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Thiophosphate as a Probe for Mitochondrial Oxidative Phosphorylation, Model Phosphorylation Reactions and Membrane Permeability

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Abstract

1. Monothiophosphate was used as a probe for substrate level phosphorylation, mitochondrial oxidative phosphorylation, and membrane permeability.
2. Thiophosphate does not support ATP synthesis either in mitochondria or model enzymic reactions, conservation of oxidative energy occurring through formation of ATP(γ)S.
3. Thiophosphoryl transfer is slower compared to phosphoryl (group) transfer. Such behavior is compatible with an addition-elimination mechanism.
4. Thiophosphate accumulates in mitochondria by an energy-dependent process and substitutes for phosphate in the catalysis of metabolite transport. Relative to phosphate, a slower and less extensive permeation was observed.

Abbreviations used: TMPD, N,N,N′,N′-tetramethyl-*p*-phenylene-diamine dihydrochloride; MES, 2 (N-morpholino)ethane sulfonic acid; TES, N-*tris* (Hydroxymethyl) methyl-2-aminomethane sulfonic acid; PEI, polyethyleneimine; ATP(γ)S, thiophosphoryl ADP; DTT, dithiothreitol; RLM, rat liver mitochondria; SP, thiophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; MOPS, morpholinopropane sulfonic acid.

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Introduction

The energy derived from the mitochondrial redox reactions is coupled to the production of adenosine triphosphate, providing that ADP and orthophosphate are present. The reaction may involve an intermediate formation of a phosphorylated derivative, X ∿ P but, however plausible, this proposal is not sufficiently substantiated to warrant general acceptance. Indeed, the strongest evidence in its favor are observations of Cross, Cross and Wang [1] showing that incorporation of ^{32}P into rat liver mitochondria is greater in the presence of aurovertin compared to that noted using oligomycin. Significantly, the presumed ^{32}P-labeled intermediate is labile and is rapidly discharged on treatment with uncouplers. Thus, the above procedure does not seem promising for identification of ^{32}P-derivative. Seeking a method which would lead to the formation of an intermediate of enhanced stability, we have therefore directed our attention to the possible use of monothiophosphate as a probe of phosphorylation mechanisms.

The rationale of this approach is that relative to the organophosphate derivatives the corresponding organothiophosphate compounds should be more stable providing that the rate-limiting step of their solvolysis involves a nucleophilic attack at the phosphorus atom.

The use of thiophosphate also offers other advantages. Thus, monothiophosphate shares with orthophosphate similar acid-base properties; but it is readily assayed, either directly, exploiting its distinctive absorption spectra; or by using the typical -SH reagents. In addition, thiophosphate, labeled at the phosphorus or sulfur, is readily available.

Materials

Mitochondria were prepared as described previously [2], except that the isolation medium contained 0.25 M sucrose and 1 mM EDTA.

Thiophosphate (Ventron Corp., Na_3PO_3S) was recrystallized from water and dried. The resulting anhydrous powder was assayed by ferricyanide oxidation at pH 7.0 and found to be 98% Na_3SPO_3. Elemental analysis found: P 16.69%, S 17.00%; expected: P 17.21%, S 17.81%.

Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, phosphoglycerate kinase, type IV from yeast, and DL-glyceraldehyde-3-phosphate were obtained from Sigma Chemical Co. All other chemicals were of the highest available purity.

Methods

Glyceraldehyde-3-phosphate dehydrogenase was activated with dithiothreitol and assayed essentially as described by Velick [3].

The concentration of D-isomer, in DL-glyceraldehyde-3-phosphate was determined enzymically in the presence of arsenate [3].

Mitochondrial protein was determined by the Biuret method using bovine serum albumin as standard.

Oxygen uptake by mitochondrial suspensions was determined polarographically with a Clark-type electrode, at 25°.

Mitochondrial ATP-synthesis was estimated by measuring absorbance at 260 nm after separation of ATP by column chromatography according to Pressman [4]). ATP-P_i exchange was investigated using ^{32}P labeled orthophosphate, the separation of the nucleotides being obtained by the method of Hagihara and Lardy [5].

