Biochimica et Biophysica Acta, 981 (1989) 358–362
Elsevier

BBA 74442

Erythrocyte bisulfite transport

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(Received 4 October 1988)

Key words: Band 3 protein; Bisulfite; Bicarbonate; Sulfate; Colloid osmotic lysis; Anion transport; (Human erythrocyte)

The wide range of transport rates for anions of differing chemical structure by the human erythrocyte anion transport protein (Band 3 protein) suggests that this protein is highly selective for anions that chemically resemble its natural substrate bicarbonate. To test this hypothesis, the influx of bisulfite (HSO_3^-), a bicarbonate analog, was compared to influxes of chloride, sulfate, and bicarbonate, as measured by the technique of colloid osmotic lysis in isotonic ammonium salt solution. The lysis time induced in chloride solution ($\gg$ 10 min) was markedly accelerated to 0.6 min by the addition of small amounts (5 mM) of bicarbonate, an effect characteristic of colloid osmotic lysis induced by the anion transport pathway. Lysis in bicarbonate solution was extremely rapid (0.09 min), and was markedly inhibited by acetazolamide (2.9 min). Lysis in bisulfite solution occurred spontaneously (2.2 min) but was markedly accelerated to a time similar to that of chloride (0.56 min) by addition of 5 mM bicarbonate. In contrast, sulfate induced lysis was extremely slow (< 10% lysis at 40 min in the presence of bicarbonate). Preincubation of erythrocytes with SITS, an inhibitor of anion exchange, prevented lysis by chloride, but had no effect on lysis by bicarbonate, indicating that lysis by bicarbonate was predominantly through diffusion and not anion transport. SITS treatment of erythrocytes eliminated the catalytic effect of bicarbonate during lysis by bisulfite, indicating that anion transport of bisulfite and diffusion of the conjugate acid in the form of SO_2 both contribute to the total membrane flux. When the contribution of diffusion is taken into account, the rate of bisulfite influx through the anion exchange pathway is at least 100-fold faster than that for sulfate.

Introduction

The anion transport protein (Band 3) is responsible for the extremely rapid, electrically neutral transmembrane exchange of Cl^- for HCO_3^- in the respiratory cycle of the erythrocyte. Moreover, essentially all anion transport in the red cell is effected by the anion transport protein. This protein has a great deal of selectivity toward the chemical nature of the transported anion. For example, transport of divalent anions (e.g., SO_4^{2-}) is about 100-times slower than that of monovalent anions of the same size (I^-) [1]. Additionally, transport of smaller anions is favored over larger anions. We believe that the anion transporter is highly selective for anions that structurally resemble bicarbonate, the natural substrate for this protein.

In the present study, we test this hypothesis by using the technique of colloid osmotic lysis to compare the influx of bisulfite (HSO_3^-), a chemical analog of bicarbonate, to that of HCO_3^- and Cl^-, as well as to the dissimilar anion SO_4^{2-}.

Methods

Osmotic lysis solutions. NH_4HSO_3 was prepared by combining 1.0 M H_2SO_3 and 5 M NH_4OH in stoicheiometric ratio. When diluted to isotonicity (297 mosM) this solution had a concentration of 180 mM and a pH of 7.11. Isotonic solutions of the following salts were prepared from reagent grade solids: NH_4HCO_3, 179 mM, osmolarity 297 mosM (pH 7.80); NH_4Cl, 150 mM, osmolarity 298 mosM (pH 7.11); $(NH_4)_2SO_4$, 140 mM, osmolarity 301 mosM (pH 7.14); $NaHSO_3$, 160 mM, osmolarity 323 mosM (pH 7.11). The pH values were adjusted with NH_4OH to 7.11–7.15 to match the pH of the bisulfite solution, and the salt concentrations were chosen to render the solutions approximately isotonic. Solutions were deoxygenated by bubbling with N_2 to minimize the oxidation of bisulfite to sulfate and to remove dissolved CO_2. The osmolarities were measured

Abbreviations: S.E., standard error of the mean; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

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on an Osmette S freezing point depression osmometer. The pH values were measured on a Corning 130 pH meter following deoxygenation.

