144. *Studies on Phosphorylation. Part XXIII.* Oxidative Phosphorylation leading to Adenosine-5′ Pyrophosphate.*

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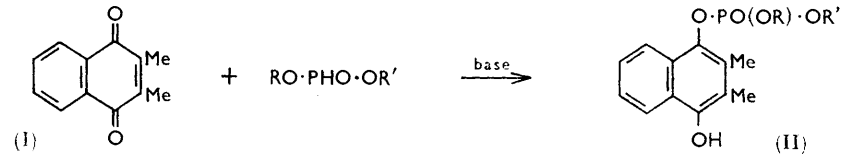
Oxidation of the monophosphate of a naphthaquinol in presence of a salt of adenosine-5′ phosphate or of a naphthaquinol ester of adenosine-5′ phosphate in presence of inorganic phosphate yields adenosine-5′ pyrophosphate (ADP).

The important rôle played by the coupling of substrate oxidation to the synthesis of adenosine-5′ triphosphate (ATP) in the functioning of animal cells is well known.[1] The demonstration *in vitro* of energetic coupling between the oxidation and reduction of carriers in the respiratory chain and ATP synthesis requires particulate preparations containing either mitochondria or multifunctional mitochondrial fragments. The detailed mechanism of this energetic coupling in the respiratory chain is essentially unknown and its elucidation constitutes a major problem of contemporary biochemistry. Recent evidence [2] suggests that benzoquinone derivatives [3] of the coenzyme Q type or naphthaquinone derivatives [4] related to vitamin K play an important part in phosphate transfer during oxidation–reduction processes.

In the preceding paper we reported the oxidation of a variety of phosphorylated quinols by a range of oxidising agents. In many of these reactions inorganic pyrophosphate was formed, and it was suggested that the effective acylating agent was related to the monomeric metaphosphate which, though as yet unknown, is assumed to be produced. Nucleophilic attack on the " metaphosphate " by orthophosphate can then produce the pyrophosphate.[5] Monoesters of quinol phosphoric acids should yield esters of " metaphosphate " which, in the presence of inorganic phosphate, should give rise to esters of pyrophosphoric acid.

Using reactions of this type, we have synthesised adenosine-5′ pyrophosphate by two routes, in one of which phosphate was transferred to adenosine-5′ phosphate as substrate, and in the other a naphthaquinol ester of adenosine-5′ phosphate was oxidised to transfer the nucleotide residue to added inorganic phosphate.

The esters of quinol monophosphates are now readily available by interaction of a quinone and a diester of phosphorous acid.[6,7] Reaction of 2,3-dimethyl-1,4-naphthaquinone with the nucleoside phosphite [8] (I; R = CH_2Ph, R′ = 2′,3′-*O*-isopropylideneadenosine-5′)



led, after anionic debenzylation by sodium thiocyanate,[9] to the naphthaquinol ester of the nucleotide (II; R = H, R′ = 2′,3′-O-isopropylideneadenosine-5′), and this was oxidised

* Part XXII, preceding paper.

[1] Lehninger, " Harvey Lectures," 1955, Series 39, p. 176.
[2] Green, D. E., " Quinones in Electron Transport," Ciba Foundation Symposium, Churchill, London, 1960, in the press.
[3] Wolf, Hoffman, Trenner, Arison, Shunk, Linn, McPherson, and Folkers, *J. Amer. Chem. Soc.*, 1958, **80**, 4752.
[4] Brodie and Ballantine, *J. Biol. Chem.*, 1960, **235**, 226.
[5] Cf. Vernon, *Chem. Soc. Spec. Publ.*, 1957, No. 8, p. 23.
[6] Ramirez and Dershowitz, *J. Org. Chem.*, 1957, **22**, 1282.
[7] Clark, Hutchinson, Kirby, and Todd, preceding paper.
[8] Corby, Kenner, and Todd, *J.*, 1952, 3669.
[9] Clark and Todd, *J.*, 1950, 2030; Morrison and Atherton, B.P. 675,779.

by bromine water to 2′,3′-*O*-isopropylideneadenosine-5′ phosphate and 2,3-dimethyl-1,4-naphthaquinone.

Bromine oxidation of 4-hydroxy-2,3-dimethyl-1-naphthyl phosphate (II; R = R′ = H) in dry *NN*-dimethylformamide in presence of the mono(tetrabutylammonium) salt of adenosine-5′ phosphate led to adenosine-5′ pyrophosphate, which was isolated as the barium salt and converted into the free acid. The quinone was extracted from the reaction mixture by addition of cyclohexene which also removed excess of bromine and the hydrogen bromide generated in the reaction. Omission of this step led to the breakdown of the adenosine pyrophosphate by the acid present. It was expected that adenosine triphosphate might also be formed in this reaction, but none was detected by paper chromatography or paper electrophoresis. However, the behaviour on ion-exchange chromatography did suggest that a small amount of higher polyphosphates was present.

