SUBMITOCHONDRIAL LOCALIZATION AND FUNCTION OF ENZYMES OF GLUTAMINE METABOLISM IN AVIAN LIVER

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ABSTRACT
Glutamine synthetase (EC 6.3.1.2) was localized within the matrix compartment of avian liver mitochondria. The submitochondrial localization of this enzyme was determined by the digitonin-Lubrol method of Schnaitman and Greenawalt (35). The matrix fraction contained over 74% of the glutamine synthetase activity and the major proportion of the matrix marker enzymes, malate dehydrogenase (71%), NADP-dependent isocitrate dehydrogenase (83%), and glutamate dehydrogenase (57%). The highest specific activities of these enzymes were also found in the matrix compartment.

Oxidation of glutamine by avian liver mitochondria was substantially less than that of glutamate. Bromofuroate, an inhibitor of glutamate dehydrogenase, blocked oxidation of glutamate and of glutamine whereas aminooxyacetate, a transaminase inhibitor, had little or no effect with either substrate. These results indicate that glutamine metabolism is probably initiated by the conversion of glutamine to glutamate rather than to an α-keto acid. The localization of a transaminase activity within avian liver mitochondria plus the absence of an active mitochondrial glutamine transaminase is consistent with the differential effects of the transaminase and glutamate dehydrogenase inhibitors. The high glutamine synthetase activity relative to glutaminase activity (40:1) suggests that mitochondrial catabolism of glutamine is minimal, freeing most of the glutamine synthesized for purine (uric acid) biosynthesis.

Glutamine synthetase and carbamyl phosphate synthetase-I appear to perform parallel functions in liver of uricotelic and ureotelic species, respectively (2, 3, 8, 39). These mitochondrial enzymes catalyze the primary ammonia-detoxifying step in pathways leading to the synthesis of urea or uric acid. During the catabolism of amino acids, ammonia released within the mitochondrion by the action of glutamate dehydrogenase is converted either to citrulline (via initial formation of carbamyl phosphate) in the case of ureoteles or to glutamine in uricoteles. Citrulline and glutamine then leave the mitochondrion and enter the cytosol where they are converted to either urea or uric acid for excretion. Confirmation of this cellular mechanism for ammonia detoxification by uricoteles was recently obtained through heavy isotope tracer studies (3).

The results reported here further emphasize the analogy between uricotelic glutamine synthetase and ureotelic carbamyl phosphate synthetase-I by establishing yet another correlation between these enzymes, namely that of a common submitochondrial localization. Additional studies indicate that avian mitochondria possess the capacity to oxidize glutamine, albeit at a slow rate, and that any
mitochondrial metabolism of glutamine probably occurs through the coupled reactions of a glutaminase and glutamate dehydrogenase.

MATERIALS AND METHODS

Materials

Cytochrome c, succinic acid, oxalacetic acid, pyruvate, α-ketoglutarate, isocitrate, glyoxylate, phenylpyruvate, glutamine, glucose-6-phosphate, deoxyribose triphosphate, ATP, ADP, AMP, digitonin, rotenone, aminoxyacetic acid, glutamate dehydrogenase, hexokinase, and glucose-6-phosphate dehydrogenase were products of Sigma Chemical Co. (St. Louis, Mo.). 4-Hydroxy-3-methoxy-benzylamine was purchased from ICN Pharmaceuticals, Inc. (Cleveland, Ohio); 5-bromofuroic acid, from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); Lubrol WX, from I. C. I. America, Inc. (Stanford, Conn.); L-amino acid oxidase, from Calbiochem (San Diego, Calif.); and the defatted bovine serum albumin for the mitochondrial isolating medium, from Miles Laboratories Inc. (Elkhart, Ind.). [u-laC]Glutamine was obtained from New England Nuclear (Boston, Mass.); the [U-14C]-α-ketoglutarate used as a substrate in the glutamine transaminase assay was synthesized by the α-amino acid oxidase-catalyzed oxidation of [U-14C]glutamine according to the procedure of Meister (29). All other reagents were of the highest commercial quality.

