labeling of the cell surface. This suggests that PMV's contain at least certain antigens that are represented over the entire cell membrane. In addition, no significant staining of cytoplasmic or nuclear components can be observed in fixed cell preparations (*20*). The results reported here, therefore, establish that PMV's have the general characteristics of plasma membranes.

Plasma membrane vesicles isolated by this new method should provide a valuable source of material to study changes in membrane composition and in the modulation of linkages of cell surface receptors and membrane enzymes. It should also be possible to study differences in the phosphorylation of membrane proteins of various cell populations, in view of our recent observation that L_6 myoblast PMV's contain an adenosine 3′,5′-monophosphate-dependent protein kinase (*21*). This technique may also serve as a model system to study the physiological shedding of the cell surface reported in lymphocytes and other mammalian cells (*22*).

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6. Guinea pig macrophages from sterile peritoneal exudates, induced by Marcol 50, were washed and incubated in RPMI 1640 for 1 hour before vesiculation to allow the cells to attach and spread. The J-111 leukemic human monocytes were obtained from the American Tissue Culture Collection. Rat L_6 macrophages were kindly provided by D. Shubert and were grown in Dulbecco's modified essential medium (DMEM) containing 10 percent fetal calf serum (FCS). The 3T3 and SV3T3 cells were obtained from G. Todaro, and AKR mouse embryo cells and methylcholanthrene-transformed AKR cells were obtained from H. Moses. The 3T3 cells were grown in DMEM and 10 percent FCS and AKR cells were grown in McCoy's medium in 10 percent FCS. Continuous cell cultures were routinely performed at 37°C in a humidified atmosphere containing 10 percent CO_2. All cell lines were negative for mycoplasma as determined by duplicate culture by Flow Laboratories and the Mayo Microbiology Laboratory and by electron microscopy.
7. Formaldehyde was prepared fresh by dissolving paraformaldehyde in H_2O at 65°C with continuous stirring, followed by the addition of NaOH to clear the solution. This 10 percent formaldehyde solution was cooled and then added to either phosphate-buffered saline or tissue culture medium to achieve the desired concentration.
8. Intact cells dislodged from the cell monolayer can be removed by filtration through a glass wool column or by centrifugation on a Ficoll or sucrose gradient adjusted to a density at which PMV's band at the interface and whole cells form a pellet at the bottom of the tube.
9. Plasma membrane vesicle pellets were fixed in 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer for 12 hours at 4°C. Pellets were then washed and postfixed in 2 percent osmium tetroxide in 0.1*M* phosphate buffer for 1 hour. Specimens were dehydrated in ethanol, stained en bloc in 2 percent uranyl acetate, and embedded in Epon. Sections were than stained in uranyl acetate and lead citrate. Thin sections were examined in a Philips EM 201 electron microscope.
10. Preliminary results suggest that vesicles can be disrupted by nitrogen cavitation and that the contents of the vesicles can be removed by washing in 10 m*M* EDTA under appropriate conditions.
11. Cells for scanning electron microscopic studies were grown on glass cover slips, treated with 25 m*M* formaldehyde and 2 m*M* dithiothreitol for 30 minutes at 37°C, then either fixed in 1 percent calcium permanganate in 0.1*M* phosphate buffer for 10 minutes at 4°C and postfixed in 2.5 percent glutaraldehyde in 0.1*M* cacodylate buffer for 12 hours at 4°C or fixed solely in 2.5 percent glutaraldehyde–0.1*M* cacodylate buffer for 90 minutes at 37°C. Specimens were coated with gold-palladium and examined in an ETEC scanning electron microscope.
12. Vesicle protein content was routinely determined as described by O. Lowry, J. S. Rosebrough, A. C. Farr, and R. J. Randall [*J. Biol. Chem.* **193**, 265 (1951)]. Since dithiothreitol (DTT) interferes with the Lowry reaction, vesicle protein determinations by the Lowry procedure were performed only on specimens that had been extensively dialyzed. To further alleviate this problem, protein assays were also performed by the fluorescamine method. Vesicles isolated from approximately 10^8 cells and centrifuged at 30,000*g* for 30 minutes contain up to 500 μg of protein. Some of this protein represents intravesicle content. Plasma membrane enzyme activities expressed per milligram of protein, therefore, represent minimal values.
13. Plasma membrane vesicles to be analyzed by gel electrophoresis were dialyzed against water or 10 m*M* tris buffer, *p*H 7.4, for 36 hours at 4°C to remove bound formaldehyde. Our studies have shown that this treatment removes all detectable bound [^{14}C]formaldehyde from PMV's. Similar reversible binding of formaldehyde to RNA and DNA has been reported by E. J. Ering and J. Ofengand [*Biochemistry* **6**, 2500 (1967)]. Vesicles were then centrifuged at 30,000*g* at 4°C for 30 minutes, and the wet pellet was solubilized in 1 percent sodium dodecyl sulfate, 10 m*M* DTT, and 8*M* urea and heated at 90°C for 5 minutes. In occasional samples some material remained insoluble. It was removed by centrifugation at 30,000*g* for 15 minutes at room temperature. Aliquots were then placed on 7.5 percent acrylamide gels. A 0.1 percent SDS–0.1*M* PO_4 buffer (*p*H 7.4) was used. Gels were subjected to electrophoresis at 8 ma per tube. They were then fixed overnight and subsequently stained with Coomassie blue for protein or with periodic acid–Schiff reagent for carbohydrate (not illustrated).
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17. Plasma membrane vesicle phospholipid composition was analyzed by two-dimensional thin-layer chromatography of the vesicles extracted in chloroform and methanol (2 : 1). Elution in the first dimension was in chloroform, methanol, and 28 percent aqueous ammonia (130 : 70 : 10) and in the second dimension in chloroform, acetone, methanol, acetic acid, and H_2O (100 : 35 : 25 : 20 : 10). The percentages of individual phospholipids were determined by inorganic phosphorus assay of individual thin-layer chromatography spots according to the method of L. F. Eng and E. P. Noble [*Lipids* **3**, 157 (1968)].
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23. This work was supported solely by the Mayo Foundation. I thank R. G. Perkins, P. Maerckelein, M. Zschunke, B. Hoerl, and D. Florine for assistance in these experiments and for helpful discussions.

