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2 August 1973

Diffusion of Weak Acids across Lipid Bilayer Membranes: Effects of Chemical Reactions in the Unstirred Layers

Abstract. *Chemical reactions in the aqueous unstirred layers of solution adjacent to a membrane can have dramatic effects on the diffusion of solutes across that membrane. This is demonstrated by the diffusion of labeled salicylate and salicylic acid across a phospholipid bilayer membrane. Two types of chemical reactions are considered. The first is an isotopic exchange reaction between the ionic and nonionic forms of a weak acid,* $HA + {}^*A^- \rightleftarrows H{}^*A + A^-$. *This reaction provides a way of estimating the true membrane permeability to a highly permeant weak acid and also a way of estimating the thickness of the unstirred layers. The second chemical reaction, the dissociation of a weak acid,* $HA \rightleftarrows H^+ + A^-$, *can be used to show how the presence or absence of buffers in the unstirred layers controls the net transport of permeant weak acids across a membrane. In principle, the addition of appropriate "antacid" buffers to salicylates can increase their rate of absorption from the stomach.*

Unstirred layers of solution adjacent to a biological membrane often constitute the rate-limiting barrier for the diffusion of highly permeant substances, for example, water, alcohols, fatty acids, anesthetics, bile acids, and various uncouplers of oxidative phosphorylation (*1–3*). If the permeability of the unstirred layers to a particular substance is much less than the permeability of the membrane, then it is impossible to measure directly the true membrane permeability to that species (*4*). However, by taking advantage of a chemical reaction within the unstirred layers, we demonstrate here a way of estimating the true permeability of a membrane to a highly permeant substance. We also show how the presence or absence of buffers in the unstirred layers can control the net transport of permeant weak acids across a membrane.

Salicylic acid was chosen for these experiments because the nonionic form of this weak acid is a highly permeant, lipid-soluble species with an appropriate *pK* (negative logarithm of the dissociation constant) of about 3.0. Furthermore, recent reports in *Science* and elsewhere (*5*) have described dramatic effects of salicylates on the permeability of both biological membranes and lipid bilayer membranes. Finally, the controversy concerning the relative rates of absorption of buffered versus unbuffered salicylates from the stomach is still unresolved (*6*). Thus, we wished to find out whether chemical reactions in the unstirred layers might be involved in the absorption process.

Lipid bilayer (optically black) membranes were made by the brush technique of Mueller *et al.* (*7*). The membranes were formed from a mixture of egg lecithin and decane on a spherical hole in a polyethylene partition. The partition separated two electrolyte solutions which were stirred magnetically (*8*). The solutions contained sodium chloride, sodium salicylate, and salicylic acid, buffered with sodium phosphate, sodium acetate, or sodium citrate. We manipulated the salicylic acid concentration by varying the *p*H, as described by the Henderson-Hasselbalch equation (*9*).

After a stable membrane had been formed, 2 to 5 μc of [^{14}C]salicylate were injected into the rear compartment. Then the rate of appearance of radioactivity in the front compartment was measured by continuous perfusion (1 to 2 ml min^{-1}) and collection of samples at 5-minute intervals. The rear compartment was sampled with a microsyringe. The samples were dried, the radioactivity was counted in a low-background planchet counter, and the one-way fluxes of solute were calculated (*10*).

We calculated the membrane resistance, using Ohm's law, from the membrane potential produced by applying a known voltage pulse across the mem-

Table 1. Effects of *p*H and an "antacid" buffer on the net flux of salicylic acid across a lipid bilayer membrane. Results in the last column are given in the sequence: mean ± standard error and, in parentheses, the number of membranes. The *p*H adjustments were made with hydrochloric acid. The membrane voltage was clamped at 0 mv, and the zero-potential current was always less than 0.1 percent of the net flux, when expressed as ionic current.

Rear solution					Front solution		Net flux ($\times 10^{-9}$ mole cm^{-2} sec^{-1})
*p*H	Sodium salicylate (m*M*)	Salicylic acid (m*M*)	Sodium chloride (m*M*)	Sodium citrate (m*M*)	*p*H	Sodium phosphate (m*M*)	
3.9	35.7	4.3	150		7.4	150	7.9 ± 0.3 (3)
4.9	39.5	0.5	150		7.4	150	0.9 ± 0.1 (3)
4.9	39.5	0.5		150	7.4	150	39 ± 4 (3)

brane in series with a known resistance. The membrane potential was recorded as the potential difference between two calomel–potassium chloride electrodes which made contact with the front and rear solutions. The membrane resistance was measured at 5- to 10-minute intervals throughout each experiment.

