SULPHUR ACQUISITION IN *NEISSERIA MENINGITIDIS*

by

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ABSTRACT

Ph.D. Jennifer L. Port Microbiology

Sulphur Acquisition in Neisseria meningitidis

Neisseria meningitidis is able to utilize a variety of biologically relevant compounds as its sole source of sulphur. Sulphate transport in the meningococcus is temperature-, pH-, concentration-, and energy-dependent. Group VI anions and sulphur-containing amino acids affect sulphate uptake. Selenate is a competitive inhibitor of sulphate transport and enters the meningococcus via the sulphate transporter. L-cysteine, a noncompetitive inhibitor of sulphate transport, exerts allosteric control on the system. L-cysteine has its own energy-dependent uptake system. Thiocyanate, a unique sulphur source for a heterotrophic bacterium, appears to be transported in the meningococcus by a system other than that for sulphate or L-cysteine uptake. Sulphur starvation enhances transport capacity and causes variations in some of the enzymes in the meningococcus. The sulphur source used for growth causes changes in the protein profiles of the envelope and cytosol fractions. The soluble sulphur pool in N. meningitidis regulates transport and metabolism of sulphur-containing

compounds. The presence of thiosulphate reductase may be to allow thiosulpahte to serve as an alternate electron acceptor during *in vivo* anaerobic growth.

RÉSUMÉ

Ph.D Jennifer L. Port Microbiologie

Acquisition du soufre chez Neisseria meningitidis

Neisseria meningitidis est un organisme capable d'utiliser une variété de composes biologique comme seule source de sulfure. Chez les meningococcus, le transport du sulfate est influencé par le temperature, le pH, la concentration, et l'enérgie. Les anions du groupe VI et les amino acides contenant du soufre affectent aussi l'absorption cellulaire du sulfate. Le selenate, inhibiteur compètitif du transport du sulfate, est absorbé par le meningococcus via un transporteur du sulfate. L-cysteine, inhibiteur non compètitif du transport du sulfate, exerce un contrôle allostérique sur le système. En outre, la L-cystéine possède son propre système d'absorption dependant de l'enérgie. Le thiocyanate, source unique de soufre chez les organismes hétérotrophies, semble être absorbé par le meningococcus par un système autre que celui utilisé pour l'absorption du sulfate ou de la L-cystéine. Une carence en sulfure augmente la capacité de son transport et provoque des fluctuations chez quelques enzymes produits par le meningococcus. Suivant la source de soufre utilisée les

profils des protéines de l'enveloppe et du cytosol varient. La resérve soufre soluble chez N. meningitidis régularise le transport et la métabolisme des composés sulfurés. In vivo le thiosulfate peut être utilisé comme une alternative et servir d'accepteur d'electrons lors la croissance en anaérobiose. .

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. N. meningitidis, cultured in a chemically defined medium, was able to use a variety of biologically relevant inorganic and organic sulphur compounds as sole sources of sulphur for growth.

2. Sulphate transport in the meningococcus was shown to be dependent on temperature, pH, concentration, and energy. Group VI anions, other inorganic sulphur compounds, sulphur-containing amino acids, and glutathione affected sulphate transport.

3. Kinetic studies clearly showed that selenate was a competitive inhibitor of sulphate uptake and that both ions were transported by the same mechanism.

4. L-cysteine was shown to be a noncompetitive inhibitor of the sulphate transport system.

5. L-cysteine, the endproduct of the sulphate pathway, was shown to be an allosteric inhibitor of the sulphate transport mechanism.

6. L-cysteine was transported by a system separate from the sulphate uptake system in N. meningitidis.

Uptake of L-cysteine was energy-dependent and displayed Michaelis-Menten kinetics. Uptake in starved cells was unaffected by inorganic sulphur compounds.

7. Thiocyanate, a unique sulphur source for a heterotrophic bacterium, appeared to have its own uptake system.

8. Sulphate and L-cysteine transport and metabolism were regulated by the low molecular weight soluble sulphur pool.

10. Sulphur starvation induced changes in the meningococcus, including enhanced transport rates and altered levels of some enzymes.

11. The sulphur source used for growth of the meningococcus resulted in differences in the protein profiles of the envelope and cytosol fractions subjected to SDS-PAGE.

12. Thiosulphate reductase was identified in N. meningitidis and may be present to allow thiosulphate to serve as an alternate electron acceptor during in vivo anaerobic growth.

