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Transport and Membrane Binding of the Glutamine Analogue 6-Diazo-5-oxo-L-Norleucine (DON) in *Xenopus laevis* Oocytes

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Summary. We have examined transport and membrane binding of 6-diazo-5-oxo-L-norleucine (DON, a photoactive diazo-analogue of glutamine) and their relationships to glutamine transport in *Xenopus laevis* oocytes. DON uptake was stereospecific and saturable (V_{max} of 0.44 pmol/oocyte · min and a K_m of 0.065 mM). DON uptake was largely Na^+ dependent (80% at 50 μM DON) and inhibited (>75%) by glutamine and arginine (substrates of the System $B^{0,+}$ transporter) at 1 mM. Glutamine and DON show mutual competitive inhibition of Na^+-dependent transport. Preincubation of oocytes in medium containing 0.1 mM DON for 24 or 48 hr depressed the V_{max} for System $B^{0,+}$ transport (as measured by Na^+-dependent glutamine uptake), this effect was highly specific (neither D-DON nor the System $B^{0,+}$ substrates glutamine and D-alanine showed any independent effect) and required Na^+ ions. Glutamine (1 mM in preincubation medium) protected transport from inhibition by DON. The possibility that specific inactivation of System $B^{0,+}$ by DON reflects attachment of DON to the transporter was tested by examining the binding of [^{14}C]DON to *Xenopus* oocyte membranes. Oocytes incubated in 100 mM NaCl in the presence of [^{14}C]DON for up to 48 hr showed 2.4-fold higher ^{14}C-binding to membranes than oocytes incubated in choline chloride. Na^+-dependent DON binding (31 ± 11 fmol/μg membrane protein) was suppressed by external glutamine, arginine or alanine and was largely confined to a membrane protein fraction of 48–65 kDa (as assessed by SDS-polyacrylamide gel electrophoresis). The present studies indicate that DON and glutamine uptake in oocytes are both mediated by System $B^{0,+}$ and demonstrate that DON binding to a particular membrane protein fraction is associated with inactivation of the transporter, offering the prospect of using [^{14}C]DON as a covalent label for the transport protein in order to facilitate its isolation and subsequent biochemical characterization.

Key Words *Xenopus* oocyte · amino acid · Na^+-dependent transport · membrane binding

Introduction

Several Na^+-dependent amino acid transport systems with different substrate specificities have been described in animal cell membranes (Christensen & Kilberg, 1987; Christensen, 1990). At present most of these systems have been characterized only in kinetic terms and the membrane proteins that effect transport have not been isolated, although a cloned retrovirus receptor-protein has recently been shown to have functional homology with a Na^+-independent transporter for cationic amino acids (system y^+: Kim et al., 1991; Wang et al., 1991). Two main lines of approach have been adopted by those attempting to elucidate the molecular structure of amino acid transporters: (i) purification of transport proteins identified either by reconstitution assay (Quesada & McGivan, 1988; Fafournoux, Dudenhausen & Kilberg, 1989), photoaffinity labeling (Segel et al., 1989) or antibody probe (McCormick & Johnstone, 1988), and (ii) isolation of transporter *c*DNA *via* expression cloning in *Xenopus* oocytes (e.g., Taylor & Rennie, 1989; Tate et al., 1989; Palacin et al., 1990; Tarnuzzer et al., 1990).

A prerequisite for use of the oocyte expression technique in cloning of amino acid transporters is an understanding of the endogenous amino acid transport systems; we and others recently reported (Taylor, Hundal & Rennie, 1989; Campa & Kilberg, 1989) that the predominant Na^+-dependent amino acid transporter in the oocyte membrane had a broad range of substrates (neutral and cationic), including glutamine and arginine, and appeared to be identical to the System $B^{0,+}$ transporter characterized in mouse blastocysts and other early conceptuses (Van Winkle, 1988). The *Xenopus* oocyte thus represents a convenient model system for studies of Na^+-dependent amino acid transport at the cellular level (e.g., Jung, Schwarz & Passow, 1984; Campa & Kilberg, 1989; Taylor et al., 1989), which could provide important information on the structure-function relationships of amino acid transport proteins.

Two diazo-analogues of glutamine, azaserine

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and 6-diazo-5-oxo-L-norleucine (DON), are known to inactivate key enzymes involved in cellular glutamine metabolism by binding covalently to the substrate recognition site on the protein *via* reactive side-chain groups (Hartman, 1963; Clark, Shapiro & Curthoys, 1982; Prajda, 1985). It is therefore possible that these compounds (especially if radioactively or fluorescently labeled) could be used as marker ligands in the isolation and subsequent characterization of membrane proteins responsible for cellular amino acid exchange. Unfortunately, recent studies in our laboratory (Low et al., 1991) indicate that neither DON nor azaserine are recognized as substrates by the major Na^+-dependent glutamine transporters in liver and skeletal muscle, although Segel and co-workers (1989) have reported that azaserine binding to human T-lymphocyte membranes is associated with inhibition of the uptake of BCH (2-amino-2-carboxybicyclo[2.2.1]heptane), a paradigm substrate for the Na^+-independent System L in this cell type.

The work presented here has been concerned with investigating the uptake and membrane binding of DON (and their relationships to glutamine uptake) in *Xenopus laevis* oocytes, in order to assess the feasibility of using labeled DON to isolate and further characterize the System $B^{0,+}$ transporter.

Materials and Methods

Chemicals were obtained from Sigma (Poole, UK) unless otherwise specified; radiochemicals were obtained from Amersham International (Aylesbury, UK): [^{3}H]-glutamine at a specific activity of 1.5 to 6.0 TBq/mmol and custom synthesized [^{14}C]-DON at a specific activity of 2.1 GBq/mmol.

Female toads (*Xenopus laevis*) were supplied by Griffin & George (Loughborough, UK) or Blades Biological (Edenbridge, UK) and maintained in fresh water aquaria at 15°C. Ovarian tissue was removed by surgery from toads anesthetized in 0.1% aminoethylbenzoate (as described by Taylor et al., 1989), rinsed in modified Barth's medium (MBM) containing in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 5.0 HEPES, pH 7.6 with Tris base, penicillin and streptomycin at 10 mg/liter each) and disaggregated using collagenase A (Boehringer Mannheim, Lewes, UK) at 2 mg/ml MBM for 3 hr at 20°C. Stage VI (prophase-arrested) oocytes were selected and incubated in MBM for 20 to 24 hr at 20°C (BDH cooled incubator) unless otherwise stated.

