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Citrulline Synthesis in Rat Tissues and Liver Content of Carbamoyl Phosphate and Ornithine

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Rat liver ornithine carbamoyltransferase appears to be located exclusively in the mitochondria; the activity that is found in the soluble fraction is indistinguishable from mitochondrial ornithine carbamoyltransferase by simple kinetic criteria, and seems to result from breakage of mitochondria during homogenization. Of several rat tissues studied, only the liver and the mucosa of small intestine contain significant amounts of ornithine carbamoyltransferase; the activity in intestinal mucosa is less than one thousandth of that in liver. Qualitatively, this distribution coincides with that of carbamoyl phosphate synthetase I and its cofactor, acetylglutamate. The rat liver contents of carbamoyl phosphate and ornithine were 0.1 and 0.15 μmol/g wet wt. of tissue respectively. On the basis of these values, it is proposed that *in vivo* the ornithine carbamoyltransferase activity of liver may be much lower than its maximal activity *in vitro* might suggest.

The regulation of arginine and urea biosynthesis in mammals is not well understood (Jones, 1965). Although studies by Schimke (1962*a*,*b*, 1963) have revealed some of the mechanisms that may underlie long-term adaptations to various nitrogen loads, the importance of the problem of ammonia toxicity, either acute or chronic, demands that we understand the regulation of urea synthesis in much greater detail. This paper presents my initial studies on that subject and touches on three areas of ornithine and carbamoyl phosphate metabolism.

Knowledge of the liver content of urea-cycle intermediates under various conditions, together with data on some other metabolites of related pathways, should prove valuable in assessing the activities *in vivo* of the enzymes involved in urea biosynthesis. The carbamoyl phosphate and ornithine contents of rat liver, reported here, allow some conclusions to be made about the activity of ornithine carbamoyltransferase and aspartate carbamoyltransferase *in vivo*.

The presence in tissues of ornithine carbamoyltransferase, possibly cytoplasmic and distinct from the mitochondrial enzyme of liver, could be of importance, as regards arginine synthesis, for protein synthesis in tissues other than the liver. The latter has long been known to be the organ where most of the urea produced by mammals is synthesized (Bollman *et al.*, 1924); however, since the enzymes required for arginine synthesis from citrulline are present in all tissues studied, it was thought that the entire pathway for the synthesis of arginine and urea *de novo* might exist in tissues other than the liver (Kemp & Woodbury, 1965). Preliminary studies did not support that possibility, which would be excluded by the demonstration of the absence of ornithine carbamoyltransferase from tissues, since biosynthesis of citrulline (and therefore arginine) could not occur in the absence of that enzyme. The ornithine carbamoyltransferase activity of rat tissues other than the liver was re-investigated by using an assay approximately 1000-fold more sensitive than that used previously by Jones *et al.* (1961).

Finally, the synthesis of carbamoyl aspartate, a precursor of the pyrimidines, in liver cytoplasm would be affected by the presence of ornithine carbamoyltransferase in that compartment. The subcellular distribution of that enzyme in liver was also re-investigated in an effort to establish whether the ornithine carbamoyltransferase activity found in soluble fractions of rat and human liver (Schnaitman & Greenawalt, 1968; Snodgrass, 1968) reflects the distribution *in vivo* or artifacts occurring during the disruption of the tissue and the separation of the subcellular fractions.

Materials and Methods

Materials

Male Wistar rats weighing approx. 125g were purchased from Simonsen Farms, Gilroy, Calif., U.S.A. Ornithine carbamoyltransferase (carbamoyl phosphate–L-ornithine carbamoyltransferase, EC 2.1.3.3), from *Streptococcus faecalis* A.T.C.C. 8043, was prepared by the method of Nakamura & Jones (1970); the preparations used synthesized between 280 and 600 μmol of citrulline/min per mg of protein

under the conditions used by those authors. Crystalline glutamate dehydrogenase, carbamoyl phosphate (dilithium salt), NADH, ADP and α-oxoglutarate were purchased from the Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. Carbamoyl phosphate labelled with ^{14}C was purchased from New England Nuclear Corp., Boston, Mass., U.S.A., and was recrystallized as described by Adair & Jones (1972); its specific radioactivity was 5.78Ci/mol. Ornithine, uniformly labelled with ^{14}C, was purchased from New England Nuclear Corp.; its specific radioactivity was 180Ci/mol. All other materials were commercially available products of analytical quality.

