176. *The Constitution of the Purine Nucleosides. Part IV. Adenosine and Related Nucleotides and Co-enzymes.*

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In previous communications (*Nature*, 1933, **132**, 782; Part II, J., 1934, 1639) comparisons of the ultra-violet absorption spectra of aqueous solutions of xanthosine and monomethylated derivatives of xanthine at different p_H values provided evidence that in this nucleoside, in guanosine, and in the related nucleotides the pentose groups are attached to position 9 of the nitrogenous nucleus.

The method has now been extended to the determination of the constitutions of adenosine, obtained from yeast nucleic acid, and of inosine prepared from it by deamination with nitrous acid. The details of the constitutions of these nucleosides are known, except for the decision between positions 7 and 9 as the point of attachment of the ribose radical to the adenine and the hypoxanthine molecule.

It is clear from Figs. 1 and 2 and Table I that the spectra of adenosine very closely resemble those of 9-methyladenine (I) but not those of 7-methyladenine (II). Similarly, the spectra of inosine (from adenosine) resemble more closely those of 9-methylhypoxanthine than those of 7-methylhypoxanthine (Figs. 5 and 6).

The evidence presented above indicates that the ribose radicals of adenosine (III, R = H), inosine, and by inference adenosine-3-phosphoric acid of yeast nucleic acid, are attached to the 9-position of adenine and not to the 7-position, as is commonly represented. There is thus an interesting parallel between the nucleosides and flavine, in which the ribityl residue is attached to the corresponding nitrogen atom (v. Euler, Karrer, Malmberg, Schöpp, Benz, Becker, and Frei, *Helv. Chim. Acta*, 1935, **18**, 522; Kuhn, Reinemund, Weygand, and Ströbele, *Ber.*, 1935, **68**, 1765).

N═══C·NH_2
CH C───N>CH
N───C───N
Me
(I.)

N═══C·NH_2 Me
CH C───N<>CH
N───C───N
(II.)

N═══C·NH_2
CH C───N>CH
N───C───N
CH·[CH·OH]$_2$·CH·CH_2·OR
└───O───┘
(III.)

Muscle Adenylic Acid and Co-enzymes.—The formulation of muscle adenylic acid as an adenine-riboside-5-phosphoric acid is fully established by the following series of changes: its deamination to inosinic acid, which yields hypoxanthine and ribose-5-phosphoric acid on hydrolysis by acid (Embden and Schmidt, *Z. physiol. Chem.*, 1929, **181**, 130; Levene and Jacobs, *Ber.*, 1911, **44**, 746; Levene and Stiller, *J. Biol. Chem.*, 1934, **104**, 299); the neutral hydrolysis of the barium salt of inosinic acid to barium phosphate and inosine (Levene and Jacobs, *Ber.*, 1909, **42**, 336); the preparation of inosine by deamination of adenosine (Levene and Jacobs, *Ber.*, 1910, **43**, 3161). These facts alone might also indicate that muscle adenylic acid is a derivative of yeast adenosine. This is indeed the case, as will be shown

below, and muscle adenylic acid has now been smoothly hydrolysed to adenosine by means of bone phosphatase; fission by chemical means is impracticable, since the phosphoric ester linkage is more stable to acid hydrolysis than is that of the riboside. Nevertheless the recognition of muscle adenylic acid as a 9-ribose derivative of adenine is based on the identification of the compounds in the reactions mentioned above, and this line of argument

