145. THE SPECIFICITY OF ARGINASE: ACTION UPON ARGININIC ACID

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IN such arginine derivatives as have hitherto been tested, an unmodified guanidino group has appeared to be a condition prerequisite for the action of liver arginase [Thomas *et al.* 1922; Felix *et al.* 1928; Felix & Schneider, 1938]. Until recently an unmodified carboxyl group was thought to be in this respect of equal importance; but according to Richards & Hellerman [1940] agmatine is not, as formerly asserted, completely resistant to the enzyme, although it contains no carboxyl group. As for the third functional group of arginine—the α -amino group—it has been known for many years that modifications obstruct, but do not always completely block, the attack of arginase [cf. Hunter, 1938]. More recently it has become evident that the amino group may even be dispensed with. Although to Felix *et al.* [1928] argininic acid, in which the α -amino group of arginine is replaced by hydroxyl, appeared to be quite unaffected by arginase, Calvery & Block [1934] observed it to yield (under conditions rather imperfectly specified) as much as 85% of the theoretically possible amount of urea. Felix & Müller [1936] and Felix & Schneider [1938] later agreed that argininic acid was indeed hydrolysed by arginase, although much more slowly than arginine. Meanwhile Hellerman & Perkins [1935] had stated that a 'highly specific' arginase preparation failed to catalyse the hydrolysis of argininic acid; but Hellerman & Stock [1938], using high concentrations of enzyme acting over long periods of time, obtained clearly positive results. Taken as a whole the work cited permits only one conclusion—that sufficiently active preparations of arginase do actually produce urea from argininic acid. With this conclusion our own experience is in complete conformity.

In the present paper the action of arginase upon argininic acid is examined further from the following points of view:

(1) In all previous work proof of hydrolysis has rested solely upon the production of urea. Although the cogency of such evidence cannot reasonably be doubted, it seemed worth while to demonstrate the appearance of the other obligatory product of arginase action, α -hydroxy- δ -amino-*n*-valeric acid. This was the more desirable, because Hellerman & Stock [1938] have expressed doubt concerning the constitution of the substrate itself.

(2) According to Felix & Schneider [1938] the optimum *pH* for the action of arginase on argininic acid is 6.8–7.0. So great a divergence from the optimum for arginine itself (*pH* 9.8) would, if confirmed, have considerable theoretical interest.

(3) The precise quantitative effect upon arginase efficiency of various substitutions in the substrate molecule is known for only one or two examples. To these we have now attempted to add the case of argininic acid.

EXPERIMENTAL

Preparation of the substrate. Argininic acid in 58% yield was prepared from *l*(+)-arginine hydrochloride [Felix & Schneider, 1938]. The product, which gave a negative ninhydrin test, melted at 225° (Felix & Schneider give 228°). Found: N, 23.8% (Kjeldahl); $C_6H_{13}O_3N_3$ requires 24.0%. $[\alpha]_D^{25} = -11.85^\circ$; $c = 10.0$; $l = 2.2$; $\alpha = -2.61^\circ$; Felix & Müller [1928] found $[\alpha]_D^{20} = -12.5^\circ$ (no details).

Preparation of the enzyme. A stable, arginase-rich and relatively pure dry powder was prepared by heating a 1:1 aqueous extract of liver to 58°, filtering from coagulated protein, and precipitating with 1 to 1½ volumes of acetone. The precipitate was separated by centrifuging, and extracted repeatedly with water. The combined extracts were clarified and again precipitated with acetone. This procedure was repeated four or five times. The final acetone precipitate was then dried *in vacuo* and ground to a fine powder. In some preparations each intermediate acetone precipitate was dried before being re-extracted. This gave a product somewhat richer in arginase, but involved a greater over-all loss of the enzyme.

Active arginase solutions were obtained from the dry powder by shaking a portion for 30–45 min. with two or more times its weight of water. The extract was clarified by centrifuging, and, if a maximal recovery of enzyme was desired, the residue was extracted twice more. The several extracts were united, and diluted to any convenient volume. The quantities of water used at each extraction and the ratio of the final volume to the weight of powder extracted would depend upon the activity of the powder and the desired concentration of enzyme. With a triple extraction the highest conveniently attainable concentration was that in which each ml. of the final dilution contained the enzyme extractable from 0.2 g. of powder.

