42. *The Structure of Adenosine Di- and Tri-phosphates.*

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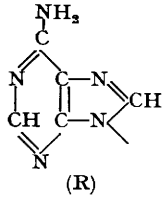
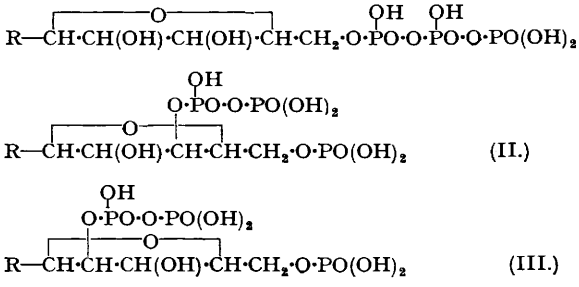
The existing experimental evidence for the constitutions of adenosine di- and tri-phosphates leaves some doubt as to the exact disposition of their acid-labile phosphoryl groups. The action of cold aqueous alkali and of the phosphatases of Russell's viper venom on these compounds has been investigated and the results are discussed in relation to the possible structures. Lohmann's conclusions are confirmed.

The structure of adenosine triphosphate, first isolated in 1929 (Lohmann, *Naturwiss.*, **17**, 624; Fiske and Subbarow, *Science*, **70**, 381), has long been a matter of dispute, and the point of attachment of the labile pyrophosphoryl group to the adenosine-5-phosphate is not yet known with certainty. The existence of inosine triphosphate as a deamination product of adenosine triphosphate (Kleinzeller, *Biochem. J.*, 1942, **36**, 729) proves conclusively that the amino-nitrogen of the adenine is not involved, and therefore the structure proposed by Barrenscheen and Filz (*Biochem. Z.*, 1932, **256**, 115) need not be considered. The pyrophosphoryl residue must therefore be united with adenosine-5-phosphate either through the stable 5-phosphoryl group as in (I) (Lohmann, *Biochem. Z.*, 1932, **254**, 381; 1935, **282**, 120) or through one of the hydroxyl groups at C_2 or C_3 of the ribose as in (II) (Satoh, *J. Biochem. Japan*, 1935, **21**, 19) or (III).



(R)



(I.)

(II.)

(III.)

Lohmann's formula is based on the results of electrometric titrations of adenosine di- and tri-phosphoric acids before and after acid hydrolysis, and on the behaviour of the compound towards hydrolytic reagents. Alkaline hydrolysis with baryta yielded adenosine-5-phosphate and inorganic pyrophosphoric acid, and N-acid at 100° " easily hydrolysed " two of the phosphoric acid residues at a rate closely similar to that of hydrolysis of inorganic pyrophosphate under similar conditions. In this communication the terms " easily hydrolysable " (acid labile) and " difficultly hydrolysable " (acid stable) phosphorus are used in accordance with Lohmann's definition.

The ability of adenosine triphosphate to form a soluble copper complex with dilute copper sulphate and lime water and to give a positive Böeseken reaction with boric acid (Lohmann, *loc. cit.*; Makino, *Biochem. Z.*,

1935, **278**, 161; Barrenscheen and Jachimowicz, *ibid.*, 1937, **292**, 350), thus resembling adenosine-5-phosphate and differing from adenosine-3-phosphate (Klimek and Parnas, *ibid.*, 1932, **252**, 392; *Z. physiol. Chem.*, 1933, **217**, 75), offers strong support for Lohmann's formula in which the adjacent hydroxyl groups at C_2 and C_3 are unesterified. Lohmann points out, however, that these reactions are not fully understood and that the results are not clearly predictable in the case of a compound containing in its molecule a congestion of hydroxyl groups and an amino-group.

