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Elasmobranch glutamine synthetase: Synthesis and subcellular localization of tissue-specific isozymes

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Rice University, 1988
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ABSTRACT

Elasmobranchs (sharks, skates and ray) synthesize and retain high amounts of urea for the purpose of osmoregulation. Ureosmotics utilize a pathway for the production of urea that incorporates enzymes used in the formation of urea and uric acid in higher vertebrates. Ammonia is produced, and the first steps in its detoxification and processing for excretion occur, in the mitochondria of the liver of these animals, as it does in ureoteles and uricoteles. The enzymes involved in these steps are therefore required to be localized in the mitochondria. Most mitochondrial enzymes are encoded by nuclear genes, synthesized in the cytoplasm, and imported into the organelle. In some instances, mitochondrial enzymes have a cytosolic counterpart. The majority of compartmental isozymes are generally not related at either the structural or genetic level.

The objectives of this work were to investigate the tissue-specific expression and localization of glutamine synthetase, the enzyme that detoxifies intramitochondrially-generated ammonia in elasmobranchs. It was determined that glutamine synthetase was mitochondrial in the liver, but cytosolic in the brain of elasmobranchs and a holocephalan. These compartmental isozymes were shown to have different subunit sizes. This work described the first report of glutamine synthetase isozymes in vertebrates. The two compartmental isozymes have the same isoelectric points and antibody specificity, suggesting a similar molecular derivation. Mitochondrial glutamine synthetase was shown to be translated with a leader sequence that is removed during import, while the cytosolic form is translated as the mature size. In the stingray and shark, it was found that a single dominant RNA species gives rise to the tissue-specific isozymes in each animal. Preliminary work suggests the isozymes are encoded by a single gene.
ACKNOWLEDGEMENTS

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DEDICATION

This thesis is dedicated to my husband, Bill Ward and to my mentor, Dr. John Ennever.
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INTRODUCTION

Environmental water stress has been a strong selective force in the development by animals of many types of physiological and biochemical adaptations to conserve or conform to available water (Smith, '61; Bently, '71; Campbell, '73; Bligh, et al., '76; Smellie and Pennock, '76; Schmidt-Neilson, '79; Wilson, '79; Young, '81; Yancey, et al., '82; Eckert and Randall, '83; Campbell, et al., '87). Metabolic factors that are affected by water availability include regulation of internal water, the balance of salts and nutrients in tissues, and removal of toxic waste (Schmidt-Neilson and Mackey, '72; Yancey, et al., '82; Eckert and Randall, '83). Establishment of a stable internal environment was one of the most significant advances in the evolution of organisms, and was a principle prerequisite for the evolution of terrestrial vertebrates (Smith, '61).

Homeostasis of body fluids is vital because of the narrow range of osmotic conditions permissive to cellular metabolism (Schmidt-Neilson and Mackey, '72; Wilson, '79). In order to inhabit environments other than the primitive seas, animals have devised osmoregulatory systems that continually maintain their internal osmotic balance (Schmidt-Neilson and Mackey, '72; Yancey, et al., '82; Eckert and Randall, '83). In addition, they have acquired mechanisms for the detoxification of ammonia through its conversion to compounds that require minimal water for excretion (Campbell, '73; Cohen, '76; Campbell, et al., '87). The combination of these two systems allows animals to cope with a wide variety of environmental changes.

According to Smith ('61), the evolution of vertebrates is due entirely to the development of osmoregulatory mechanisms. In terrestrial vertebrates, the kidney is the principle site of osmotic regulation; it maintains the balance of salts, water and nutrients through mechanisms of filtration, secretion and resorption (Smith, '61; Schmidt-Neilson and Kerr, '70; Wilson, '79; Eckert and Randall, '83). In aquatic vertebrates, this function is shared with the gills,
integument and salt glands. Depending on the salinity of the water, aquatic organisms may be relatively hyper- or hypoosmotic to the environment. To maintain osmotic equilibrium, freshwater fish actively absorb salts via the gills and skin, and produce a hypotonic urine. Animals that live in or depend on saltwater, on the other hand, have excess salt to eliminate. Marine mammals excrete this via the kidneys, while marine-dependent terrestrials also utilize salt glands. Marine teleosts excrete monovalent ions at the gills and divalent ions in the kidney (Bligh, et al., '76; Eckert and Randall, '83).

Osmotic balance may also be achieved through the use of osmotically active solutes. With the exception of the cyclostome Myxine, which retains free amino acids, most aquatic vertebrates in hypertonic environments that utilize organic osmolytes retain high concentrations of urea and methylamines (Yancey, et al., '82). Although these animals have a balanced osmotic gradient, the concentrations of salts in their tissues are not equal to that of seawater. Ureosmotic tissues have the same inorganic composition as mammals (Bedford, '83). Excess electrolytes are eliminated via kidneys and salt glands (rectal glands) (Malo, '79; Eckert and Randall, '83).

Elasmobranchs (sharks, skates and rays) are probably the best known ureosmatics, although the coelacanth Latimeria (Lutz and Robertson, '71) and the crab-eating frog Rana cancrivora (Gordon, et al., '61) also synthesize and retain urea for osmoregulation. Marine elasmobranchs may retain as much as 0.4 M urea in their tissues to offset the osmotic strength of seawater (Smith, '36). Freshwater elasmobranchs, which face a different type of environment, do not retain high concentrations of osmolytes (Thorson, et al., '67). The decrease in urea concentration during transfer of euryhaline elasmobranchs to dilute seawater presumably mimics the evolutionary invasion of fresh water by elasmobranchs (Goldstein, et al., '68; Goldstein and Forster, '71).

Urea is a bond destabilizer, and to counteract this disruptive effect on cell proteins, elasmobranchs also retain high amounts (up to 80 mM) of
trimethylamine oxide (TMAO) (Yancey and Somero, '79; '80; Yancey, et al., '82; Hochachka and Somero, '84). Together, urea and TMAO can raise tissue osmolarity to 1000 mOsm (Bedford, '83). Urea and TMAO are filtered in the kidney, but 95% is resorbed in the renal tubules (Forster, '67).

There is a close link between osmoregulation and nitrogen excretion (Campbell, '73; Eckert and Randall, '83). Animals normally take in excess amino acids, and the carbon-skeleton is either converted to glucose or oxidized directly. The first step in the utilization of amino acids is removal of the alpha-amino moiety as ammonia. Since ammonia is toxic to the cell (Hindfelt, '75), it must be detoxified or eliminated. Animals can excrete nitrogen in three forms—as ammonia (NH₃), urea (CN₂H₄O) or uric acid (C₅N₄H₄O₃). An important excretory difference among these compounds is the amount of water necessary for their elimination. To excrete 1 gram of nitrogen as uric acid requires 10 ml of water, as urea, 50 ml, and as ammonia, 500 ml (Eckert and Randall, '83). This leads to a potential dilemma for some organisms—to conserve water or to detoxify ammonia.

From their emergence, it appears that vertebrates had the ability to produce urea (Brown and Brown, '85; Campbell, '87). The enzymes necessary for urea synthesis are seen in representatives of the earliest vertebrates, the elasmobranchs (Baldwin, '60; Watts and Watts, '74) and holocephalans (Read, '67), and are also present in teleosts. The ability to synthesize urea persisted through primitive vertebrates to the stem reptiles (cotylosaurians) that gave rise to the higher tetrapod vertebrates. During evolution of the tetrapods, the mammalian-like reptiles (theropsids) selected ureagenesis, while the line leading to the birds and reptiles (sauropsids) developed uricogenesis (Campbell, et al., '85; '87). Squamate reptiles, birds and crocodiles have the enzymes necessary to produce uric acid (Campbell, et al., '87), which gives them a significant advantage in a water-stressed habitus. Marine vertebrates exhibit two important adaptations with regard to
nitrogen metabolism and urea. The use of urea as an osmolyte is advantageous metabolically since this is an end-product of amino acid catabolism (Campbell, '73). Also, urea can be produced as a detoxicant when there are restrictions on ammonia elimination (Goldstein, '72).

Whether the final product is urea or uric acid, ammonia destined for excretion is detoxified in liver (Campbell, '72; Freiden, '76; Lehninger, '82). There, the first steps leading to end-product synthesis occur in the mitochondria. The formation of urea in higher vertebrates is shown in Figure 1 and the formation of uric acid, in Figure 2. In both cases, the glutamate dehydrogenase reaction is the primary source of ammonia. In ureoteles, carbamylphosphate synthetase-I is the primary detoxifying enzyme, whereas in uricoteles, it is glutamine synthetase.

While it has been known for many years that ureosmotics synthesize urea (Smith, '36; Baldwin, '60; Schooler, et al., '66; Goldstein, '67; Huggins, et al., '69; Campbell, '72), the biochemical steps involved in urea production in these animals have only recently become understood (Anderson, '80; Webb and Brown, '80; Anderson, '81; Casey and Anderson, '82; Anderson and Casey, '84; Ballantyne, et al., '86; Anderson, '86a; '86b). In hepatic mitochondria of elasmobranchs, ammonia generated by glutamate dehydrogenase is detoxified by glutamine synthetase. One nitrogen from glutamine is then assimilated with carbonate by the action of carbamylphosphate synthetase-III to yield carbamylphosphate. Ornithine transcarbamylase uses this to form citrulline, which is then used for urea synthesis following the ureotelic pathway.

As seen in Figure 3, the pathway used by elasmobranchs has features of both the mammalian and avian nitrogen processing systems. The key enzyme responsible for the "merged" systems is carbamylphosphate synthetase-III. First described in invertebrates by Trammel and Campbell ('70), it utilizes glutamine as a substrate (like carbamylphosphate
FIGURE 1: Illustration of urea synthesis in ureotelic vertebrates.
FIGURE 2: Illustration of uric acid synthesis in uricotelic vertebrates.
FIGURE 3: Illustration of urea synthesis in ureosmotic vertebrates.
synthetase-II) but requires N-acetyl-glutamate as a co-factor (like carbamylphosphate synthetase-I). Anderson found this enzyme in largemouth bass ('76), then later in dogfish ('80). Subsequent studies in Anderson's laboratory localized carbamylphosphate synthetase-III, along with glutamine synthetase and ornithine transcarbamylase, in the matrix of hepatic mitochondria in dogfish (Casey and Anderson, '82, Casey and Anderson, '85). So, while the initial detoxification of intramitochondrially-generated ammonia is by glutamine synthetase, as in birds, crocodilians and reptiles, it is subsequently converted to urea, as in mammals. Elasmobranchs may thus occupy an intermediate position in the development of nitrogen excretion mechanisms that ultimately lead to the evolution of ureotelism and uricotelism.

A significant feature in the evolution of ammonia-processing mechanisms is the compartmentation of the enzymes involved in the different systems. Because glutamate dehydrogenase, the enzyme that liberates ammonia, is in the mitochondrial matrix it is necessary that the subsequent detoxification steps also be present in this compartment (Campbell, et al., '87). The mitochondrial genome codes for a very limited number of mitochondrial proteins (Schatz and Mason, '74; Schatz, '79; Neupert and Schatz, '81), so the majority (90%) of mitochondrial proteins are encoded by the nuclear genome. They are translated in the cytoplasm on free ribosomes for import into mitochondria (for reviews see Doonan, et al., '84; Reid, '85; Harmey and Neupert, '85; Douglas, et al., '86; Hurt and van Loon, '86).

Many of the proteins that are translocated across membranes are translated with an N-terminal leader sequence that signals the peptide's final destination (Wickner, '79; Kreil, '81; Sabatini, et al., '82). This leader sequence may be up to 10 kilodaltons (Hay, et al., '83). In liver of higher vertebrates, ornithine transcarbamylase and carbamylphosphate synthetase-I were the first enzymes that were shown to be translated in the cytoplasm with a leader sequence that is cleaved during mitochondrial import (Conboy, et al., '79;
Mori, et al., '79; Raymond and Shore, '79). However, not all proteins that are imported into mitochondria have a detectable leader sequence (Watanabe and Kubo, '82; Mori, et al., '85; Reid, '85). Glutamine synthetase, which is located in hepatic mitochondria in birds and alligators (Vorhaben and Campbell, '72; Smith and Campbell, '87), appears to be imported without the loss of a detectable leader sequence (Smith and Campbell, '83; '87). Presumably, these proteins have an internal signal region that aids in membrane translocation (Douglas, et al., '86; Hurt and Schatz, '87).

Smith and Campbell ('83) found glutamine synthetase to be cytosolic in avian neural tissue, although in the liver it is mitochondrial. Further, though localized in different compartments in these species, the two forms of glutamine synthetase have similar molecular weights, isoelectric points and peptide maps. In addition, both are immunologically cross-reactive. The mitochondrial isozyme is translated in the cytoplasm, then imported; however, the primary translation products of both peptides are the same size as mature form. Generally, compartmental isozymes are significantly different in one or more of these characteristics (Lehninger, '82). For example, phosphoenolpyruvate carboxykinase (Hod, et al., '82) and aspartate transaminase (Graf-Hausner, et al., '83) compartmental isozymes are encoded by separate genes; only the mitochondrial form contains the leader signal sequence required for translocation.

In order for a cytosolic protein to become localized in mitochondria, it would then appear to have to acquire a signal sequence during evolution (Campbell, et al., '87). This is seen with carbamylphosphate synthetase-I. In yeast, the enzyme is cytosolic (Urrestarazu, et al., '77) and its gene does not encode a signal sequence (Nyunoya and Lusty, '84). In vertebrates, the liver enzyme—which is mitochondrial—has acquired a signal sequence. Other urea cycle enzymes that are cytosolic do not have signal sequences (Bock, et al., '83; Lambert et al., '86; Dizikes, et al., '86). Ornithine transcarbamylase, which
is mitochondrial, is translated with a leader sequence.

Chondrichthyan fish are the predecessors of the stem reptiles (Young, '81). Elasmobranchs have been shown to have high levels of glutamine synthetase in the liver (Webb and Brown, '76; Webb and Brown, '80). As described above, elasmobranchs occupy an intermediate position with respect to the biochemical pathway used for urea synthesis. Glutamine synthetase, along with carbamylphosphate synthetase-III and ornithine transcarbamylase, has been demonstrated in the mitochondrial matrix in the dogfish shark *Squalus acanthias* (Casey and Anderson, '82; '85).

