172. A COMPARATIVE STUDY OF ARGINASE AND CANAVANASE

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(Received 9 August 1940)

VERY little is known about the enzyme canavanase which brings about the decomposition of canavanine into canaline and urea:

The existence of the enzyme was assumed by Kitagawa & Tomita [1929] because it was found that in the presence of liver tissue or extract canavanine gave rise to urea according to the above equation. It was the object of this investigation to study the reaction in more detail and especially to compare it with the hydrolysis of the closely similar amino-acid arginine by arginase as there are several grounds for suspecting the possible identity of the two enzymes. Canavanine has not been shown to be a constituent of proteins, in spite of the fact that the properties of this amino-acid are such that it should readily be identifiable in protein hydrolysates. It appears doubtful if the organism would contain an enzyme for dealing with a substance which it is not likely to encounter in metabolism. Further, modern studies on enzyme specificity have amply demonstrated that susceptibility to enzymic decomposition of any substance is determined not by the empirical composition but by the chemical groupings present in it.

The experiments to be described concerned the following questions: kinetics of the hydrolysis of arginine and canavanine by liver preparations and by jack bean meal which latter has been recently shown to contain arginase [Hellerman & Perkins, 1935-6; Stock *et al.* 1938]; the influence of activators and inhibitors; comparison of arginase and canavanase activities of preparations obtained by different methods, and the relative distribution of the two enzymes in different sources.

In kinetics the two enzymes showed only such differences as could be explained by the difference in the substrates. Using dehydrated preparations obtained by precipitating aqueous liver extract with acetone, the optimum pH for the splitting of arginine was found to be about 9.4 (Fig. 3) and for canavanine about 7.5 (Fig. 3), the range of enzymic activity in the former case being pH 5.5-11.5 and in the latter 5.5-10.5. This however provides no evidence for the existence of two separate enzymes as it has been shown by Felix & Schnieder [1938] that while arginase acts on arginine at a pH-optimum of 9.5 the pH-optima for its action on other substrates, viz. argininic acid, methylaminoarginine and

arginylarginine lie between 7 and 8. When the effect of pH was examined in the presence of cobalt ions (shown by Hellerman & Perkins [1935-6] to activate arginase) it was found that the pH -optima for the hydrolysis of both arginine and canavanine coincide at about 7.5 (Fig. 4). Further, the enzyme of jack bean meal also showed approximately the same optimal pH (Fig. 6) in its action on these two substrates in the presence of cobalt chloride. For both the reactions it was found that the initial reaction velocity was approximately proportional to the enzyme concentration (Tables 1 and 2). From the pS -activity curves the optimal substrate concentration for arginine is seen to be $M/15$ and for canavanine $M/3$, the values for the Michaelis constant for the two reactions being 0.0118 M and 0.0371 M respectively (Fig. 5).

The evidence from the preparative experiments is wholly in support of the identity of the two enzymes. Not only were all attempts to separate the two enzymes unsuccessful but in no case was it possible to obtain a preparation which catalysed the hydrolysis of canavanine at a higher rate than the hydrolysis of arginine. The kinetic experiments show that under optimal conditions for each enzyme the initial velocity of hydrolysis of canavanine is about 1/6th of the velocity of hydrolysis of arginine. On account of experimental difficulties it was not possible to determine the velocities of the two reactions with different preparations under strictly comparable conditions, i.e. under optimal conditions for each enzyme. But it was always found that whatever the procedure adopted for preparation, the effect on canavanase and arginase was always in the same direction and approximately to the same extent. Thus autolysis of liver tissue as a preliminary to precipitation of the enzyme tended to decrease the activities of both the enzymes (Table 3) and in preparations obtained after various periods of autolysis, the ratio of the amount of arginine hydrolysed to canavanine hydrolysed remained constant (Tables 3, 4 and 5). Autolysis of liver at different pH gave similar results. Autolysis at pH 7 yielded a more active preparation than at the natural pH of the liver (6.2), but here again the relative velocities of the two reactions were unaffected. Autolysis at pH 9 for 24 hr. destroyed both the enzymes.