Methods

SUBMITOCHONDRIAL FRACTIONATION

Mitochondria were prepared from 48-h-fasted white Leghorn chickens and were further fractionated by the digitonin-Lubrol procedure of Schnaitman and Greenawalt (35), as later modified by Kalra and Brosnan (22); isocitrate dehydrogenase, according to Plant and Sung (34); malate dehydrogenase, according to Ochoa (31), except that Tris-hydrochloride buffer was used for the glycylglycine buffer and the samples were activated by Lubrol as described by Schnaitman and Greenawalt (35); and glutamate dehydrogenase, according to Olson and Anfinsen (32).

ASSAYS FOR THE ENZYMES OF GLUTAMINE METABOLISM: Glutamine synthetase was assayed according to Curthoys and Kuhlenschmidt (12) using α-ketoglutaric acid, methionine, and glutamine as glutamyl acceptors; glutamate transaminase (kidney form), according to Kupchik and Knox (26); and phosphate-dependent and phosphate-independent glutaminases, according to Curthoys and Weiss (13).

Phosphate-dependent glutaminase activity was also determined radiometrically. Reaction mixtures at pH 8.6 contained 20 mM [14C]glutamine of specific radioactivity 0.002 μCi/μmol, 0.15 M potassium phosphate, 0.2 mM EDTA, and 50 mM Tris-hydrochloride. The conditions were similar to those used by Curthoys and Weiss (13) in the spectrophotometric assay of phosphate-dependent glutaminase. Reactions were initiated by enzyme addition and were terminated by perchloric acid addition. Incubations were carried out for 1 h at 37°C. After centrifugation, the supernatant fluids were neutralized with potassium hydroxide and the precipitated perchlorate was removed by centrifugation. Glutamate and unreacted glutamine were separated by means of electrophoresis in 0.05 M ammonium formate, pH 7.2, and counted as previously described (38).

Glutamine transaminase (liver form) was measured by two radiometric procedures. In the first, enzyme samples were incubated with 20 mM [U-14C]glutamate (specific radioactivity 0.05 μCi/μmol), 20 mM glyoxylate, and 50 mM Tris-hydrochloride buffer, pH 8.4, for 1 h at 37°C (9). Controls were reaction mixtures without enzyme or with heat-treated enzyme. The [14C]α-ketoglutarate was separated from unreacted [14C]glutamine by elution from a Dowex 50 column (Dow Chemical Co., Midland, Mich.) according to procedure two of Cooper and Meister (9). Instead of counting an aliquot of the column effluent as described, additional separation of α-ketoglutarate and glutamine was achieved by paper electrophoresis (38). Under these conditions, α-ketoglutarate migrates 10.5 cm from the origin compared to 5.5 cm for glutamine.
and counted in a Permablend-toluene mixture (38) (Packard Instrument Co., Inc., Downers Grove, Ill.). Heat-treated enzyme and reaction mixtures which did not contain enzyme were used as controls. A second procedure for assaying glutamine transaminase utilized \([14C]\alpha\text{-keto-glutarate}\) (0.02 \(\mu\text{Ci/\mu mol}\)) as the amino group acceptor and glutamine as the amino donor. Reaction conditions and controls were essentially those just described except that the Dowex column step was omitted. Instead, the reaction was terminated by adding absolute ethanol to precipitate protein. After centrifugation, unreacted \([14C]\alpha\text{-keto-glutarate}\) and \([\text{U-14C}]\alpha\text{-keto-glutarate}\) were separated by electrophoresis as described above. Other enzymes assayed were glucose-6-phosphatase (18) and lactate dehydrogenase (39).

In assays where the activity was low or not detectable in chicken liver mitochondria (adenylate kinase, glutamine transaminase, and \(\gamma\text{-glutamyltranspeptidase}\)), the same assays were applied to rat liver mitochondria or rat kidney preparations to verify reaction conditions.

**Oxygen Uptake**

Oxygen consumption was measured polarographically by means of a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The respiration medium was that of Greenawalt (16) except that the ADP concentration was 0.25 mM.