Since glutamine synthetase exists as compartmental isozymes in higher vertebrates, and the mitochondrial form is imported without a detectable leader sequence, it was of considerable interest to examine glutamine synthetase in lower vertebrates. Because the enzyme is a "typical" eukaryotic glutamine synthetase (Vorhaben, et al., '82) and not the prokaryotic enzyme (Ginsberg and Stadtman, '73), this suggests that the import mechanism was acquired during evolution (Margulis, '81). If so, investigation of glutamine synthetase in representatives of the most primitive vertebrates could yield significant information about the changes this enzyme has undergone with evolution. Moreover, in higher vertebrates glutamine synthetase exhibits a tissue-specific compartmentation. It was of interest to determine if this expression is seen in lower vertebrates, since the events which give rise to tissue-differentiation during development may be reflected in phylogenetic changes.

The objectives of this study were to determine the compartmentation of glutamine synthetase in the brain and liver tissues of the Gulf Coast stingray *Dasyatis sabina*, and in the brain of the dogfish *Squalus acanthias*. The sizes of the tissue-specific forms were determined, a partial characterization was performed. Results obtained with the two elasmobranchs lead to the investigation of glutamine synthetase in a holocephalan, *Hydrolagus colliei*. A
comparison was conducted of the elasmobranch glutamine synthetase and ornithine transcarbamylase translation products versus the mature subunits of each. Some aspects of translation and processing in vivo and in vitro were investigated. The RNA species that give rise to the tissue-specific forms in stingray and shark were identified. Finally, elasmobranch glutamine synthetase was examined at the gene level.
MATERIALS AND METHODS

I. Materials

A. Animals and Tissue

Gulf Coast stingrays (*Dasyatis sabina*) were captured by local shrimp fishermen and held in live bait tanks for 0-7 days at either the Marine Biomedical Institute or Baiten Place Bait and Tackle Shop in Galveston, TX. Animals were then transferred to holding tanks at Rice University, where they could be held for up to 6 months in artificial seawater (Fritz Super Salt Mix, supplemented with Fritz-Zyme #9, Fritz Chemical Co., Dallas, TX.). Nitrates and nitrites were monitored weekly with Quantofix sticks (Gallard-Schlesinger Industries, Inc., Carle Place, N. Y.) The animals were fed every two weeks with live ghost shrimp.

Dogfish shark (*Squalus acanthias*) were collected from the waters off Mt. Desert Island, ME. and Friday Harbor, WA. Ratfish (*Hydrolagus colliei*) were also collected from Friday Harbor, WA. These animals were held in circulating natural-seawater holding facilities until use.

B. Reagents

All reagents were of the highest commercially-available purity and were purchased from either Sigma, Fisher, Mallinckrodt, Baker, or Matheson, Coleman and Bell. Enzymes and secondary antibodies were purchased from Sigma. Radiolabelled chemicals were from I.C.N. or New England Nuclear. Restriction endonucleases and their corresponding buffers were from Bethesda Research Laboratories.

II. Methods

A. Subcellular fractionation

Excised tissue samples were blotted, weighed, then immediately placed on ice. Homogenates were prepared in nine volumes of one of the three following buffers (pH was measured at room temperature): (A.) 0.25 M
sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.4 (Vorhaben and Campbell, '72); (B.) 0.25 M sucrose, 20 mM HEPES, pH 7.5, 1 mM EDTA, 0.3 M urea, 0.15 M trimethylamine oxide (TMAO), 0.15 M potassium chloride (Casey and Anderson, '82); or (C.) 0.8 M sucrose, 10 mM HEPES, pH 7.4. The tissue was homogenized either by hand (five to seven strokes) or with a motor-driven (four to five strokes, 325 rpm) Potter-Elvehjem homogenizer with a loose-fitting pestle. All buffers and homogenizing techniques gave the same results.

The homogenate was filtered through six to eight layers of cheesecloth to remove some of the copious lipid. In the case of ratfish brain, this decreased the volume by roughly 50%. In all cases, the filtrate was labelled "homogenate." Homogenates were centrifuged at 30 x g for 10 min. (elasmobranch) or 40 x g for 5 min. (ratfish) to yield the "debris" fraction. For brain, this fraction sedimented, so the supernatant fluid was decanted. For liver, the lipid and some debris floated, so the supernatant fluid was aspirated from beneath the fat layer. These were centrifuged at 250 x g (elasmobranch) or 800 x g (ratfish) for 10 minutes, to sediment the "nuclear" fraction. The supernatant fluid from these were centrifuged at 15,000 x g for 1 hour (elasmobranch) or 14,500 x g for 2 hours (ratfish) to isolate the "mitochondrial" fraction. Supernatant fluid from the mitochondrial pellets was centrifuged at 135,000 x g (elasmobranch) or 48,200 x g (ratfish) for 1 hour to pellet the "microsomal" fraction. The final supernatant fluid was called the "cytosolic" fraction. Except for the final step, all pellets were washed once with 10 ml of fresh buffer, which was pooled into the subsequent step. All procedures were carried out at 4°C. The debris, nuclear, mitochondrial, and microsomal pellets were suspended in minimal volumes of buffer and held at -90°C. The dogfish and ratfish fractions were shipped on solid CO₂, for less than 24 hours.

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone.
B. Enzyme assays

Where detergents were used, a portion of each fraction was diluted with an equal volume of a 0.15% solution of the detergent, then held on ice for at least 15 min. prior to assay. Substrates, co-factors and other reaction mixture components were adjusted to the pH of the reaction mixture with potassium hydroxide, as necessary.

Glutamate dehydrogenase, a mitochondrial matrix enzyme, was assayed as described by Casey, et al. ('83). The oxidation of NADH was monitored at 340 nm. Final reactant concentrations were, in 1 ml : 1 mM EDTA, 2-5 mM ADP, 0.125 mM NADH, 10 mM alpha-ketoglutarate, and 200 mM ammonium acetate, in 75 mM imidazole, pH 7.4. Reactions were initiated by the addition of appropriate amounts of the fraction in 0.15% Triton X-100. The controls were minus alpha-ketoglutarate and minus ammonium acetate. One unit of enzyme activity was that amount oxidizing 1 μmol of NADH in 1 hr. at 25°C.

Cytochrome c oxidase, a mitochondrial outer membrane enzyme, was assayed as described by Wharton and Tzagoloff ('67). The oxidation of reduced ferricytochrome c (prepared by reduction with excess ascorbic acid, then dialysis against 10 mM potassium phosphate, pH 7.2) was measured at 550 nm. Aliquots of each fraction, diluted 1:1 in 0.15% Lubrol, were assayed in 1 ml of 10 mM potassium phosphate with 0.07 ml of ~1% reduced ferricytochrome c. Cytochrome c oxidase activity was calculated using a molar extinction coefficient of 19.6 x 10⁻³ M⁻¹cm⁻¹. One unit of enzyme activity was that amount oxidizing 1 μmol of reduced ferricytochrome c in 1 hour at 25°C.

Lactate dehydrogenase, a cytosolic enzyme, was assayed according to Vorhaben and Campbell ('72). The oxidation of NADH was monitored at 340 nm. The final concentration of the reactants, in 1 ml., were: 3 mM sodium pyruvate and 0.125 mM NADH in 20 mM potassium phosphate, pH
7.4. Reactions were initiated by the addition of aliquots of each fraction in 
0.15% Triton X-100. Controls were minus pyruvate. One unit of activity was 
that amount oxidizing 1 μmol of NADH in 1 hour at 25°C.

Glucose-6-phosphatase, a microsomal enzyme, was assayed 
essentially as described by Harper ('63). The reaction mixture contained, in 0.5 
ml, 16 mM glucose-6-phosphate, 40 mM citrate (pH 6.5), and an aliquot of each 
fraction. Incubation was for 1 hour at 30°C; phosphate standards were run 
concurrently. The reaction was terminated with the addition of 1 ml of 10% 
trichloroacetic acid. Precipitated protein was removed by centrifugation at 
13,500 x g for 5 minutes. The supernatant fluid was assayed for phosphate by 
the method of Fiske and SubbaRow ('25). The final reaction mixture contained, 
in 1 ml: 1 mM ammonium molybdate, 4.2 mM 1-amino-2-naphtol-4-sulfonic acid 
(freshly prepared), and 0.4 ml of the sample. The color was allowed to develop 
for 1 hour, then the absorbance was measured at 660 nm. One unit of enzyme 
activity was that amount liberating 1 μmol of phosphate in 1 hour.

Citrate synthase, a mitochondrial matrix enzyme, was 
determined by the method of Srere, et al. ('63). The production of citrate was 
determined by the formation of CoASH, which complexed with 
dithionitrobenzene to yield mercaptide. Formation of the colored mercaptide 
ion was monitored at 412 nm. The reaction mixture contained, in 1 ml: 200 mM 
Tris-HCl (pH 8.1), 0.1 mM dithionitrobenzene, 0.047 mM acetyl CoA, 0.23 mM 
oxalacetate, and 0.6 ml of the fraction. Citrate synthase activity was calculated 
using a molar extinction coefficient (for mercaptide ion) of 13.6 x 10³ M⁻¹cm⁻¹. 
Controls were minus oxalacetate, minus acetyl CoA, or minus both. One unit of 
enzyme activity was that amount producing 1 μmol of citrate in 1 hour at 25°C.

Glutamine synthetase was assayed essentially as described by 
Vorhaben, et al. ('82) by measuring the formation of 
gamma-glutamylhydroxamate from glutamine and hydroxylamine. The reaction 
mixture contained, in 0.5 ml: 80 mM magnesium chloride, 50 mM potassium
glutamate, 125 mM hydroxylamine, 40 mM ATP, 1 mM creatine phosphate, 1.5 units creatine kinase, 0.18% Triton X-100, and 0.01% oligomycin, in 50 mM imidazole (pH 7.4). Incubation was for 1 hour at 30°C. Controls were minus glutamate, hydroxylamine, and ATP; gamma-glutamylhydroxamate standards were run concurrently. The assay was terminated with the addition of ferric chloride reagent (0.37 M ferric chloride, 0.67 N HCl, 0.2 M trichloroacetic acid—Palmijans, et al., '62). The color was allowed to develop for 45 minutes, then the absorbance was measured at 500 nm. One unit of enzyme activity was that amount producing 1 μmol of gamma-glutamylhydroxamate in 1 hour.

Ornithine transcarbamylase was assayed essentially by the method of Marshall and Cohen ('72) by measuring the formation of citrulline from ornithine and carbamyl phosphate. The reaction mixture contained, in 1 ml: 5 mM L-ornithine, 5 mM diammonium carbamyl phosphate (dissolved just prior to use), 5 mM Tris-acetate (pH 8.6), and aliquots of each fraction. Incubation was for 15 minutes at 30°C. The reaction was terminated by the addition of 0.05 ml of 70% perchloric acid. The amount of citrulline formed was determined by the method of Archibald ('44), with 5.0 ml citrulline acid reagent (19% sulfuric acid, 25% phosphoric acid, and 2 x 10^-4% CuSO_4) and 0.25 ml of 3% diacetyl monoxime. The tubes were heated for 30 minutes, then the absorbance measured at 490 nm. Controls were heat-killed enzyme; citrulline standards were run concurrently. One unit of enzyme activity was that amount producing 1 μmol of citrulline in 1 hour.

Carbamyl phosphate synthetase-III was assayed by measuring the formation of [14C]carbamyl phosphate from [14C]bicarbonate and L-glutamine. The reaction mixture contained: 5.02 mM [14C]bicarbonate (0.02 μmoles sodium [14C]bicarbonate and 5.0 μmoles potassium bicarbonate, to yield 2 μCi/μmol), 10 mM magnesium sulfate, 5 mM N-acetyl-L-glutamate, 10 mM L-glutamine, 5 mM L-ornithine, 2.5 mM potassium phospho-enol-pyruvate, 5 mM ATP, and 40 mM HEPES (pH 7.5). The reaction mixture also contained
6.7 units (μmol·min⁻¹) pyruvate kinase and 3.6 units beef liver ornithine transcarbamylase. Incubation was for 20 minutes at 30°C, and the reaction was stopped by the addition of 100 μl of 20% trichloroacetic acid. The tubes were then gassed with carbon dioxide to remove the [¹⁴C]-substrate, then a portion of the mixture was counted. CPS-III activity is that amount producing 1 μmol of [¹⁴C]carbamyl phosphate in 1 hour.

Protein was determined by the method of Lowry, et al. (51) or Bradford (76). The Lowry assay mixture contained, in 1.5 ml: 2% sodium carbonate (in 0.1 N sodium hydroxide), 0.01% CuSO₄, 0.02% sodium tartrate, and aliquots of each fraction. After 10 minutes at 25°C, 0.125 ml of 1 N Folin-Coicalteu reagent was added, and color was allowed to develop for 30 minutes. The absorbance was read at 650 nm. Concentrations were determined against a bovine serum albumin standard curve, run concurrently. The Bradford assay mixture contained, in 1 ml: 0.2 ml of Dye Reagent Concentrate (Bio-Rad Biochemicals) and 0.8 ml of each fraction or bovine serum albumin standard curve. The absorbance was read at 595 nm.

