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The Heterogeneity of Arginases in Rat Tissues

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Arginase reactions in rat tissues were shown to be catalysed by three isoenzymes which can be separated by bidirectional electrophoresis on polyacrylamide gels. Anodic electrophoresis reveals a migrating band (isoenzyme I) present in all non-hepatic tissues except submaxillary gland and a non-migrating band found in all tissues. The latter is resolved by cathodic electrophoresis into isoenzyme III (characteristic of liver and submaxillary gland) and a non-moving band (isoenzyme II), present in kidney, intestine and pancreas. Sequential electrophoresis, in the two directions, of mixtures of liver and kidney extracts in the same gel columns separated all three isoenzymes. Differences in the solubilization properties, heat-sensitivity and substrate specificity of arginases from different tissues could be correlated with their electrophoretic behaviour. L-Canavanine could replace arginine as substrate in extracts of kidney but not of liver. Both kidney isoenzymes hydrolysed L-canavanine equally well, whereas isoenzyme III from submaxillary gland showed only very low activity. Antiserum against liver arginase interacted with the enzyme from submaxillary gland, but did not inactivate or adsorb arginase from kidney, intestine or pancreas. The distribution of arginase among 16 normal adult rat tissues is presented; the improved, sensitive, assay method was applicable to tissues containing as little as 0.1% of the hepatic activity.

The occurrence of at least two forms of arginase (EC 3.5.3.1) in mammalian tissues, one in liver and another in kidney and mammary gland, is well documented (Glass & Knox, 1973; Kaysen & Strecker, 1973; Reddi *et al.*, 1975). Their distinction had rested primarily on differences found in the stabilities of the enzymes to chemical procedures (Glass & Knox, 1973; Kaysen & Strecker, 1973; Gasiorowska *et al.*, 1970), temperature (Glass & Knox, 1973) and alkaline pH (Glass & Knox, 1973), in their interaction with antisera (Glass & Knox, 1973; Kaysen & Strecker, 1973) and their electrophoretic mobilities on starch (Farron, 1973) or polyacrylamide gels (Reddi *et al.*, 1975).

Previous results from our laboratory showed that kidney, intestine and mammary gland contained an isoenzyme that migrates toward the anode (Reddi *et al.*, 1975), absent from liver and submaxillary gland, and that extracts of kidney, intestine and mammary gland did not interact with antiserum against liver arginase (Glass & Knox, 1973; Reddi *et al.*, 1975). However, only about 30% of the isoenzyme that migrated towards the anode and less than 5% of the non-moving variant were recovered from the gels. It was uncertain whether such low recoveries could be representative of the distribution of arginase variants in tissues. The low recovery of the non-moving arginase also made it impossible to determine whether that arginase was identical in all tissues or whether it could be resolved into more than one zone of activity in a more effective electrophoretic system. A more elaborate procedure culminating in sequential bidirectional electrophoresis revealed that two isoenzymes (I and II), which are present in most non-hepatic tissues, both differ from the single isoenzyme (III) found in liver and submaxillary gland. Neither isoenzyme I nor isoenzyme II interacted with antiserum against liver arginase.

Although the distribution of total arginase activities in rat tissues has been reported before (Reddi *et al.*, 1975; Greengard *et al.*, 1970), tissues with activities ranging from 100units/g to less than 2units/g have now also been studied, as such activities can be determined accurately with the sensitive assay method described in the present paper.

Experimental

Materials

Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine (8.36M) and thiosemicarbazide were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.). L-Arginine, L-canavanine, butane-2,3-dione monoxime, glycine, L-asparagine and imidazole were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and Triton X-100 and riboflavin were obtained from Calbiochem (La Jolla, Calif., U.S.A.). All other reagents were of standard analytical grade.

Animals and tissue preparations

Tissues were obtained from adult male Kx rats (90 days old) or foetal Kx or CDF (Charles River Breeding Colony, Wilmington, Mass., U.S.A.) rats. Intestines were washed with 0.9% NaCl after excision. Duodenum is defined as the first 5cm posterior to the stomach, small intestine as the next 5cm and colon as the first 7cm after the caecum.

Antiserum against liver arginase was prepared by Dr. R. Glass as described by Glass & Knox (1973), and stored at −20°C. Normal serum was obtained from New Zealand rabbits.

Freshly excised tissues were homogenized in 4 or 9vol. of 56mM-imidazole/56mM-$MnCl_2$ buffer, pH7.4, in glass–Teflon homogenizers, and activated for 10min at 50°C in 1.0–2.0ml samples in 10mm× 75mm polypropylene tubes. The activated preparations were centrifuged at 105000*g* for 30min. Supernatants were used for electrophoresis or antibody titrations. Intestinal homogenates were pretreated (before activation) with Triton X-100 at a final concentration of 0.5% at 0°C for 30min to solubilize more of the arginase activity. Activities of arginase preparations were stable for at least 10 days at 4°C as activated homogenates or supernatants, but preparations older than 3 days were not used for electrophoresis, since some aggregation had occurred.

