Isotope Flows and Flux Ratios in Biological Membranes

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ABSTRACT Precise evaluation of permeability of biological tissues is often prevented by imprecise knowledge of operative forces. This problem has been approached by analysis of fluxes of isotopic species applied to opposite surfaces of a membrane. A simple and rather general flux ratio equation has been derived which may permit evaluation of membrane permeability, even without knowledge of forces, or of the nature of active transport processes. Permeability as thus defined should be insensitive to coupled flows, either of other species or of metabolism. In appropriate circumstances application of the equation may permit evaluation of the contributions of the various processes to the transport of the examined species. Composite series membranes would be expected to obey the unmodified general equation. Heterogeneous parallel pathways would alter the relation in a predictable manner. The effect of isotope interaction is specifically incorporated. The formulation is applied to consideration of energetics of active transport.

INTRODUCTION

Despite extensive use of isotopic tracers in the study of permeability and transport processes, disagreement persists concerning the applicability and interpretation of such studies in biological systems (13, 19, 21). To some extent this disagreement reflects the different terminology and concepts of physical and biological methodology; to some extent it is a consequence of the incomplete state of the theory of isotope flux applicable to systems of membranes. For these reasons, as well as the desirability of providing a formal treatment amenable to further development, it is desirable to reinvestigate the theory of isotope flow.

A reexamination of the Ussing flux ratio equation has recently been published by Hoshiko and Lindley, who provide a derivation on the basis of phenomenological equations of irreversible thermodynamics (8). Certain of the underlying assumptions are interpreted in terms of a frictional model,

and the formulation is applied to some biological problems of interest. In their derivation Hoshiko and Lindley assume an inverse proportionality between concentration and resistance to flow of test species. Consideration is limited to permeation *via* identical pathways. It is further assumed that there is no interaction between isotopes in the membrane; analysis of phenomena such as "exchange diffusion" and "file diffusion" is left for later formulation of specific kinetic models.

The present treatment approaches these problems from a somewhat more general viewpoint, aiming at formulations which should be applicable in a variety of biological systems. No assumptions are made concerning the concentration dependence of the resistance terms.

The treatment is applied first to a homogeneous array of parallel elements; forces considered to be acting on test species are electrochemical potential gradients, and coupling to flows of other species (*e.g.*, "solvent drag" (1)) and metabolism ("active transport"). Consideration of the related thermodynamic and kinetic properties of isotopic species permits derivation of integrated equations applicable to the intact membrane treated as a whole. For this case, the treatment leads to a simple and rather general flux ratio equation, permitting evaluation of membrane permeability, even without precise knowledge of the operative forces. Deviations from "normal" ratios predictable from electrochemical potential differences are specifically accounted for on the basis of coupled flows.

In biological systems, however, flows are often likely to be influenced by isotope interaction (8, 19) and the existence of heterogeneous parallel pathways (27). When these additional complexities are incorporated in the treatment they lead to further modifications of the flux ratio equation. Such effects must, of course, be taken into account in attempts to characterize the mechanism of permeation and the energetics of transport in biological membranes.

GLOSSARY

c concentration

$\bar{c}$ average concentration $\dfrac{\Delta c}{\Delta \ln c}$

f flux ratio

F Faraday's number

J net flow of test species per unit area of membrane in the x-direction

J_i flow of species i
$i = 1$ for abundant test substance
$i = 2, 3$ for tracer species of test substance
$i = 4, \cdots, n$ for flow of other substances

J_m local flow of metabolism

J_r over-all rate of metabolic reaction, *e.g.*, oxygen uptake

K $-\dfrac{J_2}{\Delta c_2}$

n $\dfrac{R_a^x}{R_p}$

p pressure

r_{ij} local phenomenological coefficient

R integral phenomenological coefficient, $\int_0^{\Delta x} r_{00}\,dx$

R^x $\int_0^{\Delta x} (r_{00} - r_{ik})\,dx$

$\overline{R^x}$ $\dfrac{RT \ln f}{J}$

R gas constant

T temperature (°K)

$\bar{v}$ partial molar volume

X $\tilde{\mu}_O - \tilde{\mu}_I$

X^0 $\Delta\psi_{\Delta c=0, J=0}$

z ionic charge

γ activity coefficient

μ° standard chemical potential; $\tilde{\mu}$ electrochemical potential

ρ_i c_i/c

ψ electrical potential

ω permeability coefficient

ω^x isotope permeability coefficient

A. Flow through a Homogeneous Array

For sufficiently slow processes it is generally assumed that the electrochemical potential is everywhere definable and that forces and flows are linearly related at every point in a membrane (3, 12). We consider first an array of parallel pathways, identical with respect to the factors influencing transport. Considering the x-axis to be perpendicular to the membrane, the driving force acting on a substance is given by the gradient of its electrochemical potential, $-(d\tilde{\mu}_i/dx)$. In general, following Kirkwood (12), we may, for isothermal systems, express this force as a function of all the flows:

$$-(d\tilde{\mu}_i/dx) = \sum_{j=1}^{n} r_{ij} J_j\,, \tag{1}$$

where i may refer either to an ion or to an uncharged molecule. The flows J_j include not only those of all species passing through the membrane, as, for example, water, but also the movement of mobile membrane components. The sum may also contain terms $r_{im}J_m$, representing the direct contribution of metabolism to oriented solute flux.

For the present purpose it is convenient to rewrite equation (1), expressing flows of test species as functions of their driving forces and coupled flows. (Here the subscript 0 refers to total test substance, 1 to abundant isotope, and 2 and 3 to tracer isotopes of test substance.) Then

$$J = -\frac{1}{r_{00}}\left(\frac{d\tilde{\mu}}{dx} + \sum_{j=4}^{n} r_{0j} J_j\right) \tag{2a}$$

$$J_1 = -\frac{1}{r_{11}}\left(\frac{d\tilde{\mu}_1}{dx} + \sum_{j=4}^{n} r_{1j} J_j\right) \tag{2b}$$

$$J_2 = -\frac{1}{r_{22}}\left(\frac{d\tilde{\mu}_2}{dx} + \sum_{j=4}^{n} r_{2j} J_j\right) \tag{2c}$$

$$J_3 = -\frac{1}{r_{33}}\left(\frac{d\tilde{\mu}_3}{dx} + \sum_{j=4}^{n} r_{3j} J_j\right) \tag{2d}$$

It was assumed here that the flows of abundant and tracer species do not influence each other. The influence of isotope interaction will be considered in Section *B*.

