pressure, and 1 ml. of ammonium hydroxide (sp. gr. 0.90) was added to the residue. After 15 minutes at room temperature, the solution was diluted with 2 ml. of water and subjected to continuous extraction with chloroform for 2.5 hours. Evaporation of the chloroform under reduced pressure gave 17 mg. of an oil which was evaporatively distilled at *ca.* 140° (1 mm.). The distillate crystallized from acetone–ether giving 10 mg. of material melting at 125–127°. Recrystallization from the same solvents raised the melting point to 151–152°.

Anal. Calcd. for $C_9H_{14}N_2O_3$: C, 54.54; H, 7.12; N, 14.14. Found: C, 54.24; H, 7.01; N, 14.29.

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Specificity and Mode of Action of Rattlesnake Venom Adenosinetriphosphatase[1]

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Zeller[2] observed that several snake venoms liberate 1 molecule of inorganic orthophosphate when incubated with ATP.[3] He suggested two possible pathways for the reaction: (1) the terminal phosphate is split off, giving orthophosphate and ADP; (2) inorganic pyrophosphate is split off, leaving AMP, which is then hydrolyzed to adenosine and orthophosphate by the specific 5'-phosphomonoesterase present in snake venoms. Zeller concluded that the conditions of the reaction favored one or the other of the two pathways.[4] In 1953 Johnson, *et al.*,[5] showed that when ATP was incubated with cobra venom the second pathway prevailed. Because we wanted to use whole as well as fractionated rattlesnake venom (*Crotalus ademanteus*) for the determination of the structure of nucleotides and related compounds, it was important to ascertain its mode of action on the nucleoside polyphosphates.

In order to detect the formation of pyrophosphate, the substrates were incubated with whole snake venom in the presence or absence of purified yeast inorganic pyrophosphatase (which by itself has no action on ATP) and the amount of inorganic orthophosphate produced was determined. Table I shows that the venom by itself liberated 2 μmoles of phosphorus per μmole of ADP and 1 μmole of phosphorus per μmole of ATP. In the presence of both venom and pyrophosphatase nearly the theoretical amount of 3 μmoles of phosphorus per μmole of ATP was released. It is of interest that the same results were obtained if UTP was substituted for ATP in these experiments, and that GDP was degraded in the same manner as ADP. The specificity of the enzyme, therefore, seems to be directed only toward the pyrophosphate moiety of the substrates.

TABLE I

LIBERATION OF INORGANIC ORTHOPHOSPHATE FROM NUCLEOSIDE DI- AND TRIPHOSPHATES BY SNAKE VENOM IN THE PRESENCE OR ABSENCE OF YEAST INORGANIC PYROPHOSPHATASE (SEE TEXT FOR EXPERIMENTAL CONDITIONS)

Subtrate 4 μmoles	Enzyme	P, μmole Present	P, μmole Released
ADP	Venom	8	7.75
GDP	Venom	8	7.7
ATP	Venom	12	4.8
ATP	Venom and pyrophosphatase	12	11.0
UTP	Venom	12	4.5
UTP	Venom and pyrophosphatase	12	11.8
ATP	Pyrophosphatase	12	0.1

The production of inorganic pyrophosphate during the incubation of ATP with the venom was further confirmed by the separation of the reaction products on a Dowex-1 (chloride) column. The eluted fractions were analyzed for inorganic ortho- and pyrophosphate and their optical density at 260 mμ determined. Figure 1 shows that the ultraviolet absorbing material (adenosine) appeared in the first tubes together with the inorganic phosphate, whereas the inorganic pyrophosphate was eluted later (tubes 9–15).

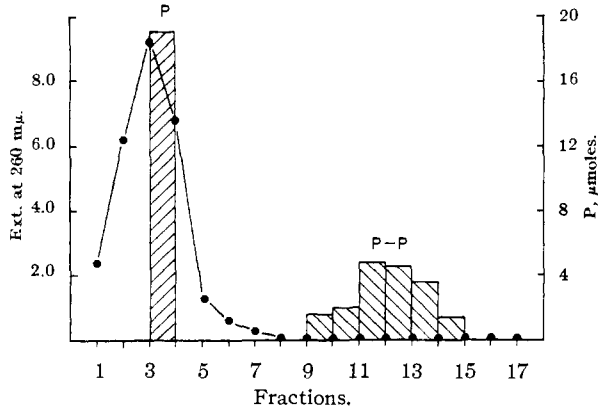

Fig. 1.—Separation of inorganic ortho- and pyrophosphate on a Dowex-1 (chloride) column after incubation of ATP with snake venom. The shaded areas represent the inorganic ortho- and pyrophosphate, respectively; the solid circles the optical density of the fractions at 260 mμ. See text for experimental conditions.