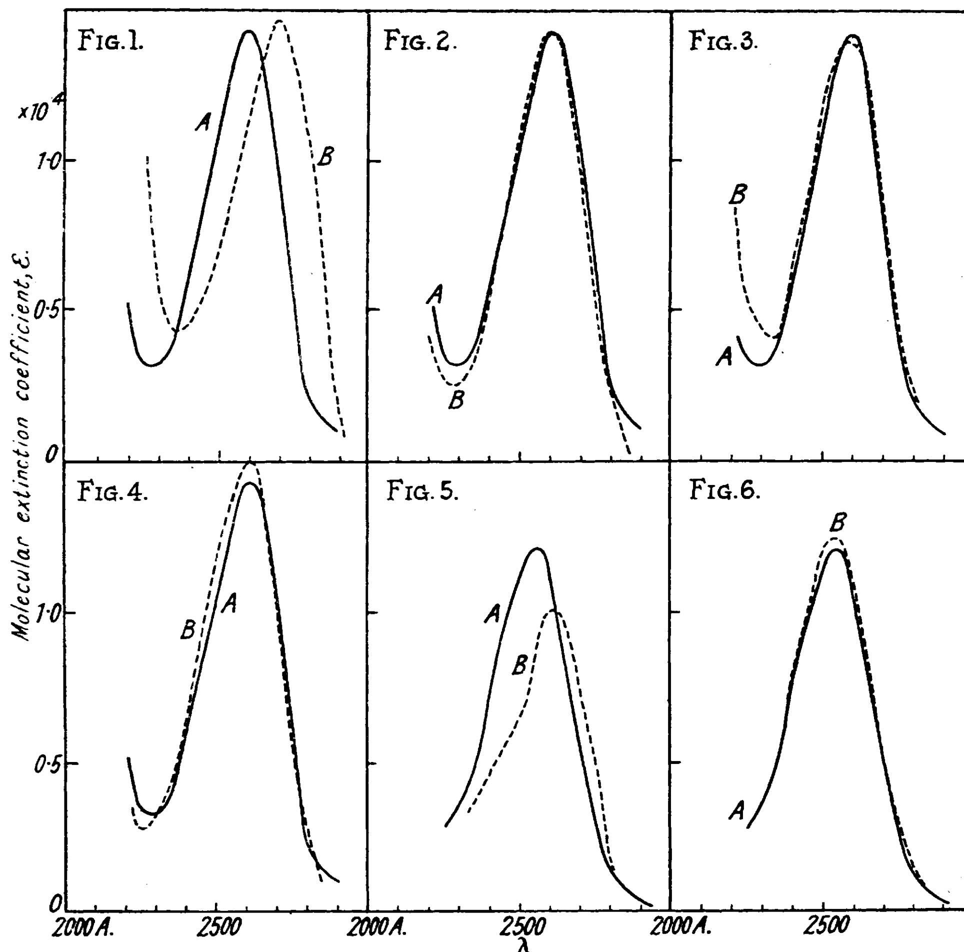

FIG. 1.—*A*. *9-Methyladenine in water, N/20-NaOH, and N/20-HCl. B. 7-Methyladenine in water and N/20-HCl.*
FIG. 2.—*A*. *9-Methyladenine. B. Adenosine in water, N/20-NaOH, and N/20-HCl.*
FIG. 3.—*A*. *9-Methyladenine. B. Muscle adenylic acid in water at* p_H *4·5, in N/20-NaOH, and N/20-HCl.*
FIG. 4.—*A*. *9-Methyladenine. B. Barium adenyl pyrophosphate in water at* p_H *6·5, in N/20-NaOH, and N/20-HCl.*
FIG. 5.—*A*. *9-Methylhypoxanthine in N/20-NaOH. B. 7-Methylhypoxanthine in N/20-NaOH.*
FIG. 6.—*A*. *9-Methylhypoxanthine in N/20-NaOH. B. Inosine in N/20-NaOH.*

is insufficient in the present connexion, since the properties of the isomeric 7 (or 9)-derivative in each case are unknown, and since the transposition of the glycosidic linkage from position 7 to 9 (or vice versa) would not in general be expected to cause marked changes in those properties, such as melting points and optical rotations, on which the identifications rest.

Myrbäck, v. Euler, and Hellström (*Z. physiol. Chem.*, 1932, **212**, 12) observed that the ultra-violet absorption spectra of aqueous solutions of adenosine and muscle adenylic acid

have peaks at 2600 A. The p_H values of these solutions must have been different (see below) and it is clear from our results that p_H values may considerably affect the absorption spectra. We have therefore confirmed and extended their results by showing that the spectra of adenylic acid are identical with those of 9-methyladenine (Fig. 3 and Table I). Muscle adenylic acid thus resembles adenosine and 9-methyladenine, and is unlike 7-methyladenine. It is therefore adenine-9-riboside-5-phosphoric acid (III, R = PO_3H_2). This conclusion links these results with the constitutions of various co-enzymes.

The system adenosine triphosphate (adenyl pyrophosphate) $\longleftrightarrow$ [adenosine diphosphate] $\longleftrightarrow$ adenosine-5-phosphate is recognised as the co-enzyme system of lactic acid formation in voluntary and heart muscles (Meyerhof and Kiessling, *Biochem. Z.*, 1935, **283**, 83; Lohmann and Schuster, *ibid.*, 1935, **282**, 104). Adenyl pyrophosphate is readily hydrolysed to adenylic acid (Lohmann, *ibid.*, 1931, **233**, 460), and it and adenosine diphosphate are therefore 9-substituted adenines from the results described above. Through the kindness of Dr. Lohmann in presenting us with a sample of barium adenyl pyrophosphate, we have been able to confirm this conclusion by showing that the ultra-violet absorption spectra of this substance closely resemble the spectra of 9-methyladenine (Fig. 4) (compare Myrbäck, v. Euler, and Hellström, *Z. physiol. Chem.*, 1932, **212**, 12). Lohmann (*Biochem. Z.*, 1935, **282**, 120) has already tentatively adopted this formulation on the basis of our earlier communications, although experimental evidence has until now been lacking.

