***S*-(*N*-Dansylaminoethyl)-6-mercaptoguanosine as a Fluorescent Probe for the Uridine Transport System in Human Erythrocytes**

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A fluorescent derivative of 6-mercaptoguanosine, *S*-(*N*-dansylaminoethyl)-6-mercaptoguanosine, was synthesized, and found to be a strong inhibitor of the uridine transport system of the erythrocyte ($K_i \sim 0.3 \mu\text{M}$). The emission spectrum of this compound has peaks at 400 and 550 nm. The emission at 550, but not that at 400 nm, is environment-sensitive. A method was devised for preparing a suspension of erythrocyte-membrane fragments with sufficiently low light scattering so that a detailed study could be made of the fluorescence of the probe when bound to membranes. Direct binding measurements showed the existence of a tight binding site, with a dissociation constant of the same order of magnitude as the inhibition constant. Binding of probe and substrate are not mutually exclusive, but the fluorescence and affinity of the bound probe are sensitive to the presence of uridine. The emission spectrum suggests that the bound probe penetrates into the bilayer region of the membrane.

Most kinetic parameters and mechanistic conclusions concerned with transport of nutrients across cell membranes have been obtained by the analysis of steady-state kinetic data (e.g. Lefevre, 1975). Some studies have made use of high-affinity inhibitors to characterize a particular transport system. Thus, *S*-substituted derivatives of 6-mercaptoneucleosides were used to investigate the nucleoside transport system of the erythrocyte (Paul *et al.*, 1975).

Fluorescent probes have long been used in protein chemistry (e.g. Teale & Weber, 1959), but only recently have specific fluorescent probes for certain membrane functions been described (e.g. Schuldiner *et al.*, 1975; Melamed *et al.*, 1976). Our purpose was to develop a suitable fluorescent probe for the uridine transport system and to use it for the study of this system. Our criteria for a satisfactory fluorescent probe were: (a) a high affinity ($K_i < 1 \mu\text{M}$) for the nucleoside transport system; (b) a high fluorescent intensity at a long wavelength ($>450 \text{ nm}$) so that even low concentrations of the probe can be detected when bound to the highly light-absorbent membrane fragments; (c) an environment-sensitive emission spectrum so that information about the microenvironment of the carrier protein can be gained.

Experimental

Materials

N-Dansylaziridine was from Pierce Chemical Co. Rockford, IL, U.S.A.; uridine, 6-mercaptoguanosine

Abbreviations used: Nbzl-sI, *S*-(4'-nitrobenzyl)-6-mercaptinosine; Dns-Aet-sG, *S*-(*N*-dansylaminoethyl)-6-mercaptoguanosine.

and 6-mercaptinosine were from Sigma Chemical Co. St. Louis, MO, U.S.A.; *p*-nitrobenzyl bromide was from Aldrich Chemical Co. Metuchen, NJ, U.S.A.; [^3H]uridine (1 mCi/ml) was from the Israel Atomic Energy Commission, Isotope Laboratory, Nuclear Research Centre, The Negev, Israel. The *S*-substituted nitrobenzyl derivative of 6-mercaptinosine [*S*-(4'-nitrobenzyl)-6-mercaptinosine] was synthesized as described by Montgomery *et al.* (1961) with the modification described by Cabantchik & Ginsburg (1977). All other chemicals were of commercially available highest purity.

Recently outdated human blood was obtained from Hebrew University, Hadassah Medical School Blood Bank, Jerusalem, Israel.

Filters (0.45 μm) and prefilters were mounted in 25 mm-diameter holders (Sartorius Membranfilter G.m.b.H., Göttingen, W. Germany) and used for the efflux measurements.

Egg-yolk lecithin vesicles were provided by Dr. H. Ginsburg, The Hebrew University, Jerusalem, Israel.