C. Immunoblots

Immunoblots were performed essentially according to the method of Towbin, et al. (79). Aliquots of each fraction, equalling 50–100 μg of protein, were electrophoresed in discontinuous (4% stacking gel/10% running gel) SDS–PAGE slab gels (Laemmli, 70). In some cases, aliquots were mixed with protease inhibitors (10 mM phenylmethylsulfonylfluoride, and 1 μg/ml of leupeptin, antipain, pepstatin and aprotinin) prior to electrophoresis. After separation, the proteins were electrophoretically transferred to nitrocellulose (Schleicher and Schuell, BA-85). Transferred proteins were stained with Ponceau S to visualize molecular weight markers. The unoccupied sites on the blot were blocked with 5% nonfat dry milk or 3% bovine serum albumin in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 8.0). The primary antibody was either rabbit anti-chicken liver mitochondrial glutamine synthetase
IgG (Smith, et al., '83), rabbit anti-bovine liver mitochondrial ornithine transcarbamylase IgG, or rabbit anti-frog (Rana catesbiana) liver mitochondrial carbamylphosphate synthetase-I IgG (Marshall and Cohen, '61). Antisera were diluted 1:100 in Tris-buffered saline with 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate) and incubated 4 hours, or diluted 1:1000 and incubated 18–20 hours. The secondary antibody was either horseradish peroxidase-conjugated goat anti-rabbit IgG, diluted 1:5000 in Tris-buffered saline with 0.05% Tween 20, or [\(^{125}\)I]iodoprotein A (10^6 cpm, 17 μCi/μg). The peroxidase reaction mixture contained 0.6% 4-chloro-1-naphthol, 5% methanol in Tris-buffered saline (pH7.5) and 0.06% H\(_2\)O\(_2\) (Hawkes, et al., '82). Radiolabelled blots were visualized by autoradiography, using Kodak XAR-5 film and an intensifying screen, at -90°C (Bonner and Laskey, '75).

D. Immunohistochemistry

Cryostat sections (16-20 μm) were prepared from snap-frozen liver tissues. These were incubated with the antisera described above (glutamine synthetase, ornithine transcarbamylase, carbamylphosphate synthetase) in 1:50 dilutions for 60-120 minutes at room temperature. Controls were with pre-immune antisera. The sections were washed three times in phosphate-buffered saline (150 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (diluted 1:50) for 60-120 minutes. After the final wash, photomicrographs were taken with Kodak Kodacolor VR film (400 ASA) with 5% N-propylgalacte in glycerol, using epifluorescence at 450-490 nm. Also, some of the sections were stained with 1% methylene blue (in 1% borax), rinsed, and photographed.

E. Isoelectric focusing

Tissue samples were homogenized in buffer with 20% glycerol and 1 mM phenylmethylsulfonyl fluoride. Isoelectric focusing was in 5% polyacrylamide gels containing 2% ampholytes (Bio-Rad) and 20% glycerol.
Localization of glutamine synthetase activity was determined as follows: gels were incubated 1 hour at 37°C in 60 mM glutamine, 6 mM magnesium chloride, 4 mM ADP, 40 mM sodium arsenate, 120 mM hydroxylamine, in 50 mM imidazole, pH 7.4 (Webb and Brown, '80). Colored product was formed by incubating gels in ferric chloride reagent (see "glutamine synthetase assay" above).

F. DNA and RNA isolation

RNA was isolated by the guanidine hydrochloride method of Deeley, et al. (77) with modifications. Tissue (either fresh or snap-frozen and held at -90°C) was homogenized in 20 volumes (w/v) of homogenization buffer (8 M guanidine-HCl, 1 mM dithiothreitol, 20 mM sodium acetate, pH 5.0), using a Tekmar tissuemizer for 1 min., on ice. Debris was removed by centrifugation at 11,000 x g for 10 min.; the supernatant fluid was filtered through sterile cheesecloth to remove lipid. To the filtrate was added 0.5 volumes ice-cold ethanol. RNA was allowed to precipitate 18-20 hours at -20°C, then was pelleted by centrifugation at 11,000 x g for 30 min. The pellet was resuspended, in one-half the homogenization volume, in extraction buffer (8 M guanidine, 1 mM dithiothreitol, 20 mM EDTA, 20 mM sodium acetate, pH 7.0). One-half of this volume of ice-cold ethanol was added, and after 18-20 hours at -20°C, the re-extracted RNA was again pelleted. This extraction step was repeated three times. With stingray liver (males particularly), dark brown pigment granules pelleted with the RNA during extraction, but could be removed in later steps.

To exchange sodium for guanidinium, the final RNA pellet was suspended in a minimum volume of 20 mM sodium acetate (pH 7.0). RNA was extracted from pigment granules by repeatedly (4 times) washing the pellet in buffer and centrifuging; the RNA was soluble but the pigment was not. The supernatant fluids were pooled, then extracted in an equal volume of chloroform:1-butanol (4:1). The lower, organic phase was re-extracted with
buffer twice. The aqueous phases were pooled, then 0.1 volume of 4 M sodium chloride, followed by 2 volumes ethanol, was added. After 18-20 hours at -20°C, precipitated RNA was pelleted by centrifugation at 8,000 x g for 10 min. This step was repeated 2 times. The final pellet was dissolved in ≤1 ml of sterile water, and held at -90°C. The ratio of $A_{260}/A_{280}$ (1.8–1.9 optimum for RNA) and the concentration (one $A_{260}$ unit = 40 μg/ml RNA) were determined. One gram of tissue would routinely yield 0.5 mg RNA.

DNA and RNA were isolated from ray and shark tissues by the guanidine isothiocyanate plus cesium chloride gradient method of Chirgwin, et al. ('79), exactly as outlined in Davis, et al. ('86). One gram of tissue would routinely yield 0.5-1.0 mg DNA and 0.2-0.4 mg RNA. DNA was stored in TE buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) at 4°C; RNA was stored in sterile water at -90°C.

Poly (A+) RNA was isolated from total RNA by affinity purification on an oligo (dT) cellulose column (Bethesda Research Laboratories) by the method of Aviv and Leder ('72). Purity and concentration of the poly (A+) RNA was determined as described above for RNA. Purified poly (A+) RNA was stored in sterile water at -90°C. One gram of tissue would routinely yield 1.0–5.0 μg.

F. In vitro translation and immunoprecipitation

Poly (A+) RNA was translated in a cell-free system essentially according to Maniatis, et al. ('82). Optimum translation conditions were determined by titrating every preparation of RNA for final assay concentration, and every batch of lysate for magnesium and potassium chloride. Routine translation mixtures (50 μl total volume) consisted of 20.0 μl rabbit reticulocyte lysate (prepared exactly according to Maniatis, et al. ('82), aliquoted, and held at -90°C), 4.0 μl potassium chloride (1 M), 1.0 μl magnesium acetate (32.5 mM), 2.5 μl EGTA (100 mM), 4.0 μl translation cocktail (6.25 mM spermidine, 100 mM creatine phosphate, 0.3 mM amino acid mixture (−methionine), 30 mM
dithiothreitol, 250 mM HEPES, pH 7.4), 5.0 μl [35S]methionine (10 μCi/μl, 1.1 x 10^3 Ci/mmol), and 3–5 μg mRNA in a volume of 13.5 μl. Translation was performed at 30°C for 1 hour. The reaction was stopped with the addition of 0.5 μg/ml cyclohexamide and 100 μM unlabelled methionine, then placed on ice. Incorporation of radiolabelled methionine into trichloroacetic acid (TCA)-precipitable proteins was determined by spotting 2 μl of translation mix onto Whatman 3 filter squares, then precipitating the proteins in ice-cold 10% TCA, followed by boiling for 10 min., rinsing in 5% TCA, dehydrating in two changes of 95% ethanol, oven-drying, then measuring the radioactivity with a scintillation counter. Background incorporation was monitored by using water in place of mRNA. Translation products were shown to be quite labile, even when stored at -90°C, so translation mixtures had to be used immediately.

Where noted in the Results, translation of commercially-prepared nuclease-treated rabbit reticulocyte lysate (Promega or Bethesda Research Laboratories), or wheat germ lysate (Promega) was performed according to the manufacture's protocol for each. Background translation was monitored in every experiment, also. Rabbit lysate from both suppliers consistently yielded poor translations; however, wheat germ lysate translated extremely well.

Immunoprecipitations were performed with the antisera described under "Immunoblots", except that glutamine synthetase antisera was affinity-purified as described in Smith, et al. (83) using chicken mitochondrial glutamine synthetase immobilized on a cyanogen bromide-activated Sepharose 4B column (provided by Dr. D. D. Smith, Jr.). The concentration of purified antibody preparation, determined by absorbance at 280 nm (1.38 A280 units = 1 mg/ml IgG), was 1.25 mg/ml. Translation mixtures were diluted to 1.0 ml with 10 mM Tris-HCl (pH 7.4), 0.1% SDS, 0.5% Triton X-100, 150 mM sodium chloride, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. In selected experiments, additional protease inhibitors (1 μg/ml each leupeptin,
peptatin, antipain, and chymostatin) were added. In most preparations, this solution was pre-cleared with 10 μl of 10% (w/v) *Staphylococcus aureus* cells (The Enzyme Center), incubated at 4°C for 15 min., then centrifuged (13,500 x g for 5 min.) to removed the cells. Either crude ornithine transcarbamylase antisera (5 μl) or purified glutamine synthetase antibodies (10 μl) were added, and the mixture was incubated at 4°C overnight. In every experiment, pre-immune antisera was used as a control. In selected experiments, an excess of unlabelled glutamine synthetase (purified from chicken liver mitochondria, as described in Vorhaben, et al., '82) was added, to compete with the labelled glutamine synthetase. To precipitate the antigen-antibody complexes, 100-150 μl of 10% *S. aureus* cells were added, tubes were incubated 2-4 hours at 4°C, then centrifuged as above. The pellets were washed four times in dilution buffer, and the final pellets were suspended in 30 μl of SDS–PAGE gel loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.01% bromophenol blue). The mixture was then heated to 90°C to dissociate the immune complexes, and centrifuged at 13,500 x g for 10 minutes. The supernatant fluid, which contained the immunoprecipitated proteins, was used for electrophoresis.

Immunoprecipitated proteins were separated on discontinuous SDS-PAGE as described under "Immunoblots." Gels were stained 12-18 hours in Coomassie blue stain (0.01% R-250 Coomassie blue in 50% methanol, 10% acetic acid), destained (methanol:H₂O:acetic acid, 5:4:1), impregnated with fluors (En³Hance, New England Nuclear), vacuum-dried onto Whatman 3 filter paper, and autoradiographed against Kodak XAR-5 film at -90°C.

G. Mitochondrial isolation

Mitochondria were isolated from stingray liver and kidney in one of the following media: (I.) 250 mM sucrose, 1 mM EGTA, 300 mM urea, 150 mM TMAO, 150 mM potassium chloride, 20 mM HEPES (pH 7.5) (Casey and Anderson, '84); (II.) 280 mM sodium chloride, 6 mM potassium chloride, 0.5
mM sodium sulfate; 1 mM sodium phosphate (monobasic), 350 mM urea, 8 mM sodium bicarbonate, 10 mM HEPES (pH 7.4), 10 mM calcium chloride, 3 mM magnesium chloride, 1% bovine serum albumin (fatty acid free) (Ballantyne, et al. ’86); or (III.) 250 mM sucrose, 150 mM potassium chloride, 1 mM EDTA, 5 mM HEPES (pH 7.4). Freshly-excised tissue was homogenized on ice in 9 volumes medium, either by motor-driven Potter-Elvehjem homogenizer with a loose-fitting pestle, or with a Tekmar tissuemizer on medium speed for 1 min. The homogenate was filtered through six layers of cheesecloth. Debris and nuclei were removed from the filtrate by centrifugation at 250 x g for 10 min.; the supernatant fluid was aspirated from between the floating fat layer and the pellet. Mitochondria were sedimented by centrifugation at 8,000 x g for 10 min.; this pellet was washed twice by resuspension in buffer in a chilled Dounce homogenizer, then recentrifuged. The final pellet was suspended in a minimal volume of buffer with or without 0.05–1% bovine serum albumin, and held on ice until use. Proteins were determined by the Bradford assay (described under “subcellular fractionation”); calculations were corrected when BSA was present. Mitochondrial concentration was routinely adjusted to be 20–50 mg protein/ml.

The respiratory viability of mitochondria was determined by measuring oxygen uptake with a Yellow Springs oxygen monitor (model 5300) equipped with a Clarke-type electrode in a 1.5 ml cell at 30°C. Respiratory control ratios and rates of oxygen consumption were determined as described by Estabrook (’67). Mitochondrial respiration was assayed in the following solutions: (i.) 2.5 mM potassium phosphate, 2.5 mM magnesium chloride, 140 mM sucrose, 0.4 mM EGTA, 1 mM EDTA, 160 mM urea, 80 mM TMAO, 80 mM potassium chloride, 11 mM HEPES, pH 7.5, with 3 mM succinate or glutamate and 1 mM ADP for respiration measurements (Casey and Anderson, ’84); (ii.) 50 mM sucrose, 80 mM potassium chloride, 60 mM urea, 30 mM TMAO, 54 mM HEPES (pH7.4), 4 mM potassium phosphate, 6 mM potassium carbonate, 2 mM magnesium chloride, 0.4 mM EDTA, 0.2 mM EGTA, 0.5 mM glutamate, 1.0 mM
ornithine, with 10 mM succinate and 1 mM ADP for respiration measurements (Anderson, '86); (iii.) 400 mM urea, 200 mM TMAO, 50 mM sucrose, 150 mM potassium chloride, 10 mM potassium phosphate, 30 mM HEPES (pH 7.2), 1% bovine serum albumin, with 5 mM succinate or 20 mM glutamate and 10 mM ADP for respiration measurements (Moyes, et al., '86); (iv.) 140 mM sucrose, 11 mM HEPES (pH 7.4), 160 mM urea, 80 mM TMAO, 80 mM potassium chloride, 2.5 mM magnesium chloride, 1% bovine serum albumin, with 5 mM succinate or glutamate and 10 mM ADP for respiration measurements.

Mitochondrial suspension equal to 1-3 mg protein was added to 1.2-1.4 ml buffer. After establishment of a baseline (exogenous oxygen uptake, usually zero), succinate or glutamate was added, and the state 4 (endogenous ADP exhausted, plus substrate) rate of respiration was determined (Chance and Williams, '56). Then ADP was added, and the state 3 (substrate and ADP present) respiration was determined. In some experiments, FCCP, DNP or oligomycin were added to determine maximum potential oxygen uptake. The best results were obtained with solution (iv.).