Membrane permeability studies were performed in a Brice-Phoenix light-scattering photometer. Particle volume changes occurring as a consequence of solute permeation were monitored indirectly by changes in the intensity of the scattering light at 90°. Reaction conditions are given in the appropriate figure legends.

Ion exchange thin layer chromatography of the products of both model and mitochondrial reactions on MN-Polygram CEL 300 PEI/UV (Macherey and Nagel, Düren, Germany), eluting with 0.75 M KH_2PO_4 adjusted to pH 3.4 with concentrated HCl, as described by Goody and Eckstein [6]. Sulfur compounds were detected by spraying the plates with azide-iodine [7]. ATP(γ)S was oxidized with H_2O_2 [6], and the resulting disulfide identified by its chromatographic mobility (see above).

Spectrophotometric measurements were carried out by using a Cary Model 14 Spectrophotometer.

Results

Model Reaction. The feasibility of using thiophosphate as an analog of orthophosphate was qualitatively assessed in the glyceraldehyde-3-phosphate dehydrogenase-phosphoglycerate kinase system.

This substrate level oxidative phosphorylation, may be expressed in terms of three partial reactions. The first step involves oxidation of D-glyceraldehyde-3-phosphate, giving an enzyme-bound thiol ester [8]. In the next stage, free glyceraldehyde-3-phosphate dehydrogenase is released through phosphorolysis of the thiol ester with the concurrent formation If 1,3-diphosphoglycerate. The final reaction, yielding ATP, is mediated by phosphoglycerate kinase, and necessarily occurs only in the presence of ADP.

Our results, shown in Figs. 1 and 2, established that an analogous scheme obtains using thiophosphate; but the rate of approach to equilibria, and the extent at equilibrium (Ae) of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase catalyzed reactions are lower compared to the corresponding reactions with orthophosphate.

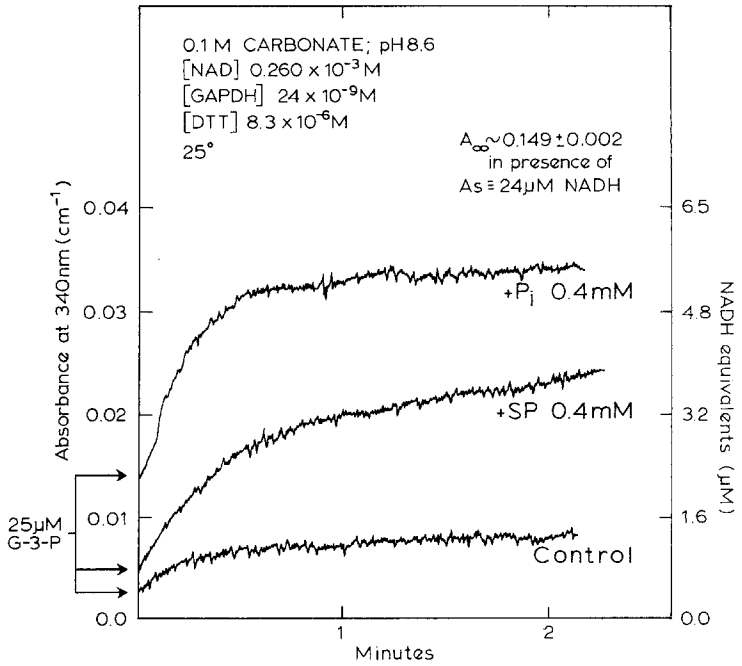
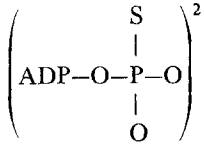


Figure 1. Kinetics of NAD[1] reduction in the glyceraldehyde-3-phosphate dehydrogenase reaction. (Note: On arsenolysis (A_∞) 24 μM NADH is obtained, i.e. nearly 100% conversion expected for 25 μM D-glyceraldehyde-3-phosphate.

The results summarized in Table 1 indicate that ATP is only a minor component of the reaction products. The major product appears to be ATP(γ)S.



The latter was identified through thin layer chromatography, using as a standard synthetic ATP(γ)S [6]. Moreover, both the synthetic and "natural" compounds give—on mild oxidation with hydrogen peroxide—derivatives which have identical chromatographic mobilities. Such oxidation involves formation of a disulfide which should be reducible to ATP(γ)S. This was confirmed using mercaptoethanol as the reducing agent.

