Purification and Properties of Arginase of Rat Kidney

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(Received 22 January 1973)

L-Arginase from rat kidney was partially purified and some properties were compared with those of L-arginase of rat liver. The kidney enzyme was firmly bound to the mitochondrial fraction and after solubilization required arginine or an unknown factor in tissue extracts for stabilization after dialysis. The two enzymes differed also in stability with respect to acetone treatment, heating or freezing. In further contrast with liver arginase, arginase from kidney was not adsorbed to CM-cellulose at pH 7.5 and its activity was not increased by incubation with Mn^{2+} . Other differences were seen in relative specificities for substrates, ratio of hydrolysis rates with high and low concentrations of arginine and effects of certain inhibitors. Antisera prepared to pure liver arginase did not cross-react with partially purified kidney arginase.

The role of arginase (L-arginine ureohydrolase; EC 3.5.3.1) in the metabolic life of cells generally has been considered in terms of its function in the urea cycle. The presence of this enzyme in organisms using other pathways for disposing of nitrogen has been explained as a consequence of functional evolutionary deletions or repressions of other enzymes of this cycle (Brown & Cohen, 1960; Watts & Watts, 1966). Similar considerations have been applied to discussions of controls of the urea cycle during development, in that the presence of arginase in organs lacking the urea cycle presumably reflects repression of other enzymes of this cycle during differentiation or development (Mora *et al.*, 1965a).

Arginase has been purified from a number of organisms, some of which excrete urea whereas others excrete uric acid. These arginases have been shown to possess different properties with respect to K_m values, inhibition by excess of substrate and antigenicity; they also have different molecular weights (Mora *et al.*, 1965b). However, a proposal to classify these arginases into two groups, 'ureotelic' and 'uricotelic' (Mora *et al.*, 1965b), does not seem justified (Reddy & Campbell, 1970).

An alternative approach to understanding the function of arginase in organisms or in tissues that do not form urea is suggested by the observations that arginine is an essential nutrient for the chicken (Klose *et al.*, 1938), for certain insects (House, 1965) and for cells in tissue culture (Eagle, 1955). Furthermore, dietary arginine apparently serves as the sole source of ornithine (Nesheim & Garlich, 1963), which in turn can be metabolized to either proline or glutamate. The growing rat also requires arginine, but this requirement is partially satisfied by proline and/or glutamic acid (Womack & Rose, 1947). Conversion of arginine into proline has been demonstrated in cells in tissue culture (Kruse, 1961; McCarty *et al.*, 1964; Eagle *et al.*, 1965) and in the silkworm

(Reddy & Campbell, 1969). These data are in accord with the concept that arginase can function physiologically also for the metabolism of arginine to proline and/or glutamate.

The purpose of the study of which this report forms a part is to determine whether the arginase that is used for catabolism of arginine to proline or glutamate is a species of protein different from the arginase operating in the urea cycle. It is possible that this second type of arginase evolved or developed independently of the urea cycle, that the genes coding for the two types of arginase are independent and that the expressions of these genes are controlled by different factors. Data in the literature already point to significant differences in the probable functions of liver and kidney arginases. Thus rat liver has an active urea cycle, rat kidney does not (Ratner & Petrack, 1953; Jones *et al.*, 1961). Steroid hormones administered to castrated male mice and rats increased kidney arginase up to eightfold without effect on the enzyme in liver (Kochakian, 1944, 1947; Kochakian *et al.*, 1948). The point of departure of the present study was a comparison of some properties of preparations of these enzymes. Accordingly, further differences between the arginases of rat liver and kidney have been shown with respect to association with subcellular fractions of tissue homogenates. The kidney enzyme has been partially purified and some catalytic differences from those of purified liver arginase have been demonstrated by kinetic measurements.

Experimental

Enzyme assays

Arginase activity was determined by measurement of urea produced, by using the reaction with Ehrlich's reagent according to the method of Hagan & Dallam

