REGULATION OF ATP SULFURYLASE IN CULTURED TOBACCO CELLS

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This is to certify that the

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ABSTRACT

REGULATION OF ATP SULFURYLASE IN CULTURED TOBACCO CELLS

By

Ziva Reuveny

The influence of changes in sulfur and nitrogen nutrition on the level of ATP sulfurylase (E.C. 2.7.7.4, ATP:sulfate adenyltransferase) in cultured tobacco cells was investigated. A new assay for the enzyme was devised, the key feature of which is the quantitative precipitation of Na_2SO_4 in ethanol:water (5:1), leaving the product, adenosine 5'-phosphosulfate (APS), in solution with an 80% yield. Using this separation procedure and $[^{35}S]$-SO_4^{-2}, the rate of synthesis of picomole amounts of $[^{35}S]$-APS can be assayed, which makes possible kinetic and regulatory studies of ATP sulfurylase based on APS formation.

Extracts from tobacco cells and several higher plants synthesized $[^{35}S]$-APS but not $[^{35}S]$-3'-phosphoadenosine-5'-phosphosulfate (PAPS). Therefore, the $[^{35}S]$-APS remaining in solution after the separation procedure is a valid assay of the rate of the ATP sulfurylase reaction.

The tobacco cell ATP sulfurylase is not induced by sulfate. It is regulated through a negative feedback mechanism by the end product(s) of the pathway. ATP sulfurylase is repressed during

growth on readily assimilated sulfur sources such as sulfate, L-cysteine, or L-methionine, but is derepressed during sulfur-limited growth on slowly assimilated sulfur sources such as djenkolate or glutathione or during sulfur starvation. The ATP sulfurylase specific activity begins to rise within 12 hours after the derepression conditions are initiated, and continues to increase up to 25-fold within 3 to 4 days.

The sulfur compounds which affect the development of ATP sulfurylase *in vivo* have no effect on the enzyme activity *in vitro*.

Derepression is inhibited by cycloheximide at a concentration which strongly inhibits incorporation of amino acids into protein, indicating that the mechanism of derepression depends, in some way, upon protein synthesis.

After addition of a repressing sulfur source to derepressed cells, a decline in total ATP sulfurylase activity can be detected, indicating a role for enzyme inactivation or degradation in the regulation of the enzyme in the tobacco cells.

Derepression does not occur in tobacco cells starved for nitrogen, a circumstance in which turnover synthesis of protein is known to continue. Upon addition of a nitrogen source derepression occurs, along with a resumption of net protein synthesis, indicating a positive role for nitrogen assimilation in the regulation of ATP sulfurylase. Therefore, it is hypothesized that ATP sulfurylase of the tobacco cells is derepressed by the absence of an end product of the sulfate pathway, SX, provided that there also is a positive effector signal, NY, from the nitrogen assimilation pathway.

Ziva Reuveny

Molybdate and selenate are structural analogs of sulfate for the reaction of APS synthesis by tobacco cell ATP sulfurylase. Either of these anions, when included in the culture media with sulfate, derepressed the ATP sulfurylase. Molybdate can cause derepression only when added at 10-fold the concentration of sulfate, a condition which inhibits growth, and net accumulation of protein is inhibited, suggesting that the derepression resulted from sulfur starvation.

Selenate, which is a competitive inhibitor of APS synthesis *in vitro*, causes a derepression of ATP sulfurylase *in vivo* when added to the culture media at concentrations at which neither growth nor protein accumulation in the cells is affected. At higher molar ratios of selenate to sulfate, selenate is toxic and derepression does not occur. Selenate is extremely toxic to derepressed cells growing on djenkolate but is much less toxic to repressed cells growing on cysteine. It is suggested that the selenate-dependent derepression of ATP sulfurylase may be via an antagonism between the hypothetical corepressor of the sulfate pathway, SX, and a hypothetical anti-corepressor, SeX.

REGULATION OF ATP SULFURYLASE IN CULTURED TOBACCO CELLS

By

Ziva Reuveny

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" קֵץ ו שָׂם לַחֹשֶׁךְ
וּלְכָל־תַּכְלִית הוּא חוֹקֵר . . .
וְהַחָכְמָה מֵאַיִן תִּמָּצֵא
וְאֵי־זֶה מְקוֹם בִּינָה
לֹא־יָדַע אֱנוֹשׁ עֶרְכָּהּ
וְלֹא תִמָּצֵא בְּאֶרֶץ הַחַיִּים ."

איוב כח: 13-12,3

"Man puts an end to darkness,
Every recess he searches. . .
But wisdom, where can it be found?
Where is the place of understanding?
Man knows not the path to it,
It is not found in the land of the living."

Job XXVIII: 3, 12-13

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LIST OF ABBREVIATIONS

APMo	adenosine 5'-phosphomyolybdate
APS	adenosine 5'-phosphosulfate
APSe	adenosine 5'-phosphoselenate
DPH	3',5' diphosphonucleoside 3' phosphohydrolase
g.f.wt.	gram fresh weight
$(N,S)^-$ MID	MID medium lacking both nitrogen and sulfur sources
OAS	O-acetylserine
PAP	adenosine 3',5'-diphosphate
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PAPSe	3'-phosphoadenosine-5'-phosphoselenate
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
PPT	precipitate
SAM	S-adenosylmethionine

INTRODUCTION

Among the few metabolic pathways of higher plants which begin with an exogenous compound is the sulfate assimilation pathway. Higher plants can utilize either sulfate or more reduced sulfur compounds to satisfy their requirements for growth (123), but sulfite and sulfide are toxic to plants (cf. 154). Higher plants incorporate sulfur predominantly into cysteine and methionine in cell protein (147). Therefore, in order to minimize accumulation of toxic intermediates, and to optimize production of end products, it can be predicted that the sulfate assimilation pathway of higher plants should be regulated by the quantities and chemical forms of sulfur available and by the overall potential for protein synthesis.

Regulation of a biosynthetic pathway usually involves the regulation of its first enzymatic step which, in the case of the sulfate assimilation pathway, after the uptake of sulfate, would be the activation of sulfate to adenosine 5'-phosphosulfate (APS), a reaction catalyzed by ATP sulfurylase. There has been only one previous attempt to investigate in higher plants the regulation of ATP sulfurylase by sulfur compounds, but negative results were obtained (104). However, regulation of this enzyme has been found repeatedly in microorganisms (24-27,126,129,185). A reexamination of the question of regulation of ATP sulfurylase in higher plants, therefore, was warranted.

The cultured tobacco cell system has been found to be useful in studies of the regulation of the nitrate assimilation pathway, because nutritional conditions are strictly controlled and can be readily manipulated (179-181). Furthermore, in earlier studies with the tobacco cells, evidence was obtained of a role for sulfur amino acids in the regulation of the uptake of sulfate (123-124). From all of these considerations, it seemed highly probable that if ATP sulfurylase were regulated in higher plants, the phenomenon could be detected and studied in the cultured tobacco cells.

Studies on the regulation of enzymes of biosynthetic pathways can involve either a genetic or physiological approach. In the absence of methods for genetic manipulation of higher plant cells, the physiological approach has to be adopted. Accordingly, the general experimental procedure was to seek changes in levels of ATP sulfurylase activity in response to specific perturbations in sulfur nutrition. Before such changes could be detected, however, an assay for ATP sulfurylase was required which would give a reliable quantitative estimate of the physiologically important reaction, APS synthesis.

The research presented in this dissertation consequently can be divided into two parts: first, development of the enzyme assay and, second, application of the assay in regulatory studies.

The regulatory studies with the tobacco cell system were guided by the following general questions:

(a) Is ATP sulfurylase induced by its substrate, sulfate?

(b) Is the enzyme subject to regulation by end product(s) of the pathway?

(c) Is there any connection between regulation of sulfate assimilation and regulation of nitrate assimilation, since the end products from both pathways flow into protein?

LITERATURE REVIEW

The Sulfate Assimilation Pathway

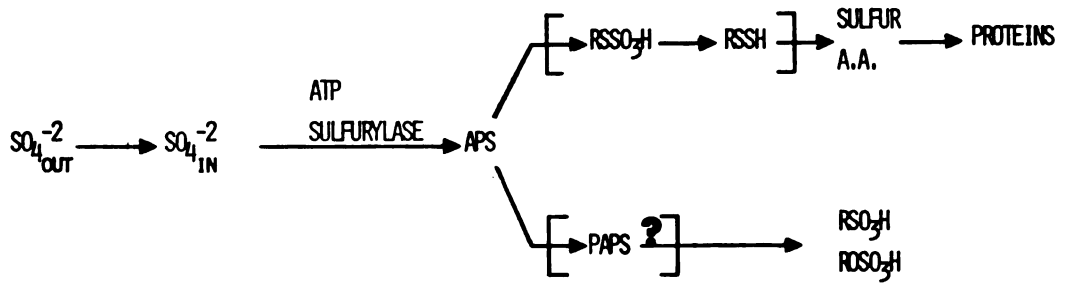
Sulfur is essential for all living organisms because of its occurrence in the sulfur amino acids of protein, in sulfur-containing bases of tRNA, and in a number of vital cofactors, e.g., thiamine pyrophosphate, biotin, lipoic acid and coenzyme A. Sulfur also occurs in sulfate esters and sulfolipids of various organisms (108,147).

While the ability to synthesize sulfate esters from sulfate, which does not involve a change in oxidation level of the sulfur, is found in all biological kingdoms, the ability to carry out the 8-electron reduction of inorganic sulfate (oxidation state +6) to the sulfide level (oxidation state -2) is restricted to plants and microorganisms (108).

Plants and most bacteria reduce sulfate to satisfy their nutritional requirement for sulfur. This is termed assimilatory sulfate reduction (148). In a small group of anaerobic bacteria, however, sulfate is reduced during anaerobic respiration and thereby serves as a terminal electron acceptor. This process is called dissimilatory or respiratory sulfate reduction.

Inorganic sulfate is the principal form of sulfur used by plants for growth. The sulfate assimilation pathway in plants is

believed to have two branches: reduction of sulfate followed by incorporation into amino acids containing sulfur on the one hand, and incorporation of sulfate into sulfate esters (and possibly sulfonates) on the other (cf. 48-49,108):



Nevertheless, cysteine and methionine, in protein, are responsible for the bulk (>90%) of the organic sulfur in plants (147). Consequently, the demand for these protein amino acids largely determines the sulfur requirements of plants.

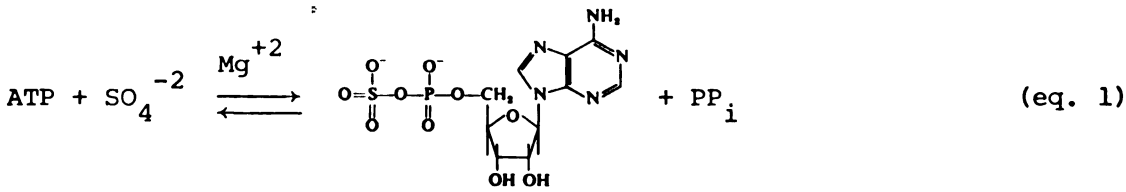
Sulfate Activation

Sulfate uptake and its control have been studied in bacteria, fungi, algae and higher plants (cf. 48).

The reduction of sulfate to sulfide is highly endergonic, requiring an input of some 180 Kcal/mole (cf. 48). Assuming that the reaction proceeds by 2-electron steps, sulfite would be the first reduced intermediate. However, reduction of sulfate to sulfite requires ca. 60 Kcal/mole (168), which is unlikely to be carried out biochemically in a single step (168). Therefore, a stepwise activation of the sulfate, prior to reduction, can be anticipated. Biochemical and genetic evidence obtained to date has

verified that activation of sulfate is a prerequisite to its utilization in either biosynthetic or energy-yielding reactions (108,148). Therefore, the ability to activate sulfate is widely distributed among living organisms.

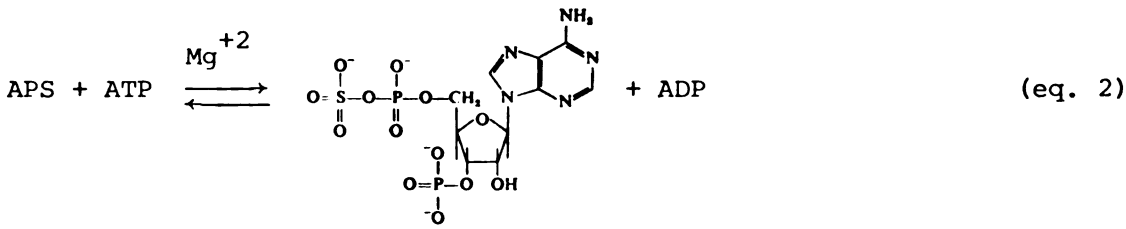
The enzymatic mechanism for the activation of sulfate, and the intermediates involved, were elucidated by Lipmann and Robbins (cf. 149) and Wilson and Bandurski (cf. 150). The first step involves the formation of the unique mixed anhydride bond between sulfate and adenosine 5'-monophosphate in adenosine 5'-phosphosulfate (APS), catalyzed by ATP sulfurylase (eq. 1).



(eq. 1)

APS

APS can be further phosphorylated on the 3'OH group of the ribose moiety, to yield 3'-phosphoadenosine-5'-phosphosulfate (PAPS). This reaction is catalyzed by APS kinase (eq. 2).



(eq. 2)

PAPS

The strongly endergonic nature predicted for the formation of the phospho-sulfate bond was evident in studies with highly purified ATP sulfurylase from yeast (140a): an apparent equilibrium constant for APS formation of the order of 10^{-8} was found (41,140a). Based on this, the group potential of sulfate in APS was calculated to be

ca. 19,000 cal/mole (140a). A similar value for the activation energy of the molybdolysis reaction, catalyzed by ATP sulfurylase purified from yeast, was recently reported (98). The activation energy was calculated from an Arrhenius plot.

The extremely unfavorable equilibrium of the proposed reaction of ATP sulfurylase is a biochemical enigma: how is the rate of synthesis of APS required *in vivo* achieved? These considerations led to the concept of the "sulfate-activating enzyme system" in which a physiologically adequate flux of APS was sustained in the face of the highly unfavorable equilibrium, by the subsequent elimination of the two reaction products from the equilibrium mixture. Pyrophosphate and APS are removed by the exergonic reactions of inorganic pyrophosphatase (eq. 4) and APS kinase (eq. 5), respectively, which meet the free energy requirement of the endergonic ATP sulfurylase reaction (eq. 3).

$$ATP + SO_4^{-2} \rightleftharpoons APS + PP_i \quad \Delta F^\circ, + 11{,}000 \text{ cal} \qquad \text{(eq. 3)}$$

$$PP_i \rightleftharpoons 2\ P_i \quad \Delta F^\circ, - 5{,}000 \text{ cal} \qquad \text{(eq. 4)}$$

$$APS + ATP \rightleftharpoons PAPS + ADP \quad \Delta F^\circ, - 6{,}000 \text{ cal} \qquad \text{(eq. 5)}$$

The finding by Robbins and Lipmann (140) that APS kinase has a high affinity toward APS is consistent with the idea that the function of this enzyme is the removal of a product of a thermodynamically unfavorable reaction.

It is noteworthy that in the presence of inorganic pyrophosphatase with the ATP sulfurylase, the equilibrium concentration of APS

was found to increase by only ca. one order of magnitude (41,154), which is lower than expected from calculations based on the reported free energy changes for the reactions of ATP sulfurylase and inorganic pyrophosphatase (see eqs. 3-4). Thus, an extremely low Km (APS) for the APS kinase reaction may be required even in the presence of inorganic pyrophosphatase.

PAPS was assumed for many years to be the chemical form of sulfate which is actually reduced in organisms which assimilate sulfate. However, APS rather than PAPS is known to be the activated substrate for the dissimilatory sulfate reduction as shown by Peck and co-workers (cf. 148), although the equilibrium constant of ATP sulfurylase isolated from these dissimilatory bacteria was very similar to the yeast enzyme (91). Furthermore, recent studies strongly suggest that APS is also the activated substrate in sulfate reduction in *Chlorella* and spinach (cf. 48). The apparent lack of participation of PAPS as the active sulfate in sulfate reduction in certain organisms indicates that there must be alternative solutions *in vivo* to the problem of the unfavorable equilibrium governing APS formation.

In contrast to the extensive documentation of the occurrence and properties of ATP sulfurylase, reports of the occurrence of APS kinase are relatively few in number. Partially purified enzyme was obtained only from yeast (140) and *Nitrobacter* (141), while its occurrence in other bacteria, fungi and algae was deduced from nutritional studies with mutants and from the synthesis of PAPS from sulfate and ATP by crude extracts (cf. 108).

The occurrence of APS kinase in higher plants was until recently a matter of dispute. Although some workers have reported the synthesis of [^{35}S]-PAPS in extracts of a few higher plant species (105,142), other workers could detect [^{35}S]-APS but not [^{35}S]-PAPS under seemingly identical conditions (104,143-144,156). The failure to detect PAPS formation in extracts of higher plants led Ellis to propose that APS rather than PAPS might be the substrate for the first reduction step (104). It now appears, however, that APS kinase does occur in higher plants but, nevertheless, APS, not PAPS, is indeed the actual substrate for reduction (cf. 48). Two possible sources of difficulty in the detection of PAPS formation in plant extracts are: (a) a lack of adequate sulfhydryl protection; and (b) rapid reconversion of PAPS to APS by enzymes that hydrolyze the 3' phosphate of PAPS. The formation of PAPS by both spinach and *Chlorella* extracts was observed upon the addition of sulfhydryl reagents (145-146). Since addition of -SH containing compounds is required to obtain APS formation with *Chlorella* extracts but not with higher plant extracts, the possible requirement for sulfhydryl protection for PAPS formation may have been overlooked with higher plants. There are two hydrolytic enzymes known which will convert PAPS to APS. The first enzyme is the 3' nucleotidase which is widely found among higher plants and has been purified from rye grass (cf. 48,155). The failure to detect PAPS formation in a strain of *Salmonella* (151) appeared to be due to the presence of a potent 3' nucleotidase, since PAPS was readily detected upon inclusion of 3' AMP in the incubation mixture. The second enzyme, which was discovered by Schiff *et al.* in *Chlorella*

(cf. 48,153) and which appears to exist in spinach as well (146), is a 3',5' diphosphonucleoside 3' phosphohydrolase (DPH). This enzyme, although it hydrolyzes the 3' phosphate group on PAPS, will not use 3' AMP efficiently as a substrate (cf. 48).

Recently, Burnell and Anderson (155) demonstrated an APS kinase activity, i.e., PAPS formation, in crude spinach extracts in the presence of 3' AMP. In the absence of the 3' nucleotide, APS was the only species detected. The absence of the 3' nucleotidase activity in the fraction which was capable of 3' AMP-dependent PAPS formation was interpreted to mean that 3' AMP was an activator of the APS kinase.

It is clear that further studies of APS kinase in the plant kingdom have to be carried out before unequivocal conclusions regarding the role of PAPS formation and APS kinase in sulfate reduction can be reached.

Assimilatory Sulfate Reduction

The exact pathway by which sulfate is reduced to sulfite and incorporated into cysteine in organisms which assimilate and reduce sulfate is still unclear.

The evidence obtained from enzymological, nutritional and genetic studies with bacteria and fungi (cf. 108) has indicated that APS, PAPS, sulfite and sulfide are all intermediates in the incorporation of sulfate into cysteine in these organisms. In addition to ATP sulfurylase and APS kinase, the enzymes catalyzing the subsequent two steps, i.e., PAPS reductase and sulfite reductase, have been studied in these organisms (108).

Most of our knowledge on the PAPS reduction comes from the studies of Wilson, Bandurski and co-workers (summarized in 150 and 154) with yeast. The PAPS reducing activity in yeast extracts has been analyzed into three protein fractions: enzyme A, enzyme B, and a low molecular weight protein, fraction C. Enzyme A catalyzed the NADPH-dependent reduction of a disulfide group in fraction C, which then participated in the reduction of the active sulfate in PAPS to sulfite, catalyzed by enzyme B.

The subsequent reduction of sulfite to the sulfide level can be catalyzed by sulfite reductase. This activity was found in many organisms (cf. 108) and has been highly purified from yeast, bacteria and higher plant sources (cf. 49 and 108). These pure sulfite reductases, which are all complex enzymes, are capable of catalyzing the 6-electron reduction in the presence of various electron donors. The enzymes purified from bacteria and yeast also possess a nitrite reductase activity which appears to be due to the same enzyme, while the sulfite reductase from higher plants has been reported to lack the nitrite reductase activity (169). This dual activity has raised some question as to the role of the sulfite reductases from yeast and bacteria in sulfate reduction (cf. 48). Nevertheless, extensive biochemical-genetic studies with mutants of *Salmonella* (170), the wide distribution of this enzyme in species capable of reducing sulfate, and its coincident repression by cysteine, along with PAPS reductase (148), indicate that it is involved in the assimilatory pathway of sulfate reduction. No such evidence exists, however, for the sulfite reductase of photosynthetic organisms.

The enzymological studies with the reductase systems of yeast by Bandurski and co-workers (cf. 108 and 154) indicated that a protein-bound sulfite, rather than a free sulfite, was the product of the PAPS reductase and the substrate for further reduction. Thus, the concept of "bound intermediates" during sulfate reduction from PAPS to cysteine emerged. Bound sulfite and bound sulfide species were predicted also because of the toxicity of the free species (49,154). Evidence in support of the concept of a bound sulfite as a product of PAPS reduction has become available with the recent isolation by Wilson (171) of a low moecular weight protein, Pr-SSO_3^-, formed enzymatically in reaction mixtures containing PAPS and enzyme B. These preliminary studies have suggested that the protein is reduced by fraction C prior to the transfer of the sulfonyl group from PAPS and, therefore, appeared to be the bound sulfite in yeast.

Recent studies with extracts of *Chlorella* and spinach, carried out by the groups of Schmidt and Schiff (cf. 48,172-174), focus on the pathway of sulfate reduction in these photosynthetic organisms. This pathway appeared to utilize APS, rather than PAPS, as the physiologically active species for reduction. This APS reducing system involves the transfer of the sulfonyl group of APS to a protein carrier to form a bound sulfite as carrier-S-SO_3^-, catalyzed by the enzyme APS sulfotransferase. This bound sulfite, which closely resembles that of yeast, is then acted upon by a second enzyme, the thiosulfonate reductase, which in the presence of ferrodoxin catalyzes the 6-electron reduction of the bound sulfite to a bound thiol group. The proposed carrier-S-SH is thought likely to participate directly

as the substrate for the cysteine forming enzymes. Thus, under experimental conditions extracts of *Chlorella* and spinach incorporated sulfate directly into cysteine (48,172-174). Because no free intermediates were found, this reductive pathway was termed the "bound intermediates pathway" (cf. 48,174). The enzyme B and $Pr\text{-}S\text{-}SO_3^-$ recently found in yeast (171) appear to be analogous to the enzyme APS sulfotransferase and $Carrier\text{-}S\text{-}SO_3^-$ found in *Chlorella* (172). However, in the photosynthetic organisms, APS appears to be the sulfonyl donor in a sulfotransferase reaction, as opposed to PAPS in yeast and bacteria. The significance of utilizing APS vs. PAPS for sulfate reduction in photosynthetic organisms is by no means clear. It is unlikely to be due to the presence of the DPH activity, nor does it appear to reflect a property of the sulfotransferase, which uses APS as a substrate, as both of these activities have been shown now to exist also in *E. coli* (152), although PAPS is the actual sulfonyl donor in this organism. The suggestion by Schiff and Hodson (48) that the use of PAPS for esterifications and APS for reduction may reflect a regulatory advantage is intriguing. Yet the proposed role for the DPH enzyme as regulating the flow between PAPS and APS is still obscure. The latter enzyme, partially purified from *Chlorella*, also has been reported to catalyze the synthesis of cyclic AMP from APS (153). Convincing evidence that the product is actually cyclic AMP has not been published, however. Additional complications come from the study of APS kinase in spinach extracts (155). The stimulation by 3' AMP of both PAPS formation and the overall rate of sulfate incorporation into cysteine led to the suggestion that PAPS is the

actual sulfonyl donor for reduction in spinach as well (155).

The characterization of the enzyme thiosulfonate reductase in *Chlorella* and spinach, which coexists with the sulfite reductase (48,174) needs some elaboration. Studies with the thiosulfonate reducatse purified from *Chlorella* (173-174) indicated that it could not reduce free sulfite, but carried out the reduction of the sulfonyl group of S-sulfoglutathione ($GS-SO_3H$) to the sulfide level in the presence of NADPH and ferredoxin. S-Sulfoglutathione could not serve as a substrate for the sulfite reductase isolated from *Chlorella*. Thus, it was proposed (172-174) that in *Chlorella* and spinach there are two pathways for sulfate reduction: both begin with the APS sulfotransferase reaction, but one pathway utilizes the thiosulfonate reductase and bound intermediates, while the other employs the sulfite reductase and free intermediates. Because the product of the APS sulfotransferase appears to be a bound sulfite, the reduction via the sulfite reductase requires prior release of the sulfonyl group as free sulfite. The hypothesis that sulfate reduction via the thiosulfonate reductase in *Chlorella* and spinach is the main pathway *in vivo* is supported by a mutant which cannot grow on sulfate, nor reduce it *in vivo*. Extracts of this mutant lack the thiosulfonate reductase activity but possess the sulfite reductase activity (174). Thus, it appears likely that the reduction of sulfate via the thiosulfonate reductase is the preferred pathway *in vivo* in *Chlorella*, while the reduction via the sulfite reductase could be functional when free sulfite is available *in vivo*. The existence of these two pathways for sulfate reduction was recently

reported for *E. coli* as well (152). Thus, studies with genetically defined mutants lacking either thiosulfonate reductase or sulfite reductase or both should now be feasible. Such studies should clarify the roles of the two enzymes in sulfate reduction *in vivo*.

Biosynthesis of Cysteine and Methionine

After its reduction, sulfide is incorporated into cysteine, and cysteine is the source of sulfur in methionine synthesis. The biosynthetic routes for cysteine and methionine in plants are generally similar to those found in bacteria (cf. 49). The formation of cysteine involves two enzymatic steps: (a) the activation of the carbon acceptor, serine, to O-acetyl-L-serine, catalyzed by serine transacetylase; and (b) the synthesis of cysteine from the activated serine and a thiol group, catalyzed by O-acetyl serine sulfhydrylase.

The formation of methionine from cysteine is carried out via the transsulfuration pathway (cf. 49) and the intermediate cystathionine, to form homocysteine which is then transmethylated to methionine.

Sulfate Esters and Sulfonic Acids

Sulfate activation is required prior to the incorporation of sulfate into sulfate esters and sulfonates as well. Sulfate esters are found among animals and plants (cf. 108), but reports on sulfate esters in bacteria are rather rare (cf. 48,133). Animal tissues contain sulfate esters such as steroids, phenols and polysaccharides (cf. 108). Their formation involves the transfer of the sulfonyl group of PAPS to suitable acceptors, in reactions catalyzed by specific sulfotransferases.

The most abundant sulfate ester in plants is choline-O-sulfate. It is found in fungi, algae, and higher plants (132). Additional sulfate esters are the flavonoid sulfates in higher plants (cf. 108), the polysaccharide sulfates in certain algae and *Chlorella* (cf. 48), and the mustard oil glycosides in certain plant species (131). There are no reports on sulfated polysaccharides in higher plants (cf. 48). Little, however, is known regarding the synthesis of the sulfate esters in plants. Incorporation of [^{35}S]-sulfate *in vivo* into polysaccharide fractions in a red alga (111-111a), *Fucus* (130) and *Chlorella* (cf. 48), or into choline-O-sulfate and flavonoid sulfates in fungi, algae and plants (58) have been documented.

Involvement of sulfotransferases and PAPS in these syntheses in the plant kingdom has not been proven. However, it seems likely on the basis of comparative biochemistry (cf. 48).

Among the sulfonates found in plants, the most important one is the ubiquitous plant sulfolipid (sulfoquinovosyl diglyceride), discovered by Benson *et al.* (cf. 135). In spite of its relatively high concentrations in green plants, particularly in the chloroplast membrane, the pathway of its biosynthesis is not known. It has been suggested to proceed from PAPS as the sulfonyl donor and phosphoenolpyruvate as the acceptor (136).

Properties of ATP Sulfurylase

ATP sulfurylase (E.C. 2.7.7.4, ATP:sulfate adenyltransferase) has been detected in bacteria (45,91-92,126,189), animals (157,162, 165), fungi (41,95-96,98-99,140-140a), algae (cf. 48) and in a wide

range of higher plants (45-47,101-105,143-144,156), where it was found in extracts of roots, leaves, shoots and chloroplasts.

Highly purified ATP sulfurylase has now been obtained from yeast (99,140-140a), *Penicillium* (95-96), spinach (45), *Nitrobacter* (107), and rat liver (162). Enzymes from several additional higher plants (46-47,156) and yeast (98) have been partially purified. These enzymes are all soluble and appear to have a broad pH optimum of 7.5-9.0, specificity toward ATP, and a requirement for a divalent cation, usually satisfied by Mg^{+2}. However, there have been reports that purified ATP sulfurylases will catalyze ATP formation (140a, 166-167), and a pyrophosphate-ATP exchange reaction (45) in the absence of added Mg^{+2}. These activities were attributed to the presumed presence of traces of divalent cations in reagent and enzyme solutions. The enzymes from plant sources are relatively independent of sulfhydryl protection compared with enzymes from *Nitrobacter* and liver.

Kinetic studies with ATP sulfurylase are rather limited and are confined mostly to the reverse reaction and the molybdolysis assay (see pp. 24, 35). Based on the formation of ATP from APS and pyrophosphate in plants (65,144,156), fungi (96) and bacteria (107), the reported Km's range from 0.3-7.0 x 10^{-6} M for APS and 0.1-7.0 x 10^{-5} M for pyrophosphate. Studies based on the sulfate-dependent pyrophosphate-ATP exchange reaction with enzyme from several higher plant sources (45-47) yielded Km's of 1-5 x 10^{-3} M for sulfate and 0.25 x 10^{-3} M for ATP. Km's for the molybdolysis reaction have been studied in yeast (66,98) and *Penicillium* (96) and are ca. 6 x 10^{-5} M

for $MgATP^{-2}$ and 2×10^{-4} M for molybdate. Studies based on measurement of accurate initial rates of APS formation have not appeared. On the contrary, many investigators commented that APS was detected only in the presence of inorganic pyrophosphatase (e.g., 45,47,156) and that, even in the presence of a large excess of inorganic pyrophosphatase, the rate of APS formation was not proportional to enzyme concentration (e.g., 47,96). In spite of the fact that the rate of APS synthesis by the ATP sulfurylase from spinach continuously declined during the 1 hour incubation period (45,47), Shaw and Anderson (45) used such data to calculate the Km of 8×10^{-3} M for sulfate and 5×10^{-3} M for ATP. A Km for sulfate of ca. 5×10^{-3} M was also presumed for ATP sulfurylase of yeast (cf. 166), based on the overall production of PAPS from sulfate.

Reported molecular weights for the highly purified enzymes are: 100,000 for yeast (140a), 900,000 for rat liver (162), 700,000 for *Nitrobacter* (107), and 440,000 for the *Penicillium* enzyme (95-96). The latter ATP sulfurylase, which has been extensively characterized (95-96), appears to be an octamer composed of 8 subunits of 56,000 molecular weight each. Additional data suggested that there are 1 cysteine and 4 cystine residues per subunit. Partially purified ATP sulfurylase from corn (156) and beetroot (46) appear to be of 42,000 and 230,000 molecular weight, respectively.

Indications for the existence of isozymes of ATP sulfurylase were found in Furth mouse mastocytoma (165) and beetroot hypocotyl (46), as 2 distinct peaks of activity appeared during ion exchange chromatography.

Specific activity values obtained with the highly purified ATP sulfurylases from different sources are comparable for a given assay. The activities obtained with the molybdolysis assay for ATP sulfurylase from yeast (140a) and *Penicillium* (95) were 42 and 19 μmoles pyrophosphate x min^{-1} x mg $protein^{-1}$, respectively. The yeast enzyme studied by Robbins and Lipmann had activity of ca. 1 μmole APS x min^{-1} x mg $protein^{-1}$. ATP sulfurylase from yeast (98) and rat liver (162) catalyzes the reverse reaction at ca. 0.65 μmoles ATP x min^{-1} x mg $protein^{-1}$.

Reports on inhibition by APS of ATP sulfurylase reactions have been published (66,96,98). However, kinetic evidence suggests that this is a simple product inhibition effect due to the unfavorable equilibrium constant of the forward reaction, as opposed to an allosteric effect (96).

The reaction of APS formation by ATP sulfurylase has generally been regarded as a nucleophilic displacement by sulfate ions on the inner phosphorus atom of ATP, resulting in elimination of pyrophosphate (154,163). However, the low nucleophilicity of the SO_4^{-2}, with the repulsion to be expected from the negatively charged ATP, even in the presence of Mg^{+2}, led to the suggestion of a more probably alternative mechanism in which enzyme-AMP complex is formed prior to the attack by SO_4^{-2} (cf. 108).

A mechanism, in which formation of an enzyme-AMP complex and pyrophosphate release occur prior to the interaction by sulfate, was suggested for highly purified ATP sulfurylase from rat liver (162). This was based largely on the finding that the enzyme catalyzed the

pyrophosphate-ATP exchange reaction in the absence of sulfate ions. This mechanism, consistent with a ping-pong type (Cleland, cf. 65), also led to the prediction of exchange between the sulfur atoms of APS and sulfate. However, this exchange could not be demonstrated in the absence or presence of other substrates (162).

In contrast, the few thorough kinetic studies of the highly purified ATP sulfurylases from *Penicillium* (96), yeast (98) and spinach (65) are all consistent with a sequential type mechanism (Cleland, cf. 65) for the reaction, in which both substrates bind to the enzyme before either product is released. The following data support a sequential mechanism: (a) initial velocity patterns obtained by the molybdolysis assay (96,98) or by the formation of ATP from pyrophosphate and APS (65) showed that the Km for each substrate varies with the concentration of the alternate substrate, and that there is no irreversible step between the addition of the two substrates; (b) initial velocity and dead-end inhibitor studies during the equilibrium conditions of the sulfate-dependent pyrophosphate-ATP exchange reaction catalyzed by the spinach ATP sulfurylase (65) likewise yielded patterns which were consistent with a sequential mechanism; (c) isotope exchange studies demonstrating that the ^{32}P exchange between Pp_i and ATP was catalyzed by ATP sulfurylase of spinach (16,45) and *Penicillium* (96), but only when sulfate was present. Likewise, the ^{35}S exchange between SO_4^{-2} and APS catalyzed by the yeast enzyme (98) was completely dependent on the presence of either pyrophosphate or $MgATP^{-2}$.

Furthermore, product inhibition studies of both the reverse reaction and exchange reaction, catalyzed by the spinach ATP

sulfurylase (65) indicated an ordered sequential mechanism, in which $MgATP^{-2}$ is the first substrate to react with the enzyme, and $MgP_2O_7^{-2}$ is the first product released. The forms of sulfate and APS in this proposed sequence of the reaction is not known, but according to this proposal, the PPi-ATP exchange could proceed in the absence of free APS (65).

Although this ordered sequential mechanism requires an exchange of ^{35}S between SO_4^{-2} and APS as well, this could not be detected under a variety of conditions, for some unknown reason, for the spinach enzyme (65). Such ^{35}S exchange was only reported for the yeast enzyme (98).

