Sulfatases of Microbial Origin

Volume II

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of

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PREFACE

Review articles on the sulfatase enzymes have appeared at fairly regular intervals, but no book devoted exclusively to these enzymes has previously appeared. Information about the enzymes and their known and potential substrates is now so vast and impinges on so many different fields of scientific endeavor that the need for collated information has become critical. The present two volumes, although confined in their scope to those sulfatases that are present in microorganisms, will hopefully contribute towards meeting this need.

In the authors' experience, few investigators enter the sulfatase field because of their basic interest in the enzymes. More frequently, they have been concerned with other problems in areas such as drug metabolism, connective tissue biochemistry, or microbial genetics, and their involvement with sulfatase enzymes has often been incidental to the main objectives of their researches. This probably goes some way towards explaining why information about microbial sulfatases is so uneven in detail and extent, and why the results of some ventures into the field leave one with a feeling that it would have paid the investigators to have consulted the literature at the outset.

In the succeeding pages the authors have collated scattered information about individual sulfatases and have endeavored to indicate their physiological roles within the microorganisms, the ways in which their synthesis is subject to genetic and physiological control, and their participation in natural processes such as the recycling of sulfur. The authors have also attempted, for the first time, to discuss at length the mechanisms of action of some of the enzymes in relation to current knowledge about the nonenzymic hydrolysis of various types of sulfate ester. Although primarily directed towards those people interested in the biochemistry and enzymology of microorganisms, it is the authors' belief that there will be much in the book that will be of interest to workers in the mammalian field.

As a final point, the authors wish to thank the many people who have helped in one way or another with the birth of these volumes, and to pay particular tribute to the fortitude and stoicism exhibited by their respective wives during their literary preoccupations.

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THE AUTHORS

Kenneth S. Dodgson received the degrees of Ph.D. and D.Sc. from the University of Liverpool in 1949 and 1961, respectively. Since 1968 he has occupied the Chair of Biochemistry in University College, University of Wales, Cardiff and is currently acting as Vice Principal (Science) for that institution. He holds an honorary appointment as Visiting Professor to the University of Georgia and served as Honorary Meetings Secretary and Committee Secretary of the Biochemical Society during the period 1964 to 1970. He was a member of the British National Committee for Biochemistry from 1967 to 1971.

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Graham F. White is presently a Lecturer in Biochemistry at University College, University of Wales, Cardiff. In 1968 he was awarded the degree of B.Sc. and the John Millar Thomson Medal for Chemistry at the University of London King's College. Postgraduate studies in enzyme kinetics at King's College led to the award of Ph.D. in 1971 whereupon he accepted a Royal Society/NATO Research Fellowship to pursue studies in enzymology at the Institute of Biological Chemistry, University of Florence, Italy.

In 1973 he joined the Department of Biochemistry in Cardiff as a Wellcome Trust Research Fellow to continue research on enzymes of sulfur metabolism, and was appointed to his present position in 1975. Dr. White is a member of the Royal Society of Chemistry.

John W. Fitzgerald is currently an Associate Professor in the Department of Microbiology at the University of Georgia. Dr. Fitzgerald obtained the BSc., MSc., and Ph.D. degrees with emphasis in Microbial Biochemistry from Queen's University, Kingston, Ontario, Canada. He left Queens in 1969 to take up a Leverhulme Postdoctoral Fellowship working with Professor K. S. Dodgson at University College, Cardiff. After serving as a Postdoctoral Associate of Professor W. J. Payne at the University of Georgia, Dr. Fitzgerald joined the faculty of the Department of Microbiology as an Assistant Professor in 1971.

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Chapter 1

HYDROLYSIS OF PHOSPHOSULFATES, SULFAMIDES, AND GLUCOSINOLATES

I. INTRODUCTION

The preceding volume dealt with enzymes acting on true sulfuric acid esters of aliphatic or aromatic C-OH groups. However this volume would be incomplete without an account of enzymes that liberate sulfate from compounds containing the $-SO_3^-$ moiety in linkages other than $C-O-SO_3^-$. The substrates that need be considered conform to one of three types: compounds that contain the $P-O-SO_3^-$ linkage (e.g., adenylyl sulfate); compounds that contain the $N-SO_3^-$ linkage (e.g., the sulfamide group of cyclohexylsulfamic acid); and finally compounds that contain the $N-O-SO_3^-$ linkage (the glucosinolates or mustard oil glycosides). While these compounds are not strictly sulfate esters and the enzymes that degrade them not true sulfatases, the relationships are certainly sufficiently close to warrant their inclusion here.

II. HYDROLYSIS OF PHOSPHOSULFATE LINKAGES

Adenosine 5′-phosphosulfate and 3′-phosphoadenosine 5′-phosphosulfate, the so-called "active forms" of sulfate, have also been referred to elsewhere as adenylyl sulfate and 3′-phosphoadenylyl sulfate, respectively. However, to relieve the text of elaborate names the authors prefer the former system of nomenclature that leads to the widely used abbreviations APS and PAPS. For consistency, the enzymes adenylyl sulfate sulfohydrolase (EC 3.6.2.1) and 3′-phosphoadenylyl sulfate sulfohydrolase (EC 3.6.2.2) that liberate sulfate from these compounds are hereafter referred to as APS-sulfatase, and PAPS-sulfatase, respectively.