(1968). To 0.9 ml of a solution containing 100 μ mol (unless otherwise noted) of arginine at pH 9.5, were added 100 μ l of enzyme solution containing 1 μ mol of MnCl_2 and 1 μ mol of Tris-HCl, pH 9.5. The final volume was 1 ml. Samples were incubated at 37°C for 10 min. The pH remained constant at 9.5 during this period. The reaction was terminated by addition of 2.5 ml of the Ehrlich reagent and the absorbance at 450 nm was determined after 20 min. Correction was made for the contribution of arginine to the colour value. When particulate preparations were used, or when more than 0.3 mg of protein was added per sample, the reaction was terminated by adding 1 ml of 10% trichloroacetic acid, the denatured protein removed by centrifugation and a portion (1 ml) of the supernatant used for the reaction with the Ehrlich reagent. Hagan & Dallam (1968) reported that zero-order kinetics were obtained until about 5% hydrolysis of arginine at an initial concentration of 0.1 M. Under our conditions of assay, which were essentially the same, this linearity was confirmed and enzyme concentrations chosen that would not hydrolyse more than 5% of the substrate in 10 min. Activity was proportional to the concentration of enzyme within this limit. For kinetic studies with purified arginase and low concentrations of arginine the spectrophotometric method of Ward & Srere (1967) was used to monitor the course of reaction. Unless otherwise noted, the experimental cuvette contained 1.0 ml of 10 mM-arginine and 0.01 M-Tris-HCl at pH 9.5. The reaction was initiated by addition of 10 μ l of a solution of arginase. Arginine is not present in saturating concentrations in this procedure. Nevertheless, in confirmation of the results of Ward & Srere (1967), it was found that by using the rate observed during the first 30 s as the initial velocity of reaction, the rate was proportional to enzyme concentration over a total change of absorbance of 0.2/min. The conditions of assays were chosen so as to stay well within this limit. A unit of activity of arginase is defined as the formation of 1 μ mol of urea/min. Spectrophotometric determinations were conducted with either the Zeiss PMQ II or the Beckman DU spectrophotometer with a Gilford recording attachment. Glutamate dehydrogenase was assayed by measuring NADH oxidation at 340 nm with $(\text{NH}_4)_2\text{SO}_4$ and α -oxoglutarate added, at pH 7.4, in 0.05 M-potassium phosphate solution (Strecker, 1955). Succinate-cytochrome *c* reductase was determined as described by Giuditta & Strecker (1959), by measuring the reduction of cytochrome *c* at 550 nm. Ornithine δ -aminotransferase and proline oxidase were assayed as described by Strecker (1965, 1971) respectively. Glutamine synthetase was assayed according to Elliott (1955). DNA was extracted by the procedure described by Schneider (1945) and determined with the diphenylamine reagent (Dische, 1954).

Preparation of tissues and of subcellular fractions

All operations were performed at 0–4°C. Kidneys and liver were removed after decapitation of the animal. The kidneys were weighed, decapsulated and homogenized in 9 vol. (v/w) of 0.25 M-sucrose–0.01 M-Tris-HCl, pH 7.5, a Teflon motor-driven homogenizer of the Potter-Elvehjem type being used. Livers were treated the same way, after removal of gross connective tissue and washing several times with the sucrose-Tris-HCl to remove blood. The homogenates were separated into subcellular fractions by the method of Schneider & Hogeboom (1950), except that the microsomal and supernatant fractions were not separated and the nuclear fraction was further purified by a modification of the method of Hymer & Kuff (1964) as follows. The nuclear fraction was suspended in 9 vol. (of the original tissue weight) of 0.25 M-sucrose–0.001 M- MnCl_2 –0.01 M-Tris-HCl, pH 7.5, and centrifuged at 1000g for 10 min. The resulting sediment was suspended in 9 vol. of the same solution containing also 0.5% (w/v) Triton X-100. This suspension was homogenized with a motor-driven Teflon homogenizer for 1 min, and the suspension centrifuged at 1000g for 10 min. The resulting sediment was treated as previously, and the two supernatants were combined. The final pellet was suspended in 2 vol. of 0.25 M-sucrose–0.01 M- MnCl_2 –0.01 M-Tris-HCl, pH 7.5.

Purification of rat liver arginase

The purification procedure, which is a modification of that of Schimke (1964), was kindly made available to us by Dr. C. Huitron, Dr. R. Palacios and Dr. G. Soberon of The University of Mexico.

The weight of liver used was 178 g. All operations were conducted in an ice bath unless otherwise noted. The homogenization, extraction, acetone precipitation and heating steps were conducted as described by Schimke (1964). The heating step was repeated on the supernatant obtained from the previous heating step. This twice-heated protein solution was then passed into a column (1.6 cm $\times$ 20 cm) of CM-cellulose prepared as described below and previously equilibrated with 0.01 M-Tris-HCl, pH 7.2. A portion (300 ml) of the same solution was then passed through the column, followed by 180 ml of 1.0 M-KCl–0.01 M-Tris-HCl, pH 7.2. The fraction eluted by the KCl solution contained the enzyme and was then dialysed against 1 litre of 0.02 M-Tris-HCl, pH 7.2, for 4 h, the medium being changed hourly. This solution was then treated with ethanol as described by Schimke (1964). The active fraction was frozen and freeze-dried. The dried protein was dissolved in 5 ml of water and an insoluble residue removed by centrifugation. To this solution was added a solution of $(\text{NH}_4)_2\text{SO}_4$, saturated at 0°C, to a final concentration of 46% saturation.

The percentage of saturation was calculated on the basis of initial and final volumes of solution. The inactive precipitate was removed by centrifugation. More $(\text{NH}_4)_2\text{SO}_4$ was added to 66% saturation. The precipitate, which contained the arginase activity, was collected by centrifugation and dissolved in 5 ml of 0.01 M-Tris-HCl, pH 7.5. The solution was dialysed for 4 h against 500 ml of 0.01 M-Tris-HCl, pH 7.5, with hourly changes. This protein solution then was passed into a column (1.6 cm $\times$ 20 cm) of CM-cellulose (Whatman CM-52) that had been equilibrated with 0.01 M-Tris-HCl, pH 7.5. The column was eluted with 30 ml of the same buffer followed by 35 ml of 0.01 M-Tris-HCl-0.10 M-arginine, pH 7.5. The enzyme appeared in two separate fractions. Each had a specific activity of 1600 units/mg, and each migrated as a single band with the same mobility during electrophoresis on polyacrylamide gel.

