The Oxidation of Thioethers by Bromine. A Model System for Oxidative Phosphorylation*

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ABSTRACT: The oxidation by bromine of a variety of thioethers including *N*-acetyl-DL-methionine, 5′-methylthioadenosine, and tetramethylene sulfide has been found to be an interesting model system for the study of oxidative phosphorylation. The product of the oxidation of *N*-acetyl-DL-methionine has been shown to be the corresponding sulfoxide. With anhydrous pyridine as solvent, a mixture of the tetrabutylammonium salts of adenosine monophosphate and inorganic phosphate, *N*-acetyl-DL-methionine, and Br_2 in the ratio of 1:2:2:4 results in 8.2 and 11.9% of the adenosine monophosphate being converted into adenosine diphosphate and adenosine triphosphate, respectively. A complex mixture of condensed inorganic phosphates is also produced including pyrophosphate, tripolyphosphate, trimetaphosphate, and probably tetrapolyphosphate. In this system, more than 75% of the phosphate reacts and 75% of this can be hydrolyzed to inorganic phosphate when heated in 1 N H_2SO_4 at 100° for 15 min.

Systems containing 5′-methylthioadenosine or tetramethylene sulfide as the thioether gave similar results. The rates of oxidation and phosphorylation are very rapid at room temperature; more than 50% of the adenosine diphosphate and adenosine triphosphate is formed in the first minute. In the absence of inorganic phosphate, 94% of the adenylic acid is converted into forms inactive in an enzymatic assay for adenosine monophosphate of which approximately 40% is P^1P^2-diadenosine 5′-pyrophosphate. These findings are interpreted in terms of a mechanism involving phosphorylated sulfonium intermediates which can react with suitable acceptors to form high-energy compounds. The possibility that thioethers and/or nonheme iron proteins are involved in biological oxidative phosphorylation is discussed.

Although the phenomenon of mitochondrial oxidative phosphorylation has been known for over 30 years, intensive efforts in numerous laboratories have failed to elucidate the basic mechanism of energy conservation. Repeated failures to isolate and characterize high-energy chemical intermediates of oxidative phosphorylation have prompted investigators to search for alternatives to the traditional hypothesis (Lipmann, 1941; Slater, 1953) for this process. Two such alternatives are the chemiosmotic coupling hypothesis formulated by Mitchell (1968) and the conformational coupling hypothesis proposed by Boyer (1965).

One approach to the theory that the energy of electron transport is conserved in the form of high-energy chemical intermediates has been the study of model reactions. Only a few model systems have been described in which electron transfer results in the activation of inorganic orthophosphate and its subsequent transfer to AMP or ADP to form the high-energy pyrophosphate bond. As yet, none of these model systems have been shown to be analogs of the mechanism of oxidative phosphorylation in biological systems. Thus, while the oxidation of naphthoquinol phosphates by bromine does result in phosphorylation of AMP in good yield (Clark *et al.*, 1961), no satisfactory mechanism has been found that would form the starting compound, (*i.e.*, quinol phosphate) without expenditure of a high-energy bond (Todd, 1960). Wang's phosphoimidazole model system (Cooper *et al.*, 1968) does not suffer from this defect and involves only compounds with close analogs in living systems. However, the phosphohistidine found in mitochondria appears to be associated with the succinic thiokinase and nucleoside diphosphokinase enzymes (Bieber and Boyer, 1966; Cha *et al.*, 1967; Wålinder, 1968).

Recently, Wieland and coworkers have investigated a number of model reactions for oxidative phosphorylation that involve sulfur compounds. They found that the oxidation of *N*-acetylhomocysteine thiolactone (Wieland and Bäuerlein, 1967a), thiazolidone (Wieland and Aquila, 1968), and *S*-alkylmonothiohydroquinones (Wieland and Bäuerlein, 1967b) by bromine in pyridine as solvent resulted in the formation of ADP and ATP in excellent yields from AMP and P_i. In the case of the former two compounds the authors postulate that oxidation of the sulfur atom leads to the formation of a carboxyl phosphate while a phosphoquinone is produced in the latter case. Wieland and Bäuerlein (1967c) have made detailed proposals as to how analogous sulfur functions might be involved in biological oxidative phosphorylation, but at present it is not clear whether the oxidized products can be reduced and condensed under biological conditions to form the original starting compounds.

Studies by Higuchi and Gensch (1966b) showed that a number of nucleophiles including orthophosphate could catalyze the reaction of iodine with thioethers to form sulfoxides. Their demonstration that phthalic anhydride was formed by the oxidation of tetramethylene sulfide in an aqueous solution containing iodine and phthalic acid indicated that the formation of high-energy bonds could be driven by such a reaction (Higuchi and Gensch, 1966a). These observations suggested the

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possibility that the oxidation of thioethers might serve as a model system for oxidative phosphorylation.

