ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

III. THE PROTEOLYTIC ENZYMES OF BEEF SPLEEN, BEEF KIDNEY, AND SWINE KIDNEY. CLASSIFICATION OF THE CATHEPSINS

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Aqueous extracts of beef spleen have been shown to contain at least three proteolytic enzymes of widely different specificity (1). Beef spleen Cathepsin I hydrolyzes carbobenzoxy-*l*-glutamyl-*l*-tyrosine and does not require the addition of an activator such as cysteine. Beef spleen Cathepsin II hydrolyzes benzoyl-*l*-arginineamide when an activator such as cysteine is added. Beef spleen Cathepsin III hydrolyzes *l*-leucineamide in the presence of activators such as cysteine or ascorbic acid. In the present communication, the identification of a fourth proteolytic component (Cathepsin IV) of beef spleen cathepsin is reported. Furthermore, counterparts of these four enzymes have also been found in beef kidney and swine kidney.

Identification of a New Proteolytic Enzyme in Beef Spleen Cathepsin

An indication for the presence of a fourth proteolytic component in beef spleen cathepsin was obtained by a comparative study of the action of a cysteine-activated spleen extract upon benzoyl-*l*-arginineamide, benzoyl-*l*-lysineamide, carbobenzoxyglycyl-*l*-phenylalanine, and carbobenzoxyglycyl-*l*-tyrosine. It was previously reported (2) that Cathepsin II is unstable at pH values more acid than 4. It will be noted from the data in Table I that the activity of beef spleen cathepsin toward carbobenzoxyglycyl-phenylalanine is more resistant to acidity than is the activity of

Cathepsin II. It must be concluded that carbobenzoxyglycyl-*l*-phenylalanine is hydrolyzed by a cysteine-activatable enzyme other than Cathepsin II. This component is the Cathepsin IV mentioned above. The data in Table I indicate also that at pH 3.5 and 3.9 the activities of spleen cathepsin toward benzoylarginineamide and benzoyllysineamide decreased in a parallel manner. This indicates that benzoyllysineamide is split by the same enzyme as is benzoylarginineamide; *i.e.*, Cathepsin II.

TABLE I

Effect of Increased Acidity on Activity of Beef Spleen Cathepsin

Samples of a solution of beef spleen cathepsin (1.40 mg. of protein N per cc. of enzyme solution) were adjusted to pH 3.9 or 3.5 with N acetic acid and kept at these pH values for 1 hour at 40°. The pH of the enzyme solutions was then brought to 5.0 with N NaOH. In a control experiment a mixture of N acetic acid and N NaOH was made up in the proportions applied above and then added to the enzyme. The control enzyme solution was also kept at 40° for 1 hour. The three above enzyme solutions were tested for enzymatic activity in the presence of cysteine (0.01 mM per cc. of test solution). The pH of the test solution was 4.8; the temperature was 40°

Substrate	$C^* \times 10^3$		
	Control	Treated enzyme	
		pH 3.9	pH 3.5
Benzoyl-*l*-arginineamide (4)	8.3	5.2	2.8
Benzoyl-*l*-lysineamide (5)	4.0	2.4	1.3
Carbobenzoxyglycyl-*l*-phenylalanine (6)	4.2	4.1	3.5

$$^*C = \frac{K \text{ (first order)}}{\text{mg. protein N per cc. test solution}} \quad (3).$$

A sample of beef spleen cathepsin was dialyzed against distilled water, and its activity upon the previously mentioned substrates was tested before and after dialysis. The data in Table II show that on dialysis the proteolytic coefficient toward benzoylarginineamide rises slightly,[1] while the proteolytic coefficients toward carbobenzoxyglycylphenylalanine and carbobenzoxyglycyltyrosine drop markedly. This decrease is of the same magnitude for both substrates. It may therefore be concluded that the hydrolysis of carbobenzoxyglycylphenylalanine and carbobenzoxyglycyltyrosine is due to the same enzymatic component of spleen cathepsin; *i.e.*, Cathepsin IV.

[1] This increase is due to the loss of protein other than Cathepsin II.