The conflict in evidence concerning the mechanism of the ATP sulfurylase reaction, obtained with the plant enzymes and the rat liver ATP sulfurylase, is difficult to account for on the basis of organism differences, because sequential addition of substrates to ATP sulfurylase of Furth mouse mastocytoma was indicated (164).

Assay for ATP Sulfurylase Activity: Difficulties in Perspective

In the past it has been difficult to conduct studies designed to elucidate the regulation of ATP sulfurylase because of the limitations of available assay procedures (cf. 108).

The most direct method to assay ATP sulfurylase activity is the determination of the rate of APS formation upon incubation of the enzyme with sulfate and ATP. However, there are two major obstacles to this assay method: (a) the very unfavorable equilibrium constant of the forward reaction, of ca. 10^{-8}, which precludes the accumulation of more than one or two nmole/ml of APS; this has been found to

be true even in the presence of inorganic pyrophosphatase; and (b) the lack of a specific chemical method for direct quantitation of APS (cf. 108,167). As a result, many of the studies of ATP sulfurylase were carried out with assays of the reverse reaction (eq. 6), which the equilibrium constant favors, the products being ATP and pyrophosphate, for which there are highly specific assays.

$$APS + PPi \xrightleftharpoons{Mg^{+2}} ATP + SO_4^{-2} \qquad \text{(eq. 6)}$$

Thus, the extensive characterization of the ATP sulfurylase of yeast, carried out by Robbins and Lipmann (140-140a), employed the reverse reaction in which ATP was measured spectrophotometrically in a coupled system with glucose-6-phosphate dehydrogenase, hexokinase, and NADP, while pyrophosphate was estimated as phosphate released after hydrolysis with inorganic pyrophosphatase. The highly purified enzyme from yeast (140a) exhibited 1:1 stoichiometry between ATP formed and pyrophosphate consumed. In crude extracts of yeast, however, the presence of inorganic pyrophosphatase activity interfered with the assay (140). The disappearance of pyrophosphate in the back reaction has been used to assay ATP sulfurylase partially purified from animal sources (157,165). The high level of inorganic pyrophosphatase found in plants (e.g., 45-47,102-104) precludes the use of the pyrophosphate disappearance assay. The hexokinase and glucose-6-phosphate dehydrogenase coupled assay of ATP production was used in studies of ATP sulfurylase purified from *Penicillium* (95,96). Assays based on the reverse reaction in which ATP formation was followed by the bioluminescence generated by the

ATP-dependent luciferin-luciferase system (of the firefly) were carried out in studies of ATP sulfurylase of *Nitrobacter* (144), spinach (107,158), corn (156) and enzyme from animal sources (162, 165). Alternatively, the formation of [^{32}P]-ATP from [^{32}P]-pyrophosphate was used for the spinach enzyme (65). Assays based on ATP formation are not, however, suitable for crude extracts from plants, because of their high ATPase activity (e.g., 43,45-47, 95,102-103,158).

In their early studies with the yeast ATP sulfurylase, Robbins and Lipmann (140-140a) also measured the disappearance of APS by the reverse reaction (eq. 6). Their assay method was based on the enzymatic conversion of APS to PAPS which is then measured by a second enzymatic assay. The latter involved the enzymatic transfer of the activated sulfate group from a carrier to a phenol acceptor, catalyzed by a phenyl sulfotransferase. In both the direct transfer (159) and the catalytic PAPS-PAP assay (160) described in eq. 7 and eq. 8, respectively, PAPS is measured by the colorimetric disappearance (eq. 7) or appearance (eq. 8) of p-nitrophenol.

$$\text{p-Nitrophenol} + \text{PAPS} \longrightarrow \text{PAP} + \text{p-Nitrophenyl-sulfate} \qquad \text{(eq. 7)}$$

$$\text{p-Nitrophenyl-sulfate} + \textbf{Phenol} \xrightarrow[\text{(or PAP)}]{\text{PAPS}} \text{p-Nitrophenol} + \text{Phenyl-sulfate} \qquad \text{(eq. 8)}$$

This indirect APS determination is, however, not suitable for assaying APS formed or consumed by crude preparations of ATP sulfurylase from which APS kinase and the transferase activities have not been removed. Also, the conversion of APS to PAPS upon addition of APS kinase would be difficult to accomplish in the presence of enzymes

which degrade PAPS (see above), as was noticed in extracts from liver (140a). Therefore, this method for APS determination was not used in subsequent studies of ATP sulfurylase.

The equilibrium barrier of the forward ATP sulfurylase reaction can alternatively be overcome by assay methods based on an exchange reaction. An ATP sulfurylase assay based on the sulfate-dependent exchange of [^{32}P]-pyrophosphate into [^{32}P]-ATP was used by Bandurski and Wilson (41,43) for the yeast enzyme, and recently was adapted by Anderson's group (161) for studies of ATP sulfurylases from higher plants (44-47,65). In addition to the interference by ATPase and pyrophosphatase, which excluded the use of this assay in unpurified extracts (45-47), this assay requires the separation of ATP from pyrophosphate. Under the conditions used by Anderson *et al.*, they could not detect exchange of ^{35}S between SO_4^{-2} and APS (65), nor was the exchange of [^{32}P]-pyrophosphate into [^{32}P]-ATP always associated with formation of an isolatable adenylate-anion mixed anhydride (47). Consequently, there is some question as to whether or not the enzymes of Anderson *et al.* actually catalyzed APS synthesis under their exchange assay conditions. Formation of the mixed anhydride need not occur if the ping-pong mechanism is correct.

The molybdolysis assay developed by Wilson and Bandurski (41, 43), in which molybdate is used as an analog of sulfate, is perhaps the simplest assay of ATP sulfurylase. Unlike the assays described above, ATP sulfurylase activity is measured in the forward reaction, as molybdate-dependent pyrophosphatase (or phosphate) released,

presumably via [APMo], a hypothetical unstable analog of APS. This assay has been widely used in studies of ATP sulfurylase (e.g., 91, 93,96-98,102). A high ATPase activity in plants (see above) has been a major obstacle to the use of this assay. Additional difficulties releated to non-enzymatic phosphate release and other molybdate effects have been reported (e.g., 75,98,190).

None of the assays mentioned above is suitable for measuring the physiologically significant reaction, APS formation, in enzyme preparations. With an equilibrium constant of ca. 10^{-8}, an assay method is required to detect and quantitate pmole amounts of APS. The enzyme coupled assay for APS developed by Robbins and Lipmann (140-140a) was, unfortunately, not sensitive to concentrations of APS below 5 nmoles/ml (140) and, therefore, was not suited for assaying APS formed from sulfate, even with the highly purified ATP sulfurylase of yeast (140a), since the equilibrium concentration of APS did not exceed 2 nmoles/ml.

The required sensitivity for assaying such low concentrations of product can be achieved by assaying the incorporation of [^{35}S]-SO_4^{-2} into [^{35}S]-APS. This radioisotope assay unfortunately has not been employed in detailed kinetic or regulatory studies of ATP sulfurylase, because of the lack of a method for rapid separation of the excess [^{35}S]-SO_4^{-2} from [^{35}S]-APS in large numbers of samples. The techniques which have been used in the past for such a separation were preparative chromatography and electrophoresis (e.g., 41,104, 156). These methods are, nevertheless, laborious for use in purification, kinetic or regulation studies in which rapid performance

of multiple assays is needed. Separation of the [^{35}S]-APS from [^{35}S]-sulfate by adsorption to charcoal was also used in conjunction with the radioisotope assay (e.g., 45,96). This, however, was not sensitive enough to detect the small amounts of APS formed (45,96). Also, many investigators indicated that the charcoal treatment caused a marked degradation of APS (cf. 69). As a result, the formation of [^{35}S]-APS has usually been used only for the rigorous identification of APS as the product of a putative ATP sulfurylase reaction, and one of the more convenient assays (such as molybdolysis, the reverse reaction, or pyrophosphate-ATP exchange reaction) has then been used in subsequent studies (e.g., 45,95-96,98,107,144).

A radioisotope assay has been used most frequently for studies with crude extracts of bacteria (e.g., 126,151,193) where the [^{35}S]-SO_4^{-2} is incorporated into [^{35}S]-PAPS via [^{35}S]-APS (154). This, however, measures the combination of ATP sulfurylase and APS kinase activities.

Control of the Sulfate Assimilation Pathway

Evidence based on biochemical and genetic studies indicated that in bacteria and fungi the sulfate assimilation pathway is regulated in response to specific changes in the availability of environmental sulfur. Studies carried out by Dreyfuss, Monty, and Kredich and Tomkins in *Salmonella typhimurium* (cf. 126), and by Pasternak, Ellis, Wheldrake, and Jones-Mortimer in *E. coli* (cf. 185), showed that the enzymes involved in sulfate assimilation are subject to both negative and positive control. Cysteine, the end product of the pathway, is an allosteric inhibitor of both the sulfate permease and serine

transacetylase. In addition, all the enzymes which catalyze the assimilation of sulfate to cysteine are subject to repression[a] control by cysteine. The syntheses of these enzymes which are dependent upon a decline in the intracellular concentration of cysteine, also require O-acetylserine (OAS) which functions as an internal inducer, and an intact *cys* B region (126,185), which appears to code for a protein (188). The nature of the interactions between OAS and the *cys* B gene product is not known, nor is the molecular mechanism of the cysteine-mediated repression.

The activity of ATP sulfurylase in *E. coli* (189-190) and *Salmonella* (126) is repressed by growth on cysteine and derepressed by slow growth on djenkolate or glutathione sulfur compared with growth on sulfate. If sulfate or cysteine is added with glutathione or djenkolate, the ATP sulfurylase level remains repressed (189). The sulfur sources do not appear to act by inhibition or activation of the enzyme. This is evident from assays of mixed extracts from repressed and derepressed cells (126), as well as by the lack of effect of sulfur compounds when added to ATP sulfurylase *in vitro* (189). Thus, the changes in the level of ATP sulfurylase activity in response to the sulfur source are likely to involve regulation of the synthesis of the enzyme (126,189). There is evidence that in

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[a]The terms, "induction", "repression", and "derepression" are used in this dissertation to describe changes in rates of development of total extractable ATP sulfurylase activity. It should be understood that the molecular bases of these changes are not yet known, either in the tobacco cell system or in any other system so far described in the literature. Thus, throughout the dissertation, changes in ATP sulfurylase activity should be considered cases of *apparent* induction, repression or derepression.

these bacteria cysteine (or a close derivative) is the corepressor and the activity of ATP sulfurylase is inversely related to the intracellular concentration of cysteine (193). The slower growth of these bacteria on glutathione or djenkolate compared with sulfate or cysteine (126,189,191-192) indicated that the ability to derepress enzymatic activity by these sulfur sources was probably due to their slower conversion into cysteine. This is in accord with the concept developed by Moyed and Umbarger (cf. 191) that slow growth of an organism on a certain nutrient may be the result of the low rate of its conversion into a repressing metabolite, and consequently may be associated with a derepression of the enzymatic pathway leading to this metabolite. Djenkolate was selected originally by Dreyfuss and Monty (191-192) because of the characteristic slow growth of *Salmonella* on it as a sulfur source.

L-Djenkolic acid (L-cysteine thioacetal of formaldehyde) is, however, of plant origin and was first isolated from the Djenkol bean (cf. 194). It is among the many cysteine derivatives which occur naturally in plants as non-protein amino acids (195). Neither its function nor its metabolism in plants is known. The assimilation of the djenkolate sulfur in *Salmonella*, though not known, was not via inorganic sulfur intermediates because it supported growth of mutants defective in the sulfate assimilation pathway (192).

In both *E. coli* and *Salmonella*, methionine, which is synthesized via cysteine, does not appear to be involved directly in the regulation of the enzymes of sulfate assimilation (196).

Changes in the level of ATP sulfurylase specific activity, in response to sulfur nutrition, have been also noticed in several fungi,

such as yeast (24-27,66-67), *Aspergillus* (129), *Penicillium* (96), and *Neurospora* (75). Among the sulfur compounds tested, neither cysteine, methionine, glutathione nor choline-O-sulfate produced any effect on the activity of these enzymes *in vitro* (66,96,98). Sulfide was the only sulfur compound found to inhibit ATP sulfurylase activity *in vitro* in yeast (66,98) and *Penicillium* (96) in a way which suggested its being an end-product inhibitor of the enzyme.

In *Aspergillus*, a decrease in ATP sulfurylase activity of 30-60% was observed in the presence of L-cysteine or L-methionine in the growth media (129). Studies with mutants, however, indicated that cysteine rather than methionine is mediating the repressive response (129).

On the other hand, in yeast (66) and *Penicillium* (96), methionine was found to be the only repressive sulfur source, since growth of these fungi on cysteine resulted in an actual increase in ATP sulfurylase specific activity compared with growth on sulfate. Examination of the studies in yeast (66) may account for these surprising results, because cysteine was a rather poor sulfur source to support growth of yeast compared with sulfate and methionine. Thus, the derepression of ATP sulfurylase by cysteine in yeast may have come abount by mere sulfur starvation. No growth data for the various sulfur sources tested in *Penicillium* were reported (96). But neither growth on djenkolate, nor sulfur starvation, had a strong derepressive effect on the ATP sulfurylase level (96), although both are very effective in derepressing the sulfate uptake system of *Penicillium* (40,71). It is therefore likely that the growth of this fungus in

this study (96) was not completely dependent on the sulfur source added.

Nevertheless, the conjectured conclusion that ATP sulfurylase activity in yeast is repressed by methionine and derepressed by cysteine (66) was interpreted by De-Robichon-Szulmajster and co-workers (cf. 23) to mean that in yeast, methionine rather than cysteine is the regulator of the sulfate assimilation pathway. That laboratory also developed the concept that in yeast, methionine synthesis is independent of the cysteine formation and linked directly to the sulfate reduction pathway by the direct sulfhydration pathway (23). This conclusion was recently refuted by Flavin *et al.* (cf. 49), who have shown that methionine is formed via the transsulfuration pathway, i.e., via cysteine, in yeast as well.

Nevertheless, the regulation by methionine and its derivatives of the sulfate assimilation pathway in yeast has been reported in many studies carried out by De-Robichon-Szulmajster's group. They presented an elaborate scheme for a 2-level control of 4 different enzymes (methionine Group I enzymes) including ATP sulfurylase, sulfite reductase and 2 enzymes of the direct pathway of methionine biosynthesis (24-28,67). The activities of methionine Group I enzymes were reported to be coordinately repressed by the addition of either methionine or S-adenosylmethionine (SAM) to the media, and coordinately derepressed by methionine-limited growth (24-28,67). According to their scheme, the mechanism of the methionine-mediated repression of these enzymes is distinct from the SAM-mediated response (26,67). The former involves binding of a "regulatory methionyl-$tRNA^{met}$" with aporepressor protein to form aporepressor-

corepressor complex which is hypothesized to act as a repressor at the transcriptional level (25,27). On the other hand, SAM is proposed to cause repression by acting upon the translational level (26,28,67). It is my opinion, however, that these interpretations of their data should be taken with caution, because none of the regulatory elements has been rigorously identified. It is nevertheless apparent from their results that ATP sulfurylase in yeast is indeed subject to regulation by sulfur nutrition as was shown earlier by De-Vito and Dreyfuss (66). However, conclusions regarding the mechanism of this control must await further studies.

Additional complications are evident from studies with *Neurospora*, by Metzenberg, Marzluf and co-workers (cf. 197). Although the process of sulfur entry into the fungal mycelia is regulated by a complex system of both positive and negative signals, in response to sulfur availability, ATP sulfurylase activity responded differently (75,197). Conditions of sulfur sufficiency (high methionine),which strongly repress the activity of the sulfate permeases of *Neurospora*, increased the activity of ATP sulfurylase (75), while sulfur-starvation, which increases the sulfate transport rate, inhibited development of the ATP sulfurylase activity.

Limited studies with higher plants are likewise confusing. There is evidence indicating that the sulfate transport system of higher plants is subject to regulation by sulfur nutrition. Hart and Filner (123) showed that both cysteine and methionine act as end-product inhibitors of the sulfate uptake in tobacco cells. Smith (124) extended this study to demonstrate an increase in the rate of

uptake upon sulfur starvation. Higher rates of sulfate transport upon sulfur deficiency were reported earlier for clover plants (128). Nevertheless, studies carried out by Ellis (104) with several higher plants, including *Lemna*, grown on either sulfate, cysteine, methionine or glutathione, failed to yield expected changes in ATP sulfurylase activities in response to the various conditions of sulfur nutrition. The activities observed after growth in the presence of these organic sulfur sources were somewhat higher than in plants utilizing sulfate. These results are difficult to account for because, in the *Lemna* cultures, unlike the seedlings, growth was dependent upon the sulfur source added. But the activity which developed upon sulfur starvation, or slower growth on cysteic acid, were not tested. Therefore, Ellis' conclusion (104) that ATP sulfurylase activity in higher plants is not repressed by cysteine or methionine, does not exclude the possibility of its regulation in response to sulfur-limited growth. Indications that sulfur nutrition influenced the ATP sulfurylase activity came from preliminary studies in developing soybean seedlings (103), where a 2- to 2.5-fold increase in activity was found in leaves of plants that grew in the absence of sulfate in the nutrient solution. These changes were detected after many days, suggesting that an endogenous supply of sulfur existed. Therefore, studies of changes in enzyme levels by nutritional factors need to be carried out only under strictly controlled nutritional conditions.

Group VI Anions and the Sulfate Transport System

Oxygen, sulfur, selenium and tellurium constitute Group VIA of the periodic table of elements. Chromium, molybdenum, and tungsten make up Group VIB.

There is abundant evidence in the literature that sulfate, selenate, molybdate, tungstate and chromate are all actively taken up by a common permease, i.e., the sulfate transport system in bacteria, fungi, algae, and higher plants.

Sulfate transport is inhibited specifically by one or more of the Group VI anions in, e.g., *E. coli* (89), *Salmonella* (77), *Neurospora* (74,84), *Chlorella* (39,81), *Euglena* (88), *Scenedesmus* (109) and higher plants (38).

The inhibition of sulfate uptake by one or more of these anions was demonstrated to be competitive in *Salmonella* (78), *Aspergillus* and *Penicillium* (40), *Neurospora* (74), *Chlorella* (39,81), the red alga *Porphyridium* (111), and barley roots (36-37,85).

The transport of the Group VI anions in general has the same dependencies, sensitivities, etc., as sulfate uptake in the above described systems, and Km's and transport rates similar to those for sulfate have been observed (40,74,81). Furthermore, the transport system for selenate, molybdate, or chromate of *Salmonella* (78), *Aspergillus* and *Penicillium* (40), *Neurospora* (74) or *Chlorella* (83) is repressed by the same sulfur sources that repress the active transport system for sulfate. On the other hand, under conditions which derepress the sulfate transport system, a coincident derepression of transport of the Group VI analogs occurs. In addition,

mutants of *Penicillium* (40), *Neurospora* (74), *Aspergillus* (76) and *Salmonella* (77-78), defective in sulfate transport, were equally defective in transporting the Group VI analogs. Other studies indicated that Group VI anions are also analogs of sulfate in the processes of efflux of internal sulfate in *Neurospora* (79) and the transinhibition of the sulfate permease in *Penicillium* (72), both of which are believed to be mediated by the sulfate transport system. The toxicological effects of Group VI anions often observed in both microorganisms (39,73-74,76-77,89,121) and higher plants (cf. 33) have been attributed to their entry via the sulfate transport system, since resistance to the toxic anion(s) was seen: (a) in mutants lacking the sulfate transport system (74,76,78); (b) during growth in the presence of sulfur sources which repressed the sulfate permease (74,78,89,121); and (c) by increasing the [sulfate]/[anion] molar ratio in the growth medium (39,60,78, cf. 33). Under all these conditions resistance to an anion involved its exclusion from the cells. The particularly strong toxic effect of chromate was utilized by Pardee and co-workers (77) in studies with *Salmonella*, and later by Marzluf's group (73-74) with *Neurospora*, for selecting mutants lacking the sulfate transport activity, which are resistant to chromate.

Interactions of Group VI Anions with ATP Sulfurylase

The concept that Group VI anions can function as structural analogs of sulfate in reactions catalyzed by ATP sulfurylase developed out of the studies by Wilson and Bandurski (41-43) with ATP sulfurylase partially purified from yeast. The enzyme acted upon either sulfate, selenate, molybdate, chromate or tungstate as substrates. The nature

of the reaction, however, varied with the added anion. In the presence of molybdate, chromate, or tungstate, no stable mixed anhydride of adenylate and anion was formed, but the enzyme catalyzed an anion-dependent cleavage of ATP to AMP and pyrophosphate. In contrast, with sulfate or selenate as substrates, only small amounts of pyrophosphate were formed. Both, however, supported an anion-dependent exchange reaction of [^{32}P]-pyrophosphate with ATP, which was not observed with the Group VIB anions. Indications that the seleno-analog of APS was formed by the yeast enzyme were provided (41), but the synthesis of APSe has still not been established rigorously.

The anion dependent release and accumulation of pyrophosphate from ATP was documented also for ATP sulfurylase of spinach (101) and bacteria (91).

Using ATP sulfurylase prepared from several higher plants, Anderson and co-workers (44-47) have verified that only selenate will substitute for sulfate in the [^{32}P]-PPi/ATP exchange reaction.

Based on their finding, Wilson and Bandurski (41) developed the so-called molybdolysis assay for ATP sulfurylase: measurement of molybdate-dependent release of phosphate from ATP, in the presence of ATP sulfurylase and inorganic pyrophosphatase. This assay has played a major role in many studies of ATP sulfurylase. Also, out of these studies came the recognition that Group VI anions are specific inhibitors of reactions catalyzed by ATP sulfurylase.

The molybdolysis assay of ATP sulfurylase has been employed in studies of the enzyme isolated from bacteria (91-92,97), animals

(93-94), fungi (41,66-67,95-96,98-100), algae (cf. 48) and higher plants (97,101-103). The molybdate-dependent release of pyrophosphate was inhibited upon addition of sulfate (41,66,96,98,102).

Group VI anions also inhibit other reactions catalyzed by ATP sulfurylase. Thus, molybdate, selenate, tungstate and chromate inhibited the incorporation of [^{35}S]-sulfate into APS (47,104,107) and PAPS (105-106) by enzymes isolated from *Nitrobacter* (107), higher plants (47,104-105) and the alga *Ochromonas* (106). Molybdate and selenate inhibited the sulfate-dependent pyrophosphate exchange reaction (45) catalyzed by ATP sulfurylase purified from spinach leaves. Anions of Group VI also inhibited sulfate reduction by extracts of bacteria and yeast (cf. 108) and the incorporation of sulfate *in vivo* into an organic cell constituent in algae (111), where sulfate uptake is not affected. These effects were attributed to the inhibition by the anion of ATP sulfurylase activity.

Among Group VI anions, molybdenum is the only one, in addition to sulfur, for which a nutritional requirement of plants has been established conclusively (116). Molybdenum is a constituent of molybdo-enzymes such as nitrogenase (120), nitrate reductase (117) and others (cf. 117). However, there is no evidence which suggests that molybdate needs to be acted upon by ATP sulfurylase in order to perform its function in these enzymes.

Chromium, tungsten and tellurium are not regarded as nutrients in plants, and they do not appear to be further incorporated into analogs of sulfur-compounds, via ATP sulfurylase (113,118-119). In contrast, the incorporation of selenate into organo-seleno

compounds is widely known in various organisms, including plants. Since 1880, when the concept of a biological analogy between sulfur and selenium was expressed by Cameron (cf. 33), it became increasingly evident that the sulfate assimilation pathway has a key role in selenium metabolism.

Selenium: Micronutrient Role in Bacteria and Animals

The discovery of selenium deficiency syndromes in both animals (7) and bacteria (2) indicated a functional role for selenium during normal growth and development of these organisms. The essentiality of selenium in these systems became evident as several distinct selenium-containing proteins have been identified. This includes the formate dehydrogenase (1,3-4,13) and Protein A of the glycine reductase (1) of bacterial origin, glutathione peroxidase of animal origin (5-6,14-15) and several others (cf. 1,16-17). As indicated by the deficiency symptoms, selenium has an indispensable role in dtermining the activity of these proteins. The selenium of the functional selenoproteins is thought to represent a unique organo-selenium compound (1,14,17), and a carrier role in electron transfer reactions has been postulated (cf. 1,4,6,14). Nevertheless, the exact chemical nature and the function of the seleno groups in these proteins remain to be elucidated.

The occurrence of specific proteins with a high content of selenium (1,6,13,15) reflects a very selective incorporation process which functions with selenium in the micromolar range of concentrations (1,3-4,6,15). This unique distribution pattern necessitates

the existence of at least one selenium-specific step (possibly branching off of the sulfate pathway).

At high concentrations, however, selenium becomes very toxic and leads to inhibition of growth and metabolic disorders in animals (12) and bacteria (11).

The biochemical events underlying the utilization of selenium as a required nutrient, though unknown, are likely to be, at least partially, different than those involved in selenium toxicity. The latter is thought to be a result of usurpation of the sulfur pathway and nonselective replacement of sulfur by selenium that causes lethality.

Selenium Metabolism in Plants: Selenium Accumulators

The pioneering work of Beath *et al.* (cf. 31), after the implication of selenium as the toxic agent of certain range plants that caused livestock disorders, led to the concept of selenium indicator plants. This small group of plants, representing only several species of a few genera, are restricted in their distribution to seleniferous soils from which they extract extremely high levels of selenium and accumulate it in organic compounds. Selenium accumulator plants characteristically contain up to 10,000 times more than the few ppm of selenium found in most plants. Moreover, whereas selenium is toxic to most plants at very low concentrations (see below), no harmful effects were detected in the selenium accumulators (31,33). Nevertheless, a micronutrient role for selenium in the accumulator species is still a matter of conjecture (cf. 32-33).

Biochemical studies indicated that the accumulator plants, in contrast to bacteria and animals, contain selenium exclusively as low molecular weight soluble organo-seleno compounds, with negligible traces in the protein fraction (34-35). The soluble compounds were identified as seleno-amino acids, of the non-protein type, and the low molecular weight peptide derivatives (32-33). In general, as was pointed out by Shrift (cf. 32-33), the dominant species was Se-methylselenocysteine accompanied by selenohomocysteine, seleno-cystathionine, γ-L-glutamyl-Se-methylselenocysteine and several other unidentified peptides.

In non-accumulator plants, however, exposure to selenium resulted in a predominant incorporation of the element into the protein fraction, presumably as selenocysteine and selenomethionine (34-35). Se-methylselenomethionine was the most abundant soluble compound in non-accumulator plants with little or no trace of the soluble seleno-compounds characteristic of the accumulator plants (32-33).

These studies indicated two important features of selenium metabolism in plants: (a) that there are physiological and bio-chemical differences between accumulator and non-accumulator plants with respect to selenium; and (b) in both classes, selenium is incorporated exclusively into analogs of sulfur compounds, known to occur in plants.

The Relationship Between Selenium Metabolism and the Sulfate Assimilation Pathway

The recovery of selenium in plants as reduced seleno-amino acid derivatives, all of which are sulfur analogs, suggested that selenate

and sulfate are likely to be coassimilated via a common pathway (cf. 33). Although it is still a matter of contention, both enzymatic studies *in vitro* and feeding experiments *in vivo* support this hypothesis.

Evidence that selenate is transported by the sulfate permease in many systems, including accumulator and non-accumulator species (38), was presented in the previous section. It is also conceivable that selenate is activated by ATP sulfurylase (see above). Anderson and co-workers (44-47) compared the activities of ATP sulfurylase purified from several accumulator and non-accumulator plants, with either sulfate or selenate as a substrate in the pyrophosphate-ATP exchange reaction. Their results can be summarized as follows: (a) selenate replaced sulfate as an alternative substrate in all the 8 enzymes tested; (b) the kinetics of sulfate-selenate competition were consistent with 2 substrates competing for a single enzyme; (c) both the selenate and sulfate activities were inseparable, and maintained a constant ratio during purification; (d) the Km for selenate in all the enzymes examined was consistently lower than the Km for sulfate; (e) the Km's of ATP sulfurylase for selenate and sulfate in accumulator plants were almost identical to those found in the non-accumulator species. The same was true for the Vmax values of the two anions; (f) the ATP sulfurylases of accumulators and non-accumulators were very similar in all other aspects studied. These results, however, are based on the ability of selenate to participate in the pyrophosphate exchange reaction only. Attempts to demonstrate an APSe formation from selenate, with enzyme from

either accumulators or non-accumulators, so far have been unsuccessful (47). This was not due to the formation of an unstable, rapidly hydrolyzed mixed anhydride of AMP and anion, as AMP was not detected either. An enzyme-bound APSe was hypothesized by the authors as compatible with the proposed mechanism for ATP sulfurylase (65).

Based on these findings, it appears likely that selenate can enter the sulfate assimilation pathway via ATP sulfurylase. Unfortunately, nothing is known about the subsequent reactions which presumably involve the reduction of selenate to the selenide level.

The predominant incorporation of labeled selenate, in a variety of plant species, into the seleno-analogs of cysteine (34-35,52), methionine (34-35,50,52-53,62), S-methylcysteine (51,62), S-methylmethionine (50-51), and the identification of the seleno-analogs of cystathionine (35,55,57,61) and homocysteine (55), in plants that actively metabolize selenium, suggested that the seleno-amino acids are synthesized in parallel with cysteine and methionine (cf. 49), possibly catalyzed by the same enzymes.

Studies with labeled precursors, in which the *in vivo* formation of Se-methylselenocysteine (the dominant seleno-product among selenium accumulators) was compared with the synthesis of the corresponding S-methylcysteine, also pointed to the likelihood of a common enzymatic path.

Thus, the *in vivo* incorporation of radioactivity from carbon precursors (56), selenium precursors (55,57) and methyl donors (55-57) into Se-methylselenocysteine were all consistent with the synthesis of S-methylcysteine by subsequent methylation of the preformed

cysteine (55-57). The *in vivo* studies further indicated that seleno-methionine participated as methionine in both, as methyl donor and as methyl acceptor for the transmethylation enzyme (55,57). The latter was also shown *in vitro*, where selenomethionine was activated, as a substrate that is competitive with methionine, by the methionyl adenosyl transferase of yeast (68, cf. 1).

Plants, however, have a wider range of sulfur-containing compounds (48,108), some of which are believed to be synthesized by a pathway separate or branched from the sulfate reduction pathway (48). Attempts to show the incorporation of selenate into seleno-analogs of either choline sulfate, flavonoid sulfates, sulfolipids (58), or glutathione (50-51) in several lower and higher plants, including selenium accumulators, were unsuccessful. The synthesis of PAPSe by the yeast enzyme (58) could not be demonstrated either. But recently, the seleno-analog of the mustard oil glucoside, sinigrin, was identified in non-accumulator plants (63).

Selenium: Resistance and Toxicity

The ubiquity of selenium toxicity throughout the plant kingdom (31,33) is believed to result also from the lethal incorporation of the seleno-analogs into cell protein (32-33). Various sulfur compounds are, therefore, able to counteract the toxic effect of selenium (33). The selenium accumulator plants, however, exhibit a unique resistance to selenium. The exact relation of the seleno-compounds found in accumulator species to the resistance phenomenon is not known. However, Peterson and Butler suggested (35) that the accumulation of seleno products in these plants represents a detoxification

mechanism, whereby selenium is shunted into certain innocuous, non-protein seleno-amino acids, as a step in a mechanism for exclusion of selenomethionine and selenocysteine from protein. This mechanism, which probably involves more than one step (35), is thought to have evolved in the accumulator plants and could be a factor in the tolerance of these species to high levels of selenium. In the absence of such a mechanism in non-accumulator plants, the incorporation of selenium into protein amino acids (34-35) would result in lethal synthesis of non-functional protein and consequent death (35). This hypothesis is compatible with numerous physiological and biochemical observations, as indicated above, but the crucial comparative studies of the specific enzyme systems which were postulated to play a role in the detoxification mechanism of accumulator plants (35) have not yet been done. For example, the suggestion of Peterson and Butler (35) that the specificity of the amino-acid activating enzymes of accumulator species might participate in the exclusion mechanism of the seleno-analogs of cysteine and methionine from protein has not been investigated. This proposal is especially attractive because: (a) an example of differentiation between a natural amino acid, proline, and its toxic analog, azetidine-2-carboxylic acid, is afforded by the prolyl-tRNA synthetases of certain species which differentiate between proline and the analog, whereas the synthetases of other plants are unable to differentiate between the two; the proline analog is toxic only to those species in which no discrimination against the proline analog occurs (Peterson and Fowden, cf. 44); and (b) the activation of selenomethionine was

shown to occur by the methionyl-tRNA synthetase, with the met-tRNA as an acceptor, purified from *E. coli* (20), rat liver (21), and more recently from yeast (68), all organisms which incorporate seleno-methionine into protein, and are subject to selenomethionine toxicity.

MATERIALS AND METHODS

Separation of Sulfate and APS by Differential Solubility of the Two Compounds in Ethanol-Water Mixture

The method for the separation of APS from sulfate (Figure 1 on p. 59) is based on the relative insolubility of Na_2SO_4 in ethanol: water (5:1) compared to Na_2APS. Ice cold absolute ethanol, 2.5 ml, is added to 0.5 ml reaction mixture containing 20 mM [^{35}S]-Na_2SO_4 (ca. 10^8 cpm), and [^{35}S]-APS in the range of 10 pmoles-10 nmoles (up to 9×10^4 cpm). The precipitate (PPT) of [^{35}S]-Na_2SO_4, which forms immediately upon the addition of ethanol, is then removed by centrifugation at 45,000 xg for 10 minutes (Sorval SS-34 rotor), and then discarded. The resultant supernatant fraction is then subjected to three additional precipitations, each consisting of the addition of 200 µmoles of carrier Na_2SO_4, in 0.2 ml water, followed by centrifugation at 45,000 xg for 10 min and discarding of the precipitate. [^{35}S]-APS is recovered in the final supernatant solution (Figure 1). All steps were carried out at 4 C using 15 ml Corex tubes.

In experiments designed to follow the efficiency by which sulfate was precipitated during the separation technique (Figure 1), the four successive precipitates were dissolved in water and aliquots were taken for the [^{35}S] determination by scintillation counting.

When the recovery of [^{35}S]-APS in the final supernatant fraction after the 4 precipitation steps (Figure 1) was investigated, radiochemically pure [^{35}S]-APS of high specific activity (see below) was used. This [^{35}S]-APS was added in 0.05 ml to the complete reaction mixtures free of [^{35}S]-Na_2SO_4 at the end of the incubation period, just prior to the addition of ethanol. Upon completion of the 4 steps of separation (Figure 1), the [^{35}S] derived from the added [^{35}S]-APS was determined in the 4 precipitates and the final supernatant solution by scintillation counting. The identity of the ethanol-soluble [^{35}S] as [^{35}S]-APS was verified by various methods (see below).

Determination of Radioactivity

Aliquots of 0.05-0.3 ml were pipetted into scintillation vials. Radioactivity was determined by liquid scintillation counting (Beckman LS-133), with 10 ml per vial of scintillation fluid prepared according to Formula II of Research Products International Corporation: 100 g of naphthalene, 5 g of PPO, 0.3 g of dimethyl POPOP, 730 ml of dioxane, 135 ml of toluene and 35 ml of absolute methanol. Formula II scintillation fluid has a high water compatibility, up to 15%, and gave a 96% counting efficiency for [^{35}S] and [^{14}C].