Preparation of tissue extracts for enzyme assays

Two homogenates were made from portions of each liver. One was obtained by thorough homogenization of the tissue in 0.25M-sucrose for 1min, and was used to determine the total activity of ornithine carbamoyltransferase and glutamate dehydrogenase, a mitochondrial marker enzyme. The second homogenate was made as described by Myers and Slater (1957) for the preparation of mitochondria, except that the mitochondrial pellet was washed twice by resuspending it in 0.25M-sucrose. The following additional fractions were obtained: the 6000–7000*g* supernatant was further centrifuged at 29000*g* for 60min; the resulting supernatant is referred to as 'soluble fraction'. The supernatants from both mitochondrial washes are referred to as 'first' and 'second mitochondrial wash'.

The procedure of Myers & Slater (1957) is designed to minimize damage to the mitochondria and does not yield maximal recoveries. In consequence, the activities of ornithine carbamoyltransferase and glutamate dehydrogenase of mitochondrial fractions, expressed as percentages of the total found in whole liver homogenates, are considerably lower than the actual percentages.

The ornithine carbamoyltransferase activity of other rat tissues was measured in 10% (w/v) water homogenates. Before assay, all tissue homogenates or liver fractions were sonically disrupted for 1min with a cooled Raytheon sonic disintegrator, model DF 101. When present, maximum ornithine carbamoyltransferase and glutamate dehydrogenase activity was obtained within 30s, but no activity was lost even after 2min of sonic disruption.

Preparation of liver extracts for the measurement of carbamoyl phosphate and ornithine

The material used for the development of the methods for the assay of carbamoyl phosphate and ornithine was the freeze-dried powder from pooled livers, which had been freeze-clamped by the method of Wollenberger *et al.* (1960) within 10s of cervical dislocation.

For actual measurements in single livers, the wafers of freeze-clamped tissue were ground in a mortar under liquid air, and a portion of the tissue was quickly weighed into a precooled tube. To 1g of tissue was added 9ml of ice-cold 2M-$HClO_4$; after quick mixing with a stirring rod, the tissue was thoroughly homogenized and the homogenate centrifuged at low speed at 4°C for 4min. The supernatant was decanted into a graduated centrifuge tube, was neutralized to approx. pH5 by adding 2M-KOH with constant mixing, and to approx. pH7 with 2M-$KHCO_3$. The mixture was left on ice for 5min, then centrifuged as above; the supernatant was collected in a graduated tube and immediately analysed for its carbamoyl phosphate and ornithine contents. The time elapsed between the addition of $HClO_4$ to the frozen liver powder and the beginning of the assay of carbamoyl phosphate was 20–25min. The recovery of carbamoyl phosphate and ornithine from this procedure was studied by adding them in amounts similar to those found in liver, as follows: the solution containing known amounts of carbamoyl phosphate or ornithine in a small volume was frozen at the bottom of homogenizer tubes, to which was then added a portion of frozen liver powder. The rest of the procedure was as described above.

Enzyme assays

A colorimetric assay of ornithine carbamoyltransferase activity was initially used when studying the subcellular distribution of this enzyme in liver. A portion of tissue extract was incubated with 12.5mM-ornithine, 5mM-carbamoyl phosphate and 100mM-Tris–HCl, pH7.5, in a final volume of 1ml. After 15min at 38°C, the reaction was stopped with 0.5ml of 5M-$HClO_4$. Tubes containing visible amounts of precipitated protein were centrifuged at low speed for 10min. The deproteinized reaction mixtures were assayed for citrulline by the method of Guthöhrlein & Knappe (1968). Samples of liver fractions containing 5μmol of sucrose or more give considerable blank values in this colour reaction. This assay of ornithine carbamoyltransferase activity is linear with time for at least 30min, and with enzyme concentrations yielding at least 0.5μmol of citrulline. For assays of liver ornithine carbamoyltransferase at pH8.5, the same incubation mixture was used, except that the buffer was replaced by 100mM-Tris–HCl, pH8.5.