The first series of experiments (Fig. 1) illustrates the effects of isotopic exchange in the unstirred layers on the one-way flux of solute. Figure 1a shows that the one-way flux is roughly proportional to the salicylic acid concentration [HA] when the salicylate concentration [A$^-$] is held constant. Conversely, Fig. 1b shows that the one-way flux is also roughly proportional to [A$^-$] when [HA] is held constant. In Fig. 1, a and b, the solid circles represent experimental data whereas the curve connecting the points is calculated from an equation which will be discussed below. In both these experiments the composition of the two bathing solutions was identical except for the addition of tracer to the rear compartment.

There are several possible explanations for these results. First, Fig. 1a suggests that HA may be simply diffusing across the membrane (about 10^{-6} cm thick) and associated unstirred layers (about 10^{-2} cm thick) (*11*). However, we can rule out this possibility by considering that the maximum observed flux of 5×10^{-8} mole cm^{-2} sec^{-1} is almost 1000 times larger than the flux of HA across the unstirred layer alone (*12*).

A second possible explanation for our results might be that A$^-$, as well as HA, can diffuse across the membrane (Fig. 1b). However, this possibility can be ruled out in virtue of the high membrane resistance, which ranged from 5×10^{-5} to 2×10^{-7} ohm-cm^2 (*13*). Using an equation which relates the ionic flux to the membrane resistance (*14*), we estimate that less than 0.1 percent of the observed flux can be due to the diffusion of A$^-$ across the membrane (*15*).

A third possible explanation might be an electrically silent transport process, that is, either the diffusion of sodium salicylate ion pairs or, alternatively, an exchange-diffusion involving a reversible reaction between A$^-$ and some component of the membrane. However, these two possibilities are both ruled out by Fig. 1a, which shows that HA, as well as A$^-$, must be present in order to generate a large one-way flux of solute.

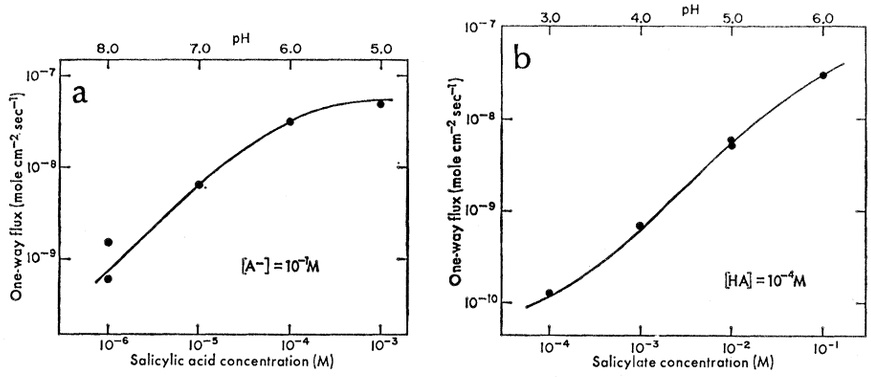

Fig. 1. (a) The one-way flux of solute as a function of [HA] at constant [A$^-$]. (b) The one-way flux of solute as a function of [A$^-$] at constant [HA]. The solutions on each side of the membrane were identical, except for the addition of tracer to the rear compartment. All solutions contained sodium chloride (50 m*M*), sodium acetate and sodium phosphate (5 m*M* each), and sodium salicylate (0.1 to 100 m*M*). Each point is a mean flux value which was calculated from four to six samples obtained from a single membrane. The variation within each group of samples was less than ± 20 percent. The solid curve is calculated from Eq. 2, which is discussed in the text.