Erythrocyte preparation. Fresh erythrocytes were prepared from whole blood obtained from adult volunteer donors by washing twice in 150 mM NaCl, to remove the plasma and buffy coat. The erythrocytes were then resuspended to a packed cell volume of 10% in 150 mM NaCl. The suspensions were incubated for 30 min at 37 °C under air passed through an alkali trap to remove CO_2. Two mM acetazolamide were then added to some cell suspensions. All suspensions were then deoxygenated by incubation under N_2 for 30 min with agitation in order to minimize any reduction of oxyhemoglobin by bisulfite during the experiments and reduce residual CO_2. The cells were then packed by centrifugation and used immediately.

Lysis times. Erythrocyte lysis was determined by measuring the change in the optical absorption at 700 nM of suspensions of 20 μl erythrocytes in 5 ml of each lysis solution, with a blank consisting of a hemolysate prepared by the addition of 20 μl deoxygenated erythrocytes to 5 ml H_2O. This optical frequency was chosen to minimize any absorption changes due to changes in the oxygenation state of hemoglobin during the course of the experiment. The spectrophotometer used was a Sequoia-Turner model 330, with a 10 mm light path. Absorbence vs. time curves were recorded on a Linear *X-Y* recorder for 10 min or longer. For some studies, 5 mM NH_4HCO_3 was added, and in some studies 1–2 mM SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) was added to the lysis solutions and the packed erythrocytes prior to the initiation of the experiment. The pH values of the lysis solutions were unaffected by the addition of 5 mM HCO_3^-. The delay time between mixing and initiation of recording of hemolysis was typically 3–4. Except as noted, each result reported represents the mean ± S.E. of three to four determinations.

Results

The colloid osmotic lysis technique measures hemolysis produced when water accompanies the net flow of solutes into the cell. Although the cation NH_4^+ is impermeant, it readily dissociates to neutral NH_3 which diffuses across the membrane [2]. In the absence of net anion flow into the cell, the NH_4^+ influx would be limited due to charge neutrality since virtually all anion transport is a 1 : 1 transmembrane exchange [3]. Thus, exchange of an extracellular anion for intracellular Cl^- (or other anion) results in no net influx of solute. If an anion, such as HCO_3^- is capable of crossing the membrane in neutral, undissociated acid form, such as CO_2, then hemolysis will occur. Alternatively, when an anion does not penetrate the membrane in undissociated form, net anion movement can be induced when catalytic amounts of HCO_3^- are present [4]. This occurs because the inflowing anion may be exchanged through the anion transport channel for intracellular HCO_3^-, which may then diffuse back across the membrane as CO_2 and be reconverted to HCO_3^-. At a given pH and HCO_3^- concentration, this latter mechanism results in hemolysis at a rate which is proportional to the transport rate of the anion by the Band 3 protein.

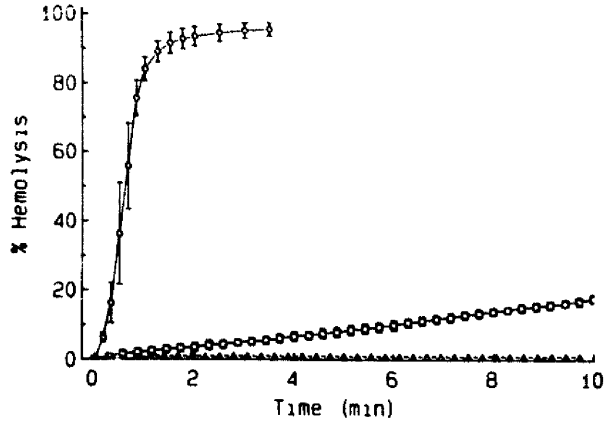

Fig. 1. Colloid osmotic lysis of erythrocytes suspended in isotonic NH_4Cl and $(NH_4)_2SO_4$ solutions. The time to 50% lysis in Cl^- solution (□; 30 min) is accelerated 50-fold by the addition of 5 mM HCO_3^- (○; 0.60 ± 0.11 min). No significant lysis is observed in SO_4^{2-}/5 mM HCO_3^- solution (△). Except as noted in the text, each curve represents the mean ± S.E. of three to four determinations.