It had previously been found (1) that carbobenzoxy-*l*-glutamyl-*l*-phenylalanine is hydrolyzed by two different enzymatic components of beef spleen cathepsin, one of which (Cathepsin I) is effective in the absence of an added activator, while the other is activated by cysteine. It has now been found that the activity of this second, cysteine-activatable enzyme was not diminished when the beef spleen extract was kept at pH 3.9 and 40° for 1 hour, but that this enzyme is lost when the spleen extract is dialyzed against distilled water for 48 hours. This behavior is similar to that previously found for Cathepsin IV, and it may be assumed that the cysteine-activatable beef spleen component that acts on carbobenzoxyglutamylphenylalanine is identical with the component

TABLE II

Loss of Cathepsin IV Activity on Dialysis of Beef Spleen Cathepsin

A solution of beef spleen was dialyzed against 1 per cent sodium chloride. The resulting Solution A was then dialyzed against distilled water for 48 hours at 4° to give an enzyme Solution B. Solutions A and B were tested for proteolytic activity with cysteine as activator (0.01 mM per cc. of test solution). Temperature, 40°; pH 4.8 to 5.1.

Substrate	$C \times 10^3$		Cathepsin component
	Enzyme Solution A	Enzyme Solution B	
Benzoyl-*l*-arginineamide	8.0	9.1	II
Carbobenzoxyglycyl-*l*-phenylalanine	3.7	1.4	IV
Carbobenzoxyglycyl-*l*-tyrosine (7)	2.3	0.9	"

that hydrolyzes carbobenzoxyglycylphenylalanine; namely, Cathepsin IV. This tentative conclusion cannot, at present, be subjected to a decisive test by comparing, under various experimental conditions, the reaction rates of the cysteine-activated catheptic hydrolyses of carbobenzoxyglycylphenylalanine and carbobenzoxyglutamylphenylalanine. Since the latter substrate is hydrolyzed simultaneously by two spleen components, rate constants for its hydrolysis cannot be calculated.

Cathepsins of Beef Kidney and Swine Kidney

Cathepsin I—Similarly to beef spleen, extracts of beef kidney and swine kidney contain enzymes (beef kidney Cathepsin I and swine kidney Cathepsin I) that hydrolyze carbobenzoxy-*l*-glutamyl-*l*-tyrosine in the absence of added activators. These

enzymes are rapidly inactivated at 50°. This thermolability was utilized to show that Cathepsin I in beef kidney and swine kidney hydrolyzes not only carbobenzoxyglutamyltyrosine but also the compound carbobenzoxy-*l*-glutamyl-*l*-phenylalanine. It will be noted in Table III that after the enzyme solutions were heated at 50° for 15 minutes the activity toward the two substrates decreased to the same degree.

Cathepsin II—The substrate for cysteine-activated beef spleen Cathepsin II, benzoyl-*l*-arginineamide, is also hydrolyzed by beef kidney and swine kidney extracts after cysteine has been added as the activator. The enzymes responsible for these hydrolyses are

TABLE III

Heat Inactivation of Cathepsin I

Solution A of beef kidney cathepsin (1.55 mg. of protein N per cc. of enzyme solution) was heated at 50° for 15 minutes and then was chilled in ice water to give a beef kidney cathepsin solution (B). A Solution A of swine kidney cathepsin (1.10 mg. of protein N per cc. of enzyme solution) was also treated as above to yield a swine kidney cathepsin solution (B). 0.5 cc. of the enzyme Solutions A and B was employed for the hydrolytic experiments. No cysteine was added. Temperature, 25°; pH 5.3 to 5.5.

