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Formation of High-Energy Phosphate Bonds Effected by Electron-Deficient Sulfides†

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ABSTRACT: Electron-deficient sulfides are postulated as intermediates in the formation of a phosphorylated sulfonium salt which has previously been suggested as the high energy phosphorylating intermediate of respiratory chain-linked oxidative phosphorylation. To test the ability of electron-deficient sulfides to effect phosphorylation of adenine nun the presence of orthophosphate, an aromatic sulfur cation radical and dication are used as models. Treatment of the tetra-*n*-butylammonium salts of adenosine 5′-monophosphate and orthophosphoric acid in anhydrous acetonitrile with thianthrene perchlorate in the molar ratio of 1:1:2 results in the rapid formation of adenosine 5′-diphosphate and triphosphate in a combined yield of 16% based on the amount of thianthrene perchlorate added or 52% yield, based on the amount of adenosine 5′-monophosphate consumed. The thianthrene perchlorate is converted to thianthrene and thianthrene sulfoxide. Similar reactions with 2,3,7,8-tetramethoxythianthrene diperchlorate in place of thianthrene perchlorate result in the rapid formation of adenosine 5′-diphosphate and triphosphate in a combined yield of 19% based on dication added or 73% yield based on adenosine 5′-monophosphate consumed. Evidence concerning the mechanisms of these reactions is presented and discussed as well as their biological significance. In particular, theoretical consideration of electron-deficient aliphatic sulfides as intermediates in oxidative phosphorylation is presented. A key suggestion is that the oxidation potential of aliphatic sulfides and the stability of aliphatic sulfur cation radicals and/or dications can be affected by neighboring group participation.

The mechanism by which energy is conserved in respiratory chain-linked oxidative phosphorylation remains unknown despite intensive investigation (Lardy and Ferguson, 1969). Three suggestions have been made for the primary energy conserving step: (1) formation of a high-energy chemical intermediate (Lipmann, 1946; Slater, 1953), (2) translocation of ions across a membrane resulting in a potential gradient (Mitchell, 1961, 1966, 1968), and (3) formation of a high-energy conformation of a macromolecule (Boyer, 1965). However, no definitive evidence has established as yet which one of these processes is the primary one.

The hypothesis that a high-energy chemical intermediate is formed in mitochondrial oxidative phosphorylation has inspired several groups to devise model systems in which oxidation is coupled to phosphorylation by such an intermediate. Oxidation

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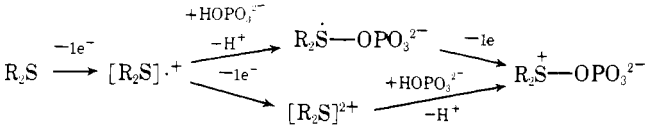
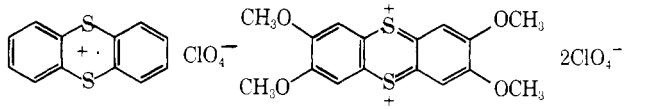
of quinol phosphates by bromine in the presence of AMP results in the formation of ADP (Clark *et al.*, 1961). Air oxidation of ferrohemochromes and imidazole in the presence of orthophosphate produces 1-phosphoimidazole which can phosphorylate AMP or ADP (Brinigar *et al.*, 1967; Cooper *et al.*, 1968). Wieland and coworkers found that oxidation of a variety of sulfur compounds in the presence of orthophosphate and adenine nucleotides results in phosphorylation of these nucleotides (Wieland and Bäuerlein, 1967a,b, 1968; Wieland and Aquila, 1968; Bäuerlein and Wieland, 1969, 1970; Bäuerlein *et al.*, 1971). Bromine oxidation of thioethers including *N*-acetyl-D,L-methionine in the presence of AMP and orthophosphate affords ADP and ATP (Lambeth and Lardy, 1969). In this case a phosphorylated sulfonium salt has been suggested as the key high-energy phosphorylating intermediate.