Protein concentrations of non-turbid solutions were determined according to the method of Warburg & Christian (1941) as described by Layne (1957), and the values obtained were in close agreement with those obtained by the method of Lowry *et al.* (1951) with dry bovine plasma albumin (obtained from Armour Pharmaceutical Co., Chicago, Ill., U.S.A.) used as a standard. The latter method was used for solutions containing particulate matter. When Mn^{2+} was present, this procedure was modified as follows.

A portion (0.6 ml) of 1 M-NaOH was added to 0.2 ml of a solution containing between 20 and 200 μg of protein. After 30 min, 0.4 ml of water was added and the solution mixed and centrifuged to remove the precipitate that formed. A portion of the supernatant was used for protein determination by the standard procedure of Lowry *et al.* (1951).

Antibodies to the purified fractions of liver arginase were prepared according to Horowitz & Scharff (1969) as follows.

A portion of each enzyme solution was diluted to a concentration of 4 mg/ml and emulsions were prepared with equal volumes of complete Freund's adjuvant. A total of 4 mg of arginase, in 2 ml of emulsion, was injected into the toe pads of a rabbit. After 4 weeks, 4 mg of a similar emulsion, freshly

prepared, was injected into the leg muscles of the rabbit. The Ouchterlony (1958) double-diffusion technique was used to test the immunological reactivities of antisera against various arginase preparations.

Sephadex was obtained from Pharmacia Fine Chemicals Inc.; DEAE-cellulose (DE-32 and DE-52) and CM-cellulose (CM-52) were Whatman grade. These materials were pretreated according to the procedure described by the distributors (Whatman Advanced Ion Exchange Celluloses Laboratory Manual, W. and R. Balston Ltd., Maidstone, Kent, U.K.). Visking casing for dialysis was always pretreated by heating for 1 h at 100°C in a solution of 10% Na_2CO_3 and 0.2% EDTA, followed by heating for an additional hour at 100°C in 10% Na_2CO_3 . The casing was then washed with deionized water until all alkali had been removed.

All reagents were commercial products of high purity and solutions were prepared in deionized or distilled water.

Results

Subcellular distribution of kidney arginase

Kidneys from adult male rats were homogenized with a solution of 0.25 M-sucrose and the homogenate was separated into subcellular fractions as described in the Experimental section. The data presented in Table 1 demonstrate that most if not all of the arginase activity of rat kidney was in the mitochondrial fraction. The activity in the supernatant-microsomal fraction and in the washings of the nuclear fraction could be derived from mitochondrial contamination. Parallel experiments, conducted with rat liver, demonstrated in confirmation of data in the literature that most of the arginase of this organ was associated with the nuclear and microsomal fractions (Schimke, 1962).

The identifications of the relevant subcellular fractions in liver and kidney were confirmed by determinations of the following marker enzymes or components. Mitochondria (liver and kidney): succinate-cytochrome *c* reductase, glutamate dehydrogenase,

Table 1. *Subcellular distribution of kidney arginase*

Fractions were prepared and activities determined as described in the Experimental section; 2.4 g of kidney was used.

Fraction	Activity		
	Total units	Units/g	%
Supernatant + microsomal	17.8	7.40	16.8
Mitochondrial	72.0	30.0	67.6
Washed nuclear	0	0	0
Washings of nuclear fraction	16.7	6.95	15.6

proline oxidase and ornithine δ -aminotransferase; nuclei (liver): DNA; microsomes (liver): glutamine synthetase. In further confirmation of earlier investigations, most of the liver arginase (83%) was extracted with a solution of 0.145 M-KCl (Rosenthal *et al.*, 1956).

Extraction of kidney arginase

In contrast with arginase of rat liver (Mora *et al.*, 1965a; Schimke, 1962, 1964), the kidney enzyme was difficult to solubilize. Homogenization with solutions containing KCl, MnCl_2 , Tris-HCl or sucrose brought into solution less than 20% of the total arginase activity. Repeated extractions with 0.25 M-sucrose solutions, up to four times, solubilized less than 50% of the total enzyme. A further difference from the arginase of liver (Greenberg, 1951) is the observation that the addition of Mn^{2+} to the homogenizing medium had no effect either on total activity of the homogenate or on the activity subsequently recovered in the fractions.

Subjecting kidney homogenates to preliminary freezing and thawing or to treatment with acetone permitted subsequent extraction of 50–65% of the total arginase activity. However, the best yield and highest specific activity were obtained by first freezing the mitochondrial fraction in sucrose solution, thawing and then sonicating. The extract obtained after centrifugation was used for the purification of the enzyme as described below.

In the course of exploring procedures for purification of kidney arginase, there emerged several differences in behaviour from the liver enzyme. These differences include stability to acetone treatment, heating or freezing as well as behaviour on ion-exchange columns.