The studies reported in this paper have shown that oxidation of the sulfur atom of a thioether by bromine can lead to the formation of ADP and ATP and a variety of condensed inorganic phosphates when the reaction is carried out in anhydrous pyridine in the presence of the TBA[1] salts of AMP and P_i. The reaction is believed to proceed through a mechanism involving a phosphorylated sulfonium ion which is in agreement with the mechanism proposed for oxidation of thioethers in aqueous solution (Higuchi and Gensch, 1966b). The known ease with which methionine and methionine sulfoxides are oxidized and reduced, respectively, and the efficiency of the phosphorylated sulfonium ion to serve as a phosphorylium ion donor suggests that a methionyl residue of a protein could be involved in oxidative phosphorylation. A specific site for such reactions might be methionine-80 of cytochrome *c*; other sites of phosphorylation might involve either methionine or the sulfur of nonheme iron proteins (Lardy and Ferguson, 1969).

Materials

Materials Were Obtained from the Following Sources. AMP, NADP, and the sodium salts of ADP and ATP were from P. L. Biochemicals, Milwaukee, Wis.; all enzymes used in the enzymatic assays, the tricyclohexylammonium salt of phosphoenolpyruvate, and NADH from Boehringer and Soehne, Mannheim-Waldorf; analytical reagent grade bromine, formic acid, ether, and benzene (thiophene free) from Mallinckrodt Chemical Works, St. Louis; tetramethylene sulfide (tetrahydrothiophene), methyl phenyl sulfide, *p*-dioxane, and a 25% titrant solution of tetrabutylammonium hydroxide (TBA-OH) in methanol, all Eastman grade chemicals, from Eastman Organic Chemicals, Rochester, N. Y.; Baker Analyzed reagent pyridine, NH_3, and sodium formate from J. T. Baker Co., Phillipsburg, N. J.; *N*-acetyl-DL-methionine and DL-methionine (A grade) from CalBiochem; sodium salts of trimetaphosphate and tripolyphosphate from Victor Chemical Works, Chicago. 5′-Methylthioadenosine was a laboratory preparation from yeast.

Methods

Preparation of TBA Salts. The procedure used was basically that outlined by Wieland and Bäuerlein (1967a). Solid reagents were dried *in vacuo* over P_2O_5 for at least 24 hr before weighing. A 1 N solution of H_3PO_4 in *p*-dioxane was prepared from 85% H_3PO_4. A 25% titrant solution of TBA-OH in methanol was diluted to a concentration of 0.4 M with benzene. Adenosine 5′-diphosphoric acid was prepared just before using from a solution of the sodium salt by passing the solution through a Dowex 50 column, H^+ form. The solution obtained was then lyophilized and dried over P_2O_5. The ADP was converted into $(TBA)_3ADP$ when used in an experiment.

In a typical experiment, 100 μmoles of solid AMP, 205 μmoles of H_3PO_4 in *p*-dioxane, and 200 μmoles of solid *N*-Ac-Met were added to a 50-ml round-bottom flask. A sufficient quantity of the TBA-OH in methanol–benzene solution was added to produce $(TBA)_2AMP$, $(TBA)_2$-P_i, and (TBA)*N*-Ac-Met. The resulting solution was then frozen and the solvents were removed by evacuating to a pressure of about 1 mm for 2 hr. The temperature of the flask was allowed to reach room temperature gradually during this step. The residual gum was then stored *in vacuo* over P_2O_5 overnight.

Reaction Conditions. The residual gum was dissolved in 10 ml of pyridine which had been dried for at least 48 hr over calcium hydride. The reaction was initiated by adding, dropwise, a specified volume of 0.4 M Br_2 in pyridine solution over a 5-min period. The mixture was magnetically stirred at room temperature for 45 min at which time 0.1–0.2 ml of cyclohexene was added in order to remove excess Br_2. After another 10 min, several ice chips were added.

Partial Purification of the Reaction Mixture. The reaction mixture was immediately cooled to 0° and placed in a rotary evaporator for 1 hr at a pressure of about 1 mm. The temperature of the mixture was kept below 20° throughout. The yellow to amber residue which resulted after evaporation of the solvents was then dissolved in about 5 ml of ice-cold water, adjusted to pH 6–8 with 1 M ammonia, and then extracted three times with 5-ml portions of ether. After removal of traces of ether by shaking *in vacuo*, the solution was diluted to 25 ml and frozen until analyzed. The ether extract was devoid of phosphorylated products.

Analysis of the Reaction Mixture. Portions of the diluted reaction mixture prepared as described above were analyzed for ATP by the hexokinase method (Lamprecht and Trautschold, 1965) and for ADP and AMP by the enzymatic method of Adam (1965).