Examples for one particular specimen of arginase powder are shown in Table 1. All activities there recorded are those obtaining in the presence of an optimum concentration of Co [Hellerman & Perkins, 1935]. They were determined by the method of Hunter & Dauphinee [1930] and are expressed in their units. The data indicate that whether the volume of water used for extraction is large or small—whether, accordingly, the arginase concentration of the extract is low or high—the yield of enzyme per mg. of powder is much the same. Only two of the calculated yields in the last column differ significantly from 4.4 units per mg. The first aberrant figure is probably to be attributed to accident. The second suggests that when the volume/weight ratio is very low, extraction is not quite complete. The ready and uniform solubility of the enzyme is none the less sufficiently evident.

Table 1

mg. powder extracted per ml. extract	Dilution at which activity of extract was determined	mg. urea-N produced in 30 min. at 37°	Units of arginase	
			per ml. undiluted extract	per mg. powder
1.6	Undiluted	1.97	6.7	4.2
10	1 in 5	2.12	37.8	3.8
26.4	1 in 15	2.18	119	4.5
49	1 in 25	2.36	226	4.6
102	1 in 50	2.31	435	4.3
204	1 in 125	1.80	725	3.6

The activity of the preparation of Table 1 is only moderate and we have prepared others having extractable activities up to 8.5 units per mg. From these it was easy to obtain clear arginase solutions with activities of > 1000 units per ml.

Sometimes it is convenient to use the powder directly without extraction. On such occasions we have assumed the activity of the whole powder to be the same as that of its triple extract: a not wholly justifiable assumption, for a suspension of the powder is usually somewhat more active than a centrifuged extract.

Isolation and identification of α -hydroxy- δ -amino-n-valeric acid. The method already used in the analogous case of carbamido-arginine [Hunter, 1938] was most suitable. Urea was destroyed by urease as it was formed, and after an adequate period of digestion the mixture, freed from inorganic ions, was concentrated, until organic solutes crystallized. The material thus obtained was then recrystallized until free from any unaltered substrate.

In one successful experiment 10.8 g. argininic acid, 1.6 g. arginase powder (5 units per mg.), 15 ml. of a highly active urease solution, and 0.5 mg. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, were dissolved in 250 ml. water. The mixture was made just alkaline to phenolphthalein with NH_3 , treated with toluene and incubated at 37° for 72 hr. It was then found to contain a total of only 0.326 g. of $\text{NH}_3\text{-N}$. Since part of this had been added as such, it was evident that decomposition of argininic acid was proceeding very slowly. Accordingly 4.8 g. more of arginase powder were added, making a total of 6.4 g. or 32,000 units of the enzyme. After a further incubation period of 120 hr. $\text{NH}_3\text{-N}$ had risen to 1.046 g., a gain of 0.72 g. Since the complete hydrolysis of the substrate would have yielded urea containing 1.725 g. of N, it appeared that the degree of hydrolysis now attained was $>42\%$, but $<60\%$. For 50% , the quantity of α -hydroxy- δ -amino-valeric acid to be expected was 4.1 g.

On removal from the incubator the mixture was made just acid to Congo red with H_2SO_4 , boiled to coagulate proteins, treated with an excess of baryta water, filtered, freed from NH_3 by vacuum distillation and then from Ba^{++} with the exact amount of H_2SO_4 . The filtrate from the BaSO_4 , after concentration *in vacuo*, was decolorized with norite, further concentrated to a volume of about 8 ml., and treated with enough alcohol (23 ml.) to produce a slight turbidity. Crystallization, which soon became evident, was promoted by adding more alcohol and cooling to -10° : first crop 0.59 g. On evaporating the mother liquor to dryness, taking up in a small quantity of water, and adding alcohol to a concentration of about 70% , the second crop (0.6 g.) separated; it consisted of large glittering cubes, which gave a completely negative response to the Sakaguchi test for the guanidine group. The first crop gave a positive reaction, and was therefore presumably contaminated with unaltered argininic acid; but after a single recrystallization the Sakaguchi reaction disappeared, and the material presented the same rather characteristic appearance as the second crop. The total yield of pure product was 0.86 g., 21% of that calculated.