A valid explanation for our results involves a consideration of the reversible, isotopic exchange reaction between A$^-$ and HA which occurs in the aqueous unstirred layers as well as in the bulk solutions. This isotopic exchange between the ionic and nonionic forms of a weak acid can be written as follows:

$$HA + {}^*A^- \rightleftarrows H^*A + A^- \qquad (1)$$

In most of our experiments [A$^-$] is much higher than [HA]. Thus, when a labeled H*A molecule diffuses across the membrane from the rear to the front, it is quickly destroyed by the exchange reaction with A$^-$. Conversely, when a nonlabeled HA molecule diffuses across the membrane from the front to the rear, it is quickly converted into the H*A form. Thus, the exchange reaction tends to maintain the values of [H*A] and [HA] at the membrane surfaces close to those in the bulk bathing solutions. In fact, when the ratio [A$^-$]/[HA] is large, virtually all of the tracer moves through the unstirred layers as *A$^-$, even though *A$^-$ cannot cross the membrane at a significant rate. Thus A$^-$ "facilitates" the diffusion of tracer through the unstirred layers.

If the exchange reaction is fast by comparison with the rate at which HA diffuses across the membrane, then the one-way flux will be given by the following series equation (*16*):

$$\frac{1}{J_A} = \frac{1}{P^{UL}_{HA}[HA] + P^{UL}_{A^-}[A^-]} + \frac{1}{P^{M}_{HA}[HA]} \qquad (2)$$

where J_A is the total flux of HA plus A$^-$; P^{UL}_{HA} and $P^{UL}_{A^-}$ are the permeability coefficients of the unstirred layer to HA and A$^-$ respectively; and P^{M}_{HA} is the permeability coefficient of the membrane to HA. By choosing the values

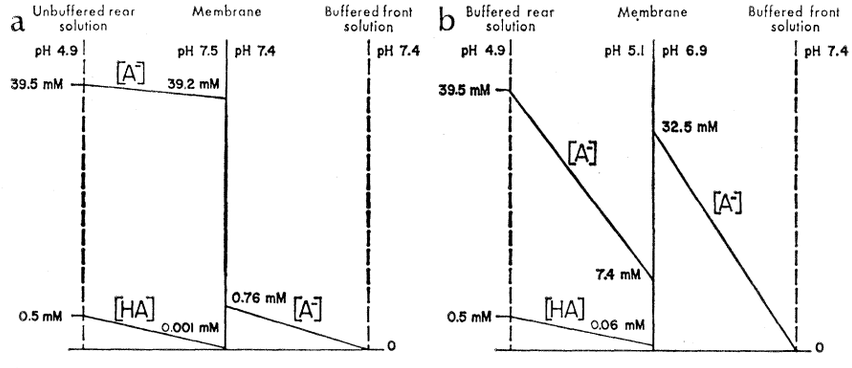

Fig. 2. Concentration profiles for HA and A$^-$ across a lipid bilayer membrane and associated unstirred layers. (a) Diffusion from a poorly buffered, acidic solution into a well-buffered neutral solution. (b) Diffusion from a well-buffered, acidic solution into a well-buffered neutral solution. The flux values and the complete composition of the solutions are given in Table 1. The concentration profiles are not drawn exactly to scale.

$P^{UL}_{HA} = P^{UL}_{A-} = 6 \times 10^{-4}$ cm sec^{-1} and $P^{M}_{HA} = 0.7$ cm sec^{-1}, we obtained the two curves shown in Fig. 1. Thus the agreement between the model and the data is good.

In this treatment we assume that the permeability of the membrane to salicylate (P^{M}_{A-}) is negligible. This assumption is justified by the high membrane resistance of about 10^6 ohm-cm^2, from which we estimate that P^{M}_{A-} is less than 10^{-7} cm sec^{-1}, that is, seven orders of magnitude less than P^{M}_{HA} (*17*).

The permeability of the membrane to HA (0.7 cm sec^{-1}) is about 1000 times larger than the combined permeability of the unstirred layers (6×10^{-4} cm sec^{-1}). Thus we could never hope to measure directly the true membrane permeability to this highly permeant solute. However, by adding A^- to the aqueous solutions, we provide a parallel pathway for tracer diffusion through the unstirred layers and, in effect, we lower the resistance of the unstirred layers to the diffusion of tracer. Thus, the exchange reaction provides a way of estimating the true membrane permeability to this highly permeant species (*18*).

Our model also provides a way of estimating the thickness of the aqueous unstirred layers. From the equation (*2*)

$$2\delta = \frac{D_{A^-}}{P^{UL}_{A^-}}$$

we estimate the combined thickness of the unstirred layers to be 1.3×10^{-2} cm. This value agrees with the values obtained by others using lipid bilayer systems (*11*).

