipid membranes and on the permeability to water and non-electrolytes (Tien, 1971; Jain, 1972), but only a few reports have been made on the permeability to various drugs (Bean, Shepherd & Chan, 1968; Howard & Burton, 1968; Gutknecht & Tosteson, 1973; Wolosin & Ginsburg, 1975).

To obtain further information on the mechanisms of the intestinal drug absorption in connection with the studies of membrane lipids, the transport of drugs across black lipid membranes formed from the intestinal lipid and egg phosphatidylcholine was systematically investigated. Drugs used were salicylamide, salicylic acid, *p*-aminobenzoic acid and L-tryptophan because of their diverse physicochemical properties.

MATERIALS AND METHODS

Materials

Total lipids from the mucosa of the small intestine of male Wistar albino rats, 150–180 g and fasted for 16–20 h, were prepared according to Folch, Lees & Sloanestanley (1957). L-α-Phosphatidylcholine (Type III E: from egg yolk) was purchased from Sigma Chemical Co. Cholesterol, cholesterol acetate and n-decane were obtained from Nakarai Chemicals, Ltd. Phosphatidylcholine, cholesterol and cholesterol acetate gave a single spot by thin-layer chromatography using solvent mixture of hexane–ethyl ether–acetic acid (70:30:1) or chloroform–methanol–water (65:25:4). Lipids were stored for not longer than 4 weeks, dissolved in chloroform, under N_2 gas in sealed tubes below 0°.

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All other chemicals used were of analytical grade and were obtained commercially.

Membrane formation

The apparatus and procedure, used were essentially similar to those described by Hanai, Haydon & Taylor (1964). Black lipid membranes were formed by brushing lipid solutions across a 1·5 mm diameter hole in a Teflon cup. The volume of the inner compartment was about 7 ml and of the outer one about 73 ml. The aqueous solution was either pH 4·5 (citric acid-Na_2HPO_4) or pH 6·5 (NaH_2PO_4-Na_2HPO_4) isotonic buffer. Except where specified otherwise, the experiments were carried out at 25 ± 1°. Phosphatidylcholine (10 mg) or intestinal lipid (10 mg) in chloroform solution was mixed with the desired amounts of cholesterol, dried under N_2 gas and finally dissolved in 1 ml of n-decane for membrane forming solution. The membrane resistance was obtained by measuring the current across the membrane during application of an external electrical potential (up to 100 mV). The results are expressed in units of $\Omega\,cm^2$ obtained by multiplying the membrane resistance by the area of the bilayer in cm^2. A Keithley 610C electrometer was used to measure current. Membrane area measurements were made with the aid of crossed scales in the microscope eyepiece.

Permeability measurements

The drugs used in the permeability measurements were dissolved in isotonic buffer (1·5–7·5 mg ml^{-1}). After membranes turned completely black and gave a constant resistance, the drug solution (0·2 ml) was added to the inner compartment and buffer solution (1·25 ml) was added to the outer one. The period of drug diffusion was 30 or 60 min. During the ensuing diffusion of the drug across the lipid membrane, the membrane area is checked at 10 min intervals. Sometimes, by visually monitoring the membrane through the microscope, a drop or two of buffer solution was added to one side to keep the membrane planar. In this way, bulging of the membrane and subsequent breakage due to hydrostatic pressure differences was avoided. At the completion of the experiment, the inner and outer solutions were sampled as follows. After the visual measurement of the membrane area, the hole was quickly sealed by the brush with a small quantity of liquid paraffin to prevent mixing of the inner and outer solutions. The inner solution was first withdrawn into the volumetric cylinder by suction, and the outer solution was then drawn into another one. The aliquots were assayed spectrophotofluorometrically as described below. To confirm whether any leak of the concentrated inner solution exists due to incomplete sealing by the brush with liquid paraffin, the amount of drug transfered during 30 s was measured similarly. However, its concentration in the outer solution was not detectable. Therefore, there appeared little possibility of mixing of the inner and outer solutions when these solutions were completely withdrawn for analysis.

Total flux, J, and apparent permeability coefficient, P, were calculated from the relation:

$$J = \frac{Co\ Vo}{At} = P\ (Ci - Co)$$

where A is membrane area in cm^2, Vo is the volume of the outer compartment, Ci and Co the drug concentrations of the inner and outer compartments, respectively, and t, the time of diffusion.

Apparent partition coefficients

Five ml of the buffered drug solution (pH 6·5) was added to an equal volume of chloroform in a glass stoppered tube, and equilibrated at 25° by vigorous shaking. The separated aqueous phase was analysed. The apparent partition coefficient of a drug was calculated from the decrease of concentration in the aqueous phase.