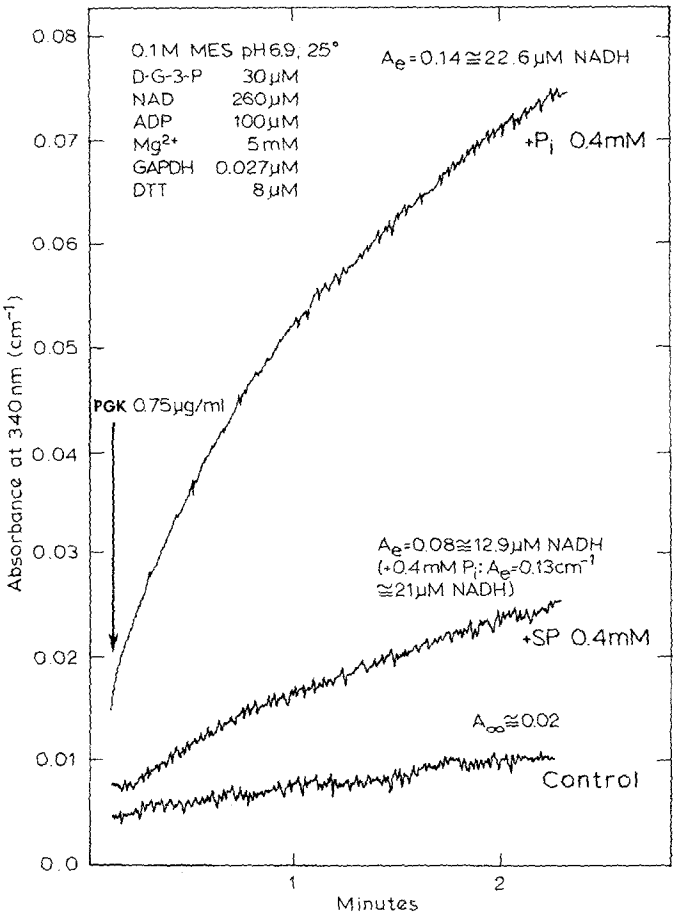


Figure 2. Kinetics of phosphoryl and thiophosphoryl transfer in the coupled glyceraldehyde-3-phophate dehydrogenase-phosphoglycerate kinase catalyzed reaction. T = 25°C.

Mitochondrial Oxidative Phosphorylation. In state IV, the respiratory rate of rat liver mitochondria is not appreciably affected by thiophosphate. This is so regardless of the nature of the oxidizable substrate (Figs. 3A and 3B) and is not dependent on the level of orthophosphate. However, the extent of respiratory rate stimulation upon addition of ADP, i.e., transition from state IV to state III is a function of the ratio of thiophosphate to orthophosphate concentrations (Fig. 3A left hand side). This is clearly illustrated in Fig. 3A (right hand side) showing that

TABLE 1. Effect of phosphate and thiophosphate on ATP synthesis by the GAPDH-PGK coupled system

	NADH nmoles ml^{-1}	ATP* nmoles ml^{-1}	(ATP:NADH) x 100
0.5 mM P_i	100	84	84
0.5 mM SP	80	9	11

The composition of the reaction mixture was: 0.1 M TES buffer, pH 7.6, 5 mM $MgCl_2$, 2.1 mM NAD, 0.5 mM ADP, 0.225 mM D-glyceraldehyde-3-phosphoric acid, 71 nM glyceraldehyde-3-phosphate dehydrogenase and 0.22 μM phosphoglycerate kinase, at 25° C.

* Estimated through hexokinase-glucose-6-phosphate dehydrogenase assay.