Although Cl^- exchange by the Band 3 protein is extremely rapid, the net flow (permeability) across the membrane is five orders of magnitude lower [5], and therefore in the absence of HCO_3^- hemolysis is expected to be quite slow. Fig. 1 depicts the colloid osmotic lysis rates for isotonic NH_4Cl solution at pH 7.1. In the absence of added HCO_3^-, only 17% hemolysis occurs by 10 min, and the 50% lysis point is not reached until 30 min. However, with the addition of 5 mM HCO_3^-, hemolysis is accelerated approx. 50-fold, so that the 50% hemolysis point is reached at 0.60 ± 0.11 (S.E.) min.

Because H_2SO_4 is an extremely strong acid, sulfate exists in essentially completely dissociated form SO_4^{2-} at the pH region studied in this experiment (pH 7.1). Transport of this divalent anion has been reported to be four orders of magnitude slower than that for Cl^- [6]. When the colloid osmotic lysis was measured for $(NH_4)_2SO_4$ in the presence of 5 mM HCO_3^- (Fig. 1), no measurable hemolysis was observed after 10 min. When the observation time was extended to 40 min, < 10% hemolysis occurred. Therefore, under these conditions SO_4^{2-} transport is more than 160-fold slower than Cl^- transport.

HCO_3^- transport by the Band 3 protein has been reported to be faster than that of Cl^- [7]. While the

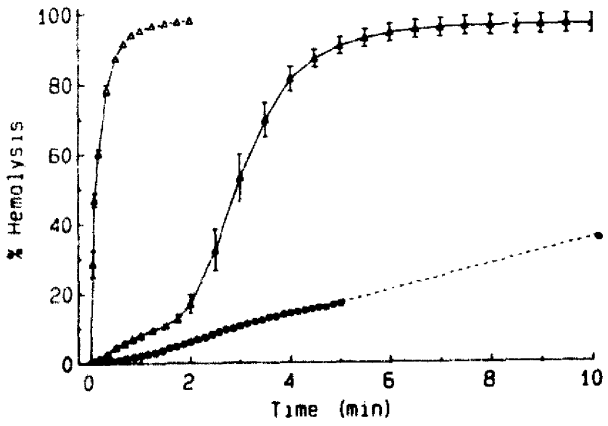

Fig. 2. The effect of inhibition of erythrocyte carbonic anhydrase on colloid osmotic lysis. The 50% lysis time in isotonic NH_4HCO_3 solution (△; 0.09 ± 0.02 min) is prolonged 30-fold by preincubation of erythrocytes in 2 mM acetazolamide (▲; 2.91 ± 0.02 min). Lysis of acetazolamide-treated erythrocytes in Cl^- solution with 5 mM HCO_3^- (●) is only 37% complete by 10 min.

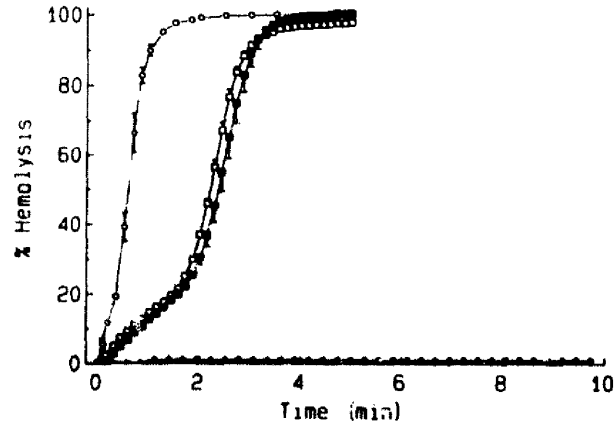

Fig. 3. Colloid osmotic lysis in isotonic sulfite solutions. The 50% lysis times are: NH_4HSO_3 (□), 2.19 ± 0.03 min; NH_4HSO_3 + 5 mM HCO_3^- (○), 0.56 ± 0.03 min; NH_4HSO_3 + 2 mM acetazolamide (■), 2.36 ± 0.07 min. No hemolysis was observed in $NaHSO_3$ solution (◇).

