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Mitochondrial Ammonia Metabolism and the Proton-Neutral Theory of Hepatic Ammonia Detoxication

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Much of the ammonia (ammonia is used as a generic term for NH_3 or NH_4^+ or a combination of the two) that is detoxified in vertebrate liver comes from the α-amino function of amino acids that are catabolized during amino acid gluconeogenesis. This functionality is released as ammonia, either directly in the cytosol of hepatocytes by specific deaminases or via the combined actions of the aminotransferases and glutamate dehydrogenase (GDH). GDH is localized exclusively in the matrix of tetrapod liver mitochondria (McGivan & Chappell, 1975; Vorhaben & Campbell, 1977; Campbell et al., 1985), so it is within this compartment that ammonia is released via the latter route. Dietary protein is a major source of amino acids for hepatic gluconeogenesis and glucose formed from the carbon skeleton of these amino acids is either used directly by extrahepatic tissues for energy or is converted to glycogen for storage (Krebs, 1972). The process of uricogenesis in uricoteles is especially tightly linked to hepatic gluconeogenesis because xanthine dehydrogenase in the urate pathway may be a major source of cytosolic reducing equivalents for glucose formation (Coolbear et al., 1982; Campbell, 1995). Ontogenetically, development of the capacity for hepatic ammonia detoxication via either the urea or urate pathway coincides with the alimentation of protein (see Campbell, 1994). In omnivorous species, hepatic levels of the enzymes for ureagenesis and uricogenesis are directly correlated with dietary protein intake (Schimke, 1962; Campbell, 1995) whereas carnivorous species generally maintain high rates of hepatic gluconeogenesis irrespective of protein intake (Silva & Mercer, 1986; Herzberg, 1991).

In addition to diet, muscle is also a source of amino acids for hepatic gluconeogenesis. This is especially true during caloric restriction and other glucocorticoid-induced protein catabolic states. Even during a normal intake of dietary protein, caloric restriction itself causes a marked increase in the capacity for hepatic ureagenesis (Tillman et al., 1996). Normal working muscle in both birds (Wu et al., 1989) and mammals (Goldberg & Chang, 1978; King et al., 1983) produces alanine and glutamine as nitrogenous end-products of metabolism. The former is quite gluconeogenic and its transport to the liver for conversion to glucose for return to muscle and other extrahepatic tissues has been termed the "alanine cycle" (Felig et al., 1970). Glutamine is also produced by other tissues such as brain via the action of glutamine synthetase (Duffy et al., 1983). Kidney and intestine are major sites of glutamine utilization but any that is returned to the liver in mammals is taken up by a specific transport system in hepatocytes (Kilberg et al., 1980) and eventually translocated into mitochondria (Soboll et al., 1991). Here it is hydrolyzed to NH_3 and glutamic acid by a phosphate-dependent glutaminase. The latter enzyme may be loosely attached to the inner mitochondrial membrane (McGivan, 1988) so, as with GDH, ammonia is again released within the mitochondrial matrix compartment. Mitochondrial glutaminase activity is very low in chicken liver relative to that in mammalian liver and the enzyme is located on the outer mitochondrial membrane (Vorhaben & Campbell, 1977). Therefore any glutamine returned to the liver in uricoteles is presumably converted directly to urate in the cytosol (see Compartmentation of the Urate Pathway below). Working muscle and other extrahepatic tissues also produce ammonia (Lowenstein, 1972; van Waarde, 1988), which must be returned to the liver for detoxication. Because both the ureotelic

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and uricotelic mechanisms for ammonia detoxication are localized within the matrix compartment, this ammonia must traverse both the plasma and mitochondrial membrane systems to be detoxified. Jungas et al. (1992) have made quantitative estimates of the amounts of ammonia formed via the different pathways of amino acid catabolism in man. These estimates indicate that extrahepatic ammonia is a major source of the ammonia that is converted to urea by the liver.

COMPARTMENTATION OF THE UREA CYCLE

The urea cycle consists of the enzymes carbamyl phosphate synthetase (CPS)-I, ornithine transcarbamylase (OTC), argininosuccinate (ASA) synthetase, ASA lyase, and arginase. The first two enzymes, CPS-I and OTC, form citrulline. Citrulline is then converted to arginine via ASA synthetase and ASA lyase. Arginase acts to convert arginine to urea for excretion, regenerating ornithine for citrulline synthesis. Early studies of these reactions using rat liver homogenates fractionated by differential centrifugation served to localize the synthesis of citrulline in the particulate fraction and the synthesis of arginine in the soluble fraction (Cohen & Hayano, 1948). Subsequent studies served to localize CPS-I and OTC in mitochondria and ASA synthetase and lyase in the cytosol (see Caravaca & Grisolia, 1960; Shambaugh et al., 1969; Ratner, 1976). CPS-I and OTC were then shown to be localized exclusively within the matrix of rat liver mitochondria (Gamble & Lehninger, 1972; Clarke, 1976). CPS-I and OTC were, in fact, two of the first vertebrate enzymes to be studied with respect to their translation in the cytosol and import into the mitochondrial matrix (Raymond & Shore, 1979; Conboy, Kalousek & Rosenberg, 1979; Mori et al., 1980) and several of the paradigms for mitochondrial targeting and import were established using these enzymes, especially OTC, as model systems. The subcellular localization of arginase has been complicated by the fact that the predominant form of the rat liver enzyme is cationic (Hirsch-Kolb et al., 1970) and associates with acidic macromolecules, including nucleic acids and membrane phospholipids. In low ionic-strength media, the enzyme has therefore been variously localized in the nuclear fraction, in microsomes (endoplasmic reticulum), and in mitochondria (Skrzypek-Osiecka et al., 1980). Most (90%) of the arginase present in rat liver mitochondria preparations can be solubilized in high ionic-strength media; the remaining 10% cannot (Cheung & Rajiman, 1981). Recently, ASA synthetase and lyase were found to be closely associated with the outer mitochondrial membrane (Cohen, 1996; Cohen & Kuda, 1996), an association that could account for the observed channeling of citrulline to the arginine synthetase system (Cheung et al., 1989). There is also channeling of exogenous ornithine to OTC (Cohen et al., 1987). Were the tightly-bound mitochondrial arginase part of a urea cycle complex as depicted hypothetically in Figure 1, then it could conceivably function to channel endogenous ornithine to OTC.