The substance thus isolated was freely soluble in water, hardly at all in ethanol. Its aqueous solution was acid to litmus. From water or from 70% ethanol it crystallized in remarkably hard, clear, shining parallelepipeds with rectangular (often square) outlines, reaching sometimes a length of 2–3 mm. Its composition agreed with that of the expected α -hydroxy- δ -amino-valeric acid. (Found: C, 45.6; H, 8.25; N (Dumas), 10.7; amino-N (Van Slyke), 10.6%. $\text{C}_5\text{H}_{11}\text{O}_3\text{N}$ requires: C, 45.1; H, 8.33; N, 10.53; amino-N, 10.53%.) It melted (after another recrystallization) at 196.4° (corr.), and the aqueous solution was laevorotatory ($[\alpha]_D^{25} = -17.1^\circ$; $c = 10.02$; $l = 1 \text{ dm.}$, $\alpha = -1.71^\circ$).

According to Fischer & Zemplen [1909] α -hydroxy- δ -amino-valeric acid, heated for a short time at its m.p., is converted into an anhydride, 3-hydroxy-

2-piperidone, which is readily soluble in hot ethyl acetate. When treated in this way, our enzymic product was observed to yield a sublimate, which condensed in needles several mm. long. A hot ethyl acetate extract of the unsublimed residue deposited upon cooling a further crop of crystals identical with the sublimate. This material was an anhydride. (Found: C, 52.16; H, 7.85; N, 12.11%. $C_5H_9O_2N$ requires: C, 52.16; H, 7.88; N, 12.16%.) It was extremely soluble in water and in ethanol, freely soluble in hot, but sparingly in cold, ethyl acetate. From water it crystallized sometimes in the form of hexagonal plates, but usually as long thick prisms with obliquely pointed ends. From alcohol or ethyl acetate it separated as a close meshwork of long and delicate needles, M.P. 171.5° (corr.). The aqueous solution was neutral and laevorotatory ($[\alpha]_D^{25} = -6.0^\circ$; $c = 7.66$; $l = 1$ dm., $\alpha = -0.46^\circ$).

An amino-acid of formula $C_5H_{11}O_3N$ and M.P. $193-194^\circ$ has been described by Felix & Müller [1928] as the product of the decomposition of *l*-argininic acid by $Ba(OH)_2$. This acid, when melted, yielded, partly as a sublimate, an anhydride, M.P. 169° . There can be no doubt that these substances, taken to be respectively α -hydroxy- δ -amino-valeric acid and 3-hydroxy-2-piperidone, are the same as those now obtained through the use of arginase. Felix & Müller make no mention of optical activities, but otherwise their description, including M.P., tallies exactly with our own observations.

On the other hand, our products differ in certain respects from the synthetic (optically inactive) α -hydroxy- δ -amino-valeric acid and 3-hydroxy-2-piperidone of Fischer & Zemlen [1909], with original samples of which we were able directly to compare them. Before the comparison was made, the samples were repeatedly recrystallized, the acid from water and alcohol, the anhydride from ethyl acetate, to constant M.P. The acid then resembled our own in its solubilities, in its conversion upon melting into an anhydride and in the fact (not recorded by Fischer & Zemlen) that the anhydride readily sublimed. On the other hand, the M.P. was 188.2° (according to Fischer & Zemlen, $188-191^\circ$) not 196.4° , and it crystallized in a variety of shapes—thick hexagonal or rhomboid plates, long straight-ended hexagonal prisms, oblong plates with bevelled edges and corners—but never in rectangular parallelepipeds. As for the anhydride, it had the same general properties as ours, and, like it, crystallized from ethyl acetate in a characteristically dense meshwork of long fine needles; but it melted at 134.3° (Fischer & Zemlen say $141-142^\circ$) instead of at 171.5° . The observed differences, whether of M.P. or of crystalline form, are just such as might exist between an optically active substance and its racemate. The evidence suggests therefore that Fischer & Zemlen's substances are racemic compounds, of which ours (as well as Felix & Müller's) are the respective laevorotatory components.

