Proof of the Imidoyl Phosphate Intermediate for Oxidative Phosphorylation in [^{18}O]Dimethylformamide Solution

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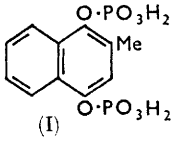
The bromine oxidation of 2-methylnaphthalene-1,4-diol diphosphate in anhydrous [^{18}O]-dimethylformamide resulted in orthophosphate enriched in oxygen-18. Appropriate controls showed no exchange. The observed incorporation indicated 26% formation of the imidoyl phosphate intermediate, $Me_2\overset{+}{N}{=}CH{\cdot}O{\cdot}PO_3H^-$, derived by solvation of monomeric metaphosphate from P–O bond fission.

The 2-methyl-1,4-naphthaquinone produced in this oxidation was also enriched in oxygen-18, corresponding to 5% formation of intermediate solvated ketal analogues from C–O cleavage.

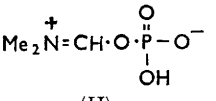
The [^{18}O]-dimethylformamide was prepared by direct exchange with enriched water.

ATTEMPTS have been made to elucidate the mechanism of oxidative phosphorylation in biological systems by comparison with chemical processes.[1-4] The present work reports the further application of isotopic tracer techniques as part of a study [5,6] of chemical oxidative phosphorylation.[2]

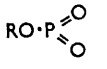
Monomeric metaphosphate (III) [7] and trialkyl trimetaphosphate (VI) [8] have been suggested as the reactive intermediates in a variety of phosphorylation reactions. There is now good evidence for direct participation in the phosphorylation by solvent pyridine, *i.e.*, for the formation of the 1-phosphorylpyridine entity (IV).[9,10]



(I)



(II)



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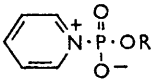
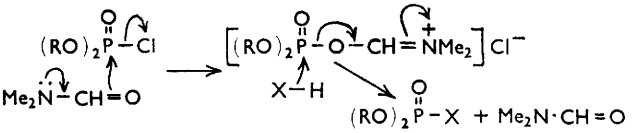


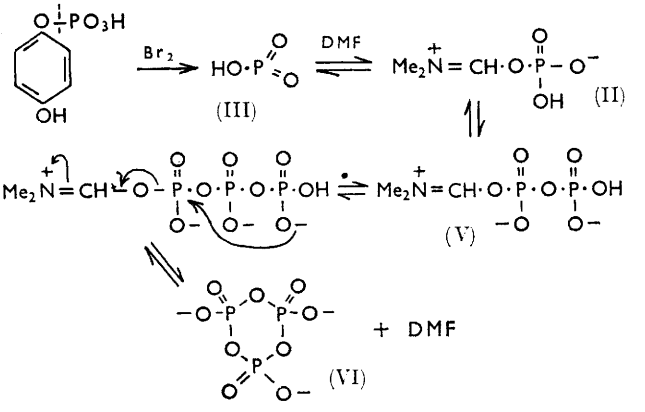
(IV)

Participation by dimethylformamide (DMF), another common solvent for phosphorylation reactions, has also been indicated. It was shown that dimethylformamide catalyses the reactions between dialkyl phosphorochloridate and nucleophiles.[11] Further, this effect was attributed to phosphorylation of the carbonyl oxygen (Scheme 1),[11,12] comparable to the mode of solvation by dimethylformamide suggested in the case of acyl chlorides.[13] Recently a crystalline product was isolated from the reaction between phosphorus trichloride and dimethylformamide,[14] and is an example of a class of addition compounds formed by dimethylformamide,[12,15,16] *viz.* $[Me_2N\cdots\overset{+}{C}H\cdots Y]X^-$.