For the measurement of ornithine carbamoyltransferase activity in other tissues, a radioactive assay utilizing [^{14}C]carbamoyl phosphate was used. The standard incubation mixtures contained the following in 1ml: 5mM-ornithine, 20mM-dilithium

[^{14}C]carbamoyl phosphate, 100mM-Tris–HCl, pH7.5, and the homogenate. After incubating at 38°C for 10min, the reaction was stopped by the addition of 0.2ml of 2M-$HClO_4$. Precipitated protein was centrifuged down; a measured portion of the supernatants (about 1ml) was transferred into graduated conical tubes capped with marbles and placed in a boiling-water bath for 10min. After cooling, CO_2 was bubbled through the samples for 30min, at the end of which time water was added to restore the samples to their original volume. The samples were adjusted to approx. pH7 with 2M-$KHCO_3$, left on ice for 20–30min, then centrifuged at low speed in a clinical centrifuge. Samples (1ml) of the supernatants were used for the measurement of citrulline by arsenolysis as described by Hager & Jones (1967), with minor modifications. The samples were transferred into scintillation vials kept at 0°C, containing: citrulline, 40μmol; sodium arsenate, pH6.25 (measured at 1M-arsenate), 800μmol; ornithine carbamoyltransferase, 280 units. The total volume was 2.3ml and the final pH6.6. The vials were sealed with serum caps fitted with plastic centre wells containing 0.2ml of a CO_2-trapping mixture of ethylene glycol–ethanolamine (2:1, v/v). The vials were incubated at 38°C overnight; the reaction was stopped by carefully injecting 0.5ml of 2M-$HClO_4$ through the rubber cap and into the incubation mixture with a syringe fitted with a thin needle. CO_2 was allowed to diffuse from the acidified mixtures for 1h (at 38°C), after which the rubber caps were removed, the centre wells dropped into scintillation vials, and their contents counted for radioactivity.

In control experiments with known amounts of [^{14}C]citrulline, time-curves showed that the decrease in citrulline of the incubation mixtures, measured colorimetrically, paralleled the decrease in ^{14}C as well as the increase in the latter fixed as $^{14}CO_2$ in the centre wells, and that the arsenolysis reaction proceeded to 90–95% of completion.

By this method, the synthesis of 1.5nmol of citrulline could be accurately measured when the specific radioactivity of the [^{14}C]carbamoyl phosphate was 38.53mCi/mol. Greater sensitivity could have been achieved by increasing the specific radioactivity of the [^{14}C]carbamoyl phosphate. The results obtained by using this assay of ornithine carbamoyl transferase are comparable with those obtained by the colorimetric assay.

Glutamate dehydrogenase was measured spectrophotometrically at approx. 25°C; assay mixtures contained 100mM-ammonium acetate, 30mM-α-oxoglutarate, 5mM-ADP, 5mM-disodium EDTA, 0.17mM-NADH, 100mM-sodium phosphate, pH7.4, the sample to be assayed, and water to a final volume of 3ml. NADH was added to the blank cuvette so that the initial absorbance of the experimental sample would be approx. 0.5 at 340nm.