The physiological consequences of the intracellular compartmentation of the urea cycle enzymes were first emphasized by Gamble & Lehninger (1973) when they raised the question of the nature of the movement of ornithine into mitochondria and the subsequent outward movement of citrulline. Because of the known catalytic effect of ornithine on urea synthesis in perfused liver and isolated hepatocytes (Krebs et al., 1973) and on citrulline synthesis in isolated mitochondria (Cohen et al., 1980; see also Beliveau Carey et al., 1993), the major emphasis was, and has been, on the inward translocation of ornithine. There have been various formulations of the mechanism for this (Gamble & Lehninger, 1973; Aronson & Diwan, 1981), but it now appears that it is via an energy-independent ornithine-citrulline antiporter (Bradford & McGivan, 1980; Indiveri et al., 1992).

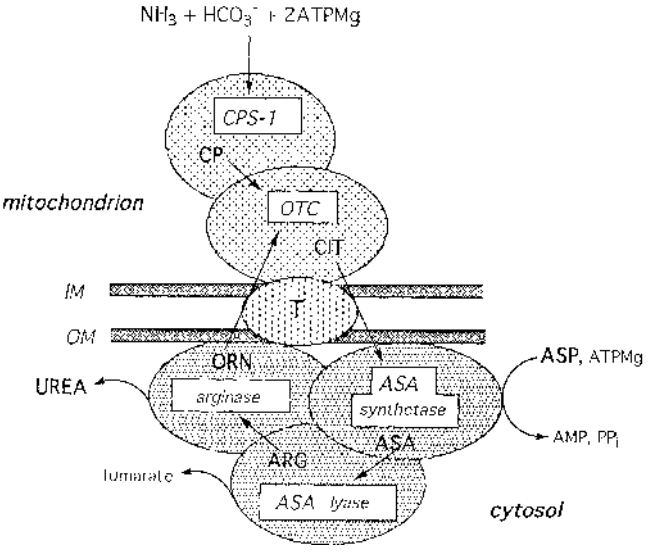

Fig. 1. Hypothetical organization of urea cycle. CP, carbamyl phosphate; CPS-1, CP synthetase-I; OTC, ornithine transcarbamylase; ASA, argininosuccinic acid. T indicates the ornithine-citrulline antiporter.

It thus seems reasonable to predict that this antiporter may serve as a kind of transmembrane link between the intramitochondrial and extramitochondrial complexes of the urea cycle as shown in Figure 1.

COMPARTMENTATION OF THE URATE PATHWAY

The original studies of Buchanan and co-workers showed that the soluble fraction of pigeon liver was capable of incorporating all of the major N precursors of the purine ring (glycine, glutamine, and aspartate) into IMP. Ammonia itself was not incorporated (Sonne et al., 1956). In studying the role of chicken liver mitochondria in supplying one-carbon units (as 5, 10-methenyltetrahydrofolate) for hypoxanthine synthesis in a reconstituted system consisting of mitochondria and cytosol, Yoshida and Kikuchi (1971) showed a marked requirement for glutamine by this system. This requirement could, however, be replaced by gutamate plus NH_4^+. This served to focus on the role of glutamine synthetase in purine (and urate) biosynthesis in uricotelic liver. The level of activity of glutamine synthetase in this tissue was then known to be much higher than in ureotelic liver tissue (Katunuma et al., 1970). At the time, the only known subcellular localization for glutamine synthetase was in mammalian liver where the enzyme is in the cytosol (Wu, 1963). However, an examination of two uricotelic species showed that glutamine synthetase is, by contrast, mitochondrial in liver of these species (Vorhaben & Campbell, 1972). Unlike many other species- or tissue-specific compartmental isozymes, the kinetic and physical properties of the cytosolic and mitochondrial glutamine synthetases are almost identical (Vorhaben et al., 1982). There do, however, appear to be at least three critical amino acid substitutions at the N terminus of the avian enzyme, which may allow this domain to function as an internal mitochondrial targeting signal following translation of the subunit peptide in the cytosol (Campbell & Smith, 1992).

The localization of glutamine synthetase in uricotelic liver mitochondria thus served to explain some of the early observations on the synthesis of purine by this tissue. The enzyme was later localized in the matrix of chicken liver mitochondria (Vorhaben & Campbell, 1977) and shown to function in situ in converting ammonia derived from glutamate deamination via GDH to the amide function of glutamine (Campbell & Vorhaben, 1976). ^{15}N from [amino-^{15}N]-L-glutamate was also incorporated into the amino function of glutamine and it was estimated that approximately one-half the glutamate taken up by isolated chicken liver mitochondria serves as a direct substrate for mitochondrial glutamine synthesis whereas the other one-half is deaminated via GDH. Glutamate thus functions both as a major source of matrix ammonia and as the carrier for this ammonia after it has been "detoxified." Its function in the latter context is therefore analogous to that of ornithine in the ureotelic system. The transport of glutamate by uricotelic liver mitochondria has not been characterized despite its obvious importance in their metabolism. Glutamine is taken up by chicken liver mitochondria by a process unsaturable at physiological concentrations and this is followed by an extremely rapid efflux in vitro (Campbell & Vorhaben, 1976). This suggests that the transit of glutamine across the mitochondrial membranes is via an energy-independent, non-carrier mediated process. In the cytosol, the amide function of glutamine contributes to N-3 and N-9 of the purine ring. The formation of IMP, from which urate for excretion is derived, involves ten enzymatic steps, all of which have been localized in the cytosol in keeping with the original observations of Sonne et al. (1956) on pigeon liver. While the first step, PRAT (see Fig. 2 for abbreviations), which adds N-9, may utilize NH_3 to a limited extent,[1] it is maximally active with L-glutamine. FGAMS (also called FGAR amidotransferase), the fourth step in the pathway which adds N-3, has an absolute requirement for L-glutamine (Rowe et al., 1978).

The isolation of a loosely-bound protein complex from pigeon liver capable of IMP synthesis has been reported (Rowe et al., 1978). Although this has not been confirmed, at least three of the proteins involved in cytosolic IMP biosynthesis in avian liver are multifunctional (Zalkin & Dixon, 1992; Zalkin, 1993; Firestone & Davisson, 1994). Based on kinetic data and theoretical considerations, there is definitely channeling of certain intermediates of the pathway (Rudolph & Stabbe, 1995). There is thus every reason to believe some sort of complex for IMP synthesis exists in the cytosol of uricotelic liver. If so, the complex might also be predicted to be associated with mitochondria, the source of glutamine. A hypothetical organization of the IMP pathway is shown in Figure 2. IMP formed by this pathway is then converted

[1]Ammonia-dependent phosphorylamine synthesis has been reported in avian liver (Reem, 1968; Rowe et al., 1978) but this may be non-enzymatic (Buchanan, 1973).