**Electron Microscopy**

Mitochondria were fixed for 1-2 h in 1.5% glutaraldehyde in a 1:1 dilution of Millonig's buffer, pH 7.4, at 4°C (30); the osmolarity of this fixative was 300 mosM. The mitochondria were then washed four times with undiluted Millonig's buffer, 300 mosM, pH 7.4. The mitochondria were allowed to remain in the buffer solution for 15 min at 4°C before centrifuging. After fixing for 1 h in undiluted Millonig's buffer containing 1% osmium tetroxide, the sample was dehydrated with a graded series of ethanol-water mixtures, embedded in low viscosity epoxy embedding media and polymerized at 70°C for 12-24 h. Sections were made with a diamond knife on a Sorvall MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.). These were stained with 2.5% aqueous uranyl acetate followed by 0.4% aqueous lead citrate. Osmolarity was determined with an Advanced Instruments osmometer (Advanced Instruments, Inc., Needham Heights, Mass.).

**Disc Gel Electrophoresis**

Electrophoresis was carried out on gels prepared by chemical polymerization of solutions containing 7% acrylamide, 0.18% \(N,N\text{-methylene-bisacylamide}\), 0.375 M Tris-hydrochloride buffer, pH 8.9, and 0.07% ammonium persulfate as the catalyst. Samples were diluted before application to the columns in 50% sucrose containing 0.0004% bromphenol blue as a tracking dye. The electrode buffer was 0.005 M Tris-glycine, pH 8.9, and the current applied was 2.5 mA/gel column. Electrophoresis was continued for 30 min after the dye had migrated off the gel. Previous experiments had shown the absence of proteins migrating faster than bovine serum albumin. Gels were stained for glutamine synthetase activity by incubating them at 37°C for 10-15 min in solutions containing 50 mM glutamate, 40 mM ATP, 125 mM hydroxylamine, 40 mM magnesium chloride, and 50 mM imidazole buffer, pH 7.4, containing 2.5 mM 2-mercaptoethanol. After incubation, the reaction solution was decanted and the gels were washed once with distilled water before addition of the ferric chloride reagent. The chromogenic product formed between ferric chloride and \(\gamma\text{-glutamylhydroxamate}\) was visible almost immediately as a sharp brown band near the top of the gel. Gels were stained for protein with Coomassie brilliant blue, destained by diffusion in 7% acetic acid, and scanned with a Gilford model 2520 gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Identification of the protein peak corresponding to glutamine synthetase was accomplished as follows. The brown band identifying glutamine synthetase migration was marked by means of fine copper wire before diffusion had broadened the band. The gel was then washed with distilled water and stained with Coomassie blue. When scanned, the wire appeared as an off-scale spike in the spectrum and was readily correlated with a protein peak in gels stained only for protein. Gels treated with ferric chloride reagent could be scanned directly for glutamine synthetase activity at 500 nm. However, correlation of glutamine synthetase with a protein band was impeded by differences in swelling between gels treated with ferric chloride reagent and those treated with Coomassie blue reagent. The percentage of the total matrix protein which glutamine synthetase represents was estimated by comparison of total peak area to glutamine synthetase peak area. Scans were traced on Albanene tracing paper (Keuffel & Esser Co., Morristown, N. J.) and integrated by weight. Corrections were made for bovine serum albumin present in the matrix sample.