Modifications of arginase assay

The arginase assay, based on that of Schimke (1962), was modified to increase its sensitivity. Incubation mixtures, in total volumes of 0.8ml, contained 100μmol of L-arginine, 60μmol of glycine buffer (both adjusted to pH9.5 with 1.0M-NaOH), and 0.05–0.2ml of appropriately diluted enzyme (0.02–0.3unit of arginase).

After incubation at 37°C for 10min, the reactions were stopped with 0.2ml of 50% (w/v) trichloroacetic acid. After centrifugation, 0.05–0.1ml samples of the supernatants were analysed for urea content as described by Geyer & Dabich (1971). The samples were diluted to 0.5ml with water; 1.0ml of 36mM-butane-2,3-dione monoxime/61.7mM-thiosemicarbazide solution and 1.5ml of 3.6M-H_2SO_4/0.12mM-$FeCl_3$/38.6mM-H_3PO_4 were added; the tubes were covered with marbles and heated in a boiling-water bath for 10min. After cooling for 10min, the absorbances were read at 520nm in a Zeiss PMQ II spectrophotometer. Blanks consisted of unincubated complete mixtures. The molar extinction coefficient for the urea derivative under these conditions was determined to be 16950±1200litre·mol^{-1}·cm^{-1}. The absorbance was linear between 0.01 and 0.3mM-urea. Urea, added to the substrate-free mixtures containing extracts of liver, kidney, submaxillary gland or intestine, was recovered quantitatively. A unit of arginase is defined as 1μmol of urea formed/min at 37°C.

Antibody precipitation

Soluble activated enzyme extracts, containing 1.4–7.8units of arginase, were incubated for 0 and 45min at 37°C with 25–50μl of control rabbit serum or antiserum against liver arginase (Glass & Knox, 1973) in total volumes of 1.0ml. Samples (in duplicate) of 0.05ml were removed immediately after mixing the serum with the enzyme extract and assayed for initial arginase activity. After incubation, precipitates were allowed to aggregate at 0°C for 30min; the reaction mixtures were centrifuged for 30min at 105000*g*, and samples of the supernatants were analysed for arginase activity.

Electrophoresis

Electrophoresis on polyacrylamide gels was carried out at 25°C in a vertical Buchler electrophoresis chamber (Buchler Instruments, Fort Lee, N.J., U.S.A.). Glass electrophoresis tubes (5mm× 65mm; Bio-Rad Laboratories, Rockville Centre, N.Y., U.S.A.) were cleaned with $Na_2Cr_2O_7$ solutions and coated with a 10% (v/v) Triton X-100 solution. Gels were polymerized at 25°C in three separate layers by modifications of published procedures (Gabriel, 1971; Davis, 1964; Maurer, 1971). After the lower gel (0.5ml of appropriate mixture, 2.5cm high) was polymerized, it was overlaid with 0.1ml of sample gel, containing the marker dyes and 0.04ml of tissue extract (0.08–1.0unit of arginase); after photopolymerization of this gel, the upper gel (0.5ml of mixture) was photopolymerized on top of the sample gel. For anaerobiosis during polymerization, each gel was overlaid with 0.1ml of cold water. After polymerization, the water layers were removed carefully with a Pasteur pipette and tested for arginase activity. When ammonium persulphate was used as a catalyst, the excess was removed by pre-electrophoresis of the gel in the usual buffer system at 3mA/tube for 30min. Photopolymerization occurred within 30min with two 20W fluorescent bulbs, 3–5cm above the electrophoresis tubes. For the compositions of the stock solutions and working mixtures used for electrophoresis see Tables 1 and 2.

Electrophoreses were continued (at 2–2.5mA/tube) until the marker dyes migrated to within 0.5cm of the bottom of the gels (115–160min). Kidney extract was always run as a standard tissue for anodic migration; submaxillary-gland extract was the standard for cathodic electrophoresis.

Anodic electrophoresis. In anodic electrophoresis, the separation buffer contains Cl^- so that negative ions flow towards the positive pole (anode) attached to the lower buffer compartment.

Cathodic electrophoresis. In cathodic electrophor-

Table 1. *Stock solutions for gels for electrophoresis*

All solutions except solutions V and VI were stored at 0°C for at least 1 month. Stock solutions were modifications of those suggested by Gabriel (1971), Davis (1964), Maurer (1971) and Rodbard & Chrambach (1971).

	Solution		
Gel-forming agents	I	22.2% Acrylamide/0.6% *NN'*methylenebisacrylamide	
	II	20% Acrylamide/5% methylenebisacrylamide	
Marker dyes			
Anodic	III	0.5% Bromophenol Blue	
Cathodic	IV	0.5% Methyl Green	
Polymerization reagents	V	0.065M-Ammonium persulphate	(prepared fresh daily)
	VI	0.02mM-Riboflavin	
	VII	Concentrated *NNN'N'*-tetramethylethylenediamine (8.36M)	
Diluent	VIII	40% Sucrose	
Gel buffers	IX	0.15M-Maleic acid, 0.21M-KOH, 0.039M-Tetramethylethylenediamine	pH6.4
	X	0.11M-Maleic acid, 0.176M-KOH, 0.039M-Tetramethylethylenediamine	pH6.2
	XI	0.15M-Maleic acid, 0.196M-KOH, 0.039M-Tetramethylethylenediamine	pH6.1
	XII	0.494M-Trizma base, 0.48M-HCl, 0.039M-Tetramethylethylenediamine	pH7.8
	XIII	0.375M-Trizma base, 0.06M-HCl	pH6.7

Table 2. *Working solutions for electrophoresis*

Stock solutions were as in Table 1.