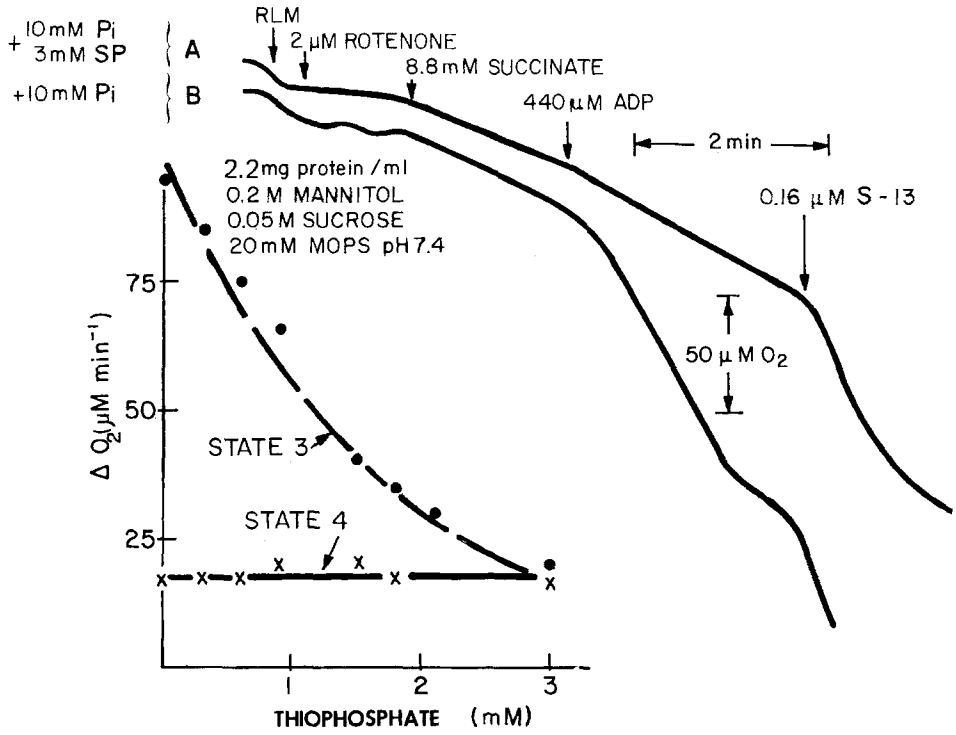


Figure 3A. Effect of thiophosphate on the ADP-induced state IV-III transition accompanying succinate oxidation in the presence of 10 mM P_i. Incubation medium contains 2.2 mg protein/ml, 0.2 M mannitol, 0.05 M sucrose, 20 mM MOPS buffer at pH 7.4, and the other additions as shown. (The data plotted on the left hand side derive from different series of experiments than that illustrated on the right hand side). T = 25°C.

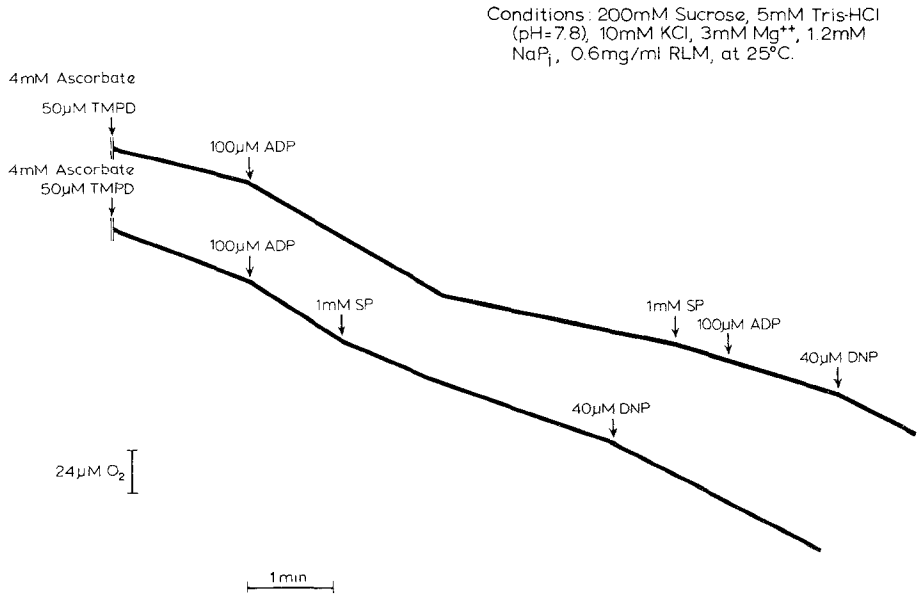


Figure 3B. Effect of thiophosphate on the ADP-induced state IV–III transition accompanying ascorbate-TMPD oxidation. The incubation medium contained: 200 mM sucrose, 5 mM tris-HCl pH 7.4, 10 mM KCl, 3 mM Mg^{++}, 1.2 mM NaP_i, 0.6 mg/ml mitochondria at 25° C.

in the presence of 8.8 mM succinate, 10 mM P_i and 3 mM thiophosphate, an increase in the respiratory rate elicited by 440 μ M ADP is, at most, equivalent to approximately 10% of that observed in the absence of thiophosphate. The results are comparable when ascorbate-TMPD is used instead of succinate (Fig. 3B).