Enzyme solution	Carbobenzoxy-*l*-glutamyl-*l*-tyrosine (8)		Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine	
	$K \times 10^4$	$\frac{K(B)}{K(A)}$	$K \times 10^4$	$\frac{K(B)}{K(A)}$
Beef kidney, A	6.2		2.6	
" " B	3.0	0.48	1.2	0.46
Swine " A	7.0		3.5	
" " B	5.3	0.76	2.6	0.74

designated beef kidney Cathepsin II and swine kidney Cathepsin II. The first order velocity constants for the hydrolysis effected by these enzymes are proportional to the enzyme concentration within the limits employed (Table IV). The value of the proteolytic coefficient for benzoylarginineamide (C_{BAA}) for beef spleen extract was previously found to be about 0.008, corresponding to 4 enzyme units per mg. of protein nitrogen, when an enzyme unit is defined according to a previous paper (3). On the same basis, the beef kidney extract used in the experiments reported in Table IV contained 3.6 units per mg. of protein nitrogen, while the swine kidney extract contained 12 units per mg. of protein nitrogen.

Beef kidney Cathepsin II and swine kidney Cathepsin II were also found to hydrolyze benzoyl-*l*-lysineamide. The experimental data will be presented in a succeeding section of this paper.

Cathepsin III—Both beef kidney and swine kidney extracts contain an enzyme (Cathepsin III) that hydrolyzes *l*-leucineamide and *l*-leucylglycine at pH 5 when ascorbic acid or cysteine has been

TABLE IV

Hydrolysis of Benzoyl-l-arginineamide by Cathepsin II of Beef and Swine Kidney

0.01 mM of cysteine per cc. of test solution. Temperature, 40°; pH 4.7.

Source of enzyme	Protein N per cc. test solution	$K \times 10^4$	$C \times 10^3$
	mg.		
Beef kidney	0.13	9	6.9
	0.20	14	7.0
	0.33	24	7.3
Swine kidney	0.05	12	24
	0.10	26	26
	0.21	51	24

TABLE V

Comparison of Cathepsin III Activity in Beef Spleen, Beef Kidney, and Swine Kidney

Temperature, 40°; pH 5.0 to 5.2.

Substrate	Activator, 0.01 mM per cc. test solution	Beef spleen cathepsin*		Beef kidney cathepsin†		Swine kidney cathepsin†	
		$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$
l-Leucineamide	Cysteine	33	9.2	3.0	1.0	11	3.7
	Ascorbic acid	7	1.9	0.6	0.2	2.2	0.7
l-Leucylglycine	Cysteine	16	4.4	1.2	0.4	5	1.7

* 0.36 mg. of protein N per cc. of test solution.

† 0.30 mg. of protein N per cc. of test solution.

added as activator (Table V). It will be noted that the Cathepsin III activity per mg. of protein nitrogen is much smaller in beef and swine kidney than in beef spleen.

Cathepsin IV—Extracts of beef kidney and swine kidney have been found to contain cysteine-activatable enzymes (beef kidney Cathepsin IV and swine kidney Cathepsin IV) that hydrolyze

carbobenzoxyglycylphenylalanine. Swine kidney extracts show a particularly high Cathepsin IV activity. It may be calculated from the data in Table VI that beef spleen extracts contain 1.5 Cathepsin IV units per mg. of protein nitrogen, beef kidney 3 Cathepsin IV units, and swine kidney 16.5 Cathepsin IV units.

TABLE VI

Hydrolysis of Carbobenzoxyglycyl-l-phenylalanine by Beef Spleen, Beef Kidney, and Swine Kidney

Cysteine, 0.01 mM per cc. of test solution. Temperature, 40°; pH 4.9.

Source of enzyme	Protein N per cc. test solution	$K \times 10^4$	$C \times 10^3$
	mg.		
Beef spleen	0.14	4	2.9
	0.28	8	2.9
	0.56	15	2.7
" kidney	0.21	12	5.7
	0.28	16	5.7
	0.42	25	6.0
Swine "	0.05	16	32
	0.10	34	34
	0.21	71	34

TABLE VII

Effect of Cysteine on Hydrolysis of Carbobenzoxy-l-glutamyl-l-tyrosine

Temperature, 25°; pH 5.3 to 5.5.

Source of enzyme	Protein N per cc. test solution	Hydrolysis in 2 hrs.	
		No cysteine	Cysteine*
	mg.	*per cent*	*per cent*
Beef spleen	0.29	22	24
" kidney	0.31	15	37
Swine "	0.18	10	36

* 0.01 mM per cc. of test solution.

