combinations of components derived from the two dietary groups were constructed so that each individual component was added at equivalent levels. In 1978 Nerurkar *et al.* [17] investigated the effect of low protein diet by using a reconstituted system technique. However, the fractions used were from rats treated with phenobarbital. So the effect observed was not only due to dietary protein. We have shown that induction leads to significant changes not only in cytochrome P-450 isoenzymes but also in lipid composition of microsomes from rats fed a LP diet [18] and that changes of the MO system introduced by LP diet in animals treated with inducers are completely different from those in untreated animals (Amelizad *et al.*, manuscript in preparation). In the present study we have therefore used a more recently developed method which allows the isolation of the very low cytochrome P-450 from animals treated with a LP diet and not treated with any cytochrome P-450 inducers. Moreover Nerurkar and coworkers used weanling rats fed 10 days with unbalanced diets, whilst in this investigation the animals were treated for 60 days with the experimental diets. Substitution of St lipid fraction with the LP lipid decreased *N*-demethylase activity by 10–20% (Table 1). A low protein intake had been demonstrated to alter the phospholipid composition or microsomal membranes [6]. In rats fed 60 days with a 5% casein diet, phosphatidyl ethanolamine and phosphatidyl serine were decreased by 30% and sphingomyelin was increased 2.5 times compared to rats fed a 18% casein diet. Our results can at least in part be explained by a change in phosphatidyl serine content. Haaparanta *et al.* [19] reported that vesicular MO reconstituted systems were up to 6 times more active in deethylation of 7-ethoxycoumarin when prepared with phosphatidyl serine than when prepared with phosphatidyl choline or phosphatidyl ethanolamine. Thus the decrease in demethylation when LP lipids were added to a St system and the increase when St lipids were added to an LP system may in part be explained by changes in phosphatidyl serine content of the microsomal phospholipids.

Substitution of cytochrome P-450 caused higher changes in monooxygenase activity of reconstituted systems compared with other substitutions. Thus the major part in decrease of specific MO activity after treatment with LP diet can be related to changes in cytochrome P-450 (about 60%). However, the change in cytochrome P-450 reductase also plays a considerable role. The contribution of the reductase to the decrease of the MO activities with ethylmorphine and benzphetamine as substrates was about 30%.

Several mechanisms underlying the observed decreases in monooxygenase activities by substituting LP components in the reconstituted system may be envisaged. Besides changes relating only to individual components, changes relating to the interaction between the components may prove to be important. The exact nature of these changes remains to be established.

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Effect of 1-anilino-8-naphthalene sulfonate (ANS) on transferrin and iron uptake by reticulocytes

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Nucleated erythroid cells and reticulocytes are able to acquire iron from plasma transferrin by receptor-mediated endocytosis [1]. Any derangement of membrane structure could markedly affect this iron transport system [2]. A membrane probe, 1-anilino-8-naphthalene sulfonate (ANS), interacts with phospholipid and protein regions of the erythrocyte plasma membrane and creates a negative surface potential of sufficient magnitude to repulse anion transport from the permeation site [3]. In view of the influence of ANS on membrane charges and carrier-mediated transport, the process of transferrin and iron uptake by reticulocytes might be disrupted. The present study investigates this possibility and attempts to find out in what way it could interfere with the transport mechanism.

Materials and methods

ANS was purchased from the Sigma Chemical Co (St. Louis, MO, U.S.A.) and was used without further purification. The pH of the ANS solution was adjusted to 7.4 by adding 0.1 M NaOH prior to use in the experiments.

Rabbit transferrin was prepared from pooled serum by ammonium sulfate fractionation and column chromatography [4] and was labeled with ^{59}Fe and ^{125}I as described previously [5]. Reticulocytosis was induced by three to five daily subcutaneous injections of neutralized phenylhydrazine (6 mg/kg). Two days after the last injection the animals were bled from a marginal ear vein. Blood cells were washed with HEPES–saline buffer (20 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid, 0.15 M NaCl, pH 7.4) and stripped of endogenous transferrin by incubation at 37° for 20 min. The reticulocyte count was generally between 40 and 80%. For brevity the cells of reticulocyte-rich blood will be referred to as "reticulocytes".