If a phosphorylated sulfonium salt is the high-energy phosphorylating intermediate in oxidative phosphorylation then how does such an intermediate arise *in vivo*? Given the known constitution of mitochondria, the minimum requirements for converting ADP to ATP *via* a phosphorylated sulfonium salt are a divalent sulfide, an electron acceptor, and orthophosphate. Possible mechanisms for forming a phosphorylated sulfonium salt under these minimum conditions are



These mechanisms postulate the formation of electron-deficient sulfur intermediates. The ability of such species to effect phosphorylation is the subject of this paper. Since no aliphatic sulfur cation radical or dication has been isolated and well characterized as yet, our studies have been concerned with an aromatic sulfur cation radical, thianthrene perchlorate (**1**) (Lucken, 1962), and an aromatic sulfur dication, 2,3,7,8-tetramethoxythianthrene diperchlorate (**2**) (Glass *et al.*, 1973), both of which have been isolated and well characterized.



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Experimental Section

Materials. Thianthrene was prepared according to the method of Gilman and Swayampati (1956) and purified by sublimation. Thianthrene perchlorate was synthesized following the procedure of Shine and Murata (1969). Preparation of 2,3,7,8-tetramethoxythianthrene diperchlorate was accomplished as previously reported (Glass *et al.*, 1973). AMP (free acid) and ATP (disodium salt) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Sigma Chemical Co., St. Louis, Mo., supplied ADP (disodium salt), TPN, DPNH, phosphoenolpyruvate (tricyclohexylamine salt), and all the enzymes used in this work. The disodium salt of ADP was converted to the free acid by elution through a Dowex 50-X8 ion exchange column in the acid form. All organic solvents were analytical reagent grade and were dried and distilled from the appropriate desiccants and stored under nitrogen. Bio-Rad Laboratories, Richmond, Calif., supplied labeled water containing 50% ^{18}O.

Methods. The TBA[1] salts were prepared by the method of Lambeth and Lardy (1969). Except as noted, 50 μmol of AMP (free acid), a solution containing 50 μmol of orthophosphoric acid dissolved in 1,4-dioxane, and a solution containing 150 μmol of TBAOH dissolved in methanol–benzene were mixed. The solvents were removed *in vacuo* and the residue was dried over phosphorus pentoxide overnight. The residue was dissolved in acetonitrile (5 ml) and treated dropwise with a solution containing 100 μmol of $ThClO_4$ dissolved in acetonitrile (5 ml). The reaction mixture was stirred for 15 min except where otherwise indicated. The solvent was then removed *in vacuo* and the residue partitioned between water and dichloromethane. The aqueous extract was frozen until assayed. The aqueous phase was assayed for ATP by the method of Lamprecht and Traütschold (1965), for ADP and AMP by the method of Adam (1965). These nucleotides as well as AppA were identified by their elution volumes from a Bio-Rad AG1X10 ion exchange column eluting with an ammonium formate gradient from 0 to 2.0 M (Lambeth and Lardy, 1969) and R_f on thin-layer chromatography on silica gel. Qualitative analysis for 3′,5′-cyclic AMP was performed using thin-layer chromatography on silica gel. Inorganic ortho- and condensed phosphates in the aqueous phase were analyzed by the method of Sumner (1944) adapted for spectrophotometer use.

To account quantitatively for all of the moieties used in this reaction (adenine, phosphate, and thianthrene regardless of the specific compound involved) the following assays were done. Ultraviolet spectrophotometric analysis of the aqueous phase for adenine derivatives was performed by measurement of the optical density at 259 nm. This determined the amount of adenine (incorporated, of course, into various derivatives) present if all of the adenine rings are assumed to have the same extinction coefficient regardless of the specific adenine derivative present. In addition to the assays for phosphate-containing compounds recorded above, the phosphates in the organic extract were assayed after digestion of the dried residue with 1 N sulfuric acid for 30 min. The organic extracts were assayed for Th and ThO by the ultraviolet spectrophotometric method of Murata and Shine (1969). Pure Th and ThO were both isolated by preparative layer chromatography on silica gel.