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Inhibition by Anions of Human Red Cell Carbonic Anhydrase B: Physiological and Biochemical Implications

In a study of inhibition by anions of the enzymatic activity of human erythrocyte carbonic anhydrases B and C, Maren *et al.* (*1*) measured inhibitory behavior for both hydration of CO_2 and dehydration of HCO_3^-, tabulating their results as I_{50}, the inhibitor concentrations that halve the observed velocities of the enzymatic reactions. With but a single exception, values of I_{50} for the dehydration reaction were reported to be substantially greater than for the hydration reaction; for Cl^-, the ratio of values is 16. The experimental conditions were stated to be such that the I_{50} values should not be much different from the true K_I values. Maren *et al.* say that these results do not violate the Haldane relation (*2*): "Our finding of different inhibition constants between hydration and dehydration would only violate considerations of equilibria, that is, the Haldane relation, if the inhibiting mechanism were the same in the two directions."

However, the results of Maren *et al.* do violate the principle of microscopic reversibility, that is, the Haldane relation. That this is so can be seen from the following. For a solution of CO_2 and HCO_3^- in buffer at *p*H 7.2—the mean value used in (*1*)—at 25°C, the ratio of $[CO_2]$ to $[HCO_3^-]$ is 1:10, from the known pK_E of 6.2 (*3*). The lifetime of these two species must be in the same ratio to maintain equilibrium; for example, in the absence of enzyme, the mean time of hydration of a CO_2 molecule is about 20 seconds and the time is independent of *p*H below *p*H 9; the mean time of dehydration of a HCO_3^- anion must then be about 200 seconds (*3*). The addition of enzyme to the solution can only decrease the lifetimes—that is, increase the rates of interconversion of CO_2 and HCO_3^-—by the identical factor for each direction. This is

by definition, since an enzyme does not change the ratio of the equilibrium concentrations of the reactants. Subsequent addition of an inhibitor of enzymatic activity, whatever the mechanism of inhibition, must decrease both catalyzed rates by the same (new) factor if equilibrium is to be maintained, and the Haldane relation obeyed. The inhibition data of Maren *et al.* violate this last requirement. Their data indicate [table 1 in (*1*)] that addition of 6 m*M* Cl^- will halve the rate of hydration of CO_2, while the rate of dehydration of HCO_3^- will be decreased by a negligible amount (< 6 percent), since 106 m*M* Cl^- is required to halve the dehydration rate.