Paper Electrophoresis

Samples of 10-100 µl were run on 45 x 6 cm strips of Whatman 3MM paper in a 0.1 M solution of sodium acetate-NaOH (pH 4.5) at 10 v per cm for 5 hr at 5 C, using a flat bed electrophoresis

apparatus (E-C Apparatus Corporation). Nucleotides were visualized by their fluorescence quenching at 254 nm. Radioactivity was located with a Radiochromatogram Scanner (Packard Model 7200), and quantitated by cutting the paper into 0.5 cm segments which were placed in scintillation vials and immersed in 10 ml Formula II scintillation fluid, for scintillation counting.

Thin Layer Chromatography

Aliquots of 5-10 µl were applied to 20 x 2.5 cm thin layer plates precoated with either cellulose or polyamide 6 (Baker-flex, J. T. Baker Chemical Company). The plates were developed in n-propanol:ammonia:water (8:2:3) at 25 C according to Schmidt (146).

Nucleotides and radioactivity were determined as described above for paper electrophoresis.

Silica gel coated plates, both commercial and laboratory made, were unsatisfactory because a radioactive breakdown product always formed during the run.

The Cultured Tobacco Cell System

The XD line of tobacco cells used throughout these studies was derived from stem pith of *Nicotiana tabacum* L.cv.Xanthi (178). Sterile cultures were maintained continuously in a chemically defined liquid medium, MID, containing 2.5 mM nitrate as the sole source of nitrogen and 3.0 mM sulfate as the sole source of sulfur (178). Cells were grown in 500 ml shake cultures at 28 C. Subcultures were started by diluting an aliquot of a 12- to 16-day-old culture 20-fold into fresh media.

For the *in vitro* studies, i.e., the development of an assay for ATP sulfurylase, and characterization of the enzyme activity, extracts were prepared from tobacco cells which had been grown on MID for 3-5 days.

In experiments in which the regulation of the development of ATP sulfurylase *in vivo* was investigated, the cells were grown on various modified MID media.

Special Modified Media

Media lacking both nitrogen and sulfur [$(N,S)^-$ MID] were prepared by replacing the nitrate and the sulfate of the MID media with the corresponding chloride salts, as described earlier (123,179). In addition, the anionic sulfur which contaminates the reagent grade sucrose was removed by passage through an anion exchange resin, Dowex AG 2X8 prior to use in the culture media (123). The following compounds were used, or tested, as nitrogen sources: 2.5 mM potassium nitrate, 3 mM urea (180), 0.05-0.1% casein hydrolysate (vitamin free) (179), and a mixture of 15 amino acids (L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan and L-valine) each at 0.1 mM.

To meet the sulfur requirement for growth, the following sulfur containing compounds were used, each at 0.1 mM: sodium sulfate, L-cysteine, L-methionine, glutathione (reduced), and L-djenkolic acid. The addition of L-methionine to cells growing on nitrate was always accompanied by equimolar L-arginine in order to prevent the

inhibition of growth and development of nitrate reductase activity caused by methionine (179).

Nitrogen and sulfur compounds were added to the media as aliquots of sterile stock solutions by a proper dilution. Stock solutions of 0.5 M KNO_3, 0.01 M Na_2SO_4, 10% casein hydrolysate and 5 mM L-djenkolic acid were sterilized by autoclaving. Stock solutions of 0.01 M of amino acids and 0.3 M urea were sterilized by filtration through Millipore GS filters.

In studies of the effects of Group VI anions *in vivo*, aliquots of the following autoclaved solutions were added to media supplemented with nitrogen and sulfur: 0.1 M $Na_2WO_4 \cdot 2H_2O$, 0.1 M $Na_2CrO_4 \cdot 4H_2O$, 0.1 M $Na_2MoO_4 \cdot 2H_2O$, 0.025 M $Na_2TeO_4 \cdot 2H_2O$ and 0.1-0.0005 M Na_2SeO_4. Solutions of 5 mM seleno-DL-methionine and 1 mM seleno-DL-cystine were sterilized by filtration prior to their addition to the media. Equimolar L-arginine was added with the methionine analog. Media and supplemented solutions were adjusted to pH 6.0-6.3 prior to sterilization.

Maintenance of Tobacco Cells on the Modified Media

Cells for studies of ATP sulfurylase activity *in vivo* were routinely maintained on $(N,S)^-$ MID supplemented with the following mixtures of compounds: 2.5 mM KNO_3 plus 0.1 mM Na_2SO_4, 3.0 mM urea plus 0.1 mM Na_2SO_4, or 0.05-0.1% casein hydrolysate. Experiments were started by either subculturing or by sterile harvesting, washing and resuspending (181) stationary phase cells (in which both the nitrogen and the sulfur sources had been completely exhausted) into

fresh $(N,S)^-$ MID in which the nitrogen supplement was unchanged, and the sulfur source was varied.

Growth of Seedlings

Seeds of pea, tomato or cucumber were surface sterilized with 1% sodium hyphochlorite (commercial bleach diluted 5-fold with distilled water), rinsed with sterile distilled water, and germinated under sterile conditions on a Whatman no. 1 filter disc in 400 ml beakers covered with petri dishes. The seeds were supplied every few days with autoclaved nutrient solution. The nutrient solution contained, in mg/liter: $Ca(NO_3)_2 \cdot 4H_2O$, 590; KH_2PO_4, 68.5; KNO_3, 253.3; $MgSO_4 \cdot 7H_2O$, 125; $ZnSO_4 \cdot 7H_2O$, 0.112; $MnSO_4 \cdot H_2O$, 0.782; $CuSO_4 \cdot 5H_2O$, 0.042; H_3BO_3, 1.45; MoO_3, 0.008; sequestrene Na_2Fe [13% Fe (w/w)], 76.88. The seedlings were grown at 23 C with a 16 hr photoperiod and harvested after 14 days of growth.

Growth of *Lemna* Plants

Aseptic cultures of *Lemna gibba* were grown in 2.5 L low form Fernbach flasks, containing 750 ml of autoclaved nutrient solution (104) in which 0.2 mM Na_2SO_4 is the only sulfur source. The cultures were grown at 28 C with 16 hr photoperiod and harvested after 17 days of growth.

Preparation of ATP Sulfurylase

Tobacco cells were harvested by vacuum filtration on Whatman no. 1 filter paper. After determining the fresh weight, the cells were suspended in 0.1 M ice-cold glycine-NaOH buffer (pH 9.0) containing 5 mM ascorbic acid. Five or ten milliliters of the buffer were used

per gram fresh weight. The cells were then homogenized by 30 strokes of a motor driven Thomas teflon-glass homogenizer at 3 C. The homogenate was centrifuged at 27,000 xg for 10 min at 4 C. The resulting supernatant fraction was used as crude ATP sulfurylase after appropriate dilution with the homogenizing buffer. As little as 0.1 g of cells was sufficient for the enzymatic assay.

Crude ATP sulfurylase extracts of seedlings and *Lemna* plants were prepared as described above for the tobacco cells except that homogenization of plant material was carried out in a pre-chilled micro-blender (Eberbach Corporation) for 15 seconds.

Assay of ATP Sulfurylase Activity

ATP sulfurylase activity was measured by the enzymatic incorporation of [^{35}S] from sulfate into APS. Radioactivity in [^{35}S]-APS was determined after its separation from [^{35}S]-sulfate. The complete reaction mixture contained: 20 mM glycine-NaOH (pH 9.0); 1 mM ascorbic acid; 10 mM $MgCl_2$; 20 mM [^{35}S]-Na_2SO_4 (5 µCi/µmole); 10 mM Na_4ATP; 7-8 units of yeast inorganic pyrophosphatase; 1-400 µg protein of enzyme extract. For each assay, 0.5 ml of the reaction mixture was prepared at ice temperature, the last addition being enzyme. The reaction was started by placing the tubes in a water bath at 30 C. Incubation times were 0 to 30 min. The reaction was stopped by adding 2.5 ml of ice-cold absolute ethanol and mixed with a vortex stirrer. The enzymatically formed [^{35}S]-APS was then separated from [^{35}S]-sulfate (see Figure 1) and quantitated by determining the radioactivity in the final ethanol supernatant solution, using zero time and boiled enzyme assays as controls. Boiled enzyme was

prepared by incubation of reaction mixture less ATP, [^{35}S]-sulfate and inorganic pyrophosphatase in boiling water for 5 min prior to the assay.

ATP Determination

ATP was determined by means of the luciferin-luciferase enzyme system with a Lab-Line ATP-Photometer (Lab-Line Instruments, Inc.). A commercial luciferin-luciferase extract, partially purified from the firefly, was dissolved in cold 0.04 M glycylglycine buffer (pH 7.4) containing 3 mM $MgCl_2$, as described earlier (182), at 1 ml/5 g. The solution was then sedimented for 2 min at 1,000 xg to pellet insoluble material. The resultant supernatant was used as the enzyme source.

ATP assays were conducted at room temperature in scintillation vials which contained 0.1-1.0 ml aliquots of unknowns in 2.0 ml of the glycylglycine-$MgCl_2$ buffer. To initiate the light producing reaction, 100 µl of cold luciferin-luciferase mixture was rapidly injected into the vial with an Eppendorf pipette. Standard ATP in the range of 5-100 pmoles resulted in a reproducible response which was proportional to the ATP concentration.

Column Chromatographic Separation of the Reaction Mixture on DEAE A-25 Sephadex

The method of Wilson (171) was adapted. DEAE A-25 sephadex was packed in a 0.7 x 7.0 cm column and then equilibrated at 4 C with 0.05 M of ammonium formate (pH 7.0-7.5). The complete reaction mixture, which had been incubated for 60 min, was placed in boiling water for 90 seconds to precipitate the protein. Protein was sedimented by

a subsequent centrifugation at 1,000 xg for 5 min. The resultant supernatant of the assay mixture was then applied to the column. Compounds were eluted with a linear gradiant of 40 ml NH_4HCO_3, ranging from 0.5 to 1.1 M at a flow rate of 2.5 ml per hr. Fractions of 1.0 ml were collected. An aliquot from each fraction was taken for the [^{35}S] determination by scintillation counting.

Chemical Characterization of the [^{35}S]-Product Eluted from the DEAE Sephadex Column

Aliquots from each of the fractions which contained the [^{35}S]-product eluted from the column at the APS region were also analyzed for their adenine and phosphate content. Adenine was determined by the absorbance at 260 nm after it had been corrected for the base-line absorption between 245 and 275 nm. Total phosphate was measured by a micro adaptation of the assay described by Ames (184), using 5' AMP as a standard. These determinations were used to compute the molar ratio of adenine:sulfur:phosphate in the product.

Column Chromatographic Separation of the Sulfur Compounds from the Reaction Mixture on Dowex-1-Nitrate

The following is essentially the method developed by Iguchi (176) and modified later by Levinthal and Schiff (175) and Schmidt and Schwenn (145).

Dowex-1-chloride x 8 (200-400 mesh) was converted to the nitrate form, packed in a 20 x 0.9 cm column and equilibrated, as described previously (175). The sample, 0.5 ml of a complete reaction mixture, was diluted 1:10 with water after a 30 min incubation period and then applied to the column under gravity pressure. Stepwise elution was

carried out at room temperature, under gravity flow rate of about 0.65 ml per min, with the following: (A) 105 ml of 0.07 M NH_4NO_3 in 9% acetone (pH 9.7), which elutes sulfite and sulfide, in that order; (B) 200 ml of 0.1 M $NaNO_3$, which elutes sulfate; (C) 90 ml of 0.3 M $NaNO_3$, which elutes thiosulfate and additional unknown compounds; (D) 54 ml of a gradient of 0.3-2.0 M $NaNO_3$, which elutes PAPS and APS in that order (145); (E) 18 ml of 2.0 M $NaNO_3$. Radioactivity in the 3.0 ml fractions collected was determined in 0.1 ml aliquots by scintillation counting. The identities of the [^{35}S]-compounds which eluted from the Dowex-1-nitrate column were verified by electrophoretic analysis as described above. The peak eluted by high salt (D) required desalting prior to electrophoresis because of the interference of high salt with electrophoresis.

The fractions in this peak area were combined, concentrated by flash evaporation and desalted by adsorption and elution from charcoal (see below).

Desalting on Charcoal

Samples of 1.0-2.0 ml containing [^{35}S]-APS and salts were mixed by vortexing with 100 mg per ml of activated charcoal. The charcoal was collected on a fiberglass filter (Millipore AP) under vacuum. Salts were washed with ca. 5-7 ml water. Nucleotides were eluted by a few milliliters of ammonia:ethanol:water (2:50:50), with a yield of 88%. The solution of eluted nucleotides was evaporated to dryness and the residue was redissolved in either water or 50% ethanol.

Preparation of [^{35}S]-APS of a High Specific Radioactivity

[^{35}S]-APS is not available from commercial sources. Consequently, the method which has been developed to assay ATP sulfurylase activity in extracts from tobacco cells (see above) was employed to prepare radiochemically pure [^{35}S]-APS of a high specific radioactivity.

In order to prepare [^{35}S]-APS, the basic reaction mixture was modified to include 0.1 mM [^{35}S]-Na_2SO_4 (1 mCi/μmole) and 320 μg protein of tobacco cell extract. Eight tubes, each of which contained 0.5 ml of the modified reaction mixture, were incubated for 60 min at 30 C. The [^{35}S]-APS formed was recovered in the final ethanol supernatant after it had been separated from the [^{35}S]-sulfate as described above (see Figure 1). The ethanolic fractions containing the [^{35}S]-APS were combined, concentrated by flash evaporation and desalted by adsorption and elution from charcoal. The latter would also remove the residual contaminant of [^{35}S]-sulfate. The [^{35}S]-APS which eluted from the charcoal was found by electrophoresis and chromatography to have a radiochemical purity of 99%.

The procedure described above yielded ca. 2.5-3 nmoles [^{35}S]-APS of 1 μCi/nmole. This [^{35}S]-APS of high specific radioactivity was used for the determination of the recovery of [^{35}S]-APS and in some of the procedures used to characterize the [^{35}S]-product of the reaction catalyzed by tobacco cell extracts.

Determination of Protein Content

Protein content of plant extracts was determined by the method of Lowry *et al.* (177), using bovine serum albumin dissolved in 1.0 M

NaOH as a standard. Protein in unknowns was precipitated with 10% (w/v) trichloroacetic acid, heated at 100 C for 5 min, sedimented, washed with 95% ethanol, dried and dissolved in 1.0 M NaOH.

Determination of Total Uptake and Incorporation of Radioactive Amino Acid into Protein

Tobacco cells were harvested and homogenized as described earlier in this section. Protein content of the total homogenate was determined (see above). Total radioactivity recovered in the trichloroacetic acid supernatant, plus the ethanol wash solution, plus the 1.0 M NaOH redissolved protein fraction was used as the measure of total uptake of the labeled amino acid. The radioactivity recovered in the protein fraction was used as the measure of total incorporation into protein.

The counting efficiency (96%) was not affected by 0.1 ml of either 10% trichloroacetic acid or 95% ethanol. The quenching caused by 0.1 ml of 1.0 M NaOH was corrected by the addition of 0.1 ml of 2.0 M HCl to the scintillation vials prior to the addition of the scintillation fluid.

Chemicals

ATP, APS, L-cysteine, L-methionine, L-djenkolic acid, reduced glutathione, seleno-DL-cystine, seleno-DL-methionine, cycloheximide, firefly luciferin-luciferase extract, ATP sulfurylase purified from yeast (6 units/mg), and yeast inorganic pyrophosphatase (type III, 647 units/mg) were obtained from Sigma Chemical Company. $Na_2TeO_4 \cdot 2H_2O$ and Na_2SeO_4 were purchased from the Ventron Corporation Alfa Products. $[^{35}S]$-Na_2SO_4, $[^{35}S]$-H_2SO_4 and $[^{35}S]$-PAPS were from

New England Nuclear Corporation (NEN). [^{14}C]-arginine (UL) was obtained from ICN. Activated charcoal ("Darco" G-60) was from Sargent-Welch Scientific Company. Sequestrene Na_2Fe (13%) was from Geigy Industrial Chemicals. Casein hydrolysate was from Difco Laboratories, and the Dowex anion exchange resins were from Bio-Rad Laboratories.

RESULTS

PART A:

DEVELOPMENT OF ATP SULFURYLASE ASSAY BASED ON THE DIFFERENTIAL SOLUBILITY OF SULFATE AND APS IN ETHANOL

The objective of this study was to develop a rapid quantitative assay for total extractable ATP sulfurylase activity, based on the physiologically significant reaction of the enzyme, APS formation. The new assay, which was developed as described below, is based upon the differential solubilities of the substrate, sulfate, and the product, APS.

I. Differential Solubility of Sulfate and Sulfate-Containing Nucleotides in Ethanol-Water Mixture

Sodium sulfate, 20 mM, is insoluble in ethanol:water (5:1). Twenty mM [^{35}S]-Na_2SO_4 (5 µCi/µmole) was completely removed from the ethanol supernatant fraction of complete reaction mixture by the 4 successive precipitation steps described in Figure 1 (Table 1). Although [^{35}S]-Na_2SO_4 from NEN could be completely precipitated by this procedure, [^{35}S]-H_2SO_4 from the same company contained a radioactive impurity which could not be coprecipitated with excess Na_2SO_4. This resulted in up to a few thousand cpm in the final ethanol supernatant when [^{35}S]-H_2SO_4 was used. Therefore, it is essential

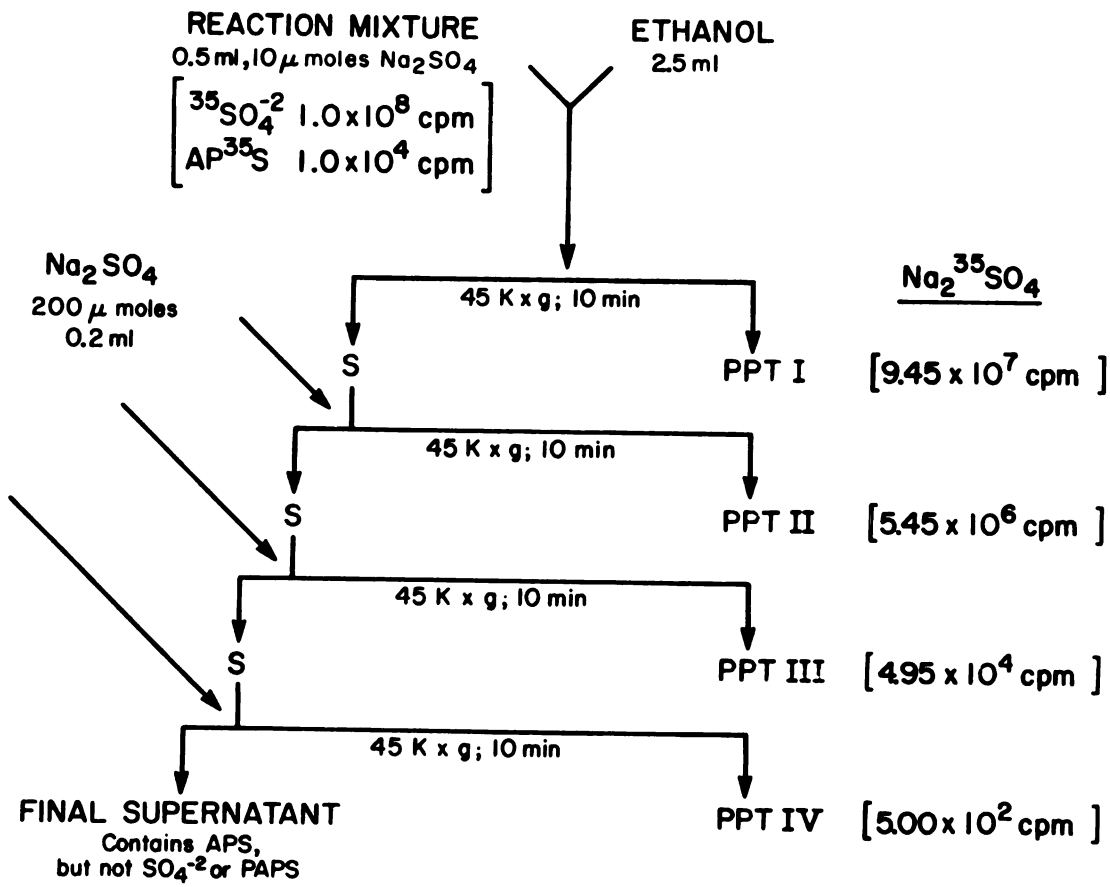

Figure 1. Procedure for separation of sulfate and APS by differential solubility in ethanol:water (5:1).

Table 1. Quantitative precipitation of [^{35}S]-sulfate by the separation method

	CPM x 10^{-2}		
	Complete zero time	Complete boiled	Complete minus enzyme
Total added	1,000,000	1,000,000	1,000,000
Fraction			
PPT I	946,920(95)[a]	931,000(93)	942,250(94)
PPT II	61,600(99)	66,110(99)	53,280(99)
PPT III	445(99)	568(99)	422(99)
PPT IV	5(100)	7(100)	6(100)
SUPERNATANT	0	0	0

[a]Numbers in parentheses are efficiency of precipitation at each step, in percent.

Reaction mixture contained: 30 mM glycine-NaOH (pH 9.0); 1.5 mM ascorbic acid; 10 mM $MgCl_2$; 20 mM [^{35}S]-Na_2SO_4 (5 µCi/µmole); 10 mM ATP; 7.2 units of yeast inorganic pyrophosphatase; 0.011 mg protein of tobacco cell extract, in total volume of 0.5 ml. Tubes were incubated for 0 or 20 min, at 30 C. At the end of the incubation 2.5 ml of absolute ethanol were added, followed by the 4 precipitation steps described in Figure 1. Precipitates were redissolved in water, and aliquots of all fractions were analyzed by scintillation counting.

to use radiochemically pure [^{35}S]-Na_2SO_4 in order to achieve the maximum sensitivity possible with this procedure.

The efficiency of sulfate precipitation is not influenced by the presence of either native or denatured extract in the complete reaction mixture. On the other hand, sodium APS, up to 50 µM, is soluble in ethanol:water (5:1). To examine the efficiency of the recovery of APS by the separation technique (see Figure 1), complete reaction mixtures containing unlabeled sulfate were incubated for various times. At the end of the incubation periods, radiochemically pure [^{35}S]-APS (1 µCi/nmole) was added to the tubes, followed immediately by the addition of ethanol. The 4 precipitation steps resulted in the recovery of over 80% of the [^{35}S], added initially as [^{35}S]-APS, in the final supernatant fraction (Table 2). This ethanol-soluble [^{35}S] was verified by electrophoresis to be entirely in APS (Figure 2-A, 2-B).

When [^{35}S]-APS was added to reaction mixtures which had been incubated with two different concentrations of extract to allow the synthesis of unlabeled APS in the range of 0-20 µM, the recovery of [^{35}S]-APS added in the ethanol supernatant remained rather constant, at ca. 80% (Table 2). Thus, the recovery of [^{35}S]-APS by this separation method is not influenced by the concentrations of extract, nor by the concentrations of APS which are in the range expected for enzyme assays.

These results also indicate the stability of the added [^{35}S]-APS under the conditions of the separation technique.

Table 2. Recovery of added [^{35}S]-APS from complete reaction mixtures by the separation technique

	^{35}S, CPM RECOVERED				
	0.1 ml enzyme zero min	0.1 ml enzyme 10 min	0.1 ml enzyme 20 min	0.2 ml enzyme 10 min	0.2 ml enzyme 20 min
Total	61,200	63,780	63,140	62,480	62,130
Fraction					
PPT I	8,240(13)[a]	6,070(10)	7,460(12)	7,080(11)	6,830(11)
PPT II	1,700(3)	1,850(3)	1,670(3)	1,760(3)	1,490(3)
PPT III	1,370(2)	1,380(2)	1,400(2)	1,430(2)	1,330(2)
PPT IV	1,150(2)	1,200(2)	1,310(2)	1,270(2)	1,340(2)
SUPERNATANT	48,740(80)	53,280(83)	51,300(81)	50,940(82)	51,140(82)

[a]Numbers in parentheses are [^{35}S] recovered in each fraction, as percent of total.

Enzyme assay mixtures without [^{35}S]-SO_4^{-2} contained: 50 mM glycine-NaOH (pH 9.0); 2.5 mM ascorbic acid; 10 mM $MgCl_2$; 20 mM Na_2SO_4; 10 mM ATP; 7.2 units of inorganic pyrophosphatase; and tobacco cell extract, 12 µg protein / 0.1 ml in total 0.5 ml volume. Tubes were incubated at 30 C for 0, 10 and 20 min. At the end of the incubation, 50 µl of [^{35}S]-APS (ca. 1 mCi/µmole) in water was added to each tube, followed immediately by the addition of 2.5 ml ethanol. The 4 precipitation steps were carried out as described in Figure 1. Analysis of [^{35}S] in the fractions was performed as described in Table 1.

Figure 2. Recovery of the [^{35}S]-APS added in the final ethanol fraction: verification by electrophoresis.

Supernatant and PPT I fractions, described in Table 2, were evaporated to dryness, redissolved in water, and aliquots were taken for electrophoretic analysis as described in Materials and Methods. Authentic APS standard was detected by its fluorescence quenching at 254 nm. Arrows indicate [^{35}S]-labeled standards.

A. [^{35}S]-APS added.

B. [^{35}S] recovered in the ethanol supernatant fraction.

C. [^{35}S] precipitated in PPT I fraction.

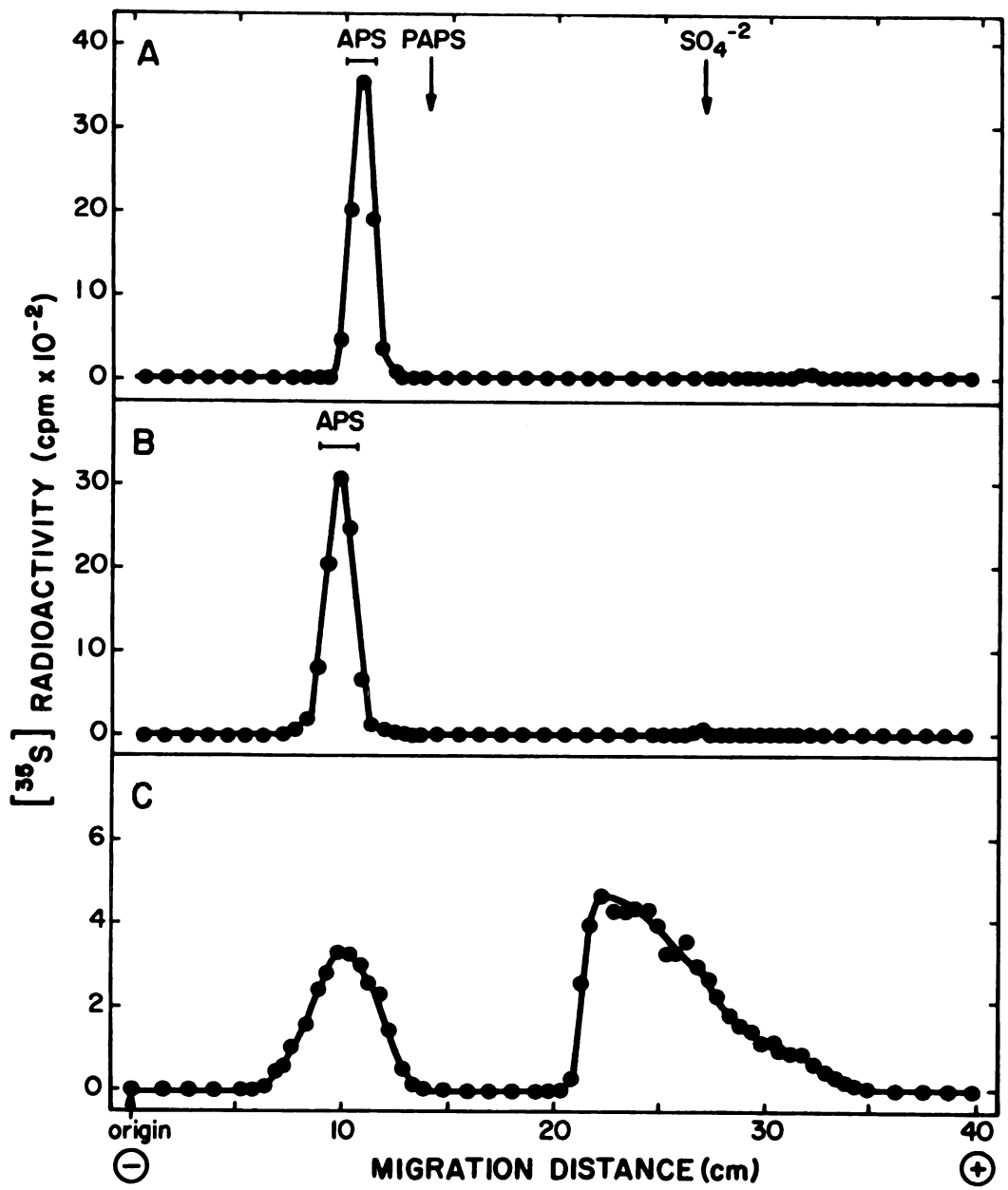
A
APS
PAPS
SO_4^{-2}
B
APS
C
40
30
20
10
0
6
4
2
origin
10
20
30
40
⊖
⊕
MIGRATION DISTANCE (cm)
[^{35}S] RADIOACTIVITY (cpm x 10^{-2})

Figure 2

The 10-13% [^{35}S] derived from the added [^{35}S]-APS, which was ethanol-insoluble and precipitated largely during the first step (Table 2), was mostly (70%) [^{35}S]-sulfate as shown by electrophoresis (Figure 2-C). The wide, asymmetrical peak (Figure 2-C) is probably due to the high level of salts and protein which are also found in this fraction.

Unlike APS, sodium PAPS was completely insoluble in the ethanol: water mixture (5:1). The addition of ethanol to complete reaction mixture containing unlabeled sulfate and ca. 35 μM [^{35}S]-PAPS resulted in the precipitation of 99% of the [^{35}S] added as PAPS (Table 3).

Therefore, sulfate and PAPS exhibit solubility properties, in ethanol:water mixture (5:1), which are markedly different from those of APS. These findings suggested that these characteristics could be further exploited as a basis for a rapid and simple method for the separation of APS from sulfate, and thus replace the conventional methods of chromatography and electrophoresis which are inherently slow. Such a separation procedure may then be used in conjunction with the radioisotope assay of ATP sulfurylase, to quantitate the amount of [^{35}S]-APS synthesized from [^{35}S]-sulfate if the enzymatically formed APS is stable and is not further converted to PAPS or other sulfur-containing derivatives. Investigation of these ideas in the cultured tobacco cells was consequently initiated.

II. Formation of an Ethanol-Soluble Product by Tobacco Cell Extract

Tobacco cell extract, incubated with [^{35}S]-sulfate and ATP in a complete reaction mixture, catalyzed the formation of a [^{35}S]-product, which coelectrophoresed with authentic APS (Figure 3).

Table 3. Precipitation of [^{35}S]-PAPS by ethanol

	[^{35}S], CPM recovered
Total	257,200
Fraction	
PPT I	253,500(99)[a]
SUPERNATANT	3,700(1)

[a]Numbers in parentheses are [^{35}S] recovered, as percent of total.

Reaction mixture contained: 50 mM glycine-NaOH (pH 9.0); 2 mM ascorbic acid; 10 mM $MgCl_2$; 40 mM $NaSO_4$; 7.2 units inorganic pyrophosphatase; ca. 100 µg protein; and ca. 35 µM [^{35}S]-PAPS. Two and five-tenths milliliters of ethanol were added to tubes maintained at 4 C, followed by centrifugation for 10 min at 45,000 xg, and separation of the precipitate from the supernatant. The [^{35}S] derived from [^{35}S]-PAPS was determined by scintillation counting of aliquots of the redissolved precipitate and the supernatant.

Alternatively, if ethanol is added to such an incubated mixture, and the precipitation procedure outlined in Figure 1 is carried out, an ethanol-soluble [^{35}S]-product, which was formed during incubation, is found in the ethanol supernatant fraction (Table 4). Furthermore, the formation of this ethanol-soluble [^{35}S] with time was completely dependent upon the addition, to the incubated mixture, of native extract, ATP and magnesium ions (Table 5). Thus, the requirements for the enzymatic synthesis by tobacco cell extract of the [^{35}S]-product which is recovered in the ethanol supernatant after precipitation of [^{35}S]-sulfate, is consistent with APS formation by ATP

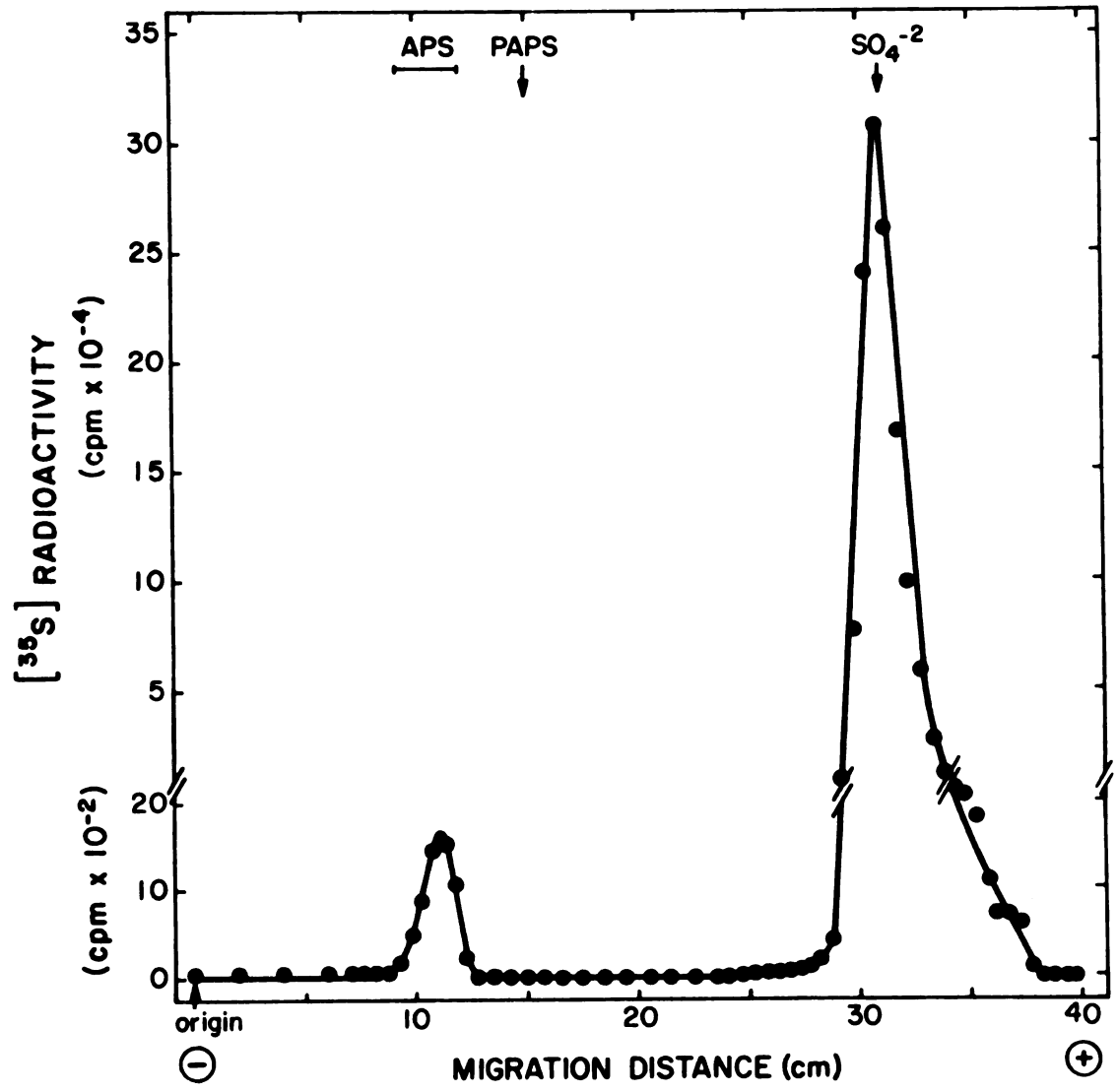

Figure 3. Radioelectrophoretogram of complete reaction mixture showing the [^{35}S]-product synthesized by tobacco cell extract.