In an earlier section of this paper evidence was presented to indicate that Cathepsin IV, when activated, hydrolyzes the typical substrates of Cathepsin I (carbobenzoxy-*l*-glutamyl-*l*-phenylalanine and carbobenzoxy-*l*-glutamyl-*l*-tyrosine). This explains why tissues that are relatively rich in Cathepsin IV show a marked

increase in the hydrolysis of carbobenzoxyglutamyltyrosine on addition of cysteine (Table VII). Beef spleen, which is relatively poor in Cathepsin IV, shows no appreciable increase in the hydrolysis of carbobenzoxyglutamyltyrosine when cysteine is added.

Classification of Cathepsins in Revised System of Proteolytic Enzymes

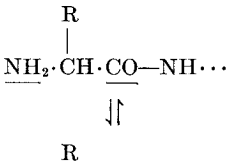
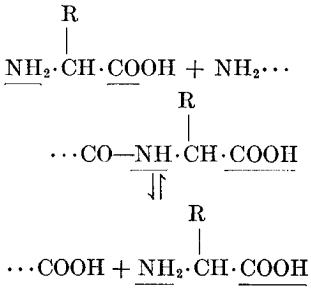
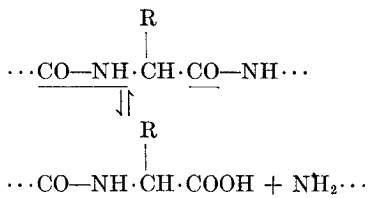
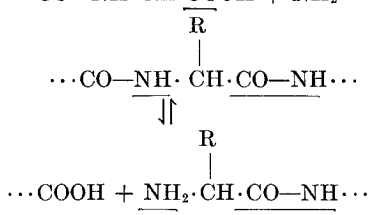
In the past, proteolytic enzymes were classified as dipeptidases, polypeptidases, and proteinases (9). Consequently, the catheptic enzymes, as classified according to this scheme, included one dipeptidase, one aminopolypeptidase, one c oxypolypeptidase, and one proteinase (10). However, during recent years information has been gathered that requires revision of the above classification. In particular, the specificity and mechanism of the action of proteinases has been studied further (11). Moreover, it has been observed repeatedly that aminopeptidases and carboxypeptidases may also split dipeptides, and consequently the existence of a separate group of dipeptidases may be questioned (6, 12–14).

The revised classification proposed in Table VIII is based on the nature and position of the chemical groups in the peptide chain of the substrate that are required for the action of various proteolytic enzymes. However, the sensitivity of a substrate to a given proteolytic enzyme is determined not only by the groups in the peptide chain of the substrate but also by the nature of the constituent amino acids. Consequently, each of the classes in Table VIII may be subdivided further on the basis of the amino acid residues in the substrates that are essential for the action of specific enzymes.

It will be noted that pepsin and trypsin belong to the group of carbonylproteinases, while chymotrypsin is an imidoproteinase. The Cathepsins I of beef spleen, beef kidney, and swine kidney fall into the group of carbonylproteinases. This conclusion is based on the fact that glutamyltyrosine is not split by these enzymes and no substrate has been found for them that does not have a peptide linkage adjacent to the carbonyl side of the sensitive peptide bond. Cathepsin II is also a carbonylproteinase. Cathepsin III splits *l*-leucineamide besides *l*-leucylglycine and therefore must be an aminopeptidase. Cathepsin IV appears to be a carboxypeptidase, since carbobenzoxyglycylphenylalanineamide is not split appreciably by a cysteine-activated swine kidney extract that is rich

TABLE VIII

Revised Classification of Proteolytic Enzymes

Class	Enzyme	Requisite groups in peptide chain
Peptidases (exopeptidases (15))		
Aminopeptidases	Intestinal aminopeptidase Cathepsin III	
Carboxypeptidases	Pancreatic carboxypeptidase Cathepsin IV	
Proteinases (endopeptidases (15))		
Carbonylproteinases	(*a*) Pepsin Cathepsin I (*b*) Trypsin Papain* Cathepsin II	
Imidoproteinases	Chymotrypsin†	

* The component of papain that hydrolyzes benzoyl-*l*-arginineamide.