Scheme 1

In two recent Publications,[3,5] dealing with the oxidation of quinol phosphate in anhydrous dimethylformamide, it was suggested that an imidoyl phosphate intermediate



Scheme 2

P–O bond fission in dimethylformamide (DMF)

(II) is formed by solvation of the hypothetical monomeric metaphosphate resulting from P–O bond fission. This could account, by the equilibrium processes illustrated in Scheme 2,[3] for the formation of condensed

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[1] V. M. Clark, D. W. Hutchinson, G. W. Kirby, and A. R. Todd, *J. Chem. Soc.*, 1961, 715; V. M. Clark, D. W. Hutchinson, and A. R. Todd, *ibid.*, p. 722.

[2] G. M. Blackburn and J. S. Cohen: Chapter in "Topics in Phosphorus Chemistry," ed. E. J. Griffith and M. Grayson, Interscience, New York, vol. 6, 1967.

[3] G. E. Tomasi and R. D. Dallam, *J. Biol. Chem.*, 1964, **239**, 1604.

[4] A. Lapidot and J. S. Cohen, *J. Biol. Chem.*, 1966, **241**, 4060.

[5] A. Lapidot and D. Samuel, *J. Amer. Chem. Soc.*, 1964, **86**, 1886.

[6] A. Lapidot and D. Samuel, *Biochim. Biophys. Acta*, 1962, **65**, 164.

[7] A. R. Todd, *Proc. Nat. Acad. Sci. U.S.A.*, 1959, **45**, 1389.

[8] H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," Wiley, New York, 1961.

[9] N. K. Hamer, *J. Chem. Soc.*, 1965, 46.

[10] W. E. Wehrli and J. G. Moffatt, *J. Amer. Chem. Soc.*, 1965, **87**, 3760, and others in the series.

[11] F. Cramer and M. Winter, *Chem. Ber.*, 1961, **94**, 989.

[12] R. Ratz and O. Sweeting, *J. Org. Chem.*, 1963, **28**, 1608.

[13] H. K. Hall, jun., *J. Amer. Chem. Soc.*, 1956, **78**, 2717.

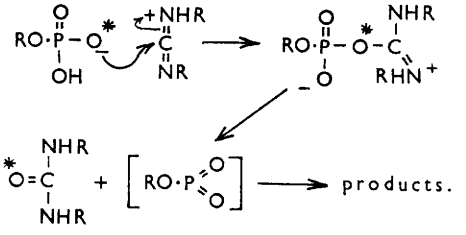
[14] T. D. Smith, *J. Chem. Soc.* (*A*), 1966, 841.

[15] A. Vilsmeier and A. Haak, *Ber.*, 1927, **60**, 119.

[16] H. Brederek, R. Gompper, K. Klemm, and H. Rempfer, *Chem. Ber.*, 1959, **92**, 837, and others quoted in ref. 14.

polyphosphates as the major products in such oxidations, as opposed to the aqueous reactions.[2,3,6]

Such an intermediate is analogous to those thought to be formed in a host of other phosphorylations (for a Review see ref. 17). In these cases the imidoyl phosphate is generated by the activation of a phosphate monoester. The oxygen atom linking the phosphorus atom and the imide group is derived from the phosphate starting material. This fact was used in the preparation of [^{18}O]urea as an analytical technique for oxygen-18 in orthophosphate,[18,19] as illustrated in Scheme 3 with dialkylcarbodi-imide.[8]



Scheme 3
(R = alkyl)

By contrast, on formation of (II), the bridge oxygen-atom linking the phosphorus to the imide group would be derived from the dimethylformamide molecule. Therefore, incorporation of oxygen from the solvent into the phosphate product would be expected to result. We have, in fact, found that the orthophosphate produced on the bromine oxidation of 2-methylnaphthalene-1,4-diol diphosphate in 17% [^{18}O]-dimethylformamide is labelled to the extent of 1·1 atom-% excess of oxygen-18. (See Table.) This is direct proof of the formation of the imidoyl phosphate intermediate (II) in this reaction.