Properties of kidney arginase

Acetone treatment. Precipitation with acetone is one of the steps commonly used for purification of rat liver arginase. The enzyme from kidney is partially extractable into aqueous media after treating the homogenate with acetone. The arginase activity obtained in this way divided into two fractions on passage through a column of DEAE-cellulose. The first fraction, containing about 60% of the total activity, came through in the void volume; the second fraction was retained and had to be eluted with 0.3 M-Tris buffer, pH 7.2. The appearance of fraction II was suppressed by addition of 0.01 M-dithiothreitol to the initial extracting solution and to the eluting solutions. Dialysis of fraction I for 4 h against a Tris buffer solution through which air was bubbled converted part of fraction I into fraction II, as judged by rechromatography on DEAE-cellulose columns. Arginase extracted from kidney mitochondria without

previous acetone treatment, as described below, passed through DEAE-cellulose columns as one fraction and was not affected by dithiothreitol. It seems therefore that acetone treatment of kidney arginase, in contrast with liver arginase, converted the enzyme into a form that was relatively easily oxidized.

Chromatography. Liver arginase in solution at pH 7.5 is not adsorbed on DEAE-cellulose but is retained on CM-cellulose, from which it can be eluted with salt solutions. The kidney enzyme under the same conditions was not retained by either DEAE- or CM-cellulose.

Stability. Arginase from kidney retained full activity at 60°C for 10 min in the presence of glycine and Mn^{2+} , a property also exhibited by the liver enzyme (Schimke, 1962, 1964). Longer periods at this temperature resulted in progressive decrease of activity with a half-life of 100 min. Rat liver arginase is completely stable to 60°C for at least 20 min (Schimke, 1962, 1964). In further contrast with the enzyme from liver, arginase from kidney lost activity when dialysed against solutions of Tris-HCl, and MnCl_2 and KCl in combination or separately, or upon passage through a column of Sephadex G-25.

Thus, for example, dialysis of kidney arginase for 3 h against 0.01 M-Tris-HCl–0.01 M- MnCl_2 , pH 7.5, with hourly changes of this solution resulted in losses of activity up to 50%. Since no loss of activity was observed with a control solution of enzyme in the same buffer maintained in the cold for the same period of time, it appeared that a protective diffusible factor(s) was present in the extract. Indeed, when the enzyme solution was dialysed against a supernatant obtained after heating a homogenate of rat kidney in 0.01 M-Tris-HCl–0.01 M- MnCl_2 , pH 7.5, at 97°C for 60 min, only 20% of the activity was lost. This protective factor was replaceable by arginine or ornithine. Only 5–10% of the activity was lost upon dialysis against 0.01 M-Tris-HCl containing 0.005 M-arginine or ornithine.

During the course of preparation and purification of arginase from rat kidney, it was observed that this enzyme lost activity while frozen even with arginine added. Thus a solution of kidney arginase in 0.01 M- MnCl_2 –0.005 M-arginine–0.005 M-Tris-HCl, pH 7.5, lost 75% of total activity in 12 days at –15°C. A duplicate solution maintained at 0–4°C lost no activity during the same period.

The information gained from these preliminary experiments was used to develop the following method of purification of arginase of rat kidney. All steps, unless otherwise noted, were carried out at 0–4°C.

Purification of kidney arginase

Extraction from mitochondrial fraction. A suspension of mitochondria was prepared from a homo-

genate of rat kidney as described in the Experimental section and maintained frozen overnight at -15°C . After the suspension was thawed, a solution of 2M- MnCl_2 was added to bring the concentration to 0.01M with respect to MnCl_2 . This procedure facilitated the removal of mitochondrial debris after sonication. The suspension was sonicated in 15ml portions by using six periods of sonication for 15s at 50W with the model W 185 Sonifier (Heat Systems-Ultrasonics Inc., Plainview, N.Y., U.S.A.). The solution was kept in an ice bath during treatment with 30s periods of cooling between each 15s period of sonication. The fragmented mitochondria were centrifuged at 105000g for 2h and the supernatant was removed.

First $(\text{NH}_4)_2\text{SO}_4$ fractionation. The above supernatant was adjusted to pH 7.5 with 2M-Tris base and solid $(\text{NH}_4)_2\text{SO}_4$ was added to 58% saturation. The pH was maintained at 7.5 during this step by the intermittent addition of suitable portions of Tris base. After the suspension had been stirred for 45min it was centrifuged at 20000g for 30min. The precipitate was dissolved in 0.005M-Tris-HCl-0.005M-arginine-0.01M- MnCl_2 , pH 7.5, and dialysed for 4h against hourly changes of 500ml each of the same solution.

Heat step. A solution of 1M-glycine at pH 7.5 was added to a final concentration of 0.025M. The solution was brought rapidly to 58°C by immersion in a water bath held at 65°C , maintained at 58°C for 1min by subsequent transfer to a bath at 58°C and then rapidly chilled in an ice bath. The precipitate was removed by centrifugation at 10000g for 20min.

Second $(\text{NH}_4)_2\text{SO}_4$ fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 46% saturation with the pH maintained at 7.5 by suitable additions of 2M-Tris base. After 45min the precipitate was removed by centrifugation as before; more $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant, to 62% saturation. The suspension was centrifuged as before and the pellet, which contained the activity, was dissolved in 0.005M-Tris-HCl-0.005M-arginine-0.01M- MnCl_2 , pH 7.2. The solution was dialysed for 4h against four

hourly changes of 500ml each of the same buffer solution as was used to dissolve the pellet obtained previously.

