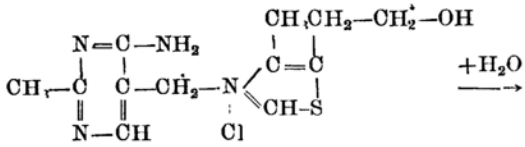
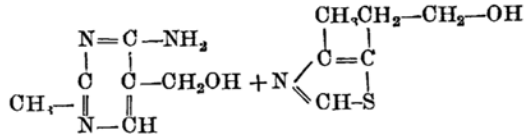
On the Specificity of Aneurinase

By Kiku MURATA

(Received September 14, 1949)

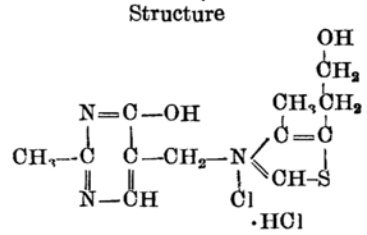
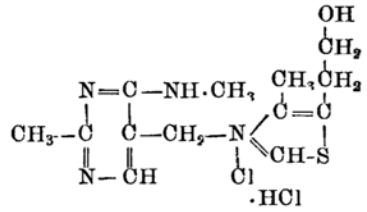
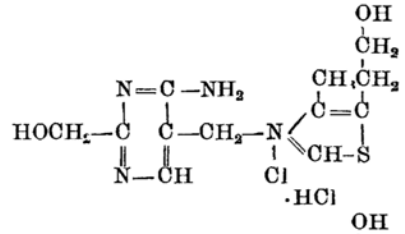
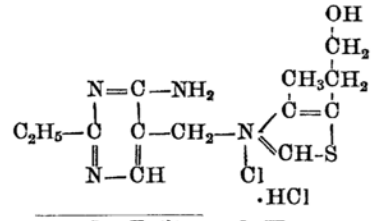
Aneurinase, which decmposes aneurin, has been found in shell-fishes by Fujita[1] and in fishes by Sealock.[2] The mode of the enzymatic decomposition has been studied by Fujita,[3][4][5] Yamasaki[6] and also by Woolley.[7] According to their results aneurin is splitted into pyrimidine and thiazole as follows;





To study the more precise mechanism of the action of aneurinase against aneurin, it is desirable to have some knowledge on the specificity of the enzyme in relation to the chemical constitution of the substrate. The author has, therefore, studied on the action of aneurinase on various aneurin analogues as described below, and the results are summarised in Table 1.

Table 1

Expt. No.	Substrate: Name	Substrate: Structure	Decomposition by Enz.
1, 2	4-methyl-5-β-hydroxyethyl-N-[(2′-methyl-4′-hydroxy-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride (I)		−
3	4-methyl-5-β-hydroxyethyl-N-[(2′-methyl-4′-aminomethyl-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride (II)		−
4, 5, 6	4-methyl-5-β-hydroxyethyl-N-[(2′-hydroxmethyl-4′-amino-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride (III)		+
7, 8	4-methyl-5-β-hydroxyethyl-N-[(2′-ethyl-4′-amino-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride (IV)		+

(1) Fujita and Numata, *J. Biochem. Soc. Japan*, **17**, 156 (1943); **18**, 63 (1944).

(2) Sealock, *J. Am. Chem. Soc.*, **65**, 935 (1943).

(3) Fujita and Numata, *J. Biochem. Soc. Japan*, **18**, 327 (1944).

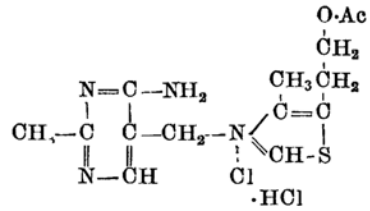
(4) Fujita, Description of the 14th meeting on special committee of V. B_1, (1947).

(5) Fujita and Hasegawa, Description of the 29th meeting on special committee of V. B_1, (1948).

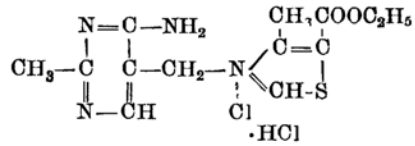
(6) Yamasaki, *J. Jap. Soc. Food and Nutr.*, **1**, 8 (1948).

(7) Woolley, unpublished (it is introduced on *Ann. Review of Biochem.* (1944)).