TABLE

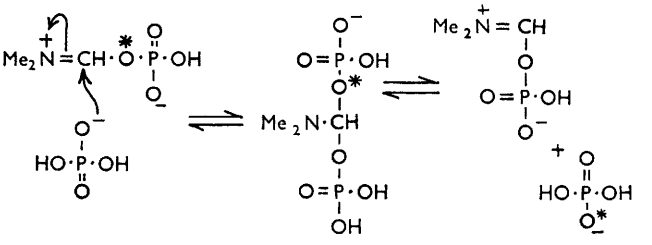
Atom-% excess of ^{18}O

Dimethylformamide	17·0
Inorganic phosphorus product	1·1
Total phosphate product	1·1
Methylnaphthaquinone product	0·9
2-Methylnaphthalene-1,4-diol diphosphate control	0·05
Orthophosphoric acid control	0·01

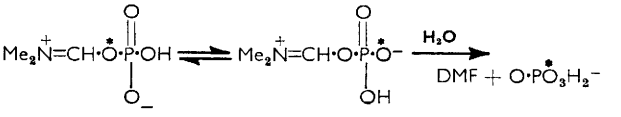
Negligible incorporation was found for controls of 2-methylnaphthalene-1,4-diol diphosphate and orthophosphoric acid after solution in 17% [^{18}O]-dimethylformamide.

Assuming that (II) is not a stable entity, intra- and inter-molecular scrambling processes can be envisaged to occur as indicated in Schemes 4 [3] and 5.

Addition of water to terminate the reaction could result in attack either at the carbon or the phosphorus atom of (II). Thus, it is clear that, on P–O bond formation by interaction with the solvent, labelled oxygen should be retained in the phosphate moiety. From the observed incorporation into inorganic phosphate and total phosphates the extent of formation of (II) can be estimated



Scheme 4



(II)

Scheme 5

as follows; assuming the [^{18}O]dimethylformamide to be present in excess solvent:

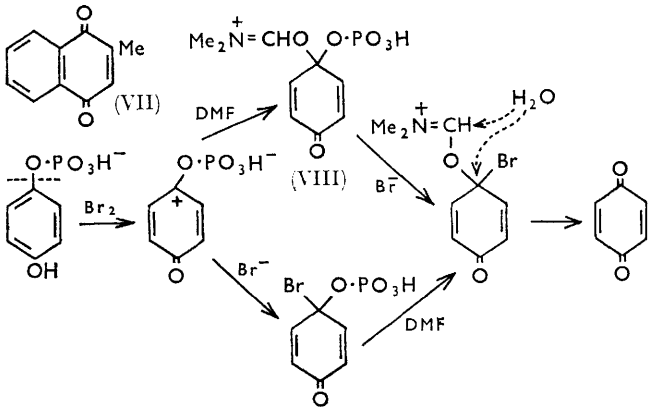
$$\frac{4 \times 1{\cdot}1}{17} \times 100 = 26\%$$

This value corresponds well with the previous estimate for P–O cleavage (*ca.* 35%) for oxidative phosphorylation in anhydrous dimethylformamide obtained by incorporation of added [^{18}O]-inorganic phosphate into condensed phosphate product.[5]

The methylnaphthaquinone (VII) produced on the bromine oxidation of 2-methylnaphthalene-1,4-diol diphosphate in 17% [^{18}O] dimethylformamide solution was enriched to the extent of 0·9 atom-% excess of ^{18}O, indicating the formation of adducts of the type (VIII). Such a formulation is feasible since it is known that dimethylformamide associates with, and stabilises oxonium ions.[20] Also the isolation of dimethyl ketal from ceric ion oxidation of quinol phosphate in methanol [21] indicates that moderately strong nucleophiles can readily attack the oxidised position. This, of course results only on oxidation giving C–O bond fission, which is almost exclusively found with Ce^{4+}. Compared to the extent of C–O cleavage expected (*ca.* 65%) on oxidation of quinol phosphate by bromine in dimethylformamide,[5] it appears that only a small proportion of the quinone resulting from C–O bond fission is labelled. This might be expected for the following reasons: (i) Only partial formation of the intermediates such as (VIII); (ii) the replacement of dimethylformamide on formation of the intermediate by the stronger nucleophile Br^- (a process inherently less likely to occur at phosphorus to give a phosphorobromidate); (iii) the lack of possible intramolecular rearrangements whereby