A. Active Sulfates

APS and PAPS occupy a central position in the biochemistry of the sulfate ion in animals, plants and microorganisms, and their specific roles have been extensively reviewed elsewhere.[1-5] Briefly, bacteria use these compounds (see Figure 1 for structures) mainly in dissimilatory or assimilatory sulfate reduction processes, while fungi, algae, and plants, although retaining the ability to reduce sulfate for assimilatory purposes, make additional use of the nucleotides for the production of sulfate esters and sulfonates. In higher organisms, reduction of sulfate virtually disappears and formation of sulfate esters predominates. Dissimilatory reduction of sulfate in microorganisms generally involves APS as the activated intermediate,[6] as does assimilatory sulfate reduction in phototrophic bacteria and algae,[7] also. In contrast, PAPS is the activated intermediate in assimilatory sulfate reduction in nonphotosynthetic bacteria and other microorganisms,[2,3] and appears to be the "active sulfate" used by all types of cell in sulfate transfer reactions leading to the formation of sulfate esters.[5] Clearly APS and PAPS together play key roles in both kinds of reductive process and in sulfate esterification.

It is generally accepted[1-3,5] that APS is first synthesized in the following reaction catalysed by ATP-sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4).

$$ATP + SO_4^{2-} \rightleftharpoons APS + pyrophosphate \qquad (1)$$
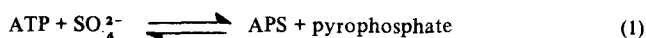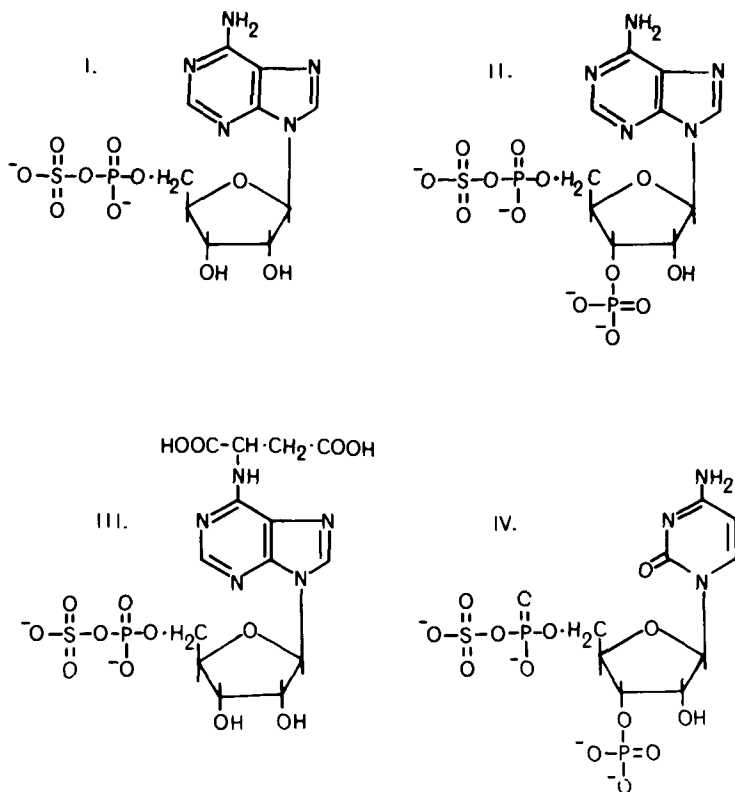
FIGURE 1.   Naturally occurring phosphosulfates. I, Adenosine 5'-phosphosulfate (APS); II, 3'-phosphoadenosine 5'-phosphosulfate (PAPS); III, 6-succinyladenosine 5'-phosphosulfate; IV, 3'-phosphocytidine 5'-phosphosulfate.

Conversion of APS to PAPS then occurs by phosphorylation of the 3'-ribose OH of APS at the expense of a second molecule of ATP (reaction 2) catalysed by APS-kinase (ATP:adenylylsulfate 3'-phosphotransferase, EC 2.7.1.2 5).

$$\text{APS} + \text{ATP} \;\rightleftharpoons\; \text{PAPS} + \text{ADP} \tag{2}$$

PAPS is therefore an expensive compound (in terms of the ATP required for its synthesis) for the cell to elaborate, and this together with the aversion of nature to wasteful synthesis is perhaps testimony to the importance of PAPS in the overall metabolism of the cell. The importance of APS and PAPS is also reflected in the attention which has been devoted to the study of the regulation of their syntheses[5] and, therefore, of sulfate reduction and transfer. However, little attention has been paid to the possibility that hydrolytic enzymes, including sulfatases, acting on APS and/or PAPS might also be involved in this regulation.

Before describing these sulfatase enzymes, three other naturally occurring phosphosulfate compounds need mention. First, a derivative of APS, 6-*N*-succinyladenosine 5'-phosphosulfate (Figure 1) was isolated from salmon liver and its structure investigated.[8] Although the compound was associated in some way with a peptide, no role was assigned. Secondly an active-sulfate nucleotide was isolated from the seaweed *Pelvetia canaliculata* and characterized as 3'-phosphocytidine 5'-phosphosulfate,[9] a cytosine analogue of PAPS (Figure 1), which also contained a tightly bound Mn^{2+} ion. The role of this compound was discussed in terms of the need for such a marine organism

to synthesize anionic polysaccharide sulfate esters to protect the cell against the influx of excess salt from sea water. Finally, a nucleotide containing cytosine, ribose, phosphate, and sulfate in approximately equimolar amounts has been isolated[10] from the skin of newborn rats. An interesting link exists here with each of the two compounds just described in that this cytosine-phosphosulfate is, first, associated with a peptide, and second, its presence in the skin was considered in relation to mucopolysaccharide synthesis. Because no further studies have been reported for any of these interesting nucleotides, their susceptibility to degradation by sulfatases is unknown and the remainder of this section is, therefore, confined to enzymes acting on APS and PAPS.