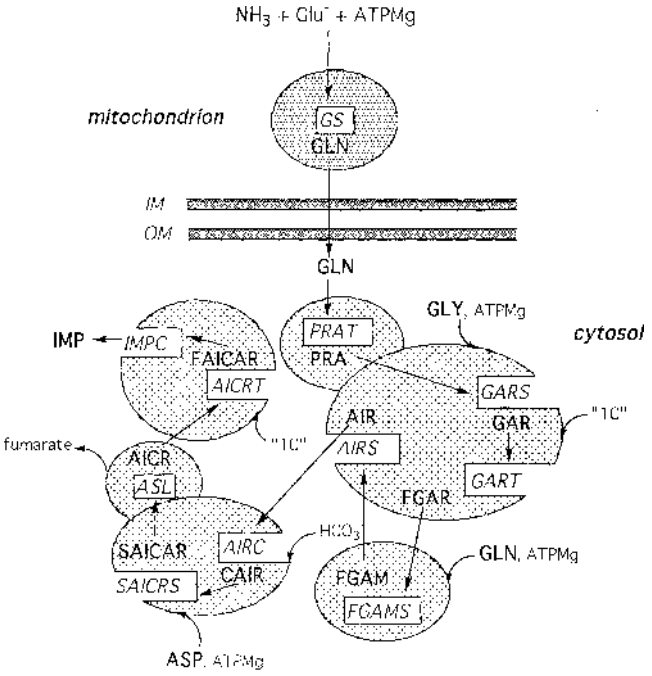

Fig. 2. Hypothetical organization of de novo pathway for IMP biosynthesis in uricoteles. GS, glutamine synthetase; PRPP, 5-phosphoribosyl-1-pyrophosphate; PRAT, glutamine-5-phosphoribosyl-1-pyrophosphate amidotransferase; PRA, 5´-phosphoribosylamine; GAR, 5´-phosphoribosylglycinamide; GARS, GAR synthetase; GART, GAR transformylase; FGAR, 5´-phosphoribosyl N-formylglycinamide; FGAM, phosphoribosyl N-formylamidine; FGAMS, FGAM synthetase; AIR, 5-phosphoribosylaminoimidazole; AIRS, AIR synthetase; AIRC, AIR carboxylase; CAIR, 5´-phosphoribosyl-5-aminoimidazole carboxylate; SAICAR, 5´-phosphoribosyl 4-(N-succinocarboxamide)-5-aminoimidazole; SAICARS, SAICAR synthetase; ASL, adenylosuccinate lyase; AICAR, 5´-phosphoribosyl 4-carboxamide-5-aminoimidazole; AICRT, AICAR transformylase; FAICAR, 5´-phosphoribosyl 4-carboxamide-5-foramidoimidazole; IMPC, IMP cyclohydrase. One carbon units ("1C") are as 5, 10-methenyltetrahydrofolate.

via the sequential actions of 5´-nucleotidase, purine nucleoside phosphorylase, and xanthine dehydrogenase to urate for excretion. The latter enzyme is also "multifunctional" in a sense because it utilizes both hypoxanthine and xanthine as substrates during urate formation.

SIMILARITIES BETWEEN THE UREOTELIC AND URICOTELIC MECHANISMS

With the localization of glutamine synthetase within the matrix of uricotelic liver mitochondria, it was possible to compare the basic properties of the uricotelic and ureotelic mitochondrial ammonia detoxication systems. Some of the properties shared by the two systems are: the primary ammonia-fixing enzymes, glutamine synthetase and CPS-I, are in the matrix of liver mitochondria. Both utilize NH_3 as a substrate for which they show high affinities and both are inhibited by Ca^{2+} (Vorhaben et al., 1982). The efflux of glutamine from uricotelic liver mitochondria is via a non-energy-dependent process like that of citrulline from uricotelic liver mitochondria (Gamble & Lehninger, 1973; Campbell & Vorhaben, 1976). CPS-I is not expressed in avian hepatocytes whereas glutamine synthetase is. This mutually exclusive expression pattern is also seen in the perivenular hepatocytes in mammalian liver. These hepatocytes, to which glutamine synthetase expression is restricted (Gebhardt & Mecke, 1983), do not express CPS-I (Gaasbeek-Janzen et al., 1984). However, this mutually exclusive expression of one of the other enzymes is not seen in other vertebrate hepatocytes (Smith & Campbell, 1988). Both enzymes are encoded in the nucleus, translated in the cytosol, and imported into the mitochondrial matrix (Raymond & Shore, 1979; Campbell & Smith, 1992). High dietary protein intake causes an increased transcription of both hepatic glutamine synthetase (see Campbell, 1995) and CPS-I (Morris et al., 1987) genes.

Perhaps the most physiologically significant similarity between the two detoxication systems is the form in which the ammonia present in the matrix exits to the cytosol for conversion to the excreted end-products. In uricoteles, this is as the amide function of glutamine and in ureoteles, as the carbamyl function of citrulline. As shown in Table 1, the pK_as for glutamine and citrulline are very similar. Glutamine and citrulline show no protonation of their R chains over a wide range of pHs and titrate like glycine which has no R chain (see titration curves in Campbell, 1991). This is unlike amino acids such as histidine, which show a third pK_a due to protonation of the imidazole function. *The R chains of glutamine and citrulline are therefore proton-neutral*. The chemistries of the glutamine amide and the citrulline carbamyl functionalities are most likely similar to that of urea, which shows an extremely acid pK_a

TABLE 1. Dissociation constants and isoelectric points compared[1]

	pK_a^1 (COOH)	pK_a^2 (NH_2)	pK_a^3 (R)	pI
L-Citrulline	2.43	9.71	—	5.92
L-Glutamine	2.17	9.13	—	5.65
Glycine	2.43	9.60	—	5.97
L-Histidine	1.78	8.97	5.97	7.47

[1]From Greestein & Winitz (1961).

of 0.18. The latter may reflect protonation of the keto oxygen rather than the amino nitrogen (Kennedy, 1976). The proton-neutrality of the R chains of glutamine and citrulline results in similar isoelectric points for the two compounds so they are difficult to separate by routine electrophoresis. The physiological consequence of the similar chemistries of citrulline and glutamine is that NH_3 is the mitochondrial matrix, whether arising by deamination of glutamate via GDH, the direct entry of NH_3 or NH_4^+, or, in mammals, the hydrolysis of glutamine by phosphate-dependent glutaminase, exits to the cytosol as a proton-neutral function for eventual conversion to either urate or urea for excretion.