**RESULTS**

The localization of glutamine synthetase within avian liver mitochondria was determined by the digitonin-Lubrol method of Schnaitman and Greenawalt (35). In this fractionation procedure, centrifugation of digitonin-treated mitochondria yields a pellet representing the mitoplast fraction (inner membrane + matrix) and a supernatant fraction which may subsequently be separated by high-speed centrifugation into the intracristal space and outer membrane fraction. The inner membrane and matrix compartments are separated by Lubrol treatment of the mitoplast fraction. This method was applied to chicken liver mitochondria. The marker enzymes used to char-
characterize the fractions obtained were those commonly used for rat liver submitochondrial fractions: cytochrome oxidase and succinate-cytochrome c reductase for the inner membrane; malate dehydrogenase, glutamate dehydrogenase, and NADP-dependent isocitrate dehydrogenase for the matrix; and rotenone-insensitive NADH-cytochrome c reductase and monoamine oxidase for the outer membrane. Unfortunately, no suitable marker for the intracristal space in avian mitochondria has been found. Adenylate kinase, the most commonly used marker for this compartment in rat liver mitochondria, was below our level of detection in avian mitochondria, and nucleoside diphosphokinase, also reported to be an intracristal space enzyme, followed the distribution of the matrix marker enzymes. Although adenylate kinase activity in avian liver mitochondria has been described by others (7), the activity reported is much too low to be of value as a marker enzyme. However, the matrix and inner membrane fractions obtained here were probably free of major contamination by the intracristal space because they were obtained by fractionation of isolated mitoplasts. Rat liver mitoplasts have a distinctive morphology characterized by pseudopodlike extensions of the inner membrane and have been shown by biochemical techniques to be devoid of outer membrane and intracristal space enzymes (16, 35). An electron micrograph of an avian liver mitoplast pellet is shown in Fig. 1. These mitoplasts are similar in appearance to rat liver mitoplasts, displaying the characteristic projections of the inner membrane.

Our initial attempts to fractionate avian liver mitochondria resulted in substantial solubilization of the outer membrane. In one such experiment, the digitonin concentration was sufficiently high (digitonin/protein ratio in excess of 0.16) to solubilize over 69% of the monoamine oxidase activity and 59% of the rotenone-insensitive NADH-cytochrome c reductase activity. The high digitonin concentration did not, however, cause release of mitoplast enzymes since the major proportion of the matrix and inner membrane markers was recovered in the mitoplast pellet and subsequently was present in the expected submitochondrial sites. Contamination of the mitoplast pellet by outer membrane was minor as indicated by a low percentage of monoamine oxidase (12.5%) and rotenone-insensitive NADH-cytochrome c reductase (6.8%) recovered in this fraction. Distribution of glutamine synthetase activity followed that of the matrix marker enzymes when mitoplasts were ruptured with detergent. Although the fractionation was not absolute, based on the solubilization of the outer membrane markers, these initial results clearly indicated a matrix localization of glutamine synthetase. Parenthetically, it may be noted that monoamine oxidase activity of avian liver mitochondria (23 nmol/h per mg protein) was about 3% that of rat liver mitochondria (35), and kynurenine hydroxylase, another outer membrane marker for some mitochondria (16), could not be detected. An additional difference between avian and rat liver mitochondria observed in these experiments was the submitochondrial localization of nucleoside diphosphokinase. In rat liver this activity is found in the intracristal space (35), whereas in avian liver its submitochondrial distribution pattern followed that of the matrix marker enzymes.

Table I contains data from a fractionation experiment in which the digitonin/protein ratio was decreased to 0.15. The lower ratio resulted in a cleaner separation of the mitochondrial subfractions as judged by enzyme assay. Outer membrane contamination of the mitoplast fraction was slightly lower (10.8%) and solubilization of the outer membrane was reduced. Although the rotenone-insensitive NADH-cytochrome c reductase activity was again distributed between the outer membrane and intracristal space, the greatest percentage of this activity (55%) was associated with the outer membrane, in contrast to the results obtained in the initial studies. The higher recoveries of isocitrate, malate, and glutamate dehydrogenases in the matrix fraction indicate that fractionation of the mitoplast was also cleaner although the same detergent/protein ratio was used to fractionate the mitoplast in all studies. As anticipated, cytochrome oxidase recovery and specific enzyme activity were highest in the inner membrane fraction. Similar observations regarding the localization of glutamine synthetase were noted: the highest percentage of the total as well as specific enzyme activity were found in the matrix compartment. This fraction also exhibited the highest specific activities for the matrix marker enzymes. The data presented above represent results from two of six mitochondrial fractionations; in each of the six fractionations, the distribution of glutamine synthetase activity paralleled that of the matrix marker enzymes.