In the second synthesis the 4-hydroxy-2,3-dimethyl-1-naphthyl ester of 2′,3′-*O*-isopropylideneadenosine-5′ phosphate was oxidised under the same conditions with bromine in the presence of added orthophosphate. Adenosine-5′ pyrophosphate was again produced and isolated from the reaction mixture as the free acid.

Experimental

Tetrabutylammonium Adenosine-5′ Phosphate.—Silver nitrate (1·05 g., 0·06 mole) in water (15 ml.) was added to a solution of adenosine-5′ phosphate (1·14 g. of dihydrate, 0·03 mole) in warm water (50 ml.), and the silver salt was then precipitated by addition of an equal volume of ethanol. The salt (1·25 g., 0·027 mole) was removed by centrifugation, washed with ethanol and ether, air-dried, and shaken for 18 hr. with tetrabutylammonium iodide (1·0 g., 0·027 mole) in 50% aqueous methanol (50 ml.). The precipitated silver iodide was filtered off and the solvent removed *in vacuo*. The *product* (1·2 g., 75%), recrystallised from ethanol–ethyl acetate, formed needles, m. p. 187° (Found: C, 51·5; H, 8·3; N, 14·1. $C_{26}H_{49}N_6O_7P,H_2O$ requires C, 51·5; H, 8·4; N, 13·9%).

Oxidation of 4-Hydroxy-2,3-dimethyl-1-naphthyl Phosphate in Presence of Adenosine-5′ Phosphate.—4-Hydroxy-2,3-dimethyl-1-naphthyl phosphate [7] (2·5 g., 0·0095 mole) and mono(tetrabutylammonium) adenosine-5′ phosphate monohydrate (1·8 g., 0·003 mole) were dissolved in anhydrous *NN*-dimethylformamide [10] (15 ml.), and benzene (15 ml.) was added. The benzene was removed *in vacuo* with exclusion of moisture. A further 10 ml. of anhydrous benzene were then added and removed *in vacuo* as before.

Bromine (0·7 ml., 0·0125 mole) was introduced and the mixture left at room temperature for 1 hr., after which cyclohexene (20 ml., 0·2 mole) was added and the mixture left at room temperature for a further 15 min. Solvents were removed under reduced pressure below 40° and the residue stirred with water. 2,3-Dimethyl-1,4-naphthaquinone was filtered off and the filtrate extracted with ether (2 × 25 ml.). The aqueous layer was shaken *in vacuo* to remove dissolved ether and washed on to an Amberlite IR-120 column (H^+ form; 2 cm. × 30 cm.). The column was washed with water until the washings had a negligible optical density at 260 mμ and the eluate and washings were then freeze-dried. The residual gum was dissolved in freshly distilled ethanol (5 ml.) and transferred to a 250 ml. centrifuge bottle with further small portions of ethanol (total, 15 ml.). Dry ether (200 ml.) at 0° was added and the solid centrifuged off. This solid was redissolved in ethanol (15 ml.) and the precipitation procedure repeated three times. The solid obtained was dissolved in a small volume of water and adjusted to pH 6 with sodium hydroxide solution. The solution of sodium salts was washed on to a Dowex-1 column (Cl^- form; 1·5 × 25 cm.), and the subsequent elution followed by measuring light absorption at 260 mμ, adenosine mono- and pyro-phosphate being assumed to have ε 14,200 at this wavelength.[11] Elution with 0·005N-hydrochloric acid gave 165 ml. of solution with an average optical density 3·3; this was unchanged adenosine-5′ phosphate (0·038 mmole, 1·3%). Elution with 0·01N-hydrochloric acid containing 0·1M-sodium chloride gave two fractions: the first (165 ml.; average optical density 16·0) contained adenosine-5′ phosphate (0·186 mmole, 6·3%); the second (1500 ml.; average optical density 4·81) contained adenosine-5′ pyrophosphate (0·52 mmole, 17%). Further elution with 0·01N-hydrochloric acid containing 0·20 ml. of

[10] Thomas and Rochow, *J. Amer. Chem. Soc.*, 1957, **79**, 1843.
[11] Chargaff and Davidson, " Nucleic Acids," Academic Press, New York, 1955, Vol. I, p. 513.

sodium chloride (1600 ml.; average optical density 1·7) and 0·1M-hydrochloric acid containing 0·2M-sodium chloride (900 ml.; average optical density 1·2) gave adenosine-5′ pyrophosphate (0·27 mole, 9%). From ultraviolet-absorption measurements the total yield of adenosine-5′ pyrophosphate was 26%.