Cozymase, the co-enzyme of alcoholic fermentation, is a derivative of adenosine-5-phosphate (Myrbäck, *Ergeb. Enzymforsch.*, 1933, **2**, 139; *Z. physiol. Chem.*, 1934, **225**, 199; v. Euler, Albers, and Schlenk, *ibid.*, 1935, **237**, 180, I). Myrbäck, v. Euler, and Hellström (*ibid.*, 1932, **212**, 12) showed that the ultra-violet absorption spectrum of cozymase has its maximum at 2580—2600 A., and these observations indicate that the pentose group is attached to position 9 of the adenine molecule.

The co-enzyme of the lactic acid dehydrogenase of heart muscle (Banga and Szent-György, *ibid.*, 1933, **217**, 39) is an adenine nucleotide, closely related to or identical with cozymase.

Warburg, Christian, and Griese (*Biochem. Z.*, 1935, **282**, 1) showed that the hydrogen-transporting co-enzyme of Warburg's oxidation system is composed of 1 mol. of adenine, 1 mol. of nicotinamide, 3 mols. of phosphoric acid, and 2 mols. of pentose. The assumption that the molecule contains an adenine-pentoside-phosphate group is almost unavoidable. The ultra-violet absorption spectrum has a marked peak at 2600 A., composed of the combined absorptions of the adenine and the nicotinamide residues. Reduction eliminates the absorption due to the pyridine ring, and leaves the peak at 2600 A., now composed solely of the absorption due to the adenine residue. These facts taken in conjunction with our results suggest that the co-enzyme is a derivative of adenine-9-pentoside.

Cocarboxylase (Auhagen, *Biochem. Z.*, 1933, **258**, 330) may also be related to adenylic acid.

EXPERIMENTAL.

Preparation of Materials.—7-Methyladenine was prepared from 2 : 6-dichloro-8-hydroxypurine by methylation (Fischer and Ach, *Ber.*, 1897, **30**, 2208), amination of the resulting 2 : 6-dichloro-8-hydroxy-7-methylpurine, replacement of the 8-hydroxyl by chlorine, and reduction of the chlorine atoms (Fischer, *Ber.*, 1898, **31**, 104).

9-Methyladenine was prepared from 9-methyldichloroadenine (Fischer, *Ber.*, 1897, **30**, 2226).

7-Methylhypoxanthine was obtained by deamination of 7-methyladenine (Fischer, *Ber.*, 1898, **31**, 104), but was more conveniently prepared from theobromine (Fischer, *Ber.*, 1897, **30**, 2400).

9-Methylhypoxanthine was prepared by deamination of 9-methyladenine (Fischer, *Ber.*, 1898, **31**, 104).

Adenosine was prepared from the hydrolysis products of yeast nucleic acid by processes which differed but slightly from those of Levene (" Nucleic Acids," 1931, Chemical Catalog Co., New York). After being dried at 110° over phosphoric oxide, it melted at 230° (Found : C, 45·2; H, 5·0; N, 26·3; amino-N, 5·1. Calc. for $C_{10}H_{13}O_4N_5$: C, 45·0; H, 4·9; N, 26·2; amino-N, 5·0%).

Inosine was prepared by deamination of adenosine with barium nitrite and acetic acid

(Levene and Tipson, *J. Biol. Chem.*, 1935, **111**, 313). In this preparation, deamination proceeds at room temperature for 3 days, to be followed by crystallisation of the inosine when the solution is cooled. It is our experience that barium nitrate, formed by aerial oxidation of the nitrous acid, separates in characteristic prisms during the deamination period and that this is not avoided by using boiled solutions and filling the loosely-corked vessel with nitrogen at the beginning of the reaction. The aqueous solution must be decanted from the barium nitrate before the inosine is allowed to separate. Mention is made of these facts, because we were at first led astray by obtaining with the barium nitrate precipitate the characteristic purple-red colour of the phloroglucinol–hydrochloric acid test for pentoses (Tollens). The colour is formed by the nitrate ion, however, and the coloured material does not give the characteristic absorption band given by pentoses in this test, although it is extracted by amyl alcohol. This is also the behaviour of galactose. In the orcinol–hydrochloric acid test (Bial), nitrates give a brown coloration, ultimately red-brown, which might be confused with that given by fructose, and thus differ from pentoses, which give a greenish-blue colour.