For comparison, rat liver mitochondria were prepared and assayed. Rat liver was homogenized on ice in 9 volumes of 250 mM sucrose, 5 mM HEPES (pH 7.2) with a motor-driven Potter-Elvehjem homogenizer, 4 passes at 325 rpm. Debris and nuclei were removed by centrifugation at 250 x g for 10 minutes. Mitochondria were sedimented by centrifugation of the supernatant fluid at 15,000 x g for 10 minutes. The pellets were washed one time by gentle resuspension in buffer and recentrifugation. The final pellet was suspended in a minimal volume of buffer, and assayed for proteins by the Bradford assay (as described above). The mitochondrial concentration was usually adjusted to 40-60 mg protein/ml. Respiration was assayed as above, using 1-3 mg protein and a solution of 250 mM sucrose, 5 mM HEPES, 2.5 mM potassium phosphate buffer (pH 7.4), 1 mM magnesium chloride, with 10 mM succinate followed by 200 μM ADP.
For some experiments, isolated mitochondria were solubлизed. Mitochondria were suspended in buffer with 1% Triton X-100, then sonicated (Heat Systems–Ultrasonics model C-2, with microprobe tip) with 3 x 30 sec. pulses, on ice. The lysate was centrifuged at 100,000 x g for 1 hour. Proteins were determined with the Bradford assay (described under "Subcellular fractionation"); soluble and insoluble fractions were held at -90°C until use.

H. Translation and processing experiments

Experiments to determine the effect of whole mitochondria or mitochondrial lysates on translation products were performed as follows. Translation mixtures were prepared, incubated and counted as described under "In vitro translation and immunoprecipitation." Mitochondria were prepared as described under "Mitochondrial isolation." In experiments where lysed mitochondria were required, this was achieved by suspending mitochondria in 0.2% Triton X-100 for 1 hour on ice, or repeated freeze-thawing, or sonication on ice (as above), or a combination of these.

Typically, 50 μl of mitochondrial suspension (200 μg protein) were incubated with 50 μl of translation mixture at 30°C for 30–60 min. The assays were terminated by dilution to 1.0 ml with dilution buffer and processed for immunoprecipitation, electrophoresis and autoradiography as described under "In vitro translation and immunoprecipitation." Pre-immune antisera was used for monitoring immunoprecipitation, and heat-inactivated mitochondrial samples were included as processing controls.

Selected experiments included an ATP-regenerating system consisting of 1.2 mM ATP, 0.3 mM mM GTP, 8 mM creatine phosphate, 2 mM dithiothreitol, and 300 μg/ml creatine kinase (Ono and Tuboi, '86). Import of precursor peptides into whole mitochondria was tested in several variations of the media described above for mitochondrial isolation and respiration. In some experiments, sample tubes were not precleared with S. aureus cells, but pre-immune controls showed no non-specifically binding proteins correlating
with any of the proteins being investigated.

Mitochondrial respiration uncouplers and inhibitors were assayed for their effect on import and processing of peptides by addition of the following amounts to the translation product/mitochondrial mixtures described above: 1 μM–10 μM FCCP or DNP (uncouplers); 1 mM–10 mM Rhodamine 123 or cyanide (inhibitors). The effects of protease inhibitors and metal chelators were assayed by addition of the following amounts of each: 1 mM–10 mM p-aminobenzamidine, o-phenanthroline, 8-hydroxyquinoline, or 1 mM–100 mM of EDTA or EGTA (metal chelators); 1 mM–10 mM phenylmethylsulfonyl fluoride, p-chloromercuribenzoate, or 1 μg–10 μg per ml. of antipain, leupeptin, pepstatin, and chymostatin (protease inhibitors). In one set of experiments, the processing of precursor peptides at 4°C, 10°C, and 30°C in 10 min. intervals was investigated.

I. Hepatocyte preparation

Stingray hepatocytes were isolated by the method of Shuttleworth and Goldstein ('84) as adapted by Ballantyne, et al. ('86). After the animal was pithed and the spine severed, the liver was exposed. Sutures were used to tie-off all of the vessels anterior of the liver (secured together as a bundle). Perfusion was initiated via the hepatic-portal vein with a solution containing 280 mM sodium chloride, 6 mM potassium chloride, 0.5 mM sodium sulfate, 1 mM sodium phosphate (monobasic), 350 mM urea, 8 mM sodium bicarbonate, 10 mM HEPES, pH 7.4, which had been sparged in a "glass lung" with O₂:CO₂ (95:5) for 15 min. at 30°C. To the initial perfusion medium was added 2 units/ml heparin. Within seconds of starting perfusion, the lower tips of the liver were clipped to allow free flow of the perfusate, which was not recirculated (to allow removal of blood cells). Within 5-10 min. the liver blanched; small areas where blood remained could be gently massaged to stimulate circulation. Collagenase (100 mg, to yield ~0.1%, of Sigma Type IV) was added to 100 ml perfusion medium (minus heparin), dissolved, and pooled
with the solution in the perfusion reservoir. Recirculating perfusion was then continued for 40-60 min., until the liver was soft (indicating digestion).

The perfused liver was excised, and placed in a flask with 100 ml perfusate. The atmosphere was purged with O₂:CO₂ (95:5), the flask stoppered, and slowly circulated for 60–90 min., with re-gassing every 15 min. From this step on, all material was kept on ice, and handled gently to prevent cell lysis. Cells were then loosened from connective tissue by slowly teasing the tissue with forceps in a petri dish. The entire mixture was strained through wire gauze (to remove undigested tissue), and the gauze rinsed with fresh perfusion medium (collagenase-free) to which had been added calcium chloride (5 mM), magnesium chloride (3 mM), and bovine serum albumin (1%, fatty acid free) (this is "elasmobranch medium").

The filtrate was centrifuged at 50 x g for 1-2 min. to sediment the cells. (If the liver contained copious fat, most of the cells would float, so this procedure was best performed on males collected in the early summer.) The loose pellet was resuspended in fresh elasmobranch medium, and recentrifuged. The final pellet was resuspended in a minimum volume of medium, and held on ice until use. The concentration and viability of cells were determined by counting (on a Neubauer cell-counting slide) serial dilutions of cells suspended in 0.6% trypan blue in elasmobranch medium. The best preparations were from medium animals (8"–10" diameter), where 1.0–5.0 x 10⁸ cells could be obtained, with 96% viability (as shown by trypan blue exclusion).

J. Cell and tissue labelling

Pulse labelling of isolated hepatocytes, essentially as described by Mori, et al. ('81), was performed as follows: 1.0–3.0 x 10⁷ cells were placed in a small glass vial with 0.1 mM amino acid mix (−methionine) in elasmobranch medium in a total volume of ≤ 2 ml. The assay began with the addition 0.5 mCi/ml [³⁵S] methionine (1.1 x 10² Ci/mmol). The vials were purged with O₂:CO₂ (95:5), capped, and slowly rocked during incubation at
room temperature. At the indicated time points, aliquots of the suspension were removed, placed in small tubes, and snap-frozen in LN$_2$. In addition, 10 µl were spotted onto Whatman 3 filter squares, and precipitated and counted as described under "In vitro translation."

Pulse-chase experiments were performed by labelling the cells (as above) for a designated time, then adding 10 µg/ml cyclohexamide and 5 mM unlabelled methionine. Aliquots were removed at 5 min. intervals and snap-frozen, with 10 µl spotted for counting incorporation. Pulse-inhibitor experiments were performed by pre-incubating the cells 5–15 min. in 0.5 mM, 1.0 mM, or 10.0 mM of either DNP or cyanide, 0.5 µM–2 µM FCCP, or 1µg/ml, 5 µg/ml, or 10 µg/ml cyclohexamide, chloramphenicol, or Rhodamine 123 (Rhodamine was titrated up to 50 µg/ml). The assays were initiated with the addition of 0.5 mCi/ml [$^{35}$S] methionine (1.1 x 10$^5$Ci/mmol), and aliquots were removed and counted as above.

Freshly-excised kidney and liver were minced into 50 mg pieces with a scalpel on dental wax, and placed (handling the cubes with electron microscopy forceps to minimize tissue damage) into elasmobranch medium and held on ice. Much of the connective-tissue capsule of the kidney could be dissected away. Pulse labelling was performed in elasmobranch medium (100 µl/50 mg tissue) to which was added 10 µM amino acids (–methionine); labelling was initiated with the addition of 0.5 mCi/ml [$^{35}$S]methionine, then purging the atmosphere in each vial with O$_2$:CO$_2$ (95:5). The vials were capped, and incubated at room temperature with gentle rocking. In some experiments, tissue was preincubated 10–30 min. in 1 mM cyclohexamide or chloramphenicol, 5–25 µM FCCP, 10–100 µg Rhodamine 123, or 1–10 mM cyanide. In pulse–chase experiments, the chase consisted of 1 mM cyclohexamide and 10 mM unlabelled methionine.

Pieces of labelled tissue were removed at selected timepoints, and snap-frozen in LN$_2$. The tissue was subsequently suspended in 50 µl
dilution buffer (described under "immunoprecipitation") with 1% SDS and 100 
µM phenylmethylsulfonyl fluoride, and sonicated (Model SC-40, Sonicor Instr. 
Corp., Copiague, N.Y.) 20 minutes in an ice bath. The samples were heated to 
90°C for 10 minutes, then hand-homogenized in each tube with a plastic 
micropestle. Ten µl of each sample were spotted, precipitated and scintillation 
counted as described under "In vitro translation." The samples were then diluted 
to 1.0 ml with dilution buffer. Glutamine synthetase was immunoprecipitated, 
electrophoresed, and subject to autoradiography as described earlier.

K. Northern blotting

Stingray and dogfish liver, kidney and brain RNA and poly (A+) 
RNA, isolated by the guanidine isothiocyanate method described earlier, were 
Northern blotted as follows. Ten µg mRNA or 40 µg RNA were denatured in 1 M 
glyoxal, 10 mM sodium phosphate (pH 7.0), 50% (v/v) dimethyl sulfoxide at 
50°C for 1 hour (Thomas, '80). Two µl loading buffer (50% (v/v) glycerol, 10 mM 
sodium phosphate buffer, pH 7.0, and 0.01% (w/v) bromophenol blue) was 
added to each sample, which was then electrophoresed through a 1% agarose 
gel in phosphate buffer (10 mM, pH 7.0) with recirculation; 18S and 28S rRNA 
was visualized by UV shadowing on a thin-layer chromatography plate 
containing fluorescent indicators. Nucleic acids were electrophoretically 
transferred to Genescreen (New England Nuclear) in TAE buffer (12 mM Tris, pH 
7.5, 6 mM sodium acetate, 0.3 mM EDTA), then crosslinked to the matrix under 
direct UV illumination, 5 min. The blots were de-glyoxalated in 50 mM sodium 
hydroxide for 30 seconds, then neutralized in 0.2 M Tris (pH 7.5),1XSSC (150 
mM sodium chloride, 15 mM sodium citrate). The blots were prehybridized 
under one of the following conditions: (a.) 50% formamide, 1% SDS, 5XSSC 
(750 mM sodium chloride, 75 mM sodium citrate), 5X Denhardt's reagent (0.1% 
Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 µg 
heat-denatured salmon sperm DNA, for 24 hours at 42°C; or (b.) 1% SDS, 
6XSSC (900 mM sodium chloride, 90 mM sodium citrate), 5X Denhardt's
reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 μg heat-denatured salmon sperm DNA, and 10% dextran sulfate, for 24 hours at 65°C.

Heat–denatured glutamine synthetase bovine retina cDNA $^{[32P]}$-labelled probe [390 base pairs, 2 x $10^8$ c.p.m./μg specific activity, prepared by D. D. Smith, as described in Smith and Campbell ('87)] was hybridized to the blots in the above solutions ($10^6$ c.p.m./ml), in (a.) for 48 hours at 42°C, or in (b.) for 48 hours at 65°C. Blots were rinsed in 2 changes of 2XSSC (300 mM sodium chloride, 30 mM sodium citrate) at room temperature for 15 min. each, then 3 changes of 2XSSC with 0.1% (v/v) SDS at 65°C for 30 min. each, and finally 2 changes of 0.1XSSC (75 mM sodium chloride, 7.5 mM sodium citrate) at room temperature for 30 min. each. Wet blots were wrapped in Saran Wrap, and autoradiographed with an intensifying screen, as described earlier.

L. Southern blotting

Stingray and shark DNA, isolated as described earlier, were dialysed (under sterile conditions) against TE buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) at 4°C. The DNA was cut with restriction enzymes in a final volume of 50 μl that included 10 μg DNA, 4 mM spermidine, and the following endonucleases and appropriate buffers (supplied with the enzymes), in 1:10 dilutions: Eco R1, Hind III, Bam H1, Pvu III, and Pst I. Sterile water was used as a digestion control. Restriction digestes were incubated 18–20 hours at 37°C. To each sample was added 5 μl loading buffer (described under "Northern blots"), and the samples were heat-denatured at 90°C for 1 min.

Restricted DNA was electrophoresed through 1% agarose gels which contained 0.5 μg/ml ethidium bromide, in TBE buffer (89 mM Tris, 89 mM borate, 2 mM EDTA). DNA standards [3 μl DNA ladder (Bethesda Research Laboratories), 5μl loading buffer, 40 μl water] were run concurrently. After electrophoresis, the gels were denatured in 1.5 M sodium chloride, 0.5 M
RESULTS

Subcellular Fractionation

Glutamine synthetase was found to be predominantly mitochondrial in the liver of both elasmobranchs and holocephalans, and predominantly cytosolic in the brain of these species.

The results of fractionation of stingray liver are in Table 1. The localization of glutamine synthetase activity (87%) follows that of the mitochondrial markers glutamate dehydrogenase (90%) and cytochrome c oxidase (92%). Only 5% of the cytosolic marker lactate dehydrogenase is seen in the mitochondrial fraction. These results are comparable to what has been shown for the dogfish shark liver (Casey and Anderson, '82).