Equations (2*a*, *b*, *c*, *d*) are related, as the isotopes are assumed identical in all thermodynamic and kinetic properties. Then, for total test substance,

$$\frac{d\tilde{\mu}}{dx} = RT\frac{d\ln c}{dx} + RT\frac{d\ln \gamma}{dx} + \frac{dzF\psi}{dx} + \bar{v}\frac{dp}{dx}, \tag{3a}$$

and for one of the tracer species,

$$\frac{d\tilde{\mu}_2}{dx} = RT\frac{d\ln c_2}{dx} + RT\frac{d\ln \gamma_2}{dx} + \frac{dz_2F\psi}{dx} + \bar{v}_2\frac{dp}{dx}, \tag{3b}$$

where $\gamma = \gamma_2$, $z = z_2$, and $\bar{v} = \bar{v}_2$. Hence, denoting $\frac{c_2}{c}$ by ρ_2,

$$\frac{d\tilde{\mu}_2}{dx} - \frac{d\tilde{\mu}}{dx} = RT\frac{d\ln \rho_2}{dx} \tag{4a}$$

Similarly, for species 3,

$$\frac{d\tilde{\mu}_3}{dx} - \frac{d\tilde{\mu}}{dx} = RT\frac{d\ln \rho_3}{dx} \tag{4b}$$

We limit consideration to systems in which no isotope separation is brought about by the coupled processes. If the specific activities are constant throughout the membrane the ratio of the flows of isotopes must then be identical with the ratio of their concentrations, whatever the other flows; further, the driving forces $-(d\tilde{\mu}/dx)$, $-(d\tilde{\mu}_1/dx)$, $-(d\tilde{\mu}_2/dx)$, $-(d\tilde{\mu}_3/dx)$ are equal. Thus

$$\frac{J_2}{J} = \frac{r_{00}}{r_{22}}\frac{\frac{d\tilde{\mu}}{dx} + \sum_4^n r_{2j}J_j}{\frac{d\tilde{\mu}}{dx} + \sum_4^n r_{0j}J_j} = \rho_2, \tag{5}$$

and similarly for $\frac{J_3}{J}$.

As equations (5) must hold for all values of J_j, it follows that

$$r_{2j} = r_{3j} = r_{0j} \tag{6}$$

and

$$r_{22}\rho_2 = r_{33}\rho_3 = r_{00} \tag{7}$$

Introducing (4), (6), and (7) into (2) the flow equations for total and tracer

species become

$$J = -\frac{1}{r_{00}}\left(\frac{d\tilde{\mu}}{dx} + \sum_4^n r_{0j} J_j\right) \tag{8a}$$

$$J_2 = -\frac{\rho_2}{r_{00}}\left(\frac{d\tilde{\mu}}{dx} + \sum_4^n r_{0j} J_j + \mathrm{R}T\frac{d \ln \rho_2}{dx}\right) \tag{8b}$$

$$J_3 = -\frac{\rho_3}{r_{00}}\left(\frac{d\tilde{\mu}}{dx} + \sum_4^n r_{0j} J_j + \mathrm{R}T\frac{d \ln \rho_3}{dx}\right) \tag{8c}$$

Here the tracer flows are expressed as functions of specific activity and variables determining total flow of test substance.

The relation of the total flow and the difference of the electrochemical potential across the membrane, X, is obtained by integration of equation (2*a*).

$$X = -\int_0^{\Delta x} d\tilde{\mu} = JR + \int_0^{\Delta x} \sum_4^n r_{0j} J_j \, dx, \tag{9}$$

where the integral resistance is defined by

$$\int_0^{\Delta x} r_{00} \, dx = R \tag{10}$$

($x = 0$ at the outer surface of the membrane; $x = \Delta x$ at the inner surface of the membrane). Here we assume that J is constant throughout the membrane in the steady state, and that both surfaces of the membrane are at equilibrium with adjacent solutions. It is assumed also that $\tilde{\mu}$ is continuous throughout the membrane. It is permissible that there be a finite number of discontinuities of $\frac{d\tilde{\mu}}{dx}$, as would be the case in a biological membrane, which would be expected to be characterized by discontinuities of local resistances and metabolic flows. We limit consideration to systems in which the test species is present on both sides of the membrane, so that X is always finite.

Often flow is characterized by a permeability coefficient rather than by a resistance. The permeability coefficient ω, conveniently defined as

$$\omega = \frac{J}{\bar{c}X_{(J_4, \cdots, J_n=0)}} \tag{11a}$$

is related to R by

$$\omega = \frac{1}{\bar{c}R}, \tag{11b}$$

where $\bar{c}$ is the average concentration of test substance, defined as $\frac{\Delta c}{\Delta \ln c}$(10).

ω is the permeability coefficient that would be measured if all flows other than that of test substance could be stopped without altering the properties of the membrane.

For non-electrolytes, or in the absence of an electrical field, equation (11*a*) is equivalent to that given previously (10); *i.e.*,

$$\omega = \frac{J}{RT\Delta c} \tag{11c}$$

(We ignore the small influence of a possible pressure gradient. For considerations in non-ideal solutions see reference 11.)

From equation (9), determination of R, and thus ω, requires knowledge of both the driving force and the contribution of coupled flows. In biological studies this information is often unavailable.

EVALUATION OF PERMEABILITY FROM NET FLOW AND ONE TRACER FLOW

Whenever ρ varies with x, the tracer flow will not be equal to ρJ, but will differ from this value because of isotope exchange (19). From equations (8),

$$J_2 - \rho_2 J = -\frac{RT}{r_{00}}\frac{d\rho_2}{dx} \tag{12a}$$

and

$$J_3 - \rho_3 J = -\frac{RT}{r_{00}}\frac{d\rho_3}{dx} \tag{12b}$$

Equations (12) show that, while the tracer flows and J depend on all the $r_{0j}J_j$, the flow of tracer relative to total test substance depends only on the gradient of ρ and the resistance r_{00}.

