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MODEL PATHWAYS FOR ENZYMATIC OXIDATIVE DEMETHYLATION—II

POLONOVSKI REACTION OF N,N-DIMETHYLANILINE N-OXIDE, PUMMERER REACTIONS OF DIMETHYL, n-BUTYL METHYL AND METHIONINE SULPHOXIDE WITH ACETYLATING AGENTS AND THEIR IMPLICATIONS IN ENZYMATIC DEMETHYLATION*

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Abstract—The reaction of N,N-dimethylaniline N-oxide with acetylphenylphosphate proceeds under a mild condition giving *o*-acetoxy-N,N-dimethylaniline. Similarly, acetylphenylphosphate is also effective in causing the Pummerer reaction of dimethyl sulphoxide giving the corresponding acetoxymethyl sulphide. The reaction of methionine sulphoxide with acetic anhydride gives α-acetoxy derivative of methionine. The resulting ester hydrolyses in the hydrochloric acid into homocysteine, formaldehyde and acetic acid. This demethylation reaction is discussed as a possible model pathway for the enzymatic oxidative demethylation of methionine to homocysteine.

THE PRESENCE of small amounts of t-amine oxides in both animals and plants have been considered to have a function as intermediates in some cellular reaction sequences rather than as merely the terminal products of amine oxidation[1]. Horning and his co-workers have shown that the rearrangements of various t-amine oxides containing at least one N-methyl group take place in aqueous solution at pH 2–7 in the presence of a transitional metal ion such as ferric ion under mild conditions yielding corresponding s-amines and formaldehyde, suggesting that this reaction could provide a possible pathway for the biological dealkylation of t-amine.[2] Very recently Terayama and his co-workers reported that dimethylaminoazobenzene N-oxide was readily demethylated in the presence of boiled rat-liver homogenate and haemoglobin.[3]

As another model pathway for enzymatic oxidative demethylation we have been interested in the mechanism of the Polonovski reaction,[4] whereby t-amine oxides containing at least one methyl group are converted by acetic anhydride into s-amines (as acetyl derivatives) and formaldehyde, and suggested on the basis of ^{18}O tracer experiments of N,N-dimethylaniline N-oxide with ^{18}O-labelled acetic anhydride that the reaction proceeds by solvent-caged free radical pair process.[5]

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[1a] C. C. J. Culvenor, *Rev. Pure. Appl. Chem.* **3**, 84 (1953).

[b] M. S. Fish, N. M. Johnson and E. C. Horning, *J. Amer. Chem. Soc.* **77**, 5892 (1955).

[2a] M. S. Fish, C. C. Sweeley and E. C. Horning, *Chem. & Ind.* 24 (1956).

[b] C. C. Sweeley and E. C. Horning, *J. Amer. Chem. Soc.* **79**, 2620 (1957).

[c] J. C. Craig, F. P. Dwyer, A. N. Glazer and E. C. Horning, *J. Amer. Chem. Soc.* **83**, 1871 (1961).

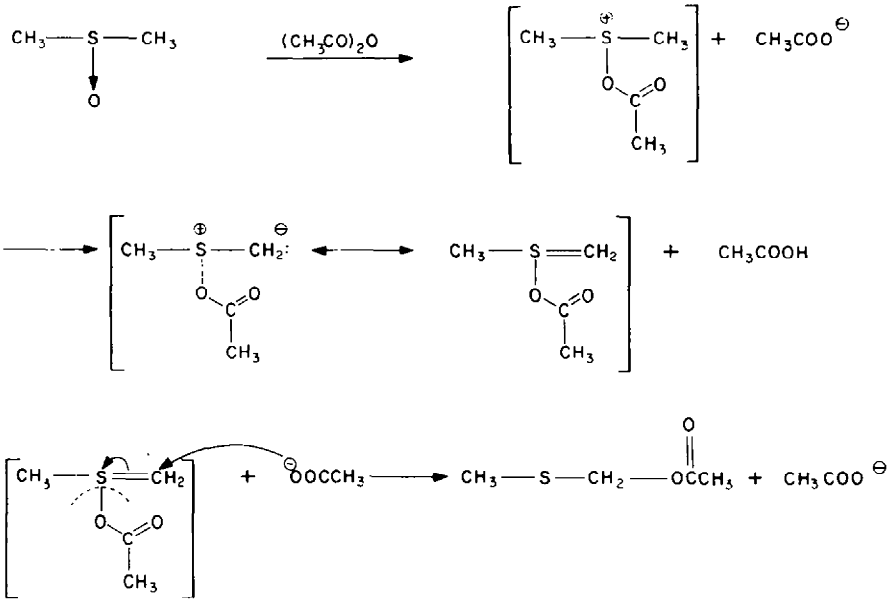
[3] H. Terayama, M. Matsumoto, T. Kurihara and A. Hanaki, 34*th General Meeting of Japanese Biological Society* Osaka, Oct (1961).