Prof. M. A. Peacock of the Department of Mineralogy, University of Toronto, has been kind enough to compare for us some of the crystallographic properties of the two acids. In his report he says: 'The optical properties and the X-ray powder photographs of the tabular crystals prepared by Hunter & Woodward and the prismatic crystals of Fischer & Zemlen are similar, but not identical. In view of the dissimilarity of the crystal structures of tartaric and racemic acids [Astbury, 1923, 1, 2], the differences noted in the present compounds do not conflict with the conclusion that they are the active and the racemic forms respectively.'

The evidence therefore confirms the constitution hitherto assigned to argininic acid. No doubt then remains that arginase can attack, however feebly, a substance in which the α -amino group is lacking.

Effect of pH on the action of arginase upon argininic acid. In numerous experiments we determined the effect of varying pH upon the amount of argininic acid decomposed within a given time by a given quantity of arginase. In some the enzyme was activated by additions of Co, in others it was not. Since all gave concordant results, it is needless to report more than two. In each of these a series of appropriate $M/4$ phosphate-phenolsulphonate buffers [Hunter & Morrell, 1933], in 2 ml. portions, were mixed with 2 ml. of a 3.125% solution of argininic acid and 2 ml. of a fresh extract of arginase powder. The added extract contained, in Exp. A, 400 units of arginase together with 0.35 mg. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; in Exp. B, $3\frac{1}{2}$ times as much enzyme, but no Co. All mixtures were made up in duplicate. One member of each pair was used for the determination of pH by the glass electrode (Beckman). The others were incubated at 37° for 24 hr., the urea produced being determined by the method of Van Slyke & Cullen [1914]. It will be evident that each mixture contained, in a total volume of 6 ml., 62.5 mg. of argininic acid. The maximum amount of urea-N to be expected was therefore 10 mg.

Fig. 1. Relation between pH and the action of arginase upon argininic acid. Curve A, with its urea scale on the left, shows this relation in the presence of Co; curve B (urea scale on the right) in its absence.

The results are shown graphically in Fig. 1. The curves merely represent enzyme actions for equal times and are not strictly pH-activity curves, but they suffice to show the general nature of the pH effect. Both curves, it is evident, are highly unsymmetrical, with descending limbs much steeper than the ascending. For the unactivated enzyme (curve B) the peak of action is at or near pH 9.9, the very point at which arginase shows its maximal activity towards arginine [Hunter & Morrell, 1933]. In the presence of Co the whole curve (A) is shifted to the left, so that the optimum pH is now about 9.1. A similar, if rather more pronounced, effect of Co has been observed in the action of the enzyme upon arginine [Hellerman & Stock, 1938; Damodaran & Narayanan, 1940]. Whether then the substrate be arginine or argininic acid, the relation between pH and arginase action is shown to be essentially the same.

Relative activities of arginase towards arginine and argininic acid. It is evident, from the experiments already reported, that in any liver extract or arginase concentrate the ratio (activity towards argininic acid)/(activity towards arginine)

must be very small. In an endeavour to estimate it, we have applied the principle already used [Hunter, 1938] with the substrate carbamido-arginine. This involves a determination and comparison of the quantities of enzyme required to perform, in some chosen time, identical amounts of work upon equivalent quantities of the respective substrates. On the assumption that the velocity curve of the reaction has the same form with each of the substrates, the ratio of the enzyme quantities found will be the reciprocal of the activity ratio sought.

In the present instance this principle was utilized in the following manner. An arginase solution was prepared containing (after activation by Co) about 750 units of enzyme per ml. 1 ml. of this solution was pipetted (a) into a urea tube, (b) into an ordinary test-tube. To each 1 ml. portion were added 7.5 mg. $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 0.15 ml. water (a 5% solution). This addition was in the nearly optimum proportion of 1 mg. per 100 units of enzyme. Since, according to unpublished observations, the activation is progressive, the mixtures were left at room temperature overnight to 'ripen'.