The fractions containing adenosine-5′ pyrophosphate were adjusted to pH 6—7 with N-sodium hydroxide and evaporated to dryness, the last traces of water being removed by freeze-drying. The solid residue was dissolved in the minimum amount of water (5 ml.), and 20% w/v barium acetate solution (1·5 ml.) was added. Precipitation of solid was completed by addition of ethanol (10 ml.). The precipitate was centrifuged off and washed with water (1 ml.), 50% aqueous ethanol (2 × 20 ml.), absolute ethanol (20 ml.), and ether (20 ml.).

The product was dried *in vacuo* over phosphorus pentoxide (yield, 250 mg.); further crops of barium adenosine pyrophosphate were obtained by evaporating later fractions (yield, 350 mg., slightly contaminated with chloride). The barium salt was examined by paper electrophoresis at pH 4 and by chromatography in butanol–acetic acid–water and trichloroacetic acid–propan-2-ol–water–ammonia.[7] In each system the compound migrated as a single ultraviolet-absorbing, phosphorus-containing spot indistinguishable from authentic adenosine-5′ pyrophosphate.

The barium adenosine pyrophosphate was converted into the free acid by exact neutralisation with 0·1N-sulphuric acid. Barium sulphate was centrifuged off and the supernatant liquor brought to pH 7·5 with sodium hydroxide. A small amount of precipitate was removed by centrifugation and the supernatant liquor was poured on an Amberlite IR-120 column (H^+ form; 1 × 8 cm.). The column was washed with water until the washings had negligible optical density at 260 mμ; the washings were freeze-dried, giving adenosine-5′ pyrophosphate (60 mg., 84% based on Ba salt) (Found, in material dried over P_2O_5/0·1 mm.: C, 28·4; H, 4·1; N, 16·0; P, 14·1. Calc. for $C_{10}H_{15}N_5O_{10}P_2$: C, 28·1; H, 3·5; N, 16·3; P, 14·5%).

Enzymic assay using phospho-enol pyruvate–pyruvate kinase and myokinase showed the sample to have 83% of the activity of an authentic sample supplied by the Sigma Chemical Co., St. Louis.

Benzyl 4-Hydroxy-2,3-dimethyl-1-naphthyl 2′,3′-O-Isopropylideneadenosine-5′ Phosphate.—To a solution of benzyl 2′,3′-*O*-isopropylideneadenosine-5′ phosphite [8] (9·43 g., 0·02 mole) and 2,3-dimethyl-1,4-naphthaquinone in dry, thiophen-free benzene (50 ml.), potassium t-butoxide in t-butyl alcohol (7 ml. of 1·3N-solution) was added. The mixture was warmed at 40° for 30 min., then left at room temperature for 5 hr. Solvents were then removed *in vacuo* and the residue was dissolved in chloroform (200 ml.) and washed with an equal volume of water, then twice with an equal volume of 0·001N-hydrochloric acid, and again with water. The chloroform layer was dried ($MgSO_4$) and concentrated to small bulk. Pentane was added; the oil which separated crystallised on trituration (9·6 g.). The product was not further purified but was used immediately for the next stage of the synthesis.

4-Hydroxy-2,3-dimethyl-1-naphthyl 2′,3′-O-isopropylideneadenosine-5′ Hydrogen Phosphate.—The product from the previous experiment (3·97 g., 1 mol.) and anhydrous sodium thiocyanate (1·0 g., 2 mol.) were heated in ethyl methyl ketone (25 ml.) for 2 hr. and then left at room temperature overnight. Solvent was removed *in vacuo*, the residual sodium salt dissolved in warm water (200 ml.), insoluble matter centrifuged off, and the cloudy supernatant liquid acidified. The precipitated *4-hydroxy-2,3-dimethyl-1-naphthyl 2′,3′-O-isopropylideneadenosine-5′ hydrogen phosphate* was centrifuged off and dried over phosphoric oxide; it (yield, 1·05 g. 30%) had m. p. 64—65° (Found: C, 50·1; H, 5·4. $C_{25}H_{28}N_5O_8P,2H_2O$ requires C, 50·4; H, 5·4%).

Oxidation of 4-Hydroxy-2,3-dimethyl-1-naphthyl 2′,3′-O-Isopropylideneadenosine-5′ Hydrogen Phosphate.—(a) *In aqueous solution.* The product from the previous reaction (100 mg.) was dissolved in 0·1N-aqueous lithium hydroxide (5 ml.), and saturated bromine water was added to give pH 5. The quinone produced was removed in chloroform (3 × 10 ml.), and the aqueous layer was evaporated to dryness *in vacuo*. The residual solid was triturated with 5 : 1 acetone–ethanol and washed with acetone and pentane, to leave *2′,3′-O-isopropylideneadenosine-5′ dilithium phosphate* (30 mg., 45%) (Found: C, 34·4; H, 4·8. $C_{13}H_{16}Li_2N_5O_7P,3H_2O$ requires C, 34·2; H, 4·8%). The product ran as a single spot (R_F 0·54) in butan-1-ol–acetic acid–water (5 : 2 : 3) on Whatman No. 1 paper.