These results show that the ATP-ase of rattlesnake venom specifically splits the pyrophosphate linkage between the phosphate group attached to the ribose and the next phosphate. In this manner AMP and inorganic orthophosphate are produced from ADP; and AMP and inorganic pyrophosphate from ATP. The specific 5'-monoesterase of the venom then splits off the phosphate from the AMP that had been produced.

Experimental

Incubation of Nucleoside Polyphosphates with Snake Venom.—The incubation mixture was made up of 4 μmoles of substrate in 2 ml. of water and 1 ml. of 0.5 *M* glycine buffer *p*H 8.0 (containing Mg^{++} at a concentration of *M*/

(1) Supported by grants from the Damon Runyon Memorial Fund for Cancer Research, Inc., and the National Cancer Institute, National Institutes of Health, United States Public Health Service.

(2) E. A. Zeller, *Experientia*, **4**, 194 (1948); *Arch. Biochem.*, **28**, 138 (1950).

(3) The following abbreviations are used: AMP = adenosine-5-phosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; GDP = guanosine diphosphate; UTP = uridine triphosphate. These materials were obtained from Pabst Laboratories, Milwaukee, Wisconsin.

(4) E. A. Zeller, "The Enzymes," Vol. 1, part 2, Academic Press, Inc., New York, N. Y., 1951, p. 1010.

(5) M. Johnson, M. A. G. Kaye, R. Hems and H. A. Krebs, *Biochem. J.*, **54**, 625 (1953).

1600) to which 3 mg. of lyophilized snake venom[6]) in 1 ml. of 0.2% NaCl was added. The tubes were incubated for 2 hours at 38° at which time the addition of venom was repeated and the incubation continued for 2 more hours. 0.1 ml. of purified yeast pyrophosphatase[7] was added twice, together with the venom, to those tubes which receive it. The reaction was stopped by the addition of 0.5 ml. of 10% trichloroacetic acid, the denatured protein spun off and the inorganic orthophosphate determined on the supernatant.[8]

Separation of Reaction Products on a Dowex-1 (Chloride) Column.—Twenty μmoles of ATP was incubated with 30 mg. of lyophilized snake venom as described above. At the end of the incubation the mixture was adsorbed on a Dowex-1 (chloride) column (6 cm. × 0.7 cm.²). The column was washed with a little water followed by 0.01 *N* HCl containing 0.05 *N* NaCl[9] to elute the products of the incubation. About 20 fractions of 10 ml. each were collected and their optical density at 260 mμ determined. Inorganic orthophosphate and pyrophosphate were determined on aliquots of the fractions, yeast pyrophosphatase being used for the pyrophosphate assay.

Acknowledgment.—We wish to thank Dr. J. M. Buchanan for his continued interest in this work.

(6) Obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

(7) L. A. Heppel and R. J. Hilmoe, *J. Biol. Chem.*, **192**, 87 (1951).

(8) C. H. Fiske and Y. SubbaRow, *ibid.*, **66**, 375 (1925).

(9) A. Kornberg and W. E. Pricer, Jr., *ibid.*, **191**, 535 (1951).

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Identifying Isomeric Substituted 5-Aminotetrazoles by Means of Infrared Spectroscopy

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1-Methyl-5-(2,6-xylyl)-aminotetrazole was reported[1] to be the product obtained from the reaction of 2,6-dimethylacetophenone with hydrazoic acid under the conditions of the Schmidt reaction[2] and from the cyclization of 1-(2,6-xylyl)-2-methyl-3-azidoguanidine in aqueous base. This latter result seemed unusual since earlier work[3] had demonstrated conclusively that the cyclizations of less highly substituted 1-aryl-2-alkyl-3-azidoguanidines yielded the isomeric 1-aryl-5-alkylaminotetrazoles. However, the 1-alkyl-5-arylaminotetrazoles are thermodynamically more stable at the temperatures normally employed during the cyclization,[4] and the possibility existed, therefore, that the cyclization of 1-(2,6-xylyl)-2-methyl-3-azidoguanidine actually yielded the more stable isomer. In order to investigate this possibility, and to confirm or refute the structure assignment of Schwartzman and Corson, the synthesis of the methyl-(2,6-xylyl)-5-aminotetrazole was repeated and the product studied.