Analyses for unreacted orthophosphate and acid-labile (15-min) phosphate were accomplished by adding a 5-ml portion of the diluted reaction mixture to a Dowex 50 column (2 × 1 cm, H^+ form). The column was washed with 250 ml of water and portions of the eluate were analyzed for unreacted orthophosphate using the method of Sumner (1944). To determine acid-labile phosphate, aliquots of the eluate were made 1 N in H_2SO_4 and heated for 15 min in a boiling-water bath, then analyzed for P_i by the method of Sumner. Subtraction of the amount of orthophosphate gave the amount of acid-labile (15-min) phosphate present in the aliquot. Under the conditions used, less than 1% of any AMP present is hydrolyzed.

Reaction mixtures were further analyzed by chromatography on Dowex 1-formate by a procedure similar to that described by Hurlbert *et al.* (1954). A 5-ml portion of the diluted reaction mixture was placed on a 19 × 1.4 cm Dowex 1-X10 formate column and then washed into the column with 5 ml of water; 10-ml fractions were collected by using a linear gradient of 1500 ml of 0–2.0 M ammonium formate (pH 4.94). The column was run at room temperature at an average flow rate of 1.5 ml/min. The distribution of nucleotides was observed by reading the optical density of each fraction at 250 nm in a Beckman DU spectrophotometer equipped with an optical density converter. The amounts of unreacted orthophosphate and acid-labile phosphate in each tube were determined by the method of Sumner. For the measurement of acid-labile phosphate, sufficient sulfuric acid was added to give an excess of 1 N (over the ammonium formate present). A longer time (90–120 min) was allowed for color develop-

[1] Abbreviations used are: TBA, tetrabutylammonium; APPA, P^1P^2-diadenosine 5′-pyrophosphate; PPP_i and $PPPP_i$ represent tripolyphosphate and tetrapolyphosphate, respectively.

ment in tubes containing concentrated ammonium formate. The same Dowex formate column was used repeatedly by regenerating with several bed volumes of 6 N formic acid and 1 N sodium formate followed by several bed volumes of 88% formic acid. The column was then washed with at least 4 l. of distilled water.

Results

Reaction of Thioethers with Br_2 in Anhydrous Pyridine. Data presented in Table I show that the yields of ADP, ATP, and acid-labile (15-min) phosphate reach a fairly constant level when the ratio of Br_2:*N*-Ac-Met is about 1.5 or greater. In addition to the reaction with thioether, there is evidence that Br_2 may react with AMP directly at a somewhat slower rate. Indeed, in the absence of thioether (expt 11, Table II), AMP is completely converted into a form not detectable by enzymatic assay. Chromatography of a portion of this mixture on Dowex 1-formate (see Methods) showed significant absorption peaks at 259 nm only in the first ten fractions where the absorbancy is due primarily to nonphosphate containing compounds such as pyridine. While a typical reaction mixture containing thioether normally remains light yellow throughout the 45 min allowed for reaction, the absence of thioether results in the development of a green color which gradually changes to red. Some loss of nucleotide still occurs in the presence of thioether as seen in the less than 70% recovery of nucleotides in expt 4–6. However, part of this loss is probably due to formation of nucleotide species such as APPA which would not be detectable by the enzymatic assays used. The nature of the apparent direct reaction between Br_2 and AMP has not been investigated further.

In accordance with the work of Lavine (1947), the product of the oxidative reaction should be *N*-Ac-Met sulfoxide. To verify this, a portion of the reaction mixture of expt 4 was heated at 100° in 1 N NaOH for 10 hr in order to hydrolyze the amide linkage of the *N*-Ac-Met derivatives. Analyses of the hydrolysate for amino acids (Spackman *et al.*, 1958; Aminex Q150S resin) showed that all of the *N*-Ac-Met had been oxidized for no free Met was present. Almost all of hydrolysis products was the sulfoxide with only a trace of sulfone. Nearly quantitative recovery of methionine was realized when the same procedure was applied to the reaction mixture from expt 1 of Table I; methionine sulfoxide was found in amounts corresponding to <2% of the methionine recovered.

It is also seen from Table I that 10–25% of the added phosphate is not accounted for in the acid-labile (15-min) phosphate and unreacted orthophosphate categories. Heating in 1 N H_2SO_4 for up to 1 hr did not increase the amount of phosphate detectable by more than about 5% and this is probably due in part to hydrolysis of AMP. These results suggest that 10–25% of the phosphate is incorporated into a form which is not very sensitive to acid hydrolysis; one possibility is phosphorylation at the 2′- or 3′-OH group of adenine nucleotides.