Assuming specific activity at the surfaces equal to that in the contiguous solutions, we may call the specific activity ρ_o at $x = 0$ (Outside) and ρ_I at $x = \Delta x$ (Inside). Integration of equations (12) then gives, with equation (10),

$$\frac{J\int_0^{\Delta x} r_{00}\,dx}{RT} = \frac{JR}{RT} = \ln\frac{J_2 - \rho_{2I}J}{J_2 - \rho_{2O}J} \tag{13a}$$

and

$$\frac{JR}{RT} = \ln\frac{J_3 - \rho_{3I}J}{J_3 - \rho_{3O}J} \tag{13b}$$

If tracer 2 was added only to the outside compartment, and the volume of the inside compartment is sufficiently large, $\rho_{2I} \simeq 0$. Equation (13*a*) then

simplifies to:

$$\frac{JR}{RT} = \ln \frac{\frac{J_2}{\rho_{2O}}}{\frac{J_2}{\rho_{2O}} - J}. \tag{14}$$

Therefore, in the absence of isotope interaction, determination of one tracer flux and net flow determines also the resistance to net flow, R. The resistance R can thus be determined without knowledge either of the driving force or of the nature of coupled flows. The permeability coefficient ω is then given by equation (11*b*).

NET FLUX FROM TWO TRACER FLUXES

Equations (13*a*) and (13*b*) relate the three flows J, J_2, J_3 :

$$\frac{J_2 - \rho_{2I} J}{J_2 - \rho_{2O} J} = \frac{J_3 - \rho_{3I} J}{J_3 - \rho_{3O} J} \tag{15}$$

If each tracer is added to only one compartment and $\rho_{2I} \simeq \rho_{3O} \simeq 0$, equation (15) reduces to:

$$J = \frac{J_2}{\rho_{2O}} + \frac{J_3}{\rho_{3I}} \tag{16}$$

In terms of the accepted terminology defined by Ussing and Teorell (22, 24), $\frac{J_2}{\rho_{2O}}$ is the influx, $\frac{-J_3}{\rho_{3I}}$ the outflux, and J the net flux. Thus equation (16) is the widely used relation:

$$\text{Net flux} = \text{influx} - \text{outflux}$$

For the derivation of J from the tracer flows it is not essential, however, to employ infinite sinks, though measurements are, of course, most accurate in this case. Denoting $\rho_I - \rho_O$ by $\Delta\rho$, equation (15) gives:

$$J(\rho_{2I}\rho_{3O} - \rho_{2O}\rho_{3I}) = J_3\Delta\rho_2 - J_2\Delta\rho_3,$$

or

$$J\left(\frac{\rho_{3I}}{\Delta\rho_3} - \frac{\rho_{2I}}{\Delta\rho_2}\right) = \frac{J_3}{\Delta\rho_3} - \frac{J_2}{\Delta\rho_2} \tag{17}$$

THE FLUX RATIO

Ussing's flux ratio, $\frac{\text{influx}}{\text{outflux}}$ (24), denoted here by f, can be expressed as a

function of the net flux and the integral resistance, by use of equations (16) and (13*a*). Assuming infinite sinks,

$$\ln f = \ln \frac{\frac{-J_2}{\rho_{20}}}{\frac{J_3}{\rho_{31}}} = \ln \frac{\frac{J_2}{\rho_{20}}}{\frac{J_2}{\rho_{20}} - J} = \frac{JR}{RT} \tag{18a}$$

or, equivalently,

$$\ln f = \frac{J}{RT\,\omega\bar{c}} \tag{18b}$$

Alternatively the flux ratio can be written as a function of the electrochemical potential difference and coupled flows, using equations (9) and (18*a*):

$$f = \exp.\left(\frac{X - \int_0^{\Delta x} \sum_4^n r_{0j} J_j \, dx}{RT}\right) \tag{19}$$

This equation, analogous to the flux ratio equation of Ussing (24), incorporates the influence of active transport. From the derivation given above it is clear that the equation applies to a wide variety of systems. It was assumed, however, that flows of abundant and tracer isotopes are independent, and that permeation is by way of identical pathways.

B. The Influence of Isotope Interaction

In many biological situations of interest flows of abundant and tracer species are not independent, but coupled (8, 19). Under these circumstances the flux ratio is no longer normal (*i.e.*, exp. (X/RT)), even in the absence of active transport or coupled flows of other species. "Abnormal" flux ratios have been adduced as evidence for such mechanisms as "porous flow," "single-file diffusion," and "exchange diffusion" (1, 7, 16, 27).

For purposes of general development, and in order to provide criteria which must be satisfied by appropriate models, it is useful to describe such coupled flows phenomenologically. The flow equations will therefore be rewritten to incorporate the interdependence of flows of abundant and tracer species. Development of the modified flow equations then leads to a generalized form of the flux ratio equation, explicitly incorporating the influence of isotope interaction.

As previously, equation (2*a*) applies to total flow of test species, *i.e.*,

$$-\frac{d\tilde{\mu}}{dx} = r_{00} J + \sum_{j=4}^{n} r_{0j} J_j \tag{20a}$$

For the isotopic components, however, we now have:

$$-\frac{d\tilde{\mu}_1}{dx} = r_{11} J_1 + r_{12} J_2 + r_{13} J_3 + \sum_{j=4}^{n} r_{1j} J_j , \tag{20b}$$

$$-\frac{d\tilde{\mu}_2}{dx} = r_{21} J_1 + r_{22} J_2 + r_{23} J_3 + \sum_{j=4}^{n} r_{2j} J_j , \tag{20c}$$

and

$$-\frac{d\tilde{\mu}_3}{dx} = r_{31} J_1 + r_{32} J_2 + r_{33} J_3 + \sum_{j=4}^{n} r_{3j} J_j \tag{20d}$$

Here the non-zero r_{ik}'s ($i \neq k$; $i, k = 1, 2, 3$) allow specifically for the influence of isotope interaction.

Examining equation (20*b*), considerations of kinetic indistinguishability require that for given values of $d\tilde{\mu}_1/dx$ and

$$\sum_{j=4}^{n} r_{1j} J_j ,$$

J_1 must depend not on the individual values of J_2 and J_3, but only on their sum, $J_2 + J_3$. Hence $r_{12} = r_{13}$, and must be independent of the ratio of concentrations, (c_2/c_3). Similarly, from equations (20*c*) and (20*d*), $r_{21} = r_{23}$, and $r_{31} = r_{32}$.