	Cathodic		Anodic		Bidirectional	
	Solution	ml	Solution	ml	Solution	ml
Lower gel	I	2.0	I	2.1	I	2.1
	VI	0.5	V	0.5	V	0.5
	VII	0.01	VII	0.05	VII	0.015
	IX	4.5	XII	5.0	XII	5.0
	Water	2.0	Water	2.25	Water	2.25
Sample gel	II	0.6	II	0.5	II	0.6
	IV	0.01	III	0.005	III	0.005
	VI	0.5	VI	0.5	IV	0.01
	VIII	0.4	VIII	0.5	VI	0.5
	X	0.5	XII	0.5	VIII	0.4
					X	0.5
Upper gel	II	1.8	II	1.0	I	2.0
	VI	1.5	VI	1.0	VI	0.5
	VIII	1.4	VIII	1.0	VII	0.01
	XI	1.5	XIII	1.0	IX	4.5

Compartment buffers

		pH		pH	
Upper	0.075M-Maleic acid, 0.011M-Trizma base, 0.01M-L-Asparagine	6.1	0.043M-Trizma base, 0.046M-Glycine	9.0	First direction; both buffers same as in cathodic system
Lower	0.02M-Maleic acid, 0.045M-KOH	8.2	0.062M-Trizma base, 0.05M-HCl	7.2	Second direction; both buffers same as in anodic system

esis, the separation buffer contains K^+ and the flow of positive ions is towards the negative pole (cathode) attached to the lower compartment. The buffer system used for cathodic electrophoresis was a compromise: at pH values lower than 6.0, only liver and submaxillary-gland arginase was stable; none of the arginase isoenzymes of any tissue tested migrated when the separation pH exceeded 6.6.

Bidirectional electrophoresis. For bidirectional electrophoresis, the gel in which anodic separation was to occur was polymerized first. After polymerization the gel was pre-run as for the anodic system. The tissue extract (0.04ml) and 0.1ml of the sample gel were photopolymerized as the next layer. A third layer of gel was photopolymerized over the sample gel. For the run in the first direction (cathodic) the tubes were inserted into the chamber with the layer of gel polymerized first, containing ammonium persulphate on top. The cathodic run was carried out at 25–45V for 120min. The tubes were then inverted (so that the gel containing Tris/HCl was now the lower, separating gel) and the compartment buffers were exchanged for those used in the anodic system. Electrophoresis in the anodic direction was run for 60min at 50–100V. The current for both runs was 2.5mA/tube. Both marker dyes reached the extreme gel slices.

Activity assays after electrophoresis

The gels were removed in ice-cold water and cut into 0.5cm sequential sections [the most anodically moving denoted as Segment 1 (*Enzyme Nomenclature*, 1973)], which were analysed individually for arginase activity.

The segments were immersed in 0.3ml of 0.2M-glycine buffer, pH9.5, and 0.3ml of water, mixed thoroughly with a Vortex mixer (Vortex-Genie, scientific industries, Springfield, Mass., U.S.A.) and 0.1ml of 1.0M substrate was added. The mixtures were incubated for 20min at 37°C. The top-most and bottom-most segments of the columns served as blanks. After addition of 0.15ml of 50% trichloroacetic acid and centrifugation (6000*g*/10min), 0.1ml samples of the supernatants were used for urea determinations.

Extractions of arginase from gels

Arginase activity could be extracted from the gel sections by homogenizing the segments in 0.6–0.75ml of 50mM-imidazole buffer, containing 0.25% Triton X-100, and allowing extraction to proceed for 2–18h at 4°C. After centrifugation at 105000*g* for 30min the supernatants had essentially the same activity as that found when the gel segments were analysed under conditions where the substrate diffused into the gels during the catalytic assay (Reddi *et al.*, 1975) (see under 'Activity assays after electrophoresis'). The extraction procedure was used only when gel-free supernatants were required to determine the properties of isolated isoenzymes (see Tables 5 and 6).

Results

The distribution of arginase among adult rat tissues is shown in Table 3. The greater sensitivity and flexibility of the assay allowed reliable determinations of very low activities. The present observations confirm values for activities reported elsewhere (Reddi *et al.*, 1975) and show that two other tissues, pancreas and duodenum, are among organs with relatively high arginase activities, whereas the activities in skin, adrenals and thymus were among the lowest. An irradiation-induced transplantable solid lymphoma contained a vestige of arginase activity. In relation to its tissue of origin, its activity was high (25% of that in thymus), whereas that of Morris hepatoma 7777, with a growth rate only slightly slower than that of the lymphoma, was only 0.2% of the activity of its cognate tissue, liver.