In other experiments, using "inside-out" vesicles from *Micrococcus denitrificans,* which show excellent respiratory control [21], John and Schonbaum have shown in this laboratory that thiophosphate also abolishes the state IV–III transition. This result indicates that the inhibitory effect of thiophosphate on respiratory control seen in mitochondria is probably not due to any permeability barriers.

Apparently thiophosphate competes for the phosphate binding site. And, if a phosphorylated intermediate is formed, it does not react sufficiently rapidly with ADP to effect a change in the respiratory rate. In this context, note that the uncoupler-stimulated respiration is not inhibited by thiophosphate. In accord with the above observations is the previously noted inhibition of ATP-P_i exchange [9]—which was confirmed in an independent series of experiments (Table II). Table III shows that net ATP formation is inhibited by thiophosphate.

TABLE II. Effect of thiophosphate on the ATP-P_i exchange reaction by rat liver mitochondria

Thiophosphate (mM)	ATP-$^{32}P_i$ Exchange (μ moles/mg protein/10 min)
0.00	0.66
0.07	0.64
0.13	0.58
0.26	0.45
0.39	0.22
0.66	0.12

Mitochondria (6 mg protein) were incubated in a medium (5 ml) containing 0.2 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 5 mM tris-HCl pH 7.4, 0.1 mM EDTA, 3 mM Na-ATP, 3 mM Na phosphate ($^{32}P_i$, 0.1 μCi) and SP as indicated. Incubation: 10 min at 25° C.

TABLE III. Effect of thiophosphate on ATP formation by rat liver mitochondria

	ATP (nmoles)
– Phosphate	68
+ Phosphate	3400
+ Phosphate + Thiophosphate	540
+ Thiophosphate	108

Mitochondria (3 mg/ml), 0.2 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM Na succinate, 3 mM Na-ADP; as indicated 3 mM Na phosphate and 1.3 mM Na thiophosphate in 5 ml of reaction medium. Incubation: 10 min at 25° C.

Membrane Permeability—Permeability of phosphate and thiophosphate.

Complementary to the study of metabolic effects is the assessment of the ability of thiophosphate to reach metabolizing sites at the inner mitochondrial membranes, as well as its capacity to substitute for phosphate in the catalysis of metabolite transport. Photometric techniques have been extensively used in oscillatory and steady-state systems to monitor osmotically dependent particle-volume changes occurring during solute permeation. This methodology has contributed valuable information in studies of the dicarboxylate carrier by Chappell and Crofts [10] and Chappell and Haarhoff [11].

Light-scattering changes accompanying the energy-dependent uptake of phosphate and thiophosphate by mitochondria are compared in Fig. 4. A low concentration of succinate was provided as an energy