The phosphorylated products formed during the reaction of *N*-Ac-Met with Br_2 have been studied further by fractionation on a Dowex 1-formate column. As seen in Figure 1(I), oxidation in the presence of $(TBA)_2P_i$, but absence of $(TBA)_2$-AMP, results in the formation of several condensed inorganic phosphates including PP_i (c), PPP_i (e), trimetaphosphate (h), and probably $PPPP_i$ (g). In addition, three smaller peaks, designated as b, d, and f, are seen in 1(I) and these also appear in Figures 1(II) and 1(III). An experiment in which one-half of the 200 μmoles of orthophosphate normally used was replaced by methyl phosphate resulted in a severalfold increase of the acid-labile phosphate in peaks b, d, and f. It is, therefore, probable that these three peaks are methyl pyro-, methyl tripoly-, and methyl tetrapolyphosphate, respectively. Their origin results from the incomplete removal of methanol during the preparation of the TBA salts. The precursor of the methyl polyphosphates, methylphosphate, has been shown to chromatograph just before orthophosphate but does not appear in Figures 1(I), 1(II), or 1(III) since it is stable to acid hydrolysis.

TABLE I: Effect of Varying the Amount of Bromine on the Yields of ADP, ATP, and Acid-Labile (15-min) Phosphate and on the Recovery of AMP and Unreacted P_i.[a]

		Products (μmoles)			Reactants Recovd (μmoles)	
Expt	Br_2 Added (μmoles)	ADP	ATP	Acid-Labile (15-min) Phosphate	Orthophosphate	AMP
1	0	0.0	0.0	5.0	197	98
2	200	10.1	2.7	81	92	72
3	300	9.4	9.1	106	65	61
4	400	8.2	11.9	120	47	47
5	600	7.8	10.5	104	47	47
6	1000	9.0	11.1	107	38	45

[a] The initial amounts of the other reactants in each experiment were: $(TBA)_2AMP$, 100 μmoles; $(TBA)_2P_i$, 205 μmoles; and *N*-acetyl-DL-methionine, 200 μmoles. The conditions of the reaction and the methods of analysis are described under Methods.

The values given for micromoles of phosphate per fraction probably underestimate the true amount of phosphate present because of incomplete hydrolysis.

Figure 1(II) shows three major peaks in the A_{259} trace that are not seen in Figure 1(I). These peaks are due to unreacted AMP (peak A) and its phosphorylated products, ADP (peak B) and ATP (peak C). The same condensed phosphates are seen as in Figure 1(I) although the amounts of PP_i and PPP_i are greater while trimetaphosphate and $PPPP_i$ have decreased. This would be expected since the total concentration of phosphoryl acceptors has increased. The acid-labile phosphate in the ADP and ATP peaks can be seen except where obscured by overlapping with other condensed phosphates.

Figure 1(III) represents chromatography of a reaction mixture in which ADP was substituted for AMP as the starting material (expt 13, Table II). The yield of ATP (relative to ADP used) was determined enzymatically to be 29%. In addition, about 12% of the ADP was converted into AMP which may be due to hydrolysis of ADP or to the intermediate formation of P^1P^4-diadenosine 5′-tetraphosphate which then breaks down to AMP and ATP (Khorana and Smith, 1958).

As demonstrated by Figure 1(IV), the absence of inorganic phosphate (see expt 10, Table II) results in the formation of a

TABLE II: Effect of Altering Conditions of the Reactions on the Yields of Products and Recoveries of Reactants.[a]

Expt	Condition Altered	Products (μmoles) AMP	ADP	ATP	Acid-Labile (15-min) Phosphate	Reactants Recovd (μmoles) Ortho-phosphate	AMP	ADP
7[b]	Reaction time, 1 min	NA[c]	7.9	7.9	93	92	68	NA
8	Reaction time, 6 min	NA	7.4	11.2	112	71	61	NA
9	AMP omitted	NA	NA	NA	135	28	NA	NA
10	P_i omitted	NA	0.8	0.3	<1	NA	6.0	NA
11	*N*-Acetyl-DL-Met omitted	NA	0.0	0.0	10	188	0.0	NA
12	Run in presence of 2.0 mmoles of added H_2O	NA	5.2	1.7	38	150	75	NA
13	Substitute ADP for AMP[d]	11.9	NA	29		67	NA	21

[a] Except for the changes noted in expt 7–13, the conditions of the reaction were the same as for expt 4 in Table I, *i.e.*, 400 μmoles of Br_2, 200 μmoles of *N*-acetyl-DL-methionine, 100 μmoles of AMP, 205 μmoles of P_i, and reaction time of 45 min. [b] The bromine was added very rapidly; 1 min after initiation of the reaction, 0.10 ml of cyclohexene was added followed 10 sec later by 2 ml of water. [c] NA, abbreviation for not applicable. [d] 100 μmoles of $(TBA)_3$ ADP were substituted for 100 μmoles of $(TBA)_2$AMP.