In ray brain, as seen in Table 2, glutamine synthetase is predominantly cytosolic. The distribution of glutamine synthetase is significantly different from that of the mitochondrial marker enzymes; less than 5% of the activity of cytochrome c oxidase and citrate synthase is found in the post-mitochondrial (i.e. microsomal and soluble) fractions, while 65% of glutamine synthetase activity is seen there. The presence of glutamine synthetase activity in the mitochondrial and microsomal fractions may be attributed to the highly membranous nature of brain tissue (Sellinger and de Balbain, '62). Of the amount of glutamine synthetase seen in the mitochondrial fraction (34%), most of it can be accounted for by entrapment in synaptosomes (or other membranous vesicles) by comparison with the cytosolic marker lactate dehydrogenase. The glutamine synthetase activity seen in the microsomal fraction (17%) may be due to the hydrophobic character of the enzyme; in mammalian liver, glutamine synthetase associates with microsomes during fractionation (Wu, '63). Or, as the microsomal pellet is not washed, it could be contamination from residual supernatant fluid. Since a comparable amount (15%) of lactate dehydrogenase activity is in the microsomal fraction, and it is a soluble enzyme, this is a distinct possibility. As
### TABLE 1: Subcellular Fractionation of Stingray Liver Tissue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units/gm tissue*</th>
<th>Fractions (% of total recovered)</th>
<th>Total % Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nuclear</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>410.8</td>
<td>8.0</td>
<td>87.0</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>210.0</td>
<td>5.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>510.0</td>
<td>8.0</td>
<td>92.0</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>150.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>120.0</td>
<td>3.0</td>
<td>89.0</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>13.8</td>
<td>2.0</td>
<td>27.0</td>
</tr>
<tr>
<td>Protein</td>
<td>85.1</td>
<td>6.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

*Based on homogenate value. Enzyme activities are in hour units and protein in mg.
TABLE 2: Subcellular Fractionation of Stingray Brain Tissue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units/gm tissue*</th>
<th>Fractions (% total recovered)</th>
<th>Total % Recovered</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nuclear</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>184.2</td>
<td>4.0</td>
<td>34.0</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>924.0</td>
<td>18.0</td>
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<td>Lactate dehydrogenase</td>
<td>1290.0</td>
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<td>Citrate synthase</td>
<td>363.0</td>
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<td>48.0</td>
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<td>Glucose-6-phosphatase</td>
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<tr>
<td>Protein</td>
<td>88.1</td>
<td>6.0</td>
<td>46.0</td>
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</tbody>
</table>

*Based on homogenate value. Enzyme activities are in hour units and protein in mg.
shown by the amount of glucose-6-phosphatase activity in the mitochondrial fraction (48%), there was some contamination of this fraction with microsomes, which would also contribute to the amount of glutamine synthetase seen there. No ornithine transcarbamylase or carbamylphosphate synthetase-III activity was seen in the brain.

Subcellular fractionation of dogfish brain is shown in Table 3. As in the stingray, glutamine synthetase is predominantly cytosolic in the dogfish brain. Glutamine synthetase has been shown previously to be mitochondrial in dogfish liver (Casey and Anderson, '82). As in other brain fractionations, some of the glutamine synthetase appears to have sedimented with the nuclear and mitochondrial fractions. The amount of lactate dehydrogenase in these fractions (25.4%) clearly demonstrates entrapment. However, only 50% of the amount of glutamine synthetase activity seen in dogfish brain mitochondria can be accounted for by this phenomenon. It remains a possibility that in dogfish brain, but not in stingray brain, some glutamine synthetase is actually mitochondrial.

The subcellular distribution of ratfish liver enzymes is shown in Table 4. In the liver, glutamine synthetase is clearly mitochondrial (72.0%). Although some microsomal contamination of the mitochondrial fraction has occurred [as evident by the amount (14.6%) of glucose-6-phosphatase present], glutamine synthetase does not appear to have associated with the microsomal membranes in this preparation, since very little (3%) activity is seen in the microsomal fraction. Therefore, the amount of glutamine synthetase present in the mitochondrial fraction is not due to contaminating microsomes. Some mitochondrial breakage has occurred, as is evident by the amount of matrix and membrane marker enzymes seen in the post-mitochondrial fraction (24.6% of cytochrome c oxidase and 20.9% of glutamate dehydrogenase). In another experiment, though, 9% of the glutamine synthetase activity, 7% of the glutamate dehydrogenase activity,
### TABLE 3: Subcellular Fractionation of Dogfish Shark Brain Tissue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units/gm tissue*</th>
<th>Fractions (% total recovered)</th>
<th>Total % Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nuclear</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>488.7</td>
<td>14.0</td>
<td>28.4</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>117.6</td>
<td>20.9</td>
<td>68.4</td>
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<tr>
<td>Cytochrome oxidase</td>
<td>477.0</td>
<td>19.8</td>
<td>77.3</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>7716.0</td>
<td>11.5</td>
<td>13.9</td>
</tr>
<tr>
<td>Protein</td>
<td>113.3</td>
<td>15.9</td>
<td>26.7</td>
</tr>
</tbody>
</table>

*Based on homogenate value. Enzyme activities are in hour units and protein in mg.
### TABLE 4: Subcellular Fractionation of Ratfish Liver Tissue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units/gm tissue</th>
<th>Fractions (% total recovered)</th>
<th>Total % Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Debris</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>27.7</td>
<td>14.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>2130.9</td>
<td>9.5</td>
<td>11.7</td>
</tr>
<tr>
<td>Carbamylphosphate synthetase</td>
<td>2.7</td>
<td>6.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>69.0</td>
<td>14.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>66.0</td>
<td>18.5</td>
<td>16.0</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>2.3</td>
<td>9.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>81.6</td>
<td>7.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Protein</td>
<td>32.8</td>
<td>5.0</td>
<td>10.3</td>
</tr>
</tbody>
</table>

*Based on homogenate value. Enzyme activities are in hour units, protein in mg.*
and only 1% of the cytochrome c oxidase activity, was seen in the soluble fraction. Ornithine transcarbamylase (Read, '67) and carbamylphosphate synthetase-III (Anderson, '80) have been previously demonstrated in ratfish liver. These two enzymes are shown here to be located in the mitochondria of this tissue (60.2% of ornithine transcarbamylase, 78.4% of carbamylphosphate synthetase).

The results of ratfish brain subcellular fractionation are shown in Table 5. Glutamine synthetase is predominantly cytosolic (55.6%). Again, some entrapment of cytosolic enzymes appears to have occurred (17.6% of lactate dehydrogenase activity was in the nuclear and mitochondrial pellets). Ratfish brain shows an anomalous distribution of glucose-6-phosphatase. Only a small percentage of the enzyme activity (2.5%) is seen in the microsomal fraction; most of the recovered activity (63.9%) was in the mitochondrial and soluble fractions. This pattern was also seen in other brain preparations from this species. Glucose-6-phosphatase is not strictly associated with microsomes in all cases. In molluscan hepatopancreas tissue (Vorhaben and Campbell, '79), glucose-6-phosphatase is cytosolic. Also, glucose-6-phosphatase has been shown to be present in nuclear membrane (Gunderson and Nordlie, '75).

**Western Blots**

To confirm the distribution of glutamine synthetase in the subcellular fractions, and to determine the size of the enzyme's subunits, Western blots (immunoblots) of the fractions prepared above were performed. An immunoblot of stingray liver and brain fractions for glutamine synthetase is shown in Figure 4. The distribution of immunoreactive protein among the fractions follows the localization of activity determined by enzyme analysis. Previously it was shown that rabbit antibodies raised to purified chicken liver mitochondrial glutamine synthetase cross-react with dogfish liver glutamine synthetase (Smith, Vorhaben and Campbell, '83). Results reported here
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units/gm tissue*</th>
<th>Fractions (% total recovered)</th>
<th>Total % Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Debris</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>76.2</td>
<td>14.8</td>
<td>10.5</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>33.6</td>
<td>10.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>178.8</td>
<td>12.3</td>
<td>12.7</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>2.2</td>
<td>22.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>1420.0</td>
<td>16.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Protein</td>
<td>46.1</td>
<td>14.4</td>
<td>17.9</td>
</tr>
</tbody>
</table>

*Based on homogenate value. Enzyme activities are in hour units, protein in mg.
FIGURE 4: Western blot of stingray liver and brain fractions for glutamine synthetase. A = liver soluble; B = liver microsomal; C = liver mitochondrial; D = liver nuclear; E = brain cytosolic; F = brain microsomal; G = brain mitochondrial; H = brain nuclear. Each lane is 100 μg protein. Except where noted, molecular mass markers are in kilodaltons (kDa).
indicate that rabbit antibodies to purified bovine liver ornithine transcarbamylase and rabbit antibodies to purified frog liver carbamylphosphate synthetase-1 cross-react with the elasmobranch and holocephalan enzymes.

In Figure 4 the localization of glutamine synthetase in ray brain and liver fractions was determined using rabbit anti-chicken liver glutamine synthetase antibodies. In this blot, equal amounts of protein from each fraction were used. In the liver fractions, specific activity ranged from 0.2 to 12.5 micromoles/mg protein/hr. Among brain fractions the range was only 1.7-4.2 micromoles/mg protein/hr., which is reflected in the similarity of staining intensity across all of the lanes on the blot.

In Figure 5, ornithine transcarbamylase distribution is shown using rabbit anti-bovine liver ornithine transcarbamylase antibodies. In Figure 6 carbamylphosphate synthetase-III distribution is shown, using rabbit anti-frog carbamylphosphate synthetase-1 antibodies. Both blots demonstrate that immunoreactivity intensity follows enzyme activity in each fractionation. The multiple bands seen in the mitochondrial lane in Figure 6 were due to degradation of the large carbamylphosphate synthetase peptide by repeated freeze-thawing of the preparation. This was confirmed by immunoblotting freshly-isolated mitochondria, as can be seen in Figure 6. Ray ornithine transcarbamylase subunit is shown to be 38 kDa; this compares favorably with the 36 kDa subunit in chicken kidney (Tsuji, '83), the 36–39 kDa subunit in rat liver (Mori, et al., '80; Conboy and Rosenberg, '81) and the 38 kDa subunit in bovine liver (Marshall and Cohen, '73). Ray liver carbamylphosphate synthetase-III subunit size is shown here to be 159 kDa. Dogfish shark (Anderson, '81) and large-mouth bass (Anderson '76) carbamylphosphate-III subunits have both been reported to be 160 kDa.

The data presented in Figure 4 reveal the presence of isozymes of glutamine synthetase in the stingray. This was the first demonstration of
FIGURE 5: Western blot of stingray liver fractions for ornithine transcarbamylase. A = soluble; B = microsomal; C = mitochondrial; D = nuclear. Each lane contains 100 μg of protein.
FIGURE 6: Western blot of stingray fractions for carbamyl phosphate synthetase-III. A = freshly isolated mitochondria; B = soluble; C = microsomal; D = mitochondrial; E = nuclear. B–E were from fractions frozen and thawed several times. All lanes contain 100 µg of protein.
Isozymes of glutamine synthetase in vertebrates; in plants, many isozymes of glutamine synthetase are expressed in different tissues (Gebhardt, et al., '86). In the stingray, the liver form is approximately 45 kDa, while the brain form is approximately 42 kDa. Figure 7 presents a comparison of dogfish and stingray isozymes. Dogfish brain cytosolic fraction yields a 45 kDa band, while the brain mitochondrial fraction shows both 45 kDa and a 47 kDa bands. The latter is in agreement with the size reported by Shankar and Anderson ('85) for glutamine synthetase purified from dogfish liver mitochondria of 46 kDa. Stingray brain cytosolic and liver mitochondrial fraction immunoblots are included in Figure 7, demonstrating the species-difference in the sizes of elasmobranch glutamine synthetase isozymes of brain and liver.

Glutamine synthetase has been localized in dogfish kidney mitochondria (King and Goldstein, '83) and stingray retinal Müller cells (Linser and Moscona, '82). Immunoblots from stingray kidney and retina are shown, along with brain and liver, in Figure 8. Kidney glutamine synthetase corresponds to the liver (mitochondrial) size of 45 kDa while retina glutamine synthetase is of the brain (cytosolic) size of 42 kDa. Immunoblots of other stingray tissues indicated heart, gill, and rectal gland glutamine synthetase is the smaller brain isozyme.

To see whether glutamine synthetase isozymes were unique to elasmobranchs, another Chondrichthyean fish, the holocephalan Hydrologus colliei (ratfish), was examined. Immunoblots of ratfish liver and brain fractions are shown in Figure 9. Again, the distribution of immunoreactive protein followed that of glutamine synthetase activity. While among the liver fractions a 12-fold difference in the specific enzyme activity (per mg protein) of glutamine synthetase is seen, only a 2-fold difference is seen among the brain fractions, corresponding again to the staining intensity observed on the blot. Figure 9 also reveals the presence of glutamine synthetase isozymes in the ratfish. The liver mitochondrial form is 45 kDa, while the brain cytosolic form is
FIGURE 7: Comparison of stingray and dogfish glutamine synthetase isozymes. A = dogfish brain cytosolic fraction; B = dogfish brain mitochondrial fraction; C = stingray brain cytosolic fraction; D = stingray liver mitochondrial fraction. Each lane contains 100 μg protein.
FIGURE 8: Western blot of stingray tissues for glutamine synthetase. A = retina; B = kidney; C = brain; D = liver. Each lane contains 100 μg protein.
FIGURE 9: Western blot of ratfish liver and brain fractions for glutamine synthetase. A = liver cytosolic; B = liver microsomal; C = liver mitochondrial; D = liver nuclear; E = brain cytosolic; F = brain microsomal; G = brain mitochondrial; H = brain nuclear. Each lane contains 75 µg of protein.
43.5 kDa. The brain soluble fraction contains only the 43.5 kDa size, while the liver mitochondria appears to contain both a 45 kDa and a 43.5 kDa form.

The presence of double bands in both the dogfish brain and the ratfish liver mitochondrial fractions deserves some consideration. In ratfish liver, the mitochondrial fraction contains the 45 kDa liver form and a 43.5 kDa brain-sized form. Figure 10 more clearly demonstrates the difference of these two sizes. The smaller form could actually be derived from hepatic neural tissue, which would be included in a whole liver homogenate. Mammalian liver is richly supplied with nerves of the autonomic nervous system (Sasse, '86). The smaller form of glutamine synthetase seems to be characteristic of neural tissue, as seen by its presence in brain and retina. Elasmobranch liver also contains many granulocytic leucocytes (Hamlett, et al., '86), which may contain the smaller form of glutamine synthetase (Schmidt and Thompson, '79). In the dogfish brain, the derivation of the larger liver form is more difficult to determine. While an increase in molecular mass can result from post-import modification of the protein (Wold, '81), the expression of a mitochondrial glutamine synthetase in the brain is not expected. As with the liver, though, the brain homogenate includes many cell types, some of which may express the mitochondrial isozyme. An explanation of the apparent dual expression of glutamine synthetase isozymes in the same tissue will depend on further investigation of the molecular events involved.