Complete reaction assay, 0.5 ml, contained: 40 mM glycine-NaOH (pH 9.0); 2 mM ascorbate; 10 mM $MgCl_2$; 0.1 mM [^{35}S]-Na_2SO_4 (1 mCi/µmole); 10 mM ATP; 7.2 units inorganic pyrophosphatase; and 319 µg protein of tobacco cell extract. After 60 min incubation at 30 C, the reaction was stopped by placing the tube in ice, and 10 µl aliquots were taken for electrophoresis analysis as described in Materials and Methods. Standard APS, [^{35}S]-SO_4^{-2}, and [^{35}S]-PAPS are shown.

Table 4. Time dependent formation of ethanol-soluble [^{35}S]-product by tobacco cell extract

	CPM x 10^{-2}	
	Complete 0-min	Complete 60-min
Total added	1,009,370	1,011,955
Fraction		
PPT I	647,970	501,340
PPT II	357,900	498,600
PPT III	3,428	5,704
PPT IV	50	247
SUPERNATANT	22	6,064

Complete reaction mixtures as described in Figure 3 were incubated at 30 C for 0 and 60 min. The reactions were stopped by the addition of 2.5 ml absolute ethanol, followed by the four precipitation steps described in Figure 1. Aliquots of all fractions were analyzed for [^{35}S]-content by scintillation counting.

sulfurylase. The relative ineffectiveness of the inorganic pyrophosphatase on the accumulation of the [^{35}S] in the ethanol is not unexpected if the [^{35}S] that formed was synthesized by ATP sulfurylase at initial rates before equilibrium is approached.

The indication that extract from tobacco cell incorporates [^{35}S]-sulfate into a single product (Figure 3), which can be recovered in the ethanolic fraction after the precipitation of [^{35}S]-sulfate by ethanol, prompted a detailed effort to characterize this product.

Table 5. Requirements for the enzymatic synthesis of the ethanol-soluble [^{35}S]-product by tobacco cell extract

Reaction mixture	Incubation time (min)	[^{35}S] in supernatant (CPM)	
COMPLETE	0	0	(0)[a]
COMPLETE	20	9,872	(100)
boiled extract	20	0	(0)
minus extract	20	0	(0)
minus ATP	20	0	(0)
minus Mg^{+2}	20	992	(10)
minus pyrophosphatase	20	7,104	(72)
COMPLETE, 3X pyrophosphatase	20	9,440	(96)

[a]Numbers in parentheses represent [^{35}S], as percent of complete assay.

Assays were performed as described in Table 1. The formation of the [^{35}S]-product was proportional to time of incubation.

III. Characterization and Identification of the Ethanol-Soluble [^{35}S]-Product Formed by Tobacco Cell Extract as APS

The objectives of the following studies were to determine whether the ethanol-soluble [^{35}S] formed by tobacco cell extract is APS, and if it is the only product formed during the incubation.

(a) Electrophoretic Analysis

The ethanol-soluble [^{35}S] formed by incubation of tobacco cell extract in complete reaction mixture (see Table 5)

coelectrophoresed as a single peak with authentic APS, but not with [^{35}S]-PAPS or [^{35}S]-sulfate (Figure 4). The [^{35}S]-sulfate and [^{35}S]-PAPS standards each electrophoresed as a single peak during separate runs, indicating radiochemical purity and lack of breakdown products.

(b) Thin Layer Chromatography

This ethanol-soluble [^{35}S]-product appears as a single peak during thin layer chromatography as well. It cochromatographed with authentic APS, but separated from [^{35}S]-PAPS (Figure 5), and from [^{35}S]-sulfate (Figure 6). The purity and stability of the [^{35}S] in the standards PAPS and sulfate during the analysis was determined in separate runs.

When the ethanol-soluble [^{35}S] was eluted from the thin layer plate (Figures 5-A and 6-A) and was subjected to a second analysis by either thin layer chromatography or electrophoresis, the same Rf values were obtained.

(c) Charcoal Adsorption

This ethanol-soluble [^{35}S] was adsorbed by charcoal and could be eluted by a mixture of ethanol:ammonia:water, which is consistent with its being a sulfur-nucleotide. The [^{35}S] eluted from charcoal coelectrophoresed and cochromatographed with authentic APS, exactly as did the ethanol-soluble [^{35}S] before the treatment with charcoal (see Figures 4, 5 and 6).

(d) Acid Lability

Upon incubation with 1.0 N HCl at 37 C for 60 min, all the [^{35}S] derived from the ethanol-soluble product appeared as sulfate

Figure 4. Radioelectrophoretograms showing the ethanol-soluble [^{35}S]-product derived from assay mixture with tobacco cell extract after precipitation with ethanol.

The ethanol supernatant fractions of complete reaction mixtures incubated for 60 min (see Table 4) were combined, concentrated by evaporating the ethanol, and aliquots were subjected to electrophoretic analysis (see Materials and Methods).

A. The [^{35}S]-product recovered in the ethanol supernatant fraction.

B. A with standard [^{35}S]-PAPS.

C. A with standard [^{35}S]-SO_4^{-2}.

D. A with authentic APS, [^{35}S]-PAPS, and [^{35}S]-SO_4^{-2} as standards.

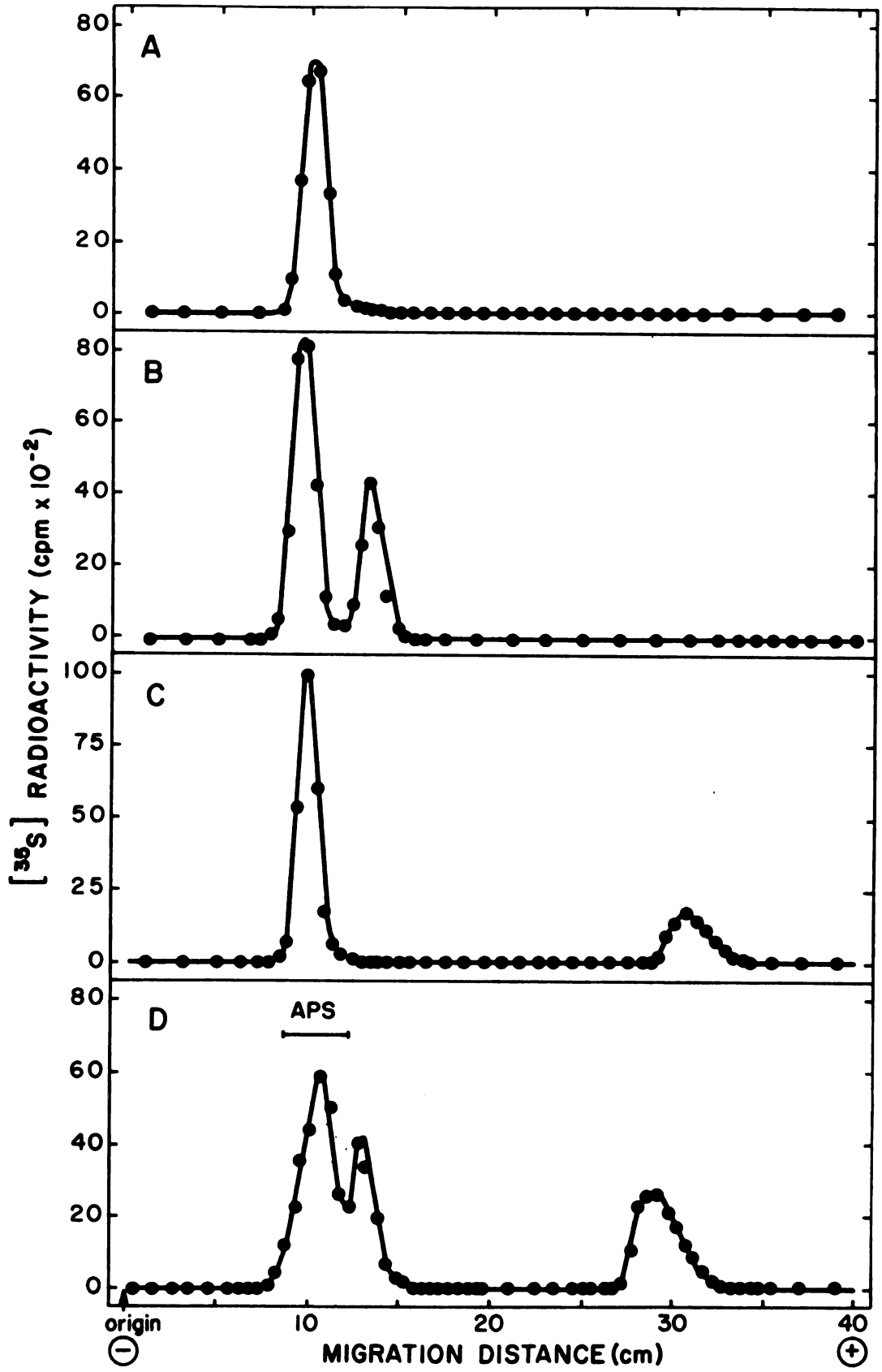
A
B
C
D
APS
80
60
40
20
0
100
75
50
25
origin
10
20
30
40
MIGRATION DISTANCE (cm)
[35S] RADIOACTIVITY (cpm x 10-2)

Figure 4

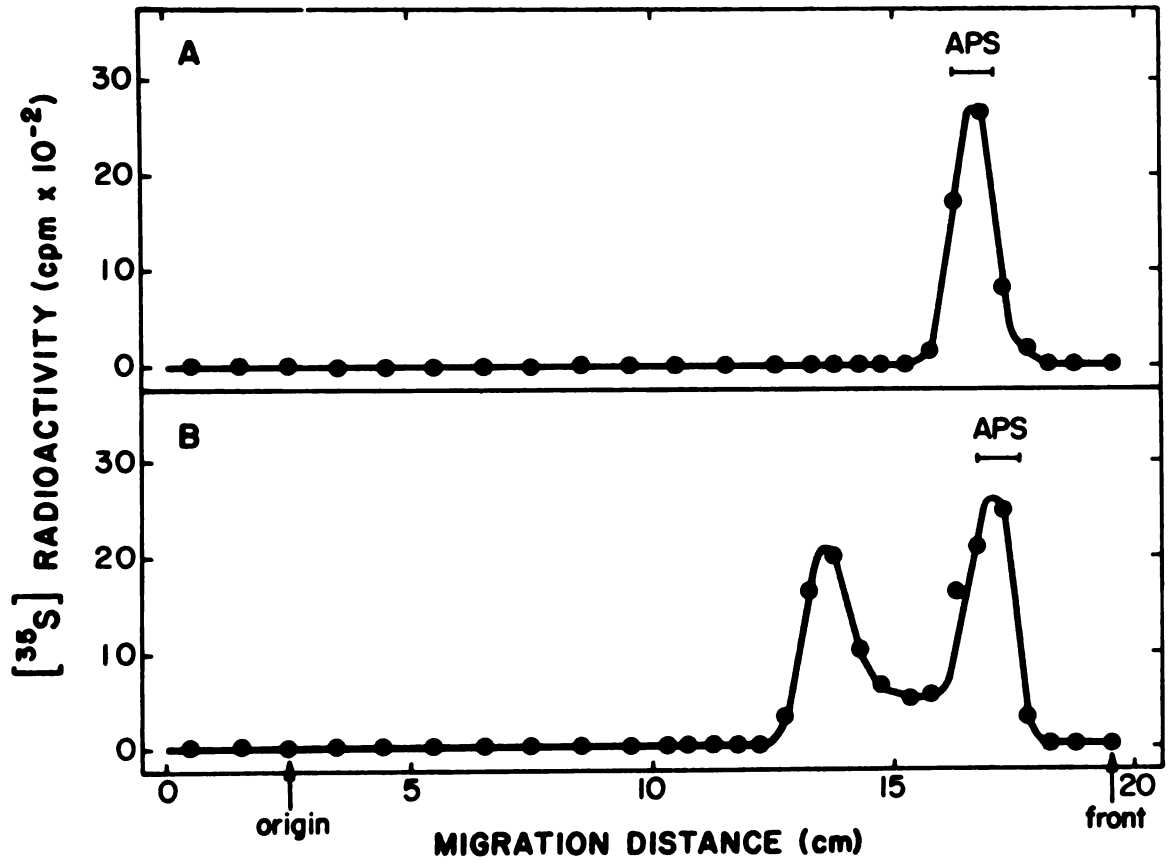

Figure 5. Radiochromatograms showing absence of PAPS in the ethanol-soluble [^{35}S]-product formed by tobacco cell extract.

Aliquots of the concentrated ethanol supernatant fractions (see Figure 4) were chromatographed on polyamide 6 precoated thin layer plates (Baker-flex) and developed in propanol:ammonia:water (8:2:3), as described in Materials and Methods.

A. The [^{35}S]-product recovered in the supernatant fraction, run with standard APS.

B. A with standard [^{35}S]-PAPS.

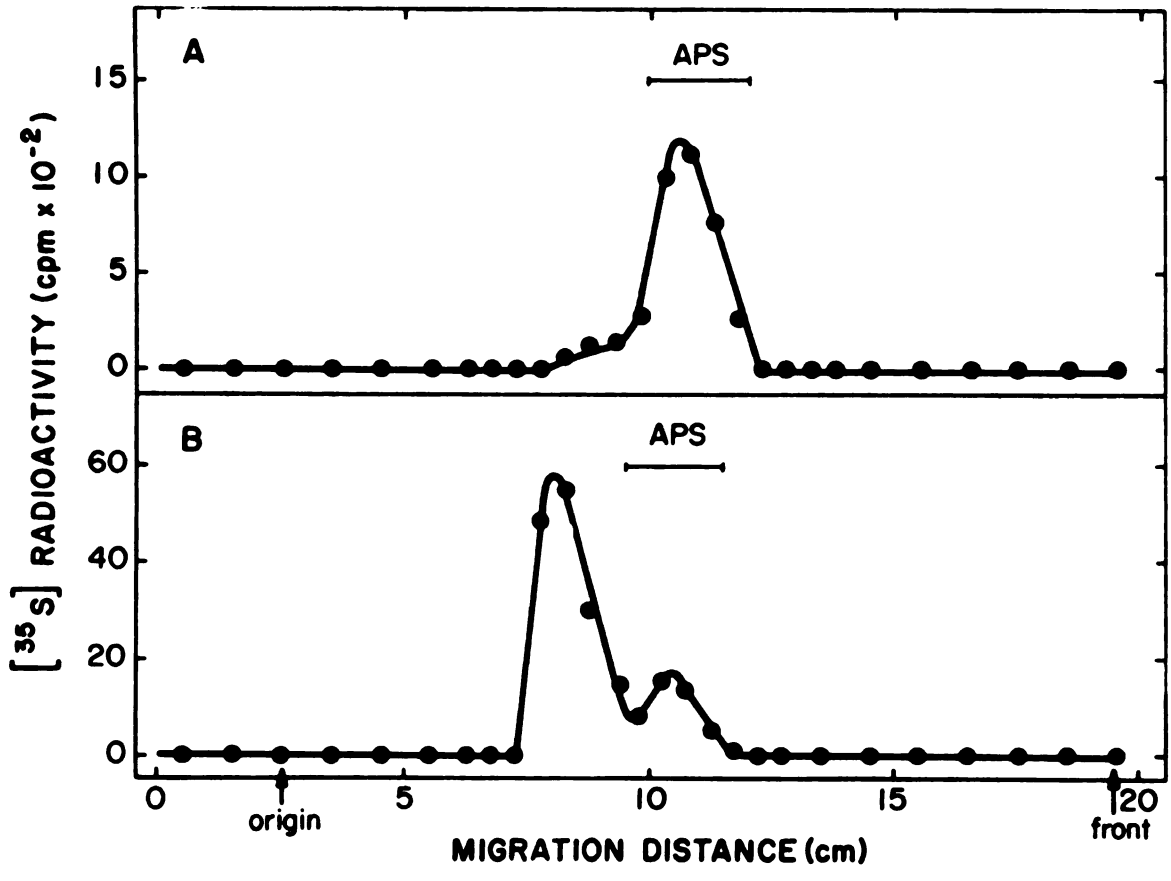

Figure 6. Radiochromatograms showing absence of sulfate in the ethanol-soluble [^{35}S]-product synthesized by extract from tobacco cells.

Aliquots of the evaporated supernatant fractions (see Figure 4) were subjected to chromatographic analysis on cellulose pre-coated thin layer plates (Baker-flex) and developed in propanol: ammonia:water (8:2:3), as described in Materials and Methods.

A. The ethanol-soluble [^{35}S]-product, run with APS standard.

B. A with [^{35}S]-Na_2SO_4 standard.

(Figure 7). These conditions are known to hydrolyze authentic APS to sulfate and AMP (163).

(e) Attempted Identification of the Ethanol-Soluble [^{35}S]-Product as APS by the Reverse Reaction of ATP Sulfurylase

Extracts of tobacco cells catalyzed the formation of [^{35}S]-sulfate in an enzyme-dependent reaction upon incubation with the isolated ethanol-soluble [^{35}S]-product, pyrophosphate and Mg^{+2} (Figure 8). However, because a crude extract was used, this reaction could not be rigorously attributed to ATP sulfurylase. Consequently, the experiment was repeated with commercial ATP sulfurylase purified from yeast, and the formation of both [^{35}S]-sulfate and ATP from the ethanol-soluble [^{35}S]-product was studied. Sulfate was determined as the [^{35}S] which precipitated by ethanol, added at 5:1. In contrast to the assay which is described for the forward reaction of APS formation, the reverse ATP sulfurylase assay employed here does not have a huge excess of [^{35}S]-sulfate. Therefore, two precipitation steps after ethanol was added were sufficient to separate the sulfate from APS. ATP was assayed by the luciferin-luciferase enzyme system, with the assistance of Mr. B. Whitaker in this laboratory.

Incubation of the purified ATP sulfurylase with the ethanol-soluble [^{35}S] resulted in the formation of [^{35}S]-sulfate which was dependent upon addition of native enzyme (Table 6). However, a simple interpretation of these results as being due solely to ATP sulfurylase is difficult because: (a) the formation of sulfate was independent of the pyrophosphate present, and (b) ATP could not be detected in the complete reaction mixture (Table 6). Because the original

Figure 7. Radioelectrophoretograms of the acid hydrolysis product of the ethanol-soluble [^{35}S]-product.

The ethanol supernatant fractions (see Figure 4) were evaporated to dryness and redissolved in water. One hundred microliter aliquots, containing approximately 90,000 cpm of the [^{35}S]-product, were incubated with 1.0 N HCl at 37 C for 60 min in a total volume of 125 µl. Control tubes containing standard [^{35}S]-Na_2SO_4 were treated in the same way. After the incubation, the HCl was evaporated, samples were redissolved in 50 µl water, and aliquots of 25 µl were subjected to paper electrophoresis as described in the text.

A. The [^{35}S]-product before acid hydrolysis, run with authentic APS.

B. The [^{35}S]-product after acid hydrolysis, run with authentic APS.

C. B run with standard [^{35}S]-SO_4^{-2}.

D. Standard [^{35}S]-SO_4^{-2} after acid hydrolysis, run with authentic APS.

The arrows indicate the Rf for PAPS and SO_4^{-2}, determined separately.

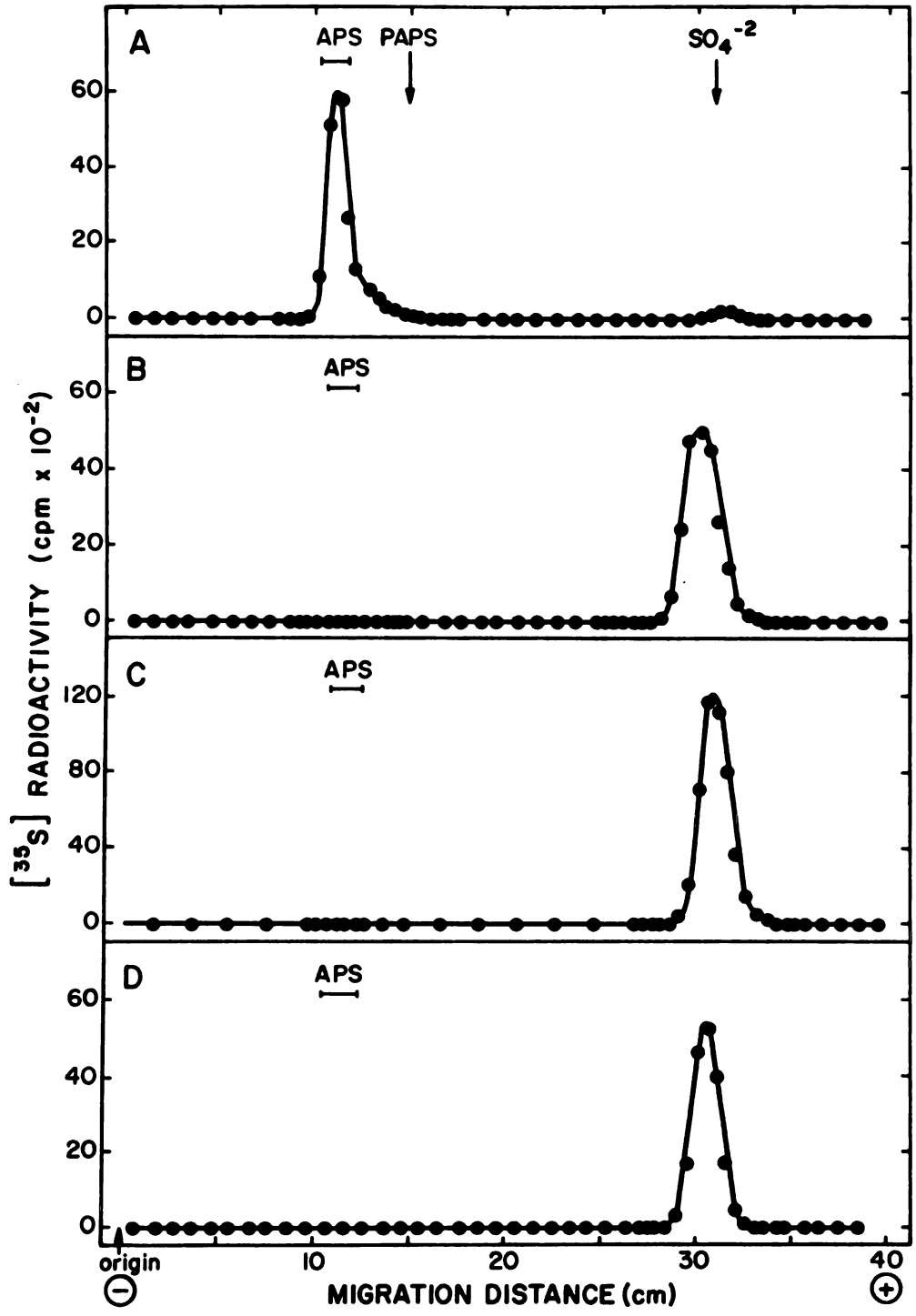
A
APS
PAPS
SO_4^{-2}
B
APS
C
APS
D
APS
[35S] RADIOACTIVITY (cpm x 10-2)
origin
10
20
30
40
MIGRATION DISTANCE (cm)

Figure 7

Figure 8. Radioelectrophoretograms showing enzymatic conversion of the [^{35}S]-product to [^{35}S]-sulfate by tobacco cell extract.

Reaction mixtures, 125 µl, contained: 10 mM glycine-NaOH (pH 9.0); 2 mM ascorbate; 10 mM $MgCl_2$; 3 mM sodium pyorphosphate; [^{35}S]-product (see Figure 7), approximately 45,000 cpm; and 90 µg protein of tobacco cell extract. Control assays containing standard [^{35}S]-PAPS were assayed in the same way. At the end of 60 min incubation at 30 C, 50 µl aliquots were analyzed by electrophoresis.

A. [^{35}S]-product before incubation, run with standard APS.

B. [^{35}S]-product after incubation with tobacco cell extract, run with standard APS.

C. [^{35}S]-product after incubation with boiled tobacco cell extract, run with APS.

D. Standard [^{35}S]-PAPS after incubation with tobacco cell extract, run with APS.

Arrows indicate the Rf for standard PAPS and sulfate.

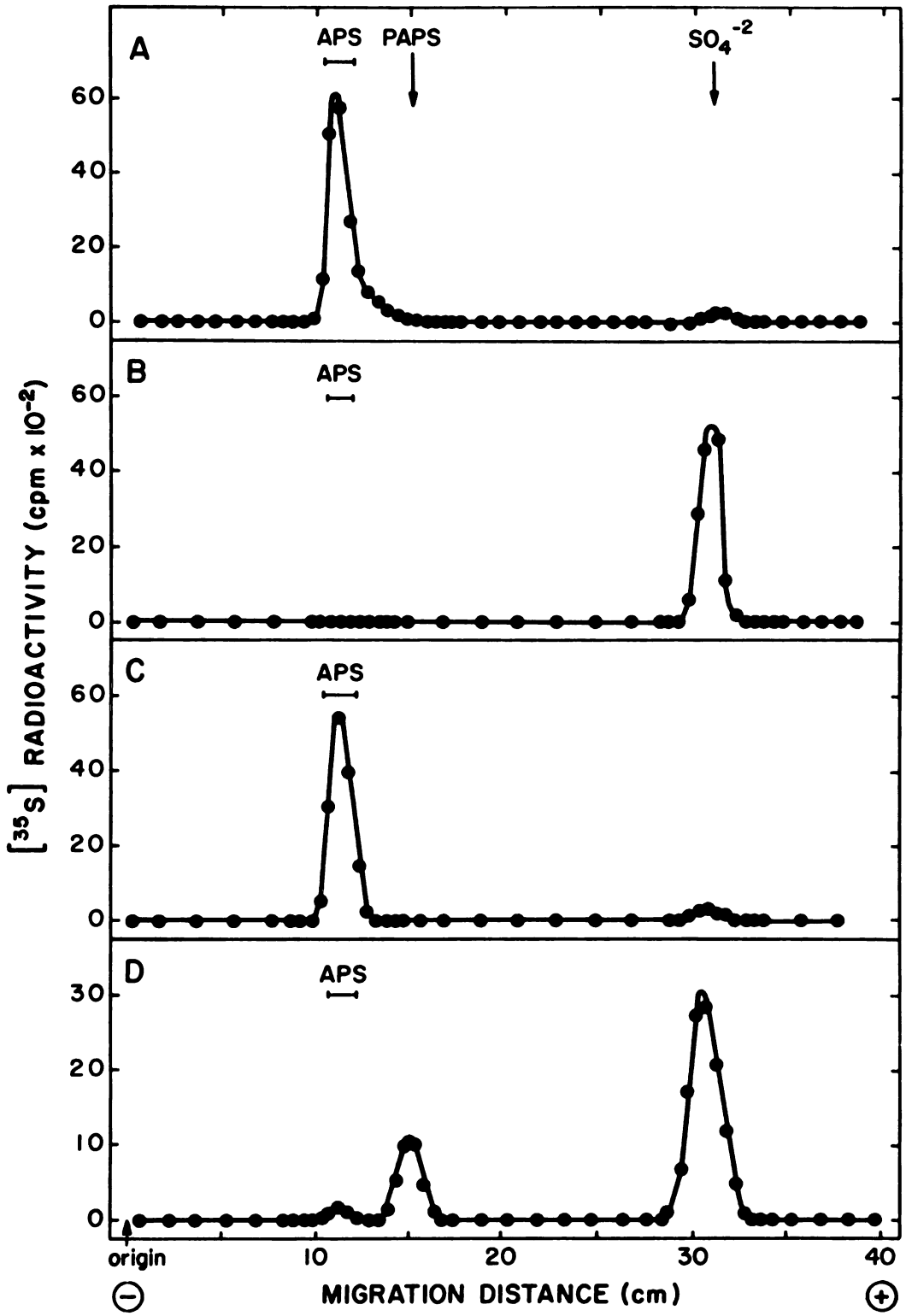
A
APS
PAPS
SO_4^{-2}
B
APS
C
APS
D
APS
60
40
20
0
30
10
origin
10
20
30
40
⊖
MIGRATION DISTANCE (cm)
⊕
[35S] RADIOACTIVITY (cpm x 10-2)

Figure 8

Table 6. Formation of sulfate from the ethanol-soluble [^{35}S]-product catalyzed by ATP sulfurylase purified from yeast

Reaction mixture	PMOLES PER REACTION MIXTURE			
	[^{35}S] added	APS recovered	Sulfate formed	ATP formed
Complete	35.5	0.8(2)[a]	33.8(98)	1.1
boiled enzyme	35.5	26.7(74)	9.2(26)	5.0
minus enzyme	35.5	26.5(78)	7.5(22)	4.8
minus PP_i	35.5	0.9(3)	32.9(97)	0.5
minus Mg^{+2}	35.5	0.4(1)	32.2(99)	12.4

[a]Numbers in parentheses are percent of total [^{35}S] recovered in each assay.

Complete reaction mixture, 0.25 ml, contained: 20 mM glycine-NaOH (pH 9.0); 10 mM $MgCl_2$; 2 µM sodium pyrophosphate; ca. 0.14 µM [^{35}S]-product (see Figure 8), of specific activity of ca. 560 cpm/pmole; and 1.5 units of commercial ATP sulfurylase purified from yeast. After incubation at 30 C for 30 min, reactions were stopped by adding 1.25 ml absolute ethanol, followed by two precipitation steps, with 10 and 50 µmoles carrier Na_2SO_4, added in 50 µl, respectively. Precipitation fractions were dissolved in 1.5 ml of 0.04 M glycylglycine, 3 mM $MgCl_2$ (pH 7.4). Three-tenths milliliter aliquots of all fractions were analyzed for [^{35}S]-content by scintillation counting. ATP measurements, by the luciferin-luciferase assay described in the text, were conducted with 1.0 ml of the precipitate fractions and 0.1 ml of the ethanolic solution.

[^{35}S]-product used as a substrate appeared to contain ca. 5 pmoles of ATP initially (Table 6), an actual net loss of ATP was observed in complete reaction mixture, indicating the presence of at least one ATP consuming enzyme in the commercial ATP sulfurylase preparation. Because net formation of ATP was observed only when Mg^{+2} was omitted from the reaction mixture (Table 6), it may be assumed that the ATP consuming reaction was Mg^{+2} dependent. Nevertheless, even in the absence of Mg^{+2}, the amount of ATP formed was considerably less than what was expected based on the formation of sulfate. Since the commercial ATP sulfurylase contained ca. 0.2% ATPase activity (Sigma specifications), the net loss of ATP observed could be accounted for, if one assumes a Km (ATP) for the ATPase reaction of ca. 0.1 mM.

Although the results of this section [(e)] do not strongly support any single conclusion with regard to the chemical nature of the [^{35}S]-product formed by tobacco cells, they are nevertheless worth noting because they shed some light on similar inconsistencies reported in the literature for ATP sulfurylases from other sources (see Discussion).

(f) Characterization of the Chemical Composition of the [^{35}S]-Product

An attempt to determine directly the molar ratio of adenine to sulfur in the ethanol-soluble [^{35}S]-product, by a double-label experiment with [^{3}H]-ATP and [^{35}S]-sulfate in the incubation mixture, was unsuccessful. Abundance of ethanol-soluble [^{3}H]-derivatives was consistently found in the final supernatant fraction, in addition to the [^{3}H],[^{35}S]-double labeled product.

A different approach was therefore attempted. A complete assay mixture containing the reaction product(s) formed by tobacco cell extract was chromatographed on a DEAE A-25 sephadex column, which has been shown to separate APS from sulfate, ATP, ADP, 5'AMP, 3'AMP and PAPS (171), prior to the chemical characterization. These studies, described below, were performed by Mr. G. Dilworth from the Department of Botany and Plant Pathology, Michigan State University.

The elution profile of the complete reaction mixture from the DEAE A-25 sephadex column showed a single [^{35}S] containing product with a Rf characteristic of APS (fractions 13-16), well-separated from the [^{35}S]-sulfate which was eluted in Fractions 5-9 (results are not presented).

When each of the fractions which contained the eluted [^{35}S]-product was analyzed for the content of [^{35}S]-sulfur, adenine, and phosphorus (see Materials and Methods), a molar ratio of adenine: sulfur:phosphorus of 0.97:1:1.07 and 0.99:1:1.08 was obtained for Fractions 14 and 15, respectively (Figure 9), which is consistent with the 1:1:1 ratio expected for APS.

(g) Determination of APS as the Only Sulfur-Containing Product Synthesized by Tobacco Cell Extract

The finding that the complete reaction mixture yielded a single ethanol-soluble [^{35}S]-product was not, however, sufficient for determining this product as the sole sulfur-containing product synthesized by the tobacco cell extract. Thus, the formation of [^{35}S]-derivative(s) which are insoluble in ethanol was conceivable.