The distribution of the enzymes in liver, kidney and blood of various animals and in certain seeds showed the same general tendency. Livers of rat, guinea-pig, rabbit and ox are rich in arginase and canavanase while kidneys of the above animals contain moderate amounts of both. Both the enzymes are absent from the blood of rat, guinea-pig and rabbit and both are present in the blood of ox and cow.

Jack bean meal recently shown by Stock *et al.* [1938] to contain arginase was also found to act on canavanine. As the presence of arginase in urease preparations used for urea determinations is a matter of practical importance a number of sources of urease recently studied in this laboratory [Damodaran & Sivaramakrishnan, 1937] were examined for arginase and canavanase activities. Seeds of *Cucurbita maxima* (common gourd), *Citrullus vulgaris* (water melon), *Dolichos biflorus* (horse-gram) and *Soja hispida* (soya bean) showed no appreciable action on either arginine or canavanine, while the seeds of *Canavalia obtusifolia* and *C. ensiformis* (jack bean) acted vigorously on both, the action on canavanine again being slower than that on arginine.

Activation and inhibition experiments also lead to the same conclusion that the two enzymes are identical. Cobaltous ions activate and cyanide inhibits both reactions; and further in the case of liver the range of enzymic activity in the presence of cobalt for both enzymes is shifted by about one pH unit towards the acid side. With the enzymes from both the sources after activation with

cobalt the maximum activity for the hydrolysis of arginine and canavanine is found to be near pH 7.7. The balance of evidence appears thus to be in favour of the identity of the two enzymes.

EXPERIMENTAL

Substrates. $l(+)$ -Arginine monohydrochloride was prepared from gelatin by the method of Cox [1928] and canavanine from the seeds of *Canavalia obtusifolia* according to Damodaran & Narayanan [1939]. Analytically pure specimens of both were used.

Enzymes. A dehydrated liver preparation was obtained according to Edlbacher [1917] with slight modifications. Ox liver, which was brought in ice from the slaughter-house, was cut into small pieces, washed free from blood with cold water and passed through a mincer twice. The minced tissue (500 g.) was ground for half an hour in a mortar with acid-washed sand and distilled water (1 ml. per g. of the moist tissue) and after adjusting to pH 7, by the addition of a few ml. of $N/20$ NaOH, kept in the refrigerator overnight under toluene. The mixture was centrifuged, and after removing toluene the filtrate poured into 10–15 vol. of acetone with stirring. The supernatant liquid was decanted, the precipitate filtered at the pump, washed with further quantities of acetone, dried first in a current of air and then in a vacuum desiccator over H_2SO_4 . The dry material was ground finely and sieved through muslin. Such a preparation was very active both on arginine and canavanine and retained its activity unimpaired for long periods.

From the dry enzyme preparation the enzyme solution for each experiment was prepared as follows: weighed amounts of the powder were ground up in a glass mortar to a thin paste with water, brought to pH 7, and the mixture made up to a known volume with water. After 2 hr. at 37° under toluene it was filtered and the filtrate mixed with an equal volume of glycerol. The solution, kept in the refrigerator under toluene, retained its activity for more than a month.

Enzyme action was in general determined as follows. The neutralized substrate solution was mixed with the buffer of appropriate pH (McIlvaine's phosphate-citric, Sørensen's phosphate and Sørensen's glycine buffers were used for the ranges of pH 3–5.8, 5.8–8.0 and 8.0–12.5 respectively) and after addition of enzyme solution was made up to known volume. Toluene was used as antiseptic. In aliquots withdrawn at intervals enzyme action was arrested by the addition of 1 ml. of $N H_2SO_4$ and keeping in a water bath at 80° for 10 min. Urea was determined according to Conway & Byrne [1933] using water-melon urease [Damodaran & Sivaramakrishnan, 1937]. Two controls were run, (i) enzyme solution incubated without the substrate, and (ii) substrate solution incubated with boiled enzyme.