[4] M. Polonovski and M. Polonovski, *Bull. soc. chim.* **41**, 1190 (1927).

[5] S. Oae, T. Kitao and Y. Kitaoka, *J. Amer. Chem. Soc.* **84**, 3366 (1962).

The demethylation of methionine is also an extremely essential step among biological reactions and yet no adequate model reaction has been shown on this demethylation reaction. Boiling methionine with concentrated hydroiodic acid is known to produce methyliodide and homocysteine.[6]

Decomposition of methionine by U.V. irradiation is also known to give homocystine as one of many products identified, such as methionine sulphoxide, α-amino butyric acid, homocysteic acid, homoserine, methyl mercaptan and ammonia.[7] However, these reactions are so drastic as to be considered as adequate reactions of enzymatic demethylation. Meanwhile, the Pummerer reaction,[8] whereby sulphoxides with at least one S-methyl group are converted by means of acetic anhydride into α-acetoxymethyl sulphides can be considered as a model reaction of the demethylation of methionine to homocysteine, since the reaction takes place quite readily and the resulting α-acetoxymethyl sulphides hydrolyse very readily in the presence of water into mercaptan, formaldehyde and acetic acid, and it was suggested recently through tracer experiments with ^{18}O-labelled acetic anhydride that the reaction with dimethyl sulphoxide proceeds by an intermolecular rearrangement by nucleophilic attack of acetate anion as shown below:[9]



Both Polonovski reaction and Pummerer reaction have many common features because of the similar chemical and physical properties of t-amine oxides and sulphoxides;[10] and could take place under mild conditions in which ordinary biological

[6] E. E. Reid, *Organic Chemistry of Bivalent Sulfur Vol III* p. 265, Chemical Publishing Co., New York (1960).

[7] G. Hirohashi, *Nagasaki Igakukai Zasshi* **31**, 706 (1956), *Ibid.* **31**, 761 (1956); *Chem. Abstr.* **51**, 6358i (1957).

[8a] L. Horner and P. Kaiser, *Liebigs Ann.* **626**, 19 (1959).

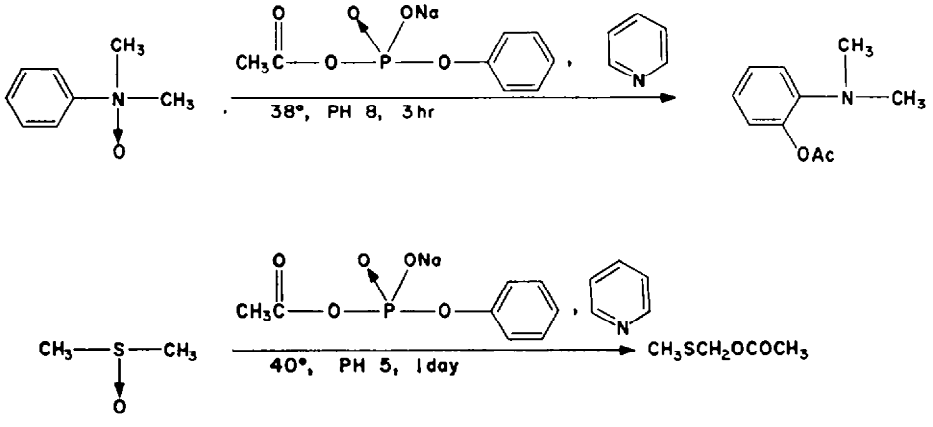
[b] F. G. Bordwell and B. M. Pitt, *J. Amer. Chem. Soc.* **77**, 572 (1955).

[9] S. Oae, T. Kitao, S. Kawamura and Y. Kitaoka, *Tetrahedron* **19**, 817 (1963).