If the hydration rate is halved and the dehydration rate is unchanged, the ratio of $[CO_2]$ to $[HCO_3^-]$ must assume a new equilibrium value of 1 : 5. Thus, according to (*1*), the addition of 6 m*M* NaCl in the presence of enzyme alters the equilibrium constant of the reaction $CO_2 \rightleftharpoons HCO_3$ by a factor 2, in violation of equilibrium mass action principles and the Haldane relation. (This small amount of salt added to the buffer already present is far too small an amount to produce a "salt effect," that is, a shift in equilibrium constant due to a change in ionic strength.) Therefore, the data of Maren *et al.* cannot be a correct measure of anion inhibition of carbonic anhydrase activity at equilibrium.

It should be clear from the above that the requirements of equilibrium will be met in the presence of enzyme only if, regardless of the extent to which the enzyme may be inhibited, the enzyme alters the rates of the uncatalyzed forward and backward reactions by the same factor. This is nothing more than a restatement of the Haldane relation. In its usual form, the Haldane relation states that, in the *limit of zero substrate concentrations*, the ratio of initial velocities for both directions of an enzymatically catalyzed, reversible reaction is equal to the equilibrium constant for the reaction. Maren *et al.* suggest, incorrectly, that the Haldane relation may be violated for anions "which inhibit competitively for dehydration and noncompetitively for hydration." There is implicit in this view the (incorrect) thought that equilibrium can be established by the catalysis of hydration along one pathway and dehydration along another. This view violates the well-established principle of detailed balance, according to which every mechanism that can serve as a pathway for reaching thermodynamic equilibrium in a system must have its forward and backward rates balance in detail; each mechanism by itself must produce the same equilibrium conditions with the same equilibrium constant. The principle of detailed balance is, in essence, another statement of the ideas of microscopic reversibility and the Haldane relation. Moreover, under conditions of low substrate concentration, such that initial velocities are linear in substrate concentration and the Haldane relation holds, there is no distinction possible between competitive and noncompetitive inhibition; there is not enough substrate present to compete with added inhibitor. Application of equilibrium arguments to the enzymatic mechanism of carbonic anhydrase has been made before (*4*).

It is not clear where the inhibition experiments (*1*) fail, but two methodological procedures are suspect. First, during a hydration experiment, the *p*H is allowed to change from 7.6 to 6.9 and, during a dehydration experiment, from 6.95 to 7.5. The enzymatic activity of carbonic anhydrase is very sensitive to *p*H (*3*), particularly in this range, as is the equilibrium ratio of $[CO_2]$ to $[HCO_3^-]$. The experiments can only yield data that are complex averages over *p*H. Second, Maren *et al.* claim that phosphate inhibits the hydration reaction but not the dehydration reaction; they use phosphate buffer in their dehydration experiments but not in hydration experiments. The point has already been made that an inhibitor cannot inhibit only one direction of a reaction in the regime where the Haldane relation holds.

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We raised the issue of the Haldane relation in our report, with a note that "There is work in progress on this unusual problem" (*1*). The problem arose when we found lower I_{50} values (the concentration that decreases the catalyzed rate by 50 percent), not K_I's (the dissociation constant between substrate and enzyme), for all of nine inhibitory anions (there were no exceptions) when measuring initial rates of catalytic hydration of CO_2, compared to dehydration of HCO_3^-. We are aware of the principle cited by Koenig and Brown, but the apparent departure from "laws" should not inevitably be considered as experimental failures. Rather, such findings may yield new and unexpected insights. Koenig and Brown offer no data, and, if they repeated these or analogous experiments, they would have the same results; they do not mention work which bears this out (*2, 3*). Of course we agree that at equilibrium two opposing reaction rates are equal.