Arginase activities have usually been determined after activating the enzyme with Mn^{2+} at temperatures between 50° and 60°C for 5min or longer (Glass & Knox, 1973; Reddi *et al.*, 1975; Schimke, 1962). Activation in Mn^{2+}/imidazole buffer at 50°C for 10min was chosen as the standard procedure, because arginase was not inactivated in any tissue and only liver and submaxillary-gland arginase was activated sub-optimally. In liver and submaxillary gland, maximal activities were obtained by activation for 10min at 60°C, whereas the activities in the other

Table 3. *Distribution of arginase in adult rat tissues*

All determinations were on activated homogenates as described in the Experimental section. The numbers in parentheses show the number of animals used. Values given are means±S.D.

Normal tissues		Arginase (units/g of tissue)
Liver	(32)	2545 ±356
Submaxillary gland	(12)	170 ± 15
Small intestine	(18)	129 ± 21
Epididymis	(3)	125 ± 11
Pancreas	(13)	103 ± 9
Duodenum	(4)	85 ± 20
Kidney	(18)	56.5± 7
Colon	(4)	7.9± 5.7
Lung	(4)	5.4± 1.9
Brain	(13)	2.5± 0.5
Skin	(2)	2, 1.6
Thymus	(1)	1.9
Spleen	(4)	1.1± 0.2
Adrenals	(1)	0.7
Heart	(3)	0.6± 0.03
Skeletal muscle	(3)	0.4± 0.3

Table 4. *Arginase activities at different activation temperatures in homogenates and cytosols and distribution of isoenzymes*

The numbers in parentheses indicate the number of animals used. Activation occurred at 50°C for 10min, (all columns except column 2); for column 2 homogenates were heated to 60°C for 10min. The preparations of supernatant fractions, with or without Triton treatment, are described in the Experimental section. Isoenzyme I is the most anodically moving area of arginase activity. Activities are expressed as a percentage of homogenate activity and isoenzyme values as a percentage of the total activity recovered from the gel.

		Activity (%)			Isoenzymes (%)			
Tissue (1)	Homogenates (units/g) (2)	Activation at 60°C (3)	Supernatant (4)	Supernatant +Triton (5)	I (6)	II (7)	III (8)	Recovery from gels (with or without Triton) (%) (9)
Adult liver (10)	2549±368	140±18	88±14		0	0	96±0.5	52±17
Foetal liver (2)	141	166	92		0	0	96.5	42
Hepatoma 7777 (2)	5.7	58						
Submaxillary gland (10)	124±15	134	113±6	108±6	0	0	96.5±1.5	54±4
Submaxillary-gland primary tumours (3)	10.4±1.6	86±20						
Kidney (9)	56.5±7	61±13	67±12	85±18	58±8	30±5	0	56±4
Small intestine (8)	129±27	61±15	61	88±8	77±1	18±2	0	53±15
Pancreas (8)	105±12	45±12	68±12	100	46±8	41±10	0	50±14
Epididymis (2)	129		50		48.5	39	0	46
Lactating mammary gland (2)	163		58		56	32	0	47.5

tissues declined significantly during such treatment (Table 4). It is apparent that a tumour originating from liver (hepatoma 7777) had lost the heat-stable property of its cognate tissue.

Table 4 shows that homogenization in Mn^{2+}/imidazole buffer and activation as described above released 90% or more of the liver and submaxillary-gland arginase into the cell cytosol, whereas only 50–70% of the enzyme activities of the other tissues were extracted into the soluble fractions. Pretreatment of the homogenates with 0.5% Triton X-100 before heat activation did not increase arginase activity in the homogenates but released a greater proportion of it into the soluble fractions. Columns (6)–(8) of Table 4 anticipate evidence to be shown in Figs. 1 and 2. The isoenzymes of liver and submaxillary gland were electrophoretically identical and different from those found in the other tissues. The distribution of isoenzymes in all tissues was the same whether or not the preparations were pre-treated with Triton. The sensitivity to heat and ease of solubilization of arginase activity may provide a rough index to the arginase variant that one can expect to isolate by electrophoresis.

Anodic polyacrylamide electrophoresis had identified a variant (isoenzyme I) in some non-hepatic tissues (Reddi *et al.*, 1975). Poor recoveries of the non-migrating band in that system were due to the escape, during electrophoresis, of the more basic proteins into the upper buffer compartment when the tissue extracts were applied to the top of the gel columns. A system for trapping the tissue extracts in a loosely polymerized 'sample' gel, overlaid with a denser upper 'trapping' gel (see the Experimental section) led to recoveries of 50% or more of the activities in all tissues after anodic electrophoresis (column 9, Table 4). In this way it was confirmed that almost all the arginase activity of liver and submaxillary gland remained at the site of sample incorporation, but it was also shown that in kidney, intestine, pancreas, epididymis and lactating mammary-gland arginase activity was distributed over two peaks, one moving anodically as reported before (Reddi *et al.*, 1975) and comprising at least half the total recovered activity, the other indistinguishable in this electrophoretic system from the arginase of liver and submaxillary gland. The two major peaks were separated by at least 1cm (two slices) in pancreas and kidney (Fig. 1*a*) or intestine (Fig. 1*b*).