DON and glutamine uptake into oocytes was measured using a radiotracer method as described previously (Taylor et al., 1989). Oocytes were incubated in experimental medium (containing in mM: 100 NaCl or ChoCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/Tris, pH 7.5; Cho = choline) containing radioactive substrate for a fixed time period at 20°C. The effect of amino acids on the uptake of 50 μM [^{14}C]DON or [^{3}H]glutamine was investigated by their inclusion in the experimental medium at 1 mM (unless otherwise stated). On termination of an uptake experiment, oocytes were rinsed twice in distilled water (DW) then transferred individually in 0.2 ml DW to Beckman scintillation vials; they lysed within 0.5 hr of transfer (presumably by osmotic swelling). 0.2 ml of 2% Triton-X100 and 3 ml of scintillant (BDH, Poole, UK) were subsequently added to each vial and sample radioactivity was assayed by liquid scintillation counting (Beckman LS 1800), with automatic quench correction to give disintegrations per min (dpm). In some experiments oocyte total protein was isolated from the osmotically-lysed cell suspension by precipitation with 5 volumes of ice-cold acetone: the protein pellet was resuspended in 0.4 ml of 1% Triton/0.5 M NaOH, transferred to a vial containing 3 ml scintillant and assayed for radioactivity as described above.

Putative transport-inactivation effects of DON and binding of [^{14}C]DON to oocyte membranes was assessed using groups of oocytes incubated in experimental medium (100 mM NaCl or 100 mM ChoCl) containing DON for periods of up to 48 hr. We assessed the condition of incubated oocytes by their ability to synthesize protein (measured as incorporation of [^{3}H]-activity into oocyte protein during 4 hr incubation in NaCl buffer containing 0.5 mM [^{3}H]glutamine). We found that tracer associated with protein represented ≈30% of total radioactivity in oocytes preincubated for 48 hr in either NaCl or ChoCl buffers ± 0.1 mM DON (a similar % incorporation as for freshly cultured oocytes; Taylor et al., 1989), and concluded that experimental incubation conditions were not harmful to oocytes. In [^{14}C]DON-binding studies, approximately 25 oocytes were removed from the incubation medium at timed intervals and subjected to subcellular fractionation by a method adapted from that of Colman (1984). Oocytes were transferred to a hand-held glass homogenizer, rinsed in experimental medium and then disrupted in 1 ml of ice-cold T-buffer (in mM: 150 NaCl, 10 Mg acetate, 20 Tris, pH 7.6 with HCl). 0.1 ml of the resulting cell suspension was retained for analysis, and the remaining 0.9 ml homogenate was layered onto 0.6 ml 50% (wt/vol) sucrose in T-buffer and spun in a microcentrifuge (10,000 × *g* for 30 min, 4°C) to pellet the oocyte yolk. The total supernatant was transferred to a second microcentrifuge tube, mixed by vortexing to yield 1.5 ml solution with a nominal sucrose concentration of 20% (wt/vol) and recentrifuged (30 min at 10,000 × *g*, 4°C) to pellet oocyte membranes. The final supernatant (containing the cytosolic and mitochondrial fractions) was retained and the membrane and yolk pellets were washed thoroughly with 1 ml T-buffer by vortex mixing and repelleting (30 min at 10,000 × *g*, 4°C). The membrane and yolk pellets were finally resuspended in 0.5 and 1 ml ice-cold T-buffer, respectively. The enrichment of the membrane fraction with oocyte plasma membrane was assessed from the potassium-stimulated *p*-nitrophenyl-phosphatase (KpNPPase) activity in our purified membrane fraction and the crude cell homogenate using a method described previously (Ahmed, Taylor & Rennie, 1990). Duplicate 50-μl aliquots of homogenate and fractions were taken for measurement of [^{14}C] activity as described above, and similar aliquots were taken to measure protein concentration using the bicinchoninic acid method (Smith et al., 1985): the binding of [^{14}C]DON to membranes was calculated as fmol DON/μg of membrane protein using the nominal specific activity of the tracer.

The pattern of [^{14}C]DON binding to oocyte membrane proteins was investigated using polyacrylamide gel electrophoresis. Fifty μg aliquots of membrane protein were denatured in 1% SDS/0.5% 2-mercaptoethanol buffer and the components separated (alongside protein mol wt standards) on 12% polyacrylamide/0.1% SDS slab gels at 100V for 1.5 hr using a Mini-Protean II electrophoresis apparatus (Bio-Rad Laboratories, Richmond CA)

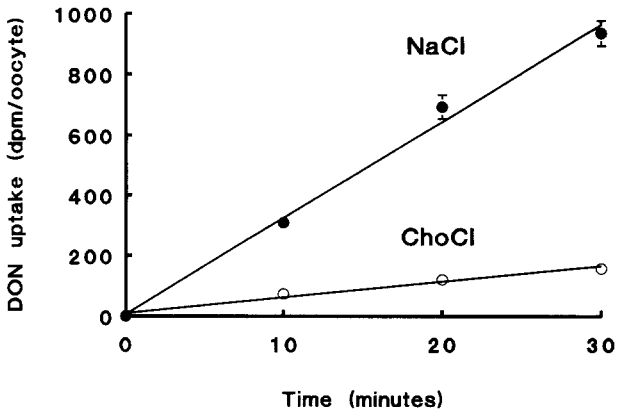

Fig. 1. Time course of uptake of 50 μM L-[^{14}C]DON (100 dpm/pmol) into *Xenopus* oocytes in 100 mM NaCl and ChoCl media; values are mean ± SEM for 8 to 15 oocytes at each time point (error bars not shown fall within a symbol).

Table 1. Effects of amino acids and analogues (1 mM in NaCl medium) on uptake of 0.05 mM DON or glutamine in *Xenopus* oocytes[a]

Amino acid or analogue	% of control uptake	
	DON	Glutamine
DON	29 ± 9[b]	22 ± 2[b]
D-DON	117 ± 22	—
Glutamine	25 ± 3[b]	22 ± 8[b]
Arginine	16 ± 2[b]	23 ± 4[b]
Azaserine	23 ± 3[b]	15 ± 4[b]
MeAIB	89 ± 11	87 ± 19
D-Alanine	—	41 ± 6[b]

[a] Results are presented as mean ±1 SEM for uptake in 10 oocytes. Control uptakes of DON and glutamine were 0.171 ± 0.015 pmol/oocyte · min and 0.229 ± 0.040 pmol/oocyte · min, respectively. MeAIB, 2-(methylamino)isobutyrate.
[b] Value indicates significant inhibition of uptake by amino acid or analogue ($P < 0.001$).

with a discontinuous buffer system (Laemmli, 1970). Membrane proteins and standards were visualized by staining with Coomassie Brilliant Blue R-250. Unstained gel lanes were cut into 5-mm fractions which were suspended in 0.8 ml water containing 0.5% SDS and 0.1% Sigma antifoam A using a motorized Teflon-glass homogenizer. Each gel-fraction suspension was taken up in 6 ml of scintillant and counted as described above. A background count obtained using a gel lane containing unlabeled protein was subtracted from appropriate gel fractions. The recovery of ^{14}C activity from the gels was >90% of applied activity in all cases.