In a second series of experiments (Table 1 and Fig. 2) we tested the hypothesis that chemical reactions in the unstirred layers may be involved in the *net* transport of HA across lipid bilayer membranes. The relevant chemical reaction in this case is the association-dissociation of HA, as well as of the protonated buffers in the unstirred layers. The reaction may be written as follows:

$$HA \rightleftarrows H^+ + A^- \qquad (3)$$

These experiments were designed to roughly simulate the absorption of salicylates by the gastrointestinal tract. Using essentially the same techniques as before, we measured the HA flux from several acidic "stomach" solutions into a neutral, well-buffered "blood" solution. In these experiments the one-way flux was equal to the net flux, because the HA was present only in the rear compartment.

The results and experimental conditions are shown in Table 1 and Fig. 2. First we measured the net flux of HA from a poorly buffered solution at *p*H 3.9 into a well-buffered solution at *p*H 7.4. The net flux was 7.9×10^{-9} mole cm^{-2} sec^{-1}, which is roughly the rate at which HA (4.3 m*M*) can diffuse across one unstirred layer. In addition, the diffusion of A^- (with H^+) makes a small contribution to the total flux through the unstirred layer. After HA crosses the membrane it is rapidly converted into A^-, which then diffuses out of the unstirred layer into the bulk solution in the front compartment.

Next we measured the net flux of HA from a poorly buffered solution at *p*H 4.9 into the same well-buffered solution at *p*H 7.4. As expected, the flux dropped to 0.9×10^{-9} mole cm^{-2} sec^{-1}, which is about one-ninth the previous value. This reduction is due chiefly to the ninefold reduction in [HA] at *p*H 4.9. Once again the net flux is approximately equal to the rate at which HA (0.5 m*M*) can diffuse across the unstirred layer.

Finally, we measured the net flux of HA from a well-buffered solution at *p*H 4.9 into the same well-buffered solution at *p*H 7.4. The flux in this case was 39×10^{-9} mole cm^{-2} sec^{-1}, which is 43 times higher than in the previous experiment. This flux is five times higher than the net flux at *p*H 3.9, even though [HA] in the bulk solution is only one-ninth the original level. This increase in the net flux is due to the presence of buffer, which maintains a higher value of [HA] in the unstirred layer. Because P^{M}_{HA} is so much larger than P^{UL}_{A-} a sizable gradient in [A^-] now develops in the unstirred layer.

Figure 2 shows approximate concentration profiles for A^- and HA under the last two of the three experimental conditions. The profiles were calculated from Fick's first law, the Henderson-Hasselbalch equation, and the fact that the total flux ($J_{HA} + J_{A-}$) is everywhere constant in the steady state (*19*). The two concentration profiles show schematically the large concentration gradients which exist in the unstirred layers in both poorly buffered and well-buffered solutions. The major difference is that in the poorly buffered solution the largest gradients exist in [H^+] and [HA] (Fig. 2a), whereas in the well-buffered unstirred layer there are large gradients in [A^-] and [HA] (Fig. 2b).

Pharmacologically, these results may apply to the absorption of salicylates and other weakly acidic drugs by the stomach, where thick unstirred layers of mucus are likely to exist. The controversy concerning the relative merits of buffered aspirin versus plain aspirin is apparently unresolved (*6*). However, our results show that, in principle, an "antacid" buffer can increase the rate of absorption of a weakly acidic drug, even though the concentration of the permeant species in the bulk stomach solution may be *decreased* by the addition of buffer.

The diffusion of other weak acids and weak bases, for example, carbon dioxide and ammonia, is also biologically important. In the light of our results, the possibility that chemical reactions in the aqueous unstirred layers play an important role in these processes must now be considered.