[^{18}O]Orthophosphoric acid was prepared by adding a slight excess of cold [^{18}O]water to phosphorus pentachloride. After cessation of the reaction, the mixture was evacuated exhaustively and then dissolved in anhydrous 1,4-dioxane. Analysis of the mass spectrum of the [^{18}O]orthophosphoric acid indicated 35% ^{18}O. Thianthrene sulfoxide prepared in coupling reactions with [^{18}O]orthophosphate was analyzed for ^{18}O by mass spectrometry.

The method used for condensation of orthophosphate and AMP effected by 2,3,7,8-tetramethoxythianthrene diperchlorate was similar to that used for condensations effected by $ThClO_4$. The only differences were the use of nitromethane as solvent and addition of a nitromethane solution containing 50 μmol of 2,3,7,8-tetramethoxythianthrene dication instead of $ThClO_4$. A modification of this reaction was performed in which a solution of $(TBA)_2HPO_4$ in nitromethane was added to a solution of 2,3,7,8-tetramethoxythianthrene diperchlorate in nitromethane. After stirring this reaction mixture at room temperature for 5 min, a solution of (TBA)(AMP) was added.

Results

Thianthrene perchlorate effects phosphorylation of AMP in the presence of orthophosphate to give ADP and ATP in 14 and 2% yield, respectively, based on the amount of $ThClO_4$ added or 47 and 5% yield, respectively, based on the amount of AMP consumed (see expt 4 in Table I). Similarly, ADP is

[1] Abbreviations used are: TBA, tetra-*n*-butylammonium; iso-ADP, AMP phosphorylated at N-6, O-2′, or O-3′; AppA, p^1,p^2-diadenosine 5′-pyrophosphate; Th, thianthrene; ThO, thianthrene sulfoxide; $ThClO_4$, thianthrene perchlorate.

TABLE I: Effect of Varying the Amount of Base on the Production of ADP and ATP and on the Consumption of AMP.

Expt	TBAOH Added (μmol)	Products ADP (μmol)	Products ATP (μmol)	AMP Consumed (μmol)	Yield[a] (%)
1[b]	0	1.1	0.03	12	9.4 (2.3)
2	50	5.5	0.35	13	45 (12)
3	100	7.0	0.40	16	46 (15)
4	150	7.0	0.80	15	52 (16)
5	200	6.5	0.60	18	39 (14)
6	250	4.0	0.20	19	22 (8.4)

[a] Yield of ADP and ATP formed based on the amount of AMP consumed and that based on the amount of $ThClO_4$ added, in parentheses. [b] This reaction mixture was stirred for 1.5 hr after the addition of $ThClO_4$.

phosphorylated by $ThClO_4$ and orthophosphate to afford ATP in 12 or 70% yield based on the $ThClO_4$ added or ADP consumed, respectively.

Coupling of AMP and orthophosphate effected by $ThClO_4$ consumes 15 μmol of AMP but the combined yield of ADP and ATP is only 7.8 μmol. Thus, 7.2 μmol of AMP is unaccounted for. Ultraviolet spectrophotometric analysis of the aqueous phase accounts for essentially all (49.8 μmol) of the adenine moiety added. This suggests that the AMP is converted into derivatives which though undetectable enzymatically still have an intact adenine ring and can thereby be detected spectrophotometrically (there is, however, the possibility that the spectrophotometric assay is misleading because other compounds devoid of an adenine ring or containing a modified adenine ring absorb at 259 nm). These derivatives, whose total amount accounts for *ca.* 5.2 μmol of adenine moiety (7.2 μmol less 2 μmol which are lost in a control experiment, *i.e.*, with no $ThClO_4$ added), may be 3′,5′-cyclic AMP, iso-ADP, AppA, or a Th-AMP compound.