Further, since equations (20*b*), (20*c*), (20*d*) relate conjugate forces and flows of the dissipation function of the examined process, the Onsager reciprocal relation applies; *i.e.*, $r_{ik} = r_{ki}$. Taken in conjunction with the conclusions of the preceding paragraph it is seen that all r_{ik}'s are equal ($i \neq k$; $i, k = 1, 2, 3$).

Remembering that $J = J_1 + J_2 + J_3$, equation (20*c*) can now be rewritten:

$$-\frac{d\tilde{\mu}_2}{dx} = (r_{22} - r_{ik})J_2 + r_{ik} J + \sum_{j=4}^{n} r_{2j} J_j \tag{21a}$$

Similarly, from equation (20*d*),

$$-\frac{d\tilde{\mu}_3}{dx} = (r_{33} - r_{ik})J_3 + r_{ik} J + \sum_{j=4}^{n} r_{3j} J_j \tag{21b}$$

As previously, we assume that no isotope separation occurs. Then again, for constant specific activity ρ_i throughout the membrane, $J_i = \rho_i J$ and $d\ /dx = d\tilde{\mu}_i/dx$. Hence, from equations (20*a*) and (21*a*),

$$r_{00} J + \sum_{j=4}^{n} r_{0j} J_j = (r_{22} - r_{ik})J_2 + r_{ik} J + \sum_{j=4}^{n} r_{2j} J_j ,$$

$$\frac{J_2}{J} = \frac{r_{00} - r_{ik}}{r_{22} - r_{ik}} + \frac{\sum_{j=4}^{n} (r_{0j} - r_{2j})}{(r_{22} - r_{ik})} \frac{J_j}{J} = \rho_2 , \tag{22}$$

and analogously for J_3/J. (It is easily shown that with species 2 and 3 present only in tracer amounts the denominators of equations (22) can never equal zero.) Since these equations must hold for all values of the independent variables it follows that

$$r_{0j} = r_{2j} = r_{3j} , \tag{23}$$

$$\frac{r_{00} - r_{ik}}{r_{22} - r_{ik}} = \rho_2 , \tag{24a}$$

and

$$\frac{r_{00} - r_{ik}}{r_{33} - r_{ik}} = \rho_3 \tag{24b}$$

The sign of r_{ik} may be misleading and should be kept in mind. Where there is mutual drag between the isotope flows, r_{ik} is negative; where flow of species i diminishes flow of species k, r_{ik} is positive. We shall refer to the case in which $r_{ik} < 0$ as positive coupling and to that in which $r_{ik} > 0$ as negative coupling.

Exchange diffusion *via* a mobile carrier has been suggested as a mechanism of negative coupling (16). While this is a plausible mechanism in the systems investigated, the phenomenon and the model should not be identified. Similarly, "single file" passage is not justifiably inferred merely from the demonstration of positive coupling, as has been pointed out by Hodgkin and Keynes (7). The clearest example of positive coupling of isotope flows, movement of water through pores, is certainly not an instance of single file passage.

As in Section A, the total flow of examined species can be related to its electrochemical potential difference across the membrane. Integrating equation (20*a*), we again obtain equation (9),

$$X = JR + \int_0^{\Delta x} \sum_{j=4}^{n} r_{0j} J_j \, dx$$

EVALUATION OF EXCHANGE RESISTANCE FROM NET FLOW AND ONE TRACER FLOW

As above, when ρ varies with x the tracer flow will exceed ρJ by the value of isotope exchange. Now, however, the resistance to exchange flow will be modified by isotope interaction. Subtracting equation (21*a*) from equation

(20*a*), and introducing equations (4*a*) and (23),

$$-\frac{d\tilde{\mu}}{dx}+\frac{d\tilde{\mu}_2}{dx}=(r_{00}-r_{ik})J-(r_{22}-r_{ik})J_2=RT\frac{d\ln\rho_2}{dx}$$

Dividing by $r_{22}-r_{ik}$, and introducing the value of ρ_2 from equation (24*a*), we obtain

$$J_2-\rho_2 J=-\frac{RT}{r_{00}-r_{ik}}\frac{d\rho_2}{dx} \tag{25a}$$

Similarly,

$$J_3-\rho_3 J=-\frac{RT}{r_{00}-r_{ik}}\frac{d\rho_3}{dx} \tag{25b}$$

Integrating in the steady state, as in Section A, we now have

$$\frac{J\int_0^{\Delta x}(r_{00}-r_{ik})\,dx}{RT}=\ln\frac{J_2-\rho_{2I}J}{J_2-\rho_{2O}J},$$

and similarly for species 3. Thus, in the presence of isotope interaction, measurement of isotope flows no longer gives R, the resistance to net flow of test substance, but rather

$$\int_0^{\Delta x}(r_{00}-r_{ik})\,dx.$$

We shall denote this quantity R^x, and refer to it as exchange resistance. Then

$$\frac{JR^x}{RT}=\ln\frac{J_2-\rho_{2I}J}{J_2-\rho_{2O}J} \tag{26a}$$

and

$$\frac{JR^x}{RT}=\ln\frac{J_3-\rho_{3I}J}{J_3-\rho_{3O}J} \tag{26b}$$

Similarly, the isotope permeability ω^x can be defined, in analogy to equation (11*a*), by

$$\omega^x=\frac{J_2}{\bar{c}_2 X_{2(J,J_{3,4},\ldots,n=0)}} \tag{27a}$$

For non-electrolytes or in the absence of an electrical field it can readily be

shown that, with an infinite sink and $\Delta c = 0$

$$\omega^x = \frac{1}{\bar{c}R^x} \tag{27b}$$

It is to be noted that ω^x is not equal to the permeability ω. For negative coupling ω^x is larger than ω, for positive coupling smaller (but always >0, from general thermodynamic considerations).

NET FLUX FROM TWO TRACER FLUXES

From equations (26*a*) and (26*b*), with $\rho_{2I} = \rho_{3O} = 0$, one again obtains equation (16). Thus the relation

$$\text{Net flux} = \text{influx} - \text{outflux}$$

remains valid, as expected, despite isotope interaction.