TABLE III: Ability of Various Thioethers to Support Oxidative Phosphorylation in the Model System.[a]

Expt	Substance Oxidized	Products (μmoles) ADP	ATP	Acid-Labile 15-min Phosphate	Reactants Recovd (μmoles) Ortho-phosphate	AMP
4[b]	*N*-Acetyl-DL-methionine	8.2	11.9	120	47	47
15	5′-Methylthioadenosine	9.1	10.8	125	33	63
16	Tetramethylene Sulfide	8.9	9.5	104	53	67
17	Methyl phenyl sulfide	2.6	5.1	100	99	17

[a] The conditions of the reaction were the same as for expt 4 of Table I; 200 μmoles of thioether, 100 μmoles of AMP, 205 μmoles of P_i, and 400 μmoles of Br_2. See Methods for further information on procedures and methods of analysis. [b] Same as expt 4 in Table I.

material which is indistinguishable chromatographically from a sample of APPA made by the procedure of Khorana and Smith (1958). This provides evidence that AMP can be activated in the same manner as phosphate. By enzymatic analysis, the amount of AMP found was 6 μmoles. Based on this value, the ratio of the areas under the peaks indicated that there are approximately 38 μmoles of adenine in the peak, leaving more than 50% of the original AMP unaccounted for. The small peak centering on fraction 21 is in the same position as adenosine. It is of interest that the same color changes were observed during this reaction as seen in expt 11 of Table II, which supports the conclusion that considerable reaction of bromine with AMP occurred. Analyses for orthophosphate and acid-labile (15-min) phosphate were negative.

A time study of the rate of ADP, ATP, and acid-labile (15-min) phosphate formation showed that oxidation of *N*-Ac-Met occurs very rapidly, with more than two-thirds of the products formed within the first minute (expt 7, Table II) Experiment 12 of Table II demonstrates the susceptibility of the phosphorylation reactions to water; the addition of 2 mmoles of water to a reaction mixture identical with that of expt 4 in Table I decreased the yield of ADP by 35%, ATP by 85%, and acid-labile phosphate by 70%.

Data presented in Table III show that other thioethers can support oxidative phosphorylation in a model system. *N*-Ac-Met, 5′-methylthioadenosine, and tetramethylene sulfide gave remarkably similar yields of ADP, ATP, and acid-labile (15-min) phosphate, while methyl phenyl sulfide was less efficient in producing ADP and ATP (approximately 3 and 5%, respectively). With methyl phenyl sulfide, the amount of acid-labile phosphate was comparable with that with other thioethers, but the total recovery of adenine nucleotides (enzymatic assay) was only about 25%. These observations, together with the fact that the same color changes were seen as

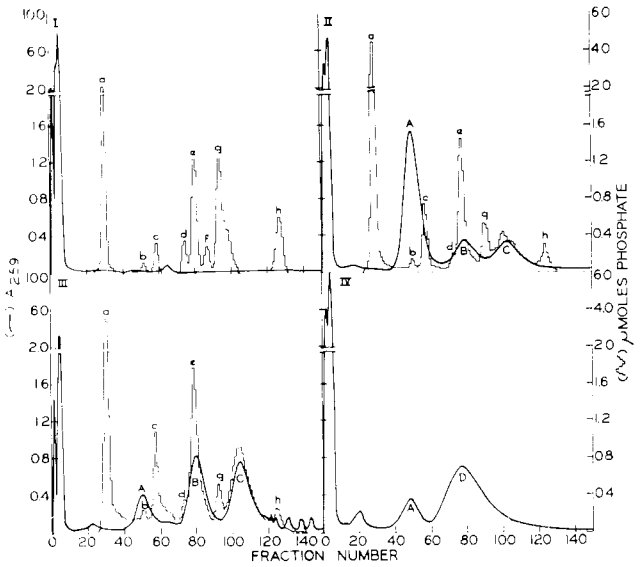


FIGURE 1: Chromatographic separation of adenine nucleotides and acid-labile (15-min) phosphate in reaction mixtures. In each experiment shown, a 5-ml aliquot of the reaction mixture which had been diluted to 25 ml was added to a 19 × 1.4 cm Dowex 1-formate column. Each column was developed using a linear gradient of 1500 ml of 0–2.0 M ammonium formate (pH 4.94). Fractions (10 ml) were collected at an average flow rate of 1.5 ml/min. The initial amounts of reactants were: I, 400 μmoles of Br_2, 200 μmoles of *N*-Ac-Met, and 205 μmoles of P_i; II same as I + 100 μmoles of AMP; III, same as I + 100 μmoles of ADP; IV, 400 μmoles of Br_2, 200 μmoles of *N*-Ac-Met and 100 μmoles of AMP. See Methods for further details on the reaction procedures, chromatography, and analysis for acid-labile (15-min) phosphate. By comparison with chromatography of authentic samples, the following peaks have been assigned: A, AMP; B, ADP, C, ATP; D, APPA; a, P_i; c, PP_i; e, PPP_i; and h, trimetaphosphate. Peak g is probably $PPPP_i$. Peaks b, d, and f have been assigned to methyl pyrophosphate, methyl tripolyphosphate, and methyl tetrapolyphosphate, respectively (see text).