Chromatographic data suggest the presence of AppA and absence of 3′,5′-cyclic AMP but small amounts of the latter compound would have escaped detection. No direct evidence for the formation of iso-ADP has been obtained. However, the following experiment supports the feasibility of producing small amounts of iso-ADP on treatment of AMP with orthophosphate and $ThClO_4$. Treatment of adenosine and (TBA)-H_2PO_4 with $ThClO_4$ affords small but detectable amounts of AMP (0.2%), ADP (0.1%), and ATP (0.01%). Since the 5′-OH of adenosine undergoes phosphorylation this suggests but does not prove that the 2′- or 3′-OH of adenosine or AMP may likewise undergo phosphorylation. Note that phosphorylation of adenosine with other reagents has been shown to give mixtures resulting from attack at O-2′, -3′, and -5′ (Waehneldt and Fox, 1967; Schwartz and Ponnamperuma, 1968).

In the coupling reaction of AMP with orthophosphate induced by $ThClO_4$, 50 μmol of phosphate moiety is initially added as orthophosphate (the phosphate moiety in AMP is not counted for these purposes). After the coupling reaction and subsequent work-up, analysis of the aqueous solution reveals 14.3 μmol of orthophosphate, an additional 10.6 μmol of orthophosphate after hydrolysis of the acid-labile condensed phosphate, and 8.6 μmol of phosphate moiety entrapped as nucleotides (this does not count the phosphate moiety of the original AMP; thus, 7.0 μmol of ADP and 0.8 μmol ATP are considered to entrap 7.0 and 1.6 μmol of phosphate moiety, respectively). A further 12.5 μmol of orthophosphate has been detected after acid hydrolysis of the mixture obtained from the organic extracts. This accounts for a total of 46.0 μmol (92%) of the phosphate moiety originally added as orthophosphate. The remaining amount of phosphate moiety may be associated with iso-ADP or 1- or 2-thianthrene phosphate which may be formed in the coupling reaction.

Ultraviolet spectrophotometric analysis of the organic extracts reveals the presence of 48.8 μmol of thianthrene and 44.6 μmol of thianthrene sulfoxide. Isolation of these products affords 38.9 μmol of pure thianthrene and 42.8 μmol of pure thianthrene sulfoxide. The spectrophotometric assay accounts for 93.4 μmol (93%) of the thianthrene moiety initially added as $ThClO_4$ and the isolated products account for 81.7 μmol (82%). The high yield of thianthrene sulfoxide proves that attack by orthophosphate or adenosine nucleotides on a ring carbon of $ThClO_4$ to produce 1- or 2-thianthrene phosphate or Th-AMP compounds is not extensive if it occurs at all.

The stoichiometry of the coupling of two materials each with a $\equiv P\text{-}O^-$ moiety, effected by $ThClO_4$, is presumed to be that shown in eq 1. Thus for each μmole of ADP or ATP produced

$$2\equiv P{-}O^- + 2Th^{\cdot+} \longrightarrow \equiv P{-}O{-}P\equiv + Th + ThO \quad (1)$$

2 or 4 μmol, respectively, of $ThClO_4$ are consumed. This means that the ADP produced in the coupling reaction requires the consumption of 14.0 μmol of $ThClO_4$, ATP requires 3.2 μmol, acid-labile condensed phosphate (assuming 1 $Th^{\cdot+}$ consumed per phosphate moiety) requires 23.1 μmol, and unidentified adenine products (assuming 2 $Th^{\cdot+}$ consumed per adenine moiety as expected for 3′,5′-cyclic AMP, iso-ADP, or Th-AMP compounds although AppA requires only 1 $Th^{\cdot+}$ per adenine moiety) require 10.4 μmol. This only accounts for a total of 50.7 μmol (51%) of $ThClO_4$ initially added.