THE FLUX RATIO

Combination of equations (26*a*) and (16) gives the flux ratio, expressed as in Section A as a function of the net flow and a resistance term.

$$\ln f = \ln \frac{\dfrac{-J_2}{\rho_{2O}}}{\dfrac{J_3}{\rho_{3I}}} = \ln \frac{\dfrac{J_2}{\rho_{2O}}}{\dfrac{J_2}{\rho_{2O}} - J} = \frac{JR^x}{\mathrm{R}T} \tag{28}$$

This equation, formally identical with equation (18*a*), predicts a proportionality between J and $\mathrm{R}T \ln f$ just as in the absence of isotope interaction. The proportionality factor, however, now is given not by R, the resistance to net flow, but by R^x, the resistance to exchange flow.

Introducing equation (9) into equation (28) we obtain

$$f = \exp. \frac{R^x}{R} \left(\frac{X - \int_0^{\Delta x} \sum_4^n r_{0j} J_j \, dx}{\mathrm{R}T} \right) \tag{29}$$

Here the factors promoting deviation from the normal flux ratio, exp. ($X/\mathrm{R}T$), are clearly seen: coupling with flows of other species and/or metabolism (active transport), and isotope interaction (27).

C. Comparison with "Permeability Coefficients" Derived from Flows of Single Isotopic Species

As mentioned above, although R^x reflects the influence of isotope interaction, it nevertheless represents an intrinsic characteristic of the membrane in a

given experimental situation. Membrane permeability has often, however, been evaluated in terms of coefficients derived from the flow of single isotopic species (15, 27). The permeability coefficient K is measured with isotope added to one compartment, and is defined by

$$K = \frac{-J_2}{\Delta c_2} \quad (5) \tag{30}$$

K is related to R^x, but the relation is simple only when the sole factor promoting isotope flux is the gradient of specific activity.

From equation (25*a*), when net flux is zero, isotope flow is given by

$$J_2 = \frac{-1}{r_{00} - r_{ik}} \mathrm{R}T \frac{d\rho_2}{dx}$$

Integration and introduction of the definition of R^x give

$$\frac{-J_2}{\Delta\rho_2} = \frac{\mathrm{R}T}{R^x}$$

With $c_O = c_I$ $(= \bar{c})$ and infinite sink, K is then given simply by

$$K = \frac{-J_2}{\Delta c_2} = \frac{-J_2}{\bar{c}\Delta\rho_2} = \frac{\mathrm{R}T}{\bar{c}R^x} \tag{31}$$

Further, by equations (10) and (11*b*), $K = \mathrm{R}T\omega$, but only in the absence of coupled flows, and only in the absence of isotope interaction.

In the presence of a net flow the isotope flow J_2 will be more complex. This situation is often treated by the consideration of two permeability coefficients $K_{O\to I}$ and $K_{I\to O}$

From equation (28), when $\rho_{2I} = 0$ and $\Delta c = 0$,

$$\ln \frac{\dfrac{J_2}{\rho_{2O}}}{\dfrac{J_2}{\rho_{2O}} - J} = \frac{JR^x}{\mathrm{R}T}$$

Then

$$\frac{J_2}{\rho_{2O}} = \frac{J}{1 - \exp.\left(\dfrac{-JR^x}{\mathrm{R}T}\right)}$$

and

$$K_{O\to I} = \frac{J_2}{\bar{c}\rho_{2O}} = \frac{J}{\bar{c}\left[1 - \exp.\left(\dfrac{-JR^x}{\mathrm{R}T}\right)\right]} \tag{32}$$

Expanding the exponential into a series and simplifying, one obtains

$$K_{O\to I} = \frac{RT}{R^x\bar{c}}\left[\frac{1}{1 - \frac{1}{2!}\left(\frac{JR^x}{RT}\right) + \frac{1}{3!}\left(\frac{JR^x}{RT}\right)^2 - \cdots}\right] \tag{33}$$

On the other hand, if $\rho_{2O} = 0$, $K_{I\to O}$ is given by

$$K_{I\to O} = \frac{-J_2}{\bar{c}\rho_{2I}} = \frac{J}{\bar{c}\left[\text{exp.}\left(\frac{JR^x}{RT}\right) - 1\right]} \tag{34}$$

Simplifying as above,

$$K_{I\to O} = \frac{RT}{R^x\bar{c}}\left[\frac{1}{1 + \frac{1}{2!}\left(\frac{JR^x}{RT}\right) + \frac{1}{3!}\left(\frac{JR^x}{RT}\right)^2 + \cdots}\right] \tag{35}$$

Thus, in general, both $K_{O\to I}$ and $K_{I\to O}$ may be expected to depend on all the factors influencing transport.

Flux measured in the direction opposite to active transport has often been regarded as passive (14, 25, 27). This is so only in a special case, that of two completely independent pathways, one active, the other a "passive leak." If, furthermore, the back flux in the active pathway is negligible as compared with the leak, $K_{I\to O}$ measures the permeability of the passive pathway. It cannot be assumed *a priori*, however, that this is the pattern of flows in a given biological membrane.

D. Composite Membranes

The above formulations are applicable both to an array of identical parallel elements and to composite membranes in series, provided only that the electrochemical potential be everywhere continuous. Heterogeneous membranes, composed of parallel arrays in which different factors influence flows, necessitate modification of the treatment. Since flux ratios have been commonly employed in the analysis of mechanisms and energetics of transport processes (6, 17, 20, 27, 28) it is pertinent to examine the influence of heterogeneity on the observed flux ratio.

For simplicity, consideration will be limited to a heterogeneous membrane consisting of two discrete parallel arrays, α and β, each array being composed of identical elements. Designating net flux in array α per cm² of membrane by J_α and influx by $J_{2\alpha}/\rho_{2O}$ the outflux is given by $J_{2\alpha}/\rho_{2O} - J_\alpha$ (equation 16).