Inosine was obtained in needles, m. p. 218° after being dried at 110° in a vacuum over phosphoric oxide (Found : C, 44·3; H, 4·5; N, 20·7; amino-N, 0·1. Calc. for $C_{10}H_{12}O_5N_4$: C, 44·8; H, 4·5; N, 20·9; amino-N, 0%). The estimation of amino-nitrogen was carried out for 1 hour at room temperature in a van Slyke micro-apparatus; Wilson (*J. Biol. Chem.*, 1923, **56**, 183) states that inosine shows a trace of amino-nitrogen under these conditions.

The carbon and hydrogen estimations were carried out by Dr. Ing. A. Schoeller, and we are indebted to Dr. T. F. Macrae for the estimations of nitrogen by the micro-Kjeldahl method. In analysing purine compounds by this method, it is necessary to oxidise for 2½ hours to convert all the nitrogen into ammonia.

Spectrographic Data.—Measurements were made with a Hilger medium quartz spectrograph and Spekker photometer. The light source was a condensed spark between tungsten-steel electrodes.

Samples were anhydrous, having been dried in a vacuum at 110° over phosphoric oxide, but the barium adenylpyrophosphate was the hexahydrate. Solutions were made to a strength of *M*/5000 and were examined immediately in a layer thickness of 1 cm. against controls.

TABLE I.

	λ, A.		$\epsilon \times 10^4$.	
	N/20-HCl.	*N*/20-NaOH.	*N*/20-HCl.	*N*/20-NaOH.
Adenine	2600	2580	1·32	1·36
7-Methyladenine	2690	2690	1·46	1·14
9-Methyladenine	2600	2600	1·42	1·47
Adenosine	2600	2600	1·42	1·43
Hypoxanthine	2480	2620	0·96	1·00
7-Methylhypoxanthine	2500	2610	0·98	1·01
9-Methylhypoxanthine	2480	2550	1·03	1·21
Inosine	2470	2540	1·32	1·34
Muscle adenylic acid	2600	2600	1·29	1·40
Adenyl pyrophosphate	2600	2600	1·49	1·38

Conversion of Muscle Adenylic Acid into Adenosine by Bone Phosphatase.—A solution of muscle adenylic acid (485 mg., equivalent theoretically to 43·3 mg. of phosphorus) in water (25 c.c.) and a little 2*N*-sodium hydroxide was mixed with bone phosphatase (100 mg; A/W 0·23) in water (10 c.c.). The mixture was adjusted to p_H 8·6 with sodium hydroxide, diluted to 50 c.c., and incubated for 20 hours at 37° after the addition of a little chloroform.

Time, hrs.	0	20
Total P, mg.	42·6	43·2
Free P, mg.	0·2	44·0

The solution was brought to p_H 4·4 with acetic acid, heated at 100° for 4 minutes, and centrifuged. Phosphate ions were removed by addition of a slight excess of baryta and filtration, and the clear colourless solution was freed exactly from barium by means of sulphuric acid, rhodizonic acid being used as indicator. The resulting solution was concentrated under reduced pressure and cooled. The fine colourless needles which separated in good yield were recrystallised from water, and after being dried at 110° in a vacuum over phosphoric oxide melted at 230° alone or when mixed with authentic adenosine, m. p. 230°, prepared from yeast nucleic acid and having the same crystalline appearance. $[\alpha]_D^{20°}$ in 5% sodium hydroxide solution was − 68·2° (*c* 4·12); Levene (*J. Biol. Chem.*, 1920, **41**, 492) gives − 68·5°.

The picrate of the material under examination, prepared in aqueous solution, formed yellow leaflets, sintering at 195° and melting at 198°. The picrate of authentic adenosine (*ex* yeast nucleic acid) crystallised in the same form and also sintered at 195° and melted at 198°. Levene and Jacobs (*Ber.*, **1909**, **42**, 2703) state that the picrate sinters at 180° and is molten at 185° (corr.).

The palladochloride of adenosine (Gulland and Macrae, J., **1932**, 2231) is not characteristic, consisting of minute yellow granules coalescing in clusters. The palladochlorides of the two substances under consideration were identical in appearance.

This comparison of physical properties indicates that the adenosine prepared by dephosphorylation of muscle adenylic acid is identical with that prepared from yeast nucleic acid.

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