The pH activity curves of arginase and canavanase

Reaction mixture. 10 ml. substrate solution, $M/10$ (urea-N on complete hydrolysis 28.02 mg.) + 10 ml. of $M/5$ buffer + 10 ml. of enzyme solution. Total volume 50 ml.

In the case of arginine an enzyme solution obtained by extracting 0.5 g. of the liver powder with 50 ml. of water was used and in that of canavanine one obtained by extracting 2 g. of the powder with 50 ml. of water. Figs. 1 and 2 give the activities of canavanase and arginase at various pH plotted against time. From these graphs the time taken to hydrolyse 50 % of the substrate at each pH was obtained. In Fig. 3 the reciprocal of the half-value period is plotted

against pH for each enzyme. As seen from Fig. 3 the optimum pH for canavanase is 7.5 and for arginase 9.4.

Fig. 1.

Fig. 2.

Fig. 1. Hydrolysis of canavanine by liver enzyme at various pH .Fig. 2. Hydrolysis of arginine by liver enzyme of various pH .

Fig. 3.

Fig. 4.

Fig. 3. Activity- pH curves of arginase and canavanase.Fig. 4. Activity- pH curves for liver arginase and canavanase in the presence of cobalt chloride.

Activity- pH curves of liver arginase and canavanase in the presence of cobalt chloride

Reaction mixture. 5 ml. of substrate solution ($M/10$) + 5 ml. of buffer + 2 ml. of cobalt chloride solution (6 mg.) + 5 ml. of enzyme solution (0.5 g. of the liver powder extracted with 50 ml. of water). Total volume 25 ml. The activity- pH curves for the two enzymes are given in Fig. 4.

Hydrolysis of arginine and canavanine by jack bean seed meal at different pH

Procedure. To 2 ml. of the substrate solution ($M/5$) in a 50 ml. conical flask, 5 ml. of required buffer (M), 2 ml. of distilled water and 5 ml. of a suspension of jack bean meal (0.1 g.) were added. The flask was fitted with a one-holed rubber stopper closed by a glass tap. After incubation for 3 hr. at 37° , 2 ml. of $N H_2SO_4$ were added through the tap tube, the enzyme destroyed by keeping the flask in a boiling water bath for 10 min. and the solution filtered into a 25 ml. measuring flask. Urea, the product of the reaction mixture, does not accumulate owing to the simultaneous presence of urease in the reaction mixture. Control experiments with equivalent quantity of urea solution at pH 9.0, 9.5 and 10.5 showed

Fig. 5. Activity-pS curves. I, arginase pH 9.4; II, canavanase pH 7.5.

Fig. 6. Activity-pH curves for jack bean enzyme.

that the urea was completely hydrolysed at pH 9.0 in 3 hr., while at pH 9.5 90 % was hydrolysed and at pH 10.5 no hydrolysis occurred. In experiments at pH < 9.0 the filtrate in the measuring flask was made up to the mark and ammonia determined on an aliquot. In experiments at pH > 9.0 the filtrate in the measuring flask was brought to pH 7 and further digested for 30 min. with 0.5 ml. of 10 % suspension of water-melon urease, the solution made up to the mark and ammonia determined on an aliquot. Control experiments with water-melon urease showed that it does not contain either arginase or canavanase.

The activity-pH curves for jack bean arginase and canavanase in the absence and in the presence of cobalt chloride are given in Fig. 6. The hydrolysis of arginine and canavanine by jack bean meal is accelerated by the presence of cobalt chloride and the amount of activation depends upon the pH. That the enzyme from jack bean meal behaves in a slightly different way from that of ox liver is evident from the activity-pH curves for these enzymes shown in Fig. 6. Neither arginine or canavanine is hydrolysed by jack bean meal at pH 6.6 while at the same pH liver enzyme is noticeably active towards both substrates. The optimum pH for jack bean arginase and canavanase in the presence of cobalt chloride is about 7.7 and in this respect the enzyme resembles that from ox liver in the presence of cobalt chloride.