That conversion to a neutral species is the primary mechanism for ammonia detoxication is supported by the development and regulation of CPS-I and OTC in the small intestine. These enzymes are coded for by the same genes as the liver enzymes and their concentrations in intestinal mucosa mitochondria are much the same as those in liver mitochondria (Ryall et al., 1985). However, unlike the liver enzymes, the intestinal enzymes do not respond to increases in dietary protein (Wraight et al., 1985) nor to gluconeogenic hormones (Ryall et al., 1986). In contrast to their effects in liver, these conditions do not necessarily increase mucosal ammonia production. This suggests intestinal CPS-I and OTC function primarily to detoxify ammonia formed ureolytically in the gut by converting it to citrulline. The latter can then be converted to arginine by kidney and other non-hepatic tissues for nutritional uses. The citrulline-forming system is the primary ammonia detoxifying system in interstinal mucosa since glutamine synthetase is absent, at least from that part of the tissue in which CPS-I and OTC are expressed (Moorman et al., 1988). Liver mRNAs for CPS-I and OTC in the rat do not appear until after 15 to 17 days gestation; they then show some increase just prior to birth and continue to rise post partum to the adult levels. In contrast, intestinal mRNAs for CPS-I and OTC increase rapidly prenatally to a peak at 21 days gestation and then decline over a 7-day period post partum (Ryall et al., 1986). A ureolytic microbial flora develops a few hours after birth in mammals (Visek, 1970), so the high perinatal levels of intestinal CPS-I and OTC may serve to prevent the toxic effects of ammonia on the intestine itself (Visek, 1970) as well as to divert ammonia from the neonatal liver prior to the latter's development of a full capacity for detoxification.

NH_3 AS AN UNCOUPLER OF PHOSPHORYLATION

According to the chemiosmotic model for electron transport-dependent ATP synthesis, protons are dumped out of the mitochondrial matrix during electron transport across the relatively proton-impermeable inner mitochondrial membrane. This results in a proton (pH) gradient across the membrane such that the matrix is relatively alkaline and the intermembrane space is relatively acid. Due to charge separation, an electrical gradient is also established. The electrochemical potential generated by these gradients is the proton-motive force and represents a conservation of the energy of oxidation. For the most part, protons can re-enter the matrix only through proton-specific channels of the ATP synthase complex. There are, however, leaks and slips in the inner membrane that allow for proton entry without energy conservation. This results in heat production and the extent of these leaks and slips may account for species differences in basal metabolic rates (Brown, 1992). During the re-entry of protons through the ATP synthase complex, the energy provided by the proton-motive force is utilized for ATP synthesis; this is the process of oxidative phosphorylation (Mitchell, 1979). This is depicted on the left side of Figure 3. In plants, protons are pumped *into* the thylakoid lumen of the chloroplast during light-driven electon transport and must then exit via the chloroplastal ATP synthase. Both the proton and electrical gradients are thus reversed with respect to mitochondria with the *outside* of the thylakoid being relative alkaline and the *inside* relatively acid (Malmström, 1989). During exit through the ATP synthase complex, the proton-motive force then provides the energy for ATP synthesis; this is the process of photosynthetic phosphorylation.

Ammonia was one of the first uncouplers of phosphorylation to be used experimentally and this was in the chloroplastal photosynthetic phosphorylation system by Krogmann et al. (1959). The proposed mechanism for uncoupling of this system by ammonia was its penetration of the thylakoid membrane as NH_3 and binding of the protons pumped into the lumen during photo-dependent electron transport. This prevented their efflux through the proton-specific channels of the ATP synthase complex (Crofts, 1967; McCarty, 1969; Jagendorf, 1975). The binding of H^+ by NH_3 to form NH_4^+ also effectively dissipates the H^+ gradient across the thylakoid membrane, which is necessary for development of the proton-motive force.

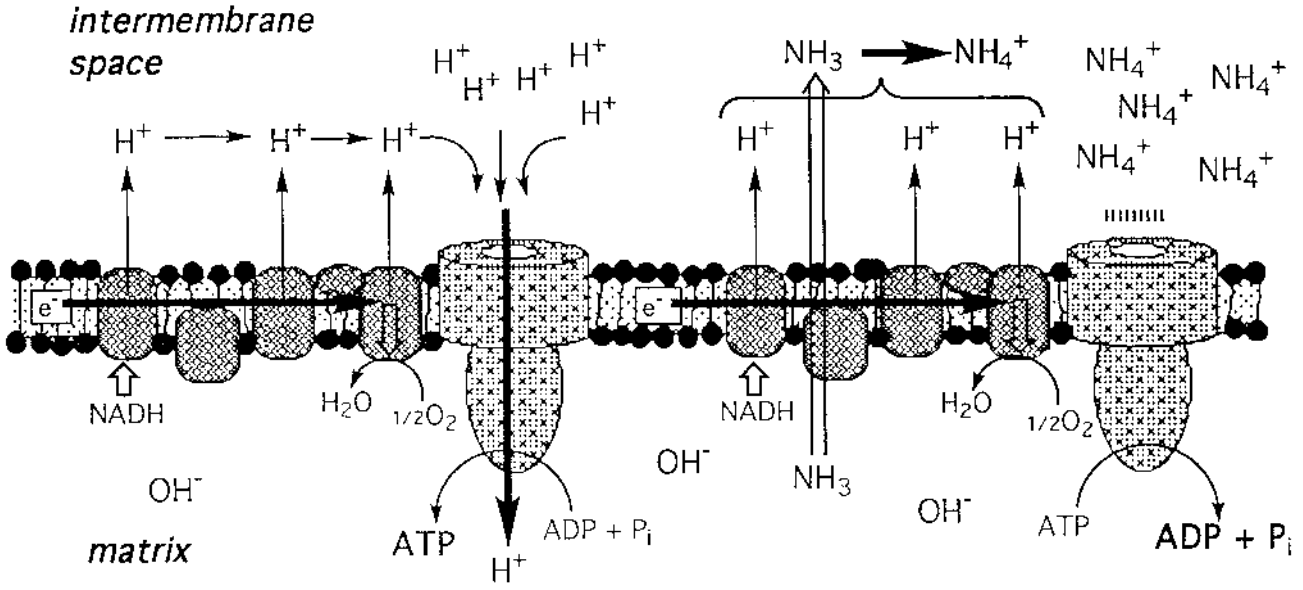

Fig. 3. Normal proton flux across the inner mitochondrial membrane during coupling of oxidative phosphorylation (left) and the uncoupling of this process by NH_3 (right).