One reasonable possibility for not accounting fully for the consumption of $ThClO_4$ is that residual amounts of water hydrolyze $ThClO_4$. However, drying the reaction mixture with molecular sieves prior to adding $ThClO_4$ does not have a beneficial effect. Dilution results in lowering the concentration of orthophosphate and AMP but, if the solvent were wet, the concentration of water remains the same. Thus, hydrolysis of $ThClO_4$ should become more important relative to coupling. However, dilution has no large effect: dilution by a factor of 10 results in a combined yield of ADP and ATP of 48 or 14% based on the AMP or $ThClO_4$ consumed, respectively. Furthermore, addition of even a large amount of water (5000 μmol), prior to coupling, does not prevent the formation of significant amounts of ADP and ATP: the combined yield of ADP and ATP is 40 or 6.4% based on the AMP or $ThClO_4$ consumed, respectively. Similarly addition of 2,4-dinitrophenol (50 μmol), a well-known uncoupler of oxidative phosphorylation, reduces the amount of ADP and ATP formed but does not preclude their formation: the combined yield of ADP and ATP is 25 or 8.0% based on the AMP or $ThClO_4$ consumed, respectively.

Additional possibilities accounting more fully for the $ThClO_4$ consumed are: (1) a condensed phosphate (or phosphoramidate) is formed which undergoes hydrolysis during the aqueous work-up, thereby escaping analysis,[2] (2) the acid-labile phosphate is a highly condensed phosphate which requires more than 1 mol of $ThClO_4$ per mol of phosphate for formation (3 mol of $ThClO_4$ per mol of phosphate as required for P_4O_{10}

[2] Incomplete acid-catalyzed hydrolysis of condensed phosphates is unlikely; furthermore, only 8% of the phosphate initially added as orthophosphate is unaccounted for.

TABLE II: Effect of Varying the Amounts of Orthophosphate and Thianthrene Perchlorate on the Production of ADP, ATP, and Acid-Labile Condensed Phosphate and on the Consumption of AMP.

	Reactants			Products				
Expt	Orthophosphoric Acid Added (μmol)	TBAOH Added (μmole)	$ThClO_4$ Added (μmol)	ADP (μmol)	ATP (μmol)	Acid Labile Condensed P_i (μmol)	AMP Consumed (μmol)	Yield[a] (%)
7	100	200	100	5.9	0.3	5.3	16	39 (12)
8	150	250	100	4.7	0.2	3.4	16	31 (10)
9	25	100	50	4.7	0.2	0.6	12	40 (20)
10	50	150	150	4.5	0.6	17	20	26 (10)
11	50	150	200	3.9	0.8	14	22	21 (9.4)
12[b]	50	150 (100)	100 (100)	9.9	0.8	24	22	46 (21)

[a] Yield of ADP and ATP formed based on the amount of AMP consumed and that based on the amount of $ThClO_4$ added, in parentheses. [b] $ThClO_4$ (100 μmol) was added and after 15 min TBAOH (100 μmol) and finally more $ThClO_4$ (100 μmol).

is the upper limit), or (3) 1- or 2-thianthrene phosphate is formed. However, the amount of thianthrene phosphates formed cannot exceed 7.2 μmol (*cf.* the amount of ThO isolated), if any is formed at all. Formation of thianthrene phosphates cannot be fully responsible for the unaccounted for consumption of $ThClO_4$. Thus, formation of highly condensed phosphates must be responsible, at least in part, for the consumption of $ThClO_4$.

Table I shows that the amount of ADP formed depends on the amount of base added initially. Without any added base (expt 1) the production of ADP is low and the rate of consumption of $ThClO_4$ is slow but substantial amounts of AMP are consumed. If this reaction is performed without adding $ThClO_4$ then 48 μmol (96%) of AMP is recovered. Thus, approximately 9 μmol of AMP are consumed in forming products which escape assay.[3] In expt 2–5 the yield of ADP varies over a relatively small range. In expt 6 this yield drops appreciably. This is due to base decomposition of AMP. If expt 6 is performed without adding $ThClO_4$ then only 28.7 μmol (57%) of AMP is recovered.