The problem of structure assignment cannot be resolved simply by chemical test since neither isomer has definitive chemical properties. Furthermore, the synthetic route,[3] which was employed to establish unequivocally the structure of the 1-phenyl-5-alkylaminotetrazoles and which involved the catalytic debenzylation of the corresponding 1-phenyl-5-benzylalkylaminotetrazoles, would probably fail in this case because of steric hindrance in one of the intermediate steps, namely, the hydrazinolysis of the 1-(2,6-xylyl)-3,S-dimethyl-3-benzylisothiourea. For example, steric hindrance markedly retards the rate of hydrazinolysis of 1-(2,6-xylyl)-S-methylisothiourea[5] and 1-(2,6-xylyl)-3,S-dimethylisothiourea, and almost completely prevents the hydrazinolysis of 1-(2,6-xylyl)-S-methyl-3-benzylisothiourea. Another test, which was developed previously and used to differentiate between 1-phenyl-5-alkylaminotetrazoles and 1-alkyl-5-phenylaminotetrazoles, was based on the almost quantitative, thermal rearrangement of the former isomers into the latter under non-equilibrium conditions. Although the methyl-(2,6-xylyl)-5-aminotetrazole formed in the cyclization of the substituted azidoguanidine does isomerize upon heating to 180°, the result was not considered to be sufficient evidence in favor of the 1-(2,6-xylyl)-5-methylaminotetrazole structure for the following reason: Since 1-methyl-5-benzylaminotetrazole will isomerize to an equilibrium mixture and since the 2,6-xylyl group is roughly comparable to the benzyl group in electronegativity,[6] the probability of the reverse isomerization occurring (1-methyl-5-(2,6-xylyl)-aminotetrazole to 1-(2,6-xylyl)-5-methylaminotetrazole) cannot be overlooked.

Lacking an unambiguous synthesis for one of the isomers and a chemical means of identification, a proof of structure based on infrared spectra was developed. Since the aryl ring is directly conjugated with the tetrazole ring in 1-aryl-5-alkylaminotetrazoles and is not so conjugated in 1-alkyl-5-arylaminotetrazoles, the infrared spectra of a pair of these isomers should show differences related to the presence or absence of this conjugation. An examination of the spectra of 1-ethyl-5-phenylaminotetrazole and of 1-phenyl-5-ethylaminotetrazole,[3] shows distinct differences in the regions attributed to phenyl[7] and tetrazole absorptions.[8] A comparison of these two spectra with the spectra of 1-methyl-5-phenylaminotetrazole, 1-phenyl-5-methylaminotetrazole, 1-cyclohexyl-5-phenylaminotetrazole and 1-phenyl-5-cyclohexylaminotetrazole reveals that the spectra of the 1-alkyl-5-phenylaminotetrazoles have characteristic patterns in the phenyl and tetrazole absorption regions and that these patterns are different from those of the isomeric 1-phenyl-5-alkylaminotetrazoles. All of the spectral patterns for the latter isomers are similar in the phenyl and tetrazole absorption regions. Finally, the spectra of two isomeric methyl-(2-tolyl)-5-aminotetrazoles

(1) L. H. Schwartzman and B. B. Corson, THIS JOURNAL, **76**, 781 (1954).

(2) One other example of this anomalous reaction has been described by K. F. Schmidt [U. S. Patent 1,599,493 (1926); *C. A.*, **20**, 3460 (1926)] and discussed in greater detail by P. A. S. Smith, THIS JOURNAL, **76**, 436 (1954).

(3) W. G. Finnegan, R. A. Henry and E. Lieber, *J. Org. Chem.*, **18**, 779 (1953).

(4) R. A. Henry, W. G. Finnegan and Eugene Lieber, THIS JOURNAL, **77**, 2264 (1955).

(5) R. A. Henry, W. G. Finnegan and Eugene Lieber, *ibid.*, **76**, 88 (1954).

(6) This statement is based on the fact that the equilibrium constants for the isomerization of 1-(2,6-xylyl)-5-aminotetrazole and of 1-benzyl-5-aminotetrazole are of the same order of magnitude (ref. 4).

(7) H. M. Randall, R. G. Fowler, Nelson Fuson and J. R. Dangl, "Infrared Determination of Organic Structures," D. Van Nostrand Co., Inc., New York, N. Y., 1949, p. 221.

(8) Eugene Lieber, D. R. Levering and Lorna J. Patterson, *Anal. Chem.*, **23**, 1594 (1951).