B. Degradation of APS and PAPS by Mammalian Enzymes

Most of the research effort on hydrolytic enzymes with activity towards APS and PAPS has centered around various mammalian systems, mainly liver and brain. In such tissues, active sulfates are used exclusively for sulfate transfer. Although the main concern of this book is with microbial enzymes, a brief review here of the mammalian active sulfate-degrading enzymes is appropriate because it forms a framework for discussion and highlights potential problems and sources of confusion. For a fuller treatment and bibliography, the reader is referred to the review by Dodgson and Rose.[11]

Following the elucidation of the structures of APS and PAPS (Figure 1) in 1955 by Hilz and Lipmann,[12] studies during the period 1958 to 1962 on PAPS synthesis indicated that there were also factors present that were responsible for degrading active sulfates. Isolated reports of the conversion of PAPS to APS, APS to sulfate, and PAPS to PAP (adenosine 3',5'-diphosphate) appeared, and it gradually became evident that the presence of these degrading activities in cell extracts was an undesirable complication in experiments in which PAPS was being produced for biosynthetic purposes. However, no detailed study emerged until the work of Balasubramanian and Bachhawat[13] in 1962 on PAPS degradation in sheep brain preparations. This work apparently demonstrated the presence of a Co^{2+} (or Mn^{2+})-activated PAPS-sulfatase. In spite of the fact that it is unusual for sulfatases to express a requirement for metal ions and, moreover, that some 5'- and 3'-nucleotidases were markedly activated by divalent metal ions, the notion of a Co^{2+}-activated PAPS-sulfatase remained unchallenged, and Co^{2+} was subsequently included routinely in the assay mixtures in most investigations that were to follow. Reports of Co^{2+}-activated PAPS-sulfatase, multiple APS-sulfatases, and PAPS-phosphohydrolases certainly indicated that a multiplicity of enzymes were involved in PAPS-degradation but, in general, confusion abounded in the wake of such reports until some work from the Cardiff laboratories began to clarify the picture.

Studies[14] designed to elucidate, in particular, the role of Co^{2+} in the degradation of PAPS in rat liver cell-sap provided a clear indication of at least three participating enzymes, namely a PAPS-sulfatase, APS-sulfatase and PAPS 3'-nucleotidase, (EC 3.1.3.7) only the last of which was activated by Co^{2+} ions. APS-sulfatase was also present in, and partly purified from, the lysosomal fraction of the cell and shown to be quite different from the cell-sap enzyme. In further studies using ox liver,[15] the three cell-sap enzymes were isolated and studied separately. Again only the PAPS-nucleotidase was activated by Co^{2+} ions. Collectively the studies established the existence in the cell cytoplasm of ox and rat liver of at least two alternative routes for the biodegradation of PAPS. The first of these involves direct desulfation of the phosphosulfate by a PAPS-sulfatase not activated by Co^{2+} (Figure 2, I). The PAP produced can lose its 3'-phosphate group to yield AMP (Figure 2, IV.). The second route involves the action of the Co^{2+}-activated 3'-nucleotidase to give APS (Figure 2, II) and subsequent desulfation of the latter by APS-sulfatase to liberate AMP and sulfate ions (Figure 2, III). It was thus becoming apparent that earlier claims for a Co^{2+}-activated
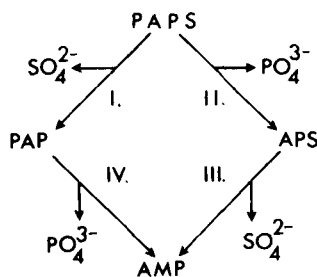
FIGURE 2. Possible alternative routes for the degradation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS). I, PAPS-sulfatase; II, Co^{2+}-activated 3'-nucleotidase; III, APS-sulfatase; IV, 3'-nucleotidase.

PAPS-sulfatase probably reflect activation of the 3'-nucleotidase and consequent stimulation of this second route for PAPS degradation, rather than activation of a simple PAPS-sulfatase. The APS-sulfatase in this second pathway was not activated by metal ions and was found to be present in both the cell-sap and lysosomal fractions of liver homogenates. Enzymes from both fractions have now been purified to homogeneity from ox liver and their properties examined in some detail.[16-18] The enzymes are quite distinct and probably the most important difference lies in their substrate specificities; the cytosol enzyme is specific for APS whereas the lysosomal enzyme will also hydrolyse, besides APS, a number of phosphoric acid anhydrides such as ATP, pyrophosphate, and bis(p-nitrophenyl)phosphate. Thus, although the discovery of a specific role for the cytosol enzyme might yet be awaited (see Volume II, Chapter 4) it seems likely that the lysosomal enzyme is a nonspecific phosphoric anhydride hydrolase participating in the general scavenging role of lysosomal enzymes. With this brief account of the mammalian degradation of active sulfates as a background, the reader's attention is now directed towards the microbial systems.

C. Microbial Degradation of APS and PAPS

During the decade or so that elapsed while the complex pattern for the mammalian enzymes was emerging, only one study of phosphosulfate hydrolysis in microorganisms appeared.[19] More recently, however, expansion in studies concerned with sulfate reduction in microorganisms has generated greater interest in these enzymes (Table 1).