The primary finding that we reported (*1*) is the relatively high inhibitory potency of Cl^- against human red cell carbonic anhydrase B in the hydration (CO_2 as substrate) reaction. This has clear implications in respiratory physiology and raises the question of the real role of this protein. Other anions are even more active, the potency of CNO^- being 10^3 times that of Cl^-. To our initial surprise, however, the anions were less active (by 4- to 24-fold) studied in the dehydration (HCO_3^- as substrate) reaction, under closely similar conditions. (We did use phosphate buffer in both tests; our table 1 (*1*) shows this for Cl^- and I^-. The *p*H range also was the same in both series.) This close comparison had not been made before, although there are data in agreement with ours (*2, 3*). The same relation was found for carbonic anhydrase C, although here the anions are much less active and the I_{50} ratio, dehydration/hydration, centers about four, rather than ten.

The determination of K_I awaits full exploration of the mechanisms, including precise determination of certain of the kinetic constants, notably K_m HCO_3^- and K_S HCO_3^- (K_m, Michaelis constant; K_S, dissociation constant for substrate) (see below). These were not determined, nor was that our primary purpose. To that extent, Koenig and Brown are justified to criticize a few sentences in our discussion (*1*, p. 471) in which we attempted, from literature values, to gauge the relation between I_{50} and K_I for the dehydration reaction.

We may now discuss the Haldane relation, in view of these data (*1*) and work of the past year. (Parenthetically, Koenig and Brown define the Haldane relation in curious ways, in terms of the "lifetime of the two species" and of the "ratio of initial velocities at limit of zero substrate.") We have clear-cut results that the anion inhibitory mechanism is different between hydration and dehydration when CO_2 and HCO_3^- are considered as substrates. With I^- and CNO^-

inhibition against both carbonic anhydrase B and C hydration (CO_2) is noncompetitive, while that against dehydration (HCO_3^-) is competitive. This is not surprising on structural grounds, and although not certain at the time, was used as the base for our discussion of the Haldane relation (*1*). This new situation in enzymology is based on the antipodal qualities of the two substrates. In these experiments, the I_{50} for I^- and Cl^- in the hydration reaction (for enzyme B) are tenfold greater at *p*H 8.2 than at 7.2, showing direct competition between OH^- and the halides for the protonated (E) form of the enzyme, an effect predicted from earlier binding studies (*4*, *5*). Inhibitors thus compete with OH^- and HCO_3^- for E, but not with CO_2.

Bicarbonate as well as OH^- has a high affinity for the B enzyme, as shown by our data (*1*). From inhibition of the hydration reaction (table 1) and the suppression of Cl^- inhibition by 15 m*M* HCO_3^-, the HCO_3^- K_S may be fairly judged to be less than 5 m*M*. The K_m of HCO_3^- has been variously reported, but our recent value is relatively low, 15 m*M*, in agreement with that cited in (*4*). Why then is it surprising that, with 30 m*M* HCO_3^- as substrate in the dehydration reaction, the I_{50} of other (competitive) anions is relatively high?

Turning specifically to the Haldane equation, we write the catalytic reaction in simplified form

$$EOH + CO_2 \rightleftarrows EHCO_3 \rightleftarrows HCO_3^- + E$$

At constant *p*H (where k_{cat} is the turnover number)

$$\frac{k_{cat}\,HCO_3^- \times K_m\,CO_2}{k_{cat}\,CO_2 \times K_m\,HCO_3^-} =$$

$$K_{equil} = \frac{(CO_2)}{(HCO_3^-)}$$

The changes on the left side are dictated by the inhibition mechanism. A given concentration of anion will decrease k_{cat} CO_2 but leave k_{cat} HCO_3^- unchanged. K_m CO_2 will be unchanged, but K_m HCO_3^- will rise. We made this point (*1*). Under the present conditions, with initial (HCO_3^-) considerably higher than its K_m or K_S (see above), this latter change may not be readily apparent in our experiments, since there may be only a small effect on velocity.