Measurement of carbamoyl phosphate and ornithine

Carbamoyl phosphate was measured as [^{14}C]-citrulline in a system containing 1ml of liver extract, 2μmol of [^{14}C]ornithine (specific radioactivity 10mCi/mol), 200 units of ornithine carbamoyltransferase, 100μmol of Tris–HCl, pH8.5, and water to give a final volume of 1.3ml. The mixtures were incubated at 38°C for 20min, after which the reaction was stopped with 0.1ml of 5M-$HClO_4$. Then 5μmol of cold citrulline was added, and the mixture was applied to a column (0.8cm×8.0cm) of Dowex 50 (X-8; 200 mesh; H^+ form). The column was washed with 20ml of water, then eluted with 0.2M-sodium citrate, pH3. Samples (4ml) were collected; citrulline was usually eluted in four fractions after approx. 35ml of citrate had been added. Very small amounts of [^{14}C]ornithine began to be eluted shortly after citrulline; several samples of eluate must be collected beyond this point, to make certain that the citrulline peak is symmetrical and contains no radioactivity from [^{14}C]ornithine. Samples (1ml) of each fraction were counted for radioactivity. The overall recovery of carbamoyl phosphate was 73–78%.

Ornithine was also measured as citrulline, in a system identical with that for the measurement of carbamoyl phosphate, except that 2μmol of [^{14}C]-carbamoyl phosphate (specific radioactivity 49.8mCi/mol) was added instead of labelled ornithine. At the end of the incubation, the acidified mixture was heated at 100°C for 10min; CO_2 was bubbled through the solutions for 30min, and the samples were transferred into vials and counted for radioactivity. The recovery of ornithine was 90–96%. The reproducibility of these methods was within 10%.

Measurements of radioactivity

In all cases, ^{14}C was measured in a scintillation mixture containing 1930ml of *p*-dioxan, 200g of naphthalene, 14g of 2,5-diphenyloxazole, and 0.6g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene. A Beckman LS-100 C or Beckman LS-245 scintillation counter was used.

Results

Carbamoyl phosphate and ornithine contents of liver

Individual measurements of the carbamoyl phosphate content of three fresh livers gave values of 0.1–0.12μmol/g fresh wt. of tissue; these values are not corrected for the recovery of the method. The content of ornithine of the same livers was 0.11–0.19μmol/g fresh wt.

The values of the carbamoyl phosphate and ornithine contents of fresh liver are in good agreement with values obtained from three different batches of

pooled freeze-clamped, freeze-dried rat livers which had been stored at −20°C for as long as 4 months.

Distribution of ornithine carbamoyltransferase among subcellular fractions of liver

The data in Table 1 show that about 2% of the total ornithine carbamoyltransferase activity is found in the supernatant fraction. This amount could be decreased to 0.5% by shortening the time of homogenization to 15s.

Should the soluble and the mitochondrial enzymes be two distinct proteins, their activities at different pH values might differ (Arashima & Matsuda, 1971; Cathelineau *et al.*, 1972), and this in turn would be reflected in the subcellular distribution of activity. The results show that the distribution of ornithine carbamoyltransferase is the same whether it is measured at pH7.5 or 8.5, and also that it parallels that of glutamate dehydrogenase, a mitochondrial matrix marker (Schnaitman & Greenawalt, 1968; Gamble & Lehninger, 1973).

Very low ornithine carbamoyltransferase activity (0.2% of the total) is found in the first mitochondrial wash, and less (0.06% of the total) in the second. If the activity found in the soluble fraction is of mitochondrial origin, it may have resulted from the breakage of some mitochondria during homogenization, or from some mitochondria remaining in the soluble fraction. To investigate the latter possibility, the soluble fraction was centrifuged at 100000*g* for 60min, after which a portion of the supernatant was assayed; 75% of the initial soluble activity remained in this supernatant. The soluble fraction was also filtered through 0.22μm Millipore filters to remove mitochondria that might be present; virtually all of the ornithine carbamoyltransferase activity of the soluble fraction was recovered in the filtrate.

Michaelis constants

The apparent K_m values for carbamoyl phosphate and ornithine of ornithine carbamoyltransferase from the mitochondrial and soluble fractions were determined at pH7.5 and 8.5 from Lineweaver–Burk plots, which were in all cases linear. The results are shown in Table 2. The effect of carbamoyl phosphate was studied at concentrations between 2μM and 0.2mM.