Chromatography on DEAE-cellulose. A column (1.6cm $\times$ 30cm) of DEAE-cellulose (Whatman DE32) was equilibrated with the same buffer solution. The dialysed kidney enzyme was passed into the column, which was then eluted with the same buffer solution. Over 90% of the arginase activity present came through with the void volume. Ammonium sulphate was added to this solution to a concentration of 75% saturation, with pH maintained at 7.5 by addition of 2M-Tris base. The suspension was centrifuged and the pellet dissolved in 0.005M-Tris-0.005M-arginine-0.01M- MnCl_2 , pH 7.2. A small insoluble residue was removed by centrifugation. The arginase activity, as shown in Table 2, was purified about 160-fold with a yield of about 35%. Further purification was difficult owing to instability of the enzyme at this stage.

Comparison of properties of kidney and liver arginases

Effect of metal ions. A variety of metal ions have been found to activate and/or stabilize arginases from different tissues (Hellerman & Perkins, 1935; Greenberg, 1951). These, in addition to Mn^{2+} , included Co^{2+} , Ni^{2+} and Fe^{2+} . Some of these ions also have been reported to shift the pH optimum (Greenberg, 1951). With respect to the enzyme from rat liver, Campbell (1966) recorded activation by Mn^{2+} , Cd^{2+} or Ni^{2+} but not by Co^{2+} .

A sample of partially purified kidney arginase was incubated in solutions of the bivalent cations shown in Table 3, as well as in EDTA, for 30min at 37°C or 20h at 5°C . The experiment with EDTA was conducted since the enzyme had been prepared in the presence of Mn^{2+} and the concentration of Mn^{2+} from the enzyme solution was 10^{-4}M in each incubation. Incubation with Mn^{2+} did not increase activity and none of the ions tested induced a shift of pH optimum. Enzymic determinations after these periods

Table 2. Purification of arginase from a mitochondrial fraction of rat kidney

The purification procedure was started with 52g of rat kidney.

Stage	Activity (units)	Protein (mg)	Specific activity (units/mg)	Recovery (%)
Homogenate	1920	12800	0.15	100
Mitochondria	1510	3940	0.38	79
Extraction	1530	1210	1.26	80
First $(\text{NH}_4)_2\text{SO}_4$ fractionation	1310	428	3.06	68
Heat step	1050	141	7.42	55
Second $(\text{NH}_4)_2\text{SO}_4$ fractionation	905	94	9.65	47
Chromatography	867	41.5	20.9	45
Third $(\text{NH}_4)_2\text{SO}_4$ fractionation	684	28.6	23.9	36

Table 3. *Inactivation of kidney arginase by metal ions*

Portions of kidney arginase, specific activity 17.5 units/mg, were incubated in solutions containing 0.005 M-Tris and 0.005 M-arginine at pH 7.5, to which each of the compounds below was added to a final concentration of 0.01 M. In each case incubations were conducted at 37°C for 30min and at 5°C for 20h.

Component present	Activity retained after 30min at 37°C (%)	Activity retained after 20h at 5°C (%)
MnCl ₂	100*	94
EDTA	98	78
MgCl ₂	97	88
CoCl ₂	89	18
HgCl ₂	65	—
CdSO ₄	50	23
FeSO ₄	46	0
NiSO ₄	38	15

* The same activity as the control incubation, which contained 0.1 mM-MnCl₂.

Table 4. *Relative substrate specificities of arginase from rat liver and kidney*

The concentration of substrates was 0.1 M. Other conditions of assay were as described in the Experimental section. The kidney arginase preparation had a specific activity of 24.9 units/mg and the liver enzyme a specific activity of 1250 units/mg. The rates are expressed relative to the rate of hydrolysis of L-arginine. —, Not tested.

Substrate	Relative activity	
	Kidney arginase	Liver arginase
L-Arginine	100	100
γ-Guanidinobutyrate	0	0
β-Guanidinobutyrate	0	—
L-Canavanine	38	6.6
L-Homoarginine	0	0.36

of incubation demonstrated that activity was fully retained in solutions of Mn²⁺ or Mg²⁺ and perhaps to a somewhat lesser extent in a solution of EDTA. Fe²⁺, Hg²⁺, Co²⁺, Cd²⁺, and Ni²⁺ all decreased enzyme activity.