colloid osmotic lysis technique cannot directly compare the rates of Cl^- to HCO_3^- influx, due to the role of HCO_3^- exchange in catalyzing Cl^- induced lysis, this technique can be used to explore the contributions of diffusion and anion transport to HCO_3^- induced lysis and the role for intracellular carbonic anhydrase in this process. The colloid osmotic lysis for erythrocytes in NH_4HCO_3 solution (Fig. 2) was found to be extremely rapid (50% at 0.09 ± 0.02 min). Because anion transport in this system allows only the futile exchange of HCO_3^- for HCO_3^- on opposite sides of the membrane, it is anticipated that HCO_3^--induced lysis will occur primarily by diffusion of CO_2. This hypothesis can be confirmed through the use of anion exchange inhibitors as described later. Preincubation of the erythrocytes with acetazolamide to inhibit carbonic anhydrase resulted in a 30-fold prolongation of the 50% lysis time to 2.91 ± 0.02 min. Therefore, the action of carbonic anhydrase in accelerating the conversion of CO_2 to HCO_3^- is to allow faster accumulation of bicarbonate, probably by reducing back diffusion of CO_2. In Cl^- solutions acetazolamide may be expected to retard HCO_3^- catalyzed lysis because the generation from CO_2 of the intracellular HCO_3^- required for Cl^- accumulation will be delayed. Fig. 2 confirms that Cl^-/HCO_3^- induced lysis in acetazolamide treated erythrocytes is only about 37% complete after 10 min incubation (single determination), compared to the 50% lysis time of 0.60 min (Fig. 1) in the absence of acetazolamide.

Fig. 3 depicts the colloid osmotic lysis times for erythrocytes suspended in bisulfite solutions. Unlike Cl^-, in the absence of HCO_3^- hemolysis occurred readily for NH_4HSO_3, with a 50% hemolysis point of 2.19 ± 0.03 min. Because bisulfite is potentially a reducing agent, we sought to determine whether hemolysis might be a result of chemical action of this compound on the membrane. If this is the case, such a mechanism would result in hemolysis even in the absence of a permeant cation. As Fig. 3 shows, no measurable hemolysis was observed for erythrocytes suspended in $NaHSO_3$ solution, indicating that bisulfite did not induce hemolysis by chemical action on the membrane. Therefore, the lysis in NH_4HSO_3 solution indicates that either minute quantities of HCO_3^- are present and acting to catalyze transport induced hemolysis or that there is substantial diffusion of this compound as the conjugate free acid SO_2. For the acetazolamide treated erythrocytes in NH_4HSO_3 solution, the 50% hemolysis time of 2.36 ± 0.07 min was similar to that for untreated erythrocytes, suggesting that trace quantities of HCO_3^- are not involved in the bisulfite-induced lysis. However, Fig. 3 also shows that the 50% lysis time for untreated erythrocytes in NH_4HSO_3 solution was markedly shor-

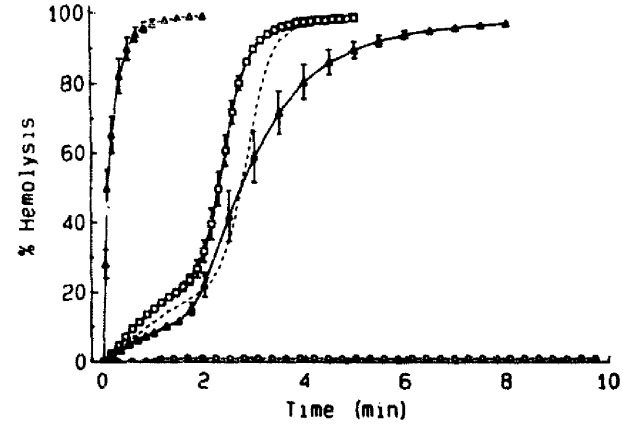

Fig. 4. Colloid osmotic lysis in erythrocytes pretreated with 1 mM SITS to inhibit anion transport. NH_4HCO_3 (△), 0.08 ± 0.03 min; NH_4HCO_3 + 2 mM acetazolamide (▲), 2.73 ± 0.22 min; NH_4HSO_3 (□), 2.26 ± 0.04 min; NH_4HSO_3 + 5 mM HCO_3^- (dashed line), 2.7 min. No lysis is observed in NH_4Cl solution (○).

tened to 0.56 ± 0.03 min by the addition of 5 mM HCO_3^-. This marked acceleration of hemolysis indicates, therefore, that anion transport of bisulfite does occur. In fact, HCO_3^- induced bisulfite lysis is essentially 100% complete by 1.3 min, a time at which diffusion mediated lysis is only 17% complete.