This mechanism of uncoupling by NH_3 is depicted in Figure 3 for the inner mitochondrial membrane. All of the commonly used uncouplers of phosphorylation such as dinitrophenol (DNP), carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), nigericin, valinomycin, etc., effectively dissipate H^+ gradients across mitochondrial and chloroplastal membranes by causing the translocation of H^+ into the matrix or out of the thylakoid lumen. As shown in Figure 4, some uncouplers act as proton carriers ($PC\text{-}H^+$) and are termed protonophores. For example, drugs of the tacrine family, which are weak bases developed for the treatment of Alzheimer's disease, are protonophoric and their uncoupling of liver mitochondria can result in hepatic malfunction (Berson et al., 1996). As discussed by Selwyn (1987), uncoupling can also be caused by leakage of OH^- through anion channels in the inner mitochondrial membrane: these include the phosphate-hydroxide antiporter and other specific

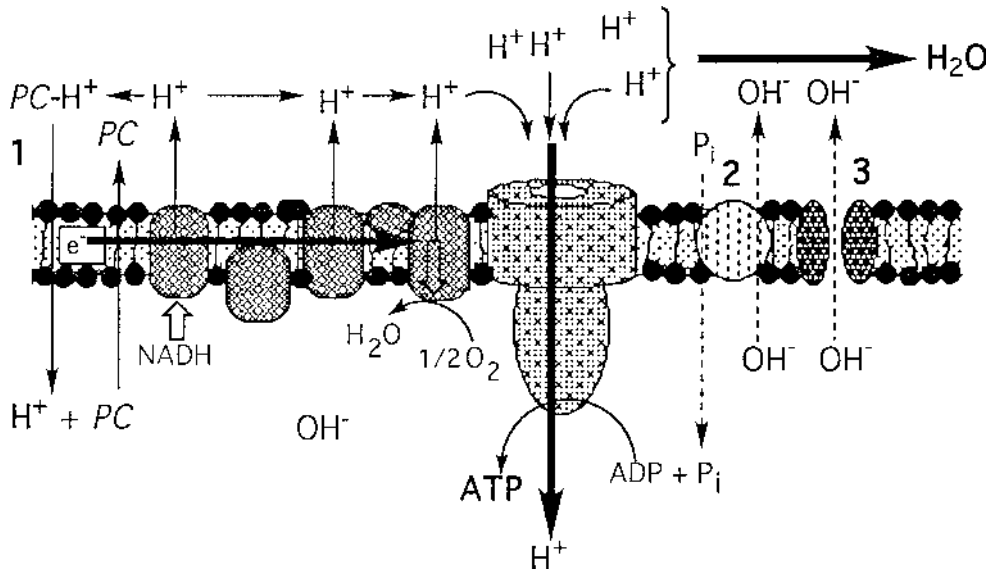

Fig. 4. Other mechanisms for uncoupling of oxidative phosphorylation. 1, inward translocation of H^+ by a proton carrier ($PC\text{-}H^+$); 2, OH^- efflux via phosphate-hydroxide antiporter; e, OH^- efflux via anion channel.

anion-conducting channels, all of which belong to the same family of transporters (Pederson, 1993). Brown fat uncoupling protein, thermogenin, acts to form anion channels through which OH^- can exit to uncouple phosphorylation, resulting in the dissipation of the proton-motive force as heat. As can be seen in Figures 3 and 4, the mechanisms of uncoupling by OH^- and NH_3 are basically the same.

With respect to the proposed mechanism for the uncoupling of electron transport-dependent phosphorylation by NH_3, Brierly & Stoner (1970) were the first to offer experimental support for it in mitochondria. This is shown in Figure 5. When the ionophore valinomycin is added to beef heart mitochondria respiring in NH_4Cl (Cl^- is not shown since the inner membrane is thought to be impermeable to it under these conditions), there is an immediate uncoupling as evidenced by depletion of the medium O_2. The postulated mechanism for this is that in the presence of valinomycin, the inner membrane (IM), which is normally impermeable to cations, becomes permeable to NH_4^+, allowing it to penetrate into the matrix (Fig. 5, Step 1). NH_3 is formed in the matrix due to the alkalinity of this compartment (Fig. 5, Step 2). NH_3 then exits to bind protons pumped out during electron transport (Fig. 5, Step 3), thereby uncoupling phosphorylation (Fig. 5, Step 4), which is indicated by a marked increase in O_2 uptake (Fig. 5, Step 5; see also graphic presentations in Brierly & Stoner, 1970; Campbell, 1991). An alternate explanation for the uncoupling by NH_4^+ is that it is simply due to the inward transport of H^+ as described above for protonophores. However, the transient alkalization of the medium following the addition of valinomycin found by Brierly and Stoner and the fact there was no osmotic swelling due to the accumulation of NH_4^+ in the matrix suggest the inward transport of protons is not a valid explanation for the uncoupling process. An explanation similar to that proposed for uncoupling of photosynthetic phosphorylation in chloroplasts by NH_3 has been used for its uncoupling of oxidative phosphorylation in inside-out mitochondrial vesicles. Like thylakoids, protons are pumped into the lumen of these vesicles so the H^+ gradient is the same in the two systems. Azzone and co-workers (Azzone et al., 1976) found NH_4Cl to be a relatively poor uncoupler of oxidative phosphorylation by inside-out beef heart mitochondria at pH 7 in comparison with some lipophilic amines. However, the presence of valinomycin markedly increased uncoupling under these conditions. Their interpretation was that NH_3 is the penetrating species and uncouples by reacting with protons pumped into the lumen of the inside-out vesicles to form NH_4^+. At pH 7, considerably less than 1% of the ammonia is present as NH_3 so a