The structure (II) was inferred by Satoh from the results of enzyme experiments. He observed that non-specific phosphatases, having no pyrophosphatase activity under the conditions of experiment, split off one-third of the total phosphorus from adenosine triphosphate as inorganic phosphoric acid and left the organic pyrophosphate as inosine pyrophosphate, deamination having occurred simultaneously with dephosphorylation. Pyrophosphatases, on the other hand, removed half the pyrophosphate phosphorus as inorganic orthophosphate, *i.e.*, one-third of the total phosphorus, yielding an adenosine diphosphoric acid, which was isolated as the calcium salt. This compound was dephosphorylated by non-specific phosphomonoesterase but was not attacked by pyrophosphatase. In agreement with Satoh, Barrenscheen and others (Barrenscheen and Lang, *Biochem. Z.*, 1932, **253**, 406; Barrenscheen and Jachimowicz, *ibid.*, 1937, **292**, 350) observed that bone phosphatase mineralised one-third of the total phosphorus of adenosine triphosphate, the inorganic phosphate originating almost entirely from the difficultly hydrolysable part, but Liebknecht (*ibid.*, 1939, **303**, 96) disagreed with Barrenscheen and claimed that bone phosphatase liberated the labile phosphorus first.

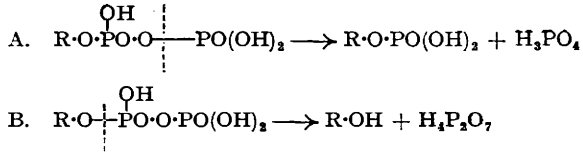
We have investigated the action of cold alkali on adenosine di- and tri-phosphates under the same conditions as those that effect the cleavage of yeast ribonucleic acid to its component nucleotides. Excess of N-sodium hydroxide acting on adenosine triphosphate at 20° for 24 hours liberated inorganic pyrophosphate but no inorganic orthophosphate. In the case of adenosine diphosphate under the same conditions, the alkali liberated half the total phosphorus as inorganic orthophosphate. These results suggested that the labile pyrophosphoryl group of adenosine triphosphate and the labile phosphoryl group of the diphosphate might be linked to muscle adenylic acid by an alkali-unstable ester link resembling the alkali-labile internucleotide linkage of yeast ribonucleic acid (Gulland and Walsh, following paper); in that case, adenosine triphosphate could have a structure (III) analogous with that of Satoh but differing from the triphosphoric acid structure of Lohmann. In order to test this hypothesis and in the hope of confirming or disproving one or other of the above formulæ, we have investigated the action of the 5-nucleotidase of Russell's viper venom (Gulland and Jackson, *Biochem. J.*, 1938, **32**, 590) on adenosine di- and tri-phosphates before and after cold alkaline hydrolysis. This enzyme has a high affinity for adenosine-5-phosphate, which is rapidly dephosphorylated, but does not attack other organic phosphates in the normal conditions of enzymic experiments.

The venom dephosphorylated adenosine triphosphate at a rapid rate, comparable with that of its action on adenosine-5-phosphate under similar conditions, but the rate diminished and action ceased when about 20% of the total phosphorus had been liberated as inorganic phosphate. Determinations of the easily hydrolysable phosphorus by heating with N-hydrochloric acid at 100° for 7 minutes (Lohmann, *loc. cit.*; Ferdmann, *Z. physiol. Chem.*, 1933, **216**, 205) indicated that most of the liberated phosphate originated from the difficultly hydrolysable part. When, however, the venom acted on adenosine triphosphate which had previously been subjected to cold alkaline hydrolysis, less phosphate was liberated, although the conditions of experiment were strictly comparable. From this, it was inferred that inorganic pyrophosphate, set free during the enzyme action or by the pretreatment with cold alkali, inhibited the 5-nucleotidase. Experiments with muscle adenylic acid and the venom in the presence of inorganic pyrophosphate confirmed this conclusion, and when the venom concentration was sufficiently high relative to the amount of inhibiting pyrophosphate present, adenosine triphosphate was rapidly attacked and the whole of the difficultly hydrolysable phosphorus liberated as inorganic phosphate.