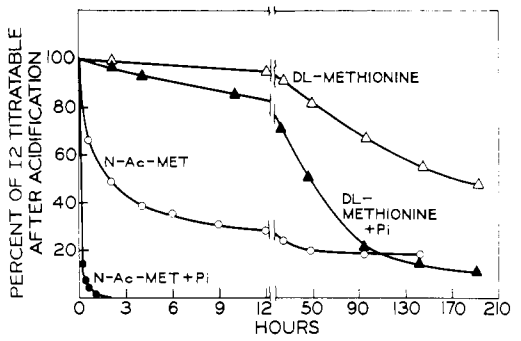


FIGURE 2: The rate of disappearance of titratable I_2 (determined in an acid medium) after incubation with DL-methionine or *N*-acetyl-DL-methionine in the presence and absence of phosphate. Aqueous solutions at pH 8.1 of 1.5 M Tris-Cl buffer, 0.125 M DL-methionine, or 0.25 M *N*-Ac-Met, and 1.0 M potassium phosphate (if used) were mixed in a 100-ml volumetric flask. The methionine solutions had been adjusted to pH 8.1 with KOH. At time zero, an iodine–potassium iodide solution was added and the contents of the flasks were immediately diluted with water (less than 5 ml) to give initial concentrations as follows: 0.25 M Tris-Cl buffer; 0.025 M DL-methionine or *N*-Ac-Met; 0.05 M P_i (if present), 0.062 M KI, and 0.025 M I_2. The solutions were incubated in a thermostated water bath at 29 ± 1°. At specified times, 5-ml aliquots were withdrawn, added to 3 ml of 5 M KI, and then acidified with 5 ml of 3 N HCl. The I_2 was quickly titrated with 0.05 M $Na_2S_2O_3$ under an atmosphere of CO_2 gas. The amount of I_2 titratable at a given time represents the amount of thioether that has not been converted into the corresponding sulfoxide.

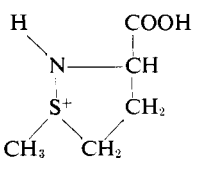
in expt 11, suggests that the rate of reaction of Br_2 with methyl phenyl sulfide is slower than with the other thioethers, allowing the reaction of Br_2 with AMP to be more significant.

Reaction of Methionine and N-Ac-Met with I_2 in Aqueous Solution. Data presented in Figure 2 show DL-methionine and *N*-Ac-Met react with I_2 in a Tris-Cl-buffered aqueous solution in very dissimilar ways. In the presence of 0.05 M potassium phosphate (pH 8.1), 0.025 M *N*-Ac-Met reacts rapidly and irreversibly with a stoichiometric amount of I_2 to form *N*-Ac-Met sulfoxide. The half-life for irreversible disappearance of I_2 is less than 10 min where the I_2 is measured by adjusting the pH to about 1 in the presence of 1 M KI and then titrating with $Na_2S_2O_3$. However, some I_2 is detectable both visually and by titration for about 1 hr. When phosphate is omitted from the reaction mixture, the disappearance of I_2 is much slower although there is a simultaneous reaction of I_2 with Tris-Cl buffer. In a control reaction where Tris-Cl, phosphate, and I_2-KI only were present, the "buffer reaction" was observed to be partially reversible upon acidification. However, less than 2% of the I_2 reacted with Tris-Cl in the first hour which makes the rate insignificant in those experiments where I_2 is quickly reduced to I^-.

In contrast to *N*-Ac-Met, DL-methionine in the presence or absence of phosphate reacts more rapidly with a stoichiometric amount of iodine, decolorizing the solution of the latter almost as rapidly as it is added. However, the reduction of iodine is readily reversed if the solution is immediately acidified in the presence of approximately 1 M KI and, as observed by Lavine (1943), more than 99% of the iodine can be recovered during the first 20 min of the reaction. This recovery of I_2 is due to reversal of the reaction resulting in re-formation of both methionine and I_2. Over a period of several days, the recovery of I_2 after acidification decreases and the data plotted in Figure 2 agree with recent studies by Gensch and Higuchi (1967) that phosphate catalyzes this type of process. Though these results indicate that *N*-Ac-Met is the preferred compound to use in a model system for oxidative phosphorylation, a direct test of DL-methionine under conditions similar to those of expt 4 in Table I, gave yields of ADP and ATP similar to those for *N*-Ac-Met.