The electrical-potential difference across the oocyte membrane (E_m) was measured with an electrometer (World Precision Instruments S-7071A) using glass microelectrodes filled with 1 M KCl (≈5 MΩ tip resistance).

The study was performed using oocytes from 12 different toads. All results are presented as the mean ± 1 standard error (SEM); for transport studies, n represents the number of oocytes (from the same toad) used in a single experiment (unless otherwise specified); for binding studies, n refers to the number of preparations (each with about 25 oocytes) from different toads. Differences between sample means were tested for significance using Student's unpaired t-test, the paired t-test or analysis of variance (ANOVA: one-way classifications) as indicated; differences were considered significant if $P < 0.05$. Lines or curves were fitted to data (by least-squares and iterative methods, respectively) using commercial software (Barlow, 1982) run on an Apple *IIe* microcomputer. Values for K_m and V_{max} of transport were estimated from Hanes linear transformation of raw data (plot of $[S]/V$ against $[S]$, where $[S]$ is substrate concentration and V is transport rate).

Results

Characteristics of L-DON Transport in Oocytes

The rate of 50 μM L-[^{14}C]DON uptake into oocytes was linear over the first 0.5 hr of incubation (Fig. 1) and was largely Na^+ dependent, the uptake rate in ChoCl being on average only 20% of that in NaCl. For convenience we used an incubation period of 20 min for subsequent uptake experiments. In the experiment shown in Fig. 1 only 23 ± 4 dpm of total oocyte [^{14}C] activity was associated with the protein pellet after 30 min incubation (2.5% of total uptake). This component of total uptake was unaffected by inhibition of oocyte protein synthesis (8 hr preincubation of oocytes with 0.1 mM cycloheximide; Colman, 1984), in contrast to a 70% reduction in the amount of [^{3}H]glutamine associated with the protein pellet (normally 15% of total uptake) observed in a similar experiment. We concluded that, as expected, DON was not incorporated into oocyte protein; therefore, DON associated with the oocyte protein pellet was in some way bound to proteins.

DON uptake into oocytes was inhibited substantially (>70%) by the presence of unlabeled L-DON, L-glutamine, L-arginine and azaserine (another glutamine analogue) at 1 mM in the incubation medium (Table 1). Saturable L-glutamine uptake into oocytes was markedly inhibited by L-DON, and also by azaserine, arginine and D-alanine (Table 1); an extensive study of the effects of naturally occurring amino acids on glutamine uptake has already been reported (Taylor et al., 1989). The saturable component of DON uptake was stereospecific because the presence of D-DON in the experimental medium did not inhibit uptake of L-DON (Table 1). The relationship between external concentration and transport of DON was investigated in both NaCl and ChoCl media using oocytes from a single toad (Fig. 2): in these oocytes Na^+-dependent L-DON uptake (i.e., uptake in NaCl minus uptake in ChoCl) had a maximal rate

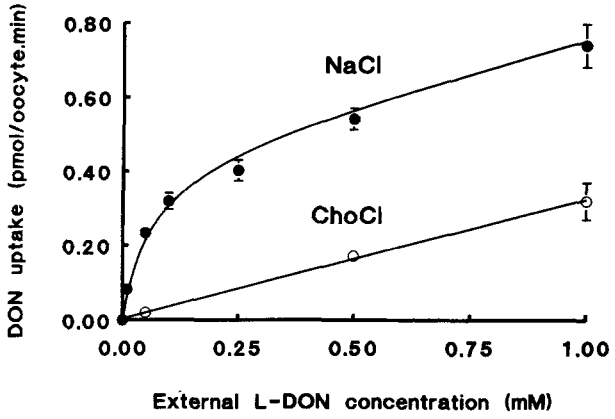

Fig. 2. Influx of L-DON in *Xenopus* oocytes as a function of external DON concentration. Results (mean ± 1 SEM for 10 oocytes) were obtained using oocytes from a single toad (error bars not shown fall within a symbol).

(V_{max}) of 0.44 ± 0.02 pmol/oocyte · min and the concentration at which L-DON transport was half-maximal (K_m) was 0.065 ± 0.010 mM. A similar K_m value (0.078 mM) was obtained when the experiment was repeated using oocytes from a second toad, but the V_{max} value (0.71 pmol/oocyte · min) was greater than that in the first experiment. The V_{max} for Na^+-dependent DON transport into oocytes from seven toads (estimated from rate of transport at an apparently saturating DON concentration of 1 mM) ranged from 0.30 to 0.95 pmol/oocyte · min.

In the presence of 0.5 mM L-DON, the V_{max} for Na^+-dependent glutamine transport in a single batch of oocytes was unaffected (V_{max} = 1.2 pmol/oocyte · min) but the apparent K_m was increased from 0.08 to 0.37 mM (*data not shown*), indicating that DON was inhibiting Na^+-dependent glutamine transport by a competitive mechanism. In subsequent experiments the K_i for this inhibition (0.05–0.09 mM; Fig. 3*a*) was shown to be of similar magnitude to the observed K_m for Na^+-dependent DON transport (0.065 mM) and, furthermore, the estimated K_i for glutamine inhibition of Na^+-dependent DON transport (0.08–0.13 mM; Fig. 3*b*) was of similar magnitude to the observed K_m for Na^+-dependent glutamine transport (0.12 mM; Taylor et al., 1989). These results indicate that mutual competitive inhibition between glutamine and DON for Na^+-dependent uptake into oocytes occurs because the two amino acids interact with the same transport system.

Neither DON nor glutamine appeared to be transported to an appreciable extent by System A, because 2-(methylamino)-isobutyric acid (MeAIB), a preferred substrate for this system, did not significantly inhibit their uptakes (Table 1); indeed the activity of this reportedly ubiquitous Na^+-dependent transporter is virtually undetectable in oocytes (Campa & Kilberg, 1989; Palacin et al., 1990). Systems $B^{0,+}$ and ASC are reported to effect Na^+-dependent uptake of small neutral amino acids in *Xenopus* oocytes (Campa & Kilberg, 1989), which is confirmed by the observation (Fig. 4) that a component of Na^+-dependent alanine uptake is insensitive to inhibition by arginine (System $B^{0,+}$ substrate), but is inhibited by threonine (System ASC substrate). In contrast, Na^+-dependent uptake of DON and glutamine was almost completely inhibited by excess arginine (Fig. 4) and therefore appears to be almost exclusively *via* System $B^{0,+}$.