1. Active Sulfate Degradation in Unicellular Algae and the Cyanobacteria

The early paper previously cited[19] was concerned primarily with uptake and activation of sulfate in *Euglena gracilis*, but it also included a section on PAPS-degradation. This activity was assayed by incubating extracts with (^{35}S)-PAPS and absorbing residual nucleotide on charcoal prior to counting liberated sulfate ions. Using this assay, the PAPS-degrading activity was found to be maximally active at pH 5.5 and stimulated by the presence of Mg^{2+} or Co^{2+} ions. Sulfhydryl reagents such as BAL (British anti-Lewisite, 2,3 dimercaptopropan-1-ol) or cysteine also stimulated activity, and specific activities in photosynthesising or nonphotosynthesising cells were the same. All these properties were considered in terms of there being a single PAPS-sulfatase present in the extracts, and the possibility of dual pathways now known to exist in liver extracts (Figure 2) was dismissed rather lightly. The assay method does not register the accumulation of APS as a degradation product of PAPS since both are absorbed onto charcoal. In any case, paper electrophoretograms of incubation mixtures showed

Table 1
MICROBIAL DEGRADATION OF APS AND PAPS

Organism	Reaction catalysed	Effect of nucleotides	Other effectors	pH of assay	Location	Enzymes involved	Ref.
E. gracilis	(^{35}S) PAPS → $^{35}SO_4^{2-}$ + no (^{35}S)-APS. K_m for PAPS = 2.25 × 10⁻⁵ M	ADP powerful inhibitor, ATP, AMP, GTP also inhibit.	Cysteine, thiols, Mg^{2+} and Co^{2+} activate. EDTA no effect.	5.5 (optimum)	—	Unsubstantiated claim for PAPS-sulfatase.	16
A. cylindrica	(^{35}S)-APS → AMP + $^{35}SO_4^{2-}$	Adenine nucleotides inhibit, GTP, CTP, TTP, UTP activate.	Fluoride inhibits. Phosphate and pyrophosphate stimulate	7.5	Soluble fraction only	APS-sulfatase	17
	(^{35}S)-PAPS → $^{35}SO_4^{2-}$ + small amounts of (^{35}S)APS	Addition of unlabeled APS leads to accumulation of (^{35}S)-APS, but no $^{35}SO_4^{2-}$. Adenine nucleotides and other nucleoside triphosphates inhibit.	Sulfite, fluoride, glucose-6-phosphate, phosphate and pyrophosphate inhibit.	7.5	Soluble and pellet fractions. Pellets produce large amounts of APS besides SO_4^{2-}.	3'-nucleotidase + APS-sulfatase. Possibly a PAPS-sulfatase.	17
C. pyrenoidosa	(^{35}S) APS → AMP + $^{35}SO_4^{2-}$ in the absence of NH_4^+	ATP inhibits	NH_4^+ leads to formation of adenosine 5'-phosphoramidate.	9.0	—	APS: NH_4^+ adenylyl transferase and APS-sulfatase probably the same enzyme.	18, 19
C. reinhardti	(^{35}S)-APS → $^{35}SO_4^{2-}$ + AMP; APS → SO_4^{2-} + cAMP	—	—	9.0; 9.0	—	APS-sulfatase; APS-cyclase	23; 23
E. coli	APS → AMP + SO_4^{2-}	—	—	—	—	APS-sulfatase?	21
C. terrigena	(^{35}S)APS → AMP + $^{35}SO_4^{2-}$. K_m for APS = 1.1 mM. Distinct from ATP-sulfurylase	AMP competitively inhibits. ATP relieves inhibition by AMP	Adenosine inhibits with nonhyperbolic kinetics	8.5 (optimum)	Cytoplasm	APS-sulfatase. Inactive towards ATP, ADP, AMP, PP, PAPS, phosphodiesters, aryl sulfate, phosphoserine.	20, 73
	(^{35}S)PAPS → (^{35}S)APS + $^{35}SO_4^{2-}$	—	Co^{2+} stimulates APS and SO_4^{2-} production.	7.5	Soluble fraction → rapid SO_4^{2-} + no APS. Pellet → slow SO_4^{2-} + some APS.	3'-nucleotidase + soluble APS-sulfatase. Possibly a PAPS-sulfatase.	74
Pseudomonas Cl2B	(^{35}S)APS → AMP + $^{35}SO_4^{2-}$			7.5		APS-sulfatase?	20
P. chrysogenum	(^{35}S)APS → AMP + $^{35}SO_4^{2-}$	Inhibited by excess APS.	Stimulated by Mg^{2+}, inhibited by Mn^{2+}, Co^{2+}, EDTA.	8.0	—	Reversal of ATP-sulfurylase in absence of pyrophosphate.	22

the absence of APS. This was interpreted as showing conclusively that there was no formation of (^{35}S)-APS by this enzyme system. However, in the light of the mammalian work and in view of the stimulation afforded by Co^{2+}, it is quite possible that a PAPS-nucleotidase/APS-sulfatase route is operating in the *Euglena* extracts. APS need not necessarily accumulate if the APS-sulfatase is sufficiently active. Furthermore, the evidence for formation of PAP during the degradation of PAPS is not sufficient to eliminate other routes of degradation because stoichiometric release with SO_4^{2-} was not established. In short, this paper, like some of its contemporaries, failed to make careful allowance for the possible existence of multiple enzymes and pathways involved in PAPS-degradation.

More recently Sawhney and Nicholas[20] have made a preliminary study of the degradation of active sulfates by crude cell extracts of *Anabaena cylindrica*. In this study the authors recognised the need to make use of APS as well as PAPS in studying the overall degradation of the latter. Crude cell extracts of this cyanobacterium contained enzymes that rapidly hydrolysed both APS and PAPS with the liberation of sulfate ions. PAPS-degradation was accompanied by the production of very small amounts of APS which persisted throughout the incubation period, indicating the involvement of a 3'-nucleotidase. This was confirmed by the discovery that in the additional presence of unlabeled APS, the loss of radioactivity from (^{35}S)-PAPS could be accounted for entirely as the accumulated (^{35}S)-APS. The results therefore suggest that the 3'-phosphate group of (^{35}S)-PAPS was first attacked by a nucleotidase to produce (^{35}S)-APS. Addition of the large excess of unlabeled APS served to saturate the enzymes involved in its further degradation, resulting in an accumulation of (^{35}S)-APS. The rate of loss of PAPS under these conditions was about one third of the rate in the absence of added APS. Evidently APS, like other adenine nucleotides (ATP, ADP and 5'-AMP), inhibited the degradation of PAPS. The absence of sulfate formation direct from PAPS under these conditions does not necessarily indicate the absence of a PAPS-sulfatase; it is possible that such an enzyme is present and normally contributes to sulfate production, but that it is inhibited by excess APS. The behavior of the *A. cylindrica* system was in many ways analogous to that discovered earlier for the liver system and it is unfortunate that the authors did not draw any comparisons between the two systems.