Analytical methods

All drugs were determined spectrophotofluorometrically (Udenfriend, 1962; Barr & Riegelman, 1970). The pH of the samples was adjusted to a pH appropriate to the analysis of the drug. The maximum activation and emission wavelengths for salicylamide, salicylic acid, *p*-aminobenzoic acid and tryptophan were 330 and 415 nm, 300 and 400 nm, 300 and 335 nm, and 280 and 360 nm, respectively.

RESULTS AND DISCUSSION

Properties of black lipid membranes

The properties of black lipid membranes generated from intestinal lipid were compared to those from egg phosphatidylcholine, because little information was available on the properties of black lipid membranes formed from intestinal lipid. Tables 1 and 2 show the various properties of black lipid membranes generated from solutions of phosphatidylcholine and intestinal lipid in n-decane with and without cholesterol. Thin lipid membranes could be also formed from intestinal lipid as well as phosphatidylcholine in the isotonic phosphate buffer (pH 6·5)

Table 1. *Various properties of black lipid membranes generated from egg phosphatidylcholine.* The membranes were generated at 25° from solutions containing 1% (w/v) egg phosphatidylcholine with different amounts of cholesterol in n-decane. The aqueous phase was pH 6·5 isotonic phosphate buffer.

	Cholesterol (w/v %)				
	0	0·3	0·5	1·0	1·5
Time of formation (min)	4*	2	2	1	0·3
Breakdown voltage (mV)	150–180	200–250	250–300	300–315	300–350
Membrane resistance (Ω cm^2)	6×10^6	5×10^7	6×10^7	5×10^7	6×10^7

* The membranes were turned black by applying voltage (up to 80 mV).

and were stable for several hours. The addition of cholesterol to the membrane-forming solutions facilitated the thinning and increased the stability of their membranes. Electrical resistance of phosphatidylcholine-cholesterol membranes was approximately 10 times higher than that of pure phosphatidylcholine membranes. In the intestinal lipid membranes, the addition of cholesterol had a small effect on the electrical resistance, and the electrical resistance was considerably higher than that of phosphatidylcholine systems. Recent evidence (Van Deenen, 1972; Papahadjopoulos, Cowden & Kimelberg, 1973) suggest that the addition of cholesterol to phospholipids modifies their molecular packing, and the mixed membranes are more condensed when compared with pure phospholipids (condensing effect). The composition of intestinal lipid was mainly phospholipid, cholesterol, fatty acid, and glyceride by thin-layer chromatography using a solvent mixture of hexane–ethyl ether–acetic acid (70:30:1). Therefore, the electrical properties of intestinal lipid membranes may be compatible with their lipid composition.

Furthermore, the effect of cholesterol was compared with that of cholesterol acetate (0·5%) in the phosphatidylcholine and intestinal lipid membranes. In the presence of cholesterol acetate, lower membrane resistance was obtained (phosphatidylcholine, $0{\cdot}5 \times 10^7$ Ω cm^2; intestinal lipid, $0{\cdot}3 \times 10^8$ Ω cm^2).

Table 2. *Various properties of black lipid membranes generated from intestinal lipid.* The membranes were generated at 25° from solutions containing 1% (w/v) intestinal lipid with different amounts of cholesterol in n-decane. The aqueous phase was pH 6·5 isotonic phosphate buffer.

	Cholesterol (w/v %)			
	0	0·1	0·5	1·0
Time of formation (min)	6	6	1	0·5
Breakdown voltage (mV)	180–250	180–250	300–350	250–350
Membrane resistance (Ω cm^2)	2×10^8	2×10^8	3×10^8	3×10^8

Permeability of drugs across black lipid membranes

The permeability of intestinal lipid-cholesterol membranes to salicylic acid was studied preliminarily at several temperatures within the range of 17–29°. The results where the logarithm of the permeability coefficient is plotted against the inverse absolute temperature (Arrhenius plot) gives a negative slope. The value of activation energy was about 20 kcal mol^{-1} (83 kJ mol^{-1}). This is in agreement with a previous report indicating similar values in permeability of artificial membranes to organic solutes (Papahadjopoulos, Nir & Ohki, 1972). The rate of black lipid membrane formation decreased at lower temperatures, and the membranes formed at higher temperatures were generally less stable. Therefore the temperature during the following experiments was controlled to 25 $\pm$ 1°.