Methods

(1) *Synthesis of S*-(*N*-dansylaminoethyl)-6-mercaptoguanosine. *N*-Dansylaziridine (0.1 mmol) was dissolved in 2 ml of ethanol. A solution of 0.11 mmol of 6-mercaptoguanosine in 3 ml of water was prepared and its pH adjusted to 9.6 with Na_2CO_3 (at this pH the compound was completely dissolved). This solution was added to the dansylaziridine solution dropwise with stirring and the mixture was left stirring overnight. The solvent was removed by vacuum and the crude precipitate was redissolved in a minimum volume of hot 1:1 (v/v) ethanol/water. To this solu-

tion hot butan-1-ol was added until the solution became slightly turbid, and then hot ethanol was added until it cleared again. The solution was then cooled gradually and the resulting precipitate collected and washed three times with cold butan-1-ol. The purity of the product was checked by t.l.c. on silica gel with fluorescent indicator on aluminium cards (Riedel de Haën, Hannover, Germany), in 1:1 (v/v) methanol/chloroform and by its u.v. spectrum. Analysis calculated for $C_{24}H_{29}N_7O_6S_2$: C, 50.1; H, 5.0; N, 17.0; S, 11.1; found: C, 50.3; H, 5.2; N, 17.4; S, 10.9%. A 10mm stock solution in dimethyl sulphoxide was stored at -20°C and before an experiment was diluted 1:1000 in phosphate-buffered saline. The exact concentration was determined by u.v. absorbance.

(2) *Preparation of erythrocyte-membrane fragments.* Recently outdated blood was washed three times with phosphate-buffered saline, pH 7.4, containing: NaCl, 8g/l; KCl, 0.2g/l; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.9g/l; KH_2PO_4 , 0.2g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1g/l; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1g/l. White 'ghosts' were prepared by the procedure of Steck & Yu (1973). These ghosts were suspended in 8 times their volume of ice-cold NaOH at pH 11 and then centrifuged for 30min at 40000g at 2°C . The pellet was washed with buffered saline and the suspension centrifuged again. This treatment resulted in the loss of about 30% of the membrane proteins, as determined by the method of Lowry *et al.* (1951). (The protein content in 'ghosts' before NaOH treatment was found to be 3.0 ± 0.3 mg/ml and in the treated membrane fragments was 2.0 ± 0.2 mg/ml.) The membranes were resuspended in a volume of phosphate-buffered saline equal to the original volume of the 'ghosts', and after flushing with N_2 for 5min were sonicated for 30min at 0°C in a bath sonicator (Ladd Research Industries, Burlington, Vermont, U.S.A.) at a maximal setting. The suspension of the membrane fragments prepared in this way was clear enough to be used for fluorescence measurements.

(3) *Transport experiments.* Recently outdated blood was washed with phosphate-buffered saline and loaded with $60\mu\text{Ci}$ of $[^3\text{H}]\text{uridine}$ in phosphate-buffered saline containing 0.2 or 1 mM-uridine/ml and then with Dns-Aet-sG, at various concentrations. The equilibrium exchange efflux measurements were performed by diluting the loaded blood 1000-fold in an unlabelled solution of the same substrate and with the same inhibitor concentrations (Cabantchik & Ginsburg, 1977). Samples were aspirated by a syringe from the external solution through filters, at desired time intervals, and their radioactivity was assayed by liquid-scintillation counting. The radioactivity at equilibrium was assayed on a sample (1ml) of the erythrocyte suspension under study, to which 0.1ml of 100% trichloroacetic acid was added.

(4) *Fluorescence measurements.* Fluorescence measurements were performed on a Perkin-Elmer MPF4

spectrofluorimeter adjusted in the energy mode. The excitation wavelength was 315nm and the emission was scanned between 350 and 600nm. Excitation and emission slits were 4 and 8nm respectively. Titrations were performed directly in the fluorimeter cell, by adding 2–5 μl of concentrated Dns-Aet-sG solution. Both 0.5 and 3ml cells were used in the course of this work.

(5) *Direct binding assay.* Membrane fragments in phosphate-buffered saline (0.5ml), prepared as described above, were incubated for 1h with 0.5ml of solutions at different concentrations of Dns-Aet-sG, uridine or Nbzl-sI. The suspension was then centrifuged at 40000g for 30min, the supernatant collected and its fluorescence measured. A calibration curve was obtained by adding known amounts of Dns-Aet-sG to a supernatant of membrane fragments prepared in the absence of Dns-Aet-sG.

Results

Properties of Dns-Aet-sG

The product (Fig. 1) of the reaction between *N*-dansylaziridine and 6-mercaptopguanosine shows an u.v.-absorption spectrum (Fig. 2) and t.l.c. pattern different from that of either of the reactants. R_f values for methanol/chloroform (1:1, v/v) are: 6-mercaptopguanosine, 0.41; *N*-dansylaziridine, 0.88; Dns-Aet-sG, 0.77.