Then the flux ratio

$$f_\alpha = \frac{\dfrac{J_{2\alpha}}{\rho_{20}}}{\dfrac{J_{2\alpha}}{\rho_{20}} - J_\alpha}$$

Allowing for the possibility of isotope interaction, J_α is given by

$$J_\alpha = \frac{RT \ln f_\alpha}{R_\alpha^x}.$$

(In the absence of isotope interaction $R_\alpha^x = R_\alpha$.) Then the influx,

$$\frac{J_{2\alpha}}{\rho_{20}} = \left(\frac{f_\alpha}{f_\alpha - 1}\right) J_\alpha = \left(\frac{f_\alpha}{f_\alpha - 1}\right) RT \frac{\ln f_\alpha}{R_\alpha^x},$$

and the outflux

$$\frac{J_{2\alpha}}{\rho_{20}} - J_\alpha = RT \left(\frac{1}{f_\alpha - 1}\right) \frac{\ln f_\alpha}{R_\alpha^x}$$

The observed flux ratio for a system with two parallel arrays, α and β, will be given by

$$f = \frac{\text{Influx}_\alpha + \text{influx}_\beta}{\text{Outflux}_\alpha + \text{outflux}_\beta}$$

or

$$f = \frac{\left(\dfrac{f_\alpha}{f_\alpha - 1}\right) \dfrac{\ln f_\alpha}{R_\alpha^x} + \left(\dfrac{f_\beta}{f_\beta - 1}\right) \dfrac{\ln f_\beta}{R_\beta^x}}{\left(\dfrac{1}{f_\alpha - 1}\right) \dfrac{\ln f_\alpha}{R_\alpha^x} + \left(\dfrac{1}{f_\beta - 1}\right) \dfrac{\ln f_\beta}{R_\beta^x}} \tag{36}$$

If we consider array α to constitute a passive leak without coupling of flows, $\ln f_\alpha = \ln f_p$ is given by X/RT, and f_p by exp. (X/RT). If $X \to 0$, $f_p \to 1$, and $\ln f_p \to f_p - 1$. Denoting the ratio of resistances of the two arrays by n

$$\left(n = \frac{R_\beta^x}{R_\alpha^x} \left(= \frac{R_\beta^x}{R_p}\right)\right)$$

equation (36) then reduces to

$$f_{X \to 0} = \frac{n + \dfrac{f_\beta}{f_\beta - 1} \ln f_\beta}{n + \dfrac{1}{f_\beta - 1} \ln f_\beta} \tag{37}$$

This equation, applicable to any heterogeneous two array parallel system in which coupled flows exist in only one of the arrays, can be applied in particular to membranes capable of active transport. When such a membrane is exposed to identical solutions, equation (37) predicts the flux ratio to be observed under "short-circuit" conditions (*i.e.*, $\Delta\psi = 0$) as a function of $f_\beta = f_a$, the flux ratio in the "active" pathway, and n, the ratio of the exchange resistances of the active and passive pathways. Representative curves of f as a function of f_a and n are shown in Fig. 1. It is to be noted that only

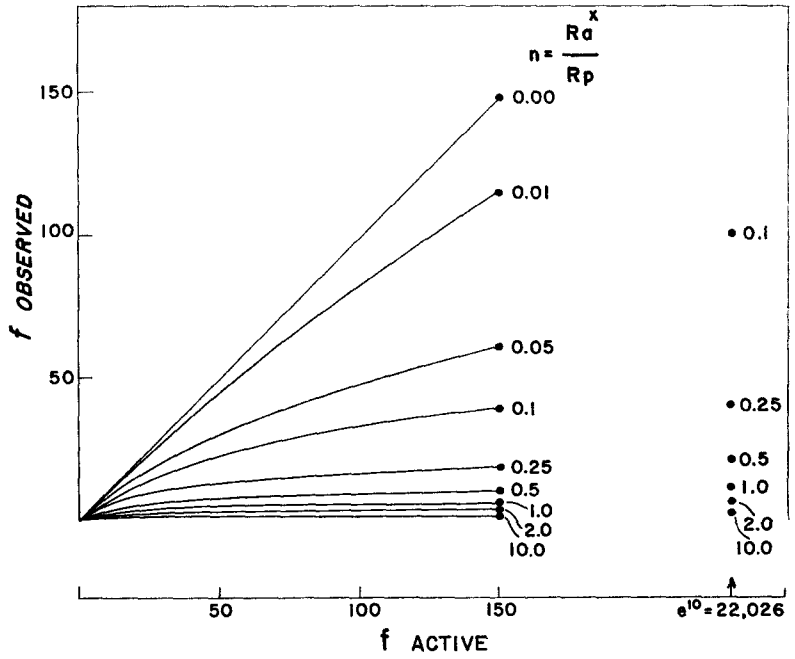

FIGURE 1. Effect of passive leak on observed flux ratio (short-circuited membrane).

when there is no passive leak ($n = 0.00$) is the observed flux ratio equal to that in the active pathway; with increasing leak deviations become quite appreciable. Large flux ratios indicate that leak must be relatively insignificant (for example, with $n > 0.5$, $f < 22$ even with f_a of 22,000).

It is useful also to consider the effect of parallel arrays on the apparent exchange resistance, defined as

$$\overline{R^x} = \frac{RT \ln f}{J} \tag{38}$$

where f is again the observed flux ratio and J the observed net flux. Since over-all flux must represent the sum of the component fluxes, equation (28)

gives

$$\frac{RT \ln f}{\overline{R^x}} = J = \sum_s J_s = \sum_s \frac{RT \ln f_s}{R_s^x}$$

Then

$$\ln f^{1/\overline{R^x}} = \sum_s \ln f_s^{1/R_s^x}$$

and

$$f^{1/\overline{R^x}} = \prod_s f_s^{1/R_s^x} \tag{39}$$

Restricting ourselves as above to consideration of membranes with two parallel arrays, α and β, we have

$$f^{1/\overline{R^x}} = f_\alpha^{1/R_\alpha^x} f_\beta^{1/R_\beta^x} \tag{40}$$

Then

$$f = (f_\alpha^n f_\beta)^{\overline{R^x}/R_\beta^x}$$

and

$$\frac{\overline{R^x}}{R_a^x} = \frac{\ln f}{n \ln f_\alpha + \ln f_\beta} \tag{41}$$

Again considering the case in which array α represents a passive leak, array β an active pathway, and $X = 0$, we have $f_\alpha = 1$ and

$$\frac{\overline{R^x}}{R_a^x} = \frac{\ln f}{\ln f_a} \tag{42}$$

Since f and f_a are explicitly related by equation (37), $\overline{R^x}/R_a^x$ can be evaluated as a function of n and f, as is shown in Fig. 2. Only in the absence of leak is $\overline{R^x}$ exactly equal to R_a^x. In the presence of leak the ratio of the apparent exchange resistance to that in the active pathway depends not only on the relative resistances in the two pathways, but also on the flux ratio, the dependence being non-linear.