Table 1. *Distribution of ornithine carbamoyltransferase and glutamate dehydrogenase activities among subcellular fractions of rat liver*

Ornithine carbamoyltransferase activity was measured by the colorimetric assay described in the Materials and Methods section. Enzyme activities are expressed as μmol of substrate converted/h per g of tissue.

	Ornithine carbamoyltransferase activity				Glutamate dehydrogenase activity	
	pH7.5		pH8.5			
	(μmol/h per g of tissue)	(%)	(μmol/h per g of tissue)	(%)	(μmol/h per g of tissue)	(%)
Homogenate	12580	100	9620	100	5250	100
Mitochondria	5810	46.2	4620	48	2620	49.9
Soluble	240	1.9	203	2.1	76	1.4

Table 2. K_m *of ornithine carbamoyltransferase for carbamoyl phosphate and ornithine at pH*7.5 *and* 8.5

The K_m of ornithine carbamoyltransferase for carbamoyl phosphate was measured as follows: 1ml mixtures containing 100mM-Tris–HCl at pH7.5 or 8.5, 12.5mM-ornithine, [^{14}C]carbamoyl phosphate at concentrations between 2μM and 0.2mM, and mitochondrial or soluble ornithine carbamoyltransferase were incubated at 38°C for 10min. The reaction was stopped with 0.2ml of 2M-$HClO_4$. The deproteinized mixtures were heated at 100°C for 30min; CO_2 was bubbled through the samples for 30min, after which they were transferred to scintillation vials and counted for radioactivity. For the determination of the K_m for ornithine at pH7.5, the mixtures contained 100mM-Tris–HCl, pH7.5, 5mM-[^{14}C]carbamoyl phosphate, 0.2–16mM-ornithine, and the enzyme in 1ml. For the determination at pH8.5, the mixtures were similar except that 100mM-Tris–HCl, pH8.5 was used, and the range of ornithine concentrations was 0.1–10mM. The rest of the procedure was described above for the determination of the K_m for carbamoyl phosphate. The specific radioactivity of the [^{14}C]-carbamoyl phosphate was between 29 and 58mCi/mol.

	K_m for carbamoyl phosphate (mM)		K_m for ornithine (mM)	
Source of enzyme	pH7.5	pH8.5	pH7.5	pH8.5
Mitochondrial fraction	0.022	0.031	1.82	0.234
Soluble fraction	0.026	0.028	1.43	0.255

The K_m values obtained for both enzyme fractions were essentially identical at each pH; average values are 0.024mM at pH7.5 and 0.03mM at pH8.5. Maximal activity at both pH values was obtained at approx. 1mM-carbamoyl phosphate; no inhibition was observed at 5mM-carbamoyl phosphate.

The K_m for ornithine varies with pH. At pH7.5, the effect of ornithine was studied at concentrations between 0.2mM and 16mM; the K_m values obtained were 1.8mM and 1.4mM for the mitochondrial and soluble enzymes respectively. Optimal activity with both enzymes was obtained with 12mM-ornithine, and a 10% inhibition was observed at 16mM. At pH8.5, the effect of ornithine was studied at concentrations between 0.1 and 10mM. The K_m values obtained were the same for both enzymes, between 0.23mM and 0.26mM. Optimal activity at this pH was obtained with 1mM-ornithine; inhibition of 20% and 53% was found at 10mM- and 50mM-ornithine respectively.

Mixtures of ornithine carbamoyltransferase from mitochondrial and soluble fractions gave K_m values for both substrates identical with those obtained with either of the enzyme preparations alone.

Effect of pH

The effect of pH on the activity of ornithine carbamoyltransferase was studied between pH7.0 and 8.5. The data in Table 3 show that at pH7.5 and at equal concentrations of imidazole and Tris, the activity of ornithine carbamoyltransferase from either source is about 15% higher in the presence of imidazole than of Tris. Maximal activity was obtained at pH7.5, above which it decreased rather slowly. The concentration of ornithine was kept constant at 12.5mM, at which concentration ornithine carbamoyltransferase is inhibited by approx. 20% at pH8.5. If a correction is made on this basis for the observed velocity at pH8.5, the decrease in velocity with increasing pH is even smaller. The behaviour of the enzymes from both sources appears to be identical with respect to pH.