[17] V. M. Clark, D. W. Hutchinson, A. J. Kirby, and S. G. Warren, *Angew. Chem. Internat. Edn.*, 1964, **3**, 678.
[18] M. Halmann, *J. Chem. Soc.*, 1959, 305.
[19] A. Lapidot, S. Pinchas, and D. Samuel, *Proc. Chem. Soc.*, 1962, 109; *J. Chem. Soc.*, 1963, 1128.
[20] N. Kornblum and R. K. Blackwood, *J. Amer. Chem. Soc.*, 1956, **78**, 4037. See also A. J. Parker, *Quart. Rev.*, 1962, **16**, 163.
[21] W. Durckheimer and L. A. Cohen, *Biochemistry*, 1964, **3**, 1948.

the labelled oxygen, once attached to the ring, can be retained in the molecule as in the case of phosphate



Scheme 6

C–O bond fission in DMF

(Schemes 4 and 5). The percentage of C–O bond fission which definitely proceeds *via* a solvated intermediate is:

$$\frac{0{\cdot}9}{17} \times 100 = 5\%$$

For the reasons stated this may be considered a minimum value for the actual formation of such intermediates.

The enriched dimethylformamide was prepared by direct exchange with enriched water. The water was removed and the dimethylformamide carefully dried by two azeotropic distillations with benzene. Finally the benzene was removed by distillation. The product was checked at each stage for its water and benzene content by use of proton nuclear magnetic resonance (n.m.r.) spectroscopy. Previously no large-scale enrichment by direct exchange has been obtained with dialkyl-amido-derivatives.[22,23]

Orthophosphate was extracted from the reaction mixture, after addition of water, by using magnesia solution. The oxygen of the orthophosphate was converted into CO_2 by using the technique of Boyer *et al.*[24] Total phosphate was also converted into CO_2 by this method, giving the same percentage of ^{18}O as found for orthophosphate, with good consistency.

It was not possible to observe a separate signal for the suggested imidoyl phosphate intermediate on using proton n.m.r. spectroscopy.[14] [^{17}O]-Dimethylformamide was also prepared in view of the possibility of using ^{17}O-n.m.r. spectroscopy [25] as a tool for this purpose.

A reaction carried out with potassium hexachloroiridate, which is known to give only P–O bond fission on oxidation of quinol phosphate in water,[26] failed to give any result due to the insolubility of this material in the dioxan—dimethylformamide used as solvent.

[22] J. S. Cohen, E. Petreanu, and D. Samuel, unpublished results.
[23] B. D. Sykes, E. B. Robertson, H. B. Dunford, and D. Konasewich, *Biochemistry*, 1966, **5**, 697.
[24] P. D. Boyer, D. J. Graves, C. H. Suelter, and M. E. Dempsey, *Analyt. Chem.*, 1961, **33**, 1906.

EXPERIMENTAL

Enriched water was supplied by the Yeda Research and Development Co., Weizmann Institute, Rehovoth.

Isotopic Analysis.—Carbon compounds were analysed for ^{18}O by the method of Rittenberg and Ponticorvo,[27] water by the method of Dostrovsky and Klein,[28] and inorganic phosphate by the method of Boyer *et al.*[24] In all cases the carbon dioxide was analysed by means of a Consolidated Engineering Corp. model 21–401 spectrometer.

[^{18}]*O- and* [^{17}O]*-Dimethylformamide.*—Anhydrous dimethylformamide (DMF) (5 ml.) was heated at 100° with 97% $H_2{}^{18}O$ (10 ml.) and potassium carbonate in a sealed ampoule. After 7 days the solution was twice distilled azeotropically with benzene, and the residual dimethylformamide in benzene was distilled. Proton n.m.r. spectroscopy indicated that no water or benzene remained in the dimethylformamide distillate. Isotopic analysis showed that the amide contained 17 atom-% excess of ^{18}O. [^{17}O]-Dimethylformamide was prepared in the same way by using $H_2{}^{17}O$ (10%) and contained 0·5 atom-% ^{17}O. The ^{17}O chemical shift was found [25] to be −540 p.p.m.