Assays with subcellular fractions of *A. cylindrica* further established that APS-degradation was almost totally confined to the soluble fraction of the cell extracts, whereas PAPS was degraded fairly rapidly by all fractions (soluble fraction and pellets obtained by differential centrifugation). However, differences were observed in the amounts of APS produced from PAPS by the different fractions. Only traces of APS were detected with crude extracts or with the soluble fraction, presumably reflecting the presence of an active APS-sulfatase. In contrast, APS accumulated when pellet fractions were used, showing the presence of a 3'-nucleotidase and relatively little APS-sulfatase. Similar results have been obtained with the soluble and pellet fractions of the soil bacterium *Comamonas terrigena* (see Section II. C. 2). Mention should be made at this point of an enzyme present in *Chlorella pyrenoidosa* which can catalyse the hydrolysis of APS. This activity[21] was inseparable from another activity which, in the presence of NH_4^+ ions, was apparently capable of eliminating sulfate from APS with concomitant cyclization to produce cyclic-AMP. More recent studies,[22] however, have shown that in the presence of NH_4^+ ions the main product of this enzyme's action is not cyclic-AMP, but adenosine 5'-phosphoramidate with which it shares many properties. The physiological significance of the enzyme's dual ability to hydrolyse APS and synthesize the phosphoramidate is unknown. However, the sulfatase was inhibited by ATP and in this respect it resembles the liver cytosol and *A. cylindrica* enzymes. The authors also concluded that the operation of a PAPS-sulfatase in the *Chlorella* system was unlikely, although the evidence for this was somewhat circumstantial.

The pattern, therefore, begins to emerge of a dual pathway for PAPS degradation for these unicellular algae as well as for the mammalian systems. The two pathways differ according to whether the first step is sulfate release from the phosphosulfate or phosphate release from the 3'-position. The predominant pathway appears to be the latter with intermediate formation of APS. However the precise contribution of the other pathway and, in particular, of the PAPS-sulfatase enzyme is uncertain and will remain so either until reliable estimates of PAP co-production in crude extracts are made or until all the enzymes can be separated and quantitated independently.

Kühlhorn and Schmidt[23] remained fascinated by the possibility arising from Schiff's work that cyclic-AMP could be formed by a cyclization of APS with concomitant elimination of sulfate ions. In a relatively recent paper they describe work pursuing this possibility using *Chlamydomonas reinhardti* as a source of enzymes. Their approach was to isolate from extracts of the alga all enzymes that could release sulfate ions from APS, and then to examine each one for ability to form simultaneously cyclic-AMP. Cells were harvested in the late-log phase and ruptured in a French press. Centrifuged extracts were treated with ammonium sulfate, and the fraction precipitating between 35 and 55% saturation was dialysed and further separated using DEAE-cellulose chromatography. Three peaks of APS-sulfatase activity were observed, the last of which only appeared in the presence of Mg^{2+} ions. The first peak to elute was able to form cyclic-AMP from APS and this activity was purified further using gel filtration chromatography. This procedure produced one peak that did not require Mg^{2+} for activity, but whether others were observed when this cation was included is not clear. Neither is it obvious how the authors conclude from their results that six different APS-sulfatases were separated. Despite its shortcomings, this paper does reemphasize that sulfate ions could be liberated from APS by one of several routes which include a simple sulfatase liberating AMP, a cyclizing enzyme producing cyclic-AMP by an elimination of sulfate, and an adenylyl transferase producing adenosine 5'-phosphoramidate. In short, whenever an enzyme liberating inorganic sulfate from APS (or PAPS) is encountered, it is important that the nucleotide co-product should be identified in order to establish whether or not the enzyme is a true sulfatase.

2. Active Sulfate Degradation in Bacteria

Following the studies on the degradation of active sulfates in mammalian liver, attention in the Cardiff laboratories was turned towards bacterial sources of these enzymes. It was anticipated that because active sulfates play a more fundamental role in microbial metabolism in assimilatory sulfate reduction, degrading enzymes might well be present and, moreover, that an understanding of the role of such enzymes might be achieved through studies of the physiological regulation of their synthesis by exogenous nutrients.