Fig. 2 is a densitometric scan of the matrix polypeptides separated by polyacrylamide gel
Figure 1. Electron micrograph of the mitoplast fraction from digitonin-treated avian liver mitochondria, postfixed in OsO₄, and stained with uranyl acetate and lead citrate as described in Materials and Methods. x 23,300; inset, x 63,875.
TABLE I
Submitochondrial Localization of Glutamine Synthetase from Chicken Liver

| Fraction                | Protein | Glutamine synthetase | Isocitrate dehydrogenase | Malate dehydrogenase | Glutamate dehydrogenase | Cytochrome oxidase | Rotenone-insensitive NADH-cytochrome c reductase |
|------------------------|---------|----------------------|--------------------------|----------------------|-------------------------|------------------|-----------------------------------------------|
|                        | %*      | % sp act             | % sp act                 | % sp act             | % sp act                | % sp act         | % sp act                                      |
| Unfractionated         | (100.0) | (100.0)              | (100.0)                  | (100.0)              | (100.0)                 | (100.0)         | (100.0)                                       |
| mitochondria           |         |                      |                          |                      |                         |                  |                                               |
| Mitoplast              | (65.3)  | (79.4)               | (74.2)                   | (71.6)               | (70.8)                  | (74.2)           | (10.8)                                        |
| Matrix                 | 39.4    | 74.2                 | 83.9                     | 71.0                 | 57.4                    | 0.14             | 0.01                                          |
| Inner membrane         | 20.9    | 10.3                 | 3.4                      | 3.5                  | 5.2                     | 72.8             | 6.97                                          |
| Outer membrane         | 17.6    | 3.8                  | 0.06                     | 10.8                 | 4.9                     | 26.9             | 3.05                                          |
| Intracristal space     | 22.1    | 11.7                 | 4.2                      | 14.7                 | 4.5                     | 26.5             | 0.11                                          |
| Recovery               | (101.4) | (96.6)               | (84.2)                   | (88.2)               | (116.7)                 | (90.4)           | (85.3)                                       |

Enzymes were assayed as described in the Materials and Methods section. All specific activities are given in micromoles per minute per milligram protein.

* Numbers in parentheses represent percentage of mitochondrial activity; numbers not enclosed in parentheses represent percentage of the sum of the activities recovered in the four submitochondrial fractions, i.e., (Mx + IM + OM + IC).

† Abbreviations are: Mx, matrix; IM, inner membrane; OM, outer membrane; IC, intracristal space.

FIGURE 2 Densitometric scan of matrix proteins separated by polyacrylamide gel electrophoresis. A 50-μl matrix sample containing 2.5 glutamine synthetase U (78 μg protein) was subjected to electrophoresis as described in Materials and Methods. BSA: bovine serum albumin.

Electrophoresis and stained with Coomassie blue. 13-15 peaks are readily distinguishable. One of the major protein bands has been correlated with glutamine synthetase activity. Identification was accomplished by directly staining gels for glutamine synthetase activity as described in the Materials and Methods section. The two fastest migrating polypeptides have been identified as bovine serum albumin added during preparation of the matrix fraction and a protein contaminant present in the commercial bovine serum albumin. By integrating peak areas and correcting for the bovine serum albumin, it has been estimated that a maximum of 6-9% of the total matrix protein is glutamine synthetase. In view of the overlapping protein bands and the known difficulties in quantitation of proteins by dye staining (21), this value is, at best, a rough approximation.

Because an earlier study had indicated some mitochondrial glutamine degradation (3), several
additional studies on glutamine metabolism in avian liver mitochondria were undertaken. Oxygen consumption by intact mitochondria with glutamine and glutamate as substrates is shown in Fig. 3. A comparison of the two substrates shows that glutamate is oxidized much more rapidly than glutamine, the rate for glutamate being 4.5 times greater than that for glutamine. In the lower recording, it can be seen that the addition of bromofuroate, an inhibitor of glutamate dehydrogenase (4), markedly inhibits oxygen uptake whereas the addition of aminoxyacetate, an inhibitor of mitochondrial transaminase (20), has little or no effect. These results suggest that glutamate oxidation by avian liver mitochondria is initiated by the conversion of glutamate to α-ketoglutarate via deamination rather than via transamination. The upper recording shows that avian mitochondria also metabolize glutamine. Oxygen consumption in the presence of glutamine is again inhibited by bromofuroate, but not by aminoxyacetate. Thus, the initial step in glutamine oxidation is presumably its conversion to glutamate through the action of a glutaminase rather than its conversion to a keto acid by a transaminase. The rate-limiting step in glutamine-dependent oxygen uptake is obviously not glutamate oxidation per se because glutamate itself is oxidized at a much higher rate.