Next morning there were added to (a) 3 ml. of a 2.084% solution of argininic acid and 2 ml. of a 0.25 *M* phosphate-phenolsulphonate buffer of pH 8.4 [Hunter & Morrell, 1933]. The mixture was covered with toluene, and placed in a thermostat at 37° for 24 hr. Meanwhile the other enzyme mixture, (b), was diluted to 1000 ml., and portions of 0.2, 0.4, 0.6, 0.8 and 1.0 ml. were measured into urea tubes, each of which already contained 3 ml. of a 2.506% solution of arginine hydrochloride, 2 ml. of the above-mentioned buffer solution, and enough water to make (after the addition of the enzyme solution) a total volume of 6 ml. To each of these mixtures there was further added, in order to ensure the maintenance of maximal activity, a drop of 2% $\text{Co}(\text{NO}_3)_2$. The arginine mixtures were then treated exactly like the argininic acid one. At the end of the 24 hr. incubation period the urea contents of all six tubes were determined in the usual way.

A parallel experiment was also made, in all respects identical except that the preliminary treatment of the enzyme with Co, as well as the addition of Co to the incubating mixtures, was omitted. Each of the 12 mixtures incubated contained in 6 ml. (or slightly more) the same quantity of buffer salts and arginine or argininic acid equivalent to 10 mg. of urea-N.

The results of two such experiments are recorded in Table 2, all enzyme quantities being there expressed in terms of the original undiluted arginase solution.

Table 2

Substrate	ml. undiluted arginase solution	mg. urea-N			
		Without Co		With Co	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
Arginine	0.0002	0.54	0.54	2.10	2.06
	0.0004	0.99	1.01	3.38	3.37
	0.0006	1.41	1.44	4.47	4.44
	0.0008	1.69	1.76	5.16	5.11
	0.001	2.00	2.06	5.89	5.88
Argininic acid	1.0	0.76	0.69	2.49	2.58

Inspection shows that in each of the four instances the work done upon argininic acid by 1 ml. of enzyme would have been accomplished upon an equivalent amount of arginine by 0.0002–0.0004 ml. Interpolating graphically for each in turn of the four groups of data available, we obtained the results in the second line of figures in Table 3. Since the volume of arginase solution used

with argininic acid was always 1 ml., these results are numerically the same as the activity ratios; but it is more convenient to express these in the form shown in the last line of the table, with the activity towards argininic acid taken as unity.

Table 3

	Unactivated		Activated	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
mg. urea-N produced from argininic acid by 1 ml. arginase	0.76	0.69	2.49	2.58
ml. arginase to produce same amount of urea-N from arginine	0.00029	0.00026	0.00025	0.00027
Activity ratio (argininic acid : arginine)	1 : 3450	1 : 3850	1 : 4000	1 : 3700

The four values found for the activity ratio are as consistent as could reasonably be expected; and it seems safe to conclude that, under the conditions given, arginase is from 3500 to 4000 times less active in the hydrolysis of argininic acid than in that of arginine. None of the estimates differs more than 8% from the average of 1 : 3750.

The precise value of the activity ratio is less important than the demonstration, here afforded, that it is not altered by Co activation of the arginase. With Co the average ratio is 1 : 3850, without it 1 : 3650. As has been argued already for carbamido-arginine [Hunter, 1938] and canavanine [Kitagawa & Eguchi, 1938; Damodaran & Narayanan, 1940], constancy of the ratio under varying conditions is strong evidence for the identity with arginase of the enzyme attacking the alternative substrate. In the present experiment the degree of activation attained was very high. From the data for Exp. 2 in Table 2 it may be seen that exactly the same amount of urea was produced from arginine by 0.0002 ml. of activated enzyme as by 0.001 ml. of the unactivated. The specific arginase activity was increased, therefore, no less than five times (and possibly slightly more in Exp. 1). That the activity ratio nevertheless remained unaltered, means that the agent attacking argininic acid was activated in precisely the same rather remarkable degree. It seems unlikely then, to say the least, that it could have been a separate entity.

(We have lately found reason to suspect, that under the prevailing conditions the effect of Co was less one of direct activation, than of protection against spontaneous inactivation. Should this prove to have been the case, the phraseology of the preceding paragraph would have to be modified, but the general argument would not be affected.)