Initially, extracts of three bacteria were examined[24] for the ability to hydrolyse APS, namely *Comamonas terrigena*, *Pseudomonas* C12B and *Escherichia coli*. The isolation of the first two of these organisms and their ability to produce multiple alkylsulfatase enzymes capable of hydrolysing alkyl sulfate surfactants has already been described (Volume I, Chapter 2). Extracts of *C. terrigena*, grown to various stages in batch culture on nutrient broth, showed that APS-sulfatase made an increasing contribution to cellular protein up to mid-log phase and thereafter the specific activity remained constant.[24] With *Pseudomonas* C12B, a peak of activity was observed at mid-log phase with maximum activity being about one third that observed with *C. terrigena*. Growth of *E. coli* on nutrient broth produced barely detectable levels of APS-sulfatase despite an earlier report[25] indicating the ability of *E. coli* to produce the enzyme. However, more recent studies in Cardiff[72] have shown that APS-sulfatase is indeed synthesised when *E. coli* is grown on defined media with sulfate as sole source of sulfur. Attention was subsequently focused on *C. terrigena*, this being the richest source.
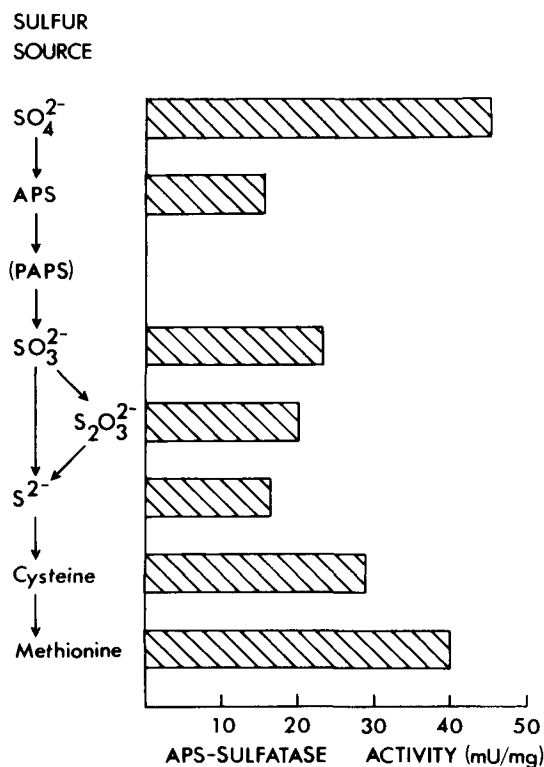
SULFUR
SOURCE

FIGURE 3. Effect of sulfur source on the synthesis of
APS-sulfatase by *Comamonas terrigena*. The results are av-
erage values for cell samples taken at two separate times
during the late-log/stationary phases. The effect of PAPS
is not known.

In contrast to the *E. coli* system, the amounts of enzyme synthesised during growth
of *C. terrigena* on a variety of sulfur compounds as sole sulfur sources fluctuated only
marginally from the level found in broth grown cells.[24] Highest activity was observed
with cells grown on sulfate or methionine (50% higher than broth grown cells), with
lower activity (50% of broth-grown cells) when intermediates in the assimilatory re-
duction pathway between APS and cysteine were employed (Figure 3). Implications of
these findings will be considered in Chapter 4 where the whole problem of the physio-
logical function of the enzyme will be discussed. For the present, it suffices to note
that the enzyme is apparently constitutive, albeit subject to partial control by the sulfur
supply to the cell. The APS-sulfatase was located entirely in the cytoplasm and was
readily distinguishable from ATP-sulfurylase in crude cell extracts by its different mo-
bility during polyacrylamide gel electrophoresis. Evidently the *C. terrigena* sulfatase
activity does not reside with the ATP-sulfurylase protein as is the case with the sulfur-
ylase of *Penicillium chrysogenum* (see following).

Recently the APS-sulfatase of *C. terrigena* has been purified to homogeneity and
partly characterized in the Cardiff laboratories.[72] Although this work is of a prelimi-
nary nature, some of the more pertinent details will be described here. The enzyme is
a single polypeptide chain (molecular weight 23,000) which appears to be quite specific
for APS for which the K_m is 1.1 mM at the optimum pH of 8.5. This K_m is considerably
lower than the value reported[24] for the enzyme in crude cell extracts. The reason for
the discrepancy is unknown, but it may be due to competitive inhibition of the enzyme
by some component in the crude cell extracts which is removed during purification.

No activity was observed towards any other compound tested (Table 1) including ATP, ADP, pyrophosphate, and phosphodiesters. This behavior is like that of the liver cytosol enzyme, and contrasts with the nonspecific lysosomal enzyme. Product inhibition by AMP was strong and, most interestingly, the inhibition was relieved by the presence of ATP although this nucleotide alone had no effect on enzyme activity. These results are suggestive of a regulation linked to the energy metabolism of the cell and this possibility is considered further in later chapters. Adenosine also inhibits the *C. terrigena* APS-sulfatase and, moreover, in the presence of this nucleoside the relationship between reaction rates and substrate concentration changes from the normal hyperbolic one to a sigmoidal dependence. The origin and purpose of this behavior are unknown, but they may be related to the effects of different adenine nucleotides described above. With the failure of various sulfur sources to affect the synthesis of APS-sulfatase, this very preliminary information indicating an elaborate regulation of enzyme activity could now prove to be a vitally important clue in the search for a realistic role for the enzyme. Clearly further experimentation on this aspect is urgent and essential.

Cell extracts of broth grown *C. terrigena* were also capable of hydrolysing PAPS.[72] Like the *A. cylindrica* system, low levels of APS were produced and persisted throughout the incubation period, suggesting the participation of a 3'-nucleotidase to produce APS from which sulfate was liberated by the APS-sulfatase described previously. This interpretation was supported by the fact that incubation in the additional presence of Co^{2+} (a known activator of 3'-nucleotidase) produced considerably higher levels of APS and accelerated the overall rates of disappearance of PAPS and appearance of sulfate. Whether or not a PAPS-sulfatase also participates in the liberation of sulfate is unknown. Incubation of the membrane fraction of the cell with (^{35}S)-PAPS produced relatively large accumulations of (^{35}S)-APS (up to 10% of the total radioactivity) as well as liberating $^{35}SO_4^{2-}$. A 3'-nucleotidase is therefore present, but because this fraction was capable of slowly hydrolysing APS to sulfate it is still uncertain whether sulfate production is due to the presence of a PAPS-sulfatase, or of a 3'-nucleotidase and APS-sulfatase acting in sequence, or of a combination of both.