The fluorescence spectrum of Dns-Aet-sG when excited at 315nm is shown in Fig. 3(a). Two emission peaks are present, at 400 and 550nm. Although the fluorescence intensity at 550nm is sensitive to the nature of the solvent, the peak at 400nm seems to be unaffected (compare curves 1 and 2 in Fig. 3a). The Dns-Aet-sG emission spectrum was measured in a solution containing egg-yolk lecithin vesicles (A_{315} 1.1), and is depicted in Fig. 3(b). The ratio between the intensities of the emission peaks (F_{550}/F_{400}) is concentration independent both in phosphate-buffered saline and in 50% propan-2-ol, and equals 1.3 and 4.4 respectively. On the other hand, this ratio in egg-yolk lecithin suspension is 1.6 for 0.6 μM - and 2.0 for 2.5 μM -Dns-Aet-sG. The fact that these ratios are between those for phosphate-buffered saline and propan-2-ol suggests that the Dns-Aet-sG is present, at least partially, in an apolar region of the vesicles. Since the peak at 400nm seems to be insensitive to the environment, it can serve as an internal standard to correct for any inner filter effects, whereas the peak at 550nm may be used as a tool to study the microenvironment of the probe. The peak at 550nm is sufficiently removed from the fluorescence and light-scattering peaks of the erythrocyte membrane fragments to enable us to study the Dns-Aet-sG fluorescence when bound to the membranes.

Fig. 1. Chemical structure of the inhibitors Nbzl-sI and Dns-Aet-sG

Fig. 2. U.v.-absorption spectrum in phosphate-buffered saline of *N*-dansylaziridine (---), 6-mercaptoguanosine (-.-) and the product of their reaction, *Dns-Aet-sG* (—)

Inhibition of uridine transport by *Dns-Aet-sG*

The effect of *Dns-Aet-sG* on the uridine transport rate of erythrocytes was determined by using the equilibrium exchange efflux technique, as applied to the same system by Cabantchik & Ginsburg (1977). For equilibrium exchange efflux, the concentration

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Fig. 3. Emission spectra of *Dns-Aet-sG* under different conditions

The excitation wavelength was 315 nm. The spectra shown are corrected for the scatter of the buffer and liposomes solutions. (a) Emission spectrum of *Dns-Aet-sG* (1.25 μ M) in (1) phosphate-buffered saline and (2) 50% propan-2-ol in phosphate-buffered saline. (b) Emission spectrum of *Dns-Aet-sG* (0.6 μ M) (1) and 2.5 μ M (2) in egg-yolk lecithin suspension in phosphate-buffered saline (0.8 mg/ml).

(or radioactivity) of substrate (C_t) appearing in the external medium at time t is given by eqn. (1),

$$\ln \frac{C_\infty - C_t}{C_\infty} = kt \quad (1)$$

where C_∞ is the concentration (or radioactivity) of the substrate at the time of full equilibration. The slope of the plot of $-\ln[(C_\infty - C_t)/C_\infty]$ versus t yields the rate constant, k , for exchange, which is equal to $v/[S]$ (the ratio between exchange rate and substrate concentration).

Experiments were performed in the absence or presence of $0.01\text{--}5\text{ }\mu\text{M}$ inhibitor in $0.025\text{--}5\text{ mM}$ -uridine. The K_m and V_{max} (mean $\pm$ S.E.) for uridine in a control experiment (no inhibitor present) were $1.4 \pm 0.2\text{ mM}$ and $7.7 \pm 1.5\text{ mM/min}$ respectively. These values are in good agreement with those reported previously: $1.29 \pm 0.11\text{ mM}$ and $7.54 \pm 0.45\text{ mM/min}$ (Cabantchik & Ginsburg, 1977).

The results, in the presence of Dns-Aet-sG, were analysed in terms of Dixon equations (Webb, 1963), which for competitive inhibition have the form:

$$\frac{[S]}{v} = \frac{1}{k} = \frac{[S]}{V_m} + \frac{K_m}{V_m} + \frac{K_m}{V_m} \frac{[I]}{K_i} \quad (2)$$

where $[I]$ is the inhibitor concentration and K_i its dissociation constant from the carrier.