We further investigated the specificity of DON and glutamine transport by examining the extent to which a variety of amino acid analogues (at 1 mM) inhibited Na^+-dependent uptake of 50 μM DON or glutamine into oocytes (Table 2). Uptake of both DON and glutamine was significantly inhibited (between 64 and 88%) by glutamate-γ-hydroxamate, glutamate-γ-hydrazide, glutamate-γ-methyl ester, *S*-carbamyl cysteine 1,2,4-triazole-3-alanine (all α-amino acids with bulky side chains), but glutamine uptake was not inhibited by either taurine or β-alanine (sulfonic and β-amino acid, respectively).

Na^+-independent uptake of L-DON (0.05 mM) was significantly inhibited by arginine and glutamine but not BCH (at 5 mM), whereas Na^+-independent uptake of glutamine (notably greater than that of DON) was inhibited to the greatest extent by BCH (Fig. 5). On the basis of these observations, we have tentatively assigned Na^+-independent uptake of DON to System $b^{0,+}$ and glutamine to Systems L and $b^{0,+}$; both of these transporters have been characterized in oocytes (Campa & Kilberg, 1989).

Inactivation of Amino Acid Transport by L-DON

The results presented above indicated that DON and glutamine were probably transported into oocytes by a common Na^+-dependent transporter; i.e., they exhibited mutual inhibition of Na^+-dependent transport (inhibition demonstrated to be competitive), and their transport was inhibited by the same group of amino acids and analogues (notably L-arginine, an identified substrate of System $B^{0,+}$ in oocytes; Taylor et al., 1989). It therefore seemed possible that reactive DON molecules could become covalently attached to the System $B^{0,+}$ transporter protein during individual transport cycles: this would result in inactivation of the transport process and binding of DON to the oocyte plasma membrane. We therefore investigated both the ability of DON to inactivate

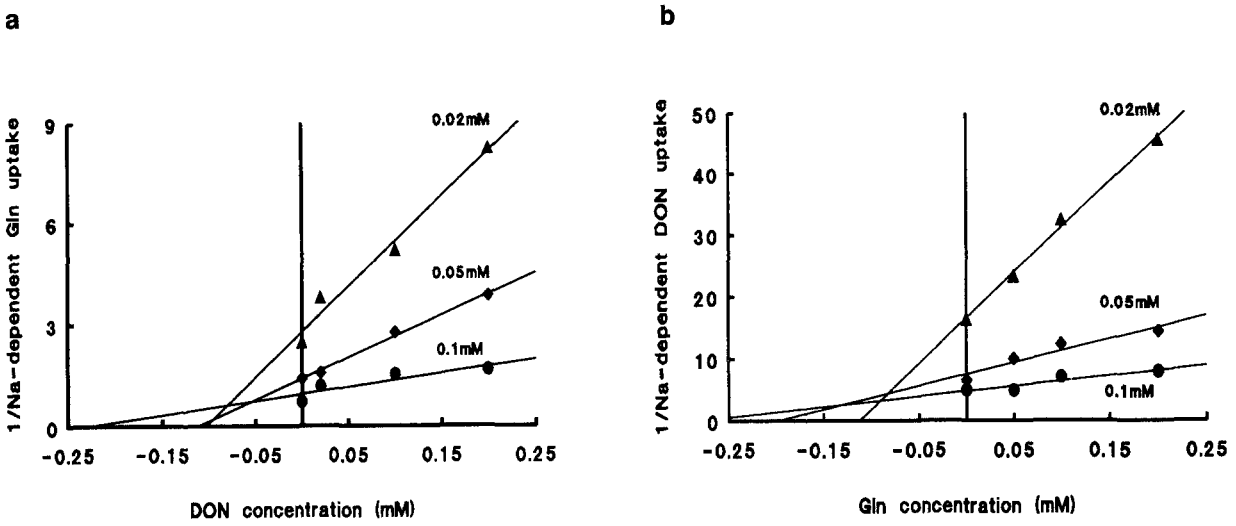

Fig. 3. Dixon plots for estimation of inhibitor K_i for Na^+-dependent glutamine and DON uptake in *Xenopus* oocytes. Each data point represents the mean value for Na^+-dependent transport in 10 oocytes (uptake in NaCl medium minus mean uptake for 10 oocytes in appropriate ChoCl medium). All lines were fitted by the least-squares method. The plots (*a,b*) show the effects of 0.02, 0.1 and 0.2 mM inhibitor on Na^+-dependent glutamine or DON transport in oocytes from two different toads; K_i values were estimated as the negative of the *x*-axis value at points of line intersection. (*a*) DON inhibition of glutamine transport (K_i = 0.05–0.09 mM); (*b*) Glutamine inhibition of DON transport (K_i = 0.08–0.13 mM).

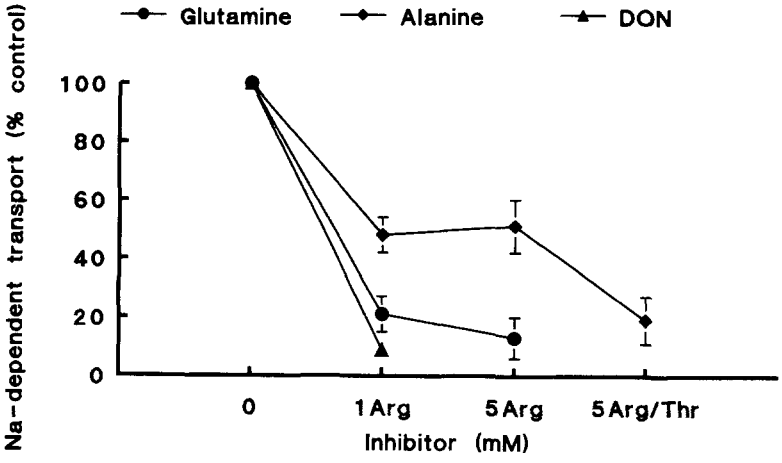

Fig. 4. Inhibition of Na^+-dependent uptake of DON, glutamine and alanine by arginine and threonine. Results are presented as means ± 1 SEM for 10 oocytes (uptake in NaCl medium minus mean uptake for 10 oocytes in appropriate ChoCl medium). Error bars not shown fall within a symbol.