[10] C. C. Price and S. Oae, *Sulfur Bonding* Chapter 4, Ronald Press, New York 1962.

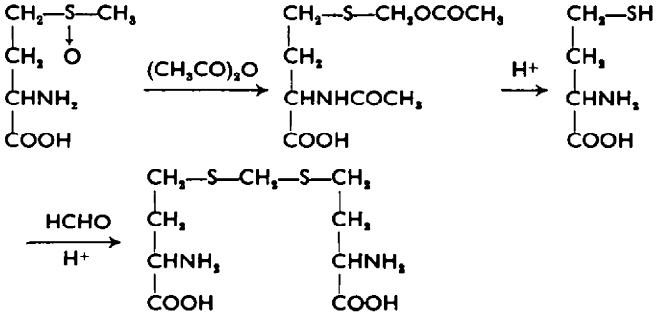
reactions are expected to occur. The initial step of both reactions is the acetylation of oxygen of the semipolar bonds by acetic anhydride, followed by the proton removal from α-methyl group by acetate anion, prior to the cleavage and rearrangement of acetoxy group. The reactions are very facile and could easily take place even without external heating.

In actual biological systems, the presence of acetic anhydride is not known, however, it could be substituted by other milder acetylating agents such as acetylphosphate which are known to be present in actual biological systems. Therefore, in this investigation, acetylphenylphosphate was at first tried as an acetylating agent for the rearrangements of N,N-dimethylaniline N-oxide[5] and of dimethyl sulphoxide.[9] When pyridine was added, not only as a catalyst for acetylation, but also as a strong base capable of removing proton from α-methyl group, the rearrangements of N,N-dimethylaniline N-oxide and of dimethyl sulphoxide underwent quite readily under very mild conditions as shown below:



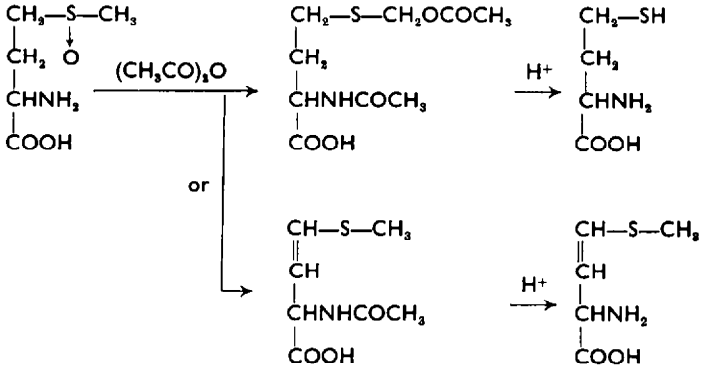
These results illustrate that the acetylation of both t-amine oxides and sulphoxides could serve as a possible scheme for oxidative demethylation.

The next approach was the application of the Pummerer reaction to methionine sulphoxide using acetic anhydride as an acetylating agent. In a mild condition, between 40–80°, however, the reaction proceeded very little and so the temperature of the reaction was raised. When methionine sulphoxide was refluxed with acetic anhydride, hydrolysed with diluted hydrochloric acid, condensed with formaldehyde, and then was subjected to paper chromatographic analysis, the predominant spot

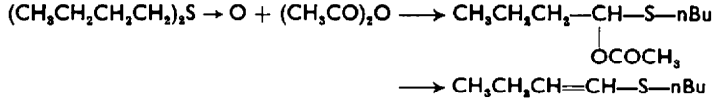


identified was that of homocysteine. A few other tiny spots also appeared: this means that other reactions also took place in the Pummerer reaction of methionine sulphoxide.

Since methionine sulphoxide is an unsymmetric sulphoxide, which is different from dimethyl sulphoxide in the previous paper,[9] there is another possibility that hydrogen atom of methylene group adjacent to the sulphur atom in methionine sulphoxide is removed with acetate anion instead of α-methyl group and the succeeding rearrangement gives the corresponding acetoxy derivative which upon elimination of acetic acid is expected to give the unsaturated product A as shown below:

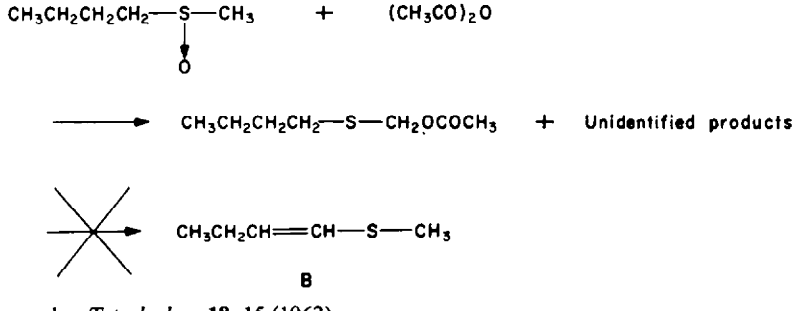


α-Acetoxy-n-butyl n-butyl sulphide is known to eliminate acetic acid at high temperature to form 1,2-butenyl derivative,[11] and also di-n-butyl sulphoxide was shown to give 1,2-butenyl sulphide when it was reacted with acetic anhydride.[8a] However, if sulphoxide bears one methyl group, the Pummerer rearrangement seems to take place



preferentially at methyl group rather than at α-position of higher alkyl group. The proton removal is known to be more favoured electronically at methyl group than at α-position of higher alkyl group.[12]

When the reaction of n-butyl methyl sulphoxide with acetic anhydride was performed α-acetoxymethyl n-butyl sulphide was isolated as a major product besides two



[11] G. Sosnovsky, *Tetrahedron* **18**, 15 (1962).

[12] C. K. Ingold, *Structure and Mechanism in Organic Chemistry* p. 429. Cornell University Press, Ithaca (1953).

other unidentified products containing sulphonate or sulphinate bonds, but there was no product having a double bond, corresponding to 1,2-butenyl methyl sulphide B. Therefore, it is not surprising that in the case of the reaction of methionine sulphoxide with acetic anhydride, the rearrangement of acetoxy anion is taking place exclusively to the methyl group forming homocysteine and the other products by succeeding reactions unknown.

Although the condition used for the Pummerer reaction of methionine sulphoxide is quite drastic, the same reaction would be substantially facilitated by a combination of successive sequences of enzymatic reactions in actual biological systems. Questions are raised how readily methionine could be oxidized to its sulphoxide leaving its other parts such as amino group intact and if there is any natural-occurring sulphoxide. It is known that methionine is readily oxidized to its sulphoxide by hydrogen peroxide under a mild condition,[13] and the occurrence of various sulphoxides in plants and animals is also quite well-known.[14] Therefore, one can safely conclude that this reaction can be a possible model pathway for the enzymatic oxidative demethylation of methionine to homocysteine in cellular reaction sequences.

EXPERIMENTAL

The reaction of N,N-dimethylaniline N-oxide hydrochloride with acetylphenylphosphate. N,N-dimethylaniline N-oxide was prepared by the oxidation of N,N-dimethylaniline with hydrogen peroxide and was converted to its stable hydrochloride.[5] Acetylphenylphosphate was prepared by the same way used by W. P. Jencks and J. Carriuolo.[15] N,N-dimethylaniline N-oxide hydrochloride, 7 g, and 22 g acetylphenylphosphate was dissolved in aqueous pyridine and this solution was maintained at pH 8 by adding sodium carbonate. The temperature was maintained at 38°. After 6 hr, the resulting O-acetoxy-N,N-dimethylaniline (ca 2 g) was extracted with ether continuously, then distilled and identified with the authentic one from I.R. spectrum and elemental analysis. (Found: C, 68·42; H, 7.18; $C_{10}H_{13}O_2N$ requires: C, 67·1; H, 7·27).

The reaction of dimethyl sulphoxide with acetylphenylphosphate. Dimethyl sulphoxide, 10 g, 3g acetylphenylphosphate and a few drops of pyridine were mixed together and warmed at 40° overnight (pH 5). The resulting α-acetoxymethyl methyl sulphide was extracted with ether and was analysed by vapour phase chromatography under the following conditions: a column used (5 mm diameter, 1 m in length) was packed with fire brick impregnated with high vacuum silicon grease (10%); flow rate of carrier gas (nitrogen), 30 ml/min; temp, 70°. When 0·05 ml of the solution obtained above was injected into the column, the retention time, 20 min, of a peak appeared was identified with the authentic α-acetoxymethyl methyl sulphide.[9]