Reversal of the electrophoretic system, to determine if the more basic isoenzyme, immobile in the anodic system, was identical in all tissues, showed that arginase in kidney and intestine was almost completely inactivated at pH values lower than 6.0; no migration was observed in any tissue at pH values greater than 6.6. The cathodic electrophoresis system finally adopted enabled us to recover 50–80% of the activities in liver and submaxillary gland and 35–45% from kidney; recoveries of intestinal arginase, however, were still low (20%). In this system, arginase activity in intestine and adult kidney (Fig. 1*c*) did not migrate at all, whereas most of that of liver and submaxillary gland

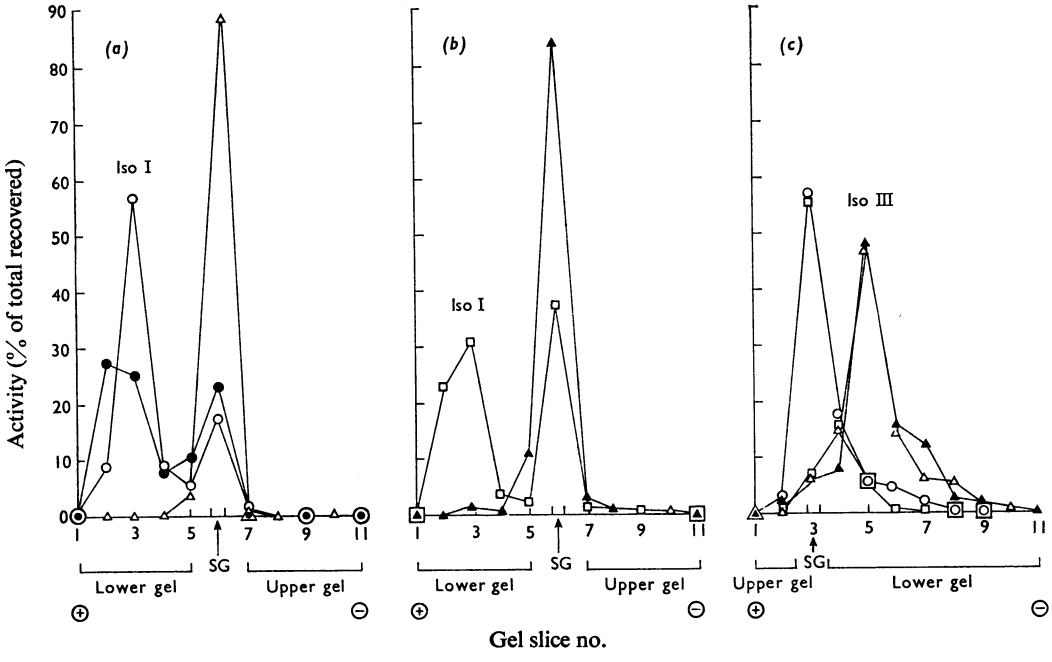

Fig. 1. *Anodic and cathodic electrophoresis patterns of arginase from adult tissues*

Arginase activities in 0.5 cm sequential gel slices in liver (△), kidney (○) and pancreas (●) (*a*), or in submaxillary gland (▲) and in small intestine after Triton treatment (□) (*b*) are shown when assayed after anodic electrophoresis. The pattern of recovered activities after cathodic electrophoresis are shown in (*c*) for intestine (□), kidney (○), submaxillary gland (▲) and liver (△). Samples were incorporated into the sample gel (SG). The electrophoresis systems are described in the Experimental section. Isoenzymes are numbered in anodic order according to the convention adopted by the Commission on Biochemical Nomenclature on the Nomenclature and Classification of Enzymes (*Enzyme Nomenclature*, 1973).

(81–90%) moved at least 1 cm toward the cathode, with a trace of activity (9%) discernible at the site of renal and intestinal arginase.

Successive electrophoresis of mixtures of liver and kidney extracts, first in the anodic direction (to isolate isoenzyme I) and then in the cathodic direction (to differentiate between isoenzymes II and III), resolved the isoenzymes present into three major bands, one corresponding to that seen in liver alone (cathodic isoenzyme III) and absent from kidney, and two characteristic of kidney (non-moving isoenzyme II and anodic isoenzyme I) but either totally absent (isoenzyme I) or only in low concentrations (isoenzyme II, present at less than 10%) in liver. Although the major isoenzymic variants were separated in this bidirectional system and recoveries were fairly good, the gels were mechanically imperfect. In the bidirectional system finally adopted, polyacrylamide gels were prepared which combined, in a single gel column, separation systems suitable for cathodic and anodic electrophoresis; they could clearly resolve the isoenzymes present in individual tissues and in mixtures of liver and kidney. The extracts were subjected first to cathodic electrophoresis; the tubes were then inverted and the compartment buffers and polarity were changed to those for anodic electrophoresis. The results shown in Fig. 2 were obtained. Liver still showed a single, major isoenzyme, moving toward the cathode (isoenzyme III), with barely a trace of activity remaining at the origin (6% of the activity recovered); kidney exhibited two major zones of activity, one migrating toward the anode (isoenzyme I) and a smaller one, still fixed at the origin (isoenzyme II) (20% of the activity recovered). The bidirectional electrophoresis of mixed liver and kidney extracts (0.35 unit of enzyme from each tissue) separated three bands of enzyme activity: kidney isoenzyme I near the anode; isoenzyme II at the origin (mostly contributed by kidney with a trace from liver); isoenzyme III near the cathode (characteristic of liver).