The uptake of transferrin and iron by reticulocytes was measured by a method described previously [5]. Prior to this measurement, the cells were preincubated with the desired concentration of ANS at 37° for 20 min. Binding studies were performed by incubating aliquots of 100 μl reticulocytes in 1 ml of Hanks' solution with various concentrations of labeled transferrin (ranging from 0.05 to 6.5 μM) for 30 min at 37°. Non-specific binding was determined by adding twenty times excess of cold transferrin to the radiolabeled transferrin. The reticulocytes were then washed, and the radioactivity remaining in the cells was measured in an LKB Rack Gamma counter.

Single-cycle endocytosis studies on transferrin in reticulocytes were adapted from methods described previously [6, 7]. Briefly, the reticulocytes in Hanks' solution containing 0.2% bovine serum albumin were first incubated with various concentrations of ANS at 37° for 10 min before being cooled to 0°. Then 0.4 μM [^{125}I]transferrin was added, and the cells were allowed to stand at 0° for another 10 min so that the labeled transferrin could bind to the cell surface. Any unbound radioactive transferrin was removed by centrifugation. The cells were then rapidly warmed to 37° for various time periods. Incubation was stopped by pipetting the cells into 3 ml of ice-cold Hanks' solution. The medium was immediately separated from the cells by centrifugation. The surface-bound [^{125}I]transferrin was removed by incubating the cells with 0.1% pronase in HEPES–saline buffer, pH 7.4, at 0° for 1 hr. The internalized transferrin molecules were protected from proteolysis and were recovered with the cell pellet [6].

Results

ANS inhibited transferrin and iron uptake by reticulocytes in a dose-dependent fashion (Fig. 1). Half-maximum inhibition of iron was achieved by a 1 mM ANS, whereas the I_{50} value for transferrin was produced only when the ANS concentration was in excess of 2 mM.

Data from the binding study for transferrin and iron were analyzed by the method of Scatchard [8], and the calculated number of transferrin binding sites (B_{max}) and the association constant (K_a) of these sites for transferrin were tabulated (Table 1). All values were reduced by addition of ANS. However, only at a 1 mM ANS concentration were the K_a of transferrin and the B_{max} values for iron uptake significantly lowered compared with the control ($P < 0.01$).

Studies on the fate of [^{125}I]transferrin during a single cycle of endocytosis in reticulocytes showed that the surface-bound transferrin disappeared rapidly within the first 4 min (Fig. 2, upper panel). Approximately 36% of the surface-bound [^{125}I]transferrin was internalized during this time, and then at least half of this protein was exocytosed into the medium in the next 10 min. A considerable amount of surface-bound ligand was dissociated directly back into the medium. However, when the cells were incubated with 0.5 mM ANS, the peak of intracellular transferrin was no longer seen (Fig. 2, lower panel). Instead, the intracellular transferrin reached a plateau after 7 min and remained unchanged for the rest of the incubation period. With a higher ANS concentration (0.8 mM) the intracellular transferrin fraction took a longer time (about 20 min) to reach the 20% level (results not shown). From the rates of disappearance of the surface-bound [^{125}I]transferrin, the $T_{1/2}$ values for internalization were calculated to be 1.6 min, 3.0 min and 4.4 min for control, 0.5 mM ANS and 0.8 mM ANS respectively.

Discussion

The inhibitory effects of ANS on transferrin and iron uptake by reticulocytes were dose-dependent, with iron being more affected than transferrin. Investigation into the site of action seems to suggest that ANS acts on both the membrane and the endotubular function of the cell. Initially, it reduced the number of surface transferrin recep-

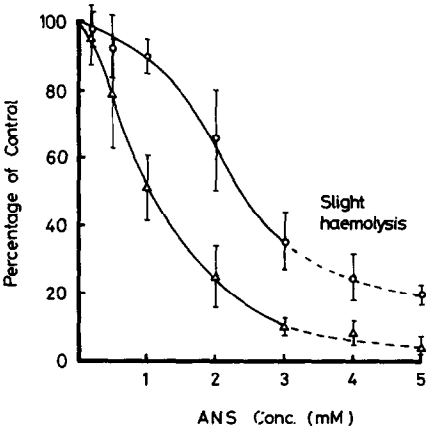

Fig. 1. Effects of different concentrations of ANS on transferrin (○) and iron (△) uptake by reticulocytes. The results are expressed as percentage of control where no ANS was added to the incubation medium. Each point represents the mean of six measurements and the bar, one standard error. The dotted line indicates the slight haemolysis that was observed.