3. Active Sulfate Degradation in Fungi

Only one report of APS hydrolysis by a fungal enzyme has appeared.[26] The activity was observed as an incidental reaction of another enzyme, namely the ATP-sulfurylase which is normally responsible for APS-synthesis in *Penicillium chrysogenum*. This enzyme has been purified and its physical, kinetic, and chemical properties examined in detail by Segel's group in California. Besides catalysing both the forward and reverse directions of reaction 1 and various associated exchange reactions, this enzyme also hydrolysed APS when incubated in the absence of the co-substrate for the reverse reaction, pyrophosphate. Few studies were made of the hydrolytic activity, but it was shown that the process was stimulated by Mg^{2+} and inhibited by EDTA, Ca^{2+}, Mn^{2+} or Co^{2+}. The K_m for APS was about 0.6 μM in the presence of 8mM Mg^{2+} at pH 8.0. These observations raise the possibility that APS-sulfatase observed in extracts of other microorganisms might also be associated with ATP-sulfurylase enzymes. In the case of *C. terrigena*, the APS-sulfatase was shown to be distinct from the ATP-sulfurylase activity. However, in view of the low molecular weight and single subunit structure of APS-sulfatase in *C. terrigena*, and the multisubunit structure of native ATP-sulfurylases (e.g., eight equivalent subunits each of molecular weight 55,000 in the *P. chrysogenum* enzyme), the possibility remains that the APS-sulfatase activity may reside in single subunits of the ATP-sulfurylase complex. No evidence is currently available to decide this issue.

D. Concluding Remarks

Many animal tissues are known to be able to hydrolyse APS and/or PAPS, and in view of the limited results so far available it seems likely that many microorganisms will prove to possess similar activities. It is also clear that the degradation of APS can be mediated either by enzymes with a broad specificity for phosphoric acid anhydrides (e.g., the liver lysosomal enzyme) or by enzymes normally engaged in some other facet of APS metabolism (e.g., ATP-sulfurylase of *P. chrysogenum*, APS:ammonia adenylyl transferase of *C. pyrenoidosa*). Such observations tend to cast a shadow of doubt across the authenticity of those cases where an apparently specific APS-sulfatase has been indicated (e.g., *C. terrigena*). The reader will recall that the liver cytosol enzyme is also highly specific for APS, and the role of scavenging for adenine nucleotides was tentatively assigned to it.[15] However, this is a rather unconvincing theory, particularly as the cellular levels of APS and PAPS are so low as to contribute negligibly to the total nucleotide pool.[27] Reference has already been made (Volume I, Chapter 2) to the view that the coexistence of mechanisms for the synthesis and hydrolysis of active sulfates in the same cell is untenable since it would merely lead to a futile hydrolysis of ATP. However, such a view fails to take account of the possibility that the catalytic activity of the synthetic and hydrolytic enzymes is likely to be closely controlled in vivo. For example, the kinetic control of the ATP-sulfurylase of *P. chrysogenum* is elaborate, while the *C. terrigena* APS-sulfatase seems to be curiously influenced by adenine nucleoside and nucleotides. Evidently, a proper discussion of the roles of APS-sulfatases needs to take an overview of all the available information including regulation of synthesis of enzymes under various conditions of growth, regulation of catalytic activity of preformed enzyme, and the mechanism of action of the enzymes. It would certainly seem premature at this time to dismiss all phosphosulfate sulfatases as experimental artifacts derived from other enzyme activities.

With these points in mind, further discussion of the role of APS-sulfatases is best deferred (Chapter 4) until regulation of synthesis and mechanism of action of these and other sulfatases have been described.

Degradation of PAPS is a somewhat more complicated problem than that of APS because two possible routes are available depending on the sequence in which phosphate and sulfate are removed (Figure 2). It will have emerged from the foregoing sections that unequivocal identification of PAPS-sulfatase in crude cell extracts is frequently made difficult by the ubiquitous 3'-nucleotidases which, while not themselves liberating sulfate, allow the apparently equally common APS-sulfatase to come into action to release SO_4^{2-} from the intermediate APS. Positive identification of PAPS-sulfatase requires either separation from, or inhibition of, APS-sulfatase and 3'-nucleotidase activities, and subsequent demonstration of stoichiometric release of PAP and inorganic sulfate from PAPS. Experiments simply showing release of sulfate from PAPS with no accumulation of APS are of little value in this respect unless it is also shown that no APS-sulfatase is present. It is to be hoped that future searches for the elusive PAPS-sulfatase will heed these problems and avoid the pitfalls that beset the path of early workers in the mammalian field.

Finally it is worth mentioning again that other phosphosulfates besides APS and PAPS have been detected in various tissues (see Section II, A and Figure 1) and it will be interesting to see whether these compounds serve as substrates either for the active sulfate degrading enzymes so far uncovered or for other specific enzymes as yet undetected.

III. HYDROLYSIS OF SULFAMIDE LINKAGES

It will be recalled that sulfamidase activity as a feature of the biodegradation of heparin and heparan sulfates has been dealt with at some length in Volume I, Chapter
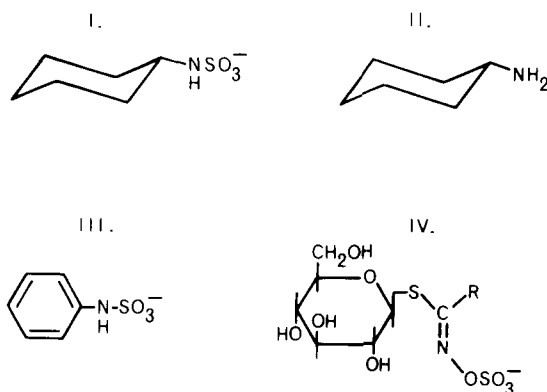
FIGURE 4. Sulfamides, glucosinolates, and related compounds. I, Cyclohexyl sulfamate (cyclamate); II, cyclohexylamine; III, phenyl sulfamate; IV, glucosinolates (mustard oil glycosides).