In the hope of avoiding the complicating factor of pyrophosphate inhibition of the enzyme, the experiments were repeated with adenosine diphosphate as substrate. The venom dephosphorylated adenosine diphosphate rapidly but not completely; the difficultly hydrolysable and the easily hydrolysable phosphorus were mineralised at closely similar rates, and the amount of difficultly hydrolysable phosphorus set free as phosphate appeared to be slightly in excess of the easily hydrolysable phosphorus. During the experiment, samples were withdrawn for the phosphate determination of alkali-labile phosphorus and acid-labile phosphorus. The results of these measurements indicated that some inorganic pyrophosphate was produced during the experiment, and since acid hydrolysis converts inorganic pyrophosphate entirely into orthophosphate whereas only half the phosphorus of the adenosine-diphosphate is so converted, there is, during the experiment, an increase in easily hydrolysable phosphorus. On the other hand, cold alkaline hydrolysis liberates as phosphate half the phosphorus of the adenosine diphosphate but does not appreciably attack inorganic pyrophosphate in the same conditions. It follows that the observed difference between the amounts of acid-labile and alkali-labile phosphorus is equivalent to the amount of inorganic pyrophosphate, and therefore to twice the increase in easily hydrolysable phosphorus, produced by the enzyme. The necessary correction can thus be made when calculating the relative proportions of easily- to difficultly-hydrolysable phosphate liberated by the venom. Having regard to the limits of accuracy of the experiment, this ratio was very nearly 1 : 1.

When the venom was incubated with adenosine diphosphate which had been previously subjected to cold alkaline hydrolysis, the remaining organic phosphate was rapidly and completely mineralised by the 5-nucleotidase.

These results can only be explained on the basis of Lohmann's formula for adenosine diphosphate (corresponding to I). Adenosine diphosphate suffers hydrolysis by the enzyme in two ways, represented by A and B:



(where R is the adenosine radical)

The main reaction (A) involves splitting of the pyrophosphate link with liberation of inorganic orthophosphate and muscle adenylic acid, which is at once hydrolysed by the 5-nucleotidase to adenosine and inorganic phosphate. The subsidiary reaction (B) produces inorganic pyrophosphate but no phosphate.

It is therefore concluded that the two phosphoryl groups in adenosine diphosphate are united as pyrophosphate, and since both labile phosphoryl groups of adenosine triphosphate are also united as pyrophosphate, Lohmann's formula (I) is correct. Whilst this work was in progress, further evidence that adenosine triphosphate is a derivative of triphosphoric acid has been furnished by Needham and his collaborators (*J. Gen. Physiol.*, **1944, 27, 355**) from their studies on myosin.

The support for the correctness of Lohmann's formula (I) for adenosine triphosphate provided in this communication has a bearing on the structure of codehydrogenase II, which was originally suggested (v. Euler and Schlenk, *Z. physiol. Chem.*, **1937, 246**, 64) as being a derivative of adenosine triphosphate. That view cannot, however, be reconciled with the conversion of codehydrogenase I into codehydrogenase II by phosphorylation in ether (Schlenk, *Naturwiss.*, **1937, 25**, 668) or with the facts that codehydrogenase II contains no readily hydrolysable phosphate and yields no alkaline degradation product active as cophosphorylase (Schlenk, Högberg, and Tingstam, *Arkiv Kemi, Min., Geol.*, 1939, 13A, No. 11). Support is thus lent to Schlenk's formulation (Symposium on Respiratory Enzymes, 1942, 104, University of Wisconsin Press) of codehydrogenase II as a derivative of adenosine diphosphate with an additional phosphoryl monoester group, and not as a derivative of adenosine triphosphate.

Experimental.

The adenosine triphosphate used was a sample of the barium salt, prepared from horse muscle. It had a N : P ratio of 5 : 3 and gave a positive Böeseken reaction, and 68% of the total phosphorus was hydrolysed to inorganic orthophosphate by N-hydrochloric acid at 100° in 7 minutes. It was further purified by washing with ice-cold water, acidified to pH 4 with dilute acetic acid, in order to remove any traces of adenylic acid and adenosine diphosphate.

Adenosine diphosphate was prepared from the triphosphate either by the action of rabbit muscle myosin (Greenstein and Edsall, *J. Biol. Chem.*, **1940, 133**, 397) as described by Bailey (*Biochem. J.*, **1942, 36**, 121), or by the action of yeast hexokinase (Meyerhof, *Biochem. Z.*, 1927, **183**, 186) in the presence of glucose according to the method of Colowick and Kalckar (*J. Biol. Chem.*, 1943, **148**, 121).

Inorganic orthophosphate was determined by Briggs's modification (*J. Biol. Chem.*, 1922, **53**, 13) of the Bell–Doisy colorimetric method.