Na^+-dependent glutamine transport in oocytes and the nature of any [^{14}C]DON binding to oocyte membranes. In pilot experiments (using oocytes incubated in MBM ± 0.1 mM DON) we attempted to increase the reactivity of DON in solution by brief (<30 min) photoactivation with a UV light source (multiband lamp type UVGL-58, without filter: Ultraviolet Products, San Gabriel CA) placed 15 cm above the sample. This procedure caused DON hydrolysis (as measured by a decay in DON absorbance at 274 nm; Clark et al., 1982) but also resulted in a marked increase in the fragility of oocytes (and in their mortality after 24 hr) both in the presence and absence of 0.1 mM DON, so it was abandoned.

We subsequently investigated oocytes incubated in DON-containing media without photoactivation. Incubation of oocytes in transport buffer containing 0.1 mM L-DON for 24 hr caused a reduction in Na^+-dependent glutamine transport (Fig. 6) and the effect became more pronounced after 48 hr incubation. Control oocytes (maintained in transport buffer) exhibited some downregulation of Na^+-dependent glutamine transport during incubation (Taylor et al., 1989), but the effect of L-DON preincubation was additional to this and

Table 2. Effects of amino acid analogues and derivatives (1 mM) on Na^+-dependent uptake of 0.05 mM glutamine or DON in *Xenopus* oocytes[a]

Analogue	% of control Na^+-dependent uptake	
	Glutamine	DON
Glutamate-γ-hydroxamate	20 ± 6[b]	26 ± 2[b]
Glutamate-γ-hydrazide	17 ± 4[b]	22 ± 4[b]
Glutamate-γ-methyl ester	12 ± 3[b]	15 ± 3[b]
S-carbamylcysteine	24 ± 4[b]	26 ± 4[b]
1,2,4-Triazole-3-alanine	26 ± 4[b]	36 ± 5[b]
Taurine	95 ± 9	—
β-alanine	125 ± 14	—

[a] Results are presented as mean ± 1 SEM for Na^+-dependent uptake in 10 oocytes (uptake in NaCl medium minus mean uptake of 10 oocytes in appropriate ChoCl medium).
[b] Value indicates significant inhibition of uptake by analogue ($P < 0.001$).

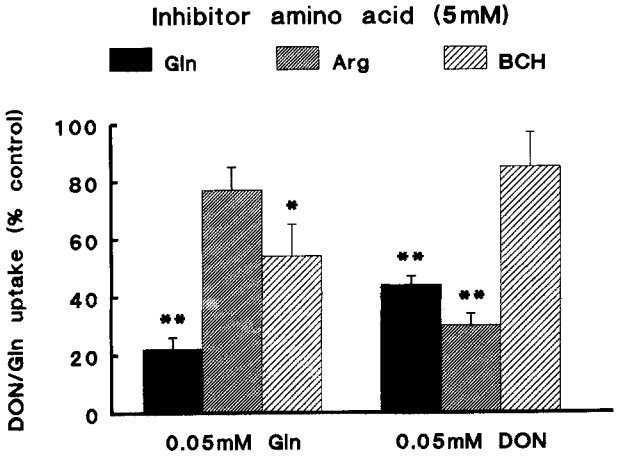

Fig. 5. Inhibition of Na^+-independent uptake of DON and glutamine by amino acids (1 mM in 100 mM ChoCl medium). Results are presented as mean ± 1 SEM for 3–4 preparations; control uptakes of DON and glutamine were 0.05 ± 0.015 and 0.17 ± 0.06 pmol/oocyte · min, respectively (four preparations). BCH, 2-amino-2-carboxybicyclo[2,2,1]heptane. Significant inhibitions of uptake are indicated (*, $P < 0.05$; **, $P < 0.01$).

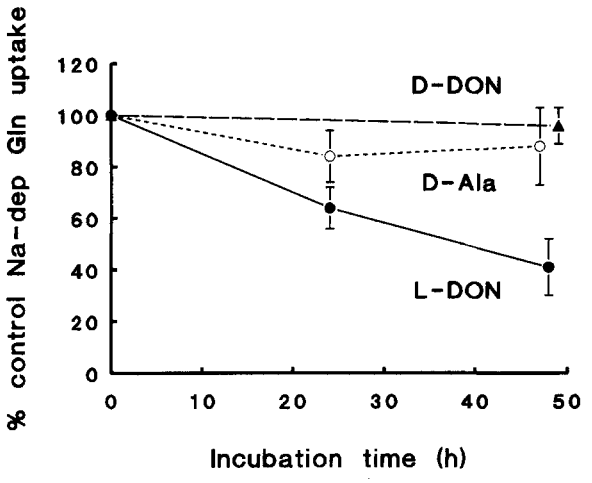

Fig. 6. The effect of preincubation with L-DON, D-DON or D-alanine (0.1 mM in NaCl transport buffer for 24 or 48 hr) on Na^+-dependent L-[^{3}H]glutamine uptake in oocytes; $n = 4$ preparations (each of 10 oocytes). Oocytes were washed free of preincubation buffer (by rinsing in transport buffer) before measurement of 0.05 mM glutamine uptake.

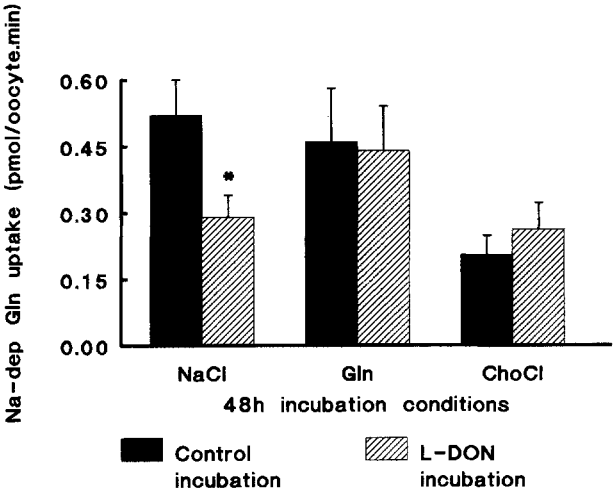

Fig. 7. The effect of Na replacement (by choline) or glutamine addition (at 1 mM) in DON-incubation buffer on Na^+-dependent glutamine (0.05 mM) transport in oocytes; $n = 5$–6 preparations (each of 10 oocytes). The DON-incubation period was 48 hr in all cases. *Uptake significantly different from control ($P < 0.05$).

was not mimicked by D-DON (i.e., it was not a nonspecific effect of the diazo group) or by System $B^{0,+}$ substrates such as D-alanine (Fig. 6) or glutamine (at 1 mM, *see* Fig. 7), (i.e., the effect did not result from downregulation of transport in response to the presence of exogenous substrate). This reduction in Na^+-dependent glutamine transport resulted from a decrease in transport V_{max} with no change in K_m (Fig. 8), indicating that DON incubation caused a time-dependent transport inactivation. Inactivation of Na^+-dependent glutamine transport by L-DON was prevented by including 1 mM glutamine in the incubation medium (Fig. 7) or by preincubating in 100 mM ChoCl rather than NaCl medium (Fig. 7). Preincubation in ChoCl medium did, however, result in an overall inhibition of Na^+-dependent glutamine transport which was associated with depolarization of the oocyte membrane (Table 3), a factor known to reduce transport activity (Jung, Lafaire & Schwarz, 1984).