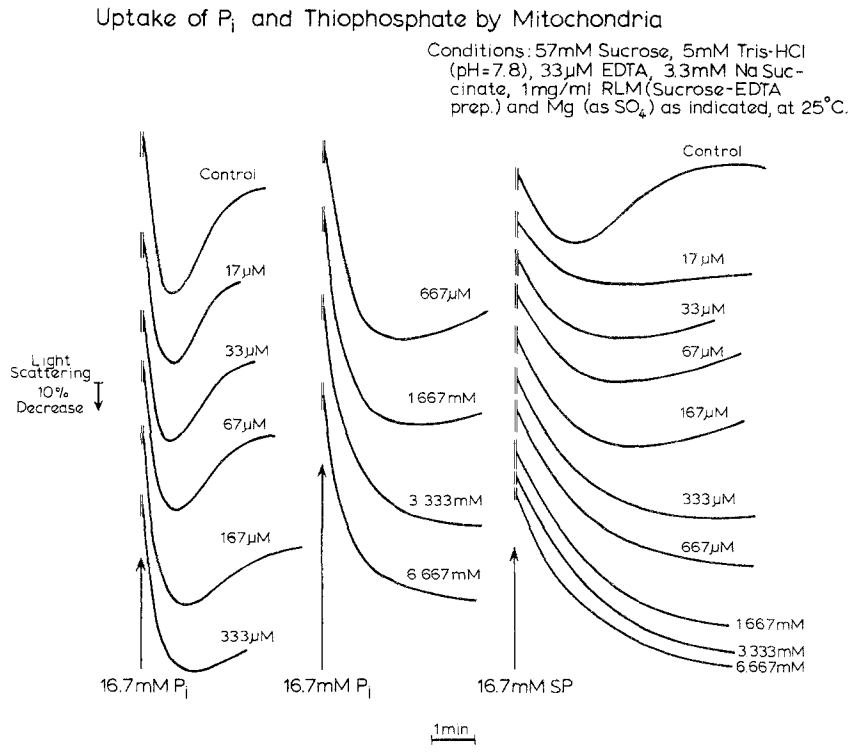


Figure 4. Uptake of phosphate and thiophosphate by mitochondria in the presence of varying levels of Mg^{2+}. The incubation medium contained: 57 mM sucrose, 5 mM tris-HCl pH 7.8, 33 μM EDTA, 3.3 mM Na succinate, 1 mg protein/ml of mitochondria and Mg^{++} (as SO_4) as indicated, at 25° C.

source. As seen in the "control" traces, the permeation of thiophosphate is markedly slower and less extensive than that of phosphate.

At pH 7.8 and in the absence of bivalent cations, ion transport follows an oscillatory pattern. As previously reported [12], in the presence of bivalent cations, phosphate transport occurs, but the oscillatory movements of these ions are damped. This was confirmed for both anions by experiments at varying Mg^{++} concentrations (Fig. 4, lower tracings). Moreover, Mg^{++} is shown to increase the extent of swelling in both cases.

Phosphate and Thiophosphate-catalyzed Metabolite Transport

Thiophosphate is shown in Fig. 5 to substitute for phosphate in the catalysis of succinate uptake by rat liver mitochondria. Addition of Mg^{++}