3, Section VII. Apart from a brief comment to be made in a later section, the enzyme in question (glucosamine sulfamidase) will not be discussed further, and the present account is restricted to sulfamidase activity towards cyclohexylsulfamic acid (cyclamate) and other related compounds (cyclohexylsulfamate sulfamidase, EC 3.10.1.2)

A. Introduction

In recent years increasing attention has been paid to the role of intestinal microorganisms in the biotransformation of xenobiotics. In earlier times researchers in the field of xenobiotic metabolism (or "detoxication" as it was then generally called) were preoccupied with establishing the overall nature of the chemical changes undergone by xenobiotics that had been administered to experimental animals by injection or (most frequently) by the oral route. With the passage of time the liver became the focus of attention of most investigations and the fact that the gastrointestinal tract, the lungs or any other organ might also be involved in xenobiotic transformations tended to be largely ignored. Enzyme systems involved in transformations later become a popular topic for study and it is only within the last 15 years that serious consideration has been given to the possibility that gut microorganisms could supply some of those enzyme systems. A number of factors have helped to stimulate interest; for example, the need to know more about the ways in which gut microorganisms can acquire drug resistance, the increased legal requirements placed on the pharmaceutical industry to ensure safety of new products, and the growing realization of the possibility that variations in the composition of the gut flora may sometimes have a part to play in the conversion of apparently innocuous compounds into potential carcinogens.

Relevant to the present story is the fact that in 1959 the noncaloric sweetener, saccharin, was largely replaced by a new sweetener, cyclamate (cyclohexylsulfamic acid, marketed as the calcium or sodium salt, Figure 4,I). Like saccharin the new sweetener appeared not to be metabolized to any significant degree by mammalian organisms and moreover, lacked the undesirable aftertaste of the former compound. The use of cyclamate pervaded the food, beverages and pharmaceutical industries on an ever increasing scale until 1969 when its use in the U.S., the U.K. and certain other countries was totally banned, following a report[28] that suggested that very high dose levels of cyclamate given to rats over a prolonged period of time may cause the animals to develop bladder cancer. This report had to be viewed in the light of an earlier investigation[29] that some conversion of cyclamate to cyclohexylamine (Figure 4, II) did occur

in some experimental animals, and the possibility existed that the amine could serve as a bladder carcinogen or precarcinogen. It was later established (see References 30 and 31 for example) that the ability to achieve the conversion of cyclamate to cyclohexylamine appeared to be absent from most normal human subjects or animals, but developed only after a period of continued intake of the sweetener over a period of several weeks. Subjects or animals that develop this capacity (and not all do) have been termed "converters".

The adaptability of mammalian liver enzyme systems to cope with some xenobiotics has long been known. However, in the case of cyclamate it quickly became evident that gut microorganisms, and not liver systems, were responsible for the relatively low levels of metabolism noted. The phenomenon of the "converter" was actually reflecting either the induction of appropriate bacterial enzymes, the selection of a mutant, or a relative increase in the number of a particular organism already possessing the capacity to metabolize the sulfamate. Thus, it was shown[32] that feces from rats, rabbits and humans known to be converters were able to produce cyclohexylamine when incubated with cyclamate under anaerobic conditions. The activity was traced to clostridia in rats, enterobacteria in rabbits, and enterococci in humans. The numbers of clostridia in the feces of rats on a cyclamate diet were greater than those in normal rat feces. Finally, the ability to metabolize cyclamate by mixed cultures from the colon and rectum of rats tended to disappear if the organisms were subcultured more than twice in media that did not contain cyclamate.

In another study[33] 15 strains of cyclamate assimilating bacteria were isolated from the feces of guinea pigs that had been subjected to continued cyclamate feeding and were excreting some cyclohexylamine in urine. The strains, which appeared to belong to the genus *Pseudomonas* and *Corynebacterium,* were all capable of utilizing cyclamate as sole source of C and N and growth on the sulfamate led to the accumulation of cyclohexylamine in culture fluids. A cell-free extract of one of the *Pseudomonas* species (strain K) was able to convert cyclamate to cyclohexanone via cyclohexylamine and this was presumed to indicate the presence of a hydrolytic enzyme capable of liberating cyclohexylamine from the sulfamate (i.e., a sulfamidase) and some form of oxygen dependent amine oxidase. This organism has since been used for the only investigations that appear to have been made of these enzymes.

B. The Sulfamidase of *Pseudomonas* sp. Strain K

For their study of this enzyme Niimura et al.[34] maintained stock cultures of the bacterium on a basal salts-vitamin medium containing sodium cyclohexylsulfamate. On a larger scale, the bacterium was grown in 6-l batches for 7 to 9 days at 30°C with shaking, or for 10 to 14 days without shaking. Cyclamate was present at a concentration of 1% and maximum sulfamidase activity was not obtained until the stationary phase of growth. The enzyme was purified 620-fold (yield 37%) from cell-free extracts by a procedure involving heat treatment, removal of nucleic acid with streptomycin sulfate, fractionation with ammonium sulfate, gel filtration chromatography on Sephadex® G-200, followed by chromatography on ECTEOLA-cellulose. The purified enzyme could be stored for lengthy periods at −20° C in 40 mM-Tris/HCl buffer, pH 7.2, containing 0.1% gelatin. However, appreciable losses were observed if gelatin was omitted or if the enzyme was stored in 4 mM-phosphate buffer at pH 7.0.

Maximum enzyme activity was obtained at 50°C and this incubation temperature was later used in all experiments. The standard assay procedure was always carried out in 40 mM-Tris/HCl buffer at pH 7.2 but, in fact, the optimum pH for enzyme activity was 6.6. A K_m value of 5×10^{-3} M was obtained, but it is not clear whether the relevant experiments were conducted at pH 7.2 or at the optimum pH. The enzyme released inorganic sulfate and cyclohexylamine in equimolar proportions so that the