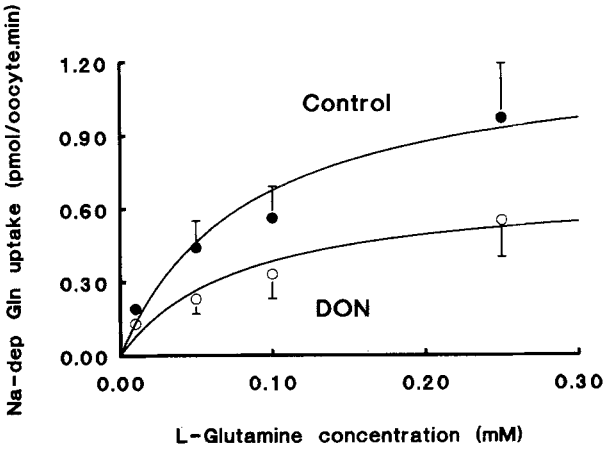

Fig. 8. Effect of 48 hr DON incubation on the kinetic characteristics of Na^+-dependent glutamine transport in oocytes. Data are presented as mean ± 1 SEM for 10 oocytes from a single toad (error bars not shown fall within a symbol). DON preincubation results in reduction of transport V_{max} (from 1.3 ± 0.15 to 0.7 ± 0.09 pmol/oocyte · min) with no change in transport K_m (0.08 ± 0.025 mM).

Table 3. The effect of 48 hr incubations on the resting membrane potential of *Xenopus* oocytes[a]

Incubation conditions	Membrane potential (mV)
NaCl (control)	−61.8 ± 2.8
NaCl + 0.1 mM DON	−58.1 ± 2.1
ChoCl	−31.3 ± 2.0[b]
ChoCl + 0.1 mM DON	−30.9 ± 4.8[b]

[a] Results are mean ± SEM for five oocytes in each case.
[b] Significantly different from control value (ANOVA; $P < 0.001$).

L-DON appeared to have no independent effect on the oocyte membrane potential (Table 3). Na^+-dependent transport of L-alanine was also inactivated by L-DON preincubation (Table 4), an effect that appeared to be restricted largely to the arginine-inhibitable fraction of Na^+-dependent alanine transport (i.e., System $B^{0,+}$). Na^+-independent uptake of glutamine, but not of alanine, was significantly reduced after 48 hr preincubation with DON (Table 5).

Binding of L-DON to Oocyte Membranes

Oocytes were incubated in [^{14}C]DON-containing transport buffer for periods up to 48 hr to determine the extent of DON binding to oocyte surface membranes. In order to evaluate the extent and specificity of DON binding to surface membranes of the oocyte, we isolated a membrane fraction from homogenized, defolliculated oocytes. This membrane fraction was enriched 16-fold in activity of KpNPPase (a marker enzyme for plasma membrane) relative to oocyte homogenate (Table 6) and appeared to be substantially depleted of oocyte yolk proteins as assessed by polyacrylamide gel electrophoresis (*data not shown*), but it includes both intracellular and surface membrane components (Colman, 1984). L-[^{14}C]DON exhibited time-dependent binding to oocyte membranes during incubations of up to 48 hr in NaCl or ChoCl transport buffer containing 0.1 mM DON (Fig. 9*a*). Oocytes incubated

Table 4. The effects of 48 hr preincubation with L-DON on Na^+-dependent alanine transport in oocytes[a]

Preincubation medium (48 hr incubation)	Na^+-dependent alanine uptake (pmol/oocyte · min)		% Inhibition by arginine
	Control	+5 mM arginine	
NaCl	0.634 ± 0.082	0.326 ± 0.088[b]	49
NaCl + 0.1 mM L-DON	0.391 ± 0.038	0.322 ± 0.062	17

[a] Results are presented as mean ±SEM for 16 oocytes pooled from two separate experiments (7 + 9).
[b] Value significantly different from control ($P < 0.05$)

Table 5. The effects of 48 hr preincubation with L-DON on Na^+-independent uptake of glutamine and alanine in oocytes[a]

Incubation conditions (48 hr incubation)	Uptake in ChoCl medium (pmol/oocyte · min)	
	Glutamine	Alanine
NaCl	0.115 ± 0.023	0.148 ± 0.024
NaCl + 0.1 mM L-DON	0.077 ± 0.017[b]	0.138 ± 0.013

[a] Results are presented as mean ±SEM for eight preparations (glutamine uptake) or for 16 oocytes[c] (alanine uptake)
[b] Value significantly different from mean uptake after NaCl incubation (paired-sample *t*-test; $P < 0.05$)
[c] Pooled from results of two experiments (9 + 7 oocytes)

Table 6. Protein yield and marker enzyme activity of membrane fraction isolated from *Xenopus* oocytes[a]

	Protein (mg/oocyte)	K-stimulated phosphatase (nmol/mg protein · min)
Homogenate	0.141 ± 0.033	0.5 ± 0.1
Membrane	0.008 ± 0.002	8.1 ± 1.2

[a] All values are mean ± 1 SEM; $n = 4$ membrane preparations.