Two experiments were performed at different concentrations of substrate and inhibitor. Parallel modified Dixon plots were obtained for $[S]/v$ versus $[I]$ at different uridine concentrations, indicating a competitive mode of inhibition. The data are presented in Table 1, and the results of one experiment are depicted in Fig. 4. $[S]/v$ is plotted as a function of $[I]$ since this

Fig. 4. Kinetics of equilibrium exchange efflux of uridine in erythrocytes, in the presence of the inhibitor Dns-Aet-sG. Rates of uridine efflux were measured at various concentrations of uridine ($[S]$) and inhibitor ($[I]$). Modified Dixon plots, in the form of $[S]/v$ versus $[I]$ (see eqn. 2), for three different uridine concentrations are shown: Δ , 0.025 mM ; $\bullet$, 0.25 mM ; $\circ$, 2.5 mM . The lines were drawn by linear regression.

Table 1. Inhibition constants of the interaction of Dns-Aet-sG and the uridine transport system

Expt. no.	[Uridine] (mM)	Dns-Aet-sG concn. range (μM)	K_i (μM)
1	1	0–2.5	0.24 ± 0.03
	0.2	0–5	0.41 ± 0.17
2	0.025		0.47 ± 0.14
	0.25	0–0.33	0.26 ± 0.05
	2.5		0.34 ± 0.1

value is directly obtained in the equilibrium exchange experiment.)

By averaging the data for all the experiments and assuming competitive inhibition, K_i $0.34 \pm 0.10\text{ }\mu\text{M}$ is given. The high affinity of the probe to the uridine transport system, as demonstrated by the kinetic study, indicates its usefulness as a fluorescent probe for this system.

Interaction of Dns-Aet-sG with the erythrocyte-membrane fragments

The binding of Dns-Aet-sG at low concentrations (up to $0.4\text{ }\mu\text{M}$) to the erythrocyte-membrane fragments as measured by the direct assay is shown in Fig. 5. As described under 'Methods', the bound (C_b) and free (C_f) concentrations were determined by measuring the fluorescence remaining in the supernatant after the erythrocyte-membrane fragments were incubated with the probe at total concentration, C_t , and then separated by centrifugation. The concentration found in the supernatant is C_f (the concentration of the free ligand) and C_b is obtained as the difference $C_b = C_t - C_f$; r is the ratio of C_b to the membrane protein concentration.

Binding of Dns-Aet-sG to the erythrocyte-membrane fragments was studied in the presence and absence of 25 mM -uridine or $5\text{ }\mu\text{M}$ -Nbzl-sI, a known inhibitor of the uridine transport system (Paul *et al.*, 1975) whose chemical structure is similar to that of the Dns-Aet-sG (Fig. 1). In Fig. 5(a), r is plotted as a function of the free inhibitor concentration. A different binding curve is found for each type of experiment; in the presence of Nbzl-sI, r is linear with C_f , indicating a non-specific interaction between the probe (Dns-Aet-sG) and the membrane. In a control experiment (results not shown) we studied the effect of 25 mM -uridine on the binding of Dns-Aet-sG to erythrocyte-membrane fragments in the presence of Nbzl-sI. No significant effect could be detected over the whole concentration range of Dns-Aet-sG ($0\text{--}0.5\text{ }\mu\text{M}$). Each value of r for the untreated membranes or the membranes saturated with uridine is higher than the corresponding value for the Nbzl-sI-treated membranes (Fig. 5a). Assuming that the

Fig. 5. Results of the direct binding study of the Dns-Aet-sG to the erythrocyte-membrane fragments

(a) The ratio of concentration of bound probe and protein concentration (r) as a function of the concentration of free Dns-Aet-sG (C_f): $\odot$, in the presence of Nbzl-sI; $\bullet$, untreated membranes; $\circ$, in the presence of uridine. The straight line was drawn by linear regression through the points obtained in the presence of Nbzl-sI. (b) A plot of r_{specific} (as defined in the text) as a function of free Dns-Aet-sG. $\bullet$, Untreated membranes; $\circ$, in the presence of uridine; inset, Scatchard plot of the same data.

Nbzl-sI has displaced the Dns-Aet-sG from its specific sites one can estimate the specific interaction of the Dns-Aet-sG with the membrane. This can be done by calculating the difference between the r measured at a certain C_f value and the corresponding value of r in the presence of Nbzl-sI, as derived from the linear-regression line in Fig. 5(a). A plot of the specific binding, r_{specific} , obtained in this way, is depicted in Fig. 5(b), and the inset to this Figure shows a Scatchard plot of the same data.