The effect of anion inhibition will therefore leave the terms of the numerator on the left unchanged, and alter the terms of the denominator in opposite directions. The result is to preserve the Haldane relation.

Finally, a critical error by Koenig and Brown (end of penultimate paragraph) is to speak, presumably of our experiments, as "conditions of low substrate concentration" . . . where "there is no distinction possible between competitive and noncompetitive inhibition." Reference to our report and to the above show that (S) for HCO_3^- is at least as high, and probably much higher than its K_m or affinity constant. The substrates were chosen in attempts to come reasonably close to physiological situations, and still acquire useful biochemical data.

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22 September 1976

No Desertification Mechanism

Jackson and Idso (*1*) have stated that there is little justification for the desertification mechanism theory proposed by Otterman (*2*). They argued (*1*) that their own "analysis tends to indicate that the denuding of soil may have thermal and climatic effects just the opposite of those that he [Otterman] has postulated" (*3*). Otterman's hypothesis was triggered by a "sharp demarcation line" seen in a Landsat-1 image of southwestern Israel and northwestern Sinai (*2*). The same line was seen on chromatic and achromatic photographs taken by one of the Apollo 7 astronauts in October 1968 (Fig. 1). Otterman suggested that the line, which coincided with the 1948–1949 armistice line between Israel and Egypt, was an indication of a desertification mechanism: namely, that the Sinai side of the

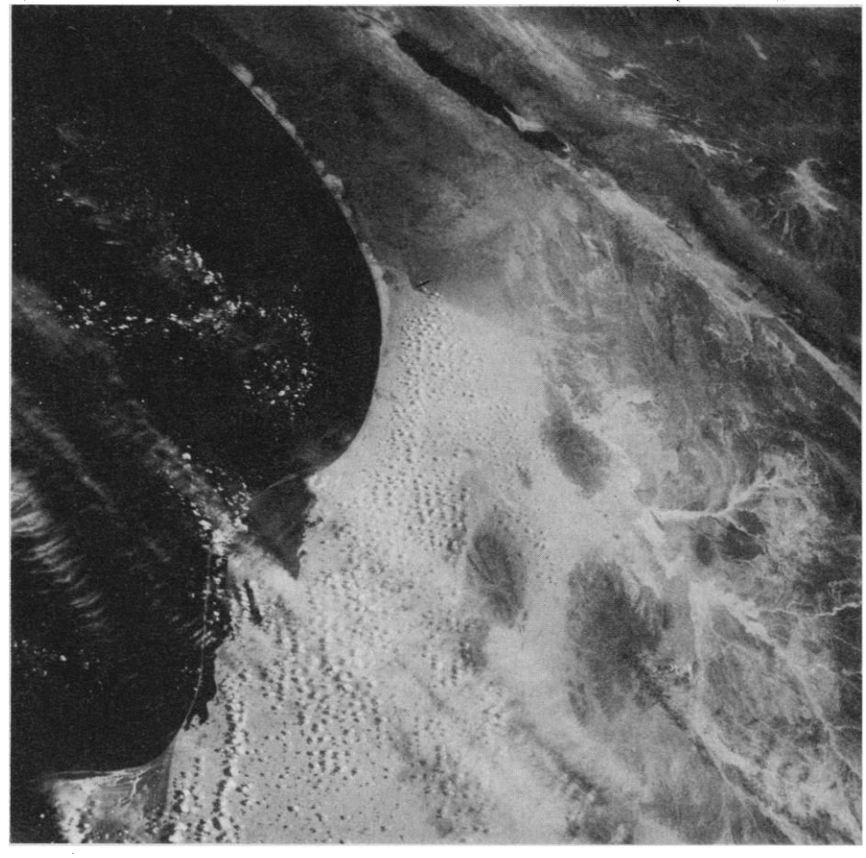

Fig. 1. The Sinai-Negev region: National Aeronautics and Space Administration photograph AS7-6-1696 taken during the 42nd orbit of Apollo 7 at an altitude of 230 km on 14 October 1968 at 11:13 local solar time with a sun elevation of 40°; the demarcation line has the coordinates 30°59′N, 33°55′E.