OCCURRENCE OF AN INHIBITOR OF ATP-CREATINE-TRANSPHOSPHORYLASE IN THE PLANT, RYANIA SPECIOSA VAHL

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Some years ago Pepper and Carruth (1945) and Wheeler (1945) reported the effectiveness of crude preparations from the plant, Ryania speciosa Vahl, as insecticides. A toxic principle, ryanodine, was isolated from these sources by Rogers et al. (1948), and has been considered to be the insecticidal agent. Edwards et al. (1948) and Hassett (1948) reported production of a flaccid type of paralysis in cockroaches with ryanodine-containing extracts. Procita et al. (1952) and Procita (1956 and 1958) have shown an irreversible rigor-like contracture to be caused by ryanodine in isolated mammalian skeletal muscle and the intact mammal. In the course of studying the mechanism of action of these extracts a second, non-ryanodine principle has been found which is a potent inhibitor of muscle ATP-creatine-transphosphorylase. Ryanodine itself has no effect on this enzyme. The enzyme inhibitor effects observed with several types of extracts from the Ryania speciosa plant are described in this report.

Crystalline ATP-creatine-transphosphorylase was prepared from rabbit muscle by the method of Kuby et al. (1954a). Physico-chemical criteria confirmed the high degree of purity of this preparation. Activity in the forward direction, i.e., production of creatine phosphate, was assayed by the method of Noda et al. as presented by Colowick and Kaplan (1955).

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Assay in the reverse direction, based on kinetic studies by Kuby et al. (1954b), was carried out as follows: A 1:25,000 (w/v) solution of the enzyme in .001 M glycylglycine buffer, pH 7.0, was preincubated with the inhibitor. An aliquot of 2 ml of this system was added to 8 ml of a reaction mixture giving final assay concentrations for ADP, creatine phosphate, $MgSO_4$, and glycylglycine buffer, pH 7.0, of, respectively, .002 M, .004M, and a .12 M. Otherwise this procedure followed that for the forward direction.

Initial recognition of the enzyme inhibitor came with use of a 22% ryanodine extract**. This was further extracted with ether, the extracted materials in turn being partitioned between ether and water. The water phase, on drying, yielded a product equivalent to approximately 80% ryanodine as measured by its absorption in the ultraviolet at 268.5 mμ. This material was a strong inhibitor of the enzyme, this inhibition being ascribed at the time to ryanodine. Later acquisition and testing of 100% ryanodine** demonstrated that the inhibition was due to an impurity, however, and not to ryanodine itself.

Varying concentrations of the inhibitor, expressed on a weight/volume dilution basis, were incubated with the enzyme for 15 minutes at 37°C and then were assayed by standard procedures. The inhibition obtained with 1:200,000, 1:20,000, and 1:2,000 preparations was, respectively, 24.2%, 50.8% and 72.9%. With incubation at room temperature, 50% inhibition was obtained with a 1:2,000 dilution.

Using the 1:2,000 inhibitor preparation, it was possible to show increasing inhibition with increasing time of incubation. With 2 minutes incubation, 34.7% inhibition was obtained; with 5 minutes, 47.5% with 15 minutes, 70.5%.

Kuby et al. (1954b) have demonstrated the magnesium requirements of this enzyme. Although, under the conditions these workers chose for assay of activity in the forward direction, the final magnesium concentration is .006 M,

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** Kindly supplied by S. B. Penick Co., New York.

the optimal concentration is .001 M and is equimolar with the ATP used. Magnesium complexes of ATP and ADP were observed to be the active substrates. Nucleotides not complexed with magnesium were inactive while excess magnesium reduced the activity slightly. In our experiments the effects of the enzyme inhibitor were noted at both magnesium levels. In inhibitor concentrations lower than 1:40,000, no difference in degree of inhibition with .001 M and .006 M magnesium was apparent. At 1:20,000 inhibitor dilution, 91.8% inhibition was obtained with .001 M Mg as compared with 47.2% with .006 M Mg.