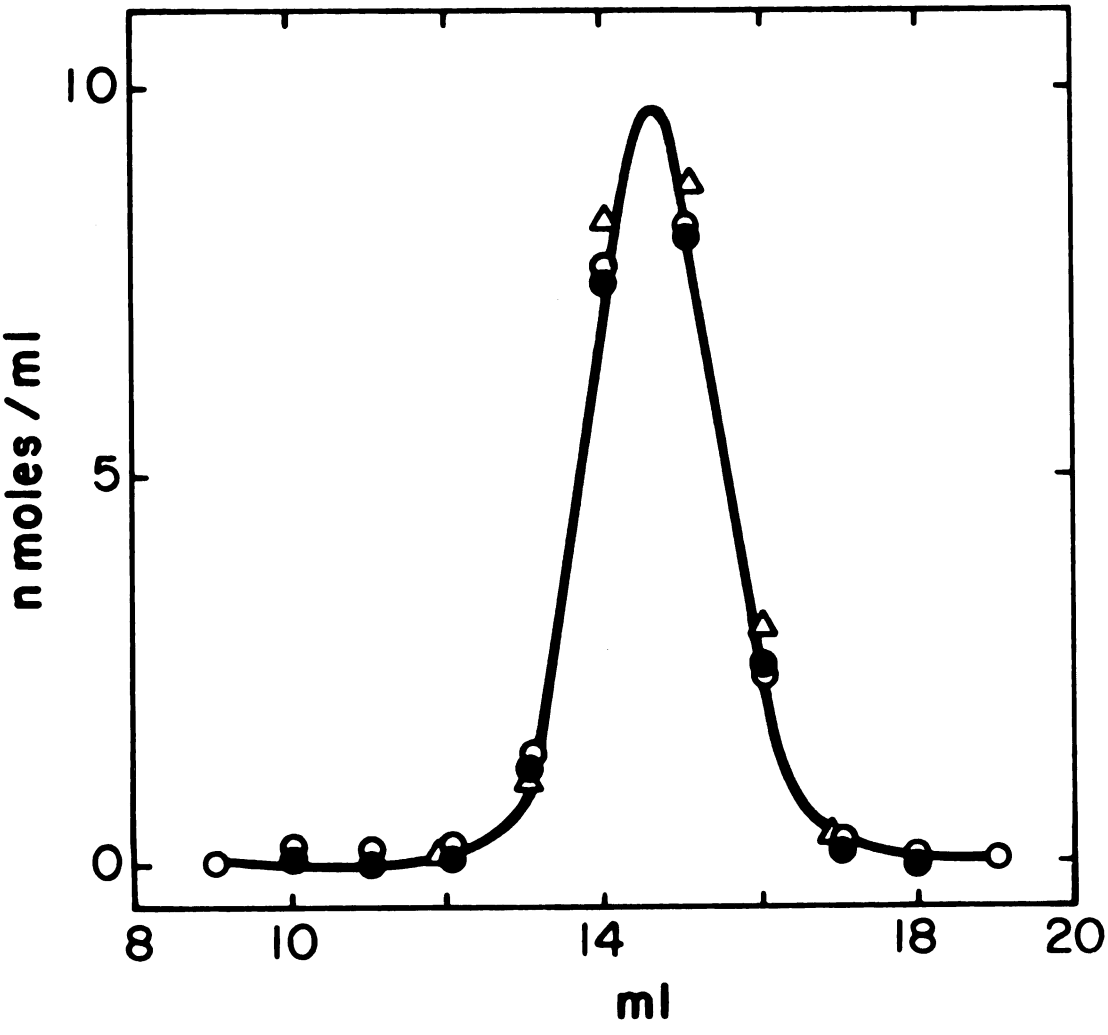

Figure 9. Chemical composition of the [^{35}S]-product eluted from DEAE sephadex column, showing the molar ratio of sulfur: adenine:phosphate.

Complete reaction mixture (see Table 1) containing approximately 100 µg protein of tobacco cell extract, incubated at 30 C for 60 min, was chromatographed on DEAE A-25 sephadex column (see Materials and Methods). Fractions containing the eluted [^{35}S]-product (Fractions 12-17) were analyzed for [^{35}S] (○); adenine content (●); and phosphate (△), as described in the text.

Such derivative(s), if formed by the conversion of APS, will result in an underestimation of the amount of APS formed in the reaction catalyzed by ATP sulfurylase. Therefore, the identification of the [^{35}S]-product(s) synthesized by tobacco cell extract was investigated.

Electrophoresis (see Figure 3), as well as chromatography on DEAE sephadex, of the complete reaction mixture, indicated that APS was the only [^{35}S]-containing product synthesized by tobacco cell extract. Nevertheless, a third independent method was used to verify this conclusion.

Chromatography on Dowex-1-nitrate resin is perhaps the most powerful method available to resolve the major sulfur-containing intermediates of sulfate reduction (146,175-176). When the incubation of the complete reaction mixture was followed by chromatography on a Dowex-1-nitrate column, only one substantial [^{35}S]-peak was found in addition to [^{35}S]-sulfate (Figure 10). The [^{35}S] in this major peak, which was eluted in the APS region, was verified as APS by electrophoretic analysis of the pooled fractions from the peak area.

In addition to APS and sulfate, a trace amount of an unknown was found in a third peak (Figure 10). However, this peak contained only 1.6% as much [^{35}S] as the major APS peak and therefore would be a negligible factor in estimates of APS synthesis.

(h) Summary of Conclusions Regarding the Measurement of Incorporation of [^{35}S]-Sulfate by Tobacco Cell Extract Into Product

It may be concluded from the results presented above that:

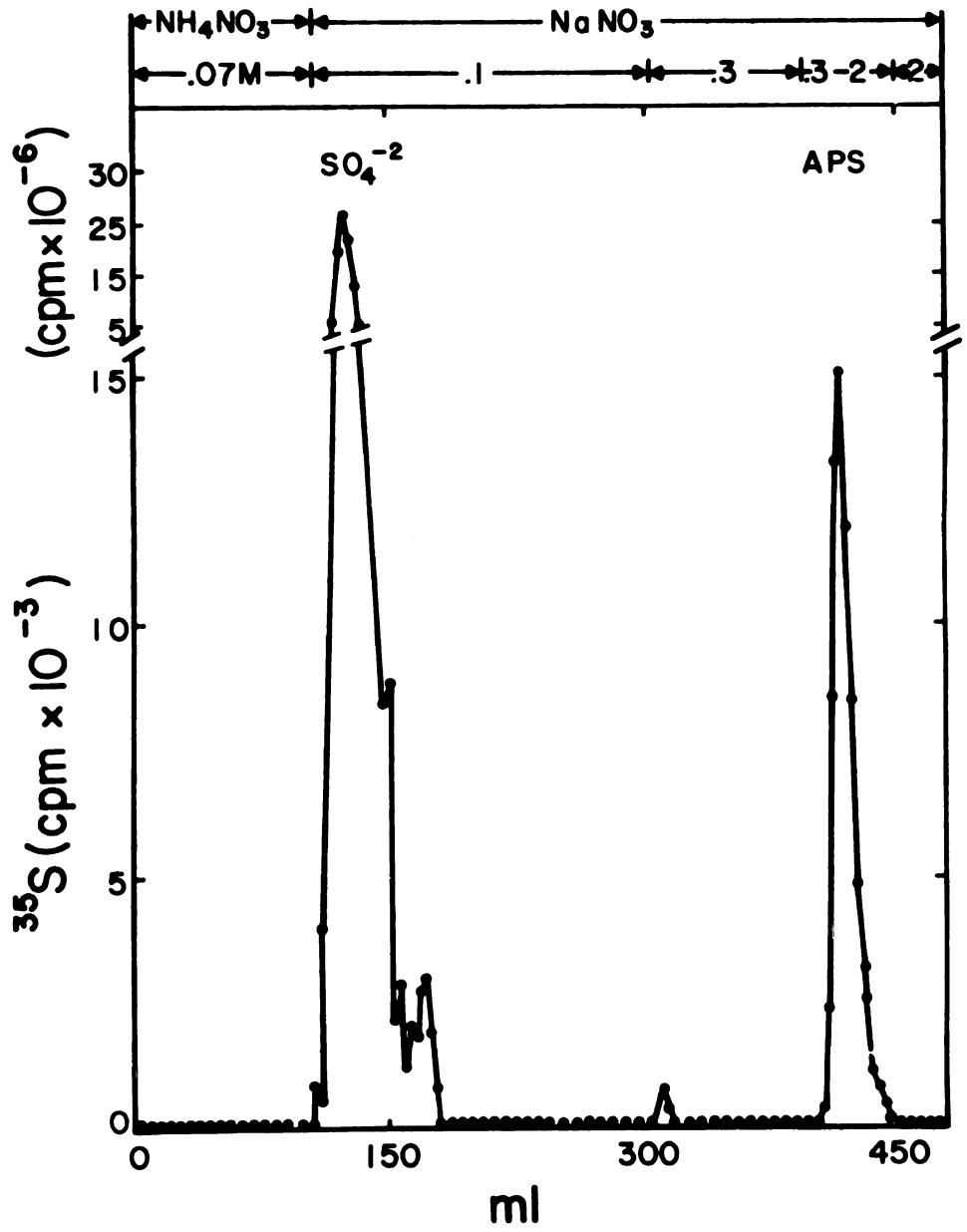

Figure 10. Analysis of the [^{35}S]-derivatives formed by tobacco cell extract: chromatography on Dowex-1-nitrate column.

Complete reaction mixture, 0.5 ml, as described in Table 1, was incubated with approximately 150 µg protein of tobacco cell extract, at 30 C for 30 min. Then the assayed mixture was diluted 1:10 with water and analyzed for [^{35}S]-derivatives by chromatography on Dowex-1-nitrate column as described in the text.

(1) Cultured tobacco cells contain a soluble enzyme which catalyzes the synthesis of a single [^{35}S]-labeled compound from [^{35}S]-sulfate, in a reaction mixture that requires ATP and Mg^{+2}.

(2) The [^{35}S]-product has solubility properties, electrophoretic and chromatographic behavior which are identical with those of APS, and distinct from those of sulfate and PAPS.

(3) The compound is acid labile and adsorbs and elutes from charcoal in the same manner as authentic APS.

(4) The enzymatic product contains adenine, sulfur, and phosphate in a molar ratio of ca. 1:1:1, as expected for APS, and behaves as a substrate for ATP sulfurylase in the reverse reaction.

(5) The [^{35}S]-product can be separated from the [^{35}S]-precursor, sulfate, by quantitative precipitation of Na_2SO_4 in ethanol:water (5:1).

Therefore, the product is APS and the enzyme is ATP sulfurylase. Moreover, the separation of APS from sulfate on the basis of solubility in ethanol-water can be used for quantitative determination of the APS formed by the crude ATP sulfurylase of tobacco cells.

IV. ATP Sulfurylase Assay Based on the Differential Solubility of Sulfate and APS

Conditions for assay of ATP sulfurylase activity, in the forward reaction, in which APS is quantitatively measured as the [^{35}S] recovered in the final ethanolic fraction (see Figure 1), were readily established. With these standard assay conditions, the rate of APS synthesis by tobacco cell enzyme is constant with time (Figure 11-A) and proportional to enzyme concentration (Figure 11-B).

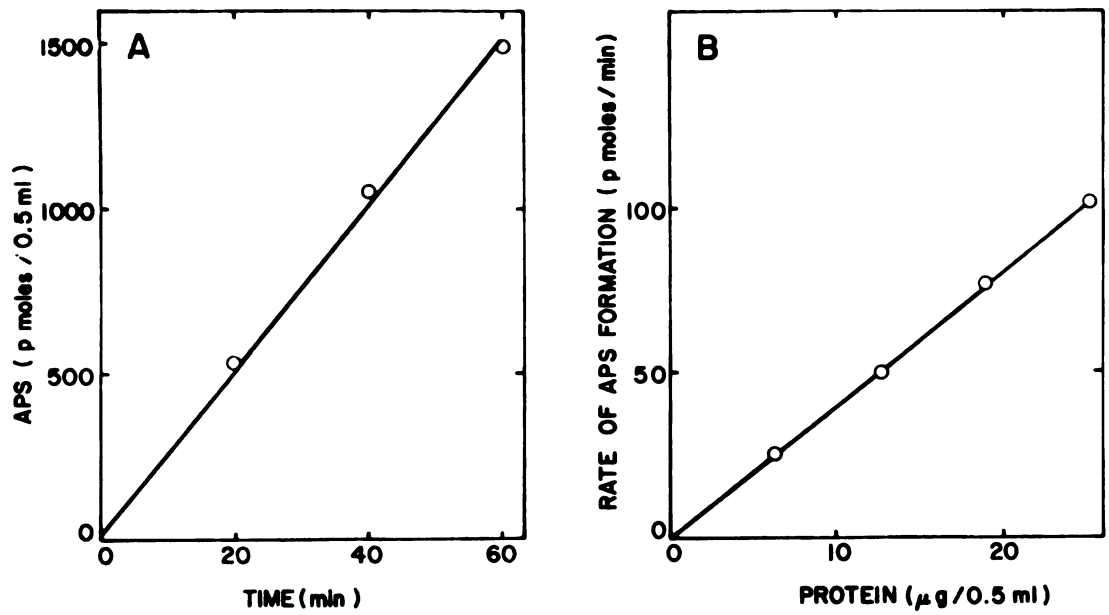

Figure 11. ATP sulfurylase assay based on APS formation: proportionality with time and enzyme concentration.

Complete reaction mixtures, 0.5 ml, contained: 45 mM glycine-NaOH (pH 9.0); 2 mM ascorbate; 10 mM $MgCl_2$; 40 mM [^{35}S]-Na_2SO_4 (2.5 μCi/μmole); 10 mM ATP; 8.5 units of inorganic pyrophosphatase; and 6.3-25.2 μg protein of tobacco cell extract. After incubation at 30 C for the times indicated, the assays were stopped by 2.5 ml cold absolute ethanol. APS was assayed by determining the soluble [^{35}S] remaining in the ethanol supernatant after precipitation of [^{35}S]-SO_4^{-2}.

A. Proportionality of APS formation with time of incubation.

B. Proportionality of the rate of APS formation with enzyme concentration.

When the concentrations of APS in the reaction mixture exceed 3 μM, a decrease in the rate of its synthesis was observed. This may be due to APS approaching the equilibrium concentration, but strict equilibrium was not reached even after 8 hr of incubation in which APS was at ca. 18 μM. Another possibility is that APS is an end-product inhibitor of the reaction.

Extension of the proportional accumulation of APS (see Figure 11) beyond the limitation imposed by the equilibrium constant, which was expected to result from the presence of excess inorganic pyrophosphatase in the assay mixture, was nevertheless not observed. Consequently, ATP sulfurylase activity was assayed under conditions confined to the range at which proportionality of APS formation occurs. This included 20 mM [^{35}S]-Na_2SO_4 (5 μCi/μmole), low amount of protein of ca. 1-40 μg, and short incubation times of 5-10 min.

When [^{35}S]-APS of a high specific radioactivity was desired for analytical purposes, APS was allowed to be synthesized in a reaction mixture containing 0.1 mM [^{35}S]-Na_2SO_4 (1 mCi/μmole), excess of extract up to 400 μg of protein for 30-60 min incubation. Under these different conditions, summarized in Table 7, the formation of APS had nevertheless the same dependencies as those described in Table 5.

The validity of this new method for assay of APS formation in crude extracts was tested with extracts obtained from several higher plants. All species examined exhibited ATP sulfurylase activity similar to the tobacco cell extract. Thus, extracts prepared from seedlings of pea, tomato, cucumber and *Lemna* plants all

Table 7. APS formation during various conditions

Reaction mixture	Total $[^{35}S]$-SO_4^{-2} added (CPM x 10^{-6})	$[^{35}S]$ in supernatant (CPM x 10^{-2})	APS formed (pmoles/0.5 ml)
Complete[a]	99.3	124	1,240
Complete[b]	101.2	6,040	302

[a]Complete reaction mixture, 0.5 ml, contained: 50 mM glycine-NaOH (pH 9.0); 2.5 mM ascorbate; 10 mM $MgCl_2$; 20 mM $[^{35}S]$-Na_2SO_4 (10^8 cpm per assay); 10 mM ATP; 7.2 units inorganic pyrophosphatase; and 33 µg of protein from tobacco cell extract. Incubation time was 10 min at 30 C.

[b]Complete reaction mixture, 0.5 ml, prepared as described above for (a) except the following modifications: 0.1 mM $[^{35}S]$-Na_2SO_4 (10^8 cpm per assay); and 320 µg protein of tobacco cell extract. Incubation at 30 C lasted for 60 min.

Both assays were stopped by 2.5 ml ethanol, and APS was determined, in the ethanol supernatant solution, after precipitation of $[^{35}S]$-SO_4^{-2}.

incorporated $[^{35}S]$-sulfate into a single ethanol-soluble $[^{35}S]$-product verified as APS (Table 8).

Therefore, APS formation can be generally assayed in plant extracts merely by determining the soluble $[^{35}S]$ remaining in the ethanol supernatant after precipitation of $[^{35}S]$-sulfate.

The assay is remarkably reproducible. The standard deviation within an experiment has not exceeded 2.5%. The high sensitivity of the assay is exemplified by the ability to detect easily as little as 20 pmoles of APS. Finally, the high suitability of this assay method for rapid performance of multiple assays was indispensable for the kinetic and regulatory studies described in the following

Table 8. APS formation catalyzed by extracts from higher plants

Enzyme source	ATP SULFURYLASE SPECIFIC ACTIVITY	
	nmole APS·min^{-1}·g.f.wt.$^{-1}$	nmole APS·min^{-1}·mg protein^{-1}
Peas	7.2	1.2
Tomato	7.6	1.2
Cucumber	7.1	1.7
Lemna	6.5	1.1
Tobacco cells	9.4	2.8

Reaction mixtures, 0.5 ml, contained: 45 mM glycine-NaOH (pH 9.0); 2 mM ascorbate; 10 mM $MgCl_2$; 40 mM [^{35}S]-Na_2SO_4 (2.5 µCi/µmole); 10 mM ATP; 8.5 units of inorganic pyrophosphatase; and 0.2 ml of crude extract. The protein content in each assay was: 248 µg for peas; 252 µg for tomato; 166 µg for cucumber; 228 µg for *Lemna*; and 33 µg for tobacco cells. Assays were conducted at 30 C for 0, 15 and 30 min. Reactions were stopped by the addition of 2.5 ml ethanol, and APS was determined from the soluble [^{35}S] remaining in the supernatant after precipitation of [^{35}S]-SO_4^{-2}.

section of this dissertation. A Sorvall SS-34 centrifuge rotor with a capacity of 8 tubes was used in order to generate the centrifugal force needed to achieve tightly-packed [^{35}S]-sulfate precipitates. Allowing for ca. 30 min for each round of precipitation, 8 to 24 samples can be assayed in 4 and 8 hours, respectively, with a single rotor.

It is noteworthy that, contrary to information in many commercial catalogs, the [^{35}S]-APS recovered in the ethanol supernatant fraction was rather stable. Thus, after storage of the ethanolic fraction at -20 C for 10 days, no breakdown product could be

detected by electrophoresis. The APS was somewhat less stable when stored at -20 C in water solution.

PART B:

THE REGULATION OF ATP SULFURYLASE IN CULTURED TOBACCO CELLS

B.1 Regulation of ATP Sulfurylase by Sulfur Compounds

Studies with bacteria and fungi indicated an apparent correlation between the ability of an organism to assimilate sulfate and the regulation of ATP sulfurylase by end-product repression (e.g., 24-27,66-67,126,129,185). The unsuccessful attempts to detect such a regulation of ATP sulfurylase in higher plants (104) were therefore somewhat surprising. The finding that sulfate transport is subject to end-product regulation in cultured tobacco cells (123-124) further suggested that the development of ATP sulfurylase in plants is likely to be regulated. Therefore, a reexamination of the influence of end-products of the sulfate pathway on the development of ATP sulfurylase was undertaken in the tobacco cell system.

Conditions in which the growth of the XD strain of cultured tobacco cells is dependent on the sulfur source added to the medium were determined earlier in this laboratory (123). This required the removal of contaminating anionic sulfur, of otherwise unknown chemistry, from reagent grade sucrose by means of an anion exchange resin. Under such conditions, 0.1 mM sulfate and 2.5 mM nitrate just meet the nitrogen and sulfur requirements of the tobacco cells to yield ca. a 30-fold increase in mass over ca. 14 days, up to ca. 30 g/l fresh weight of cells (179). Organic sulfur sources such as L-cysteine,

L-methionine, or reduced glutathione, at 0.1 mM each, can sustain growth of the tobacco cells when substituted for sulfate (123).

The parallel utilization by the cells of nitrate and sulfate supplied at a molar ratio of 25 to 1 is consistent with the relative rates of uptake for nitrate and sulfate in the tobacco cell system of 1.5 and 0.05 μmoles/hr/g.f.wt., respectively (70,123). The 25:1 ratio is also consistent with the relative abundance of nitrogen and sulfur in proteins (e.g., 199), and the fact that both nitrogen and sulfur are mostly (ca. 90%) assimilated into protein in plants (cf. 147,179). These correlations all suggest that the regulation of the rates of both nitrate and sulfate assimilation should be coupled to each other and to the rate of net protein synthesis.

I. The Effects of Sulfur Compounds on Growth and ATP Sulfurylase Activity in Tobacco Cells

Organic sulfur compounds vary in their relative effectiveness in supporting growth of the tobacco cells, as determined after 12 days of growth (Table 9). The utilization of either L-cysteine or L-methionine resulted in fresh weights similar to those obtained with sulfate. Glutathione and L-djenkolate also served as sulfur sources but resulted in lower growth rates. The elevated yield observed when cysteine and methionine were included with djenkolate in the medium (Table 9) suggested that the lower growth rate characteristic of djenkolate reflected its slow assimilation compared to L-cysteine, L-methionine or sulfate, rather than a toxic effect.

The small increase in fresh weight above the initial inoculum observed in "sulfur-free" medium prepared with purified sucrose

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The small increase in fresh weight above the initial inoculum observed in "sulfur-free" medium prepared with purified sucrose

Table 9. Growth of tobacco cells on various sulfur sources

SULFUR SOURCE(S) (0.1 mM each)	YIELD (g fresh weight/l)
Sulfate	25.0
L-cysteine	25.7
L-methionine	20.5
L-cysteine plus L-methionine	21.2
Reduced glutathione	11.5
L-djenkolate	9.9
L-djenkolate plus sulfate	23.9
L-djenkolate plus L-cysteine	24.2
L-djenkolate plus L-cysteine plus L-methionine	20.5
No sulfur added, purified sucrose	3.0
No sulfur added, reagent grade sucrose[a]	5.1

[a] $(N,S)^-$ MID in which reagent grade sucrose substitute for the purified sucrose.

Stationary phase cells were subcultured into $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM of sulfur source as indicated. Fresh weights were determined after 12 days, in duplicate cultures, and averaged. The initial inoculum was 1.7 g fresh weight of cells/l.

(Table 9), which is associated with a corresponding small increase in soluble protein, may mean that the anion exchange treatment did not remove all contaminating sulfur from the sucrose, or that other reagents were contaminated with sulfur. Regardless of its source, or chemical nature, this sulfur contaminant was sufficient to support ca. one doubling of the cells (Table 9). Consequently, in experiments in which strict dependence on an added sulfur source was desired, large initial inocula were used to rapidly exhaust the contaminating sulfur and thereby minimize its interference in experiments designed to determine the effects of specific sulfur sources on ATP sulfurylase development.

The various sulfur sources supporting growth have a profound influence on the level of ATP sulfurylase in the cells (Table 10). Thus, exponentially multiplying tobacco cells yield low ATP sulfurylase activity when grown on those sulfur sources which support rapid growth (see Table 9), while a strikingly higher activity is found in cells growing slowly on the sulfur of djenkolate or in "sulfur-free" medium (Table 10). These results also show that ATP sulfurylase of the XD cells is not induced by its substrate sulfate, which is in agreement with data reported on the enzyme isolated from other sources (e.g., 96,104,126). In contrast, sulfate represses the level of enzyme relative to the level found in sulfur starved cells (Table 10). Neither cysteine nor methionine, nor the combination of both (see Figure 12), result in complete repression of the enzyme in the tobacco cells (Table 10). Nevertheless, within any given experiment, cells utilizing either cysteine or methionine consistently

Table 10. ATP sulfurylase activity of tobacco cells grown on various sulfur sources

Sulfur source	ATP sulfurylase[a]
Sulfate	4.6
L-cysteine	3.0
L-methionine	2.7
L-djenkolate	20.1
No sulfur added	22.4

[a] nmoles APS x min^{-1} x mg $protein^{-1}$.

Stationary phase cells were subcultured into $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM of the sulfur source indicated. After 5 days of growth, ATP sulfurylase was extracted and assayed (see Materials and Methods). The initial ATP sulfurylase activity was 2.1 nmoles APS x min^{-1} x mg $protein^{-1}$.

have a somewhat lower level of ATP sulfurylase compared with cells growing on sulfate. Therefore, the tobacco cell ATP sulfurylase is highly, but not completely, repressed under optimal growth conditions. Derepressed levels appear when the sulfur supply falls short of supporting the optimal growth rate possible.

The possibility that these changes in ATP sulfurylase activities resulted by direct interactions of the sulfur compounds with the enzyme molecules was examined by determining the effects of the various sulfur sources on ATP sulfurylase activity *in vitro*. Neither cysteine nor methionine inhibited the rate of APS formation catalyzed by the tobacco cell enzyme, nor did djenkolate activate it (Table 11).

Table 11. Lack of effects of the sulfur amino acids on APS formation *in vitro*

Assay mixture	ATP SULFURYLASE ACTIVITY	
	nmoles APS/10 min	nmoles APS x min^{-1} x mg^{-1}
Complete	823	5.5
Complete plus L-cysteine	904	6.0
Complete plus L-methionine[a]	808	5.4
Complete plus L-cysteine plus L-methionine	800	5.3
Complete plus L-djenkolate	758	5.1

[a]Either with or without 1.0 mM L-arginine.

Complete reaction mixture, 1.0 ml, contained: 20 mM glycine-NaOH (pH 9.0); 0.5 mM ascorbate; 10 mM $MgCl_2$; 20 mM $[^{35}S]$-Na_2SO_4 (5 µCi/µmole); 10 mM ATP; 7.2 units of inorganic pyrophosphatase; and 0.015 mg protein of tobacco cell extract. Sulfur amino acids were added to a final concentration of 1.0 mM each. Extract, prepared from exponential cells grown on MID, dialyzed against 0.1 M glycine-NaOH buffer (pH 9.0) containing 5 mM ascorbate, for 7 hr at 4 C. Solutions of the sulfur amino acids were prepared in the assay buffer, and the pH was adjusted to ca. 9.0. After incubation for 10 min at 30 C, 5 ml of absolute ethanol were added, and APS was determined as described in Materials and Methods. The formation of APS was proportional with time and enzyme concentration.

A slight stimulation by L-cysteine of APS formation *in vitro* has been observed consistently. This phenomenon has not been investigated further.

The above results lead to the conclusion that the changes in the level of extractable ATP sulfurylase activity which develop during growth on the various sulfur sources result from the repression-derepression type of regulation rather than from the feedback inhibition type of regulation.

II. Dependence of the Regulation of ATP Sulfurylase on Growth Rate, as Determined by Nitrogen Source

The results described above further indicated that the ability of a sulfur compound to bring about changes in ATP sulfurylase level was linked to its effectiveness in supporting growth. The growth rate of cells, however, can be manipulated independently of the sulfur source by means of the nitrogen supply. It was, therefore, desirable to investigate the dependency of the regulation of ATP sulfurylase on the growth rate as determined by various nitrogen conditions.

Tobacco cells can satisfy their nitrogen requirements with urea, but the growth rate on urea is slower than on nitrate (70). Since the lower growth rate on urea should result in a lower rate of net protein synthesis and hence a lower rate of assimilation of sulfur, this raised the question of whether the ATP sulfurylase level in tobacco cells growing on urea nitrogen would also be lower than in cells grown on nitrate nitrogen. If the regulation of ATP sulfurylase is governed by the rate of sulfur assimilation in relation to protein

synthesis, slower growth on urea should yield a concomitant lower level of the enzyme. If, however, the enzyme level is determined solely by the sulfur source, independent of the rate of protein synthesis, activities on urea should be comparable to those on nitrate. Thus, experiments were performed to test these ideas. Indeed, cells grown on urea exhibited ca. a 2-fold decrease in growth rate concomitantly with a ca. 2-fold decrease in soluble protein compared with cells grown on nitrate (Table 12). This trend was found under all sulfur conditions. Thus, both growth rate and total protein are governed predominantly (although not exclusively) by the nitrogen supply. In contrast, the specific activity of ATP sulfurylase was rather independent of the nitrogen source (Table 12). This is a reflection of the fact that there was ca. a 2-fold decrease of *total* activity in cells grown on urea, which parallels the decrease in protein. Nevertheless, regardless of the nitrogen source, sulfate, cysteine and methionine resulted in repressed levels of ATP sulfurylase, while cells grown on djenkolate, or starved for sulfur, have the characteristic derepressed activity (Table 12). Thus, the regulation of ATP sulfurylase, though governed primarily by the sulfur source, appeared to be coordinated in some way as well with the rates of nitrogen assimilation into protein.

III. Kinetics of the Development of ATP Sulfurylase as a Function of the Sulfur Source

The finding of different levels of ATP sulfurylase in cells grown on various sulfur sources prompted studies of the kinetics of the development of these differences.

Table 12. Growth and ATP sulfurylase activity: dependency on both sulfur and nitrogen sources

GROWTH CONDITIONS				
Nitrogen source	Sulfur source	g fresh weight increase (% x day^{-1})	Protein (mg/g fresh weight)	ATP sulfurylase (nmoles APS x min^{-1} x mg $protein^{-1}$)
Nitrate	sulfate	62	8.2	3.7
Urea	sulfate	32	3.7	3.3
Nitrate	L-cysteine	55	6.0	2.2
Urea	L-cysteine	25	3.0	2.6
Nitrate	L-methionine	50	7.2	2.7
Urea	L-methionine	24	4.8	2.4
Nitrate	L-djenkolate	33	2.6	17.8
Urea	L-djenkolate	20	1.8	13.9
Nitrate	no sulfur added	26	2.4	18.6
Urea	no sulfur added	11	1.6	14.0

Stationary phase cells were subcultured into fresh $(N,S)^-$ MID containing either 2.5 mM nitrate or 3.0 mM urea as the nitrogen source and 0.1 mM of the sulfur source. After 4 days of growth on nitrate and 5 days on urea, cells were harvested, and ATP sulfurylase activity was determined. The initial activity was 2.1 and 3.7 nmoles APS x min^{-1} x mg $protein^{-1}$ for nitrate and urea grown cells, respectively. Growth rates are based on the determinations of fresh weights between 3 and 7 days of exponential growth.

Stationary phase tobacco cells which have consumed all of the nitrogen and sulfur initially included in the medium have low levels of ATP sulfurylase. Because such cells have access to a minimal amount of free sulfur compounds, and contain a minimal amount of the enzyme, they are particularly suitable for use in studies of the kinetics of ATP sulfurylase development as a function of exogenously supplied sulfur. When these cells are transferred to fresh medium, ATP sulfurylase develops with kinetics that depend on the sulfur source.

In the presence of readily assimilated sulfur sources such as sulfate, cysteine, or cysteine plus methionine, ATP sulfurylase specific activity remained continuously repressed at close to the initial level for 6 days (Figures 12 and 13). During this period, however, there was an increase of over 3-fold in total soluble protein in the culture (Table 13). On the other hand, in cells slowly assimilating djenkolate sulfur, as judged by the slow increase of growth and soluble protein (Tables 13 and 14), the specific activity of ATP sulfurylase began to rise within 12 hr (Figure 12) and increased steadily during the subsequent 4 days, up to 10- to 25-fold above the initial level (Figures 12 and 13). After 4 days, when the growth rate of the cells on djenkolate declined, ATP sulfurylase specific activity decayed (Figure 13, Table 14). Subsequently, when growth ceased, the level of ATP sulfurylase in cells grown on djenkolate returned to the low initial level, which is about the same as the repressed level found in cells grown on sulfate or cysteine.

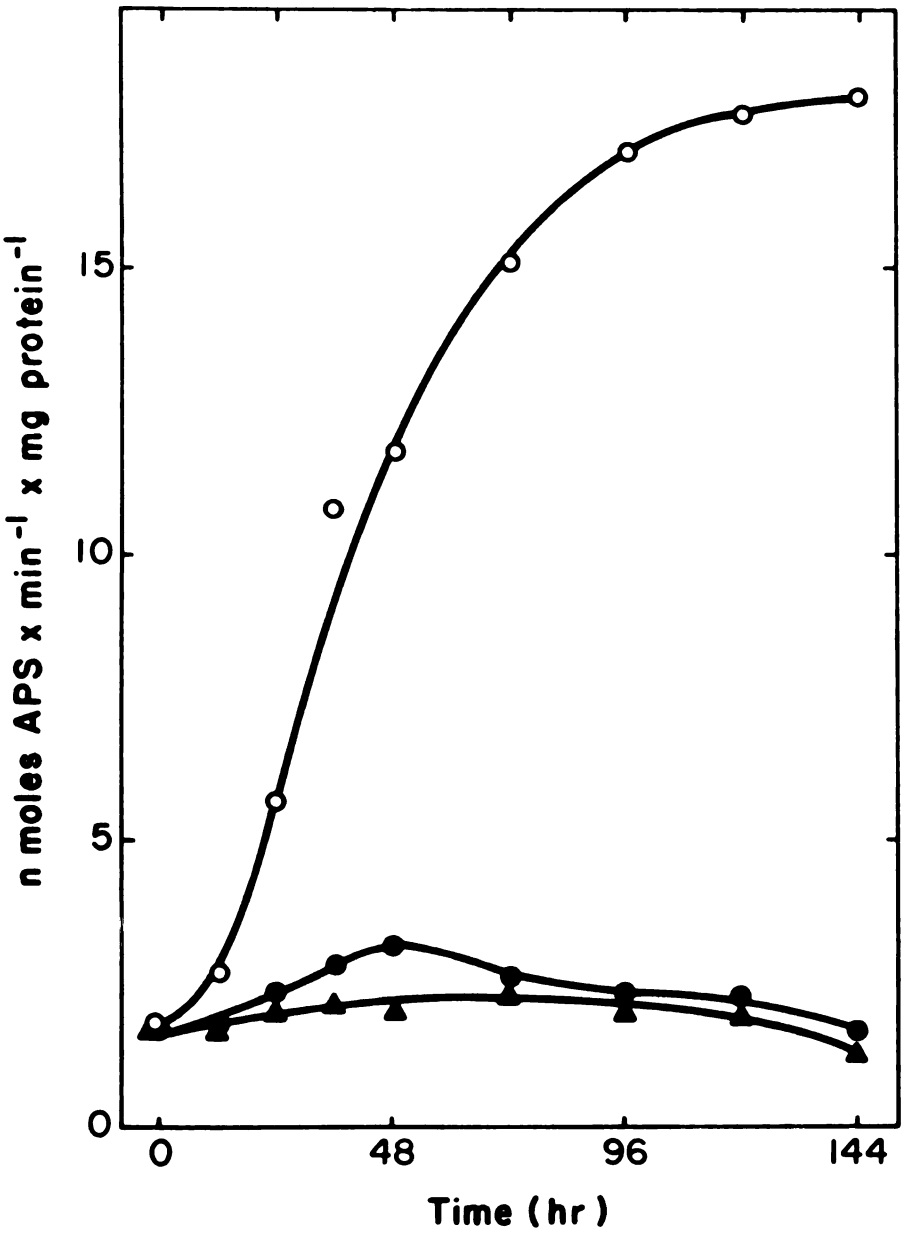


Figure 12. Kinetics of the development of ATP sulfurylase as a function of the sulfur source.

Stationary phase cells grown on $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM sulfate were aseptically harvested, washed with $(N,S)^-$ MID and resuspended in $(N,S)^-$ MID containing 2.5 mM nitrate and 0.1 mM of each of the sulfur sources: sulfate (●), cysteine plus methionine (▲), and djenkolate (O). At the times indicated, aliquots were aseptically removed for determinations of fresh weight, soluble protein and enzyme activity.