As illustrated in Table II, expt 7–9, orthophosphate and AMP are competitive nucleophiles. Note also, that although increasing the concentration of AMP relative to orthophosphate (as in expt 9) increases the combined yield of ADP and ATP based on the amount of $ThClO_4$ used, the yield based on the amount of AMP consumed decreases. This most likely means that more AppA, Th-AMP compound, cyclic AMP, or iso-ADP forms in expt 9.

Despite the fact that rather large amounts of AMP and orthophosphate are apparently left in expt 4 after consumption of $ThClO_4$, addition of more $ThClO_4$ initially, as in expt 10 and 11, does not increase but rather decreases the amount of ADP formed. This is due, at least in part, to further coupling of the ADP produced. The results of expt 12 afford further insight into this result. The additional TBAOH added in expt 12 regenerates $\equiv P\text{-}O^-$ moieties and may also hydrolyze condensed phosphates to orthophosphate.

To place the competition between orthophosphate and AMP for the electron-deficient thianthrene intermediate on a more quantitative basis, studies have been made of the coupling reaction using ^{18}O-enriched orthophosphate. The ^{18}O content of the thianthrene sulfoxide formed in such a coupling reaction is 32%. A similar reaction but without any added AMP results in the production of thianthrene sulfoxide containing 34% ^{18}O. These results demonstrate that the principal source of the oxygen of thianthrene sulfoxide is orthophosphate. These results also support the conclusion that the consumption of $ThClO_4$ unaccounted for in the coupling reaction is not due to hydrolysis by residual amounts of water, but rather to the formation of highly condensed phosphates. That orthophosphate and AMP compete for the electron-deficient thianthrene is substantiated by the observation that increasing the amount of AMP to 100 μmol (and TBAOH to 200 μmol) without increasing the amount of labeled orthophosphate in a coupling reaction results in the formation of thianthrene sulfoxide containing 26% ^{18}O.

Condensation of orthophosphate and AMP effected by 2,3,7,8-tetramethoxythianthrene diperchlorate produces 9.3 and 0.2 μmol of ADP and ATP, respectively. The combined yield of ADP and ATP in this reaction is 73 or 19% based on the AMP consumed or the thianthrene dication added, respectively. A modification of this experiment in which the dication is pretreated with orthophosphate gave 6.0 and 0.1 μmol of ADP and ATP, respectively. The combined yield of ADP and ATP is 34 or 12% based on AMP consumed or dication added, respectively. This means that either the reaction of orthophosphate with the dication is slow, *i.e.*, after 5 min there is sufficient dication left to effect the amount of coupling observed, or a relatively stable high-energy phosphorylating intermediate is formed, *e.g.*, a phosphorylated sulfonium salt. Evidence against the former explanation is that the color of the dication (sapphire blue) disappears immediately after the addition of the solution of $(TBA)_2HPO_4$. An identical experiment save that $ThClO_4$ is used in place of the dication and acetonitrile in place of nitromethane produces no detectable amount of ADP.[4]

Discussion

Either of the two mechanisms shown below (Schemes I and II) is a reasonable pathway for the phosphorylation of AMP effected by $ThClO_4$[5] (note that the anions involved depend on amount of TBAOH added and may vary during a given reaction).

[3] Possibilities for these products are 3′,5′-cyclic AMP, iso-ADP, AppA, or a Th-AMP compound.

[4] This experiment proves that ADP is not formed from the reaction of AMP with condensed phosphates which are produced by the reaction of orthophosphate with $ThClO_4$.

[5] Some ADP may arise, under the appropriate conditions, by Scheme I or II in which the roles of orthophosphate and AMP are reversed. See results with [^{18}O]orthophosphate.