(b) *In presence of di(tetrabutylammonium) hydrogen phosphate in* NN-*dimethylformamide.* 4-Hydroxy-2,3-dimethyl-1-naphthyl 2′,3′-*O*-isopropylideneadenosine-5′ phosphate (500 mg., 1 mol.) and di(tetrabutylammonium) hydrogen phosphate (600 mg., 1 mol.) were dissolved in

dry *NN*-dimethylformamide (10 ml.), and dry benzene (10 ml.) was added and removed *in vacuo* with exclusion of moisture. A further 10 ml. of dry benzene were added and again removed *in vacuo*. Bromine (0·16 ml., 3 mol.) was next added and the mixture left at room temperature for an hour; cyclohexene (10 ml.) was introduced with shaking and the mixture left a further 15 min. Solvents were removed at $<40°/0·1$ mm., the residue stirred with water (10 ml.), and the aqueous solution extracted with ether (3 × 10 ml.). A precipitate was removed from the aqueous layer (120 mg.). This was soluble in alkali, gave a quinone on oxidation, and had R_F identical with that of the starting material in butanol–acetic acid–water and trichloroacetic acid–water–propan-2-ol–ammonia;[7] this represented 24% of unchanged starting material.

The filtrate was shaken *in vacuo* to remove ether and was then transferred to an Amberlite IR-120 column (H^+ form; 1·5 × 15 cm.). The column was washed until the washings had a negligible optical density at 260 mμ, and the washings were freeze-dried. Electrophoresis of the residue at pH 4 (2 v/cm.) for 14 hr. showed three products: orthophosphate, and two ultraviolet-absorbing and phosphorus-containing materials with the same mobilities as adenosine-5′ phosphate and adenosine-5′ pyrophosphate. This freeze-dried residue was dissolved in dry, freshly distilled ethanol (10 ml.) and transferred to a 250 ml. centrifuge bottle with a further 5 ml. of ethanol. Dry ether (200 ml.) at 0° was added and the precipitate centrifuged off, redissolved in ethanol, and precipitated with ether a further three times. The final precipitate was dissolved in water (10 ml.) and brought to pH 6 with N-sodium hydroxide, then applied to a Dowex-1 column (Cl^- form; 2 × 15 cm.), and the subsequent elution was followed by ultraviolet absorption at 260 mμ. The column was washed with water (giving little ultraviolet absorbing material), then with 0·005N-hydrochloric acid. The major fraction (600 ml.; average optical density 1·7) was adenosine-5′ phosphate (10%). 0·02N-Hydrochloric acid containing 0·25M-sodium chloride gave an eluate (600 ml.; average optical density 3·75) containing adenosine-5′ pyrophosphate (yield, 22%). Finally, with 0·1N-hydrochloric acid containing 0·4M-sodium chloride, a fraction (1 l.) containing no ultraviolet-absorbing material was obtained. The fraction containing the adenosine-5′ pyrophosphate was evaporated to dryness and the solid washed with water (5 × 1 ml.). 20% w/v Barium acetate (1 ml.) was added and then ethanol (5 ml.). The precipitate was centrifuged off and washed with water (1 ml.), 50% ethanol (2 × 25 ml.), absolute ethanol (25 ml.), and finally ether (25 ml.). This barium salt was dried *in vacuo* over phosphoric oxide (yield, 85 mg.; 80% recovery of ultraviolet-absorbing material). With the metaperiodate–Schiff reagent spray[12] it gave a purple colour showing the presence of a *cis*-glycol. Electrophoretic and chromatographic examination indicated that there was only one ultraviolet-absorbing, phosphorus-containing compound. This was indistinguishable from authentic adenosine-5′ pyrophosphate.

The barium salt (85 mg.) was shaken with Amberlite IR-120 (H^+ form; 5 ml.) and water (5 ml.) until it dissolved. The solution and resin were then washed on to an Amberlite IR-120 column (H^+ form; 1 × 5 cm.), and the column washed until the eluate had negligible optical density at 260 mμ. The eluate and washings were freeze-dried to yield adenosine-5′ pyrophosphate (45 mg.) (Found, in material dried over silica gel at 12 mm.: C, 26·7; H, 4·3; N, 15·9; P, 14·3. Calc. for $C_{10}H_{15}N_5O_{10}P_2,H_2O$: C, 27·0; H, 3·8; N, 15·7; P, 14·3%).

Enzymic assay using phospho-enol pyruvate–pyruvate kinase and myokinase showed the sample to have 85% of the activity of an authentic sample supplied by the Sigma Chemical Co., St. Louis.

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Lensfield Road, Cambridge. [*Received, August 19th,* 1960.]

[12] Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.