Bromine Oxidation of 2-*Methylnaphthalene*-1,4-*diol Diphosphate in* [^{18}O]-*Dimethylformamide.*—2-Methylnaphthalene, 1,4-diol diphosphate (I) ("Synkavit"; supplied by the Hoffman La Roche Co., Grunzbach) was converted into the acid form by passing an aqueous solution through a Dowex-50 H^+ column (12 × 2 cm.). The eluate and washings were lyophilised and 0·735 g. ($2{\cdot}2 \times 10^{-3}$ mole) of the dried material was dissolved in 17% [^{18}O] dimethylformamide (1·8 ml.). Bromine (0·14 ml.; $2{\cdot}8 \times 10^{-3}$ mole) was added dropwise, with magnetic stirring. The mixture was kept at room temperature for 40 hr. in a stoppered flask. Water (10 ml.) was then added until no further yellow precipitate was produced. After being filtered, the aqueous solution was extracted with light petroleum (b. p. 30—40°) (4 × 5 ml.). Magnesia solution was added to the aqueous solution followed by dilute ammonia until a pH of 8 was reached. The solution was then filtered and stored at 0° for 12 hr. A white precipitate of magnesium ammonium phosphate was formed. Some of the filtered solid was washed with an excess of Dowex-50 H^+ resin and potassium hydroxide solution was added dropwise to the filtrate, (Methyl Orange indicator). At the end-point, acetone was added to precipitate the potassium dihydrogen phosphate. The solid, after centrifugation and being washed, was dried in a vacuum desiccator and analysed for its ^{18}O content by conversion into carbon dioxide, guanidine hydrochloride [24] being used. The mass spectrometric results showed the phosphate to contain 1·1 ± 0·1 (5 values) atom-% excess of ^{18}O. Total phosphate from the initial aqueous solution was converted into potassium salts as above and found to have an enrichment of 1·1 ± 0·1 (5 values) atom-% excess of ^{18}O.

The methylnaphthaquinone (VII) (0·3 g.; m. p. 107° decomp.) precipitated on addition of water to the initial reaction mixture, after being washed and dried, was also converted into CO_2 for mass spectrometric analysis. About 10 mg. of material were mixed with mercuric chloride [27] in each sample. The compound was found to be en-

[25] H. A. Christ and P. Diehl, *Helv. Chim. Acta*, 1961, **44**, 865.
[26] A. Lapidot and D. Samuel, unpublished results.
[27] D. Rittenberg and L. Ponticorvo, *Internat. J. Appl. Radiation Isotopes*, 1956, **1**, 208.
[28] I. Dostrovsky and F. S. Klein, *Analyt. Chem.*, 1952, **24**, 414.

riched in ^{18}O to the extent of 0·9 ± 0·1 (3 values) atom-% excess.

Controls.—(i) A small portion (0·3 ml.) of the mixture from reaction of 2-methylnaphthalene-1,2-diol diphosphate in [^{18}]O-dimethylformamide was removed before the addition of bromine. After 40 hr. the solvent was removed by lyophilisation and the residual solid was dissolved in unlabelled dimethylformamide to dilute any traces of ^{18}O-enriched solvent remaining. The process was repeated and finally the residual solid was analysed for its ^{18}O content (Found: 0·05 atom-% excess of ^{18}O). The diphosphate itself contained 0 atom-% excess of ^{18}O.

(ii) Orthophosphoric acid (0·1 ml.) was dried under vacuum and dissolved in 17% [^{18}O]-dimethylformamide (0·35 ml.). After 24 hr. the solvent was removed and the isotopic content of the residual dimethylformamide diluted as above. Finally potassium dihydrogen phosphate was prepared as described above and analysed for its ^{18}O content by the method of Boyer [24] (Found: 0·01 atom-% excess of ^{18}O).

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