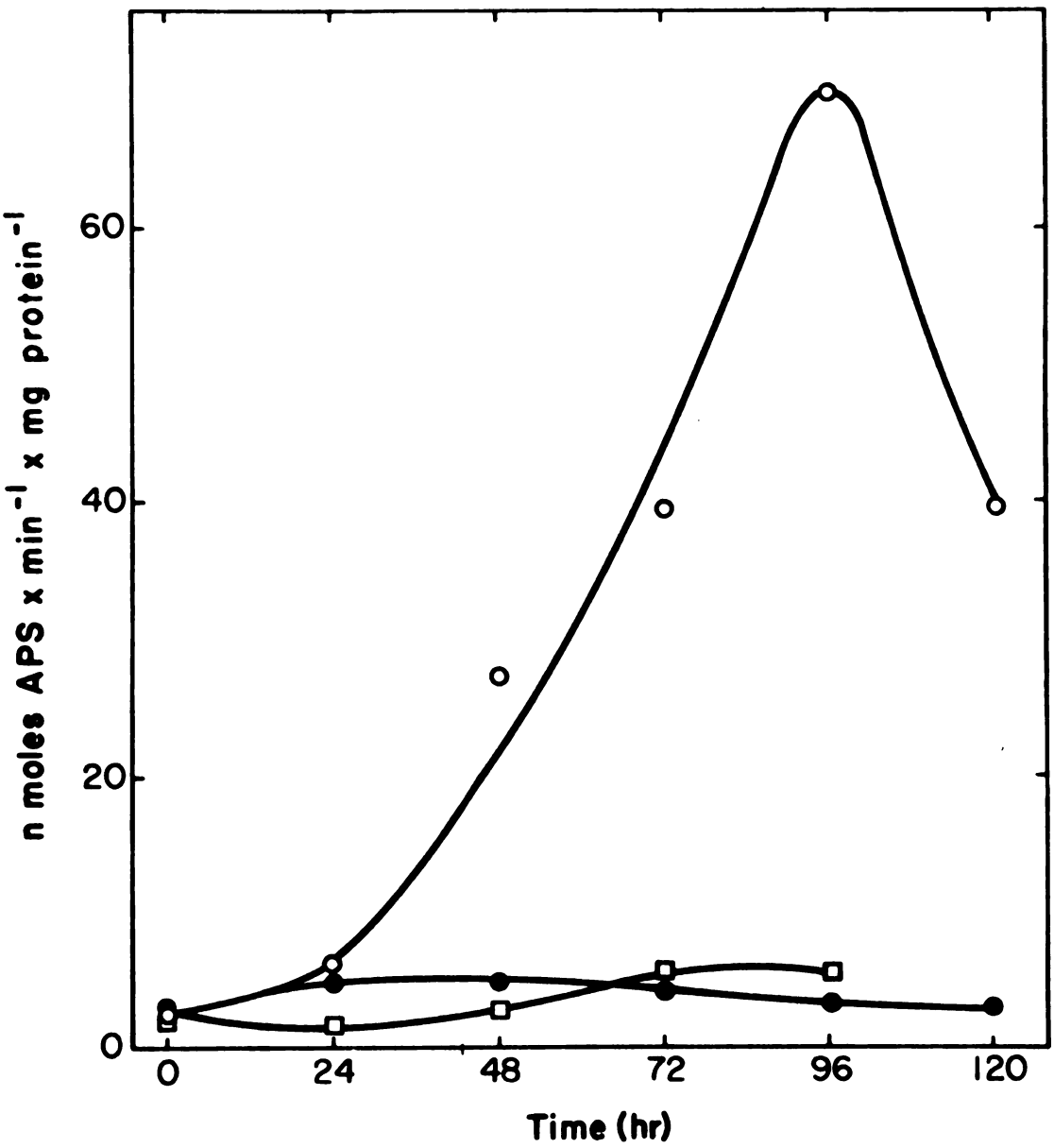


Figure 13. Kinetics of the development of ATP sulfurylase: derepression by djenkolate and repression by sulfate or cysteine.

Stationary phase cells grown for 10 days on $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM djenkolate were aseptically harvested, washed with $(N,S)^-$ MID, and resuspended in fresh $(N,S)^-$ MID containing 2.5 mM nitrate and 0.1 mM of the sulfur source: sulfate (●), L-cysteine (□), and L-djenkolate (○). At the times indicated, aliquots were taken from each culture for determinations of fresh weight, soluble protein and ATP sulfurylase activity.

Table 13. Relationship of ATP sulfurylase development to fresh weight and soluble protein during growth on various sulfur sources[a]

Time (hr)	SULFATE			L-CYSTEINE + L-METHIONINE			L-DJENKOLATE		
	Fresh weight (g/l)	Protein (mg/l)	ATP sulfurylase[b]	Fresh weight (g/l)	Protein (mg/l)	ATP sulfurylase[b]	Fresh weight (g/l)	Protein (mg/l)	ATP sulfurylase[b]
0	18.3	29.3	1.7	18.2	27.3	1.8	16.9	30.4	1.8
12	19.0	36.1	1.8	19.2	32.6	1.6	17.0	32.3	2.7
24	20.6	53.6	2.4	21.5	49.4	2.0	17.2	41.3	5.7
36	22.4	73.9	2.8	24.3	77.8	2.1	19.7	37.4	10.8
48	22.6	76.8	3.2	23.0	89.7	2.0	22.0	39.6	11.8
72	33.8	104.8	2.6	27.0	102.6	2.3	23.0	41.4	15.1
96	37.2	89.8	2.4	33.4	93.5	2.0	26.6	50.5	17.1
120	42.0	67.2	2.3	32.8	72.2	1.9	28.0	50.4	17.7
144	48.8	73.2	1.7	42.0	88.2	1.3	35.6	49.8	18.0

[a] Data from same experiment described in Figure 12.

[b] nmoles APS x min^{-1} x mg $protein^{-1}$.

These results suggest that under repressive conditions ATP sulfurylase was formed at ca. the same rate as total protein, while during sulfur-limited growth, an active enzyme accumulated at a much higher rate than that at which the average protein accumulated in the cells (see Table 13). Furthermore, the regulation of ATP sulfurylase during growth on slowly assimilated sulfur involved both derepression during active growth and a subsequent decay.

Derepression of ATP sulfurylase also occurs in cells utilizing the slowly assimilated sulfur of glutathione, or in cells starved for sulfur in a "sulfur-free" medium containing a nitrogen source. Similar results are obtained with nitrate (Figure 14) or urea (Figure 15). Moreover, the rate of the derepression is inversely related to the growth rate, i.e., the lower the growth rate the higher the rate of development of ATP sulfurylase (Figures 14 and 15; see Table 9).

If sulfate is included with djenkolate in the growth media, the level of ATP sulfurylase remains repressed (Figures 14 and 15). The derepression of the enzyme is also prevented by the presence of cysteine with djenkolate (Figure 15) or cysteine plus methionine with djenkolate, indicating that it is the absence of the readily assimilated sulfur, rather than the presence of djenkolate which results in the derepression.

Furthermore, the addition of sulfate to sulfur-starved cells in which ATP sulfurylase has been derepressed results in a rapid decline in the enzyme specific activity (Figure 16), which coincides with the resumption of net protein synthesis in response to the

Table 14. Growth and ATP sulfurylase activity during the derepression by djenkolate and the repression by sulfate or cysteine[a]

Time (hr)	SULFATE		L-CYSTEINE		L-DJENKOLATE	
	Fresh weight (g/l)	ATP sulfurylase[b]	Fresh weight (g/l)	ATP sulfurylase[b]	Fresh weight (g/l)	ATP sulfurylase[b]
0	7.0	2.6	7.0	2.6	7.0	2.6
24	8.5	4.8	11.0	1.9	9.5	6.2
48	11.5	4.7	11.0	2.7	13.0	27.1
72	17.5	4.4	18.0	5.7	13.5	39.4
96	25.0	3.2	25.5	5.7	14.0	69.8
120	34.0	3.1	-	-	16.0	39.6

[a]Data from same experiment described in Figure 13.

[b]nmoles APS x min^{-1} x mg $protein^{-1}$.

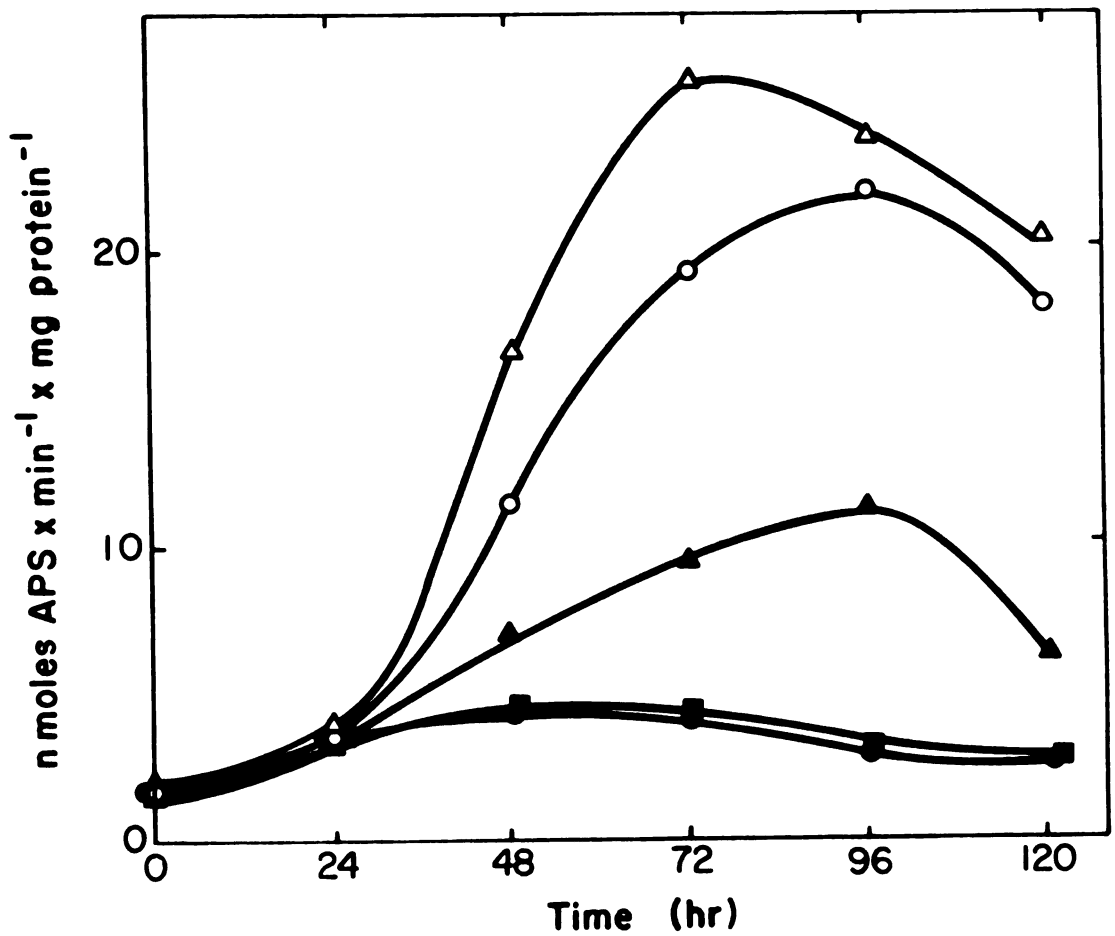


Figure 14. Derepression of ATP sulfurylase during growth on glutathione or during sulfur starvation on nitrate.

Stationary phase cells maintained continuously on $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM sulfate were subcultured into $(N,S)^-$ MID containing 1.25 mM nitrate and 0.05 mM sulfate. After 9 days, cultures which contained ca. 13 g/l fresh weight were resupplemented with 2.5 mM nitrate and 0.1 mM of each of the sulfur sources: sulfate (●), sulfate plus djenkolate (■), reduced glutathione (▲), djenkolate (O), and no sulfur added (Δ). At the times indicated, aliquots were aseptically removed for determinations of fresh weight, protein and ATP sulfurylase activity.

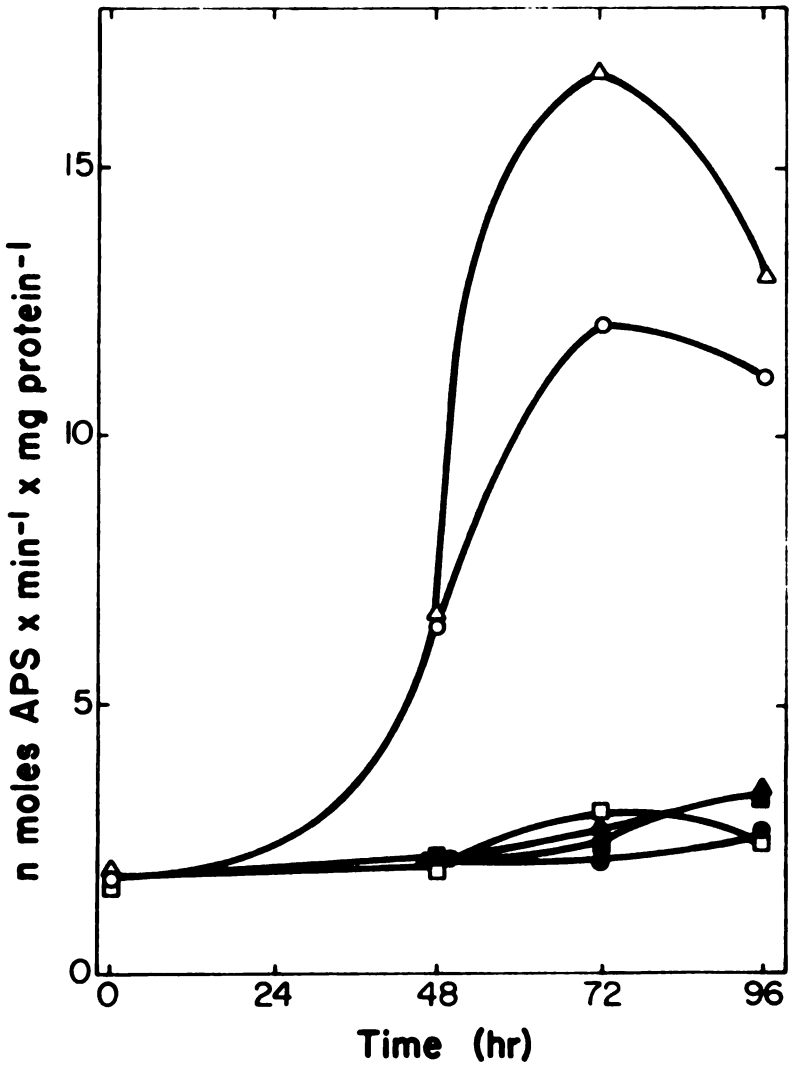


Figure 15. Kinetics of the repression-derepression development of ATP sulfurylase during growth on urea.

Stationary phase cells maintained continuously on $(N,S)^-$ MID supplemented with 3.0 mM urea and 0.1 mM sulfate were subcultured into $(N,S)^-$ MID containing 0.75 mM urea and 0.025 mM sulfate. After 7 days, cells were aseptically harvested, washed with $(N,S)^-$ MID and resuspended in $(N,S)^-$ MID supplemented with 3.0 mM urea and 0.1 mM of each of the sulfur sources: sulfate (●), sulfate plus djenkolate (□), cysteine (▲), cysteine plus djenkolate (■), djenkolate (○) or no sulfur added (△). At the times indicated, aliquots were aseptically removed for determinations of fresh weight, protein and ATP sulfurylase activity. Initial inocula were 5.5 g/l of fresh weight cells.

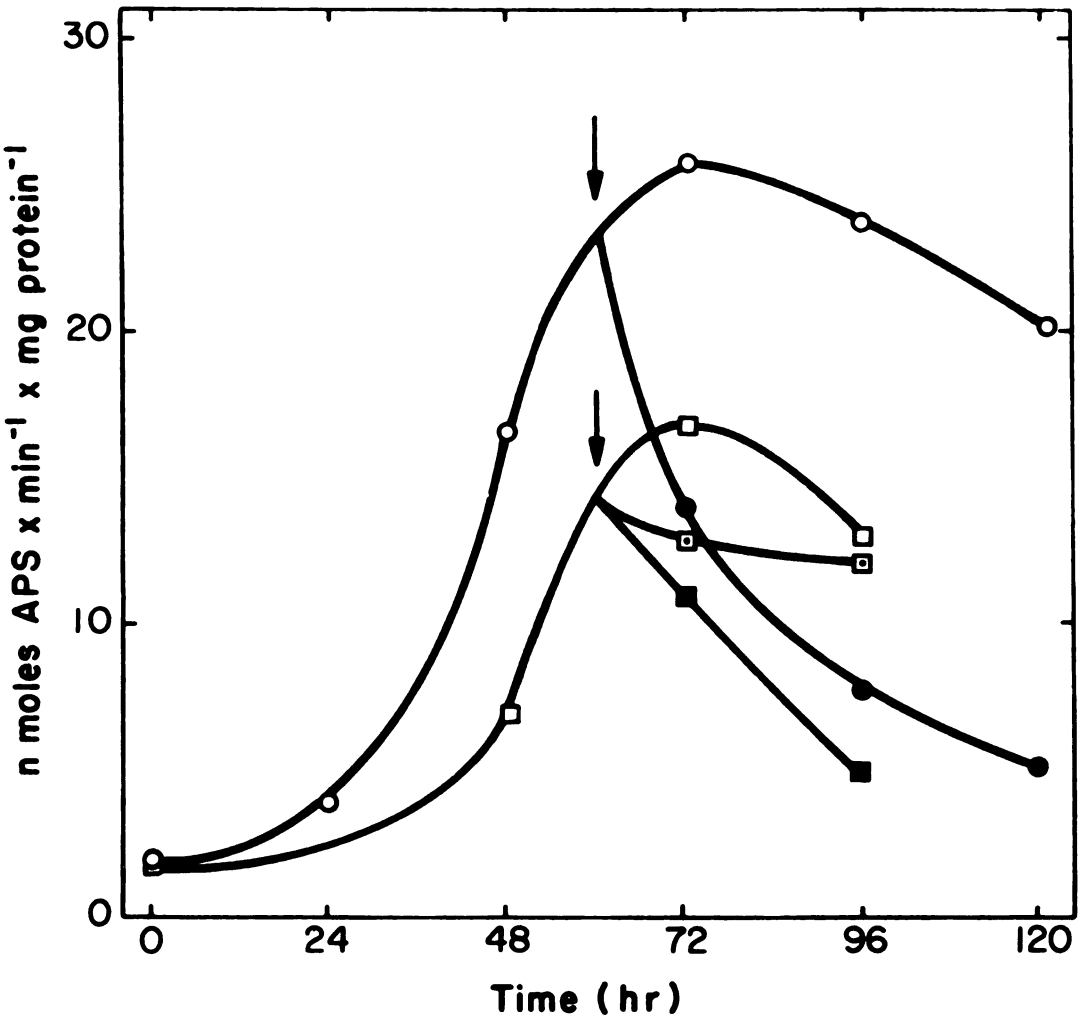


Figure 16. Kinetics of the decline of ATP sulfurylase activity upon addition of sulfate to sulfur-starved cells.

Sulfur-starved cells on nitrate (○) or urea (□) were described in the legends to Figures 14 and 15, respectively. After 60 hr (arrows), 0.1 mM of sulfate was added to aliquots of the sulfur-starved cells on nitrate (●) or urea (■). In addition, 0.1 mM djenkolate was also added to an aliquot of the sulfur-starved cells on urea (⊡). At subsequent times, aliquots from each culture were removed for determinations of fresh weight, soluble protein, and ATP sulfurylase activity.

added sulfate (Table 15). Similar results are obtained when sulfate is added to cells that have been derepressed on djenkolate (see Figure 25-C). This decline in ATP sulfurylase specific activity upon addition of a repressing sulfur source to derepressed cells reflects a decay of enzyme rather than dilution as a result of increased total protein (Table 16). Thus, enzyme inactivation or degradation also plays a role in regulation of ATP sulfurylase. Djenkolate, however, is much less effective than sulfate in promoting both the decay of ATP sulfurylase and the resumption of net protein synthesis, when added to cells derepressed by sulfur starvation (Figure 16; Table 15).

The results are, therefore, leading to the conclusion that a repressing sulfur source is effective in both the repression of ATP sulfurylase as well as the decay of the enzyme. On the other hand, a derepressing sulfur source is effective neither in repression nor in the decay of ATP sulfurylase.

IV. Specificity of the ATP Sulfurylase Repression-Derepression Mechanisms for Sulfur Amino Acids

The activity of the sulfur amino acids cysteine, methionine, and djenkolate in the regulation of ATP sulfurylase is hypothesized to be due to their sulfur content. Nevertheless, alternative hypotheses, e.g., that the α-amino group or carboxyl group determines activity, were conceivable. In an attempt to assess the specificity for the sulfur amino acids in the repression-derepression regulation, the effects of a mixture of non-sulfur amino acids on the development of ATP sulfurylase was studied.

Table 15. Resumption of net protein synthesis concomitantly with the decline of ATP sulfurylase level upon addition of sulfate to sulfur-starved cells[a]

Time (hr)	UREA — NO SULFUR ADDED: Protein (mg/l)	UREA — NO SULFUR ADDED: ATP sulfurylase[b]	Time (hr after sulfur addition)	UREA — SULFATE ADDED: Protein (mg/l)	UREA — SULFATE ADDED: ATP sulfurylase[b]	UREA — DJENKOLATE ADDED: Protein (mg/l)	UREA — DJENKOLATE ADDED: ATP sulfurylase[b]
0	15.4	1.8	-	-	-	-	-
48	29.0	6.8	-	-	-	-	-
72	21.6	16.8	12	24.7	10.9	22.8	12.9
96	18.7	13.0	36	46.5	4.9	33.0	12.3

[a]Data from same experiment described in Figure 16 for sulfur-starved cells on urea nitrogen. The sulfur source was added to aliquots removed from the sulfur-starved cells after 60 hr (see Figure 16).

[b]nmoles APS x min^{-1} x mg $protein^{-1}$.

Table 16. Decay of ATP sulfurylase upon addition of sulfate to derepressed cells[a]

Time (hr)	NITRATE NO SULFUR ADDED: Protein (mg/culture)	NITRATE NO SULFUR ADDED: ATP sulfurylase[b] (nunits/culture)	Time (hr after sulfate addition)	NITRATE SULFATE ADDED: Protein (mg/culture)	NITRATE SULFATE ADDED: ATP sulfurylase[b] (nunits/culture)
0	28.8	57.6	-	-	-
24	47.1	183.7	-	-	-
48	41.1	682.3	-	-	-
72	32.6	837.8	12	52.4	733.6
96	33.6	799.7	36	64.3	495.1
120	34.3	699.7	60	79.2	403.4

[a]Data from same experiment described in Figure 16, for derepression of ATP sulfurylase during sulfur starvation on nitrate containing medium. Sulfate was added after 60 hr.

[b]Total activity as nmoles APS x min^{-1} per culture.

Cells with a derepressed level of ATP sulfurylase were transferred to media containing 15 non-sulfur amino acids and either djenkolate or cysteine plus methionine to provide sulfur. The amino acid mixture did not affect the changes in ATP sulfurylase activity governed by the sulfur source (Figure 17). The repressing sulfur amino acids, cysteine and methionine, prompted the decay of the enzyme, while the derepressed level of ATP sulfurylase was maintained by djenkolate.

Therefore, the role of these sulfur amino acids in the regulatory processes of ATP sulfurylase in the tobacco cells is likely to be related specifically to their chemical nature as sulfur sources.

V. The Effect of Nitrogen Starvation on the Derepression of ATP Sulfurylase

The indication that nitrogen assimilation has a role in the regulation of ATP sulfurylase by sulfur compounds was tested by monitoring the enzyme changes during nitrogen starvation.

Derepression of ATP sulfurylase by djenkolate sulfur does not occur if the tobacco cells are starved for nitrogen (Figure 18). This lack of derepression cannot be attributed to a nonspecific mechanism because the rate of protein synthesis determined by incorporation of a radioactive amino acid is known to continue at the normal rate in nitrogen-starved cells (70). This must represent turnover synthesis of protein only since no net increase in total soluble protein can be detected in the cells over a 4-day period (Table 17). Within 12 hr after addition of a nitrogen source to such cells an increase in ATP sulfurylase specific activity is

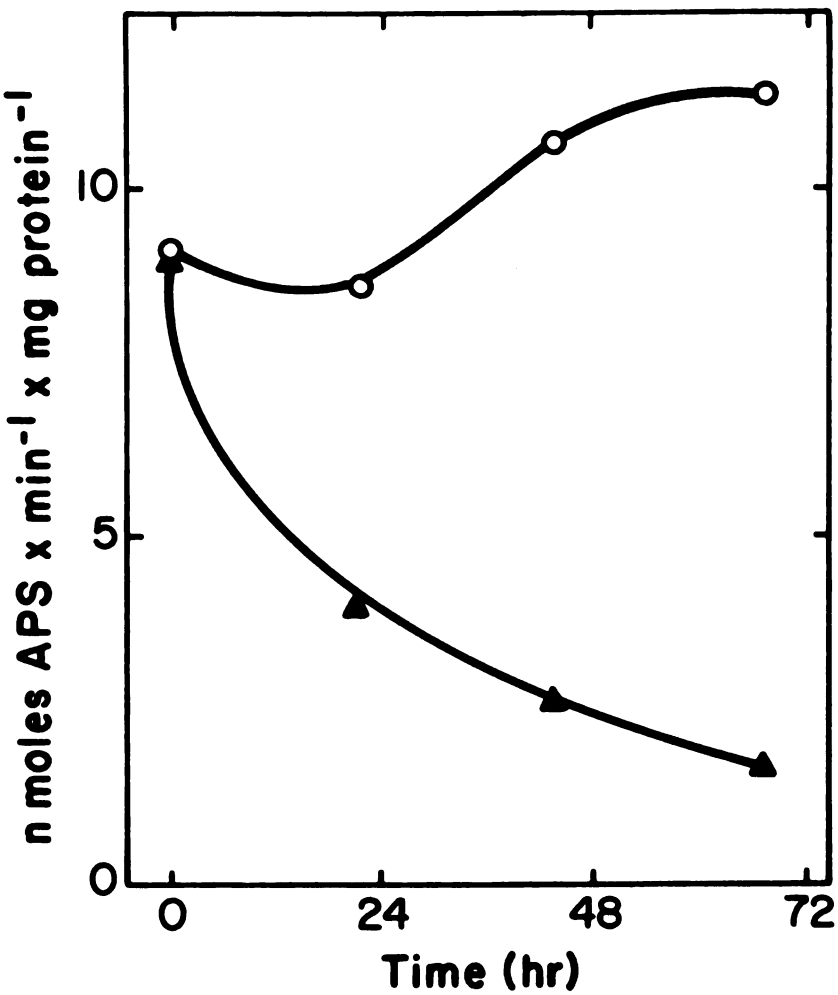


Figure 17. Lack of effect of non-sulfur amino acids on the repression-derepression regulation of ATP sulfurylase.

Cells were grown on $(N,S)^-$ MID containing 0.5 g/l of casein hydrolyzate as the sole source for both nitrogen and sulfur. Five-tenths gram per liter casein hydrolyzate contains 3.6 mM nitrogen and 0.06 mM sulfur (183). After 12 days, the cells were aseptically harvested, washed with $(N,S)^-$ MID and resuspended in $(N,S)^-$ MID containing a mixture of 15 amino acids (see Materials and Methods), each at 0.1 mM, and sulfur as either djenkolate (O) or cysteine plus methionine (▲) at 0.1 mM each. At the times indicated aliquots were aseptically removed for determinations of fresh weight, soluble protein and ATP sulfurylase activity. The initial inocula contained ca. 10 g/l of fresh weight cells.

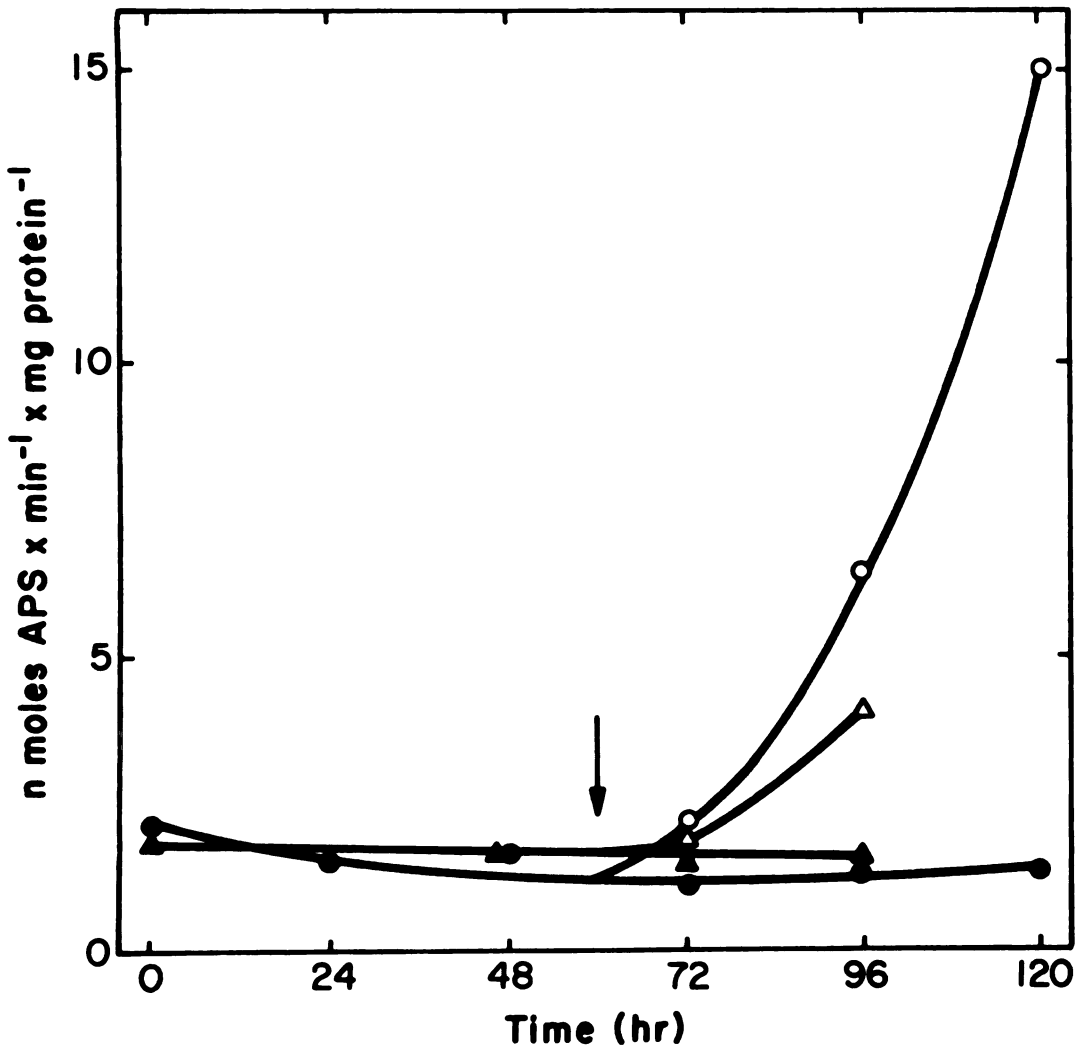


Figure 18. Dependency of the development of ATP sulfurylase activity on nitrogen assimilation: lack of derepression by djenkolate in cells starved for nitrogen.

Stationary phase cells maintained continuously on $(N,S)^-$ MID supplemented with 0.1 mM sulfate and (a) 2.5 mM nitrate or (b) 3.0 mM urea were described in the legends to Figures 14 and 15, respectively, except that: (a) culture was supplemented with 0.1 mM djenkolate but no nitrogen was added (●), and (b) cells were resuspended with 0.1 mM djenkolate but no nitrogen added (▲). After 60 hr (arrow) nitrogen was added to aliquots of the corresponding culture: (a) 2.5 mM nitrate (○), and (b) 3.0 mM urea (△). Fresh weight, soluble protein, and ATP sulfurylase activity were determined on aliquots removed from each culture at the times indicated.

Table 17. Absence of net protein synthesis in nitrogen-starved cells and its resumption upon addition of nitrogen[a]

Time (hr)	DJENKOLATE NITROGEN FREE MEDIUM Protein (mg/l)	ATP sulfurylase[b]	Time after 60 hr (hr)	DJENKOLATE UREA ADDED Protein (mg/l)	ATP sulfurylase[b]
0	16.8	1.8	-	-	-
48	17.0	1.7	-	-	-
72	16.5	1.6	12	28.6	1.8
96	10.4	1.5	36	39.0	4.0

[a]Data from same experiment described in Figure 18 for urea-starved cells. Urea was added after 60 hr. Similar results were obtained in nitrate-starved cells (not shown).

[b]nmoles APS x min^{-1} x mg $protein^{-1}$.

evident and continues with a rate characteristic of the derepression (Figure 18). The derepression of ATP sulfurylase level initiated by the addition of urea occurs concomitantly with the resumption of net protein synthesis (Table 17). Similar results are obtained upon addition of nitrate. Thus, active assimilation of nitrogen is required for the derepression of ATP sulfurylase.

The requirement for nitrogen assimilation is also consistent with the finding of low ATP sulfurylase level in stationary phase (nitrogen starved) cells, regardless of the sulfur source.

VI. The Effect of Inhibition of Protein Synthesis on the Derepression of ATP Sulfurylase

In an attempt to approach the molecular events underlying the derepression of ATP sulfurylase, the effect of inhibition of protein synthesis by cycloheximide on the development of ATP sulfurylase was investigated in cells grown on djenkolate. Cycloheximide, at 4 µg/ml, is an effective inhibitor of protein synthesis in the tobacco cells (70). The effect of cycloheximide on the development of ATP sulfurylase was studied in cells utilizing urea nitrogen in order to avoid the inhibition by cycloheximide of the induced development of nitrate reductase (181), which is essential for assimilation of nitrate nitrogen.

The effect of cycloheximide on the derepression by dkenkolate sulfur of ATP sulfurylase was studied and compared with its effect on the enzyme level in control cells grown on djenkolate plus sulfate. Cycloheximide inhibited the rate of [^{14}C]-arginine incorporation by the tobacco cells into protein by 95 - 97% within 1 hr (Figure 19-B).

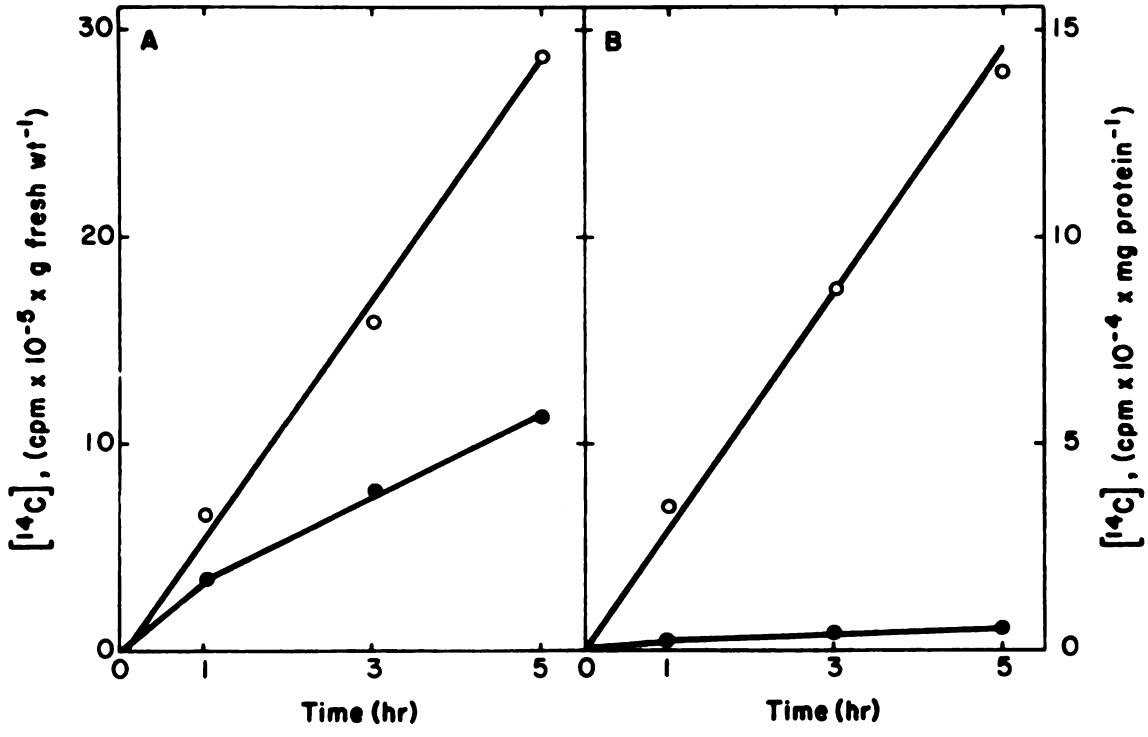


Figure 19. Inhibition by cycloheximide of the uptake and incorporation into protein of [^{14}C]-L-arginine in the tobacco cells.