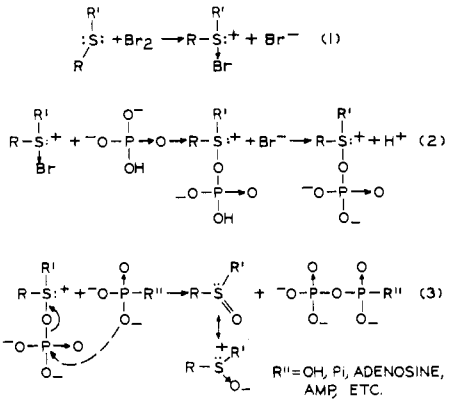
Discussion

Studies by Lavine (1943, 1945) demonstrated that the rapid oxidation by iodine of methionine in aqueous solution resulted in a cyclic product which he called dehydromethionine and which has the structure



In neutral solution, this intermediate gradually decomposes to the sulfoxide over a period of 1 week. Lavine (1945) observed

SCHEME I



that this decomposition occurred more rapidly in acid or alkaline solution and at neutrality phosphate catalyzed sulfoxide formation. Recently, Gensch and Higuchi (1967) studied the influence of several anions upon this process.

When the amino group of methionine is derivatized, as would be the case for all internal methionine residues of proteins, oxidation by iodine in aqueous solution results in direct conversion into the sulfoxide. The results shown in this paper indicate that phosphate has a pronounced catalytic effect on the rate of oxidation of *N*-Ac-Met, suggesting that a phosphorylated intermediate is formed. Although oxidation of DL-methionine by I_2 in aqueous solution does not result in immediate conversion into the sulfoxide, the good yields of ADP and ATP obtained by the oxidation of DL-methionine by Br_2 in anhydrous pyridine indicates that rapid conversion into the sulfoxide must occur.

Wieland and coworkers have found that the oxidation by bromine of *N*-acetylhomocysteine thiolactone (Wieland and Bäuerlein, 1967a), thiazolidone (Wieland and Aquila, 1968), and *S*-methylmonothiodurohydroquinone (Wieland and Bäuerlein, 1967b), in anhydrous pyridine containing P_i and AMP, results in yields of ADP and ATP similar to those reported in this paper for thioethers. They proposed that oxidation of the sulfur atom by bromine increases the susceptibility of the carbonyl function of the former two compounds to nucleophilic attack by phosphate. Subsequent cleavage is postulated to result in a carboxyl phosphate that acts as the phosphorylating agent. In the case of *S*-methylmonothiodurohydroquinones, Wieland and Bäuerlein (1967b) suggested that oxidation of sulfur eventually produces the corresponding phosphoquinone whose phosphorylating properties were first studied by Clark *et al.* (1961).

However, the results obtained with tetramethylene sulfide and 5′-methylthioadenosine prove conclusively that oxidation of the thioether function alone can support oxidative phosphorylation as these thioethers are incapable of forming phosphoquinones or carboxyl phosphates. The relatively poor yields of ADP and ATP obtained with methyl phenyl sulfide may be due to the phenyl ring decreasing electron density at the sulfonium group through resonance. The resulting slower reaction would allow Br_2 to react with a greater amount of AMP. This effect is probably overcome by an OH or other electron-withdrawing group in the *para* position of the phenyl ring which would explain the good yields obtained by Wieland and Bäuerlein (1967b) for the oxidation of *S*-methylmonothiodurohydroquinones.

In Scheme I is shown the mechanism proposed by us for phosphorylation supported by oxidation of thioethers. It is analogous to the mechanism suggested by Higuchi and Gensch (1966b) based on their studies of the effect of P_i, PP_i, arsenate, and other anions on the oxidation of tetramethylene sulfide by iodine in aqueous solution. First, bromine reacts with the sulfur atom to form a bromosulfonium ion (eq 1). This species then undergoes nucleophilic attack by orthophosphate resulting in displacement of bromide ion (eq 2). The strong attraction of the sulfonium ion for electrons activates the phosphorus atom to attack by nucleophiles. The presence of water would lead to hydrolysis to form sulfoxide and regenerate orthophosphate. However, in the absence of water, the phosphorylium ion can be transferred to a suitable acceptor (eq 3). The formation of APPA indicates that AMP can be activated in place of phosphate and the wide variety of products found supports the conclusion that a number of adenine nucleotides and condensed phosphates can function either to form the sulfonium complex or to act as a phosphorylium acceptor. Though the reactions studied by Wieland and coworkers may occur by the mechanisms they suggest, we believe they are also explainable in terms of the mechanism we propose. It is noteworthy that thioethers, thiazolidone, thiolactone, and alkylmonothioquinols give very similar yields of ADP and ATP.

Based on the experiments reported in this paper, the work of Wieland and coworkers and that of Higuchi and Gensch, it is not unreasonable to postulate that the oxidation of sulfur may be the crucial energy conservation step in oxidative phosphorylation in biological systems. In the case of thioethers, any oxidizing agent which could generate a sulfonium intermediate susceptible to nucleophilic attack by phosphate could result in generation of the high-energy phosphorylated intermediate. Sulfonium compounds have long-been recognized as high-energy compounds (Cantoni, 1952, 1960); thus the initial sulfonium intermediate formed before attack by orthophosphate could be the nonphosphorylated high-energy intermediate postulated in some hypothetical schemes (Slater, 1953).