The values for K_d and n in the presence of uridine are well defined, but not for the untreated membrane fragments. The reason for the large error is the presence of high percentage of non-specific bound probe. In the presence of uridine, the dissociation constant $K_d = 0.11 \pm 0.01 \mu\text{M}$ and the number of sites

is $30 \pm 5 \text{ pmol/mg}$ of protein. However, if one assumes that the same number of sites is available for Dns-Aet-sG in the presence as in the absence of uridine, and uses the standard error obtained for n in the presence of the substrate, the error for K_d for the untreated membranes can be calculated as $0.4 \pm 0.2 \mu\text{M}$, which is in good agreement with the kinetically obtained K_i . These parameters were used for drawing the theoretical lines of Fig. 5(b). The extension of the direct binding study to higher Dns-Aet-sG concentrations is limited by the high non-specific interaction of the probe with the membranes.

On the basis of the value of approx. $6.6 \times 10^{-10} \text{ mg}$ of protein/erythrocyte 'ghost' (Dodge *et al.*, 1963) we assumed that owing to the NaOH treatment approx. $4 \times 10^{-10} \text{ mg}$ of protein of membrane fragments was derived from one blood cell. Thus the number of binding sites for Dns-Aet-sG can be estimated to be: $30 \times 10^{-12} \times 4 \times 10^{-10} \times 6 \times 10^{23} \sim 7 \times 10^3$ sites/cell.

Fluorescence titration

A fluorescence titration of membrane fragments with Dns-Aet-sG was performed. Untreated membranes and membranes incubated with uridine were titrated with Dns-Aet-sG over a concentration range similar to that used for the binding and inhibition studies. Due to scattering of light by the membranes and their high light absorption, measurements at low concentrations of the probe (below $0.5 \mu\text{M}$) were not possible. Fig. 6 shows the spectra of a membrane preparation in the presence and absence of Dns-Aet-sG.

We have shown at the beginning of this section that the ratio of the fluorescence intensities, F_{550}/F_{400} , is sensitive to the environment of the probe. We define the ratio F_{550}/F_{400} in the system under study divided

Fig. 6. Fluorescence spectra of erythrocyte-membrane fragments in phosphate-buffered saline, excited at 315 nm. The protein concentration was 0.5 mg/ml . (a) No Dns-Aet-sG; (b) $2.25 \mu\text{M}$ Dns-Aet-sG; (c) $5.25 \mu\text{M}$ Dns-Aet-sG.

Fig. 7. Fluorescence titration of erythrocyte-membrane fragments with Dns-Aet-sG

●, Untreated membranes; ○, in the presence of uridine; ⊗, Dns-Aet-sG in egg-yolk lecithin suspension, as shown in Fig. 3. Extrapolation to zero concentration of Dns-Aet-sG was done by eye. The 'fluorescence enhancement', given by the quotient of fluorescence intensity ratios obtained with the system under study (exp.) and with phosphate-buffered saline alone (PBS), is described in the Results section.

by the same ratio measured in phosphate-buffered saline under identical conditions as the 'enhancement' of the probe's fluorescence. A plot of this 'enhancement' as a function of the total Dns-Aet-sG concentration is depicted in Fig. 7.

At low concentrations of Dns-Aet-sG, where most of the probe is bound to the specific sites, the enhancement is high. At high concentrations of Dns-Aet-sG both titration curves reach a constant value of enhancement, significantly higher than 1, which is probably due to the non-specific interaction of the probe with the membrane. The ratio obtained is similar to that obtained for Dns-Aet-sG in the egg-yolk lecithin suspension (1.55), presumably also due to non-specific binding. The Dns-Aet-sG concentrations at which the enhancement reaches half its maximal value are of the same order of magnitude as the dissociation constants derived from the direct binding assay. As with the direct binding study, the interaction of the Dns-Aet-sG with the membrane is stronger in the presence of uridine. Rough extrapolation to zero Dns-Aet-sG concentration yields estimates for the enhancement due to the specific interaction. The value seems to be slightly higher in the presence of uridine. Note that the Dns-Aet-sG in 50% propan-2-ol has an enhancement of $4.4/1.3 = 3.4$.

Discussion

In the present study we describe the synthesis and use of a fluorescent probe of the uridine transport system of the erythrocyte. The probe, which is a *S*-dansylated derivative of 6-mercaptoguanosine, has a high-intensity emission peak at long wavelengths and thus can be used in the presence of a highly absorbing medium, such as the erythrocyte-membrane fragments. The fluorescence intensities ratio, F_{550}/F_{400} , is environment sensitive and has been used here as an indicator of the microenvironment of the probe while bound to the membrane. The probe is a strong competitive inhibitor of the uridine transport system, as has been shown by the kinetic study. The similar results obtained by the kinetic study (Fig. 4), the direct binding (Fig. 5) and the fluorescent titration (Fig. 7) indicate that in all these approaches we follow the binding of a specific probe to the nucleoside transport system.