The effectiveness of cycloheximide as inhibitor of protein synthesis in the tobacco cells was studied concomitantly with its effect on inhibition of the development of ATP sulfurylase, in cells grown on 3.0 mM urea and 0.1 mM djenkolate as described in the legend to Figure 20. After 48 hr, aliquots of 30 ml were aseptically removed and incubated with 0.01 mM L-arginine plus 2 µCi of uniformly labeled [^{14}C]-L-arginine, with 4 µg/ml cycloheximide (●) or without it (○). At 1, 3 and 5 hr thereafter aliquots were removed for determinations of uptake (A) and incorporation into protein (B) of the radioactive arginine (see Materials and Methods).

This inhibition could not be attributed solely to the inhibition by cycloheximide of the uptake of arginine. The latter was inhibited by cycloheximide only by 26-39% within 1 hr (Figure 19-A). Inhibition by cycloheximide of the rates of uptake and incorporation of [^{14}C]-arginine into protein in cells grown on djenkolate (Figure 19) was identical to the control cells utilizing djenkolate plus sulfate. The cycloheximide effects are, therefore, independent of the sulfur source available to the cells.

Cycloheximide, added to cells that have been derepressed for 48 hr, results in a complete inhibition of the development of ATP sulfurylase, observed within 1 hr after its addition (Figure 20). The inhibited level of enzyme in treated cells, however, remained constant during the subsequent 24 hr. If added at 72 hr, cycloheximide likewise prevented the continuation of enzyme development. Cycloheximide has little or no effect on the repressed level of ATP sulfurylase in cultures grown on djenkolate plus sulfate (Figure 20).

These findings indicate that the mechanism of the derepression of ATP sulfurylase in the tobacco cells depends in some way upon protein synthesis.

B.2 Regulation of ATP Sulfurylase by Group VI Anions

The physiological and biochemical studies described in the preceding section indicated that the availability of sulfur to support growth of the tobacco cells governs repression-derepression of ATP sulfurylase.

In efforts to further analyze this regulatory system, use was made of the observation that Group VI anions are structural analogs

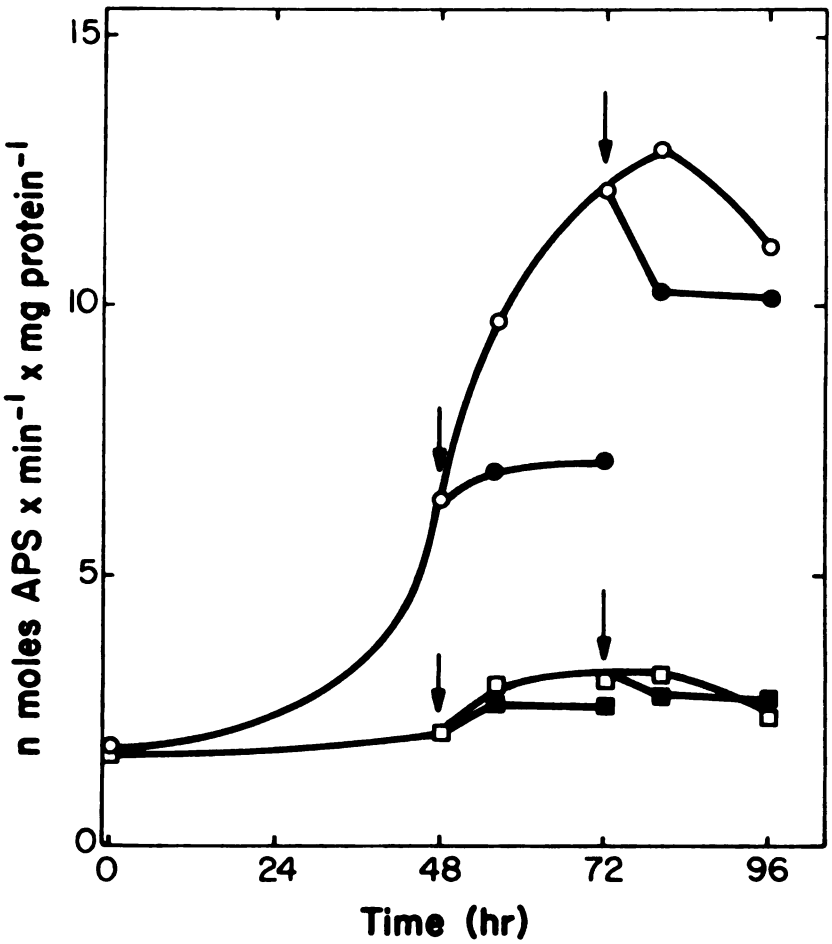


Figure 20. Inhibition by cycloheximide of the development of ATP sulfurylase during derepression on djenkolate.

Stationary phase cells maintained on $(N,S)^-$ MID supplemented with 3.0 mM urea and 0.1 mM sulfate were subcultured into $(N,S)^-$ MID containing 0.75 mM urea and 0.025 mM sulfate. After 7 days, cells were aseptically harvested, washed, and resuspended in $(N,S)^-$ MID containing 3.0 mM urea and sulfur as either djenkolate (O) or djenkolate plus sulfate (□), each at 0.1 mM. At the times indicated, aliquots were aseptically removed for determinations of fresh weight, soluble protein and enzyme activity. After 48 and 72 hr (arrows), aliquots were aseptically removed and incubated with 4 µg/ml of cycloheximide. Aliquots from the cycloheximide treated cells were assayed after 8 and 24 hr following the addition of the inhibitor. Open symbols are control untreated cells, closed symbols are cycloheximide treated cells. The initial inocula contained 5.5 g/l of fresh weight cells.

of sulfate in the ATP sulfurylase reactions *in vitro*. Logically, if these analogs of sulfate can also inhibit ATP sulfurylase activity *in vivo*, a specific perturbation of the sulfate assimilation system could be achieved. Such a perturbation could ultimately alter the level of sulfur-containing metabolites in the cells, which in turn could bring about changes in the level at which ATP sulfurylase is regulated. The specific prediction is that if ATP sulfurylase is derepressed by a decrease in the concentration of a sulfur-containing compound synthesized via ATP sulfurylase, then the Group VI anions, which are known to inhibit ATP sulfurylase activity *in vitro*, should derepress the enzyme in cells growing on sulfate, but not on cysteine or methionine. No previous attempt appears to have been made in any system to detect changes in extractable ATP sulfurylase activity as a result of growth in the presence of Group VI anions.

I. Group VI Anions as Inhibitors of APS Formation *in vitro*

The synthesis of APS, catalyzed by the tobacco cell ATP sulfurylase, is inhibited by Group VI anions *in vitro* (Table 18), as has been found before for other ATP sulfurylases (47,104,107) and for other reactions catalyzed by ATP sulfurylase (e.g., 45,102). Molybdate, tungstate and selenate inhibited the incorporation of [^{35}S]-sulfate into APS in a concentration dependent manner, while chromate inhibited completely even at the lowest concentration tested (Table 18). Although the chromate result is in agreement with the high potency of chromate found by others *in vivo* (72,74,76), it may not be inhibiting as a sulfate analog but rather in a

Table 18. Inhibition of APS formation *in vitro* by Group VI anions

Anion concentration (mM)	ATP SULFURYLASE ACTIVITY (PMOLES APS/5 MIN) Molybdate[a]	Tungstate[b]	Selenate[b]	Chromate[b]
0	560	448	448	448
20	280(50)[c]	193(43)	121(27)	0
50	125(22)	134(30)	54(12)	0
100	56(10)	54(12)	21(5)	0

[a,b]Complete reaction mixtures of 0.5 ml (see Materials and Methods) contained 20 mM sulfate and either molybdate, tungstate, selenate or chromate at the indicated concentrations. Extracts were prepared from exponential tobacco cells, grown on MID for (a) 4 days or (b) 3 days and added at (a) 29 µg protein/assay, and (b) 35 µg protein/assay. Assays were conducted at 30 C for 5 min, and APS was determined as described in Materials and Methods.

[c]Numbers in parentheses represent percent of activity observed with no anion added.

nonspecific manner, e.g., as a protein denaturant. Tellurate, at 10 mM, did not inhibit APS formation (data not shown). Higher concentrations, however, were difficult to obtain because of the insolubility of sodium tellurate in water. This could account for its rare use in biological studies involving Group VI anions.

The kinetics of inhibition of APS formation were examined in detail for selenate, the only Group VI anion which is thought to be incorporated in plants into analogs of sulfur compounds via ATP sulfurylase.

The inhibition of [^{35}S]-APS synthesis by selenate clearly exhibited competitive kinetics with respect to sulfate. The dependence of the rate of APS formation upon sulfate concentration followed Michaelis-Menten kinetics with an apparent Km for sulfate of 1.48 mM (Figure 21). The inhibition constant, Ki, of selenate ranged between 0.64 and 0.7 mM (Figures 22 and 23). Thus, selenate has a slightly greater affinity for the ATP sulfurylase of tobacco cells than the natural substrate, sulfate.

II. The Effects of Group VI Anions on Growth and ATP Sulfurylase Level of Tobacco Cells

The evidence pointing to a role for ATP sulfurylase in the toxic action of Group VI anions *in vivo* is rather limited: (a) growth of mutant strains of *Salmonella* (78), *Aspergillus* (76), and *Penicillium* (72) lacking ATP sulfurylase but functional in sulfate transport, exhibited resistance to molybdate (78) and selenate (72,76) under conditions in which the growth of the wild type was completely inhibited; (b) the inhibition of growth of *E. coli* by selenate or molybdate was completely prevented by conditions which were known to repress strongly the ATP sulfurylase activity in the absence of the Group VI anions (121); and (c) preloading the red alga *Porphyridium* with a high concentration of molybdate inhibited the incorporation of [^{35}S]-sulfate into sulfate esters under conditions that did not inhibit sulfate transport (111).

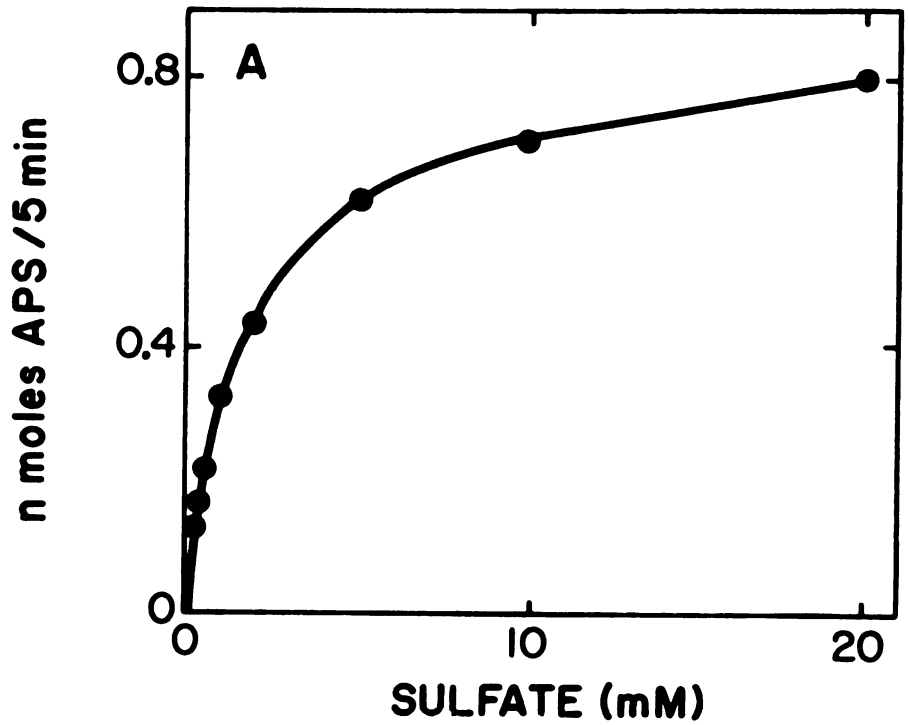
With these considerations in mind, and the availability of a convenient assay for APS formation, a study was undertaken to determine the effects of the sulfate analogs *in vivo* on growth and ATP sulfurylase in the tobacco cells.

Figure 21-A. Dependence of the rate of APS formation on sulfate concentration.

Complete assay mixture prepared as described in Materials and Methods, with various concentrations of sulfate. Crude ATP sulfurylase was prepared from exponential tobacco cells grown on MID. The tubes were incubated for 5 min, and APS was determined as described before.

Figure 21-B. Double reciprocal plot (Lineweaver-Burk) of the rate of APS formation versus sulfate concentration.

The apparent Km for sulfate (arrow) is 1.48 mM.


A
0.8
0.4
0
n moles APS/5 min
0
10
20
SULFATE (mM)

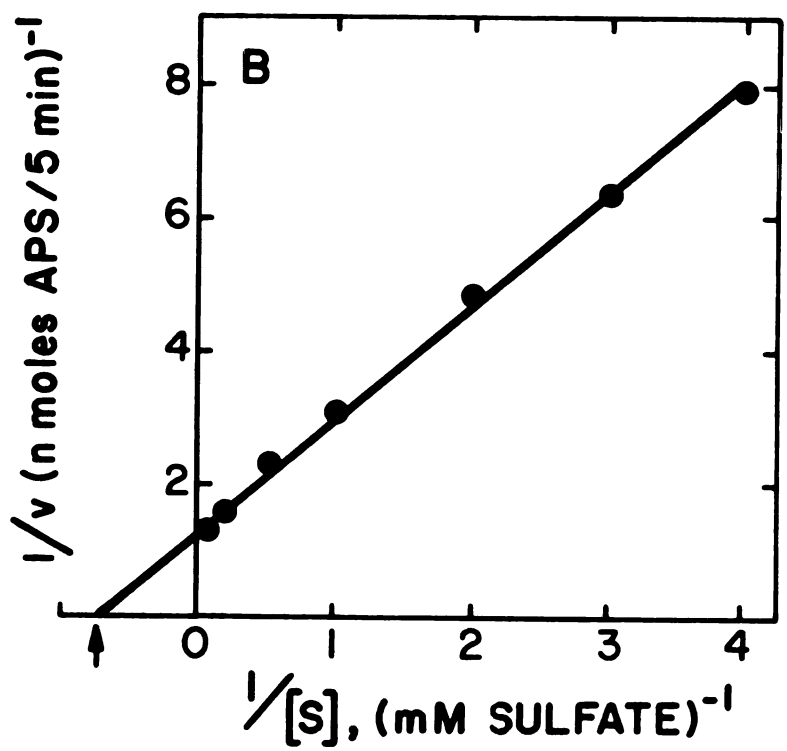

B
8
6
4
2
1/v (n moles APS/5 min)⁻¹
0
1
2
3
4
1/[S], (mM SULFATE)⁻¹

Figure 21

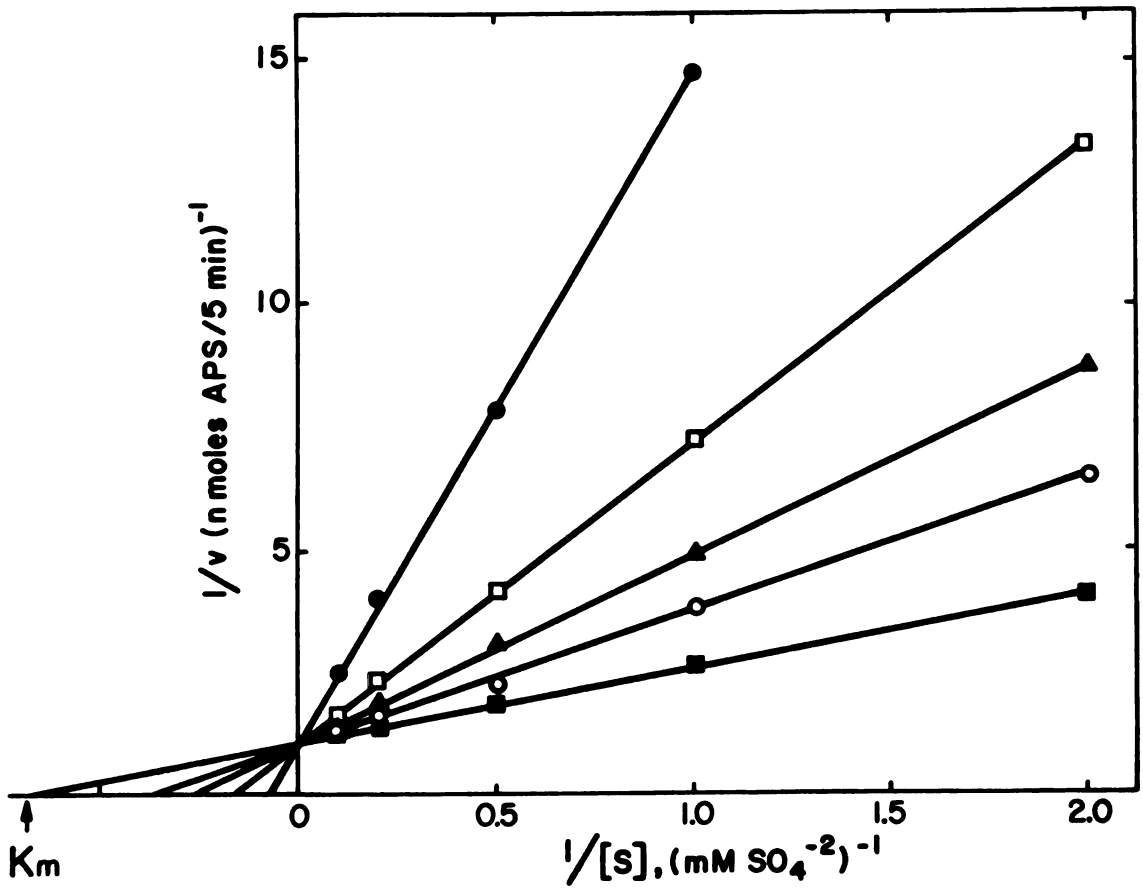


Figure 22. Double reciprocal plot of the concentration-dependent inhibition of APS formation by selenate.

Tobacco cell extracts from exponential cells grown on MID were incubated for 5 min in complete reaction mixtures with varying concentrations of sulfate, in the absence (■) or in the presence of selenate at 0.5 (○) mM, 1 mM (▲), 2 mM (□) and 5 mM (●). Km for sulfate (arrow) is ca. 1.48 mM. Ki is ca. 0.64 mM.

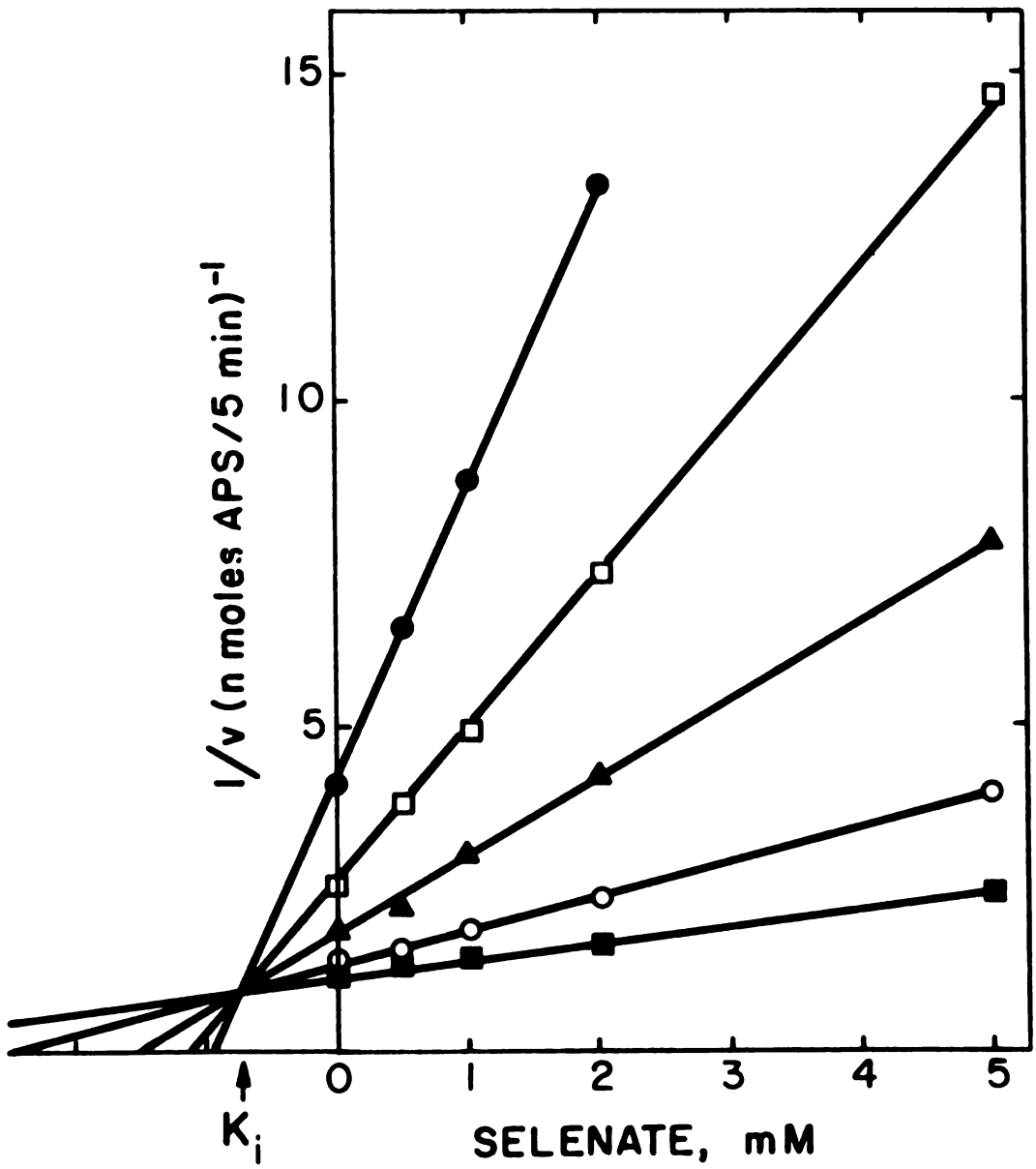


Figure 23. Dixon plot of the concentration-dependent inhibition of APS formation by selenate.

Same data as in Figure 22, but plotted according to Dixon (127). Assay mixtures contain 0.5 mM (●), 1 mM (□), 2 mM (▲), 5 mM (○), and 10 mM (■) sulfate, in the presence of various concentrations of selenate. The inhibition constant, Ki, for selenate (arrow) is ca. 0.7 mM.

Molybdate or tellurate, at concentrations equimolar with sulfate, caused relatively small changes in either growth or ATP sulfurylase specific activity (Table 19). Higher concentrations of either analog gradually inhibited growth but the specific activity of the enzyme remained relatively unchanged.

In contrast, selenate at a concentration equimolar with sulfate was toxic to the cells, but the ATP sulfurylase level increased as much as 3-fold compared with cells grown on sulfate only (Table 19). Moreover, the increase in ATP sulfurylase specific activity diminished at more toxic concentrations of selenate.

Because the cells were grown in the presence of Group VI anions, there was the possibility that the ATP sulfurylase activities in extracts were affected by direct inhibition of the enzyme by high concentrations of anions in the extracts, rather than being a simple reflection of the amount of enzyme present. This possibility was tested and excluded in the following manner. The rate of APS formation was measured at two dilutions of extract, different by a factor of 2, and found to be proportional to the dilution factor. This result means that the anions in the extracts were at too low a concentration to measurably inhibit enzyme activity.

An attempt was made also to see if an increase in activity resulted from dialysis of the extracts, which would be expected if dialyzable anions were appreciably inhibitory. No such increase was observed. However, these results were complicated by the fact that there was a substantial loss of activity after 7 hr of dialysis. This is in contrast to the complete stability of ATP sulfurylase

Table 19. Effects of Group VI anions on growth and ATP sulfurylase *in vivo*

	6TH DAY		11TH DAY	
Group VI anion added (in mM)	Fresh weight (g/l)	ATP sulfurylase[a]	Fresh weight (g/l)	ATP sulfurylase[a]
None	8.9	3.0	26.5	2.1
Molybdate, 0.1	6.8	3.2	21.5	1.7
Molybdate, 0.5	4.6	3.3	-	-
Molybdate, 1.0	3.3	3.7	11.2	1.8
Tellurate, 0.1	5.0	3.4	20.0	2.6
Tellurate, 0.5	5.2	4.1	15.5	3.6
Tellurate, 1.0	4.6	4.3	-	-
Selenate, 0.1	3.1	8.1	5.7	6.8
Selenate, 0.5	1.5	4.3	-	-
Selenate, 1.0	0.6	-	-	-
Tungstate, 0.1	0.1	-	-	-
Chromate, 0.1	0.4	-	-	-

[a] nmoles APS x min^{-1} x mg $protein^{-1}$.

Stationary phase cells were subcultured into $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM sulfate. Sodium salts of Group VI anions were added to the culture media at zero time. After 6 and 11 days, the cells were harvested, and fresh weights, soluble protein and ATP sulfurylase activities were determined. The initial fresh weight was 1.5 g/l, and ATP sulfurylase activity was 2.0 nmoles APS x min^{-1} x mg $protein^{-1}$.

activity found in undialyzed tobacco cell extracts kept frozen at -15 C for 3 days.

Both tungstate and chromate, at 0.1 mM, were very toxic to the tobacco cells and resulted in complete inhibition of growth (Table 19). The tungstate effect is believed to reflect its interference with the development of functional nitrate reductase; chromate, on the other hand, is a nonspecific toxicant to many organisms.

The enzyme assays presented in Table 19, however, were done after the cells had been growing for 6 days or more in the presence of the anions. The responses to the anions at earlier times were examined in separate experiments (Tables 20 and 21; Figure 24). In order to obtain enough cell material for multiple analyses, stationary phase cells were resuspended in the test media, but with less dilution than in the previous experiment (see Table 19), so that aliquots could be taken from one culture at various times.

A gradual increase in extractable ATP sulfurylase specific activity of up to ca. 2.5-fold above the control occurred over 4 days in cells supplied with molybdate, but only when the molar ratio of molybdate to sulfate in the medium approached 10 (Table 20). Under these conditions, the total soluble protein did not increase during the 4-day incubation with molybdate. Thus, the increase in enzyme specific activity observed upon incubation with molybdate (table 20) could have resulted from sulfate starvation imposed by the high concentration of the inhibitory sulfate analog.

The effect of selenate on the ATP sulfurylase, however, became evident in cells grown on selenate at 1/10 the concentration of

Table 20. The effect of molybdate on the development of ATP sulfurylase in cultured tobacco cells

Time (hr)	MOLYBDATE CONCENTRATIONS: NONE Protein (mg/l)	NONE ATP sulfurylase[a]	0.1 mM Protein (mg/l)	0.1 mM ATP sulfurylase[a]	1.0 mM Protein (mg/l)	1.0 mM ATP sulfurylase[a]
0	33.9	1.6	28.6	1.6	31.4	1.5
24	46.6	3.0	44.5	3.3	31.7	4.4
48	63.5	4.4	68.0	3.8	30.2	5.5
72	84.5	3.3	74.5	3.8	33.8	6.6
96	53.2	3.2	74.8	3.3	32.8	8.1

[a] nmoles APS x min^{-1} x mg $protein^{-1}$.

Stationary phase cells grown on $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM sulfate were aseptically harvested, washed with $(N,S)^-$ MID and resuspended in $(N,S)^-$ MID containing 2.5 mM nitrate, 0.1 mM sulfate and various concentrations of molybdate. At the times indicated, aliquots were aseptically removed for determinations of fresh weight, soluble protein and enzyme activity. The initial inocula contained ca. 24 g/l of fresh weight cells.

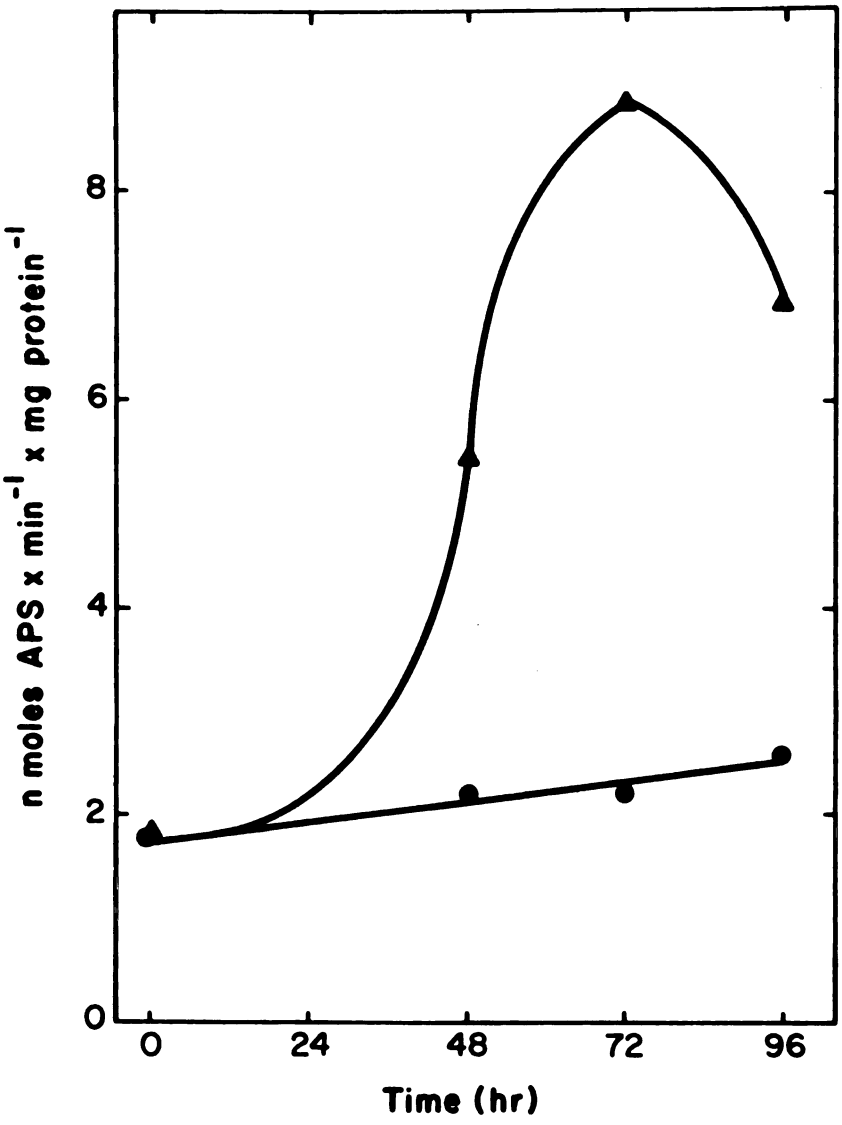


Figure 24. Derepression by selenate of ATP sulfurylase in cells grown on sulfate.

Stationary phase cells grown on $(N,S)^-$ MID supplemented with 3.0 mM urea and 0.1 mM sulfate were subcultured into fresh $(N,S)^-$ MID supplemented with 0.75 mM urea and 0.025 mM sulfate. After 7 days, the cells were aseptically harvested, washed with $(N,S)^-$ MID and resuspended in $(N,S)^-$ MID containing 3.0 mM urea, 0.1 mM sulfate and either zero (●) or 0.01 mM (▲) selenate. At the times indicated, aliquots were removed for determinations of fresh weight, soluble protein and enzyme activity (see Materials and Methods). The initial fresh weight was 5.5 g/l. After 4 days, fresh weight determinations for both cultures were ca. 15 g/l.

Table 21. The effects of selenate on soluble protein and ATP sulfurylase in cells utilizing sulfate for growth[a]

Time (hr)	SELENATE CONCENTRATION: None — Protein (mg/l)	None — ATP sulfurylase[b]	0.01 mM — Protein (mg/l)	0.01 mM — ATP sulfurylase[b]
0	15.4	1.8	15.4	1.8
48	31.5	2.2	25.0	5.4
72	54.6	2.2	53.2	8.9
96	63.0	2.6	76.5	6.9

[a]Data from same experiment as Figure 24.

[b]nmoles APS x min^{-1} x mg $protein^{-1}$.

sulfate in the medium; a 4-fold increase in enzyme specific activity developed within 3 days (Figure 24) with no apparent inhibitory effect on growth or net protein synthesis in the cells (Table 21). Moreover, in contrast to molybdate, selenate was most effective in derepressing ATP sulfurylase when it was at sub-toxic or incipiently toxic concentrations (see Tables 19 and 21).

The differences in the derepression by selenate as compared with molybdate suggest that they act by somewhat different mechanisms.

According to the published literature in a wide variety of organisms, the sulfate transport system does not discriminate

between selenate or molybdate, whereas ATP sulfurylase acts in different ways with respect to selenate and molybdate (see Literature Review). These differences suggest that the derepression by selenate is due to the synthesis, via ATP sulfurylase, of a compound SeX, an analog of a natural metabolite, SX, with a regulatory function, while derepression by molybdate is due to sulfur deprivation via inhibition of sulfate assimilation at the ATP sulfurylase step.

III. Derepression of ATP Sulfurylase by Selenate: Dependence on the Sulfur Source

The effect of selenate on the development of ATP sulfurylase activity in cells utilizing sulfate for growth was compared with its effect on cells utilizing either cysteine or djenkolic acid.

Addition of 0.03 mM selenate to a culture growing on 0.1 mM sulfate resulted in more than a 4-fold derepression of ATP sulfurylase (Figure 25-A), with little or no inhibition of growth. However, selenate at the same concentration, 0.03 mM, added to cells growing on 0.1 mM L-cysteine, did not cause derepression of the enzyme (Figure 25-B). Again, growth was not inhibited. When 0.03 mM selenate was added to a culture growing on 0.1 mM L-djenkolate, growth was totally inhibited and so was the 27-fold derepression observed in the absence of selenate (Figure 25-C). Selenate, at 0.1 mM, is more toxic to cells grown on sulfate, and resulted in much less derepression than at 0.03 mM (Figure 25-A). However, selenate at 0.1 mM was not toxic to cells grown on cysteine, nor was it effective in derepressing ATP sulfurylase in such cells (Figure 25-B). The ineffectiveness of selenate, in causing derepression when cysteine is the sulfur source, is not altered by the

Figure 25. The effects of various sulfur sources on the derepression of ATP sulfurylase by selenate.