Table 5 shows that almost all the arginase activity of liver and submaxillary gland was precipitated out with 50μl of anti-(liver arginase) serum; renal, intestinal or pancreatic arginase showed no inactivation by the antiserum (Table 5, columns 2–4). After anodic electrophoresis, the non-moving isoenzymes (in 'sample gel' slices) from submaxillary gland, kidney and intestine were eluted with Triton in imidazole buffer overnight; the supernatants were incubated for 0 and 45min with control rabbit serum or antiserum and, after centrifugation, analysed for residual arginase activity (Table 5, columns 5–7). In these partially purified fractions of arginase also, there was no interaction between antiserum and the non-moving isoenzyme of kidney or intestine, whereas the non-migrating isoenzyme of submaxillary gland was inactivated by more than 60%. The incomplete inactivation of the submaxillary-gland enzyme probably was due to the diluted form of the enzyme in the antibody incubation mixture. It had been noted previously (P. K. Reddi, unpublished work) that higher amounts of antibody were required to inactivate dilute arginase solutions of even the precipitable antigenic form.

To confirm further that anti-(liver arginase) serum does not complex with arginase in extracts of kidney or intestine, samples of extracts of submaxillary gland, kidney or intestine and either control serum or antiserum were incubated for 45min and centrifuged, then subjected to anodic electrophoresis (Fig. 3). Although submaxillary-gland extracts were inactivated by the antiserum (Fig. 3*a*), both isoenzymes of kidney (Fig. 3*b*) and intestine (Fig. 3*c*) moved similarly in the presence of control serum or antiserum, with equal recoveries under both conditions of incubation. Apparently neither isoenzyme of kidney and intestine arginase adsorbed antiserum to liver arginase. After preincubation of 3.5units of kidney or intestine extract with 12.5μl of antiserum [just enough fully to inactivate 3.5units of appropriate arginase (Glass & Knox, 1973)] for 45min, 3.5units of submaxillary-gland arginase were added to the mixtures with and without an additional 12.5μl of antiserum and incubated for another 45min. Even without the addition of more antiserum, submaxillary-gland arginase was fully inactivated after this incubation.

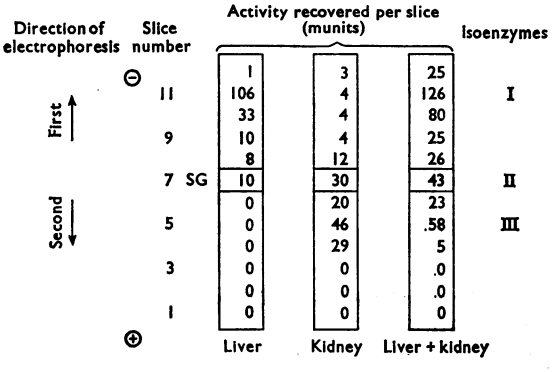

Fig. 2. *Bidirectional electrophoresis of arginase*

Arginases from liver (0.35unit), kidney (0.35unit) and 0.35unit each of a mixture of kidney and liver enzymes were subjected to bidirectional electrophoresis. Activities are expressed as munits (nmol/min) per slice. The details of the gel compositions are given in Table 2. Samples were incorporated into the sample gel (SG, slice 7).

Table 5. *Arginase activities in extracts or isolated isoenzymes remaining after incubation with antiserum*

Soluble fractions ('Whole extracts') containing 1.4–7.8units of arginase were incubated with control or anti-(liver arginase) serum, and the arginase activity remaining was measured as described in the Experimental section. To determine the antigenicity of the non-migrating isoenzyme, 0.31unit of kidney, 0.50unit of submaxillary-gland and 0.8unit of intestine enzyme were subjected to anodic electrophoresis. Arginase activities left in the sample gel ('isoenzyme II') were extracted by homogenization in 0.75ml of 0.05M-imidazole buffer containing 0.25% Triton X-100, pH7.4 (see the Experimental section). After centrifugation at 105000*g* for 20min, 0.025ml of control rabbit serum or anti-(liver arginase) antiserum was incubated with the supernatant for 45min; the mixtures were then analysed for remaining arginase activity as described. Numbers in parentheses indicate the number of extracts tested.