The results of the kinetic studies, depicted in Fig. 4, can be interpreted as a competitive inhibition of the Dns-Aet-sG for uridine transport. On the other hand, in the direct binding assay of Dns-Aet-sG to the erythrocyte membrane we find that the uridine does not replace Dns-Aet-sG from its site, suggesting the existence of distinct binding sites for these compounds. Fig. 5 indeed shows that the binding of the probe is enhanced in the presence of the substrate. This apparent contradiction can be resolved by analysing the data in terms of the one complex model of a simple carrier (Lieb & Stein, 1974). This model (Fig. 8) describes adequately the uridine transport system of the erythrocyte (Cabantchik & Ginsburg, 1977).

For the equilibrium exchange experiment the

Fig. 8. One complex model of a simple carrier (Lieb & Stein, 1974)

E_1 , free carrier at side 1 of the membrane; E_2 , free carrier at side 2 of the membrane; ES, carrier-substrate complex; s_1 , substrate concentration at side 1 of the membrane; s_2 , substrate concentration at side 2 of the membrane. f , k and b are the corresponding rate constants.

steady-state transport rate is given by the following equation (Stein & Lieb, 1973):

$$\frac{u_{12}}{[T]} (\text{equilibrium exchange}) = \frac{[S]/R_{ee}}{K \cdot \frac{R_{00}}{R_{ee}} + [S]} \quad (3)$$

where $[S]$ is the substrate concentration, u_{12} is the unidirectional flux from side 1 to side 2 and $[T]$ is the total carrier concentration.

K is given by:

$$K = k_{+1}/f_1 + k_{-1}/f_2 \quad (4)$$

and can be shown to be the average dissociation constant of the substrate from the carrier at the two faces of the membrane:

$$R_{ee} = 1/b_1 + 1/b_2 \quad (5)$$

R_{ee} can be considered the sum of the breakdown resistances of the complex and is equal to the reciprocal of the maximal velocity of transport under equilibrium exchange (ee) conditions.

$$R_{00} = 1/k_{+1} + 1/k_{-1} \quad (6)$$

R_{00} can be considered as the sum of the resistances experienced by the free carrier as it crosses the membrane in a cyclic fashion. The subscript 00 refers to conditions where no substrate is present.

It can be seen from eqn. (3) that a competitive inhibition pattern occurs for those cases in which R_{ee} is independent of the inhibitor concentration, and the term $K \cdot R_{00}/R_{ee}$ increases linearly with $[I]$. In our case (which is of the competitive type) K decreases in the presence of the inhibitor (since, as shown in Fig. 5, the affinity of the probe is higher in the presence of the substrate, the same holds for the substrate in the presence of the probe). Thus it must be the increase of R_{00} (the resistance of transport of the carrier bound with Dns-Aet-sG, but not with uridine) that is responsible for the inhibitory effect of the Dns-Aet-sG on the uridine transport system. It should be noted that Cabantchik & Ginsburg (1977) have found that the movement of the unloaded carrier is the rate-limiting step in the transport of uridine across the erythrocyte membrane.

The number of sites determined by the Scatchard plot in Fig. 5 is 7×10^3 sites/cell, which is in good agreement with previously reported value for uridine carrier molecules (Cass *et al.*, 1974). In the direct binding study it has been shown that Nbzl-sI replaces the Dns-Aet-sG. Since both inhibitors have a similar chemical structure (Fig. 1), it is probable that they affect the uridine transport in a similar manner. Cass & Paterson (1976) suggested that the Nbzl-sI sites

appear to be distinct from the nucleoside's permeation site. Cabantchik & Eilam (1976) also suggested that the Nbzl-sI interacts with the uridine transport system of a hamster cell line at a site distinct from that of the substrate.

The high value of the ratio F_{550}/F_{400} at the concentration range of specific interaction between the Dns-Aet-sG and the membranes indicates a hydrophobic nature of the microenvironment of the chromophore. This suggests that the binding site of the Dns-Aet-sG with the uridine carrier is located in the bilayer region of the membrane.

The development of an environment-sensitive fluorescent probe to a carrier protein might serve as a tool for further studies of conformational changes related to the process of transport.

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