The reaction of methionine sulphoxide with acetic anhydride. Methionine sulphoxide was prepared by the oxidation of methionine with hydrogen peroxide.[13] The crude methionine sulphoxide was conveniently recrystallized twice from its aqueous solution by adding alcohol. Methionine sulphoxide (m.p. 132°, decomposition), 10 g, and an excess of acetic anhydride were refluxed for 1 hr. Methionine sulphoxide did not dissolve in acetic anhydride at ordinary temperature but dissolved into boiling acetic anhydride and the solution became reddish brown. Excess of acetic anhydride and the other volatile matter was removed under reduced pressure. The syrup obtained was hydrolysed with 20 ml 4N hydrochloric acid, heating on a boiling water bath for about 1 hr. To this solution was added sufficient amount of formaldehyde and the homocysteine formed was completely condensed with formaldehyde by warming 80° for 30 min. The resulting solution was developed on a chromatographic paper together with the authentic homocysteine which was subjected to the same precedure, using a mixture of *n*-butanol, formic acid and water (77:10:13) as developer.[16]

[13] W. W. Westerfild *et al., Biochemical Preparations* Vol. 4 p. 80, John Wiley, New York (1959).
[14] J. F. Carson and F. F. Wong, *J. Org. Chem.* **26,** 4997 (1961).
[15] W. P. Jencks and J. Carriuolo, *J. Bio. Chem.* **234,** 1272 (1959).
[16] E. Strack, W. Friedel and K. Hambsch, *Hoppe-Sey. Z.* **305,** 166 (1956).

These results are shown below:

	R_f values (4N HCl solution)				
Product from the reaction of methionine sulphoxide and acetic anhydride[a]	0·105[c] (red)	0·21[c] (yellow)	0·35[b] (blue)	0·39[c] (orange)	
Authentic homocysteine[a]			0·35[b] (blue)		
Methionine					0·42[b] (red)
Methionine sulphoxide	0·099[b] (red)				
Authentic homocystine	0·097[b] (red)				

[a] after condensation with formaldehyde under acidic condition
[b] predominant spot which appeared by heating at 100°
[c] tiny spot which appeared by heating at 120°

The solution of the reaction product and that of the authentic homocysteine gave only one blue spot when they were mixed, developed and heated at 100° on a chromatographic paper.

n-Butyl mercaptan, 1 g, was treated under the same conditions of the condensation of homocysteine. Volatile was removed and residual oil gave di-n-butylmercaptomethane. I.R. characteristic band, 1170 cm^{-1}. (Found: C, 55·76; H, 10·53; $C_9H_{21}S_2$ requires: C, 56·2; H, 10·4.) When the authentic homocysteine was condensed with formaldehyde it gave the same characteristic I.R. band. Therefore, homocysteine forms exclusively S—CH_2—S bond by condensation with formaldehyde.

The reaction of n-butyl methyl sulphoxide with acetic anhydride. n-Butyl methyl sulphoxide was prepared by ordinary oxidation of the corresponding sulphide with 1·05 molar amount of hydrogen peroxide. Volatile was completely removed under reduced pressure and the crude sulphoxide was collected and purified. n-Butyl methyl sulphoxide, 22 g, and 96 g acetic anhydride were refluxed for 6 hr and then was fractionated by distillation: fraction A; 53–60°/15 mm, 2·7 g, B; 90–92°/1 mm, 3·4 g, C; 104°/1 mm, 0·9 g and the residual oil. The fraction A was further purified by vapour phase chromatography under the conditions: a column used (5 mm diameter, 1 mm in length) was packed with fire brick impregnated with Apiezon grease M (30%); flow rate of carrier gas (nitrogen), 8·5 ml/min; temp, 150°, and the fraction, α-acetoxymethyl n-butyl sulphide, the retention time of which was 25 min was collected (A'). I.R. characteristic band for A', 1745 cm^{-1}. (Found for A': C, 51·59; H, 8·14; $C_7H_{14}O_2S$ requires: C, 51·8; H, 8·64) I.R. characteristic bands for B, 1280, 1165 cm^{-1}. (Found for B: C, 48·57; H, 9·01) I.R. characteristic bands for C, 1340, 1155 cm^{-1}. (Found for C: C, 50·92; H, 8·39) Neither B nor C showed I.R. bands of C=C bond.

I.R. spectra

All spectra were taken on a Parkin-Elmer Model 221 spectrophotometer using Nujol for amino-acids and NaCl windows for liquid compounds.