Action of Cold Alkali on Adenosine Diphosphate and Triphosphate.—Neutral solutions of the sodium salts of adenosine di- and tri-phosphate were prepared as follows: Barium adenosine di- or tri-phosphate (*ca.* 20 mg.) was dissolved in a few c.c. of ice-cold water, acidified with a few drops of dilute hydrochloric acid, and sodium sulphate (*ca.* 10 mg.) added. The solution, freed from barium sulphate by centrifuging, was neutralised to phenolphthalein by the addition of dilute alkali, and the volume adjusted to 20 c.c. with ice-cold water. Samples (1·0 c.c.) were taken for the determinations of total phosphorus and easily hydrolysable phosphorus, *i.e.*, phosphorus liberated as inorganic phosphate by N-hydrochloric acid at 100° in 7 minutes. For the determination of alkali-labile phosphorus, samples (1·0 c.c.) were mixed with water (2·0 c.c.) and N- or 4N-sodium hydroxide (1·0 c.c.) and left for 24 hours at 20°.

	Adenosine triphosphate.			Adenosine diphosphate.		
	Phosphorus, mg. per c.c.					
Expt. No.	1	2	3	4	5	6
Total	0·112	0·073	0·096	0·082	0·132	0·132
Inorganic phosphate after 7 mins. N-HCl at 100°	0·075	0·049	0·065	0·042	0·067	0·067
Inorganic phosphate after 24 hrs. 1% NaOH at 20°	0	0	0	0·023	0·031	0·034
Inorganic phosphate after 24 hrs. N-NaOH at 20°	0	0	0	0·040	0·064	0·066

Action of Cold Alkali on Inorganic Pyrophosphate.—A freshly prepared solution of potassium pyrophosphate (1·10 g.) in cold water (500 c.c.) had a total phosphorus concentration of 0·414 mg. per c.c., of which 0·0027 mg. was present as orthophosphate. Samples (1·0 c.c.) were mixed with water (2·0 c.c.) and N- or 4N-sodium hydroxide (1·0 c.c.) and kept at 20° in a closed tube for 24 hours. After neutralisation, the orthophosphate present was determined.

P as orthophosphate.	% total P.
Original solution	0·65
After hydrolysis with 1% NaOH	1·69
After hydrolysis with N-NaOH	2·66

The total phosphorus concentration used was higher than in the other experiments, otherwise the amount of orthophosphate would have been too small to permit accurate determination.

Action of Russell's Viper Venom on Adenosine Triphosphate.—The results of two experiments, A and B, are recorded. A mixture (15 c.c.) of sodium adenosine triphosphate [from 20 mg. (A) and 30 mg. (B) of barium salt], Michaelis' veronal buffer (5 c.c.), Russell's viper venom [5 mg. (A) and 15 mg. (B)], and water was incubated in the presence of toluene at 37° and pH 8·6. The total phosphorus concentrations were 0·096 (A) and 0·143 (B) mg. per c.c. Samples (1·0 c.c.)

were withdrawn at intervals for the determination of inorganic phosphate, and simultaneously, samples (1·0 c.c.) were withdrawn, heated in a closed tube with 2N-hydrochloric acid (1·0 c.c.) for 7 minutes in the boiling water-bath, and the inorganic phosphate determined.

	Inorganic phosphate as % of total phosphorus.									
Time in minutes.	0.		15.		30.		60.		120.	
Experiment.	A.	B.	A.	B.	A.	B.	A.	B.	A.	B.
I. Phosphate liberated by enzyme action ...	0	0	19·5	28	20·4	32	22·0	35	22·6	35·5
II. Phosphate liberated by enzyme and acid hydrolysis	68	68	85·5	94	86·1	98	86·7	100	86·7	100
III. Acid-stable phosphate liberated by enzyme (II—68)	0	0	17·5	26	18·1	30	18·7	32	18·7	32
IV. Easily hydrolysable phosphate liberated by enzyme (I—III)	0	0	2·0	2	2·3	2	3·3	2	3·9	3·5

An experiment was carried out under conditions of phosphorus, buffer and enzyme concentrations identical with Expt. A, but using as substrate adenosine triphosphate which had been previously subjected to the action of N-sodium hydroxide at 20° for 24 hours. After 30 minutes at 37° and pH 8·6, only 13·9% of the total phosphorus was present as inorganic orthophosphate as compared with 20·4% in experiment A.