In order to confirm that bicarbonate-induced hemolysis occurs by diffusion and that both diffusion and transport contribute to the net bisulfite flux, colloid osmotic lysis studies were carried out in erythrocytes treated with SITS, a potent inhibitor of anion transport [1]. As shown in Fig. 4, no hemolysis was observed for erythrocytes suspended in NH_4Cl solution because influx of this compound is limited to anion transport. In contrast, hemolysis in NH_4HCO_3 solution was extremely rapid (0.08 ± 0.03 min), and was inhibited to 2.73 ± 0.22 in acetazolamide-treated cells. These rates are essentially identical to those for bicarbonate influx in erythrocytes not pretreated with SITS and indicate that hemolysis for this compound is exclusively mediated by diffusion of CO_2 and not by anion transport. This observation is consistent with the role for HCO_3^- in catalyzing hemolysis induced by other anions, because the net movement of anions into the cell is not supported by the anion transport pathway - hence, no matter how rapid the anion transport is for bicarbonate, it does not result in the accumulation of NH_4HCO_3 by the cell. The 50% hemolysis time in NH_4HSO_3 solution was 2.26 ± 0.04 min, a rate nearly identical to that observed in the absence of SITS. Moreover, the 50% lysis time in the presence of 5 mM HCO_3^- was 2.7 min (single determination), slightly longer than in the absence of bicarbonate. Therefore, inhibition of anion transport eliminates the catalytic effect of HCO_3^- on bisulfite-induced hemolysis. The significance of the longer hemolysis time in the presence of 5 mM HCO_3^- is uncertain.

Discussion

Like HCO_3^-, HSO_3^- is the monodissociated form of a volatile acid (sulfurous acid, H_2SO_3), whose anhydride (SO_2) is gaseous and is extremely soluble in water. The fully dissociated form sulfite (SO_3^{2-}) bears a resemblance to carbonate (CO_3^{2-}):

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \overset{pK\ 6.3}{\rightleftharpoons} HCO_3^- + H^+ \overset{pK\ 10.2}{\rightleftharpoons} CO_3^{2-} + 2H^+$$

$$SO_2 + H_2O \rightleftharpoons H_2SO_3 \overset{pK\ 1.8}{\rightleftharpoons} HSO_3^- + H^+ \overset{pK\ 6.9}{\rightleftharpoons} SO_3^{2-} + 2H^+$$

where the pK values are reported for titration by a strong base such as NaOH. The analogy between these two series is strengthened by their being isoelectronic and having similar chemical structure, each having three oxygen atoms. Therefore bisulfite/sulfite represents a potential chemical analog for the natural substrate for the anion exchange protein. However there are little data in the literature to indicate to what extent the membrane transport of these compounds is analogous. Deuticke [8] has studied the effects of substitution of sulfite for Cl^- in phosphate influx studies, and found that this substitution enhances phosphate influx, although this effect was also observed when Cl^- was substituted by other divalent anions such as sulfate. Becker and Duhm [9] have found that sulfite is similar to bicarbonate in promoting the anionic transport of cations, most notably Li^+. This effect was considerably more pronounced than for sulfate.

There are few analytical methods available to study the transport of sulfite/bisulfite. ^{32}S lacks an NMR signal, and the potential volatility and diffusion of SO_2 from the cell make studies with radiolabeled $^{35}SO_2$ difficult. Moreover, sulfite is readily oxidized to sulfate. Colorimetric methods for sulfite determination are available, but are also subject to losses due to volatility and oxidation.