SCHEME I

$$2Th^{\cdot+} \rightleftharpoons Th + Th^{2+}$$

$$Th^{2+} + (HO)_2PO_2^- \rightarrow Th^+\text{-}OPO(OH)_2$$

$$Th^+\text{-}OPO(OH)_2 + AMP^- \rightarrow ADP + Th^+\text{-}O^-$$

SCHEME II

$$Th^{\cdot+} + (HO)_2PO_2^- \rightarrow ThOPO(OH)_2$$

$$ThOPO(OH)_2 + Th^{\cdot+} \rightarrow Th^+OPO(OH)_2 + Th$$

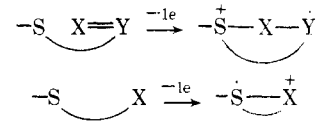
$$Th^+OPO(OH)_2 + AMP^- \rightarrow ADP + Th^+\text{-}O^-$$

In each of these mechanisms there is a conceivable alternative for the last step, *i.e.*, decomposition of $Th^+OPO(OH)_2$ to thianthrene sulfoxide and monomeric metaphosphate (Cox and Ramsay, 1964; Bruice and Benkovic, 1966; Bunton, 1970). The monomeric metaphosphate then reacts with AMP to form ADP. Scheme I is analogous to that suggested for the hydrolysis of $ThClO_4$ (Shine and Murata, 1969; Murata and Shine, 1969) and reaction of $ThClO_4$ with anisole (Silber and Shine, 1971). The validity of this mechanism for the hydrolysis of thianthrene cation radical has been challenged by Parker and Eberson (1970). These workers, on the basis of electrochemical studies using a rotating ring disk electrode, suggest a mechanism analogous to Scheme II. Recent electrochemical studies of the hydrolysis of $ThClO_4$ in acetonitrile tend to rule out Scheme I (Broman *et al.*, 1973). However, the concentration of thianthrene cation radical in the electrochemical experiments is different from those in the chemical studies.

The results of the ^{18}O-labeled orthophosphate experiments demonstrate that orthophosphate is a better nucleophile than AMP toward the electron-deficient thianthrene intermediate. This is in accord with the expectation that nucleophilicity correlates inversely with acidity (Streitwieser, 1962). Therefore, those factors which are responsible for the increased acidity of AMP relative to orthophosphoric acid (inductive and solvation effects, Kumler and Eiler, 1943) probably contribute to the decreased nucleophilicity of ions derived from the former relative to similarly charged ions of the latter. Additional reasons for the differences in nucleophilicity may be differences in interionic steric hindrance, ion pairing, or aggregation (*e.g.*, base stacking with AMP). Increased interionic steric hindrance to attack in AMP relative to orthophosphoric acid may not be of much importance because AMP and its ions may be in the anti conformation in solution (Barry *et al.*, 1971; however, see also Kainosho and Kyogoku,1972).

The fact that AMP (free acid) is a zwitterion (Kraut and Jensen, 1963) accounts for the results found in expt 1. Since the phosphate group in AMP (free acid) bears a negative charge, it would be a better nucleophile than orthophosphoric acid (protonation of AMP, free acid, by orthophosphoric acid to generate $H_2PO_4^-$ would occur to a small extent; the pK for this process in water is 1.21). Thus, AMP (free acid) may initiate attack on the electron-deficient thianthrene intermediate under the conditions of expt 1 and the intermediate so generated would undergo further reaction to give 3′,5′-cyclic AMP, AppA, or possibly a Th-AMP compound as well as small amounts of ADP and ATP.