The influence of enzyme concentration

Reaction mixture. 10 ml. of substrate solution ($M/10$) + 5 ml. of buffer ($M/5$ phosphate, pH 7.5 for canavanine and $M/5$ glycine pH 9.4 for arginine) + x ml. of enzyme solution (0.5 g. of the liver powder extracted with 50 ml. of water). Total volume 25 ml. The results are given in Tables 1 and 2 and the reaction velocity curves for the two substrates are given in Figs. 7 and 8.

Table 1. *Proportionality between enzyme concentration and urea formation from canavanine*

Enzyme quantity ml. E	Time in hr. T	ET	Urea-N formed in mg.
1	6	6	2.103
2	3	6	2.151
4	1½	6	2.101
2	2	4	1.601
4	1	4	1.552

Table 2. *Proportionality between enzyme concentration and urea formation from arginine*

Enzyme quantity ml. E	Time in min. T	ET	Urea-N formed in mg.
0.5	100	50	3.651
1	50	50	3.602
2	25	50	3.600
1	100	100	5.701
2	50	100	5.652
4	25	100	5.653

Fig. 7. Enzyme concentration and hydrolysis of canavanine.

Fig. 8. Enzyme concentration and hydrolysis of arginine.

The influence of substrate concentration

Reaction mixture. 20 ml. of substrate solution, 20 ml. of buffer ($M/10$ phosphate pH 7.5 for canavanine and $M/10$ glycine pH 9.4 for arginine), 20 ml. of enzyme solution (1 g. of the liver powder extracted with 50 ml. of water).

As shown in Figs. 9 and 10 both the reactions depend upon the substrate concentration. In the case of arginine the maximum amount of urea formed is reached at a final concentration of $M/15$, while in the case of canavanine the maximum is reached only at a final concentration of $M/3$; further, in the case of arginine on increasing the concentration to $M/6$ a slight inhibition is observed.

Fig. 9. Substrate concentration and hydrolysis of canavanine at pH 7.5.

Fig. 10. Substrate concentration and hydrolysis of arginine at pH 9.4.

Effect of autolysis on the extractability of arginase and canavanase

Fresh ox liver was extracted with water as described in the preparation of the enzyme and the mixture kept at room temperature (30°) for various periods. After 16, 40 and 64 hr. portions of the mixture were centrifuged and the filtrate dehydrated with acetone in the usual way. The arginase and canavanase activities of the three preparations thus obtained were then tested. The results are given in Table 3.

Table 3. *Effect of autolysis for different periods on arginase and canavanase activity*

Enzyme	mg. of urea-N formed after 4 hr. incubation		
	Arginine	Canavanine	Urea-N from arginine
			Urea-N from canavanine
16 hr. filtrate	9.85	7.59	1.30
40 hr. filtrate	9.02	6.91	1.30
64 hr. filtrate	9.02	6.21	1.47

Reaction mixture. 5 ml. of substrate solution (urea-N on complete hydrolysis 10 mg.) + 5 ml. buffer (pH 7.5 for canavanine and pH 9.4 for arginine) + 5 ml. of enzyme solution (1 g. in 100 ml. of water).

Effect of autolysis at different pH on arginase and canavanase activity

Portions of ox liver extract were kept at room temperature at the pH of the liver (6.2), at pH 7.0 and at pH 9.0. After 24 hr. the mixtures were centrifuged and the filtrate dehydrated with acetone. The arginase and canavanase

activities of the three preparations thus obtained were then determined, the reaction mixture being the same as in the previous experiments. The results are given in Table 4.