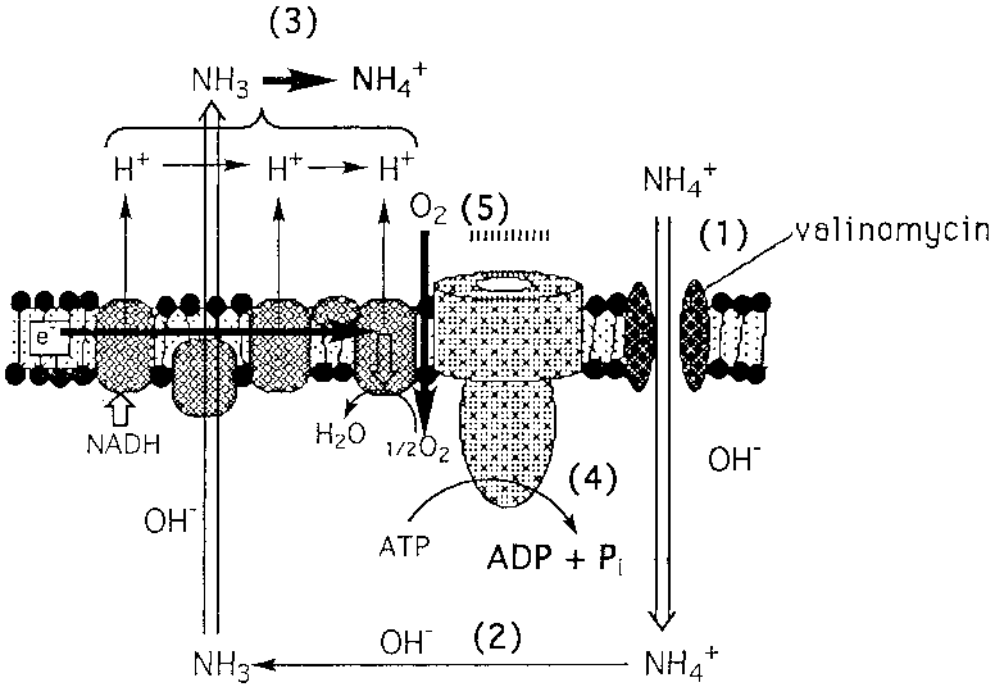

Fig. 5. Uncoupling of beef heart mitochondria respiring in ammonium chloride. See text for discussion.

low medium concentration of NH_4Cl might be expected to result in poor uncoupling. However, the addition of valinomycin would allow for the rapid efflux of NH_4^+, thereby increasing the rate of recycling NH_3 into the vesicles and, consequently, increasing the effectiveness of uncoupling.

PROTON-NEUTRAL THEORY

Based on the above experimental as well as theoretical considerations, we (Vorhaben & Campbell, 1972) proposed the *proton-neutral theory of hepatic ammonia detoxication*. Fundamentally, this is that the detoxication of ammonia within the matrix of liver mitochondria of tetrapod vertebrates involves its conversion to a proton-neutral function—either the amide of glutamine or the carbamyl of citrulline—for efflux to the cytosol. As indicated in Figure 6, during efflux of these compounds, there is no interference with the proton gradient across the inner membrane and, therefore, no uncoupling of oxidative phosphorylation.

AMMONOTELES: THE QUESTION OF AMMONIA EFFLUX FROM HEPATIC MITOCHONDRIA

Many ammonoteles, especially fresh-water teleosts, are carnivorous and sustain high rates of hepatic amino acid gluconeogenesis (Cowey et al., 1977; Bever et al., 1981). Since neither the ureotelic nor the uricotelic ammonia detoxication system is present in ammonotelic liver mitochondria, the resulting high rate of mitochondrial amino acid deamination should result in a high rate of efflux of ammonia across the inner mitochondrial membrane. Were this as NH_3, it would contradict the idea outlined above that such flux must be prevented to avoid uncoupling of oxidative phosphorylation. In terms of the mechanism for ammonia excretion by fresh-water ammonoteles such as teleosts, current dogma is that the ammonia formed in liver as well as other tissues is cleared from the blood at the gill (Heisler, 1990). Although a Na^+-H^+/NH_4^+ antiporter and the Na^+-K^+ ATPase on which NH_4^+ will substitute for K^+ may contrib-

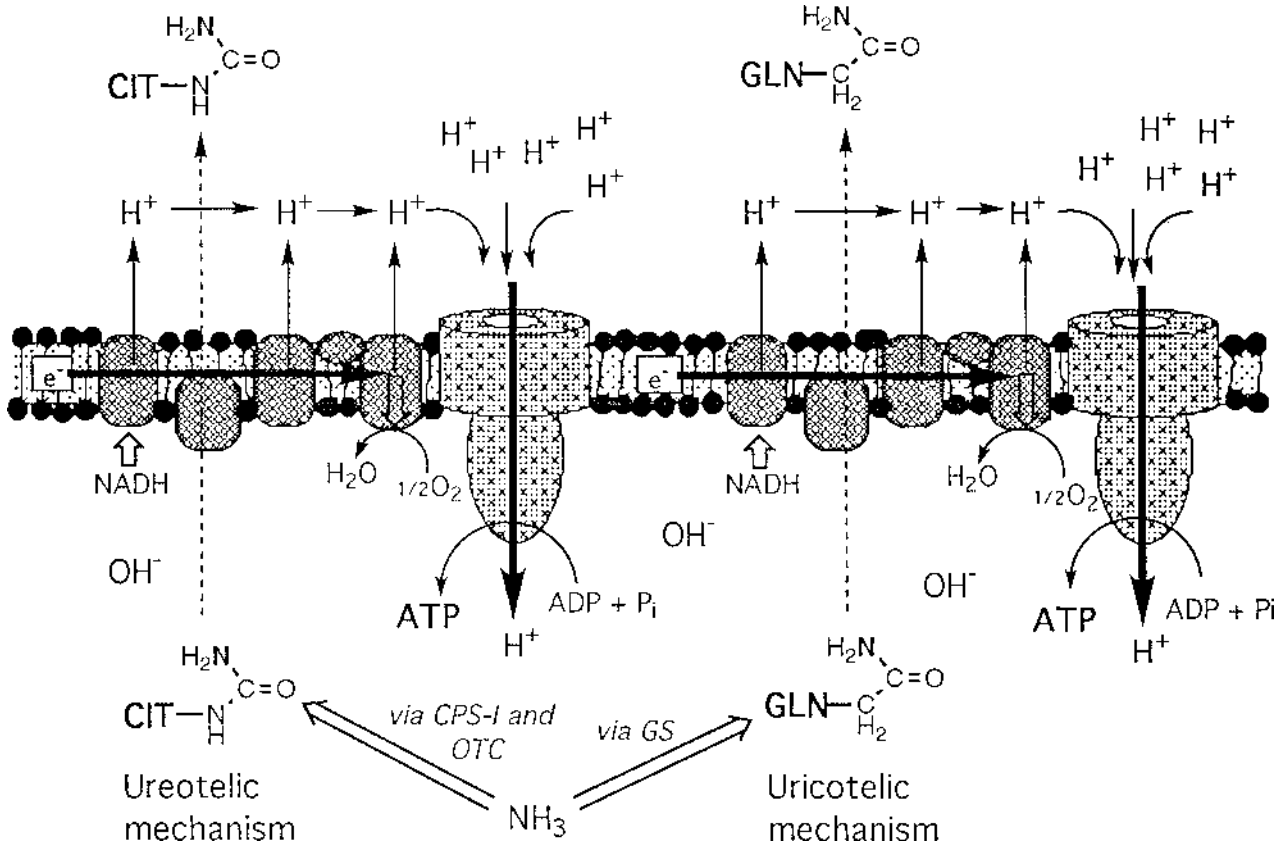

Fig. 6. Proton-neutral theory for hepatic ammonia detoxication in tetrapod vertebrates. Ammonia generated within the mitochondria matrix is converted to either the amide function of glutamine or the carbamyl function of citrulline for exit to the cytosol in order to avoid dissipation of the proton gradient across the inner mitochondrial membrane.