The results presented in this paper demonstrate the ability of an aromatic sulfur cation radical and dication to effect phosphorylation of adenine nucleotides in the presence of orthophosphate. These electron-deficient sulfur species are presumably stabilized by interaction with the π system, the additional sulfur atom, and, in the case of the dication, the methoxy substituents as well. Presumably electron-deficient alipatic sulfides, which are more likely intermediates *in vivo* than their aromatic analogs, can similarly effect condensation of adenine nucleotides and orthophosphate. Although aliphatic cation radicals have been suggested as intermediates (Meissner, 1966; Cottrell and Mann, 1969; Maycock and Berchtold, 1970; Laird and Williams, 1971) no stable isolable aliphatic sulfur cation radical (or dication) has been reported as yet. Furthermore, the half-wave potentials reported for oxidation of simple aliphatic sulfides are sufficiently anodic (positive) to render their involvement in oxidative phosphorylation remote. Both the stability of aliphatic sulfur cation radicals and the ease of oxidation of aliphatic sulfides could reasonably be increased by suitably disposed neighboring groups. Such moieties, which are known to be present in components of mitochondria, are porphyrin rings, iron atoms (such a neighboring group has been discussed in detail by Lardy and Ferguson (1969), groups containing O, N, S, or aromatic rings in the side chains of protein amino acids, and either the oxygen or nitrogen atoms of the amide groups which constitute the backbone of proteins. There are two ways in which suitably placed groups may assist oxidation at sulfur: (1) donation of one electron, or (2) donation of two electrons. Examples of these two possibilities are



Note should be made that these groups may assist two-electron oxidations as well as one-electron oxidations.

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Transfer Ribonucleic Acid Sulfurtransferase Isolated from Rat Cerebral Hemispheres†

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ABSTRACT: A tRNA sulfurtransferase system has been isolated from rat cerebral hemispheres which is capable of transferring the labeled sulfur from [^{35}S]β-mercaptopyruvate to tRNA. While such enzymes have been reported in bacteria, none has been harvested previously from mammals. In addition to the enzyme and the sulfur donor, the transsulfuration reaction has a strict requirement for ATP, magnesium ion, and tRNA as sulfur acceptor. Of all the RNA species tested, only tRNA can accept sulfur; rRNA, mRNA, and synthetic ribohomopolymers lack this capacity. Two findings indicate that the sulfur is transferred onto the tRNA molecule by the brain enzyme: (1) on Sephadex G-100 chromatography, the ^{35}S label elutes with tRNA; (2) the labeled enzymatic product is sensitive to RNase treatment. Hydrolysis of the *in vitro* labeled [^{35}S]tRNA followed by DEAE-cellulose chromatography or electrophoresis reveals the formation of several thionucleotides, none of which is 4-thioUMP, the major thionucleotide synthesized by *Escherichia coli*. Fractionation of the subcellular components of rat cerebral hemispheres indicates that the sulfurtransferase activity is present predominantly in the soluble portion of the cytoplasm.

Thionucleotides are among the minor components of bacterial, yeast, and mammalian tRNAs (Carbon *et al.*, 1965, 1968; Lipsett, 1965; Baczynskyj *et al.*, 1968; Burrows *et al.*, 1968; Eliceiri, 1970). The usual role postulated for these minor constituents is that they regulate the secondary structure and consequently the functioning of tRNA. Earlier work suggested that the amino acid accepting ability of certain species of tRNA is controlled by thionucleotides; the latter must remain in the reduced form for the tRNA molecules to function as amino acid acceptors (Carbon *et al.*, 1965; Goehler and Doi, 1968). More recent studies have revealed the presence of thionucleotides in the anticodon region of certain tRNAs of *Escherichia coli*, yeast, and rat liver, which suggests that they are crucial to precise codon recognition by tRNA (Ohashi *et al.*, 1970; Yoshida *et al.*, 1971; Kimura-Harada *et al.*, 1971; Nishimura, 1972). Because of the central role played by tRNA in protein synthesis, the manner by which these unusual nucleotides come to be present in tRNA molecules presents a challenging riddle. Two basic mechanisms may be visualized for the biosynthesis of minor nucleotides in a tRNA molecule: insertion during nucleotide polymerization, or modification of nucleotides after polymerization. Investigations in bacterial systems by others and ourselves have indicated that the sulfur of thionucleotides originates from biochemical alteration occurring after polynucleotide assembly, through enzymatic transfer of the sulfur moiety of cysteine or β-mercaptopyruvate to tRNA (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966; Wong *et al.*, 1970). The sulfur in *E. coli* tRNA has been shown to be derived from cysteine. Soluble extracts of *E. coli* capable of catalyzing such reactions were first described by

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