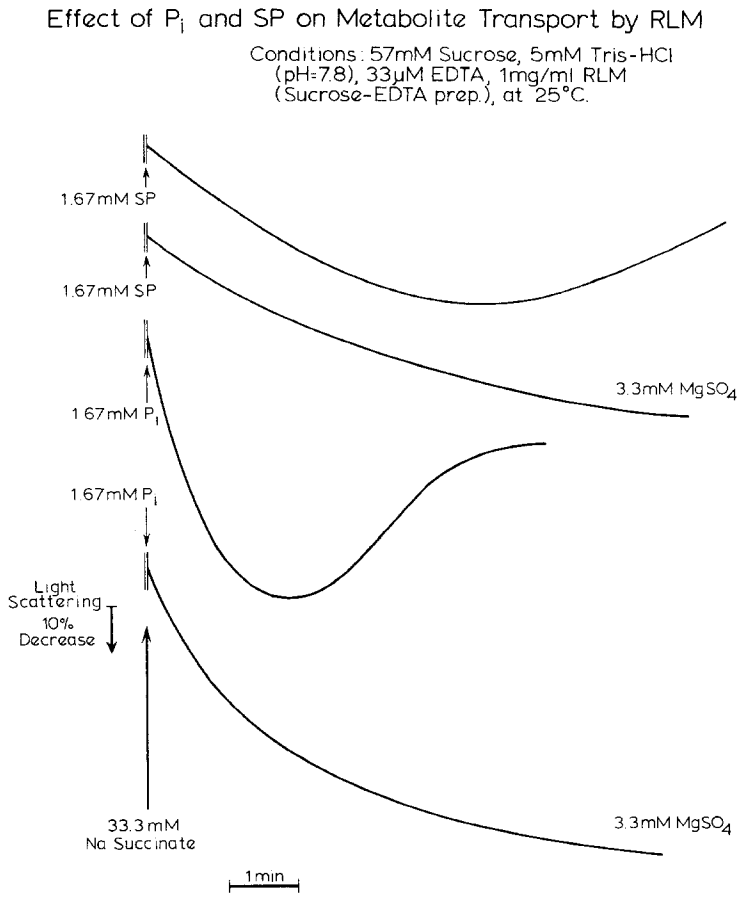


Figure 5. Effect of phosphate and thiophosphate on metabolite transport by rat liver mitochondria. The composition of the incubation medium was: 57 mM sucrose, 5 mM tris-HCl pH 7.8, 33 μM EDTA 1 mg protein/ml of mitochondria, at 25°C. Thiophosphate or phosphate was added where indicated after the initial band of light-scattering of the system had stabilized.

leads to a more extensive uptake of succinate and abolishes the oscillatory kinetics. In this experiment, energy is provided by succinate oxidation. Further studies, shown in Fig. 6, show that energy required for succinate transport can also be provided by ATP hydrolysis, when electron flow is inhibited by antimycin A and rotenone. However, in this experiment, precise interpretation is rendered difficult because of the presence of P_i released during ATP hydrolysis.

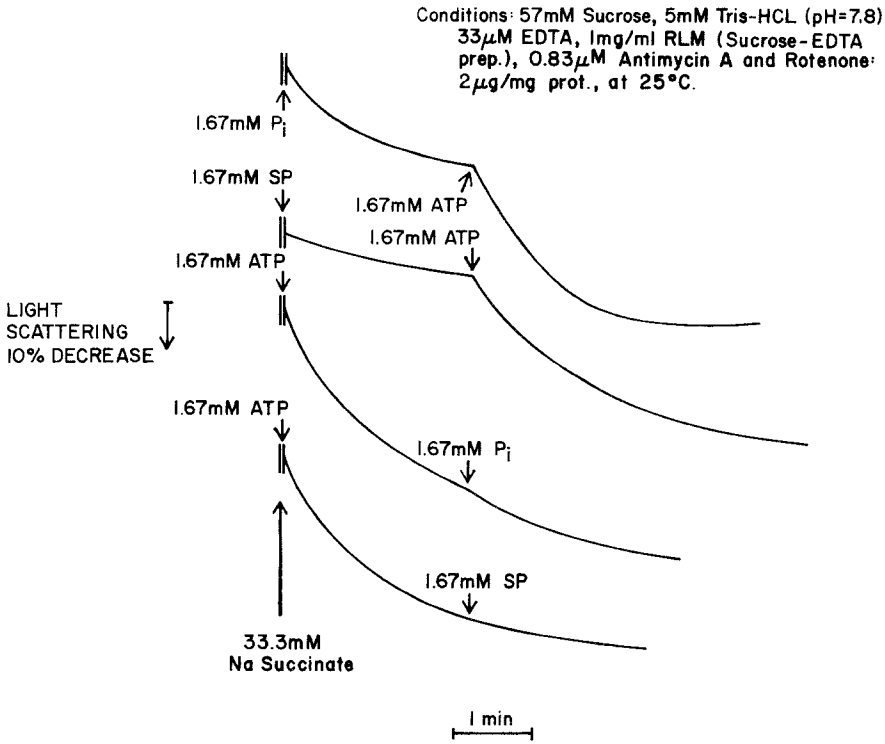


Figure 6. Effect of phosphate and thiophosphate on ATP driven metabolite transport by rat liver mitochondria. The incubation medium contained: 57 mM sucrose, 5 mM Tris-HCl pH 7.8, 33 μM EDTA, 1 mg/ml RLM (sucrose-EDTA prep.), 0.83 μM antimycin A and rotenone, 2μg/mg prot., at 25° C.