Effect of Inorganic Pyrophosphate on 5-Nucleotidase of Russell's Viper Venom.—A mixture (10 c.c.) of muscle adenylic acid (5 mg.), veronal buffer (5 c.c.), venom (3 mg.), and water was incubated in the presence of toluene at 37° and pH 8·6. The total phosphorus concentration was 0·045 mg. per c.c. Parallel experiments containing in addition potassium pyrophosphate in 0·01, 0·001 and 0·0001 M concentration were simultaneously carried out. Samples (2·0 c.c.) were withdrawn at intervals for the determination of inorganic orthophosphate.

	Dephosphorylation, %.			
Time, mins.	0.	0·01.	0·001.	0·0001M-P_2O_7''''.
0	0	0	0	0
15	93	2	52	85
30	95	4	80	95
60	98	7	92	98

The above quantities are corrected for the amount of orthophosphate produced by a weak pyrophosphatase activity of the venom demonstrable when potassium pyrophosphate and venom are included under identical conditions but without muscle adenylic acid as substrate.

Action of Russell's Viper Venom on Adenosine Diphosphate.—A mixture (15 c.c.) of sodium adenosine diphosphate (from 25 mg. of barium salt), veronal buffer (5 c.c.), venom (5 mg.), and water was incubated with toluene at 37° and pH 8·6. The total phosphorus concentration was 0·085 mg. per c.c. Samples (1·0 c.c.) were withdrawn at intervals for the determination of inorganic phosphate. Simultaneously, samples (1·0 c.c.) were withdrawn, heated in a closed tube with 2N-hydrochloric acid (1·0 c.c.) in a boiling water-bath for 7 minutes, and the inorganic phosphate determined. Samples (1·0 c.c.) were also mixed with 4N-sodium hydroxide (1·0 c.c.) and water (2·0 c.c.) containing a few drops of 0·5M-potassium cyanide to arrest enzyme action (Gulland and Walsh, *loc. cit.*), and the mixtures were kept at 20° for 24 hours, neutralised, and the inorganic phosphate determined.

	Inorganic phosphate as % of total phosphorus.							
	A.	B.	C.	D.	E.	F.	G.	H.
Time, mins.	Phosphate liberated by enzyme action.	After 7 mins. N-HCl at 100°.	After 24 hrs. N-NaOH at 20°.	Apparent stable P set free (B—51).	Apparent labile P set free (A—D).	P present as P_2O_7'''' (B—C).	Stable P set free by venom (corrected).	Labile P set free by venom (corrected).
0	0	51	—	0	0	—	0	0
15	27·6	64	60·6	13	14·6	3·4	11·3	16·3
30	41·4	73·1	69·9	22·1	19·3	3·2	20·5	20·9
60	53·7	80·8	74·3	29·8	23·9	6·5	26·6	27·1

The corrected values for stable phosphorus liberated as phosphate by the enzymes (G) are calculated by subtracting half the inorganic pyrophosphate phosphorus (F) from the apparent acid-stable phosphorus liberated (D). The corrected values for acid-labile phosphorus liberated as phosphate by the enzymes (H) are obtained by difference from A and G.

Action of Russell's Viper Venom on the Products of Cold Alkaline Hydrolysis of Adenosine Diphosphate.—A solution (2·0 c.c.) of sodium adenosine diphosphate, of total phosphorus concentration 0·14 mg. per c.c., was mixed with 4N-sodium hydroxide (0·6 c.c.), and the mixture kept at 20° for 24 hours. The excess of alkali was neutralised to pH 8·6 with dilute sulphuric acid, veronal buffer (2 c.c.) and venom (2 mg.) added, and the mixture, adjusted to 5 c.c., incubated with toluene at 37° and pH 8·6 for 30 minutes. Samples (2·0 c.c.) were withdrawn for the determination of inorganic orthophosphate. The inorganic orthophosphate, expressed as percentage of total phosphorus, was 49·2% before enzyme action began and 98·7% after 30 minutes.

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