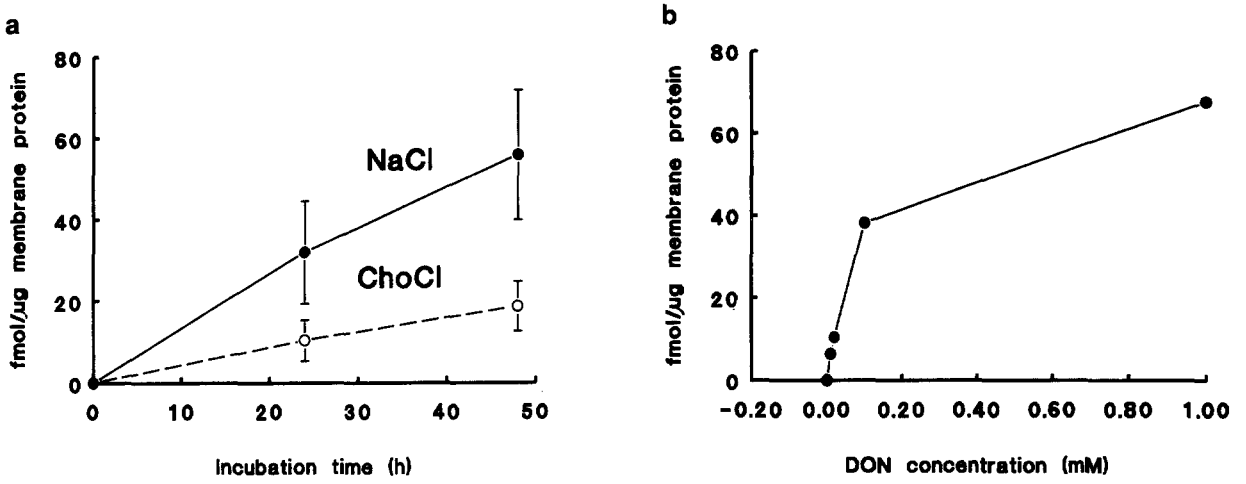

Fig. 9. (*a*) Time-course of L-[^{14}C]DON binding to oocyte membranes (mean binding ± SEM, n = 4–5 preparations); oocytes were incubated for up to 48 hr in NaCl or ChoCl media containing 0.1 mM L-[^{14}C]DON. (*b*) Effect of external L-[^{14}C]DON concentration on DON binding to oocyte membranes after 24 hr incubation (mean binding of duplicate samples in a single experiment).

Table 7. The effects of different compositions of incubation medium on binding of [^{14}C]DON (at 0.1 mM) to oocyte membranes[a]

Incubation medium (48 hr incubation)	[^{14}C]DON binding to oocyte membranes (fmol/µg membrane protein)
100 mM NaCl	54 ± 10[b]
100 mM NaCl + 1 mM glutamine	27 ± 4
100 mM ChoCl	23 ± 4

[a] All values are mean ± 1 SEM for 5–6 membrane preparations.
[b] Mean value significantly different from others (ANOVA; $P < 0.05$ for each comparison).

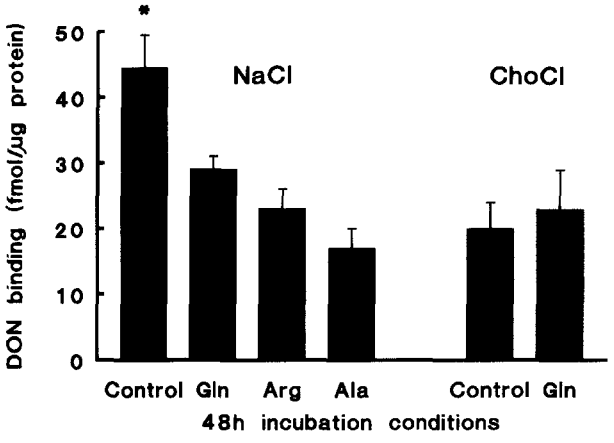

Fig. 10. Effect of amino acids (glutamine, arginine and alanine) at 1 mM in the incubation medium on binding of L-[^{14}C]DON (at 0.1 mM) to oocyte membranes. Incubation was for 48 hr, and results are presented as mean ± SEM for triplicate samples (each for membranes from 10 oocytes) from a single preparation. Similar values were obtained in a second experiment using oocytes from a different toad (*data not shown*). *Value significantly different from others in NaCl medium (ANOVA: $P < 0.05$ for each comparison).

for 48 hr in NaCl buffer exhibited significantly (2.4 times) higher [^{14}C]DON binding to membranes than those incubated in ChoCl buffer (Table 7). DON-binding was significantly reduced by inclusion of 1 mM L-glutamine in NaCl buffer (Table 7); this appeared to be due largely to competitive suppression of the "Na^+-dependent" component of binding, because glutamine did not affect DON binding to oocytes incubated in ChoCl buffer (Fig. 10). DON binding was also reduced by arginine and L-alanine (Fig. 10), both of which are substrates of System $B^{0,+}$. Na^+-dependent DON binding to oocyte membranes averaged 31 ± 11 fmol/µg membrane protein (n = 6 preparations) after 24 hr incubation, equivalent to ≈250 fmol/oocyte (assuming 8 µg membrane protein/oocyte; Table 6). There was no significant difference between the amount of [^{14}C] label associated with membranes isolated from oocytes incubated for 48 hr in NaCl or ChoCl buffer containing 0.1 mM D-[^{14}C]alanine at the same specific activity as L-[^{14}C]DON (*data not shown*). D-alanine and L-DON are concentrated into oocytes to a similar extent after 48 hr incubation (for both radiotracers at 0.1 mM, the [^{14}C]-activity of oocytes incubated in NaCl buffer is ≈11 times higher than that in ChoCl buffer);

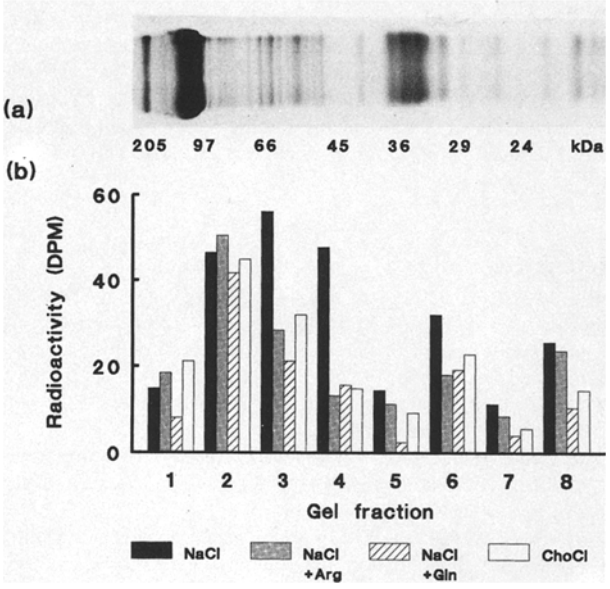

Fig. 11. Polyacrylamide gel electrophoresis of oocyte membrane proteins labeled with [^{14}C]DON. (*a*) Gel lane stained with Coommassie blue showing membrane proteins and molecular weight calibration from protein standards; (*b*) radioactivity associated with fractions from gel lanes containing proteins labeled by 48 hr incubation of oocytes under the denoted conditions.

therefore, the difference between [^{14}C]DON binding to membranes of NaCl- and ChoCl-incubated oocytes does not simply reflect the higher intracellular activity of [^{14}C]DON in NaCl-incubated oocytes. In a single experiment, [^{14}C]DON binding to membranes of oocytes incubated for 24 hr in NaCl buffer containing one of four different L-[^{14}C]DON concentrations was measured; the results show an apparently saturable increase in total binding with increased external DON concentration (Fig. 9*b*), with an external DON concentration for half-maximal binding (after 24 hr incubation) of the order 0.1 mM.