Specificity. The guanidine derivatives listed in Table 4 were each tested for substrate activity at a single concentration with the kidney and liver arginases. The major differences in specificity of the two enzymes appeared with canavanine, which was hydrolysed about five times more rapidly by the

Fig. 1. *Relative rates of hydrolysis by rat kidney and liver arginases of L-arginine at concentrations of 720 mM and 100 mM*

Incubations were conducted at 37°C for 10min and activity was determined by the method of Hagan & Dallam (1968) as described in the Experimental section. The ratios of activities at the two concentrations of arginine are plotted for the kidney enzyme (specific activity 23.9 units/mg) (●—●) and for the liver enzyme (specific activity 1600 units/mg) (○—○).

kidney enzyme, and with homoarginine, which was not attacked by the arginase of kidney. A slow rate of hydrolysis of homoarginine by the liver preparation was significant; complete hydrolysis of the compound was obtained by sufficiently prolonged periods of incubation, with a high concentration of enzyme. Double-reciprocal plots were made according to Lineweaver & Burk (1934), to compare K_m values for the liver and kidney enzymes at pH 9.5. At this pH the K_m value for kidney arginase was 18 mM, about two and a half times the K_m value for liver arginase (6.8 mM). Addition of NaCl up to 1 M or potassium glutamate up to 0.8 M had no effect on the K_m value of the kidney enzyme.

Special consideration was given to comparing the rates of hydrolysis at high and low concentrations of the substrate because of the suggestion that inhibition by high concentrations of substrate is characteristic of arginase from ureotelic animals (Mora *et al.*, 1965b). However, no inhibition of the liver enzyme was observed at concentrations of arginine up to 0.72 M in the range pH 7.4–10.0.

In contrast the kidney enzyme was inhibited by high substrate concentration at low pH values and activated by these same concentrations of substrate at high pH values. These data are summarized in Fig. 1, in which are plotted the ratios of the rate of hydrolysis at 0.72 M-arginine to the rate of hydrolysis at 0.10 M-arginine as a function of pH. With the liver enzyme this ratio was constant. With kidney enzyme this ratio fell on a line with values below 1.0 at pH

Table 5. *Inhibitors of arginase activity*

Kidney arginase of specific activity 23.9 units/mg and liver arginase of specific activity 1250 units/mg were incubated with the compounds listed at the concentrations shown under the standard conditions described in the Experimental section. The pH of the solution of each compound was adjusted to 9.5 by addition of HCl or KOH. The rate of hydrolysis of arginine in the absence of inhibitors is taken as 100.

Inhibitor	Concn. during reaction (M)	Relative rate of hydrolysis	
		Kidney arginase	Liver arginase
L-Canavanine	0.01	97	80
DL-Homocysteine	0.02	26	84
Borate	0.02	4	1.0
L-Homoarginine	0.02	96	85
L-Lysine	0.01	30	61
L-Lysine	0.003	62	75
L-Proline	0.04	33	61
L-Ornithine	0.005	40	76
L-Isoleucine	0.01	46	33
L-Methionine	0.01	99	90
L-Leucine	0.025	65	43
L-Valine	0.01	40	35
NaCl	1.00	100	69
L-Glutamate	0.800	103	—
L-Glutamate	0.65	—	77

values less than 8.9, and values above 1 at pH values above 8.9.

Inhibitors. A number of substrate analogues, amino acids, products of ornithine metabolism and other compounds reported to inhibit liver arginase were tested for inhibitory effect with both enzymes. These data are presented in Table 5. Not shown in the table are the data obtained with γ -guanidinobutyrate, δ -aminovalerate, β -guanidinopropionate, spermine, cadaverine, spermidine, putrescine and aspartate. None of these compounds at 0.01M concentration exhibited inhibition. In general, the susceptibility to inhibition by the various compounds tried was parallel for both enzymes. However, NaCl or glutamic acid in relatively high concentrations inhibited the liver enzyme without any effect on the kidney arginase. Inhibition of liver arginase by solutions of high ionic strength has been reported previously (Campbell, 1966). Canavanine and homoarginine were inhibitory to the liver enzyme only, whereas homocysteine, lysine, proline and ornithine were more strongly inhibitory to kidney arginase. Leucine was somewhat more inhibitory to the arginase from liver. Not reported in the table are experiments with *p*-hydroxymercuribenzoate, which at 0.003M decreased activity with both enzymes by about 10% after preincubation with either enzyme for 30 min.

L-Ornithine has been found previously to inhibit competitively arginase from chicken liver or *Neurospora crassa*, and to inhibit non-competitively rat liver

arginase (Mora *et al.*, 1965a). Both lysine and ornithine were reported to inhibit competitively both snail and rat liver arginases (Campbell, 1966). Fig. 2 and Fig. 3 present reciprocal plots in the presence of various concentrations of ornithine and lysine respectively. It is noted that the downward deflexion of these curves indicative of an activation by high substrate concentrations persists at 20mM-ornithine but is overcome by 10mM-lysine. Extrapolations of the straight-line portions of the curves of Fig. 2 intersect beyond the two axes, whereas extrapolations of the curves of Fig. 3 appear to intersect at the ordinate. Not shown are the data from a similar group of experiments with proline, which demonstrate that, even at a concentration of 5mM, the inhibitory action of this amino acid overcame the stimulation by high concentrations of substrate.

Immunological studies. Antisera to both fractions of purified liver arginase antigen obtained from the second CM-cellulose column were prepared as described in the Experimental section. These antisera were allowed to diffuse by the Ouchterlony (1958) technique against a solution containing either one purified fraction of arginase or both fractions, which were separable on CM-cellulose. In either case, only a single zone of precipitation was obtained. A distinct precipitin line was obtained with arginase samples containing only 0.45 unit of activity.