Figure 11 shows Western blots of ratfish liver fractions for ornithine transcarbamylase and carbamylphosphate synthetase-III. As with glutamine synthetase, immunoreactivity correlated with enzyme activity. Ratfish ornithine transcarbamylase subunit size is 39 kDa, which is the same as that in the dogfish. Ratfish carbamylphosphate synthetase-III subunit size is 147 kDa. This is slightly smaller than the stingray (159 kDa) or dogfish (160 kDa) subunit sizes.

Immunohistochemistry
FIGURE 10: Western blot comparison of ratfish liver and brain isozymes. A = liver mitochondrial fraction; B = mixture of A and C; C = brain cytosolic fraction. Lanes A and C contain 50 µg protein each; lane B contains 25 µg each of A and C.
FIGURE 11: Western blot of ratfish liver fractions for ornithine transcarbamylase and carbamyl phosphate synthetase-III. A and E = liver cytosolic; B and F = liver microsomal; C and G = liver mitochondrial; D and H = liver nuclear. Each lane contains 75 μg protein.
Another indication of subcellular localization, and investigation of the pattern of expression of glutamine synthetase at the tissue level, were both achieved by immunohistochemistry. Frozen sections of stingray liver tissue (16–20 μm) were immunoreacted with rabbit antibodies to purified glutamine synthetase, carbamylphosphate synthetase I, and ornithine transcarbamylase. For the control, frozen sections were incubated with nonimmune rabbit antisera. All sections were subsequently incubated with goat anti-rabbit antibodies conjugated to fluorescien isothiocyanate. Figure 12 contains photographs of the fluorescence of the immunoreacted sections. Figure 13 is a cryostat section of ray liver stained with methylene blue. All three enzymes displayed a punctate staining characteristic of a mitochondrial localization (Gaasbeek–Jansen, et al., '84; Groot, et al., '87 ; Smith and Campbell, '87). No specific staining was seen in the nonimmune treated sections. At lower magnification, the enzymes appeared to be distributed homogeneously throughout the tissue, without specificity for any particular region or cell type, as has been reported for avian liver (Smith and Campbell, '87). In comparison, mammalian liver displays differential expression of glutamine synthetase and carbamylphosphate synthetase (de Groot, et al., '87; Smith and Campbell, '87). In these species, glutamine synthetase is localized to a few cells around the terminal hepatic venule, while those same cells do not contain carbamylphosphate synthetase-I.

Isoelectric Focusing

To better characterize the elasmobranch glutamine synthetase isoforms, their isoelectric points were determined by isoelectric focusing. The results of this procedure, using stingray and shark tissue homogenates, are shown in Figure 14. The gels were run as a pH 3-10 gradient then activity stained for glutamine synthetase. The isoelectric points for all of the tissue isoforms appear the same at approximately pH 5.2, although some diffusion of the reaction product occurs during staining. These results suggest a
FIGURE 12: Immunohistochemistry of stingray liver cryosections. A = reaction with anti-glutamine synthetase antisera; B = reaction with anti-carbamylphosphate synthetase antisera; C = reaction with anti-ornithine transcarbamylase antisera; D = reaction with pre-immune antisera. Each section was reacted with FITC-conjugated secondary antibodies. Magnification is 250X.
FIGURE 13: Cryostat section of stingray liver stained with methylene blue. Arrow indicates fat droplet. Magnification is 250X.
FIGURE 14: Isoelectric focusing of glutamine synthetase from dogfish and stingray tissues. A = dogfish brain; B = dogfish liver; C = stingray liver; E = stingray brain; F = stingray retina; G = stingray kidney. Each lane contains 100 µg whole tissue homogenate protein. The positions of two pl markers run on separate gels is shown on the right.
strong degree of structural and compositional homology among these isoymes. Stingray and dogfish liver and brain homogenates were run in a narrower range (pH 4-7), activity stained, then immunoblotted. Comparison of these procedures, shown in Figure 15, indicates a correspondence of enzyme activity and immunoreactivity, similar to what was seen in the subcellular fractions from enzyme assays and Western blots. Multiple bands seen in the immunoblot may be due to protein degradation, since whole tissue homogenates were prepared with only a single protease inhibitor.

**In vitro translation and immunoprecipitation**

Since most mitochondrial proteins are translated as larger molecular weight precursors, this possibility was investigated in the elasmobranchs. Stingray and dogfish liver glutamine synthetase *in vitro* translation products are shown in Figure 16. In both cases the immunoprecipitable peptides are larger than the mature subunits seen in each species. These results are in contrast to those reported for avian and alligator liver glutamine synthetase, wherein the mature mitochondrial subunits (42 kDa for the former, 45 kDa for the latter) are the same size as their translation products (Vorhaben, et al., '82; Smith and Campbell, '87). In the dogfish the translation product is 50 kDa, while the stingray translation product is 48 kDa. Both are 2 kDa larger than their respective mature subunits. In the stingray, a smaller peptide can also be recovered by immunoprecipitation that corresponds to the size of the brain cytosolic enzyme (42 kDa). Due to the abundance of protease inhibitors used in this procedure, it seems unlikely that this is the result of proteolysis, although it is always a possibility. **Figure 16** includes immunoprecipitation of shark and stingray translation products in the presence of excess unlabelled purified glutamine synthetase. In both cases, excess cold glutamine synthetase competed with the labelled translation products for the antibody, which confirms the specificity of antibody recognition. This figure also includes minus mRNA and nonimmune lanes, as controls.
FIGURE 15: Correspondence between activity staining and immunochromical reactivity following isoelectric focusing of stingray glutamine synthetase. A = liver, activity stained; B = brain, activity stained; C = liver, immunoblotted; D = brain, immunoblotted. Each lane contains 100 μg of whole tissue homogenate protein.
FIGURE 16: Translation of stingray and dogfish liver mRNA and immunoprecipitation of glutamine synthetase. A = no RNA added; B = total stingray liver translation products; C = immunoprecipitation of stingray liver translation with pre-immune antisera; D = as in C, but with anti-glutamine synthetase antibodies; E = as in D, but with competing levels (50μg) of purified chicken liver glutamine synthetase; F = immunoprecipitation of dogfish liver translation with anti-glutamine synthetase antibodies. Except where noted, translations were performed in rabbit reticulocyte lysate prepared as described in "Methods."
Stingray and dogfish ornithine transcarbamylase translation products are shown in Figure 17. Each is larger by 2–3 kDa than sizes seen by Western blot in the liver mitochondria. Stingray ornithine transcarbamylase is translated as 40 kDa peptide, in comparison to a 38 kDa mature subunit; dogfish translation product is 42 kDa, in contrast to a 39 kDa mature subunit. This is similar to that reported for rat liver ornithine transcarbamylase, which is translated with a 3-4 kDa leader sequence (Mori, et al., '80; Conboy and Rosenberg, '81).

Figures 18 and 19 demonstrate the glutamine synthetase translation products of stingray and dogfish brain. Here, the translation products are the same size as the mature subunits found in this tissue. Stingray brain glutamine synthetase is translated as a 42 kDa peptide, the size of the mature form. Dogfish brain mRNA produced a glutamine synthetase translation product of 43 kDa, which is the mature form size. Although an additional 47 kDa mitochondrial peptide is seen in Western blots of dogfish brain (see Figure 7), a corresponding translation product was not recovered. Figures 18 and 19 also show that, in both species, brain translation product could be eliminated from immunoprecipitation when an excess of unlabelled purified glutamine synthetase was present. These results strongly support specificity of the antibody for glutamine synthetase.

Hepatocyte and tissue labelling

Protein translation and translocation in whole cells was investigated by radiolabelling cells and tissues. Incorporation of [35S]-methionine into isolated stingray hepatocytes is shown in Figure 20. A linear increase in labelled trichloroacetic acid-precipitable protein is seen for up to 1 hour. Addition of cyclohexamide, a cytosolic translation inhibitor (Wu, et al., '85) virtually eliminates incorporation. In contrast, chloramphenicol, a mitochondrial translation inhibitor (Wu, et al., '85), only slightly decreases overall protein synthesis. Since it is known that uncouplers of oxidative
FIGURE 17: Translation of stingray and dogfish liver mRNA and immunoprecipitation of ornithine transcarbamylase. A = immunoprecipitation of dogfish liver translation with anti-ornithine transcarbamylase antisera; B = immunoprecipitation of stingray liver translation with anti-ornithine transcarbamylase antisera; C = as in B, but with anti-glutamine synthetase antibodies; D = total stingray liver translation products; E = no RNA added. Translations were in wheat germ extract.
FIGURE 18: Translation of total RNA from stingray brain and immunoprecipitation of glutamine synthetase. A = no RNA added; B = total stingray brain translation products; C = immunoprecipitation with pre-immune antisera; D = immunoprecipitation with anti-glutamine synthetase antibodies; E = as in D, with competing levels of purified chicken liver glutamine synthetase.
FIGURE 19: Translation of total RNA from dogfish brain and immunoprecipitation of glutamine synthetase. A = no RNA added; B = total dogfish brain translation products; C = immunoprecipitation using pre-immune antiserum; D = immunoprecipitation with glutamine synthetase antibodies; E = as in D, but with competing levels of purified chicken liver glutamine synthetase.
FIGURE 20: Radiolabelling of stingray hepatocytes. Time-dependent incorporation of $^{35}\text{S}$ methionine into trichloroacetic acid–precipitable proteins. "Ethanol" was 8 µl of 50% ethanol. Rhodamine 123 (1µg/mg cells), 10 µM dinitrophenol (DNP), and 1mM chloramphenicol were in 8 µl of 50% ethanol. Cyclohexamide was 1 mM. Each point equals 5 mg (wet weight) cells.
Radiolabelling of Stingray Hepatocytes

Radiolabel incorporation (cpm x 1000)

Time (minutes)

- Pulse
- Ethanol
- Rhodamine 123
- DNP
- Cyclohexamide
- Chloramphenicol
phosphorylation inhibit the import of many mitochondrial proteins (Reid, '85; Harmey and Neupert, '85), the effects of these were tested in vivo. This proved difficult to determine, however, because Rhodamine 123 and FCCP reduced the amount of total protein synthesis, and dinitrophenol eliminated it. Some of this decrease was shown to be due to the inhibitory effects of ethanol, in which some of the reagents were dissolved due to incompatibility with aqueous solvent.

Immunoprecipitation of glutamine synthetase from cells labelled in the presence of either cyclohexamide or chloramphenicol is shown in Figure 21. The absence of glutamine synthetase from the cyclohexamide lane, but its presence in the chloramphenicol lane, indicates the peptide is cytoplasmically translated from nuclear-encoded genes (Wu, et al., '85). Immunoprecipitation of glutamine synthetase from pulse-labelled hepatocytes is shown in Figure 22. An increase in the amount of 45 kDa peptide, corresponding to the mature size, is seen with time. No precursor is detected at any time-point, suggesting rapid processing following translation of the peptide (Reid, '85; Harmey and Neupert, '85). A nonimmune precipitation confirms the specificity of the antibodies.

Figure 23 shows the results of immunoprecipitation of pulse/chasing and pulsing in the presence of inhibitors. In the chased samples, the amount of processed labelled glutamine synthetase increases after addition of cold methionine, suggesting the labelled precursor is "chased" through import. Again, though, no precursor is seen, possibly indicating rapid processing. Also as seen in Figure 23, cells pulsed in the presence of FCCP, a mitochondrial uncoupler, do not continue to accumulate processed labelled glutamine synthetase. Again, though, no precursor can be detected. This may be due to degradation of the precursor peptide in the cytosol when import is blocked (Reid and Schatz, '82; Raymond and Shore, '81).
FIGURE 21: Effect of cytoplasmic and mitochondrial translation inhibitors on glutamine synthetase synthesis in stingray hepatocytes. Immunoprecipitation of glutamine synthetase from cells pulsed 1 hour with $[^{35}\text{S}]$ methionine in the presence of 1 mM chloramphenicol (A) or 1 mM cyclohexamide (B). Each lane is from 50 mg (wet weight) of cells.
FIGURE 22: Immunoprecipitation of glutamine synthetase from pulse-labelled stingray hepatocytes. A = immunoprecipitation from 60-minute labelled cells with pre-immune antisera; B = immunoprecipitation with glutamine synthetase antibodies from 0 minute pulse; C = as in B, but from 15 minute pulse; D = as in B, but from 30 minute pulse; E = as in B, but from 45 minute pulse; F = as in B, but from 60 minute pulse; G = as in B, but from 75 minute pulse. All immunoprecipitations were from 50 mg (wet weight) cells.
FIGURE 23: Stingray hepatocytes pulse/chased in the presence and absence of a mitochondrial inhibitor. A–C = with FCCP (5µM), A = 30 minute chase, B = 20 minute chase, C = 10 minute chase; D–F = without FCCP, D = 30 minute chase, E = 20 minute chase, F = 10 minute chase. After a 30 minute pulse with [³⁵S]methionine, 10 mM cold methionine and 1 mM cyclohexamide were added to start the "chase." Immunoprecipitations were with glutamine synthetase antibodies from 50 mg (wet weight) cells.
Recovery and stability of stingray hepatocytes was significantly hampered by the amount of lipid contained in the cells, which caused them to float and spontaneously lyse. Elasmobranch liver contains extremely high amounts of lipid (Oguri, '78; Smith et al., '87), and this is magnified by seasonal increases in liver lipid stores (Rossouw, '87). The prevalence of large fat droplets can be seen in the stained section in Figure 13. In order to forgo the problems associated with floating cells, liver and kidney sections were prepared and labelled. The incorporation of \(^{35}\text{S}\)-methionine into stingray kidney sections is shown in Figure 24. The plot indicates that synthesis of proteins is linear with time. The total amount of incorporation was less in whole tissue (6.1 \times 10^3 \text{cpm}/50 \text{mg}) than in isolated cells (18.1 \times 10^4 \text{cpm}/50 \text{mg}). This is likely due to limits of diffusion in tissue slices versus individual cells. As with the hepatocytes, in the presence of uncouplers and inhibitors, incorporation of radiolabel into proteins declines. Also, cyclohexamide nearly eliminates labelling while chloramphenicol only reduces it slightly.