Figure 3 Mitochondrial respiration in the presence of glutamate and glutamine. Mitochondria were isolated as described in Materials and Methods. The reaction chamber contained 2 ml of the respiration medium defined by Greenawalt (16): 70 mM sucrose; 220 mM D-mannitol; 0.5 mM EDTA; 2.5 mM magnesium chloride; 2.5 mM potassium phosphate, pH 7.4; 2.0 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid); and 0.5 mg/ml bovine serum albumin. Additions shown by the arrows were: mitochondria, 2.5 mg/ml; glutamine, 5 mM; glutamate, 5 mM; ADP, 1 mM; amino-oxyacetate, 2 mM; and bromofuroate, 12.5 mM.
than glutamine. This conclusion is supported by the data shown in Table II. This table lists the enzymes of glutamine catabolism which have been assayed in avian liver mitochondria. A glutaminase activity which is phosphate-dependent was found, whereas glutamine transaminase activity was barely detectable. There are two distinct glutamine transaminases in rat liver mitochondria which may be distinguished by their different substrate specificities. The "liver form" is highly active toward methionine, glyoxylate, and pyruvate, whereas the "kidney form" is active with phenylalanine, tyrosine, and their corresponding α-keto acids (11). With avian liver mitochondria, only the liver-type glutamine transaminase was measurable when assayed with [14C]glutamine and glyoxylate. However, this mitochondrial transaminase activity may simply be a matter of cytosolic contamination since it represented only 5% of the homogenate activity. Also, in contrast to an assay using rat liver mitochondria, no liver-type glutamine transaminase could be detected in avian liver mitochondria using [14C]-α-ketoglutarate and glutamine. Because γ-glutamyltranspeptidase and phosphate-independent glutaminase are reported to be catalytic activities of a single protein (12, 37), assays for both were performed. Neither activity could be demonstrated.

The subcellular localization of the phosphate-dependent glutaminase was investigated by means of differential centrifugation. As shown in Table III, this activity is localized in the mitochondrial fraction. The distribution of the phosphate-dependent glutaminase was similar to that of cytochrome oxidase, the mitochondrial marker enzyme. Almost 50% of these activities were recovered in the mitochondrial pellet which also showed the highest specific enzyme activities. A substantial proportion of both activities was recovered in the nuclear fraction although the specific activities were less than those observed with the mitochondrial pellet. As expected, the major percentages and highest specific activities of glucose-6-phosphatase, a microsomal marker, and lactate dehydrogenase, a cytosolic marker, were found in the microsomal and soluble fractions, respectively.

Attempts to define the submitochondrial locali-

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1 As previously reported by others (see reference 7, p. 3109, footnote), we have frequently observed marked mitochondrial contamination of the nuclear pellet during fractionation of chicken liver.

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TABLE II

| Enzyme                        | Specific activity (nmol/min/mg protein) |
|-------------------------------|----------------------------------------|
| Glutamine synthetase          | 26,700                                  |
| P_i-dependent glutaminase     | 610                                     |
| P_i-independent glutaminase   | 0                                      |
| γ-Glutamyl transpeptidase     | 0                                      |
| Glutamine transaminase        | 0.22*                                   |

Enzymes were assayed as described in Materials and Methods.

* Represents only 5% of homogenate activity.

zation of the phosphate-dependent glutaminase in chicken liver mitochondria was complicated by the low level of enzyme present and the apparent instability of it. Data from an experiment in which activity was assayed with the radiometric assay are shown in Table IV. In this experiment, some 99% of the mitochondrial activity was recovered in the submitochondrial fractions. Of that recovered, 84% was present in the extramitochondrial fractions. In other experiments in which the enzyme was assayed spectrophotometrically, recoveries were only 20-30%. This was presumed to be due to the instability of the enzyme but the addition of borate did not result in increased yields. Borate does stabilize the glutaminase present in rat kidney mitochondria during fractionation (13). That activity recovered in the low-recovery experiments was nevertheless mainly present in the outer membrane fraction. On the basis of these results, the avian liver phosphate-dependent glutaminase is tentatively assigned to the extramitochondrial space, specifically the outer membrane.