ute to the translocation of ammonia at the gill, most of it is cleared as NH_3. H^+ generated via carbonic anhydrase at or in the external surface of the gill membrane may increase the rate of NH_3 clearance via a diffusion-trapping mechanism. This overall mechanism implies that ammonia formed by amino acid deamination or deamidation in the cytosolic and/or mitochondrial compartments of liver simply diffuses down a NH_3 gradient across the boundary membranes of that compartment. Although, as pointed out by Goldstein et al. (1982), NH_3 was early on shown to be more hydrophilic than lipophilic by its very low chloroform/water partition ratio, it, nevertheless, is the species generally thought to traverse membranes via lipid-phase diffusion (see e.g., Flessner et al., 1991). The cation, NH_4^+, is generally considered to be the species involved in aqueous-phase diffusion. There is thus the fundamental issue of which species of ammonia is permeable to ammonotelic hepatic liver mitochondria.

The study by Campbell et al. (1983) appears to be one of the few in which the issue of the form of the ammonia exiting hepatic mitochondria of fresh-water ammonoteles has been addressed. The mechanisms for ammonia release tested in this system were those proposed by LaNoue & Schoolwerth (1979) for mammalian kidney mitochondria and are shown in Figure 7. The mechanism designated *A* in Figure 7 is a simple diffusion-trapping mechanism in which NH_3 is the penetrant and is trapped on the outside of the inner mitochondrial membrane by bulk phase protons without regard to their source. According to this mechanism, any increase in medium $[H^+]$ should cause an increase in rate of ammonia efflux. However, when the pH of the medium in which catfish liver mitochondria respiring in the presence of substrate and ADP was varied between 7.4 and 6.7, there was a *decrease* in ammonia efflux with an *increase* in the $[H^+]$ of the medium. Glutamine was the substrate used in these specific experiments so the greater release of ammonia from mitochondria at the more alkaline pHs probably reflects a higher rate of its formation due to glutaminase activation and not necessarily an effect on its translocation. The addition of glutamine itself resulted in a slight acidification of the medium by the mitochondria. Based on the experimental findings of Brierly & Stoner (1970) discussed above, if the ammonia formed via deamidation of the added glutamine were exiting as NH_3, an alkalinization would be expected. NH_4^+, on the other hand, should result in the slight acidification seen due to its dissociation to NH_3 plus H^+. One source of protons for diffusion-trapping of exiting NH_3 proposed for kidney mitochondria is the electron transport system, which functions as an electrogenic proton pump. This is indicated as a simple pump (*B*) in Figure 7. Theoretically, abolishing the proton gradient formed by this pump should therefore inhibit ammonia efflux. However, the uncoupler FCCP was found to cause a marked increase in the release of ammonia formed from glutamate in the catfish liver mitochondrial system. Since FCCP is a protonophore and causes a dissipation of the H^+ gradient across the inner mitochondrial membrane, this indicates the gradient has little or no effect on ammonia efflux. In fact, there was little or no change in ammonia efflux under conditions where H^+ is made to flow inward through the ATP synthase complex by the addition of ADP to mitochondria respiring in the presence of either glutamate or glutamine (state 4 to state 3 transition). Rotenone, which directly inhibits the electrogenic H^+ pump, also had little effect on ammonia efflux from these mitochondria. Another source of protons for diffusion-trapping of NH_3 in the kidney is the glutamate-H^+ co-transporter (*C* in Fig. 7). This model predicts a stoichiometric appearance of NH_4^+ and glutamate in the medium. With glutamine as substrate, ammonia efflux in the catfish system initially exceeded glutamate efflux although, after 30-min incubation, effluxes of the two were about equal. While the co-transport of glutamate and H^+ seems an appropriate mechanism for mammalian kidney mitochondria where NH_4^+ formation from glutamine is presumably a means of excreting H^+ (Flessner et al., 1991), such a mechanism would seem inappropriate for fish liver where the deamination of

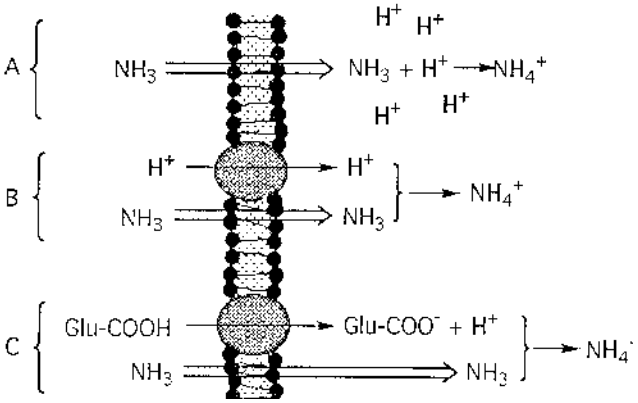

Fig. 7. Mechanisms for ammonia efflux from kidney mitochondria (LaNoue and Schoolwerth, 1979). See text for discussion.

glutamate formed via transamination is a major pathway for amino acid catabolism (Campbell et al., 1983). Therefore, the available experimental data as well as certain theoretical considerations suggest that ammonia formed during amino acid gluconeogenesis in catfish liver exits mitochondria as NH_4^+, and not as NH_3. Whether there is co-translocation of NH_3 and H^+ as shown in *B* of Figure 7 or whether NH_4^+ itself is translocated is, however, not known.

NH_4^+ TRANSPORT SYSTEMS

There is now considerable evidence for NH_4^+ translocation by several membrane systems. Some of these, such as those of cells of the ascending loop of Henle in the medulla of the mammalian kidney (Kikeri et al., 1989) are, in fact, impermeable to NH_3. Indeed, the mammalian kidney tubule and collecting duct have several different mechanisms for the translocation of NH_4^+. For example, NH_4^+ will substitute for K^+ on the ouabain-sensitive Na^+, K^+-ATPase (Kurtz & Balaban, 1986; Wall, 1996) and on the furosemide (bimetanide)-sensitive Na^+, K^+, $2Cl^-$ co-transporter (Good, 1994). Recently, there has been a report of a specific verapamil-sensitive K^+-NH_4^+ antiporter (Amlal & Soleimani, 1996). These and other NH_4^+ transport systems have also been reported in tissues other than kidney. For example, transport of NH_4^+ takes place on the Na^+, K^+-ATPase of crab gill (Towle & Holleland, 1987) and insect rectal cells have an amiloride-sensitive system for the antiport of Na^+ and NH_4^+ (Thomson et al., 1988). For the most part, these transport systems are involved in the "excretion" of NH_4^+ and/or H^+.