Immunoprecipitation from labelled kidney sections, as shown in Figure 25, recovered a 45 kDa peptide, which is the mature size in this tissue. As in the hepatocyte experiments, no evidence of precursor was seen with any of the metal chelators, uncouplers, or inhibitors used (EDTA, p-aminobenzamidine, p-chloromercuribenzoate, 8-hydroxyquinoline, Rhodamine 123, FCCP, cyanide, or dinitrophenol). Also in this figure is shown the effects of cyclohexamide and chloramphenicol on glutamine synthetase synthesis. Glutamine synthetase was translated in the presence of chloramphenicol, but not cyclohexamide. As with hepatocytes, this indicates cytoplasmic translation of nuclear-encoded mRNA.

**Mitochondrial isolation and precursor processing**

The metabolic integrity of isolated mitochondria was investigated by assaying their degree of coupling and oxygen consumption. The results of
FIGURE 24: Radiolabelling of stingray kidney slices. Time-dependent incorporation of $[^{35}\text{S}]$methionine into trichloracetic acid-precipitable proteins. The start of the "chase," indicated by the arrow, was with the addition of 10 mM cold methionine and 1 mM cyclohexamide. FCCP = 5 μM; Rhodamine 123 = 1 μg/mg tissue; cyclohexamide = 1 mM; chloramphenicol = 1 mM, in 8 μl of 50 % ethanol. Each point equals 5 mg (wet weight) of tissue.
FIGURE 25: Immunoprecipitation of glutamine synthetase from pulsed stingray kidney slices. A = immunoprecipitation from 60-minute pulse with pre-immune antisera; B = immunoprecipitation with glutamine synthetase antibodies from 10 minute pulse; C = as in B, but from 20 minute pulse; D = as in B, but from 30 minute pulse; E = as in B, but from 40 minute pulse. The effects of chloramphenicol and cyclohexamide on glutamine synthetase synthesis are seen in F and G. F = pulse in the presence of 1 mM chloramphenicol; G = pulse in the presence of 1 mM cyclohexamide.
oxygen uptake and respiratory control ratio assays of isolated stingray liver mitochondria are shown in Figure 26. For comparison, rat liver mitochondria were assayed. In the best set of experiments, stingray mitochondria utilized 8 nmol O$_2$/min/mg protein, with respiratory control ratios of 1.65, with succinate as a substrate. Different isolation media and conditions, as well as other substrates, gave similar results. Dogfish liver mitochondria, with succinate as a substrate, yield respiratory control ratios of 2.0–3.5, with O$_2$ uptake of 10-15 nmol/min/mg protein (Anderson and Casey, '84). Under similar conditions, skate liver mitochondria (Raja erinaceae) yield respiratory controls ratios of 3.5–4.7, with 12 nmol O$_2$/min/mg protein (Moyes, et al., '86). By comparison, rat liver mitochondria had respiratory control ratios of 3.7–5.5, with 20 nmol O$_2$/min/mg protein taken up. The weaker coupling seen in isolated elasmobranch mitochondria may be due to the abundance of fatty acids in the homogenates, which are known to uncouple mitochondria (Estabrook, '67). Attempts to block fatty acid uncoupling by adding increasing amounts bovine serum albumin (Estabrook, '67) were unsuccessful.

Isolated stingray liver mitochondria were incubated with labelled ray liver translation product to determine if glutamine synthetase would be imported and processed. Figure 27 shows the results of immunoprecipitation of glutamine synthetase from the separated pellet and supernant mixture of this type of experiment. For comparison, translation product alone is included. Glutamine synthetase was not imported into whole mitochondria, as is evident by the larger size present in the supernant mixture lane. Repeated experiments with intact mitochondria remained unsuccessful, even in the presence of an ATP-regenerating system, which has been shown to be neccessary for import of some mitochondrial proteins (Pfanner and Neupert, '86; Eilers, et al., '87). Others have also reported difficulty with translocation of proteins into whole mitochondria (Mihara, et al., '82; Shore, et
FIGURE 26: Comparison of oxygen uptake by isolated mitochondria. Tracings from oxygraph recordings. Upper tracing = rat liver mitochondria; lower tracing = stingray liver mitochondria. Respiratory control ratios (R.C.R.) were determined from the slopes before and after the addition of ADP. Each assay contained 2 mg mitochondrial protein.
1. mitochondria (2 mg)
2. succinate (10 mM)
3. ADP (200 μM)

R.C.R. = 3.65

20 nmol O₂/min/mg protein

RAT LIVER MITOCHONDRIA

1. mitochondria (2 mg)
2. succinate (3 mM)
3. ADP (1 mM)

R.C.R. = 1.65

8 nmol O₂/min/mg protein

RAY LIVER MITOCHONDRIA

1.9 nmol O₂

20 seconds
FIGURE 27: Attempted translocation of glutamine synthetase into isolated stingray mitochondria. Total translation products of stingray liver mRNA were incubated with whole stingray mitochondria (2 μg mitochondrial protein/μl translation mix) for 1 hour, then the mixture was separated into pellet and supernatant fluid. Glutamine synthetase was immunoprecipitated from both fractions, as well as from translation mixture without added mitochondria. A = no RNA added; B = total stingray liver translation products; C = pre-immune antisera; D = immunoprecipitation from supernatant fluid; E = as in D, but from pellet; F = as in D, but from translation mixture alone.
al., '83; Nguyen, et al., '86).

Since whole mitochondria could not translocate precursor peptides in vitro, activity of the processing protease alone was investigated. This was accomplished by lysing mitochondria, then incubating the lysate with labelled precursor peptides. If the protease were active, these precursors should be cleaved to the mature sizes. The results of immunoprecipitation of glutamine synthetase from this type of experiment are shown in Figure 28. Again, whole mitochondria showed no processing of the translation product, but when the same mitochondria were lysed, the lysate processed the 47 kDa precursor peptide to the 45 kDa mature size. So, although the precursor was unable to be imported by whole ray mitochondria, the processing protease could function when exposed to the translation product. In addition, as seen in Figure 29, the amount of processed peptide increased with time of incubation with the mitochondrial lysate.

To investigate the species-specificity of the processing protease, ray liver mitochondrial fragments were incubated with shark liver translation products. Figure 30 demonstrates the results of this experiment. Stingray mitochondria did process the shark 50 kDa translation product, but the cleavage product was the size of the mature stingray peptide (45 kDa), not the mature shark peptide (47 kDa). This suggests the size of the cleavage product was determined by the processing protease. Two types of heterologous processing have been reported. Carbamylphosphate-1 precursor is processed by mitochondria from tissues that express the enzyme, but not from tissues that do not express it (Bhat and Avadhani, '85). On the other hand, ornithine transcarbamylase is processed by mitochondria from different tissues, whether or not they express the enzyme (Miura, et al., '82). In both types of experiments, the cleavage products are the native mature enzyme size. Of interest, then, would be the reciprocal experiment, where shark liver mitochondria are incubated with ray liver translation product.
FIGURE 28: Processing of glutamine synthetase precursor by whole versus lysed stingray mitochondria. Stingray liver mRNA translation products were incubated with either whole or lysed isolated stingray liver mitochondria (2 µg mitochondrial protein/µl translation mixture) for 1 hour. Immunoprecipitation with glutamine synthetase antibodies from (A) whole mitochondria, (B) lysed mitochondria, (C) translation products alone; D = immunoprecipitation with pre-immune antiserum.
FIGURE 29: Time-dependent processing of glutamine synthetase precursor by stingray mitochondrial lysate. Stingray liver translation products were incubated with lysed stingray liver mitochondria, and samples from different timepoints were immunoprecipitated with glutamine synthetase antibodies. A = no RNA added; B = total stingray liver translation products; C = glutamine synthetase precursor; D = as in C, with lysed mitochondria for 0 minute incubation; E = 10 minutes; F = 20 minutes; G = 30 minutes; H = 40 minutes. Each sample contained 2 μg mitochondrial protein/μl translation mixture.
FIGURE 30: Processing of stingray and shark glutamine synthetase precursor by stingray liver mitochondrial lysate. A = no RNA added; B = total stingray liver translation products; C = pre-immune antisera; D = stingray glutamine synthetase precursor; E = dogfish glutamine synthetase precursor; F = stingray GS precursor with stingray mitochondrial lysate; G = dogfish GS precursor with stingray mitochondrial lysate. Both processing incubations were for 1 hour, with 2 μg mitochondrial protein/μl translation mixture.
However, this data must await further collection of shark tissue.

Attempts to inhibit the mitochondrial protease with the protease inhibitors phenylmethylsulfonyl fluoride, p-chloromercuribenzoate, antipain, leupeptin, pepstatin, and chymostatin, and the metal chelators p-aminobenzamidine, o-phenanthroline, 8-hydroxyquinoline, EDTA and EGTA, were unsuccessful. The majority of mitochondrial and chloroplast processing proteases that have been described are metalloproteases that are not blocked by inhibitors of trypsin or other serine proteases (Doonan, et al., '84; Ellis and Robinson, '85). However, a chloroplastic protease was recently reported that was unable to be inhibited by methods shown to inhibit other processing proteases, and in fact was activated by metal chelating agents (Kirwin, et al., '87).

Northern blots

The RNA species that code for the glutamine synthetase isozymes found in elasmobranch tissues were identified by Northern blot analysis of stingray and dogfish RNA with a bovine retina glutamine synthetase probe. These results are shown in Figure 31. Stingray kidney, liver, and brain all have a major 3.0 kb mRNA band. These data are comparable with the reported sizes of the dominant RNA species (2.8–3.2 kb) for glutamine synthetases from Texas tortoise, alligator, hamster, chicken, duck, rat, mouse and cow (Smith and Campbell, '88), and in 3T3-L1 adipocytes (Bhandari, et al., '86). An exception to this was found with dogfish kidney, liver, and brain, which all produce a predominant 4.1 kb mRNA. In both species, one size of message codes for the isozymes seen in the tissues. So, it seems that in elasmobranchs a single-sized message gives rise to two different sizes of translation product, apparently by some tissue-specific mechanism.

Southern blots

Since isozymes of plant glutamine synthetase are shown to be encoded by several genes (Tingey, et al., '87), the number of gene species
FIGURE 31: Northern blot analysis of elasmobranch total RNA for glutamine synthetase. A = dogfish kidney RNA; B = dogfish brain RNA; C = stingray kidney RNA; D = stingray brain RNA; E = stingray liver RNA. The probe was from a bovine retina library, as described in "Methods." Each lane contains 50 μg RNA. Sizes of the bands are noted in kilobases (kB).
coding for the glutamine synthetase isozymes in stingray tissues was examined. **Figure 32** demonstrates the results of restriction enzyme cleavage and Southern blot analysis of stingray DNA with the bovine retina glutamine synthetase cDNA probe used for the Northern blots. Restriction cutting with EcoRI, Bam HI and Hind III resulted in the formation of a single band with each enzyme (Eco RI=1.6 kB, Bam HI=2.4 kB, Hind III=2.8 kB). This lack of complexity of the restriction fragments suggests the presence of a single gene (Lewin, '83). So, unlike plant isozymes, the glutamine synthetase isozymes found in the stingray appear to be encoded by a single gene.
FIGURE 32: Southern blot analysis of elasmobranch DNA for glutamine synthetase. A = Eco RI; B = Hind III; C = Bam HI. Each lane contains 100 µg dialysed DNA. The probe was from a bovine retina library, as described in "Methods." The size of each fragment is noted in kilobases (kB).
DISCUSSION

While many isozymes of glutamine synthetase are known in plants (Gebhardt, et al., '86; Bhandari, et al., '86; Tingey, et al., '87), none have been previously identified in animals. Data presented here demonstrate the first isozymes of glutamine synthetase in animals. In chondrichthyean fish, mitochondrial glutamine synthetase (47 kDa shark, 45 kDa ray and ratfish) is larger than the cytosolic (43 kDa shark, 42 kDa ray and 43.5 kDa ratfish) isozyme. Both forms cross-react with antibodies generated to chicken mitochondrial glutamine synthetase, indicating a highly conserved epitope structure among the isozymes (Zaleski, et al., '83). The shark and stingray isozymes have the same isoelectric points (the ratfish has not been assayed). This is somewhat surprising given the 2-5 kDa size difference between the forms. However, if the additional residues are neutral, or, if charged, cancel each other, then the overall charge of the molecules could be identical (Lehninger, '83). The molecular basis for the formation of these isozymes is therefore of considerable interest (see discussion below).

The hepatic compartmentation of stingray and ratfish glutamine synthetase, ornithine transcarbamylase, and carbamylphosphate synthetase—III shown here expands the work of others (Casey and Anderson, '82; '85) in characterizing intramitochondrial detoxification of ammonia and the ultimate production of urea by ureosmotics. These primitive vertebrates have intramitochondrial enzymes that function in the first steps of both mammalian and avian/reptilian ammonia metabolism, with a form of carbamylphosphate synthetase that uses the product of the glutamine synthetase reaction as a substrate for the synthesis of carbamyl phosphate. Some have suggested (Casey and Anderson, '82) that by having these enzymes in the same subcellular compartment, ureosmotics may exploit compartmentation as mechanism for the metabolic regulation of urea production for osmoregulation. Also, ureosmotics establish the precedent of
urea formation at the beginning of the vertebrate line (Campbell, et al., '87).

The compartmentation of glutamine synthetase in elasmobranch tissues is the same as that previously shown in chicken (Vorhaben and Campbell, '72), where glutamine synthetase is predominantly cytosolic in the brain and mitochondrial in the liver. In the chicken, though, the two different compartmental forms are the same size. In addition, the two forms are immunologically cross-reactive, generate identical peptide maps, and have the same isoelectric points (Smith and Campbell, '83).