† Chymotrypsin is designated an imidoproteinase because it hydrolyzes *l*-tyrosylglycineamide at the peptide linkage joining the tyrosyl and glycyl residues (unpublished experiments) and also hydrolyzes carbobenzoxytyrosylglycineamide (7).

in Cathepsin IV and highly active toward carbobenzoxyglycylphenylalanine.

Additional support for this classification has been obtained by comparing the action of several enzymes upon two test substrates in a quantitative manner. Thus, the Cathepsins II of beef spleen, beef kidney, and swine kidney are compared with respect to their action on benzoylarginineamide and benzoyllysineamide (Table IX). The proteolytic quotient C_{BAA}/C_{BLA} was found to be essentially the same (2.2 to 2.5). New determinations of the reaction velocity constants for the hydrolysis of the above substrates by

TABLE IX

Hydrolysis of Benzoyl-l-argininеamide and Benzoyl-l-lysineamide by Several Proteolytic Enzymes

In all cases, except that of trypsin, cysteine was present in a concentration of 0.01 mM per cc. of test solution and the temperature was 40°. In the experiments with trypsin, the temperature was 25°.

Enzyme	pH	$C \times 10^3$		$\frac{C_{BAA}}{C_{BLA}}$
		Benzoylarginine-amide	Benzoyllysineamide	
Beef spleen Cathepsin II	4.7	8.3	3.8	2.2
" kidney " "	4.7	8.7	3.7	2.3
Swine " " "	4.7	27	11	2.5
Trypsin	7.4	42	20	2.1
Papain	5.0	167	78	2.1

crystalline beef trypsin (*cf.* also (5)) gave a proteolytic quotient C_{BAA}/C_{BLA} of 2.1. Furthermore, with cysteine-papain a proteolytic quotient of C_{BAA}/C_{BLA} of 2.1[2] was obtained.

The similarity of the proteolytic quotients C_{BAA}/C_{BLA} for the five enzymes is the more striking since the enzymes compared differ in their pH optimum and activation behavior. In our opinion, this similarity exists because the five enzymes all act upon their

[2] It was found that the proteolytic activity toward each substrate dropped to 30 per cent of the original value after treatment of a papain solution at pH 2 for 17 hours at 21°. The fact that the quotient C_{BAA}/C_{BLA} was unchanged indicates that the two substrates are split by the same enzymatic component of papain.

substrates by a similar reaction mechanism; they all are carbonylproteinases.

The hydrolysis of the substrates benzoylglycyl-*l*-arginineamide and benzoylglycyl-*l*-lysineamide by crystalline trypsin has already

TABLE X

Comparison of Cathepsins IV with Crystalline Carboxypeptidase

In all cases, except that of carboxypeptidase, cysteine was present in a concentration of 0.01 mM per cc. of test solution and the temperature was 40°. In the experiments with carboxypeptidase, the temperature was 25°.

Enzyme	pH	$C \times 10^3$		$\frac{C_{CGP}}{C_{CGT}}$
		Carbobenzoxyglycyl-*l*-phenylalanine	Carbobenzoxyglycyl-*l*-tyrosine	
Beef spleen Cathepsin IV	5.0	2.5	1.5	1.7
" kidney " "	5.1	6.3	4.0	1.6
Swine " " "	5.0	34	19	1.8
Carboxypeptidase*	7.7	6570	3620	1.8

* This preparation was kept at 0° for over 1 year and thus had lost some activity.

TABLE XI

Comparison of Cathepsin I Activity in Beef Spleen, Beef Kidney, and Swine Kidney

No cysteine was added. Temperature, 25°; pH 5.3 to 5.5.