Antiserum prepared to either of these two arginase fractions did not produce a zone of precipitation

Fig. 2. Inhibition of rat kidney arginase by ornithine at pH9.5

Plots are of reciprocal velocity versus reciprocal substrate concentration at these concentrations of ornithine (mM): 0 (●); 5 (○); 10 (□); 20 (×). Velocities are expressed relative to the rate of hydrolysis of 0.1M-arginine at pH9.5 and 37°C by an equal concentration of enzyme. Assays were by the method of Ward & Srere (1967) conducted as described in the Experimental section.

when allowed to diffuse against a solution of partially purified kidney arginase containing 6.5 units of activity.

Discussion

The activity of arginase in the liver of the adult male rat is about 30 times that in the kidney of the same animal. This difference in activity could mean either that kidney contains relatively little of the same arginase protein as liver or that the two arginase activities are due to different enzymes with different turnover numbers. The data presented in this communication provide support for the latter proposal. Liver arginase is readily solubilized by extraction of the tissue with ionic solutions near physiological concentrations whereas kidney arginase is relatively firmly bound to mitochondria and requires fragmentation of these organelles to bring the enzyme into

Fig. 3. Inhibition of rat kidney arginase by lysine at pH9.5

Details and conditions are similar to those given for Fig. 2, with these concentrations of lysine (mM): 0 (●); 3 (○); 5 (▲); 10 (×).

solution. Liver arginase is stable to prolonged dialysis (Schimke, 1962), but kidney arginase is inactivated during dialysis unless arginine or an unidentified tissue factor is added to the medium. The liver enzyme is adsorbed to CM-cellulose; the kidney enzyme is not. The liver enzyme is inhibited by high salt concentration; the kidney enzyme is not.

The substrate inhibition of rat liver arginase reported by other investigators (Mora *et al.*, 1965a) could not be reproduced in this laboratory. The ratio of activities at high (0.72M) and low (0.10M) concentrations of arginine was above 1 and constant over the range pH7.4–10. By contrast, rat kidney arginase with these substrate concentrations exhibited ratios of activities that varied linearly with pH. The apparent K_m values for arginine at the optimum pH for the reaction differed for the two enzymes and relative velocities with canavanine as substrate also differed substantially. In addition, there were some differences in substrate specificity and in the extent of inhibition obtained with certain compounds.

Among the inhibitors, the effects of lysine and proline on the kidney enzyme were especially interesting. The activation of this enzyme by high concentrations of arginine appeared to be abolished by these amino acids at specific concentrations. These data may indicate a second binding site, for arginine and certain other amino acids, which when occupied modifies the

catalytic site and which is dependent on pH. The extremely high concentration of arginine required to demonstrate effects at this second site raises the possibility that some other compound might be the physiological modifier.

Finally, strong evidence for the non-identity of the two arginase proteins was the lack of cross-reactivity with the kidney enzyme of antiserum prepared against the purified liver enzyme. The pure liver arginase was prepared by a modification of the method of Schimke (1964). In this modification two fractions were obtained by elution from a column of CM-cellulose with 0.1 M-arginine. Both fractions had the same specific activity, both migrated identically on polyacrylamide gel and the elution pattern was unchanged by substituting 0.1 M-NaCl for 0.1 M-arginine in the eluting solution. The conclusion that each was a single homogeneous protein was supported by the observations that each migrated as a single distinct band during electrophoresis on polyacrylamide gel and that each gave a single precipitin line in the Ouchterlony double-diffusion technique. The question of differences between these two fractions was not further investigated.

If indeed, as the data presented here indicate, the arginases of rat kidney and rat liver are different proteins it seems quite likely that the comparatively low activity of the kidney enzyme is related to a function for this enzyme other than the production of urea. The unimportance of the urea cycle in the kidney is also indicated by the low activities of two other urea-cycle enzymes, namely carbamoyl phosphate synthetase and ornithine carbamoyltransferase (Jones *et al.*, 1961).

The most likely metabolic role for arginase in kidney is to initiate the sequence of reactions metabolizing arginine to proline and/or glutamate. The importance of this pathway may be evident in other organisms, such as chickens and insects, as well as in cells in tissue culture; these do not synthesize arginine and therefore require it for survival (Klose *et al.*, 1938; Eagle, 1955; Nesheim & Garlich, 1963; House, 1965). In some of these species the conversion of arginine into proline has been reported (Kruse, 1961; McCarty *et al.*, 1964; Eagle *et al.*, 1965; Reddy & Campbell, 1969), although the importance of arginine versus glutamate as a precursor of proline has not been quantitatively assessed in all situations. In some species of cells in tissue culture the glutamate to proline pathway appears to predominate (Eagle *et al.*, 1965). In rat liver, compared with arginine glutamate is a poor precursor of proline (Rojkind & Diaz De Leon, 1970; H. J. Strecker & M. Rojkind, unpublished work). It is probable that the controls of these pathways vary with species, with tissue, with nutrition and with development. Further elucidation of structural differences and similarities between arginases isolated from different sources and under

different conditions should be helpful in obtaining an understanding of the regulation of the metabolic pathways in which these enzymes function.