Stationary phase cells grown for 10 days on $(N,S)^-$ MID supplemented with 2.5 mM nitrate and 0.1 mM djenkolate were aseptically harvested, washed with $(N,S)^-$ MID, and resuspended in fresh $(N,S)^-$ MID containing 2.5 mM nitrate as the nitrogen source and either 0.1 mM sulfate (A), 0.1 mM L-cysteine (B), or 0.1 mM L-djenkolate (C) as the sole sulfur source. Cultures also contained no selenate (○), 0.03 mM selenate (△), or 0.1 mM selenate (□). After 72 hr (arrows), 0.03 mM sulfate was sterilely added to 50 ml aliquots of cultures with no selenate (●), and cultures containing 0.03 mM selenate (▲). Fresh weight, soluble protein and enzyme activity were determined on aliquots taken at various times.

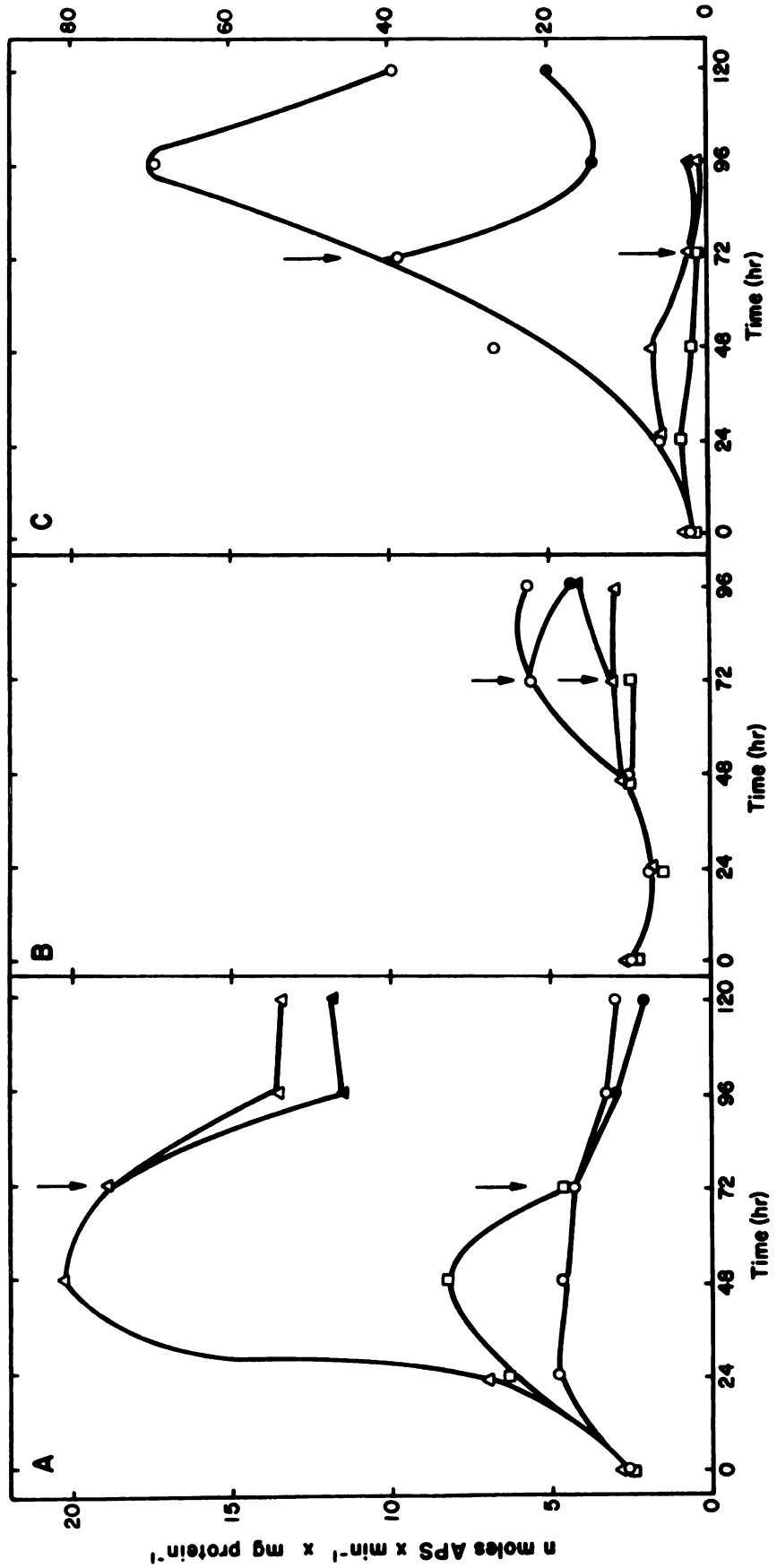


Figure 25

addition of 0.03 mM sulfate to the culture grown for 72 hr with 0.1 mM cysteine and 0.03 mM selenate (Figure 25-B). Thus, it is the presence of cysteine rather than the absence of sulfate which is responsible for this lack of a selenate effect. Also, the severe inhibitory effect of 0.03 mM selenate on cells utilizing djenkolate is not reversed upon a subsequent addition of 0.03 mM sulfate (Figure 25-C). Thus, there is more to selenate toxicity for cells growing on djenkolate than mere sulfur starvation, which would be overcome by addition of sulfate.

Regardless of the final interpretation, the effect of selenate on ATP sulfurylase development is clearly a function of the sulfur source used by the cells.

IV. Selenate Toxicity as a Function of the Sulfur Source Utilized for Growth

The experiments on the derepression of ATP sulfurylase by selenate described in the previous section suggest that the toxic effect of selenate on growth was enhanced by derepression of ATP sulfurylase and diminished by repression of the enzyme in the tobacco cell system.

In order to test the validity of this interpretation, a series of growth experiments were performed in which conditions favoring repression or antagonizing the repression were created, and the sensitivity of growth to selenate was determined.

Table 22-A shows that selenate toxicity in tobacco cells is a function of: (a) the molar ratio of [selenate]/[sulfur]; and (b) the nature of the sulfur source.

Table 22-A. Inhibition by selenate of growth of tobacco cells on various sulfur sources

Selenate (μM)	FRESH WEIGHT, G/L		
	Sulfate	Djenkolate	Cysteine
None	25.5	8.5	22.0
1	25.0(98)[a]	3.6(28)[a]	17.5(78)[a]
3	25.5(100)	1.1(<0)[b]	16.1(71)
10	9.7(34)	0.7(<0)[b]	8.7(35)
30	2.2(2)	0.7(<0)[b]	8.7(35)
100	0.4(<0)[b]	0.6(<0)[b]	5.9(21)

[a]Numbers in parentheses are percent of increase in fresh weight observed with no selenate added (for each sulfur source used).

[b]Indicates an actual net loss of initial fresh weight.

Stationary phase cell which had been maintained continuously on $(N,S)^-$ MID supplemented with 2.5 mM KNO_3 and 0.1 mM Na_2SO_4, were subcultured into fresh $(N,S)^-$ MID containing 2.5 mM KNO_3, 0.1 mM of the sulfur source indicated, and various concentrations of sodium selenate. After 12 days, cells were harvested and the fresh weights were determined in duplicate cultures, and averaged. Initial fresh weight was ca. 1.7-2.0 g/l.

Cells on cysteine exhibit resistance to selenate at concentrations which completely inhibit cells utilizing sulfate. Selenate present at 1/100 the concentration of the sulfur source has little or no effect on cysteine- or sulfate-dependent growth, but is strongly inhibitory to cells growing on djenkolate (Table 22-A).

Hart and Filner (123) showed that L-arginine inhibited the uptake of L-cysteine by tobacco cells, and consequently acted as an antagonist to the inhibition by cysteine of sulfate uptake. If cysteine protects cells from selenate toxicity by repressing the sulfate uptake system and/or ATP sulfurylase, then arginine should antagonize this protective action. Accordingly, arginine was tested as an antagonist of the cysteine-dependent resistance to selenate toxicity. Arginine, supplied in equimolar concentrations with cysteine, has no effect on yield of cells after 12 days of growth. However, it reduces their resistance to selenate to a significant extent (Table 22-B). This reduction was partially overcome by including sulfate in the medium, as would be expected if the inhibition of growth were due to a greater influx of selenate when arginine inhibited cysteine uptake.

Furthermore, cysteine protects against the severe toxicity by selenate encountered in cells grown on djenkolate (Table 22-C), and arginine is an antagonist of this protective effect of cysteine as well.

Thus, the results in Tables 22 A-C support the hypothesis that selenate toxicity in tobacco cells is mediated by the steps of the normal sulfate pathway, and conditions which repress or inhibit one

Table 22-B. The effect of L-arginine on the protection by L-cysteine of tobacco cells from inhibition of growth by selenate

	FRESH WEIGHT, G/L		
Selenate (μM)	Cysteine[a]	Cysteine, arginine	Cysteine, arginine, sulfate
None	22.0	26.0	26.5
1	17.5(78)[b]	22.7(86)[b]	25.8(97)[b]
3	16.1(71)	12.5(44)	24.9(94)
10	8.7(35)	2.3(3)	21.7(81)
30	8.7(35)	1.6(<0)[c]	7.8(25)
100	5.9(21)	1.5(<0)[c]	2.3(2)

[a]Data taken from Table 22-A.

[b]Numbers in parentheses are percent of increase in fresh weight observed with no selenate added (for each sulfur source used).

[c]Indicates an actual net loss of initial fresh weight.

The experiment was carried out as described in Table 22-A, except that in addition to L-cysteine as the sulfur source, L-arginine or L-arginine plus sulfate, each at 0.1 mM, were added at zero time.

Table 22-C. The effect of L-cysteine on the djenkolate-dependent susceptibility to growth inhibition by selenate

Selenate (μM)	FRESH WEIGHT, G/L		
	Djenkolate[a]	Djenkolate, cysteine	Djenkolate, cysteine, arginine
None	8.5	21.1	25.7
1	3.6(28)[b]	18.5(87)[b]	23.0(89)[b]
3	1.1(<0)[c]	14.6(67)	8.1(27)
10	0.7(<0)[c]	5.1(18)	3.2(6)
30	0.7(<0)[c]	3.3(8)	1.8(0)
100	0.6(<0)[c]	2.9(6)	0.7(<0)[c]

[a]Same data as in Table 22-A.

[b]Numbers in parentheses are percent of increase in fresh weight observed with no selenate added (for each sulfur source used).

[c]Indicates an actual net loss of initial fresh weight.

The experimental details are outlined in Table 22-A. L-Cysteine or L-cysteine plus L-arginine, each at 0.1 mM, were added to the djenkolate medium at zero time.

or more of the early steps of that pathway render the cells resistant to selenate.

DISCUSSION

Roy and Trudinger (108) have pointed out that the problems involved in assaying ATP sulfurylase are undoubtedly responsible to some extent for the rarity of studies of this enzyme.

It is in this context that the new assay method for ATP sulfurylase which has been developed during the course of these studies should be evaluated. This assay allows for the first time the measurement of the initial rates of APS formation (Figure 11), which until now had been considered to be practically impossible (108,166). Since there are no chemical methods available for the determination of phosphate-sulfate anhydrides (167), and the coupled enzymatic methods (140a) are not sensitive enough for the amount of APS synthesized, it is necessary to rely on radioisotope assays in which $[^{35}S]$-SO_4^{-2} is incorporated into $[^{35}S]$-APS. The unfavorable equilibrium of the reaction necessitates the use of $[^{35}S]$-SO_4^{-2} of a very high specific activity and a method for the separation of the small quantities of product, $[^{35}S]$-APS, from the vastly greater amount of $[^{35}S]$-SO_4^{-2}. Although the separation can be achieved by chromatography or electrophoresis, these methods have not been used in any routine assay in any published study of ATP sulfurylase kinetics. Attempts to use adsorption of APS to charcoal have been unsatisfactory (e.g., 45,69,96). Attempts to increase the amount of APS formed by

addition of excess inorganic pyrophosphatase resulted in less than the expected increase in APS accumulation (45,96).

The fact remains that, prior to the work presented in this dissertation, kinetic studies of ATP sulfurylase by assaying APS formation have simply not been a practical possibility. However, ATP sulfurylase activity has been demonstrated in extracts in a number of studies by measuring incorporation of [^{35}S] from SO_4^{-2} into APS, which was separated by chromatography or electrophoresis.

The assay which was developed and used in the studies presented in this dissertation will separate 10^2-10^4 cpm [^{35}S]-APS completely from 10^8 cpm [^{35}S]-SO_4^{-2}, with 80% yield of APS (Figure 1, Tables 1 and 2).

As expected for measurements of initial velocities, the rate of APS synthesis by the tobacco cell extract was not greatly affected by the presence of inorganic pyrophosphatase in the assay mixture (Table 5). Under these conditions, [^{35}S]-APS accumulated to a concentration of 2 μM. With this value, the calculated equilibrium constant for the reaction is ca. 10^{-8}.

One would predict from considerations of chemical equilibria that by the addition of an excess of inorganic pyrophosphatase to the reaction mixture, the equilibrium concentration of APS would be increased well above 2 to 3 μM, the equilibrium concentration in the absence of inorganic pyrophosphatase, so that the initial rate of APS formation could be sustained. However, such results were not obtained, either with the tobacco cell ATP sulfurylase, or with any other ATP sulfurylase previously studied (e.g., 41,47,96,154). This enigma may be explained in one of three ways: (a) APS is an allosteric

inhibitor of ATP sulfurylase; (b) APS is a product inhibitor of the reaction; and (c) inorganic pyrophosphatase malfunctions under the assay conditions.

The inhibition by APS of the molybdolysis reaction catalyzed by ATP sulfurylase has been noted by several investigators (66,96,98). APS was also stated to inhibit [^{35}S]-APS synthesis by the purified enzyme from *Penicillium* (96). Kinetic evidence obtained from the inhibition of the molybdolysis reaction by APS (96) indicated that APS is not an allosteric effector of ATP sulfurylase. These results (96) suggested, rather, that APS is a simple product inhibitor of the enzyme. The concentrations of [^{35}S]-APS reached by the forward reaction catalyzed by the *Penicillium* enzyme fall within the same range as the Ki of 40 μM for APS as an inhibitor of the molybdolysis reaction (96). This Ki is substantially higher than the Km for APS of the back reaction, which has been reported to be 0.5-7 μM (65,96, 144,156). Product inhibition by APS therefore apparently occurs when nearly all the enzyme molecules are complexed with APS.

Based on these considerations, product inhibition would begin to be detected at ca. 5 μM, so the decrease of the rate of APS formation by the tobacco cell enzyme at above 3 μM APS may have resulted from product inhibition.

There is no strong evidence to support the alternative possibility that the inorganic pyrophosphatase is inhibited under the assay conditions. The inhibition by APS of yeast inorganic pyrophosphatase has been described (30). However, the inhibition was rather non-specific and could only be detected at 1.0 mM APS. Therefore, the decline in the rate of APS synthesis by the tobacco cell

enzyme is unlikely to result from the inhibition of the excess inorganic pyrophosphatase by 3 μM APS. The possibility that the tobacco cell extract contained a low molecular weight inhibitor of inorganic pyrophosphatase (e.g., Ca^{+2}) can be excluded because neither passage of the extract through a Sephadex G-25 column, nor the addition of EGTA to the assay mixture, extended the linear range of APS formation (results not shown).

Nevertheless, it is evident that the use of any assay based on APS formation should be confined to APS concentrations below 3 μM, in order to avoid the equilibrium and product inhibition problems.

Results obtained with several independent methods, such as electrophoresis (Figure 3), DEAE A-25 Sephadex column (Figure 9), and Dowex-1-nitrate column (Figure 10), showed quite conclusively that extracts of tobacco cells, when incubated with SO_4^{-2} and ATP, synthesized APS but not PAPS, as was previously found in a variety of other higher plant extracts (104,143-144,156). The apparent absence of APS kinase activity in tobacco cell extracts has not been studied in detail during the course of this investigation. However, preliminary experiments indicated that PAPS was not synthesized by the tobacco cell extract even when a large excess of 3' AMP was included in the reaction mixture (151,155). These results are therefore implicitly adding to the controversy over the existence of APS kinase in higher plants.

During the course of these studies commercial ATP sulfurylase purified from yeast was used in an attempt to assay APS by the method of Robbins (167), who measured either ATP formation or pyrophosphate disappearance in the presence of excess enzyme. The

development of a method to separate APS from sulfate (Figure 1) provided an opportunity to follow the enzymatic synthesis of both products, ATP and SO_4^{-2}, concomitantly. However, the expected stoichiometry of formation (1 mole ATP per mole of sulfate) was not obtained. The purified enzyme from yeast appears to catalyze the release of sulfate from APS in the absence of pyrophosphate, and without ATP formation (Table 6). Two possibilities may account for these results: (a) synthesis of SO_4^{-2} from APS by ATP sulfurylase, in a ping-pong mechanism in which SO_4^{-2} is released before pyrophosphate is bound to the enzyme; and (b) hydrolysis of APS by an APS-sulfatase activity of a protein other than ATP sulfurylase to yield SO_4^{-2} and AMP.

A ping-pong type mechanism is in conflict with the sequential mechanism recently proposed for the ATP sulfurylases from yeast (98), *Penicillium* (96) and spinach (65). However, in these studies, with highly purified enzymes (65,96,98), the simultaneous exchange of both [^{32}P] between ATP and PPi, and [^{35}S] between SO_4^{-2} and APS, could not be demonstrated for a single ATP sulfurylase. Only one or the other exchange reaction was demonstrated for a given enzyme (65,96,98).

The alternative possibility, a sulfatase activity associated with, or contaminating commercial yeast ATP sulfurylase, cannot be ruled out. Little is known about such an activity (cf. 108). However, it should be pointed out that because ATP sulfurylase assays were based solely on either ATP formation or pyrophosphate consumption, a sulfatase type activity may have been overlooked previously.

There is one report in the literature describing the release of sulfate from [^{35}S]-APS by fragmented chloroplasts incubated in the

absence of pyrophosphate (144). This activity was, nevertheless, attributed to ATP sulfurylase by the authors.

In the absence of Mg^{+2}, the commercial ATP sulfurylase preparation synthesized ATP from APS (Table 6), but in the presence of Mg^{+2} the ATP did not accumulate, suggesting that the ATP was degraded by a Mg^{+2}-dependent ATPase present in this preparation. This observation is consistent with previous reports indicating that ATP sulfurylase purified from yeast synthesized ATP in the absence of added Mg^{+2} (140a,166). The apparent association of a Mg^{+2}-dependent ATPase activity with yeast ATP sulfurylase preparations necessitated the omission of Mg^{+2} from assays in which APS was measured by an excess of ATP sulfurylase (167). Thus, under these conditions, the putative APS synthesized by tobacco cell extracts could serve as a substrate for yeast ATP sulfurylase, and resulted in ATP synthesis (Table 6).

The new assay for ATP sulfurylase, based on the measurement of initial rates of APS formation, is simple, rapid, inexpensive, highly reproducible, and suitable for assaying multiple samples. Therefore, it makes possible a variety of kinetic and regulatory studies of ATP sulfurylase, which were until now difficult to approach.

The procedure for separation of APS from sulfate, and from PAPS, should also prove to be of value in the preparative synthesis of [^{35}S]-APS, because the method can be scaled up readily.

The ATP sulfurylase activity found in extracts of tobacco cells grown under optimal conditions on sulfate and nitrate is ca. 2.0 nmoles APS x min^{-1} x mg $protein^{-1}$. This activity can account for

the *in vivo* rate at which sulfur is incorporated into protein in the cells. Taking for example the data presented in Table 13 for such cells, it can be calculated, assuming 0.7% sulfur in protein, that during the first 12 hr, ca. 1.5 µmoles of sulfur were required for the synthesis of 6.8 mg of soluble protein, which is most of the protein in the cells. At the same time, the activity of ATP sulfurylase detected in the extract was sufficient for synthesis of ca. 8.8 µmoles of APS, under the standard assay conditions. The extractable, assayable activity of ATP sulfurylase therefore is about 5 times more than is required for the rate of sulfate assimilation by the cells. Since the reaction *in vivo* may occur with lower sulfate and ATP concentrations, the *in vivo* and *in vitro* rates are reasonably close.

Results presented in this dissertation show that ATP sulfurylase of the XD strain of cultured tobacco cells is subject to apparent repression-derepression regulation governed by sulfur nutrition. The enzyme is repressed under conditions of sulfur nutrition sufficient to allow optimal growth, and is derepressed when the assimilation of sulfur becomes rate limiting for growth. It is very likely, therefore, that ATP sulfurylase of the tobacco cell is subject to a negative feedback control mechanism in which an end-product of the sulfate assimilation pathway is the repressive effector. This hypothetical effector, designated as SX, is likely a metabolic derivative of cysteine or methionine which contains a reduced sulfur, and thereby constitutes the negative signal affecting the development of the enzyme.

The development of ATP sulfurylase in the tobacco cell is also regulated by a positive control mechanism governed by a signal from the nitrogen assimilation pathway. The derepression of ATP sulfurylase does not occur in cells starved for nitrogen, although turnover protein synthesis is demonstrable (70). The positive effector signal, let us call it NY, appears to be related to the reduced nitrogen level or the resultant positive rate of net protein synthesis in the cells. Therefore, the development of ATP sulfurylase in the tobacco cell requires both a deficiency in SX and a sufficiency of NY.

The formation of APS catalyzed by ATP sulfurylase has long been believed to be the first enzymatic step, following the uptake of sulfate, in the sulfate assimilation pathway of higher plants. This belief has been based on comparative biochemistry, not on direct genetic or physiological evidence. The finding that ATP sulfurylase of the tobacco cell is regulated by sulfur nutrition is, therefore, the strongest evidence obtained so far in support of the postulated role of the enzyme in sulfur assimilation in higher plants.

In contrast to what has been described in bacteria, the ATP sulfurylase of the tobacco cell is highly repressed during optimal growth on sulfate (Table 10). Again in contrast to the bacterial system, neither cysteine nor methionine, nor the two of them together, resulted in complete repression in the tobacco cells (Figures 12 and 13). The latter difference between the bacterial and tobacco cell systems may reflect differences in the functional roles of the enzyme in the two systems. There are believed to be two branches to the sulfate assimilation pathway in plants, and ATP sulfurylase is

thought to be the branch point enzyme (cf. 48). One branch leads to the sulfur amino acids, while the other branch leads to the sulfate esters and the sulfonates. Consequently, from a regulatory point of view, complete repression of the first enzyme of a branched pathway by an end-product of one branch is rather unlikely. This concept has been expressed previously for *Penicillium* (96).

It has been documented that the amino acid biosyntheses in plants follow the same general patterns elucidated previously in microorganisms (90). Nevertheless, although in microorganisms many of these biosynthetic pathways are regulated by end-product repression, attempts to demonstrate end-product-dependent changes *in vivo* in the level of some of these biosynthetic enzymes in plants have been uniformly unsuccessful (90). Repression of ATP sulfurylase by sulfur amino acids is only the second case known so far in higher plants in which an enzyme involved in amino acid biosynthesis has been demonstrated to be repressed by amino acid end-products, the first case being the nitrate pathway enzymes. Judging from the findings reported here, it is likely that the reason for the failure of many previous attempts to demonstrate repression of biosynthetic enzymes in higher plants by addition of excess end-product is that, in contrast to bacteria, the biosynthetic enzymes of higher plants may be highly repressed during optimal growth on minimal media. Based on these ideas, future studies in plant systems should be directed toward devising conditions for minimizing the build-up of end-products to bring about derepressions. In general, this could be achieved by means of inhibitors of intermediate steps of pathways.

During the course of these investigations, an attempt was made to examine the role of the nitrogen pathway in the mechanism of the regulation of ATP sulfurylase. Such a role had been predicted because the rates at which nitrogen and sulfur are assimilated have to be coordinated and coupled to the rate of protein synthesis.

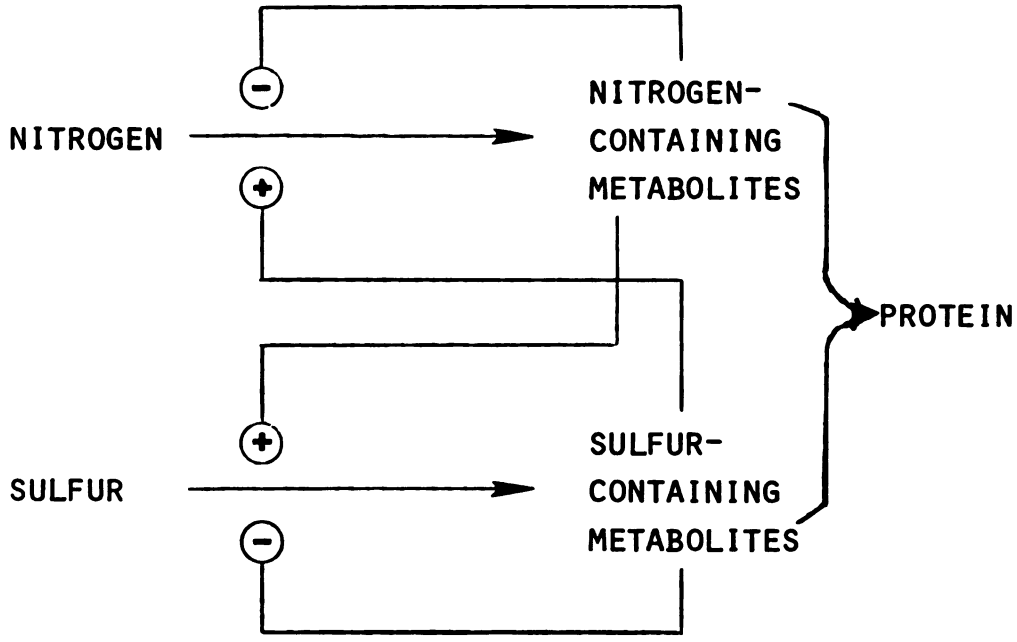
The results which point to the existence of a positive regulatory role for the nitrogen assimilation process in the regulation of ATP sulfurylase are as follows: (a) the regulation of ATP sulfurylase by sulfur compounds was dependent on the rates at which nitrogen was assimilated as shown by the comparison of the level of ATP sulfurylase in cells grown on either nitrate or urea (Table 12); (b) the derepression of ATP sulfurylase upon sulfur limitation is completely dependent upon the availability of an assimilatable exogenous nitrogen source. The development of the enzyme does not occur in cells starved for nitrogen (Figure 18). This effect is not due to a nonspecific mechanism, because the protein synthesis machinery is completely functional, as is evident from the steady rate of turnover protein synthesis under conditions of nitrogen starvation (70). ATP sulfurylase begins to develop, however, within 12 hr after the addition of a nitrogen source which simultaneously initiates net protein synthesis (Table 17).

The exact molecular basis of this positive signal, which appears to function as an internal inducer for the development of ATP sulfurylase upon sulfur starvation, is not known. The ability of urea, a reduced nitrogen source, to initiate the derepression response of ATP sulfurylase similar to that initiated by nitrate suggests, however, that this control does not involve directly the nitrate

reducing system, nor inorganic nitrogen intermediates. More likely, there is a low molecular weight compound, containing reduced nitrogen, and this compound thereby constitutes a signal which is coupled to net protein synthesis. Could this hypothetical positive signal be mediated by O-acetylserine as was shown in bacteria (126,185)? This question remains to be examined.

From the regulatory point of view, the coordination of the rates of assimilation of both nitrogen and sulfur into protein likewise requires a regulatory positive control over the nitrogen assimilation mediated by the sulfate pathway. This is consistent with the evidence that nitrate reductase of the tobacco cells is not induced by nitrate when the cells are starved for sulfur (186).

A concept is therefore emerging that the controls over the metabolic pathways of sulfur and nitrogen assimilation involve two levels: (a) regulation of each pathway by its own internal signals when the other pathway is not limiting; and (b) regulation of each pathway by external signals provided by the other pathway to coordinate the flow of both constituents into protein.



Such a model was originally proposed by Monod and Jacob (198) as one possible way by which two different inducible or repressible pathways could be interconnected via regulatory elements.

This concept appears to resolve the apparently contradictory observation that stationary phase tobacco cells, although starved for sulfur, contain a low level of ATP sulfurylase. However, under the culture conditions used, 2.5 mM nitrate and 0.1 mM sulfate, the tobacco cells are in fact starved for both nitrogen and sulfur in the stationary phase (123,179). Consequently, nitrogen starvation is overriding the signal of sulfur limitation and prevents the derepression of ATP sulfurylase.

It is worth noting that possibly similar observations have been described previously in the literature. Kredich, in his studies with *Salmonella* (126), noted that derepressed cells on djenkolate

had a very low level of ATP sulfurylase activity if they were harvested during the late log or stationary phases of growth. He could not explain the results. Our findings suggest that perhaps the bacterial cultures in the stationary phase of growth had also depleted their nitrogen supply.

The low level of ATP sulfurylase in nitrogen-starved cells proved to be rather useful in the studies presented here. Nitrogen starvation was a convenient device for obtaining low enzyme levels for initial conditions in derepression studies.

The parallel consumption by the tobacco cells of the nitrate and sulfate provided at 2.5 mM and 0.1 mM, respectively, suggested that the desired stationary phase cells could be obtained by maintaining the molar ratio of nitrogen:sulfur of 25:1, regardless of the absolute amounts. Indeed, cells grown for 9 days in media containing 1.25 mM nitrate and 0.05 mM sulfate exhibited the desired low level of ATP sulfurylase characteristic of stationary phase cells (Figure 14). Consequently, lower amounts of nitrogen and sulfur were provided at a molar ratio of 25:1 when cells were needed within a short period of time (e.g., Figure 15).

This concept also predicts that if the nitrogen supply exceeds the molar ratio of 25:1, stationary phase cells should have a high level of ATP sulfurylase activity as a result of the sulfur limitation for growth. Derepression of ATP sulfurylase has been found in stationary phase tobacco cells utilizing casein hydrolysate, 0.5 g/l, as the sole source of both nitrogen and sulfur (Figure 17). These results are not surprising because 0.5 g/l of casein hydrolysate is calculated to contain 3.6 mM nitrogen (Difco specifications), and

0.06 mM of sulfur amino acids (183), which results in a molar ratio of nitrogen:sulfur of 60:1.

The proposed influence of the molar ratio of nitrogen to sulfur on the assimilation of sulfate via ATP sulfurylase in the tobacco cell system is supported by an independent study on the sulfate transport system in the XD cells carried out by Smith (124). He observed an increase in the rate of uptake of sulfate in stationary phase cells utilizing either sulfate of cysteine as the sole sulfur source. Contrary to his interpretation that the intracellular sulfate pool is the regulating factor, I wish to suggest that his results reflect simply sulfur starvation, because the cells were grown on 2.5 mM nitrate and 0.05 mM sulfur as either sulfate or cysteine, resulting in a ratio of nitrogen:sulfur of 50:1.

The molecular bases for the described biochemical changes in ATP sulfurylase specific activity in response to specific perturbations in environmental sulfur and nitrogen are not known. Several lines of evidence suggest, however, that these changes are due to changes in the rate of synthesis and/or the rate of degradation of the enzyme molecules rather than activation-inhibition of pre-existing enzyme molecules. The evidence consistent with this idea is as follows:

(a) The sulfur sources which mediate the changes of enzyme activity *in vivo* do not affect the enzymatic activity *in vitro* (Table 11).

(b) Derepression of ATP sulfurylase is inhibited by cycloheximide (Figure 20) at a concentration which strongly inhibits incorporation of amino acids into protein (Figure 19). Previous studies with

the tobacco cells indicated that cycloheximide at such a concentration does not inhibit the conversion of inactive tungsto-nitrate reductase to active molybdo-nitrate reductase, which is a slow activation of a pre-existing inactive enzyme (70,122), suggesting that the inhibition of protein synthesis by cycloheximide in the tobacco cells is relatively specific.

The effect of cycloheximide added to derepressed cells growing on djenkolate sulfur (Figure 20) is rather distinct, kinetically, from the effect of adding sulfate to such derepressed cells (Figure 25-C). Thus, cycloheximide does not promote the decay of ATP sulfurylase, but sulfate does cause decay (Table 16). Presumably, this decay is part of the regulatory processes by which the level of ATP sulfurylase can be altered.

The results of these investigations using Group VI anions demonstrated that either molybdate or selenate, when included with sulfate in the culture medium, can cause a derepression of ATP sulfurylase (Tables 20 and 21, Figure 24). Kinetically, the derepression by these Group VI anions was rather similar to the drepression by sulfur starvation (e.g., Figure 15). Analysis of the molybdate-dependent derepression supported the idea that molybdate acted upon ATP sulfurylase *in vivo* as an inhibitor of APS formation, and thereby imposed sulfur starvation in the cells, which in turn initiated the development of the enzyme (Table 20).

On the other hand, the mechanism of the selenate-dependent derepression of ATP sulfurylase appears to be other than a mere sulfur starvation. Selenate, which is a competitive inhibitor of the activation of sulfate by the tobacco cell ATP sulfurylase *in*

vitro (Figures 22 and 23), derepressed the enzyme *in vivo* only when present at such a low concentration that neither the growth of the cells, nor the assimilation of sulfate into protein, was affected (Table 21). At higher concentrations, at which selenate becomes toxic, the derepression of ATP sulfurylase does not occur (Figure 23-A). It is suggested, therefore, that unlike molybdate, the selenate-dependent derepression was mediated by a seleno-compound synthesized via ATP sulfurylase to yield the seleno-containing antimetabolite of the hypothetical regulatory sulfur compound SX, the predicted corepressor of the sulfate pathway. I wish to suggest that the selenium analog is acting as an anti-corepressor.

The proposed mechanism for the selenate-dependent derepression response predicts that selenate is metabolized by the tobacco cells via ATP sulfurylase. This is supported by the correlation between the level of ATP sulfurylase in the tobacco cells and the toxicity of selenate to growth. Thus, selenate is extremely toxic to cells with a derepressed level of ATP sulfurylase (Figure 25-C, Table 22-A). Both the derepression of ATP sulfurylase by selenate via SeX, and the toxicity of selenate due to the incorporation of Se-selenocysteine and Se-selenomethionine into protein would require assimilation of selenate via the enzymes of the sulfate pathway.

The proposal that selenate is incorporated by the tobacco cells into a seleno-analog, SeX, of the natural sulfur-containing corepressor, SX, and that SeX interferes with the repressive response, should be put to experimental test. With the aid of $[^{75}Se]\text{-}SeO_4^{-2}$, it might be possible to isolate and identify the selenium containing anti-corepressor.

Although the actual corepressor of the sulfate pathway has not been identified as yet in any system, it is thought to be a close derivative of cysteine or methionine. It is therefore conceivable that Se-selenocysteine and Se-selenomethionine, both of which are commercially available, can be used to trace the hypothetical anti-corepressor. Preliminary attempts to use these seleno-analogs in the tobacco cell system were unsuccessful. At 0.05 mM of the seleno-analog with 0.1 mM sulfate in the culture medium, both analogs were very toxic to the growth of the cells. Lower concentrations were not tested. Based on the studies with selenate (Figures 24 and 25), the seleno-analogs are predicted to cause derepression of ATP sulfurylase only when present at sub-toxic concentrations.

If the corepressor and anti-corepressor can be isolated and identified from the tobacco cells, binding assays may be useful as an approach toward the isolation of the repressor molecule in this system. This system is probably the most attractive one currently available in higher plants if one wishes to undertake a hunt for a specific repressor molecule.

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