	Whole extracts			Non-migrating isoenzyme		
	Amount incubated	Activity remaining (%)		Amount incubated	Activity remaining (%)	
Tissue	(units)	Control serum	Antiserum	(unit)	Control serum	Antiserum
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Foetal liver (1)	3.41	100	1			
Submaxillary gland (4)	3.7–7.8	110±20	3±3	0.205	100	39
Adult kidney (4)	1.4–4.2	95± 4	101±5	0.095	98	96
Small intestine (2)	4.2–5.7	101± 4	98±8	0.092	102	100
Pancreas (2)	4.1–5.1	97	104			

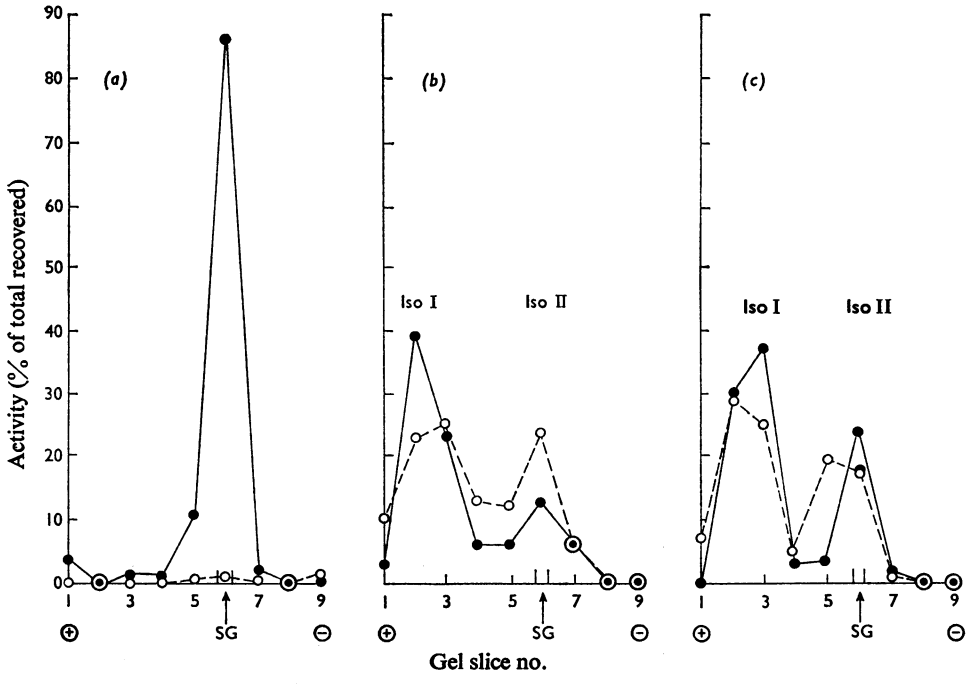

Fig. 3. *Anodic electrophoresis of supernatants from submaxillary gland, kidney or intestine after incubation with control rabbit serum or anti-(liver arginase) serum*

Submaxillary-gland (*a*), kidney (*b*) and intestine (*c*) (3.1–3.4 units each) arginase were incubated for 45 min with 25 μl of control rabbit serum (solid lines) or antiserum to liver arginase (broken lines); the mixtures were centrifuged and 0.04 ml of the supernatants were subjected to anodic electrophoresis as described in the Experimental section. Samples were incorporated into the sample gel (SG, slice 6). Recoveries for submaxillary-gland enzyme were 72% with control serum and less than 3% with antiserum; recoveries with control or antiserum were 69% for kidney and 67% for intestine. The gel slices, in anodic order, are shown on the abscissa; the percentage distribution of the recovered activities is shown on the ordinate. Slight variations in the slicing accounts for the small shifts of peaks seen in kidney and intestine with and without antibody.

Table 6. *Substrate specificity of arginase isoenzymes isolated by anodic electrophoresis*

Submaxillary-gland (0.5 unit) or kidney (0.35 unit) extracts were subjected to anodic electrophoresis; the duplicate gels were sliced, and arginase activity was determined with L-arginine or L-canavanine as substrate.

Substrate ...	Activity (unit/assay) 125 mM-L-arginine	125 mM-L-canavanine
Submaxillary-gland (isoenzyme I position)	0	0
Submaxillary-gland isoenzyme III (non-moving)	0.343	0.033
Kidney isoenzyme I	0.158	0.065
Kidney isoenzyme II (non-moving)	0.047	0.025

Additional support for the heterogeneity of the peak which does not migrate in anodic electrophoresis systems is shown in Table 6. Kaysen & Strecker (1973) have reported that L-canavanine serves as substrate for kidney but not for liver arginase. When L-canavanine replaced arginine as substrate, we observed 42% of the usual arginase activity with kidney homogenates, 9% with liver homogenates. Table 6 illustrates that after anodic electrophoresis both isoenzymes of kidney hydrolysed L-canavanine at 41–53% of the rate for arginine, whereas the activity in the submaxillary gland was only 9% of that seen with arginine as the substrate.

Discussion

Modifications of the assay for arginase activity (Schimke, 1962) by adjusting substrate concentrations, incubation volumes and times, and the volumes used for the urea determinations, allowed accurate measurements of enzyme activities in tissues with less than 0.1% of the liver activities. As little as 1 mg of even the most inactive tissues was sufficient to determine arginase activities. The

sensitive assay also enabled us to measure precisely the distribution of arginase activities in those segments of the polyacrylamide gels which contained only small fractions of the total activity.

More than half the tissues tested had very low arginase activities (0.4–7.9units/g); rat skin had less than 0.1% of the activity of rat liver. In contrast, human skin is reported to contain about 3% of the arginase activity found in human liver (Porembska & Kedra, 1971). To the previously known tissues with medium arginase activities (i.e. ranging from 56.5 to 170units/g in the present work), two others, pancreas and duodenum, have now been added. Tissues in this range of activities and, of course, liver with its uniquely high arginase activity, have been used to study the properties of arginase variants.