Ornithine carbamoyltransferase activity in rat tissues other than the liver

The results of these experiments are shown in Table 4. Of the tissue homogenates assayed, those of pancreas, testicle, adrenals and intestinal mucosa

Table 4. *Activity of ornithine carbamoyltransferase in rat tissues*

Samples of homogenates containing as much as 60mg of tissue were assayed by the radioactive method. The specific radioactivity of [^{14}C]carbamoyl phosphate was 38.53mCi/mol. Counting efficiency was 78–82%; 100 net counts (1.47nmol at 80% efficiency) were considered significant (background averaged 32c.p.m.); on that basis, the limit of detection was approx. 0.22μmol/h per g of fresh tissue. Activities are expressed as μmol of citrulline formed/h per g wet wt. of tissue. The value for liver is an average of the values obtained from six rats; all others are from three rats.

Tissue	Ornithine carbamoyltransferase activity
Brain	0
Kidney	<0.22*
Skeletal muscle	<0.22*
Spleen	<0.22*
Salivary gland	<0.22*
Pancreas	0.29
Testicle	0.32
Adrenal	0.67
Intestinal mucosa	2.60
Liver	13300

* Although these tissues clearly synthesized some citrulline, their activity was slightly below the chosen limit of detection and was not linear with tissue concentration.

Table 3. *Effect of pH on the activity of ornithine carbamoyltransferase from mitochondrial and soluble fractions*

The incubation mixtures contained 5mM-[^{14}C]carbamoyl phosphate, 12.5mM-ornithine, 100μmol of imidazole–HCl or Tris–HCl at the indicated pH, and either 20μl of a 1:100 (v/v) dilution in water of mitochondrial fraction or 50μl of a 1:10 (v/v) dilution in water of soluble fraction. The total incubation volume was 1ml. After incubation at 38°C for 10min, the reaction was stopped with 0.2ml of 2M-$HClO_4$, followed by centrifugation. Samples of the deproteinized incubation mixtures were heated at 100°C for 10min; CO_2 was bubbled through the solutions for 30min, after which the samples were transferred to scintillation vials and counted for radioactivity. The activity of each fraction in the presence of Tris buffer at pH7.5 is taken as 100%.

	Activity (%)				
Buffer ..	Imidazole–HCl		Tris–HCl		
Enzyme source pH ..	7.0	7.5	7.5	8.0	8.5
Mitochondrial fraction	62	118	100	91	77
Soluble fraction	57	114	100	87	64

from the duodenum and the upper portion of the jejunum contained measurable ornithine carbamoyltransferase; the activity was proportional to the amount of tissue used in all these cases.

Discussion

The data reported here on the subcellular distribution of ornithine carbamoyltransferase suggest that the small portion (0.5–2%) of the total activity which is found in extramitochondrial fractions of rat liver may be due to the breakage of mitochondria during the homogenization of the tissue, since milder homogenization conditions result in lower ornithine carbamoyltransferase activity in the extramitochondrial fraction. The activity present in the soluble fraction is fully recovered after filtration through Millipore filters, which retain residual mitochondria. Further, 75% of the soluble activity remains in the supernatant after centrifugation at 100000*g* for 60min. Ornithine carbamoyltransferase does not appear to leak easily out of these mitochondrial preparations, since successive washes contain diminishing amounts of enzyme activity. In view of this, the fact that the enzymes obtained from the mitochondrial and soluble fractions appear to be identical as regards their K_m for both substrates at different pH values and the effect of pH and buffer changes on their activity can be taken as inferential evidence of the mitochondrial origin of soluble ornithine carbamoyltransferase.