The permeability coefficients for various drugs across black lipid membranes formed from phosphatidylcholine-cholesterol and intestinal lipid-cholesterol at pH 6·5 are summarized in Table 3, which also lists molecular weights and the chloroform-to-phosphate buffer partition coefficients of the drugs. In both membrane systems the permeability coefficient of salicylamide, an uncharged drug with a high partition coefficient, was much larger than those of the charged drugs with lower partition

Table 3. *Permeability coefficients of various drugs across black lipid membranes generated from egg phosphatidylcholine and intestinal lipid at pH 6·5.* The membranes were generated at 25° from solutions containing either 1% (w/v) egg phosphatidylcholine-0·5% (w/v) cholesterol or 1% (w/v) intestinal lipid-0·3% (w/v) cholesterol in n-decane.

Drug	Mol wt	P.C.*	Permeability coefficient (10^{-6} cm s^{-1})** Phosphatidyl choline	Intestinal lipid
Salicylamide	137	2·38	22·7 $\pm$ 0·6	24·4 $\pm$ 1·0
Salicylic acid	138	0·01	8·9 $\pm$ 1·1	10·0 $\pm$ 0·8
p-Aminobenzoic acid	137	0·03	7·6 $\pm$ 0·5	16·4 $\pm$ 1·3
Tryptophan	204	0·01	1·5 $\pm$ 0·5	1·8 $\pm$ 0·4

* $CHCl_3$-to-phosphate buffer (pH 6·5) partition coefficients.
** Each value is the mean $\pm$ s.e. of 3–5 experiments.

coefficients. However, the permeability coefficients of acidic drugs such as salicylic acid and *p*-aminobenzoic acid were much larger than comparable values predicted from the partition coefficients, and the permeability for these drugs through intestinal lipid membranes was even larger than that obtained from phosphatidylcholine membranes.

To further examine the large permeability coefficients for acidic drugs, the pH dependence of salicylamide, salicylic acid and *p*-aminobenzoic acid permeability was studied. Table 4 compares the relation between the degree of ionization and the permeability coefficients of these drugs at pH 4·5 and 6·5. At pH 4·5, the permeability coefficients of salicylic acid and *p*-aminobenzoic acid increased but not to the degree expected from the increase of unionized form.

Table 4. *The relation between the degree of ionization and the permeability coefficients of various drugs across black lipid membranes generated from egg phosphatidylcholine at pH 4·5 and 6·5.* The membranes were generated at 25° from solution containing 1% (w/v) egg phosphatidylcholine-0·5% (w/v) cholesterol in n-decane.

Drug	Unionized form (%)* pH 4·5	pH 6·5	Permeability coefficient** (10^{-6} cm s^{-1}) pH 4·5	pH 6·5
Salicylamide	100	99·0	18·8 ± 1·1	22·7 ± 0·6
Salicylic acid	3·1	0·0	19·2 ± 1·2	13·3 ± 0·7
p-Aminobenzoic acid	66·6	2·0	20·1 ± 1·1	7·6 ± 0·5

* Calculated using the dissociation constants pKa = 8·5 for salicylamide, pKa = 3·0 for salicylic acid and pKa = 4·8 for *p*-aminobenzoic acid.
** Each value is the mean ± s.e. of 3–4 experiments.

It is evident from the results in Tables 3 and 4 that ionized molecules of acidic drugs are permeable across black lipid membranes, and their permeability cannot be explained on the basis of pH-partition theory alone. This result may correspond to the well-known phenomena that the weak acids, those with a pKa of 3 or higher, are absorbed relatively rapidly from the small intestine at physiological pH (Schanker & others, 1958; Hogben & others, 1959; Kakemi & others, 1969). In contrast, Furusawa, Okumura & Sezaki (1972) showed that the apparent chloroform-to-water partition coefficients of acidic drugs were markedly raised by addition to the chloroform of phosphatidylcholine at physiological pH. From drug transport studies using black lipid membranes, it is reasonable to assume that some of the stages in the intestinal absorption process will be limited by the extent of interactions of the ionized forms of acidic drugs and intestinal membrane constituents such as phospholipids.

On the other hand, cholesterol, which is added to the membrane-forming solutions to facilitate the thinning and increase the stability of their membranes in this study, is a major lipid component of many biological membranes. The effect of cholesterol on the permeability of phosphatidylcholine and intestinal lipid membranes to salicylic acid is shown in Fig. 1. Within the concentration range (0–1·5 %) employed, the addition of cholesterol had a small effect, usually tending to decrease the permeability of the drug.