The inter-organ heterogeneity of arginases is apparent from the differences in their solubilization properties and from the extent to which their activities were enhanced or inhibited by preincubation at 60°C (Table 4). Their utilization of an alternate substrate, L-canavanine, confirmed this distinction (Table 6). However, the new electrophoretic technique described here gave the most conclusive evidence on the variation in isoenzyme composition.

Reliable discrimination among isoenzymes requires recoveries of representative activities. The application of the samples into the middle layers of gels for the first time gave consistent recoveries of arginase activities in excess of 50%. It thus became possible to detect the non-migrating zone of arginase activity as well as isoenzyme I (e.g. kidney) after anodic electrophoresis. Cathodic electrophoresis demonstrated that the non-migrating zone of liver contained a variant different from the non-migrating one of most non-hepatic tissues. By bidirectional electrophoresis (Fig. 2) mixtures of kidney and liver arginase were separated into three distinct peaks of activity, corresponding to isoenzymes I and II (both in kidney) and isoenzyme III (liver).

The relative rates of hydrolysis of L-arginine and L-canavanine by the two kidney isoenzymes were not significantly different, indicating that the electrophoretically separated variants may be catalytically similar. Therefore we cannot exclude completely the possibility that the second, non-moving, band of arginase activity in kidney (and most non-hepatic tissues) may result from aggregations of isoenzyme I. However, treatment with Triton X-100, changes in the concentrations of porosity and cross-linkage in the sample or separation gels, variations in the time or temperature of electrophoresis or slight alterations in the ionic strengths or pH of the solutions used, did not alter the distribution of isoenzymes I, II and III.

We therefore postulate the occurrence of at least three isoenzymes of arginase in rat tissues, namely: isoenzyme I, migrating anodically; isoenzyme II, immobile in all systems used; isoenzyme III, the basic protein characteristic of liver and submaxillary gland. Some recent studies of differences in elution patterns from DEAE-cellulose columns of arginases from different rat and human tissues (Gasiorowska *et al.*, 1970; Kaysen & Strecker, 1973; Porembska & Kedra, 1971; Van Elsen & Leroy, 1975) have already pointed towards a probable heterogeneity of arginase isoenzymes within a single tissue.

Anti-(liver arginase) serum failed to inactivate arginase from kidney, intestine and pancreas. It was demonstrated that the tests were conducted under conditions which left the anti-(liver arginase) serum free to react with the appropriate antigen. Submaxillary-gland arginase was almost completely inactivated by antiserum previously preincubated with extracts of kidney or intestine. The similarity of electrophoretic migration of kidney or intestine arginase after incubation with control or antisera made it unlikely that any interaction between antiserum and enzyme had occurred.

The immunological observations are consistent with the electrophoretic observations. The anti-[liver arginase (isoenzyme III)] serum does not interact with arginase of kidney, intestine or pancreas since the latter are different protein species (isoenzymes I and II).

All the results confirm that the isoenzyme of arginase in submaxillary gland is identical with that of liver and restricted to those two tissues. Since no ornithine transcarbamoylase (EC 2.1.3.3) activity was detectable in submaxillary gland (A. Herzfeld, unpublished work; Raijman, 1974), isoenzyme III cannot be solely involved in the catalysis of the urea cycle but must function also in another pathway of ornithine catabolism.

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References

Davis, B. A. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Enzyme Nomenclature (1973) pp. 24–25, Elsevier Publishing Co., Amsterdam, London and New York
Farron, F. (1973) *Anal. Biochem.* **53**, 264–268
Gabriel, O. (1971) *Methods Enzymol.* **22**, 565–578
Gasiorowska, I., Porembska, Z., Jachimowicz, J. & Mochnacka, I. (1970) *Acta Biochim. Pol.* **17**, 19–30
Geyer, J. W. & Dabich, D. (1971) *Anal. Biochem.* **39**, 412–417
Glass, R. D. & Knox, W. E. (1973) *J. Biol. Chem.* **248**, 5785–5789

Greengard, O., Sahib, M. K. & Knox, W. E. (1970) *Arch. Biochem. Biophys.* **137**, 477–482

Kaysen, G. A. & Strecker, H. J. (1973) *Biochem. J.* **133**, 779–788

Maurer, H. R. (1971) *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, pp. 44–52, W. de Gruyter, Berlin and New York

Porembska, Z. & Kedra, M. (1971) *Bull. Acad. Pol. Sci. Ser. Biol.* **19**, 633–637

Raijman, L. (1974) *Biochem. J.* **138**, 225–232

Reddi, P. K., Knox, W. E. & Herzfeld, A. (1975) *Enzyme* **20**, 305–314

Rodbard, D. & Chrambach, A. (1971) *Anal. Biochem.* **40**, 95–134

Schimke, R. T. (1962) *J. Biol. Chem.* **237**, 459–468

Van Elsen, A. F. & Leroy, J. G. (1975) *Biochem. Biophys. Res. Commun.* **62**, 191–198