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$$\delta = D_j / P^{UL}_j$$
where δ is the thickness of the unstirred layer (in centimeters), D_j is the diffusion coefficient (in square centimeters per second) for a particular species j, and P^{UL}_j is a permeability coefficient.
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8. The concentration of egg lecithin in decane was 20 to 40 mg ml^{-1}. (The egg lecithin was purchased from Supelco, Bellefonte, Pa.) The surface area of the membrane was 2.0 ± 0.1 mm^2. The front and rear compartments each had a volume of 1.2 ml. The temperature was $23° \pm 2°$C.
9. The equation is as follows:
$$pH = pK + \log \frac{[A^-]}{[HA]}$$
Because egg lecithin is isoelectric over a wide range of *p*H [A. D. Bangham, B. A. Pethica, G. F. V. Seaman, *Biochem. J.* **69**, 12 (1958)], we assumed that altering the *p*H of our solutions did not greatly affect the permeability properties of the membranes.
10. The [^{14}C]salicylate was purchased from ICN, Irvine, Calif. The one-way flux of solute across the membrane was calculated from the equation:
$$J_A = \frac{^{14}CF}{t\,A\,SA^R}$$

where J_A is the one-way flux (in moles per square centimeter per second), $^{14}C^{T}$ is the amount of tracer (in counts per minute) entering the front compartment during the sampling time t (in seconds), A is the surface area of the membrane (in square centimeters), and SA^{R} is the specific activity of tracer in the rear compartment (in counts per minute per mole).

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12. From Fick's first law

$$J_{HA} = D_{HA}[HA]/\delta$$

we estimated the rate of diffusion of $10^{-4}M$ HA across the unstirred layer of water 10^{-2} cm thick to be about 8×10^{-11} mole cm^{-2} sec^{-1}. The diffusion coefficient for HA in water is 8×10^{-6} cm^2 sec^{-1}.
13. The membrane resistance in the presence of high concentrations of A$^-$ and HA was 10 to 50 times lower than the control level, which ranged from 10^7 to 10^8 ohm-cm^2. We will not discuss in detail these changes in membrane resistance, because in all of our experiments 99.9 percent of the one-way flux or net flux, or both, was electrically silent. The effect of salicylates on the electrical properties of phospholipid bilayers has been discussed by S. McLaughlin [*Nature* **243**, 234 (1973)].
14. The appropriate equation is

$$J_{A^-} = \frac{R\,T}{Z^2\,F^2} G_{A^-}$$

where J_{A^-} is the one-way flux of salicylate ions, R is the gas constant, T is the absolute temperature, Z is the ionic valence, F is the faraday, and G_{A^-} is the conductance of the membrane to A$^-$. Because the total membrane resistance was always greater than 10^5 ohm-cm^2, the upper limit for G_{A^-} is $< 10^{-5}$ mho cm^{-2} and the upper limit for J_{A^-} (or $J_{HA_2^-}$) is $< 2.5 \times 10^{-12}$ mole cm^{-2} sec^{-1} [see A. L. Hodgkin, *Biol. Rev. (Cambridge)* **26**, 339 (1951)]. A second argument against the possibility of an ionic diffusion process is that an applied membrane voltage of +50 mv had no effect on the one-way flux.
15. However, if *both* the ionic and nonionic forms of the weak acid are highly permeant, then the electrical properties of the membrane may be very dependent upon chemical reactions within the unstirred layers [see, for example, LeBlanc (*3*) and B. Neumcke, *T.I.T. J. Life Sci.* **1**, 85 (1971)].
16. J. Gutknecht, L. J. Bruner, D. C. Tosteson, *J. Gen. Physiol.* **59**, 486 (1972).
17. From electrostatic considerations it is not surprising that the permeability to the neutral species (HA) is orders of magnitude higher than the permeability to the charged species (A$^-$) [see Finkelstein and Cass (*2*)].
18. Our model also provides a reasonable explanation for some "anomalous" observations on the diffusion of labeled weak acids across an amphibian epithelium. H. Rosen, A. Leaf, and W. Schwartz [*J. Gen. Physiol.* **48**, 379 (1964)] found that the permeability coefficients of nonionic forms of lipophilic species appear to increase markedly with increasing *p*H. However, as the *p*H increases, the ratio [A$^-$]/[HA] increases, and A$^-$ provides a parallel pathway for tracer diffusion through the unstirred layers. Thus the permeability coefficient for HA appears to increase with increasing *p*H, whereas the true permeability coefficient for HA remains constant.
19. We assumed, as before, that the reacting species are in chemical equilibrium throughout the bulk solutions and unstirred layers. The values that we used for the permeability coefficients (in centimeters per second) were as follows:

$$P^{M}_{HA} = 0.7,\ 2P^{UL}_{A^-} = 2P^{UL}_{HA} = 1.2 \times 10^{-3},$$

and

$$2P^{UL}_{H^+} = 1.3 \times 10^{-2}.$$

The factor 2 is used because in this case we consider each unstirred layer separately.
20. Supported by PHS grant HE 12157. We thank D. Hastings, J. Graves, and D. Benos for constructive comments.
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Mapping Human Autosomes: Assignment of the MN Locus to a Specific Segment in the Long Arm of Chromosome No. 2