DISCUSSION

The localization of avian liver glutamine synthetase within the mitochondrial matrix is based on the methodology and operational definition of the various subunitochondrial compartments described by Schnaitman and Greenawalt for rat liver (35). Certain differences between rat and avian liver were found here with respect to the marker enzymes. These included nondetectable levels of adenylate kinase and kynurenine hydroxylase and low monoamine oxidase activity. In addition, nucleoside diphosphokinase, localized within the intracristal space of rat liver mitochondria (35), was associated with the matrix fraction in avian liver mitochondria. Nevertheless, the results obtained
TABLE III
Distribution of P<sub>r</sub>-Dependent Glutaminase Activity in Subcellular Fractions of Chicken Liver

| Fraction          | Protein (mg/g tissue) | P<sub>r</sub>-dependent glutaminase (U/mg protein) | Cytchrome oxidase (U/mg protein) | Glucose-6-phosphatase (U/mg protein) | Lactate dehydrogenase (U/mg protein) |
|-------------------|-----------------------|-----------------------------------------------|---------------------------------|-------------------------------------|---------------------------------------|
| Homogenate        | 156.5                 | 3610                                          | 23                              | 70.6                                | 4.50                                  |
| Nuclear           | 30.0                  | 975                                           | 37.4                            | 33                                  | 23.6                                  |
| Heavy mitochondrial | 22.7                 | 1299                                          | 49.8                            | 57                                  | 35.8                                  |
| Light mitochondrial | 3.7                  | 127                                           | 4.9                             | 35                                  | 4.8                                   |
| Microsomal        | 37.7                  | 207                                           | 7.9                             | 5                                   | 9.0                                   |
| Soluble           | 75.5                  | 0                                             | 0                               | 0                                   | 0                                     |
| Total in fractions| 169.6                 | 2608                                          | (100.0)                         | (100.0)                             | (100.0)                               |

Enzymes were assayed as described in Materials and Methods.
* Percentage of recovered activity.
† 1 U = that amount of enzyme catalyzing the formation of 1 nmol glutamate/h at 37°C.
‡ 1 U = that amount of enzyme catalyzing the oxidation of 1 µmol reduced cytochrome c/min at 25°C.
∥ 1 U = that amount of enzyme catalyzing the hydrolysis of 1 nmol glucose-6-phosphate/min at 37°C.
§ 1 U = that amount of enzyme catalyzing the oxidation of 1 µmol NADH/min at 25°C.

TABLE IV
Submitochondrial Localization of P<sub>r</sub>-Dependent Glutaminase from Chicken Liver

| Fraction      | Radiometric assay | Total U | % | sp act |
|---------------|-------------------|---------|---|--------|
| Mitochondria  |                   | 273     | 0.35       |
| Mitoplast     |                   | 101     | (37.0)*    | 0.17   |
| OM            |                   | 182     | 0.23       |
| IC            |                   | 48      | 0.23       |
| IM            |                   | 10      | 0.05       |
| Mx            |                   | 30      | 0.09       |
| Total         |                   | 270     |            |

P<sub>r</sub>-dependent glutaminase was assayed as described in Materials and Methods. Abbreviations are given in the legend of Table I. 1 U is that amount of enzyme catalyzing the formation of 1 µmol glutamate/h.
* Percentage of mitochondrial activity.
† Percentage of recovered activity.