E. Energetics of Active Transport Systems

Flux ratios have frequently been utilized as a means of analyzing the energetics of active transport (2, 6, 17, 20, 23, 27, 28). Thus Ussing, treating the frog skin as a battery, has attempted to evaluate the "electromotive force of

the sodium transport system," E_{Na}, responsible for active transport of sodium from the outer to the inner surface of the tissue. One method employed equates E_{Na} with the counter-electromotive force necessary to reduce the flux ratio of sodium to 1 (or J to zero). A second method assumes that "the electromotive force of the sodium pump affects the flux ratio of the sodium ion in exactly the same way as an applied electromotive force would affect the flux ratio of a passive ion" (28). With identical solutions on each side of the

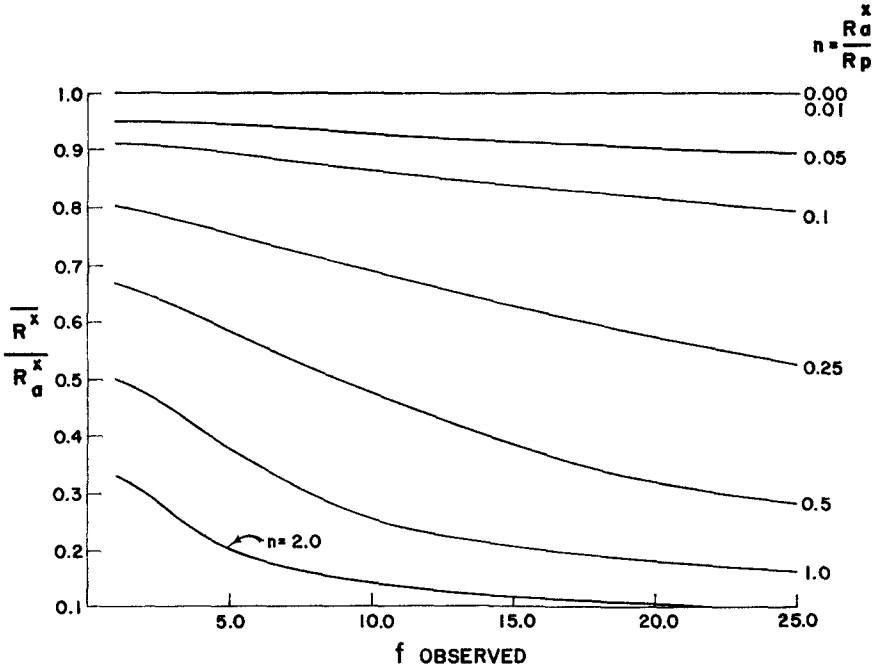

FIGURE 2. Effect of leak on observed exchange resistance (short-circuited membrane).

membrane and zero potential difference, E_{Na} is then given by

$$E_{Na}F = RT \ln \frac{\text{Influx}}{\text{Outflux}} = RT \ln f$$

As has been pointed out by Ussing, the experimental value for E_{Na} may be influenced by the method of evaluation, significant exchange diffusion, and leakiness of the membrane (17, 26, 28). The treatment assumes, however, that the frog skin behaves like a perfect battery, that is, that the flow of the metabolic reaction responsible for sodium transport is indeed entirely coupled to the flow of sodium. This has not been shown to be the case.

Even without knowledge of the degree of coupling of sodium transport and metabolism it is of interest to consider information which may be ac-

quired by application of the above methods, without thereby implying a battery model. Consideration will be limited to a membrane comprising a passive leak p and an active pathway a, bathed on its two surfaces by identical solutions. We assume further no coupling of sodium flow with flows of other species. Then

$$J = J_p + J_a \tag{43}$$

From equation (9)

$$J_p = \frac{X}{R_p},$$

and J_a, incorporating the metabolic contribution to transport, may be expressed as

$$J_a = \frac{(X - J_r R_{0r})}{R_a} \text{ (9)} \tag{44}$$

Here J_r represents the experimentally determined rate of metabolism (*e.g.* oxygen uptake) and $-J_r R_{0r}/R_a$ the contribution of this process to transport.

Consider first the maximum difference of electrochemical potential which can be produced by the active transport system, *i.e.* the value of X such that $J = 0$; we shall call this quantity X^0. Setting $X = X^0$, we have

$$J = \frac{X^0}{R_p} + \frac{X^0}{R_a} - \frac{(J_r R_{0r})_{J=0}}{R_a} = 0$$

or

$$X^0 = \frac{(J_r R_{0r})_{J=0}}{\frac{R_a}{R_p} + 1} \tag{45}$$

(It should be noted that X^0 as thus defined cannot in general be assumed equal to the value of X required to make $J = 0$ when the solutions bathing the two surfaces do not contain equal concentrations of test substance. Only experiment can establish the influence of chemical as against electrical forces on a given biological transport process.[1])

[1] For solutions and a variety of synthetic membranes it has been found that the same flow is produced by thermodynamically equivalent concentration and electrical driving forces. Nevertheless such equivalence must not be assumed *a priori* in biological membranes; experimental evidence concerning this point would be of value. Lacking such evidence, it should be appreciated that the expressions given here may define ω (and R) uniquely only for the conditions of a given experiment.

In the absence of a leak, equation (45) reduces to

$$X^0 = (J_r R_{0r})_{J=0} \tag{46}$$

Consider now the same membrane when $X = 0$ (*i.e.*, "short-circuited," since $\Delta c = 0$). Now $J_p = 0$ and

$$J = J_a = -\frac{(J_r R_{0r})_{X=0}}{R_a} \tag{47}$$

The flux ratio for this system is given by

$$\mathrm{R}T \ln f = J\overline{R^x} = -(J_r R_{0r})_{X=0}\left(\frac{\overline{R^x}}{R_a}\right) \tag{48}$$

Thus determination of f, reflecting the influence both of leak and of isotope interaction, cannot in general precisely evaluate an energetic parameter of the system. Furthermore, even in the absence of leak and isotope interaction $\mathrm{R}T \ln f$ is not equal to X^0, for with coupling of metabolism and sodium transport $(J_r)_{J=0}$ will not equal $(J_r)_{X=0}$ (29). Similarly, for the above reasons, Ussing's R_{Na} $\left(\text{equivalent in our terms to } \frac{\mathrm{R}T}{F}\frac{\ln f}{J}, \text{ or } \frac{\overline{R^x}}{F}\right)$ (28) should not be regarded as the "internal resistance of the transport mechanism," even in the absence of a leak.