Table 1. Effects of ANS on the number of receptor sites (B_{max}) and the association constant (K_a) of transferrin with rabbit reticulocytes

Reagent	B_{max} (molecules/$\times 10^{-4}$)	K_a ($M^{-1} \times 10^6$)	B_{max} (molecules/cell/hr $\times 10^{-5}$)
Control	3.51 ± 1.13	2.95 ± 1.29	2.55 ± 0.56
0.25 mM ANS	3.29 ± 0.56	2.53 ± 1.25	1.99 ± 0.74
1 mM ANS	2.83 ± 0.71	2.05 ± 1.42*	1.59 ± 0.75*

The results were derived from the binding study as described in Materials and Methods. Each value represents the mean ± standard derivation from six experiments.

* $P < 0.01$ (Student's *t*-test).

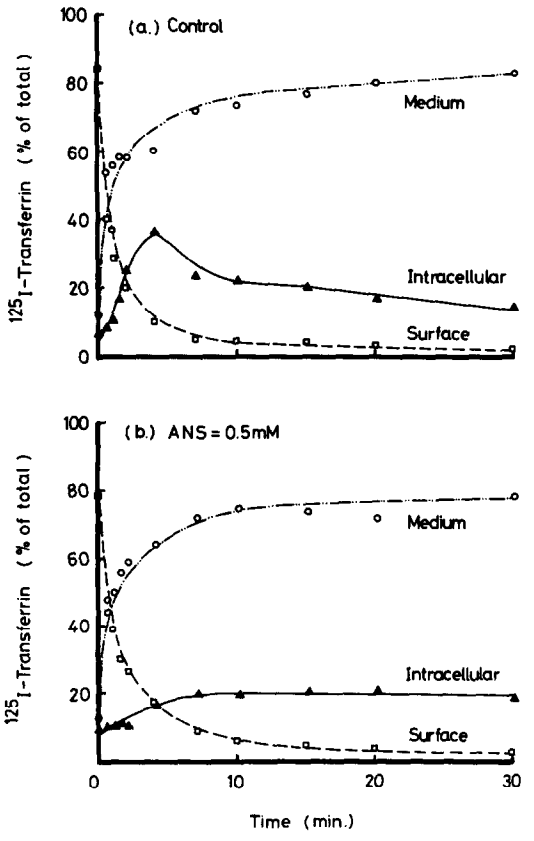

Fig. 2. Movement of surface-bound [^{125}I]transferrin into cells and medium when incubated at 37° without (upper panel) and with 0.5 mM ANS (lower panel). The radioactivity in the pronase-resistant fraction (▲—▲), the pronase-sensitive fraction (□----□) and the medium (○·---·○) was determined as described in the text.

tors and the association constant of the receptors for transferrin (K_a), although only the latter was significantly different at 1 mM ANS concentration. Subsequently, the rate of iron uptake was also reduced. It is unlikely that ANS was in direct competition with the protein for binding sites as the probe inhibited the binding of transferrin in a non-competitive manner (unpublished data). Also, ANS binds to the hydrophobic region of the erythrocyte membrane spanning from the outer cell surface to some discrete areas of the inner surfaces [9]. As the transferrin receptors are not hydrophobic [10], it is doubtful that the membrane probe could have occupied a part, or all, of the transferrin receptor site. Since ANS reduces the Coulombic repulsion of the ionic groups of the phospholipids in the membrane [11], the decrease in the binding affinity was most likely the result of an altered membrane fluidity.

By blocking the recycling of transferrin in the reticulocyte, ANS could reduce the effective number of receptor sites. It prolonged the rate of transferrin internalization as well as the rate of exocytosis. A high concentration of ANS almost completely abolished the exocytosis of transferrin (Fig. 2). This perturbation of the endotubular functions of the cell by ANS is further supported by electron microscope studies showing the presence of enlarged vesicles after incubation with ANS (unpublished data). Since the delivery of iron from the plasma transferrin to reticulocytes requires repeated recycling of transferrin-iron into the cell, it is no surprise that the effect of ANS is more pronounced on the uptake of iron than on the uptake of transferrin.

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Influence of the route of administration on the protective effect of L-carnitine on acute hyperammonemia

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Carnitine is essential for the oxidative metabolism of long-chain fatty acids. Long-chain acylcarnitines readily cross the inner mitochondrial membrane and undergo β-oxidation [1].

We found that L-carnitine, when administered prior to an LD_{100} of ammonium acetate, prevented death entirely and suppressed the symptoms of ammonia toxicity in mice [2]. Although the mechanism of ammonia toxicity is