Our colloid osmotic lysis studies show that hemolysis in NH_4HSO_3 is extremely rapid compared to that for $(NH_4)_2SO_4$, indicating that the influx of the bicarbonate-like anion is extremely rapid. Moreover, although hemolysis is accelerated by the addition of catalytic amounts of HCO_3^-, this addition is not necessary for hemolysis to occur. Hemolysis is not due to a chemical attack on the membrane by HSO_3^-/SO_2, because lysis was not observed in $NaHSO_3$ solutions. Studies with the anion exchange inhibitor SITS indicate that diffusion of neutral CO_2 provides the major pathway for the extremely rapid hemolysis in NH_4HCO_3 solution. Similar to HCO_3^-, lysis does occur in HSO_3^- solution for SITS treated erythrocytes at rates similar to untreated erythrocytes. Therefore, in the absence of bicarbonate, hemolysis is the result of diffusion across the membrane as neutral SO_2. However, it would be extremely difficult to use these lysis times to compare diffusion rates for SO_2 and CO_2 for several reasons. First, at any given pH and bisulfite concentration the free SO_2 concentration is considerably lower than that expected for CO_2 at equivalent bicarbonate concentration, because the pK for H_2SO_3 dissociation (1.8) is much lower than that for H_2CO_3 dissociation (6.3). Second, the relative permeabilities of the membrane to these two substances are not known. Third, although the equilibrium concentrations of SO_2 and CO_2 can be predicted from the Henderson-Hasselbalch equation, the relative rates of interconversion between SO_2 and HSO_3^- and between CO_2 and HCO_3^- are not known. As these experiments have shown, the action of carbonic anhydrase in catalyzing this interconversion for HCO_3^- profoundly affects the lysis times for this compound. However, it is not clear whether this enzyme has similar

activity toward HSO_3^-. In the experiment of Fig. 3, there is no clear inhibition of lysis in HSO_3^- solution for acetazolamide-treated erythrocytes, but we have observed such inhibition at lower pH values where the equilibrium SO_2 concentration is expected to be higher. Further experimentation may determine whether HSO_3^- acts as a substrate for carbonic anhydrase.

The rate of hemolysis in HSO_3^- solution at pH 7.1 ((50% lysis time)$^{-1}$, 0.46 min^{-1}) in the absence of HCO_3^- is proportional to the rate of diffusion of SO_2, whereas the rate of hemolysis in the presence of HCO_3^- (1.79 min^{-1}) is proportional to the sum of the rate of diffusion and the rate of HSO_3^--HCO_3^- exchange through the transport pathway. Therefore, in the presence of bicarbonate, the contribution of transport to the hemolysis rate is 1.33 min^{-1}, or 74% of the net flux of this compound. This rate is of a similar magnitude to that found for Cl^- (50% lysis rate, 1.67 min^{-1}), at at least 100-fold faster than that for sulfate, where < 10% lysis occurred after 40 min.

Aubert and Motais [10] in their study of the transport of organic anions have postulated that a strategic three-oxygen atom attachment occurs between the anion and the transport site, although their studies generally involved oxygen atoms belonging to neighboring anion centers in a given molecule. In our comparison of the transport rates for a series of phosphorous oxyacids [11], we found that phosphite (HPO_3^{2-}) influx was 300-fold faster than phosphate influx at pH 7, although phosphite is a stronger acid (pK 6.4) than phosphate and therefore expected to exist to a higher degree in diionized form. The significance of the three-oxygen, bicarbonate-like anion center in transport was further born out by our observation that hypophosphite ($H_2PO_2^-$) influx was actually slower that of phosphite, although hypophosphite has only two oxygens and hence has a lower molecular weight.

However, the chemical analogy between phosphite and bicarbonate is limited in that bicarbonate has a planar structure, while the structure of phosphite is more nearly tetrahedral [12]. Moreover, the phosphite proton is covalently bonded to pentavalent phosphorus, while the bicarbonate proton is not directly bonded to its carbon. In the present study we find that the anion transport of the bicarbonate-like HSO_3^- is at least two orders of magnitude faster than the phosphate-like SO_4^{2-}. The disparity in influx rates between sulfate and bisulfite further strengthens the hypothesis that the three-oxygen anion center is the preferred chemical structure for transport. Interestingly, there is an aspect of bisulfite chemistry that bridges bicarbonate and phosphite. Horner and Connick [13] have shown using ^{17}O-NMR spectroscopy that bisulfite exists at room temperature in aqueous solution as an equilibrium between two isomers, HSO_3^- and SO_3H^-. In the former isomer, the hydrogen is directly bonded to sulfur (phosphite-like), while in the latter, the hydrogen is bonded to oxygen (bicarbonate-like). It would be valuable to know whether these two isomers are handled differently by the anion transporter.

Acknowledgment

This work was supported by Grant No. R01 DK35779 from the National Institutes of Health.

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