Whereas it has long been known that most of the ornithine carbamoyltransferase of liver is in the mitochondria (see Gamble & Lehninger, 1973), variable amounts of activity have been found in 'soluble' fractions obtained from rat (Schnaitman & Greenawalt, 1968) and human liver (as high as 14%; Snodgrass, 1968) preparations. The results in the present paper indicate that those findings may have been artifacts resulting from breakage of mitochondria during homogenization.

Several types of ornithine carbamoyltransferase deficiencies have been demonstrated in children suffering from hyperammonaemia. A characteristic of many of these patients is that they excrete large amounts of orotic acid, uracil and uridine in urine (Levin *et al.*, 1969*a*,*b*). This reflects an increased synthesis of pyrimidines, which is probably not due to carbamoyl phosphate synthetase II, the pyrimidine-specific synthetase; the activity of that enzyme in adult rat liver is approximately one-thousandth of that of carbamoyl phosphate synthetase I. The rate of synthesis of carbamoyl phosphate in mitochondria may be greater than normal in these patients. The NH_3 content of normal rat liver (Williamson *et al.*, 1967) approximates to the K_m for NH_3 of carbamoyl phosphate synthetase I (Caravaca & Grisolia, 1960; Marshall *et al.*, 1961); if that were also the case in human liver, higher blood NH_3 concentrations might result in higher concentrations in liver and an increase in the rate of mitochondrial carbamoyl phosphate synthesis. This, together with the decreased ability of ornithine carbamoyltransferase to utilize carbamoyl phosphate in these patients, might result in the transport of 'excess' mitochondrial carbamoyl phosphate into the cytoplasm, where it would be a substrate for aspartate carbamoyltransferase. The absence of ornithine carbamoyltransferase from the extramitochondrial compartment would make the utilization of cytoplasmic carbamoyl phosphate for citrulline synthesis impossible. The findings just discussed support the suggestion (Bourget *et al.*, 1971) that liver carbamoyl phosphate synthetase I may (under certain pathological conditions) provide carbamoyl phosphate for pyrimidine biosynthesis; there is no evidence, however, that this occurs in normal liver.

The ornithine carbamoyltransferase activity of rat tissues other than the liver was studied by means of an assay that is about 1000 times more sensitive than others used previously (Jones *et al.*, 1961). Activity linear with tissue concentration was detectable in homogenates of pancreas, testicle, adrenal and intestinal mucosa, the amount increasing in that order. These findings are in good agreement with those of Jones *et al.* (1961), in that ornithine carbamoyltransferase activity is negligible in all tissues assayed (unless a different ornithine carbamoyltransferase with unknown requirements is present), the only exception being intestinal mucosa; they differ in that no activity was detected in kidney in the present study. Reichard (1960) studied the distribution of ornithine carbamoyltransferase in human tissues and found that the activity in the mucosa of small intestine was approx. 14% of that in liver; other human tissues also contained negligible amounts.

The acetylglutamate-dependent carbamoyl phosphate synthetase I is present in intestinal mucosa (Jones *et al.*, 1961), as are acetylglutamate (Shigesada & Tatibana, 1971) and apparently the other enzymes required for arginine biosynthesis (Levin, 1971). It is therefore quite probable that the entire urea cycle is functional in intestinal mucosa. The mucosa may well be self-sufficient as regards its own need for arginine and, although its contribution to the requirements of the whole animal for urea synthesis is not significant (Bollman *et al.*, 1924), it may utilize some of the NH_3 produced by intestinal bacteria.

The exact requirement of mammals for arginine for protein synthesis in tissues is not known; however, in view of the extremely low ornithine carbamoyltransferase and carbamoyl phosphate synthetase I activities of only a few tissues and of the apparent absence of these enzymes from most others, it must be concluded that the source (direct or indirect) of

arginine for protein synthesis in most mammalian tissues is the liver. Whether the latter provides mostly arginine as such or the precursor, citrulline, is not known with certainty. Citrulline is transported across the liver mitochondrial membrane by a respiration-independent process (Gamble & Lehninger, 1973). It is present in blood (Mallette *et al.*, 1969) and appears to permeate into and out of cells without difficulty (Tamir & Ratner, 1963; Featherston *et al.*, 1971). A preliminary report of experiments carried out with whole animals suggests that in rats the kidney utilizes citrulline for the synthesis of a major portion of the arginine required for protein synthesis by other tissues (Featherston *et al.*, 1971). This is consistent with the finding that, at least in some species, the capacity of the kidney to synthesize arginine from citrulline is comparable with that of the liver (Ratner & Petrack, 1953; Ratner *et al.*, 1960).