F. Experimental Applications

For an array of identical pathways, in the absence of isotope interaction, equation (19) applies. This equation has been partially tested by Meares and Ussing, in a system incorporating the influence of solvent drag, but not that of active transport (18). Using a cation exchange membrane, they have studied flux ratios of sodium and chloride as a function of concentration differences. Satisfactory agreement with theory was found over a range of 0.1 to 1.0 molar.

In the presence of isotope interaction equation (29) must be employed. Deviations of flux ratios from the normal (exp. $X/\mathrm{R}T$) have, of course, been observed in a variety of systems (1, 7, 13, 16, 24). Since contributions of the $r_{0j}J_j$ terms may alter the sign of the integral force, coupled flows of other species or metabolism may modify both the magnitude and sign of $\ln f$, and thus promote "uphill transport." Since R^x/R is greater than zero, isotope interaction can alter only the magnitude of $\ln f$.

If flow is not coupled to that of other species, isotope interaction can be evaluated from the flux ratio, providing the integral force is known. When such is the case R^x/R is given simply by the ratio of $\mathrm{R}T \ln f$ and force (equation 29). For water flow across epithelial membranes R^x/R may be $\gg 1$

(5, 13). Assuming water transport through the toad bladder to occur via an array of identical elements values of R^z/R ranged from 102 to 134 (5).

For identical pathways in the absence of isotope interaction, equation (18*a*) (or 18*b*) permits evaluation of R, the resistance, (or ω, the permeability) of the membrane. (In the more general case equation (38) gives $\overline{R^z}$.) Behavior of R (or $\overline{R^z}$) with change in experimental conditions is, of course, a function of the nature of a membrane, and must be determined for each given case. Few data are as yet available. In the cation exchange membrane studied by Meares and Ussing R declines appreciably with increasing $\bar{c}$ (18). For potassium flux in giant nerve fibers, $\frac{\ln f}{J}$ increases significantly with increase of force (7). For water flow, however, the data of Hays and Leaf in the toad bladder show only relatively slight variation of $\frac{\ln f}{J}$ over a range of Δc of 60 to 170 milliosmols per liter (5). Garby has cited similar results as evidence for a capillary mechanism (4). As shown above, equation (19) can be derived from very general assumptions, and cannot therefore support any specific model.

G. DISCUSSION

In the interests of precision, and in order to facilitate further theoretical development, the formalism of irreversible thermodynamics has been employed here. No specific models are implied thereby. Although the formulations remain to be tested experimentally, the broad validity of the assumptions suggests that the treatment should be applicable in a variety of experimental circumstances.

It is not necessary to assume linearity of the integrated equations, since they deal with a membrane as it stands in given experimental situations. While substantial variations in forces and flows might in general alter the r_{0j} and the R they should not alter the basic formal relations of J to the integral force, nor the validity of the flux ratio equations.

It would appear that the chemical state of permeants need not be the same in the membrane as in the external solutions. Association with members of the same or other species should be permissible, as well as association with moving components. The treatment is not limited to single phase membranes, being applicable to composite series membranes, provided that there is continuity of the electrochemical potential at phase boundaries. While heterogeneous parallel pathways modify the basic equations the general formulation should remain useful within the limits described.

We do assume that electrochemical potential is definable and continuous at every point in a membrane. While this assumption is generally made with respect to both synthetic and biological membranes, it is possible that this

may not be the case in very thin membranes of biological interest. If not, a basically different physical analysis may be necessary.

For a homogeneous array, in the absence of isotope interaction, equations (18) define the resistance R, or the permeability ω, in a given experimental situation. It is to be noted that these parameters are determined without knowledge of the integral force, whether resulting from electrochemical potential differences or coupled flows. The parameters thus defined are intrinsic characteristics of the membrane, and would be expected to be insensitive to coupled flows, either of other species or of metabolism.

In the presence of isotope interaction, although $\frac{RT \ln f}{J}$ again defines an intrinsic parameter of the membrane, this quantity no longer represents the resistance to net flow R, but rather the exchange resistance, R^x. In these circumstances R cannot be determined without direct measurements of net transport and integral forces. When this information is available R^x provides in addition an evaluation of isotope interaction.

In view of the generality of the present treatment, inferences as to mechanism are limited; the coefficients employed are purely phenomenological. While the approach may permit demonstration of positive and negative coupling, identification of such mechanisms as "bulk flow," "single-file diffusion," or "exchange diffusion" must rest upon different evidence.

H. CONCLUSIONS

1. Net flux may be determined from measurement of two isotope fluxes, both in the absence and presence of isotope interaction.

2. A relation between the flux ratio and the forces promoting transport may be derived from assumptions of broad validity. In analogy with the treatment of Ussing, for permeation by way of identical pathways, deviations from normal flux ratios are attributable either to coupling of flows, or isotope interaction. Coupled flows may influence both the magnitude and sign of $\ln f$; isotope interaction influences only the magnitude.

3. For such a homogeneous array, in the absence of isotope interaction, measurement of the net flow and flux ratio determines R, the resistance to net flow (or ω, the permeability), without knowledge of either driving forces or coupled flows. Permeability as thus defined is an intrinsic characteristic of the membrane, which should be insensitive to coupled flows either of other species or of metabolism.

4. With heterogeneity and/or isotope interaction $\overline{R^x}$, the apparent exchange resistance derived from the net flow and flux ratio, is no longer equal to R. It is, however, an intrinsic parameter of the membrane, in distinction to permeability coefficients derived from a single isotopic flux.

5. Existence of heterogeneous parallel pathways modifies predictably

both the observed flux ratio and the apparent exchange resistance. Only in the absence of leak are the observed parameters equal to those in an active pathway. With increasing leak deviations become quite appreciable; large flux ratios indicate insignificant leak.

6. In a membrane for which $\overline{R^x}$ is known, measurement of a single isotope flux suffices to determine the net flux.

7. Evaluation of the contribution of metabolism to transport processes is dependent on the experimental setting. The maximum electrochemical potential difference produced by active transport is related to the magnitude of leak. The flux ratio of a short-circuited membrane is influenced by both leak and isotope interaction.

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