Abstract. *The locus for MN blood groups,* MN, *is tentatively assigned to a specific region near the centromere of the long arm of chromosome No. 2 on the basis of a demonstrable deletion of band 2q14 from a No. 2 chromosome in a boy previously reported to be hemizygous* (M/−) *at that locus.*

A mildly retarded boy with microcephaly, facial dysmorphia, and an abnormal gait was reported in 1968 by German, Walker, Stiefel, and Allen (*1*), with evidence (*2*) that the MN blood group locus of only one chromosome was being expressed. The father's blood type was N, but the child's was M. Dosage tests with antiserums to M and N (anti-M and anti-N) suggested that the boy was not homozygous for *M*; the father, however, gave double-dose reactions with antiserum to N, confirming that he was homozygous (*N/N*). (The mother and both sibs of the propositus, who were type MN, gave single-dose reactions with anti-M and anti-N). The child, therefore, appeared to be hemizygous at the *MN* locus, or to have a condition that could not be distinguished from hemizygosity by serological methods.

The child's blood lymphocytes had a unique chromosome complement, in which a reciprocal translocation had occurred between the long arm of a chromosome No. 2 and the long arm of a No. 4; by the nomenclature of the Chicago Conference (*3*) his complement was 46,XV,t(2q−;4q+). Each of the 70 metaphase cells examined had the translocation, and no evidence of mosaicism was found. The rearrangement was considered to have occurred de nova, because the complements of the child's parents were normal. An autoradiographic study of the translocation made when it was first detected showed that the breaks, at least one in each chromosome, had occurred near the middle of 2q and near the end of 4q. Measurements of the affected chromosome segments suggested that a short segment of chromosome had been lost during the rearrangement (*2*). The maldevelopment of the child supported the conclusion that the translocation was not completely balanced, that is, that either deficiency, duplication, or both had occurred; but, position effect was considered to be another possible explanation. Similarly, loss of a segment of chromatin seemed to be the most likely explanation for the child's hemizygosity (*M/−*), but translocation of the gene to a new position near heterochromatin (leading to inactivation) was also a reasonable possibility, especially because 4q consists of prominently late-replicating chromatin.

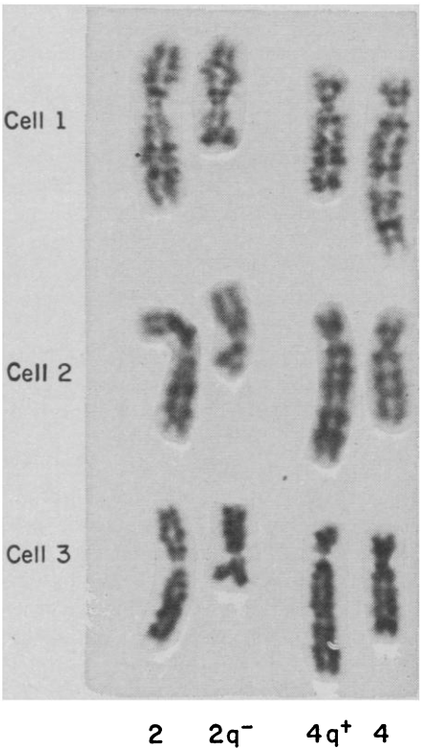

Fig. 1. G-banding of the two chromosomes No. 2 (normal and rearranged) and the two chromosomes No. 4 (normal and rearranged) in three cells.

The observations reported suggested to us that *MN* is normally located in either the 2q or the 4q, but neither confirmatory nor contradictory reports have appeared since our report. [Blood grouping in one other patient studied in our laboratory (*1*) suggested that the locus was not in the 4q.]

A fibroblast cell line, identified as HG 406, had been derived from a fragment of skin taken from the patient in 1968 and has been maintained since in liquid nitrogen. It has now been activated, and the translocation has been characterized by G-banding techniques (*4*, *5*) with the use of cells harvested at the tenth subculture generation.

We analyzed 57 cells with chromosomes showing good band patterning.