Table 4. *Effect of autolysis at different pH on arginase and canavanase activities*

Enzyme	mg. of urea-N formed after 4 hr. incubation		
	Arginine	Canavanine	Urea-N from arginine
			Urea-N from canavanine
pH 6.2 filtrate	8.54	3.83	2.23
pH 7.0 filtrate	9.06	4.03	2.24
pH 9.0 filtrate	0	0	—

Fractionation of liver extract with sodium sulphate

The precipitates obtained by treatment of liver extract with sodium sulphate to half and full saturation were tested for arginase and canavanase activities. The precipitates were suspended in water equal to the original volume of the extract and 5 ml. of the suspension were used as the enzyme solution. The reaction mixture was the same as in the previous experiment and the results are given in Table 5.

Table 5. *Effect of precipitation of liver extract with Na_2SO_4 on arginase and canavanase activities*

Enzyme	mg. of urea-N formed after 4 hr. incubation		
	Arginine	Canavanine	Urea-N from arginine
			Urea-N from canavanine
Precipitate obtained after half saturation with Na_2SO_4	9.88	3.31	2.98
Precipitate obtained after full saturation with Na_2SO_4	9.60	2.85	3.36
Filtrate from the above	1.96	0.55	3.51

Distribution of arginase and canavanase in the tissues of different animals (Table 6)

The animals tested were all adults. The guinea-pigs, rats and rabbits were killed in the laboratory. The tissues and blood of ox and cow were brought in ice from the slaughter-house.

Table 6. *Distribution of arginase and canavanase in the tissues and blood of different animals*

Source	mg. of urea-N formed after 4 hr. incubation					
	Liver		Kidney		Blood	
	Arg.	Can.	Arg.	Can.	Arg.	Can.
Guinea-pig (male)	9.381	8.412	2.113	0.671	Nil	Nil
Guinea-pig (female)	9.546	7.193	2.451	1.452	"	"
White rat (male)	9.123	8.470	3.976	1.061	"	"
White rat (female)	9.123	8.021	3.976	1.112	"	"
Rabbit (male)	9.548	8.470	1.184	0.293	"	"
Rabbit (female)	9.123	7.941	1.412	0.285	"	"
Ox	9.581	8.573	0.513	0.354	7.521*	1.925*
Cow	9.541	7.791	0.812	0.354	5.491*	2.110*

* 18 hr. incubation instead of 4 hr.

Enzyme solution. The fresh tissue (5 g.) was extracted with water (25 ml.), the mixture centrifuged and the supernatant liquid made up to 50 ml. so that 1 ml. of the solution corresponded to 0.1 g. of the moist tissue. The blood was diluted with an equal volume of water.

Reaction mixture. 5 ml. of substrate solution (urea-N 10 mg.) + 5 ml. of buffer (pH 7.5 for canavanine and pH 9.4 for arginine) + 5 ml. of enzyme solution. Total volume 25 ml.

Distribution of arginase and canavanase in different urease preparations (Table 7)

The different sources of urease tested were the seeds of *Citrullus vulgaris* (water melon), *Canavalia obtusifolia*, *Soja hispida* (soya bean), *Dolichos biflorus* (horse-gram), *Cucurbita maxima* (common gourd) and *Canavalia ensiformis* (jack bean). In all cases the ground material was used without removal of fat. In the case of water melon seeds, which were tested, the results obtained were the same as those obtained with undefatted material.

Table 7. *Hydrolysis of arginine and canavanine by urease preparations from different sources*

Source	mg. of urea-N formed after 3 hr. incubation			
	Arginine		Canavanine	
	mg.	%	mg.	%
<i>Citrullus vulgaris</i> (water melon)	0.072	1.3	0.072	1.3
<i>Soja hispida</i> (soya bean)	0.110	2.0	0.097	1.7
<i>Cucurbita maxima</i> (common gourd)	0.063	1.1	0.051	0.9
<i>Dolichos biflorus</i> (horse-gram)	0.100	1.8	0.068	1.2
<i>Canavalia obtusifolia</i>	3.880	69.4	2.970	53.0
<i>C. ensiformis</i> (jack bean)	4.460	79.6	3.452	61.7

Among the different sources of urease examined only the seeds of *Canavalia obtusifolia* and jack bean contain an enzyme which under suitable conditions hydrolyses both arginine and canavanine. Water melon urease does not possess this property.