The inhibition of the enzyme in the forward direction obtainable with other Ryania plant extracts is noted in Table I. The inhibitor was found

TABLE I

EFFECT OF VARIOUS RYANIA EXTRACTS ON THE ACTIVITY OF ATP-CREATINE-TRANSPHOSPHORYLASE

Extract	Inhibitor Concentration	% Inhibition
1. Water extract of ground dried plant***	1:25,000 (w/v) 1:10,000 (w/v)	12 80
2. Isopropyl acetate extract of dried plant**	1:50,000 (w/v) 1:20,000 (w/v)	41 80
3. Ryanodine 40%***	1:10,000 (w/v)	80
4. Mother liquor of Ryanodine crystallization**	1:10,000 (w/v) 1: 1,000 (w/v)	34 51
5. Ryanodine 100%**	1: 2,000 (w/v)	0

*** 40% ryanodine and dried ground plant (Ryanex 100%) were kindly supplied by Merck Sharpe & Dohme

in all the extraction conditions employed and was present in greatest quantity in the isopropyl acetate extract.

The isopropyl acetate extract was used to test agents which might prevent or reverse the enzyme inhibition. The potential role of sulfhydryl

groups in the activity of ATP-creatine-transphosphorylase has been noted in studies with iodoacetate by Padieu and Mommaerts (1960). Accordingly the effects of several compounds known to protect sulfhydryl enzymes were observed.

These results are presented in Table II. The inhibitor was incubated with the enzyme for the standard fifteen minute period. The test compounds were added either two minutes before or two minutes after the start of the incubation.

TABLE II

EFFECT OF POTENTIAL PROTECTIVE COMPOUNDS ON THE INHIBITION OF ATP-CREATINE-TRANSPHOSPHORYLASE

Test Compound	% Inhibition with Test Compound Added	
	Before inhibitor	After inhibitor
Glutathione 5×10^{-3}M	0	0
Mercaptoethylamine 5×10^{-3}M	5	20
Cysteine 5×10^{-3}M	10	10
5×10^{-4}M	57	80
Thiourea 5×10^{-3}M	90	90
Control (Inhibitor alone)	90	

Compounds having free SH groups prevent the enzyme inhibition, otherwise (thiourea) there is no effect. The data obtained with the test compound added after the inhibitor make it uncertain whether reversal has occurred. In the absence of inhibitor, the enzyme was slightly activated by glutathione, slightly inhibited by thiourea, and unaffected by the other two sulfur compounds. The mechanism of this protection is of considerable interest and the possible direct interaction of the phosphorylated substrates with enzyme sulfhydryl groups is under further study.

Limited observations with enzyme activity in the reverse direction indicate that this reaction might be less subject to inhibition than the forward reaction. Inhibitor concentrations (water extract of ether extract of 22% ryanodine) which caused 25% inhibition in the forward reaction produced no significant effect in the reverse direction. 40% ryanodine causing 80% forward inhibition was 57% inhibitory in the other direction. Pure ryanodine again had no effect in the reverse reaction. The underlying mechanism of these differences is not yet apparent.

Inhibitor isolation studies in progress have not as yet provided a product entirely free of ryanodine and inert plant material. Detailed studies of the effects on the kinetics of ATP-creatine-transphosphorylase will await the purification of the inhibitor.

REFERENCES

Edwards, G. A., Weiant, E. A. Slocombe, A. G. and Roeder, K. D., Science 108, 330 (1948).

Hassett, C. C., Science 108, 138 (1948).

Kuby, S. A., Noda, L., and Lardy, H. A., (a), J. Biol. Chem. 209, 191 (1954).

Kuby, S. A., Noda, L., and Lardy, H. A., (b), J. Biol. Chem. 210, 65 (1954).

Noda, L., Kuby, S. A., and Lardy, H. A., in "Methods in Enzymology" Vol. 11, 605, Colowick, S. P. and Kaplan, N. O., Eds., Academic Press Inc., New York (1955).

Padieu, P. and Mommaerts, W. F. H. M., Biochem. et Bioph. Acta 37, 72 (1960).

Pepper, B. B. and Carruth, L. A., J. Econ. Entomol. 38, 59 (1945).

Procita, L., Shideman, F. E., and Rathbun, R. C., J. Pharmacol. and Exptl. Therap. 106, 411 (1952).

Procita, L., J. Pharmacol. and Exptl. Therap. 117, 363 (1956).

Procita, L., J. Pharmacol. and Exptl. Therap. 123, 296 (1958).

Wheeler, E. H., J. Econ. Entomol. 38, 281 (1945).