...clearly indicate that glutamine synthetase is a matrix protein. This activity was latent and invariably followed the distribution pattern of the three matrix marker enzymes. Glutamine synthetase was not released under conditions which solubilized outer membrane protein but rather remained within the mitoplast vesicles (inner membrane plus matrix). Conditions which did release the matrix marker enzymes also solubilized glutamine synthetase. A matrix site for glutamine synthetase in uricotelic liver mitochondria is consistent with its proposed function. This enzyme catalyzes the reaction by which ammonia is "detoxified" via conversion to the amide function of glutamine. It should logically occur in the same compartment as glutamate dehydrogenase, the ammonia-releasing enzyme. A similar situation exists in ureotelic mitochondria. Using the fractionation procedure employed here, Gamble and Lehninger (14) localized carbamyl phosphate synthetase-I in the matrix fraction of rat liver mitochondria. Confirmation of the matrix localization of carbamyl phosphate synthetase-I was recently reported by Clarke (6) whose results also indicate that carbamyl phosphate synthetase constitutes 15-20% of the total mitochondrial protein. The concentration of avian mitochondrial glutamine synthetase appears to be much lower than this. On the basis of the polyacrylamide gel electrophoresis studies, approximately 6-9% of the matrix protein and 1.5-2.0% of the total mitochondrial protein is glutamine synthetase. Although the percentage is lower than that reported for carbamyl phosphate synthetase, glutamine synthetase is nevertheless one of the major polypeptides of avian liver mitochondrion.

As reported previously (3), the avian mitochondrial membrane is freely permeable to glutamine, allowing it to exit to the cytosol where it serves as a major precursor for purine synthesis. However, as the O₂ uptake studies indicate, avian mitochondria are also capable of metabolizing glutamine, albeit at a markedly slower rate compared to glutamate. Catabolism of glutamine to α-ketoglutarate, an electron transport-linked substrate, could occur by hydrolysis to glutamate followed by the oxidative deamination of this product to α-ketoglutarate. A second possible pathway of glutamine metabolism involves an initial transamination to α-ketoglutaramate which is then hydrolyzed to α-ketoglutarate (10). Glutamine metabolism via the...
first pathway is suggested by our results. Respiration in the presence of glutamine as well as glutamate was blocked by bromofuroate, an inhibitor of glutamate dehydrogenase, but not by aminooxyacetate, a transaminase inhibitor. Hydrolysis rather than transamination as the initial step in glutamine metabolism has been observed in several other mitochondrial systems. In addition to those of the well-characterized kidney system (13, 15), mitochondria from Ehrlich ascites tumor cells and rat liver metabolize glutamine via an initial hydrolysis to glutamate catalyzed by glutaminases localized in the mitochondria (1, 17, 19, 24, 25).

Consistent with the above interpretation of the O₂ uptake studies was the finding of a glutaminase activity but little or no transaminase activity in avian liver mitochondria. The glutaminase activity was phosphate-dependent and confirms an earlier report of this activity in chicken liver homogenates (23). In agreement with the response of this enzyme from kidney (13) and Ehrlich ascites tumor cells (25), disruption of the avian mitochondrion with detergent resulted in enzyme inactivation in most cases. Unlike the other systems, however, addition of borate failed to protect the avian liver glutaminase against inactivation. In this regard, it may be noted that borate does not protect against all methods of mitochondrial disintegration since it does not stabilize the activity in sonically ruptured tumor cell mitochondria (25).

It is generally accepted that the physiological role of the kidney glutaminase is to provide ammonia in the regulation of acid-base balance (15) and that of the rat liver enzyme to provide ammonia for urea synthesis during glutamine catabolism. Yet another function for this enzyme in Ehrlich ascites tumor cells has been suggested (25), and that is the intramitochondrial generation of reducing equivalents in the form of NAD(P)H via the oxidation of glutamate. A physiological role for the avian liver mitochondrial glutaminase is not readily evident. In one experiment in which essentially all of the mitochondrial activity was recovered in the submitochondrial fractions, the enzyme was localized in the extramitoplastal compartment, mainly the outer membrane. This compartment also consistently contained the highest specific enzyme activity, even where overall recoveries were low. An extramitoplastal localization of phosphate-dependent glutaminase is the opposite of that found in kidney (13) and tumor cells (25) but is nevertheless consistent with the function of glutamine metabolism in avian liver mitochondria.

The localization of glutaminase in the outer membrane of these mitochondria suggests a "vectorial" metabolism of glutamine. Glutamine normally exists and may be channeled directly into purine biosynthesis. However, if entering the mitochondrion, glutamine may be broken down by glutaminase.

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