Reaction mixture. 2 ml. of substrate solution (*M*/5) + 5 ml. of *M* buffer pH 7.7 + 2 ml. of cobalt chloride solution (6 mg.) + 5 ml. of an aqueous suspension of urease (0.1 g.).

Comparison of arginase and canavanase activities of liver enzyme in the absence and in the presence of CoCl₂

(I) Without addition of cobalt chloride.

Reaction mixture. 5 ml. of substrate solution (*M*/10) + 5 ml. of buffer of pH 7.5 + 5 ml. of enzyme solution (0.5 g. of the liver powder extracted with 50 ml. of water). Total volume 25 ml. The results are given in Table 8.

Table 8. *Arginase and canavanase activities in absence of CoCl₂*

Time hr.	Urea-N				Urea-N from arginine Urea-N from canavanine
	Arginine		Canavanine		
	mg.	%	mg.	%	
1	3.246	23.2	0.875	6.3	3.71
2	5.615	40.1	1.486	10.6	3.78
3	7.190	51.4	2.002	14.3	3.61
4	8.598	61.4	2.436	17.4	3.58

(II) In the presence of cobalt chloride.

The reaction mixture was the same as in the previous experiment but 2 ml. of cobalt chloride (6 mg.) solution were added before the addition of the enzyme. The results are given in Table 9.

Table 9. *Arginase and canavanase activities in presence of cobalt chloride*

Time hr.	Urea-N				Urea-N from arginine Urea-N from canavanine
	Arginine		Canavanine		
	mg.	%	mg.	%	
1	5.615	40.1	3.414	24.4	1.64
2	8.706	62.1	5.698	40.7	1.52
3	11.420	81.6	7.491	53.5	1.52
4	13.451	96.1	9.143	65.3	1.47

Effect of cyanide

The actions of liver enzyme on arginine and canavanine at their respective pH-optima were studied with and without the addition of potassium cyanide.

The results are shown in Figs. 11 and 12 from which the inhibitory influence of cyanide at high concentrations is evident. Under given conditions canavanase

Fig. 11. Effect of KCN on the hydrolysis of canavanine.

Fig. 12. Effect of KCN on the hydrolysis of arginine.

is inhibited to a greater extent than arginase. Such a preferential inhibition is probably to be ascribed to the difference in pH as shown by Edlbacher *et al.* [1932].

Reaction mixture. 10 ml. of substrate solution ($M/10$) + 10 ml. of buffer + 10 ml. of neutralized KCN solution + 10 ml. of enzyme solution (1 g. of the powder extracted with 50 ml. of water). Total volume 50 ml.

SUMMARY

A comparative study of the enzymes arginase and canavanase from both animal and plant sources has been made.

In their kinetics the two enzymes show only such differences (pH-optimum, optimum substrate concentration and Michaelis constant) as are to be expected from the nature of the substrates.

In other respects there is a close parallelism between arginase and canavanase actions. In tissues and blood of different animals they are either present together or not at all and in every case the action on canavanine is slower than that on arginine. By no procedure has it been possible to reverse this quantitative relationship and obtain a preparation in which canavanase activity is stronger than arginase activity. In any particular preparation factors affecting arginase influence canavanase in the same direction and often to the same extent, e.g. the loss of activity on autolysis and the increase in activity in the presence of cobalt ions affect both enzymes equally. In the presence of cobalt the pH-optima for the two reactions also become identical at pH 7.5.

The balance of evidence is thus in favour of the two enzymes being identical.

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