Source of enzyme	Protein N per cc. test solution	Carbobenzoxy-*l*-glutamyl-*l*-phenylalanine		Carbobenzoxy-*l*-glutamyl-*l*-tyrosine		$\frac{C_{CGP}}{C_{CGT}}$
		$K \times 10^4$	$C \times 10^3$	$K \times 10^4$	$C \times 10^3$	
	mg.					
Beef spleen	0.45	7.2	1.6	14.7	3.3	0.48
" kidney	0.31	2.6	0.84	6.2	2.0	0.42
Swine "	0.22	3.5	1.6	7.0	3.2	0.50

been studied (16) and the quotient C_{BGAA}/C_{BGLA} was found to be 1.8. This value is close to those given above for the quotient C_{BAA}/C_{BLA}.

In Table X the Cathepsins IV of beef spleen, beef kidney, and swine kidney as well as crystalline carboxypeptidase from beef

pancreas are compared with respect to their action on carbobenzoxyglycyl-*l*-phenylalanine and carbobenzoxyglycyl-*l*-tyrosine. It will be noted that the proteolytic quotient C_{CGP}/C_{CGT} was found to be 1.6 to 1.8. Here again enzymes are compared which differ with respect to pH optima and activation behavior but which belong to the same group of carboxypeptidases.

The proteolytic quotients C_{CGP}/C_{CGT} for the hydrolysis of carbobenzoxy-*l*-glutamyl-*l*-phenylalanine and carbobenzoxy-*l*-glutamyl-*l*-tyrosine by the Cathepsins I of beef spleen, beef kidney, and swine kidney were found to be 0.42, 0.48, and 0.50, respectively (Table XI), thus indicating that the phenylalanine-containing substrate is hydrolyzed at a rate one-half of that of the tyrosine-containing substrate. It will be recalled that in the case of Cathepsin IV the phenylalanine-containing substrate was hydrolyzed at nearly twice the rate of the hydrolysis of the tyrosine-containing substrate. This difference in the quotients for Cathepsin I and Cathepsin IV indicates that these two enzymes have different mechanisms of action, and serves as added support for the classification of Cathepsin I and Cathepsin IV in separate classes (Table VIII).

The authors wish to express their thanks to Mr. Maurice Rapport for valuable assistance in this investigation.

EXPERIMENTAL

The beef spleen cathepsin solutions were prepared as described in a previous paper (2). The same procedure was employed for the preparation of beef kidney cathepsin and swine kidney cathepsin. Crystalline trypsin was prepared according to the directions of Kunitz and Northrop (17). Crystalline carboxypeptidase was prepared by the method of Anson (18). The papain preparation was obtained as described in a previous paper (19).

The course of enzymatic hydrolysis was followed by means of amino nitrogen determinations and the microtitration method of Grassmann and Heyde. The substrate concentration was 0.05 mM per cc. of the test solution in all cases. The pH was adjusted by means of citrate buffers (near pH 5) and by means of phosphate buffers (near pH 7).

BIBLIOGRAPHY

1. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **138,** 249 (1941).
2. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130,** 19 (1939).
3. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138,** 231 (1941).
4. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127,** 643 (1939).
5. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130,** 81 (1939).
6. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134,** 225 (1940).
7. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118,** 405 (1937).
8. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **127,** 627 (1939).
9. Grassmann, W., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipzig, **1,** 133 (1932).
10. Waldschmidt-Leitz, E., Schäffner, A., Bek, J. J., and Blum, E., *Z. physiol. Chem.*, **188,** 17 (1930).
11. Bergmann, M., and Fruton, J. S., in Nord, F. F., and Werkman, C. H., Advances in enzymology and related subjects, New York, **1,** 63 (1941).
12. Bergmann, M., *Nature*, **131,** 602 (1933).
13. Bergmann, M., Zervas, L., Salzmann, M., and Schleich, H., *Z. physiol. Chem.*, **224,** 17 (1934).
14. Johnson, M. J., *J. Biol. Chem.*, **137,** 575 (1941).
15. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **117,** 189 (1937).
16. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **138,** 243 (1941).
17. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19,** 991 (1936).
18. Anson, M. L., *J. Gen. Physiol.*, **20,** 663 (1937).
19. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **139,** 569 (1941).

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