Transport systems for NH_4^+ uptake are quite common in prokaryotes and lower eukaryotes (Kleiner, 1992). Assuming a symbiotic origin of mitochondria, it would therefore not be unusual to find them in these organelles. That ammonia is permeable to mitochondria is evident by the fact it stimulates glutamate (Katunuma & Okada, 1965) and carbamyl phosphate (Charles et al., 1967) synthesis by intact mitochondria and activates glutaminase (McGivan et al., 1984). Although NH_4Cl has routinely been used as a substrate for citrulline synthesis in vitro by intact ureotelic liver mitochondria and for glutamine synthesis by uricotelic mitochondria (Campbell & Vorhaben, 1976; Campbell et al., 1985), there appears to be no consensus on the form in which the ammonia penetrates. Some think the mitochondrial membrane is generally impermeable to cations including NH_4^+ so that NH_3 is the penetrating species (Kleiner, 1981). On the other hand, Gutiérrez et al. (1987) claim to have demonstrated an NH_4^+ transporter in rat liver mitochondria, which shows a decreasing affinity for Na^+, Li^+, K^+ and other monovalent cations and conclude that the inner mitochondrial membrane is impermeable to NH_3. They used mitochondrial swelling in the presence of acetate as their experimental approach. As pointed out by LaNoue & Schoolwerth (1979), there are certain limitations to this system. One is that it is not always possible to identify what is actually causing the osmotic swelling. For example, when the pH of the ammonium acetate medium was varied by Gutiérrez et al. (1987), the rate of swelling decreased with increasing alkalinity although essentially the same total degree of swelling was eventually obtained at equilibrium. While $[NH_4^+]$ is decreasing under these conditions and $[NH_3]$ is increasing, $[CH_3COOH]$, the presumed penetrant is also decreasing. According to the LaNoue-Schoolwert model, there would therefore be a lower matrix $[H^+]$ available to trap NH_3. More NH_3 may then simply equilibrate across the membrane rather than accumulate in the matrix as NH_4^+ to cause osmotic swelling. Dissociation of the various salts used in these experiments might also be a factor in the concentration of the penetrating species of acetate. It should be added that if NH_4^+ is indeed the penetrating species in liver mitochondria, were the NH_3 formed from it by dissociation in the matrix allowed to recycle, this should result in uncoupling just as with FCCP and other transmembrane proton-carriers discussed above. Jungas et al. (1992) estimate the main source of ammonia for hepatic ureagenesis in man is cytosolic NH_4^+, either arriving in this compartment from the extrahepatic tissues or being formed there by deamination. They assume NH_4^+ is the penetrating species for liver mitochondria. Based on their estimates of the amount of ammonia being detoxified by conversion to urea in liver, the number of H^+ transported as NH_4^+ into the mitochondrial matrix is small relative to the number pumped out by the electrogenic pump. However, as discussed above, cycling of NH_3 under these conditions, were it allowed, would be catalytic, not stoichiometric, and could therefore result in significant uncoupling by equilibrating H^+ across the inner membrane. Normally, this is prevented by the conversion of NH_3 to either citrulline or glutamine for efflux in a proton-neutral form.

This may, in fact, be one of the most significant physiological consequences of the proton-neutral theory.

It nevertheless seems possible that a translocator may be present in ammonotelic liver mitochondria that transports NH_4^+ out of the matrix, thereby preventing dissipation of the proton gradient established by the electrogenic H^+ pump. Also, a H^+ transporter independent of the electron transport chain that acts simultaneously with the translocation of NH_3 cannot be ruled out. Both mechanisms would result in electrogenic H^+ efflux from the matrix, which would presumably increase ATP formation by the synthase complex.

ONTOGENETIC AND EVOLUTIONARY ASPECTS

The above considerations indicate that, in those species that undergo transitions between ammonotelism and either ureo- or uricotelism during their life histories, there may also be fundamental changes in the permeability properties of hepatic mitochondrial membranes to ammonia and/or in the proton flux across these membranes. Metamorphosis of aquatic, ammonotelic amphibian tadpoles into semi-terrestrial, ureotelic adults is generally thought to recapitulate the transition of vertebrates to the land. In addition to water availability, amphibian metamorphosis also represents a change from the low-protein diet of the herbivorous tadpole to the high-protein diet of the carnivorous adult. The transition in *Rana catesbeiana* includes an increased transcription of CPS-I and OTC (Tata, 1993; Atkinson, 1994) and, therefore, an increased ability to convert mitochondrially generated NH_3 to citrulline to sustain the predictably higher rate of amino acid gluconeogenesis in the adult. Because tadpoles are quite anabolic, as well as being herbivorous, they, unlike carnivorous ammonoteles, presumably have very low rates of hepatic amino acid gluconeogenesis so ammonia efflux from their liver mitochondria may not pose an uncoupling problem. Certain treefrogs of the genus *Phyllomedusa* and *Chiromantis* undergo a typical ammonotelic to ureotelic transition during metamorphosis but, as adults, can also undergo a ureotelic to "uricotelic" transition under conditions of water restriction (Shoemaker and McClanahan, 1982). However, unlike true uricoteles, glutamine synthetase is a cytosolic enzyme in liver of these frogs (Campbell et al., 1984). Presumably, ammonia formed via transdeamination must therefore exit their hepatic mitochondria to be detoxified by conversion to glutamine in the cytosol. Like typical ureoteles, their liver mitochondria contain high levels of glutaminase so there must be considerable compartmentation of the glutamine formed in the cytosol to prevent its uptake by mitochondria. This again argues for an association of the enzymes of the urate pathway in the cytosol and, in this case, also with glutamine synthetase. In any event, NH_3 is the substrate for glutamine synthetase (Tate & Meister, 1973) and its withdrawal from the NH_4^+ dissociation reaction in the cytosol, if NH_4^+ is indeed the exiting species, would effectively result in an electrogenic efflux of H^+ from mitochondria. The treefrogs apparently represent the first evolutionary experiment with uricoteley, which may have been followed by the translocation of glutamine synthetase into liver mitochondria, resulting in a dual system for hepatic ammonia detoxication as predicted to be present in the cotylosurian descendents of the amphibians (Campbell et al., 1985; 1987).

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