Discussion

Model Reactions and Oxidative Phosphorylation

The synthesis of ATP(γ)S by the coupled enzyme system (GAPDH-PGK) implies thiophosphoryl transfer from an O-acyl phosphorothioate to ADP.

$$R{-}C{-}O{-}\begin{vmatrix} O \\ \| \\ PSO_3^{=} \end{vmatrix} + ADP \xrightarrow[Mg^{++}]{PGK} \left(\begin{matrix}\text{addition} \\ \text{intermediate}\end{matrix}\right) \rightleftharpoons ATP\,(\gamma)S + RCO_2^{-}$$

Note that the apparent lower susceptibility of the presumed intermediate, 1-phosphorothioate-3-phosphoglyceric acid to attack by the terminal oxygen of ADP (Fig. 2) is not unexpected when the reaction proceeds via an addition-elimination mechanism. Thus, Ketelaar *et al.* [13] already reported several years ago that in the alkaline hydrolysis of

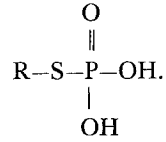
phosphate and thiophosphate tri-esters, the ratio of the characteristic second order rate constants $K_{P=S}/K_{P=O}$ is approximately 0.02 : 0.1. Similarly, the work of Breslow and Katz [14] on *p*-nitrophenyl phosphorothioate and of Mushack and Coleman [15] on arylazoaryl phosphorothioates, indicates the same type of kinetic constraint. These observations can be explained by the lesser electronegativity of sulfur as compared with oxygen, thereby rendering less favorable the ADP attack at phosphorus.

Thiophosphate is an ambident reagent and, *a priori,* we might expect it to react either via sulfur or oxygen atoms. Indeed, this is the case but the nature of the dominant derivative is contingent on the properties of the co-substrate. Thus with alkyl halides, or epoxides, thiophosphate gives the corresponding S-alkyl compounds; while in reactions with acylating reagents, the product is predominantly an O-acyl derivative [16, 17]. These examples are an excellent illustration of Pearson's thesis [18, 19], that in general, "soft" bases (RS^-) tend to react with "soft" acids (RCH_2^+); and conversely that "hard" bases (RO^-) tend to react with "hard" acids (RCO^+). The results presented in Tables I and III fully accord with these expectations.

Clearly, these results do not define the rates of ATP(γ)S formation, but indicate only that, relative to the kinetics of ATP generation, these are seemingly lower both in the glyceral-dehyde-3-phosphate dehydrogenase-phosphoglycerate kinase and the mitochondrial systems. Whether this is simply due to a difference in the absolute rates of ATP(γ)S and ATP syntheses, or in addition, reflects a significant breakdown of ATP(γ)S to ADP and a phosphorylated precursor intermediate remains uncertain. This being the case, it is not surprising that only trace amounts of ATP(γ)S are detectable, even on prolonged incubation, using concentrated suspensions of rat liver mitochondria.*

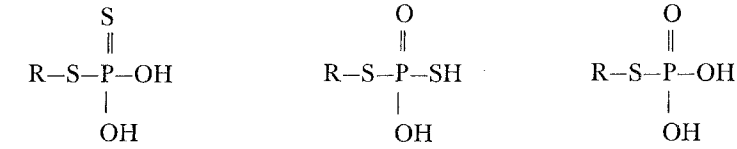
Membrane Permeability

Current views on mitochondrial permeability arising mainly from the work of Chappel *et al.* [10, 11], and of Mitchell and Moyle [20], consider the phosphate carrier as a phosphoric acid-proton symporter, hydrodehydration reactions occurring at both sides of the membrane. The phosphoric-carrier complex is represented as



* ATP(γ)S was determined after 30 min incubation of 5 mM thiophosphate with 5 mM ADP, in the presence of 0.35 M sucrose, 1 mM EDTA, 5 mM succinate, 10 mM KCl, 5 mM TES and 5 mM $MgCl_2$ at pH 7.6, using rat liver mitochondria at 70 mg/ml of mitochondrial protein. The reaction mixture was continuously aerated. T = 25° C.

Our results indicate that thiophosphate shares with phosphate an energy dependent mechanism of transport, and three limiting configurations can be assigned to the thiophosphoric-carrier complex:



(1a) (1b) (2)

Transport as in (1b) and (2) is not likely, in view of the low concentration of the thiolo form expected in a polar medium. Furthermore, mechanism (2) implies desulfuration and accumulation of orthophosphate by mitochondria. This mechanism is not favored in view of the absence of respiratory inhibition (Fig. 4) which should occur in H_2S liberation and by the synthesis of ATP(γ)S.

Transport as in (1a) agrees with the observed slower kinetics, the lesser electronegativity of the sulfur rendering the phosphorus atom in the carrier complex less susceptible to OH^- attack.

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