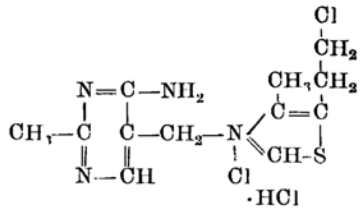
9 4-methyl-5-β-hydroxyethyl-N-[(2′-methyl-4′-amino-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride-acetate (V)



10 4-methyl-5-carboethoxy-N-[(2′-methyl-4′-amino-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride (VI)



11 4-methyl-5-β-chloroethyl-N-[(2′-methyl-4′-amino-pyrimidyl-5′)-methyl]-thiazolium-chloride-hydrochloride (VII)



From Table 1 it is clear that the side chain of the thiazole nucleus and some substitution at position 2 on the pyrimidine nucleus have no effect but the amino group at position 4 of the latter is absolutely necessary* for the hydrolytic decomposition of aneurin by aneurinase.

It may be presumed that aneurin splits into the pyrimidine and thiazole residues after the protein part of aneurinase has been joined to the amino group on the pyrimidine nucleus in the aneurin molecule. According to Woolley,[7] in the isolation of the thiazole and pyrimidine residues from the decomposition products by the action of aneurinase, the separation of the thiazole residue is followed by the detachment of the pyrimidine residue and the velocity of the detachment of the latter depends upon what species of shell-fish was used in the preparation of the enzyme. Reasons for this are, however, to be left in the future study; there may be some relation between the amino group in the pyrimidine nucleus and the enzyme action.

Experimental

Preparation of the Crude Extract of the Enzyme.—The meat and juice removed from shells were weighed together and triturated with addition of the sand. An equal quanity of $N/10$ sodium bisphosphate was added to it and the mixture was kept on standing over-night and centrifuged for 10 minutes. The clear supernatant liquid thus obtained was used as the crude enzyme extract.

Test for Enzymatic Activity.—The enzyme extract was mixed with an equal volume or a slightly excess of buffer solution of sodium acetate (pH 6.1) and a unit quantity of aneurin was added as shown in Table 2. After one hour at 60°, the reaction mixture was filtered and the content of aneurin remained in the filtrate was determined by the method of diazo or thiochrome. It was then possible to calculate the amount of aneurin decomposed by the ferment by subtracting the amount of aneurin remaining in the filtrate from what has been added originally.

Table 2

Reaction condition	Volume of enzyme extract, cc.	Substrate (V. B_1 analogues added), γ/cc.	Volume of buffer solution, cc.	Volume of $2N$ hydrochloric acid, cc.
1	6	200 γ/2 cc.	11.5	0.5
2	3	100 γ/1 cc.	6.0	——
3	10	150 γ/1.5 cc.	18.0	0.5
4	2	10 γ/1 cc.	2.0	——
5	24	120 γ/1.2 cc.	22.8	——
6	22	110 γ/1.1 cc.	20.9	——
7	20	300 γ/3 cc.	36.5	0.5
8	2	10 γ/1 cc.	17.0	——

The Main and Blank Tests.—Equal volumes of the enzyme extract were put in two round bottom flasks of about 100 cc. in volume, which are attached to reflux condensers through a ground glass joint. The one, to be used as a blank test, was boiled gently over the asbestos pad for three minutes to inactivate the enzyme. The rest of the operations was the same for the both tests. A unit quantity of the buffer and a substrate under consideration were added to each and warmed to 60° for one hour. At the end of the reaction, water droplets condensed at the neck of the flasks and the lower part of the condensers were mixed well with the reaction mixtures in order to keep the concentration constant. They were then filtered and the filtrates were subjected separately for the aneurin determination.

The determination of aneurin was made by the usual methods, the diazo and thiochrome, except in the cases of substrate (VI) and (VII), where the determinations were done in the alkaline solution because the thiochrome of these compounds were difficult to dissolve in isobutanol.

Since the value obtained by the main test indicates percentage of aneurin recovered, it is then

* Those compounds which do not possess the free amino group and undergo splitting with difficulty can be made to decompse with addition of an equal amount of V. B_1.