Separation of denatured membrane proteins by polyacrylamide-gel electrophoresis (Fig. 11) revealed that the component of [^{14}C]DON binding which was suppressed by glutamine and arginine or Na^+ replacement (by choline) was largely restricted to a gel area (fractions *3* and *4*) corresponding to proteins of relative molecular mass 48–65 kDa. The relatively large amount of radioactivity recovered from gel fraction *2* under all conditions appears to be associated largely with the residual contaminating yolk protein.

Discussion

We have observed that L-DON and L-glutamine exhibit mutual competitive inhibition of Na^+-dependent transport in cultured (Stage VI) *Xenopus laevis* oocytes. This observation, together with the finding that L-DON transport is markedly inhibited by L-arginine (a competitive inhibitor of Na^+-dependent glutamine transport in oocytes; Taylor et al., 1989), provides strong evidence that L-DON and L-glutamine are transported into oocytes by the Na^+-dependent System $B^{0,+}$, which we previously characterized in oocytes (Taylor et al., 1989). Most cationic and neutral aliphatic amino acids are taken up into oocytes via this transporter, although System ASC also serves to transport small neutral amino acids (Campa & Kilberg, 1989), but apparently not glutamine or DON. System $B^{0,+}$ is known to be an α-amino acid transporter of broad scope (Van Winkle, 1988), which is consistent with our observation that a variety of α-amino acid analogues with bulky side-chains cause substantial inhibition of DON and glutamine uptake into oocytes. Our present values for K_m and V_{max} of Na^+-dependent transport of L-DON and L-glutamine in oocytes are of the same order of magnitude (10^{-4} M, 10^{-12} mol/oocyte · min, respectively) as values reported previously for a variety of amino acids (e.g., Jung et al., 1984; Taylor et al., 1989; Campa & Kilberg, 1989).

L-DON inactivated System $B^{0,+}$ activity (i.e., Na^+-dependent glutamine transport) after preincubation. The inhibition was not a general effect (e.g. DON cleavage causing nonspecific damage to the oocyte membrane) because (i) D-DON (which does not appear to be transported by System $B^{0,+}$) did not have an independent effect on transport activity and (ii) KpNPPase activity of membranes isolated from control and DON-treated oocytes were not significantly different from one another (*data not shown*). D-alanine is a System $B^{0,+}$ substrate and, like L-DON, is not incorporated into oocyte protein; it is therefore a useful control for investigating the effects of L-DON in this type of experiment. D-alanine and glutamine were without effect on Na^+-dependent glutamine transport after preincubation; these findings indicate that DON inactivation of Na^+-dependent glutamine transport is not simply a result of downregulation of transport in response to increased exogenous substrate availability ("adaptive regulation"; Christensen & Kilberg, 1987). System $B^{0,+}$ activity in control oocytes is downregulated with prolonged incubation time (confirming previous reports), but DON has a marked inhibitory effect on transport independent of this phenomenon. Culture of oocytes in MBM results in marked changes in the type of endogenous amino acid transport activity expressed over a period of days and in the degree of this expression over much shorter periods (e.g. Jung et al., 1984; Taylor et al., 1989), this phenomenon may in part be ascribed to a gradual depolarization of the oocyte membrane, because

Na^+-dependent amino acid transport in oocytes is voltage sensitive (Jung et al., 1984).

There is a direct temporal relationship between the extent of System $B^{0,+}$ transport inactivation by DON and the amount of L-[^{14}C]DON-binding to the oocyte membrane. We have also demonstrated that DON is not incorporated into oocyte protein, that glutamine can prevent DON-induced inhibition of glutamine transport and that DON binding to oocyte membranes is stimulated in the presence of external Na^+ ions and suppressed by substrates of System $B^{0,+}$ (glutamine, alanine, arginine). Furthermore, the DON concentration for half-maximal binding ($\approx$0.1 mM) is of the same order as the K_m estimated for Na^+-dependent DON transport into oocytes (although this result in isolation is of equivocal significance). The weight of evidence presented above allows us to suggest that the correlation between inactivation and binding may reflect a specific covalent attachment of [^{14}C]DON to the amino acid receptor moiety on the System $B^{0,+}$ transporter. If this is the case, [^{14}C]DON could be used as a convenient photoaffinity label for the transporter during biochemical purification of the System $B^{0,+}$ protein. The labeling pattern of [^{14}C]DON to oocyte membrane proteins separated by electrophoresis indicates that a peptide of 48–65 kDa may include at least the amino acid binding site of the System $B^{0,+}$ transporter.

At this preliminary stage we cannot exclude the possibility that DON is also binding to other glutamine "receptors" on the oocyte membrane, including Na^+-independent amino acid transporters such as System $b^{0,+}$ for which DON appears to be a substrate (note that a component of Na^+-independent glutamine uptake is reduced by DON preincubation). We are presently conducting further investigations of the extent and specificity of DON binding to specific proteins in the oocyte membrane.

The involvement of glutamine in a number of important metabolic processes (e.g. purine, pyrimidine and urea synthesis) and its utilization as a fuel by many rapidly dividing and proliferating cell types (e.g. enterocytes, lymphocytes and tumor cells) has led to considerable interest in the use of glutamine antimetabolites (e.g. DON) as antineoplastic agents in the treatment of various mammalian tumors (Prajda, 1985; Huber, Rosenfeld & Roberts, 1988). The ability of these antimetabolites to inactivate glutamine-metabolizing enzymes by binding irreversibly to active sites has made them useful probes of enzyme structure (e.g. Hartman, 1963; Clark et al., 1982) and it is now evident that they could also be applied as covalent ligands for mammalian amino acid transporters (e.g. System L; Segel et al., 1989) and System $B^{0,+}$ in *Xenopus* oocytes.

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