DISCUSSION

Since liver extracts have now been shown to produce from argininic acid not only urea, but also a substance identifiable, with reasonable certainty, as *l*- α -hydroxy- δ -amino-*n*-valeric acid, there can remain little doubt (1) that argininic acid is actually α -hydroxy- δ -guanido-*n*-valeric acid, or (2) that it is hydrolysable by a liver enzyme having an action similar to that of arginase. The identification of this enzyme with arginase itself is justified by the facts (1) that whether the substrate be argininic acid or arginine the relation between *pH* and enzyme activity is of the same unsymmetrical form, (2) that for each substrate the optimum *pH* lies close to 9.8, (3) that with both, activation by Co shifts the optimum to a less alkaline region, and (4) that activation enhances the rate of hydrolysis in each case to an equal degree. The demonstration would

seem to be complete, that replacement of the α -amino group of arginine by hydroxyl leaves a substrate still accessible to attack by arginase.

In Fig. 1 the highest degree of hydrolysis shown is 44%; but under more extreme conditions we have found it possible to reach a figure of 91%. This is not very different from the maximum of 85.6% reported by Calvery & Block [1934].

As a striking example of the bearing of chemical structure upon the concept of specificity, the action of arginase upon argininic acid has considerable theoretical interest. Physiologically it can have little if any significance. It is known already that a single substituent in the amino group of arginine notably reduces its 'affinity' for the enzyme. In one instance in which this effect was studied quantitatively—that of carbamido-arginine—the activity of the enzyme towards the substituted compound was 165 times less than its activity towards arginine [Hunter, 1938]. The complete replacement of the amino group has, it would now appear, a depressant effect more than 20 times greater. An activity-ratio of 1:3750 implies—to put it in terms of time—that a given amount of arginase would take 3750 hr. or almost exactly 5 months, to liberate from argininic acid as much urea as it would produce in a single hour from an equivalent quantity of arginine. The calculation gives quantitative emphasis to the conclusion of Hellerman & Stock [1938]—that the α -amino group, though not wholly indispensable, is necessary for the *efficient* functioning of the enzyme.

Arginase, it is known, exerts a feeble action not only upon *l*-argininic acid, but also upon *dl*- δ -guanidino-valeric acid, which has no substituent at all in the α position [Hellerman & Stock, 1938; Richards & Hellerman, 1940], and upon *d*(-)-arginine, in which the amino group is in the 'unnatural' configuration [Edlbacher & Zeller, 1936]. No determination of activity-ratios for these substrates have yet been made; but published data, as far as they go, suggest that they will be found to be of the same order as that for argininic acid. Should this prove to be the case, one might reasonably deduce that neither an α -hydroxy nor a sterically misplaced α -amino group offers any 'point of attachment' whatever for arginase; and that the effect of that enzyme upon *d*(-)-arginine is not so much an instance of imperfect optical specificity as another proof that the amino group as such, apart altogether from its spatial position, is not an essential feature of the substrate's structure.

Some comment appears to be called for upon the discrepancy between the optimum pH as here reported and that given by Felix & Schneider [1938]. This discrepancy would seem to be another example of the fact that the apparent optimum is apt to be considerably affected by the conditions under which it is determined [Hunter & Morrell, 1933]. In any case we have been informed, in a personal communication from Prof. Felix himself (January, 1939), that a repetition of his experiments in another laboratory indicated, in sufficient agreement with our own observations, an optimum at pH 9.4. It would not be surprising if the optimum for arginase should sometimes be found to be dependent upon the nature of its substrate; but the substitution of —OH for —NH₂ in the alpha position is shown to be in this respect without appreciable influence.

SUMMARY

Argininic acid is split by liver extracts into urea and *l*(-)- α -hydroxy- δ -amino-*n*-valeric acid. The latter product has been isolated and identified. Under favourable conditions 90% of the substrate may be hydrolysed. The enzyme responsible is identical with arginase, because (1) it has the same pH optimum and (2) in the presence of Co its action is magnified in exactly the same degree.

Susceptibility to arginase is therefore not absolutely dependent upon the presence of an intact or a modified α -amino group. This group exerts, nevertheless, an extremely important influence; for argininic acid is hydrolysed by arginase about 3750 times less actively than arginine.

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