There has been some dispute about the evolutionary relatedness of holocephalans and elasmobranchs. Based on anatomical comparisons, some place the holocephalans closer to the placoderms, the extinct armored fishes (Patterson, '65; Stahl, '67). Others classify them as Chondrichthyesans, along with the elasmobranchs (Schaeffer and Williams, '77; Young, '81). In either case, they synthesize and retain high levels of urea for osmoregulation (Dakin, '31; Rasmussen, '80). Like elasmobranchs, they possess urea cycle enzymes (Read, '67), including carbamylphosphate synthetase-III (Anderson, '80). The kinetics of some of these enzymes are comparable to those in elasmobranchs (Read, '70), with high levels of glutamine synthetase (Webb and Brown, '80). The tissue-specific expression of compartmental isozymes of glutamine synthetase provides significant evidence for the ancestral relatedness of these two groups.

Proteins that are imported into the mitochondrial matrix are typically translated with an amino-terminal signal sequence that targets the precursor to the organelle. For compartmental isozymes, only the mitochondrial form is translated with this signal (Harmey and Neupert, '85). Thus, the mitochondrial form is imported while the cytosolic form is not. Data presented here show that elasmobranch liver glutamine synthetase is synthesized in the cytosol from nuclear-encoded genes (as evident from cyclohexamide inhibition) with a detectable leader sequence that is cleaved during mitochondrial import. In
the brain, the nascent glutamine synthetase translation product (42 kDa ray, 43 kDa shark) is the same size as the mature peptide, and it remains in the cytosol. This is comparable to "conventional" compartmental isozymes such as phosphoenolpyruvate carboxykinase (Hod, et al., '82) and aspartate aminotransferase (Graf-Hausner, et al., '83).

The mechanism of mitochondrial import of glutamine synthetase in higher vertebrates seems to be quite different from that in elasmobranchs. In birds (Smith and Campbell, '83) the cytosolic brain form is the same size as the mitochondrial liver form (42 kDa), and both translation products are the sizes of the mature peptides. Also, while hepatic glutamine synthetase is mitochondrial in uricoteles, it is cytosolic (43 kDa in rat) in ureotelic liver (Deuel, et al., '78). It would thus appear that the mechanism by which glutamine synthetase is imported into mitochondria has evolved from using a cleavable presequence to using some other targeting system that can be expressed in a tissue— and species—specific manner. This strongly contrasts with what is seen for another urea cycle enzyme, ornithine transcarbamylase. As shown here, elasmobranch ornithine transcarbamylase is translated as a larger molecular weight precursor (40 kDa ray) than the native mitochondrial form (38 kDa). This mechanism has been conserved in the higher vertebrates; in ureoteles, the mature subunit size is 36–39 kDa, which results from the cleavage of a 3–4 kDa precursor (Conboy, et al., '79; Mori, et al., '80; Morita, et al., '82).

Recent evidence suggests that the signal sequence itself is not as specific as previously proposed—in fact, a large number of random sequences seem to target proteins for mitochondrial import (Warren, '87). It now appears that a "domain" structure is the active feature of imported protein sequences. The "domain" is an amphiphilic helix that arranges positively-charged and hydrophobic amino acids opposite each other (Allison and Schatz, '86; von Heijne, '86; Douglas et al., '86). It may be that glutamine synthetase evolution
has been from a cleavable "domain" to an internalized "domain." Information on this possibility must await further sequence data from both the elasmobranch and avian mitochondrial enzymes.

From stingray hepatocyte pulse-labelling experiments it appears the precursor of glutamine synthetase is translocated rapidly, or is degraded when import is blocked. The half-life of some precursor proteins is very short, \( \leq 2 \) minutes (Mori, et al., '81; Raymond and Shore, '81; Morita, et al., '82; Shore, et al., '83), and only small amounts are seen in the cytosolic compartment prior to import (Raymond and Shore, '81). Also, some precursors have proved very difficult to detect in intact cells (Fenton, et al., '84; Horwich, '84). Further experiments with hepatocytes that may help to identify glutamine synthetase precursor would be to try to inhibit cytosolic degradation of non-imported precursor. For example, vanadate inhibits intracellular degradation of some proteins without adversely affecting translation (Seglen and Gordon, '81). Since vanadate has been used successfully in adipocytes (Dubyak and Kleinzeller, '80), it may work well with the high-lipid ray hepatocytes. Alternatively, the effect of specific glutamine synthetase inhibitors could be examined. The approach has been used successfully with apocytochrome c (Henning and Neupert, '81), where deuterohemin (an analog of hemin, which binds to mature cytochrome c) was shown to block import. For example, methionine sulfoxime irreversibly binds to glutamine synthetase, and is permeable to cell membranes (Rowe, et al., '69). If this compound were incubated with isolated cells under during protein labelling, perhaps it could block import of glutamine synthetase by binding to the precursor peptides. Recently, other inhibitors of glutamine synthetase have been described (Farrington, et al., '87) that could also be tested. Another method to identify precursor could be to slow down translocation by lowering the reaction temperature. If this can be done without significantly decreasing translation, precursor could possibly remain in the cytosol long enough to be
detected. Addition of an intracellular protease inhibitor (as described above) might prevent rapid degradation of the precursor, thereby increasing the chance of successfully isolating any precursor peptides.

The results of reconstitution experiments, where precursors translated in a cell-free system were combined with isolated mitochondria, suggest that translocation and processing are separate events. While intact mitochondria could neither import nor process glutamine synthetase, lysed mitochondria cleaved the precursor into mature-sized peptides. The inability of isolated mitochondria to import \textit{in vitro} synthesized precursor peptides has been reported by others (Mihara, et al., '82; Shore, et al., '83; Nguyen, et al., '86).

There are several reasons why isolated elasmobranch mitochondria may not be able to function in translocation. Inhibitors and uncouplers of oxidative phosphorylation and compounds that dissipate the mitochondrial energy charge are known to inhibit translocation (Reid, '85; Harmey and Neupert, '85). Elasmobranch liver has a high fatty acid content, and fatty acids are potential uncouplers (Estabrook, '67). Homogenization of liver to isolate mitochondria allows the fatty acids to act on the mitochondria, possibly reducing coupling. Results of oxygen uptake assays indicated the isolated mitochondria were not tightly coupled. Also, elasmobranch mitochondria function under high osmotic conditions (Anderson, '86a). Although a variety of respiration media were tested, including those reported to work for shark and skate mitochondria, isolated stingray mitochondria appeared to be weakly coupled, at best. Reconstitution experiments were tested with media of varying composition and osmotic strength, with negative results. Mitochondrial import of proteins appears to be dependent upon ATP (Pfanner and Neupert, '86; Eilers, et al., '87), but the addition of an ATP-regenerating system to the reaction mixture did not affect translocation. Since mitochondria in intact cells import glutamine synthetase \textit{in vivo}, the inability of isolated mitochondria to translocate it \textit{in vitro} must be an artefact of the preparation.
As shown by continued activity in the presence of metal chelators, the stingray mitochondrial signal sequence protease does not appear to be metal-dependent, unlike others that have been reported (Harmey and Neupert, '85; Ellis and Robinson, '85) where processing was inhibited by a number of metal chelators. The elasmobranch signal sequence protease was not inhibited by serine proteases or microbial proteases. Other mitochondrial signal processing proteases have been reported to respond similarly (Ellis and Robinson, '85). Recently, a signal sequence protease has been described in chloroplasts that was not inhibited by conventional protease inhibitors, and was in fact activated by the metal chelators EDTA and EGTA (Kirwin, et al., '87). Further characterization of the elasmobranch processing protease might better describe the relationship between translocation and processing during mitochondrial import of proteins, and could yield significant information about the evolution of the glutamine synthetase import mechanism.

The processing by ray mitochondria of glutamine synthetase precursor from both ray and shark (which are different sizes) to equal-sized peptides was quite unexpected. This suggests sequence-specific cleavage by the protease. Tissue-specific and species-specific heterologous processing of precursor peptides has been shown for some proteins. Carbamylphosphate synthetase-I can be imported only by mitochondria from tissues which express the enzyme (Bhat and Advahani, '85). On the other hand, ornithine transcarbamylase can be imported by mitochondria from a variety of tissues, even if they do not express the enzyme (Mori, et al., '81; Morita, et al., '82). In addition, ornithine transcarbamylase can be imported by mitochondria from different species (Takiguchi, et al., '83). As yet there is no evidence for sequence-specific processing of precursors, although there appears to be a requirement of arginine, serine, leucine, and the initial methionine (Allison and Shatz, '86). There are many interesting investigations of this mechanism
that can be performed with glutamine synthetase, since the sizes of both the precursors and the mature peptides vary between species. What size product results from shark liver mitochondria processing of ray precursor? Do hepatic mitochondria from uricoteles process elasmbranch precursor, and if so, what size is the cleavage product? These heterologous processing experiments, in conjunction with amino acid sequence information, could be used to determine the specificity of the elasmbranch protease for particular sequences.

The isozymes of elasmbranch glutamine synthetase appear to be translated from single dominant RNA species in individuals. In the stingray, this size corresponds to those reported for other vertebrates (3.2 kB). In the shark, however, the dominant RNA is larger than these (4.1 kB), which may be related to the fact that the shark translation product is also larger than those of other vertebrates. There are several ways a single primary transcript can give rise to two proteins. The elasmbranch transcripts are several times larger than necessary to produce the peptides seen, considering 1 kB ~ 37 kDa (which calculates to 111 kDa for ray). Most primary transcripts contain 5' and 3' untranslated regions, and many have internal splice sites (Lewin, '83). Post-transcriptional modification of the RNA can eliminate or rearrange segments of the message before it is used for protein synthesis. In elasmbranches, this could result in one transcript with a leader sequence and one without it. An example of this type of differential processing is seen in the immunoglobins. Two different 3' ends can be generated from a single RNA by differential splicing, yielding one message that codes for secreted IgM and another that codes for a membrane-associated IgM (Early, et al., '80). Two polyadenylation signals have been identified in compartmental isozymes of mouse aspartate aminotransferase (Obaru, et al., '86). Another possibility is the presence of two start sites on the RNA. Two start sites have been identified for yeast histidine tRNA synthetase, where one produces a
mitochondrial peptide with a leader sequence and the other yields a cytosolic peptide that lacks this sequence (Natsoulis, et al., '86). Najarian, et al. ('87) also report the presence of two translation start sites in yeast. Recently, magnesium control of start-site selection in viral RNA translation has been reported (Sanger, et al., '87). Also in yeast, a single RNA precursor has been identified that yields two mitochondrial proteins (Wadinger-Ness and Weiss, '87). It could be that only one of the start sites might include the signal region coding for mitochondrial import. If multiple start sites existed, then the two forms should have different amino terminal sequences.

Results obtained by Southern blot analysis suggest that elasmobranch glutamine synthetase is encoded by a single gene. However, the probe used was specific for the carboxy terminal of the sequence, whereas any differences in the isozymes might be expected at the amino terminal. Also, these data might not identify interrupted genes or closely-spaced tandem genes, if the restriction sites were outside of the entire coding region. In plants, which heretofore had the only known isozymes of glutamine synthetase, there is a family of four genes that encode the enzymes (Cullimore, et al., '84). Nonetheless, there is metabolic evidence to support the existence of a single gene in the rat (de Groot, et al., '87). In addition, the compartmental isozymes of glutamine synthetase are immunologically cross-reactive among all vertebrate classes (Smith, et al., '83), indicating highly-conserved epitopes in each molecule (Zaleski, et al., '83). A single gene has been shown to produce tissue-specific isozymes of porphobilinogen deaminase, possibly by the action of different promoters (Grandchamp, et al., '87). In a situation that may more closely describe the elasmobranch system, Elferink, et al. ('87) have indicated that a single gene and a single mRNA species generate isozymes of 5-aminolevulinate in chickens.

Several experiments could be performed with the use of either a genomic clone to glutamine synthetase, or cDNA clones to the RNA transcripts
that may provide a better understanding of the nature of the molecular-level events in the expression of the elasmobranch isozymes. S1 nuclease mapping (Berk and Sharp, '77) could be performed to see if the gene for glutamine synthetase is continuous or interrupted, and to determine the size of the coding region(s). A cDNA clone to each isozyme might be used with RNase mapping (Melton, et al., '84) to identify differences in the transcripts that give rise to the tissue-specific compartmental isozymes. The former experiments would reveal the presence of introns and exons in the glutamine synthetase gene, while the latter might identify differential splicing of RNA transcripts.

The initial objectives of this work were to determine the subcellular compartmentation of glutamine synthetase in elasmobranch tissues, to discover the nature of its translation product, and to investigate its import into mitochondria. Data acquired in pursuit of these goals lead to the first identification of tissue-specific isozymes of glutamine synthetase in animals. Based on these findings, further evidence was obtained for the evolutionary relationship between holocephalans and elasmobranchs. It was established that elasmobranch glutamine synthetase and ornithine transcarbamylase are formed as larger precursors that are cleaved with import into mitochondria. While the mechanism of mitochondrial import of ornithine transcarbamylase seems to have been conserved during evolution, the import of glutamine synthetase in higher vertebrates appears to have lost the dependence on a large, cleavable leader sequence for mitochondrial translocation. The processing protease for glutamine synthetase was shown to be inside the mitochondria, and preliminary data suggest this protease is sequence-specific. The RNA species for elasmobranch glutamine synthetase were identified, with two significant features. First, in both the shark and the stingray, it appears that one dominant RNA yields two tissue-specific subunit forms, one of which contains the information needed for mitochondrial import.
Second, while the size of stingray glutamine synthetase mRNA is similar to those reported for other animals, the shark mRNA is the largest known for vertebrate glutamine synthetase. Finally, elasmobranch glutamine synthetase isozymes were shown to be most likely encoded by a single gene. The data obtained in this project have provided the background needed for further examination of the cellular and molecular basis of glutamine synthetase expression and compartmentation during development and evolution.
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