A logical place to look for a thioether participating in oxidative phosphorylation may be a methionyl residue in one of the electron carriers of the electron transport chain. The best-characterized protein electron carrier is cytochrome *c* whose amino acid sequence has been determined in over 30 species representing all branches of the phylogenetic scale above bacteria (Margoliash and Schejter, 1966). In these species, there is an absolutely invariant sequence of eleven amino acids always located the same number of residues from the two cysteine residues which covalently bind the heme prosthetic group to the apoprotein. The carboxy terminus of this invariant sequence is Met-80 and several recent studies strongly indicate that Met-80 may serve as one of the heme iron ligands in cytochrome *c* (Harbury *et al.*, 1965; Tsai and Williams, 1965a,b; Ando *et al.*, 1966a,b; Shechter and Saludjian, 1967).

The invariancy of the 11 amino acid sequence of cytochrome *c* implies that this particular sequence fulfills some critical role in the functioning of cytochrome *c* for which evolutionary processes have not found a viable alternative. Though binding to cytochrome oxidase is essential, two proteins which must interact could maintain a functioning relationship through complementary substitutions of amino acid residues. If methionine is essential for the transfer of electrons into or from the heme

iron (Schejter and Aviram, 1969), the question still remains of why at least a conservative substitution is not permissible in one of the ten other residues. One possible explanation for the invariance of the eleven amino acid sequence is that Met-80 is involved in electron transfer and energy conservation. Then several of the other ten residues may provide binding sites for orthophosphate, ADP, or protein cofactors that do have invariant and stringent requirements over the evolutionary time scale (Margoliash, 1966). A more detailed hypothesis for how Met-80 of cytochrome *c* could interact with the heme iron and P_i to form a phosphorylated methionyl residue has been recently proposed by Lardy and Ferguson (1969). Though such a hypothesis is particularly attractive for cytochome *c*, because of the information available on the structure of this protein, it is perhaps equally applicable to cytochrome oxidase or to the cytochrome *b* region where structural information is as yet scanty. Aleem (1968) has presented convincing evidence that the phosphorylation which occurs during the oxidation of nitrite by *Nitrobacter agilis* occurs in the cytochrome oxidase region and does not involve cytochrome *c*. On the other hand, site I phosphorylation and perhaps site II phosphorylation may involve the sulfur of nonheme iron. Recent studies on nonheme iron proteins isolated from three different sources indicate that the electron taken up on reduction of the oxidized protein is shared by both iron and sulfur (DerVartanian *et al.*, 1967; Orme-Johnson *et al.*, 1968). Mechanisms for the involvement of nonheme iron in oxidative phosphorylation have been proposed recently by Russell (1968).

In order for the thioether group to be involved in biological oxidative phosphorylation, it is essential that thioethers and sulfoxides be oxidized and reduced, respectively, under biological conditions. The ability of thiols to reduce sulfoxides (Toennies and Kolb, 1939; Jori *et al.*, 1968; Wallace and Weiss, 1966) has obvious implications for the well-documented role of thiols in oxidative phosphorylation (Kurup and Sanadi, 1968). Black *et al.* (1960) have studied a system for the enzymatic reduction of L-(−)-methionine sulfoxide. This system is composed of three protein components. Acting as an electron transport system it utilizes the reducing equivalents of NADPH to reduce either disulfides or sulfoxides. A system for the reduction of methionine sulfoxide in the presence of H_2 has been reported to occur in *Escherichia coli* (Sourkes and Trano, 1953). The ability of methionine sulfoxide to replace methionine in the diet of the rat has been reported by Bennett (1939).

The widespread occurrence of sulfonium compounds (Shapiro and Schlenk, 1960) and sulfoxides (Maw, 1966; Fowden, 1964) and the ease of oxidation of thioethers and reduction of sulfoxides is being increasingly appreciated. Evidence has been presented for the enzymatic oxidation of methionine to methionine sulfoxide in the blow fly (Lucas and Levenbrook, 1966) and of *S*-methyl-L-cysteine to *S*-methyl-L-cysteine sulfoxide in broccoli and turnip (Arnold and Thompson, 1962). Dedman *et al.* (1957, 1961) found that a methionyl residue in adrenocroticotropin could be readily oxidized to sulfoxide by potassium ferricyanide (0.002 M, 60 min, pH 7.0) and then reduced by a variety of thiol compounds including cysteine (2% cysteine-HCl, 90 hr, 37°). Thus there is considerable evidence that the thioether–sulfoxide interconversion can be effected by compounds that occur naturally.

The ease with which methionyl residues are oxidized and the possible presence of methionine sulfoxide in proteins has probably been generally overlooked. Acid hydrolysis of proteins results in reversion of sulfoxide to the thioether (Ray and Koshland, 1962). Neumann *et al.* (1962) analyzed several proteins for sulfoxide by alkaline hydrolysis and concluded that none was normally present. However, none of these were proteins involved in the electron transport chain.

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