The K_m values of rat liver ornithine carbamoyltransferase for carbamoyl phosphate reported here are in good agreement with those reported for the bovine enzyme at similar ranges of carbamoyl phosphate concentration and pH (Marshall & Cohen, 1972); the values reported for the human enzyme are an order of magnitude larger (Snodgrass, 1968). The K_m values for total ornithine vary with pH; the values for the rat enzyme are of the same order of magnitude as those reported for human ornithine carbamoyltransferase (Snodgrass, 1968).

The method used for the measurement of the ornithine content of liver is simple and reproducible; that for carbamoyl phosphate, although reproducible, is laborious, and further work is needed to develop a simpler and faster procedure. The contents of ornithine and carbamoyl phosphate in liver of normal fed rats is 0.15 and 0.1 μmol/g. of tissue respectively. Schimke (1963) reported values of 0.013–0.018 μm of ornithine/g. of rat liver; a completely different method used for the preparation of liver extracts is probably the source of the discrepancy between the values obtained by Schimke (1963) and those reported here.

Ornithine must be distributed between mitochondria and cytoplasm (Gamble & Lehninger, 1973) in unknown proportions. Carbamoyl phosphate, however, can be expected to be mostly in the mitochondria. Assuming that 1 g of liver equals 1 ml, the concentration of carbamoyl phosphate in mitochondria can be estimated to approach 0.5 mM, and that of ornithine to be less than 0.57 mM. That is, carbamoyl phosphate may be present at concentrations above the K_m of ornithine carbamoyltransferase for this substrate at pH 7.5, though below saturation; ornithine is present at concentrations well below the K_m for total ornithine at pH 7.5. The ionic species of ornithine, which is the substrate of human ornithine carbamoyltransferase, is the zwitterion (Snodgrass, 1968) resulting from the deprotonation of a group with a pK of 8.7 (Batchelder & Schmidt, 1940; Snodgrass, 1968); the data in Table 2 suggest that the same is true of the rat liver enzyme. At pH 7.1, the ratio of zwitterion to total ornithine would be about 0.02; assuming this pH to be near the physiological value, the effective concentration of ornithine in rat liver would be much lower than the concentration of total ornithine, and lower than the K_m of ornithine carbamoyltransferase for the zwitterion (Snodgrass, 1968). It seems possible, therefore, that the excess of ornithine carbamoyltransferase over carbamoyl phosphate synthetase I *in vivo* may be much smaller than had been assumed on the basis of measurements *in vitro*. This may be relevant to the findings in some patients with an ornithine carbamoyltransferase deficiency in which the enzyme activity measured *in vitro* is still quite high with respect to the normal average (Levin, 1971); if the ratio of ornithine carbamoyltransferase to carbamoyl phosphate synthetase I *in vivo* approached that *in vitro* (approx. 100), urea synthesis would be expected to proceed with normal flexibility in those cases, with no blockage occurring at the ornithine carbamoyltransferase step, yet those children are unable to cope with normal protein loads, which cause enormous increases in their blood NH_3.

The data presented in this paper strengthen the enzymological basis on which the results of whole-animal studies on the synthesis of urea and arginine can be explained, and some pathological findings in humans can be understood. Obviously, much more information is needed, not only on the activity of enzymes *in vivo* and the concentration of intermediates of the urea cycle, but on the interrelationships of the cycle with, among other processes, the tricarboxylic acid cycle, the transport and metabolism of amino acids and the induction and repression of enzymes.

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