Table 3

Expt. No	Reaction condition	Test	Substrate	Aneurin content, γ/cc.	Diazo method: Enzyme extract, cc.	Substrete (calculated) aneurin, γ	Aneurin recovered, γ	Recovery, %	Decomposition, %	Thiochrome method: Enzyme extract, cc.	Substrete (calculated) aneurin, γ	Aneurin recovered, γ	Recovery, %	Decomposition, %
1	1	Enz. act.	——	10	5	50	14.9	——	70.4	——	——	——	——	——
		Main	〃	〃	〃	〃	41.5	——	3.1					
		Blank	〃	〃	〃	〃	42.8	85.6	——					
	2	Main	〃	〃	〃	〃	33.2	——	0.3					
		Blank	〃	〃	〃	〃	33.3	66.6	——					
			(I)							T. R. negative				
2	3	Enz. act.	——	5	10	50	13.3	——	70.3	0.4	2·0	0.04	——	98.0
		Main	〃	〃	〃	〃	41.6	——	4.2					
		Blank	(I)	〃	〃	〃	43.4	86.8	——	T. R. negatiue				
3	3	Enz. act.	——	5	10	50	6.8	——	86.4	——	——	——	——	——
		Main	〃	〃	〃	〃	32.7	——	0					
		Blank	(II)	〃	〃	〃	32.7	65.4	——	T. R. negative				
4	3	Enz. act.	——	5	—	—	——	——	——	0.4	2.0	0	——	100.0
		Main	〃	10	50	〃	2.3	——	95.2	——	——	——	——	——
		Blank	(III)	〃	〃	〃	47.6	95.2	——	——	——	——	——	——
5	4	Enz. act.	——	2	—	—	——	——	——	0.25	0.5	0.01	——	98.0
	5	Main	〃	2.5	20	50	5.0	——	84.4	0.4	1.0	0.16	——	69.3
		Blank	(III)	〃	〃	〃	32.1	64.2	——	0.2	0.5	0.52	104.0	——
6	4	Enz. act.	——	2	—	—	——	——	——	0.25	0.5	0	——	100.0
	5	Main	〃	2.5	20	50	0	——	100.0	0.4	1.0	0	——	100.0
		Blank	(III)	〃	〃	〃	48.1	96.2	——	0.2	0.5	0.53	106.0	——
7	4	Enz. act.	——	2	—	—	——	——	——	0.25	0.5	0	——	100.0
	6	Main	〃	2.5	18	45	0	——	100.0	0.4	1.0	0	——	100.0
		Blank	(IV)	〃	〃	〃	46.7	104.0	——	0.2	0.5	0.49	98.0	——
8	4	Enz. act.	——	2	—	—	——	——	——	0.25	0.5	0	——	100.0
	7	Main	〃	5	10	50	10.0	——	81.7	0.2	1.0	0.18	——	64.0
		Blank	(IV)	〃	〃	〃	54.4	108.0	——	0.1	0.5	0.50	100.0	——
9	4	Enz. act.	——	2	—	—	——	——	——	0.2	0.4	0	——	100.0
	5	Main	〃	2.5	20	50	0	——	100.0	0.4	1.0	0	——	100.0
		Blank	(V)	〃	〃	〃	40.0	80.0	——	0.2	0.5	0.52	104.0	——
10	8	Enz. act.	——	0.5	—	—	——	——	——	2.0	1.0	0	——	100.0
		Main	〃	〃						2.0	1.0	0	——	100.0
		Blank	(VI)	〃			D. R. negative			1.0	0.5	0 52	104.0	——
11	8	Enz. act.	——	0.5	—	—	——	——	——	2.0	1.0	0	——	100.0
		Main	〃	〃						2.0	1.0	0.01	——	99,0
		Blank	〃	〃						1.0	0.5	0.60	120.0	——
		Main	〃	〃			D. R. negative			2.0	1.0	——	——	100.0
		Blank	(VII)	〃						1.0	0.5	0.48	96.0	——

possible to calculate its decompsition percentage. The blank test shows percentage of recovery of aneurin added originally, this value was compared with that obtained by the main test. These results are shown in detail in Table 3.

Conclusion

The experimental results show that the free amino group in the pyrimidine ring of aneurin molecule is essential for the decomposition of aneurin by aneurinase, but other groups in the pyrimidine or the thiazole nuclei have little influence on this hydrolytic decomposition.

The auther wishes to express his sincere thanks to Dr. Matsukawa of Takeda Institute, who kindly furnished him with the aneurin analogues used in these experiments. Thanks are also due to Prof. Akabori at Osaka University for his painstaking revision of the paper and to Dr. Chachin, Director of this research institute, for his support in executing this work.

The Osaka Municipal Institute for Domestic Science, Osaka