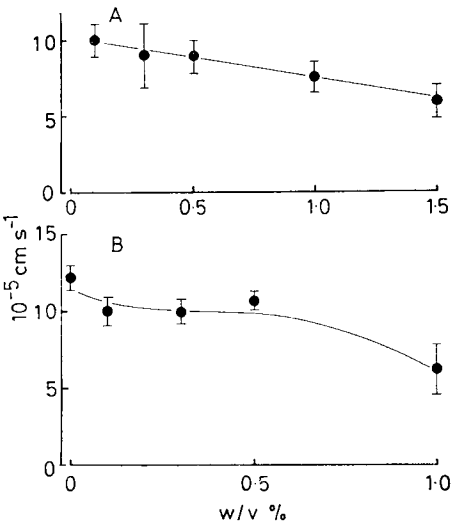

FIG. 1. Effect of cholesterol (w/v %) on the permeability coefficient (10^{-5}cm s^{-1}) of salicylic acid across black lipid membranes generated from (A) egg phosphatidylcholine and (B) intestinal lipid at pH 6·5. The conditions were as described in Tables 1 and 2. Each point is the mean ± s.e. of 3–10 experiments.

The above results relate with the effect of cholesterol in increasing the electrical resistance of black lipid membranes as described before. However, they are not so significant as earlier studies indicating a corresponding drop in permeability to water, glucose and other molecules (Finkelstein & Cass, 1967; Demel, Kinsky & others, 1968; Papahadjopoulos & others, 1972).

The use of this model system will provide a greater degree of insight into the complex process of drug absorption, as well as lead to a better understanding of the physicochemical factors influencing the process.

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REFERENCES

BARR, W. H. & RIEGELMAN, S. (1970). *J. pharm. Sci.*, **59**, 154–163.

BEAN, R. C., SHEPHERD, W. C. & CHAN, H. (1968). *J. gen. Physiol.*, **52**, 495–508.

DEMEL, R. A., KINSKY, S. C., KINSKY, C. B. & VAN DEENEN, L. L. M. (1968). *Biochim. biophys. Acta*, **150**, 655–665.

DOLUISIO, J. T. & SWINTOSKY, J. V. (1965). *J. pharm. Sci.*, **54**, 1594–1599.

FINKELSTEIN, A. & CASS, A. (1967). *Nature*, **216**, 717–718.

FOLCH, J., LEES, M. & SLOANESTANLEY, G. H. (1957). *J. biol. Chem.*, **226**, 497–509.

FURUSAWA, S., OKUMURA, K. & SEZAKI, H. (1972). *J. Pharm. Pharmac.*, **24**, 272–276.

GUTKNECHT, J. & TOSTESON, D. C. (1973). *Science*, **182**, 1258–1261.

HANAI, T., HAYDON, D. A. & TAYLOR, J. (1964). *Proc. R. Soc. (London)*, **A281**, 377–391.

HOGBEN, C. A. M., TOCCO, D. J., BRODIE, B. B. & SCHANKER, L. S. (1959). *J. Pharmac. exp. Ther.*, **125**, 275–282.

HOWARD, R. E. & BURTON, R. M. (1968). *J. Am. Oil Chem. Soc.*, **45**, 202–229.

JAIN, M. K. (1972). *The Bimolecular Lipid Membrane: A System*, New York: Van Nostrand Reinhold Company.

KAKEMI, K., ARITA, T., HORI, R., KONISHI, R., NISHIMURA, K., MATSUI, H. & NISHIMURA, T. (1969). *Chem. Pharm. Bull.*, **17**, 255–261.

LEVY, G. & MROSZCZAK, E. J. (1968). *J. pharm. Sci.*, **57**, 235–239.

MUELLER, P., RUDIN, D. O., TIEN, H. T. & WESCOTT, W. C. (1962). *Nature*, **194**, 979–980.

PAPAHADJOPOULOS, D., NIR, S. & OHKI, S. (1972). *Biochim. biophys. Acta*, **266**, 561–583.

PAPAHADJOPOULOS, D., COWDEN, M. & KIMELBERG, H. (1973). *Ibid.*, **330**, 8–26.

PERRIN, J. (1967). *J. Pharm. Pharmac.*, **19**, 25–31.

SCHANKER, L. S., TOCCO, D. J., BRODIE, B. B. & HOGBEN, C. A. M. (1958). *J. Pharmac. exp. Ther.*, **123**, 81–88.

TIEN, H. T. (1971). In: *The Chemistry of Biosurfaces*, Volume, 1, pp. 233–348. Editor: Hair, M. L. New York: Marcel Dekker.

UDENFRIEND, S. (1962). *Fluorescence Assay in Biology and Medicine*, New York: Academic Press.

VAN DEENEN, L. L. M. (1972). *Chem. Phys. Lipids*, **8**, 366–373.

WOLOSIN, J. M. & GINSBURG, H. (1975). *Biochim. biophys. Acta*, **389**, 20–33.