This work was supported by Research Grant GB 12277 from the National Science Foundation and by PHS Training Grant 5T5 GM 1674 from the National Institutes of Health. H. J. S. was recipient of United States Public Health Service Research Career Program Award 1-KG-GM-2-487 from the National Institute of General Medical Sciences.

References

- Brown, G. W., Jr. & Cohen, P. P. (1960) *Biochem. J.* **75**, 82-91
- Campbell, J. W. (1966) *Comp. Biochem. Physiol.* **18**, 179-199
- Dische, Z. (1954) in *The Nucleic Acids* (Chargaff, E. & Davidson, J. N., eds.), vol. 1, pp. 285-305, Academic Press, New York
- Eagle, H. (1955) *Science* **122**, 501-504
- Eagle, H., Washington, C. L. & Levy, M. (1965) *J. Biol. Chem.* **240**, 3944-3950
- Elliott, W. H. (1955) *Methods Enzymol.* **2**, 337-342
- Giuditta, A. & Strecker, H. J. (1959) *J. Neurochem.* **5**, 50-61
- Greenberg, D. M. (1951) in *The Enzymes* (Sumner, J. B. & Myrback, K., eds.), vol. 1, pp. 893-921, Academic Press, New York
- Hagan, J. J. & Dallam, R. D. (1968) *Anal. Biochem.* **22**, 518-524
- Hellerman, L. & Perkins, M. E. (1935) *J. Biol. Chem.* **112**, 175-194
- Horowitz, M. S. & Scharff, M. D. (1969) in *Fundamental Techniques in Virology* (Habel, K. & Salzman, N. P., eds.), pp. 253-262, Academic Press, New York
- House, H. L. (1965) in *Physiology of Insects* (Rockstein, M., ed.), vol. 2, pp. 769-813, Academic Press, New York
- Hwyer, W. C. & Kuff, E. L. (1964) *J. Histochem. Cytochem.* **12**, 359-363
- Jones, M. E., Anderson, A. D., Anderson, C. & Hodes, S. (1961) *Arch. Biochem. Biophys.* **95**, 499-507
- Klose, A. A., Stockstad, E. L. R. & Almquist, H. J. (1938) *J. Biol. Chem.* **123**, 691-698
- Kochakian, C. D. (1944) *J. Biol. Chem.* **155**, 579-589
- Kochakian, C. D. (1947) *Amer. J. Physiol.* **151**, 126-129
- Kochakian, C. D., Garber, E. E. & Bartlett, M. N. (1948) *Amer. J. Physiol.* **155**, 265-271
- Kruse, P. F., Jr. (1961) *Pathol. Biol.* **9**, 576-578
- Layne, E. (1957) *Methods Enzymol.* **3**, 447-454
- Lineweaver, H. & Burk, D. (1934) *J. Amer. Chem. Soc.* **56**, 658-666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- McCarty, K. S., Woodson, B., Amstey, M. & Brown, V. (1964) *J. Biol. Chem.* **239**, 544-549
- Mora, J., Martuscelli, J., Ortiz-Pineda, J. & Soberon, G. (1965a) *Biochem. J.* **96**, 28-35
- Mora, J., Tarrab, R., Martuscelli, J. & Soberon, G. (1965b) *Biochem. J.* **96**, 588-594
- Nesheim, M. C. & Garlich, J. D. (1963) *J. Nutr.* **79**, 311-317

- Ouchterlony, O. (1958) *Progr. Allergy* **5**, 1-78
- Ratner, S. & Petrack, B. (1953) *J. Biol. Chem.* **200**, 175-185
- Reddy, S. R. R. & Campbell, J. W. (1969) *Biochem. J.* **115**, 495-503
- Reddy, S. R. R. & Campbell, J. W. (1970) *Comp. Biochem. Physiol.* **32**, 499-509
- Rojkind, M. & Diaz De Leon, L. (1970) *Biochim. Biophys. Acta* **217**, 512-522
- Rosenthal, O., Gottlieb, B., Gorry, J. D. & Vars, H. M. (1956) *J. Biol. Chem.* **223**, 469-478
- Schimke, R. T. (1962) *J. Biol. Chem.* **237**, 459-468
- Schimke, R. T. (1964) *J. Biol. Chem.* **239**, 3808-3817
- Schneider, W. C. (1945) *J. Biol. Chem.* **161**, 293-303
- Schneider, W. C. & Hogeboom, G. H. (1950) *J. Biol. Chem.* **183**, 123-128
- Strecker, H. J. (1955) *Methods Enzymol.* **2**, 220-225
- Strecker, H. J. (1965) *J. Biol. Chem.* **240**, 1225-1230
- Strecker, H. J. (1971) *Methods Enzymol.* **17B**, 251-254
- Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384-421
- Ward, R. L. & Srere, P. A. (1967) *Anal. Biochem.* **18**, 102-106
- Watts, D. C. & Watts, R. L. (1966) *Comp. Biochem. Physiol.* **17**, 785-798
- Womack, M. & Rose, W. C. (1947) *J. Biol. Chem.* **171**, 37-50