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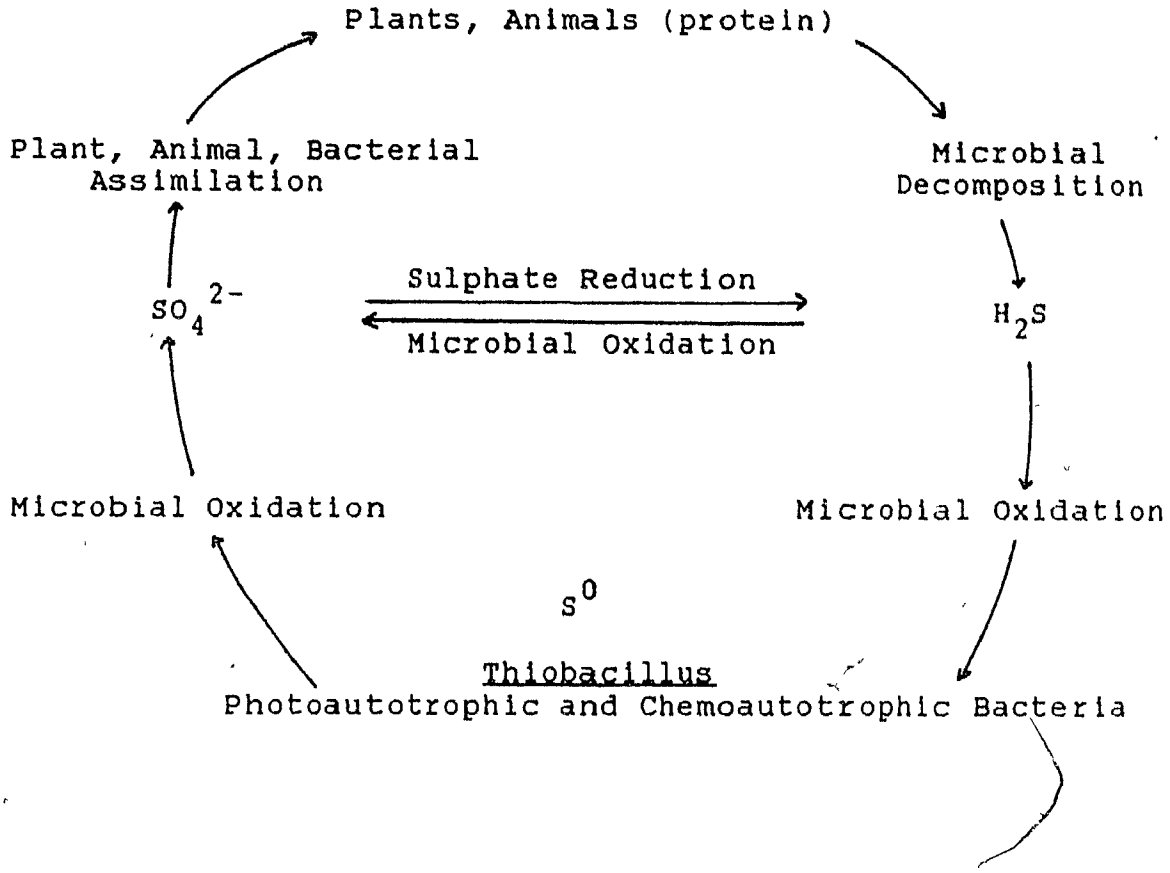
i. Introduction

The sulphur cycle in nature is a complex system of reductive and oxidative processes involving plants, animals, and microorganisms. Sulphur is transformed throughout the cycle via an eight electron change, ranging in oxidation state from 6^+ in sulphate, to S^0 as elemental sulphur, to 2^- as sulphide and sulphydryl. The majority of the Earth's sulphur is found in rocks and sediments in the form of minerals such as gypsum ($CaSO_4$) or pyrite (FeS_2). The ocean contains the largest sulphur store for the biosphere, in the form of sulphate (Brock et al., 1984). The sulphur cycle (Wistreich and Lechtman, 1984) is shown on the following page.

Sulphur is a major nutrient for all living cells. Mammals can oxidize reduced sulphur compounds such as sulphide, sulphite, and thiosulphate and incorporate the sulphate into organic molecules (Lipmann, 1958), but are unable to reduce sulphate to sulphide and depend on plants and microbes to supply them with reduced sulphur compounds (Diewiatkowski, 1954).



ii. Microbial Sulphate Reduction

Postgate (1959) divided sulphate reduction by microorganisms into two classes, assimilatory and dissimilatory. Assimilatory sulphate reduction is carried out by aerobes and facultative organisms which use sulphate as a principal sulphur source. Sulphate must be reduced as the majority of sulphur-containing compounds in the cell are at the oxidation level of H_2S. Assimilatory sulphate reduction is summarized in Figure 1.

Dissimilatory sulphate reduction is carried out only by

Figure 1. Assimilatory reduction of sulphate and formation of L-cysteine.

1 = sulphate permease

2 = ATP sulphurylase

3 = APS phosphokinase

4 = PAPS reductase

5 = sulphite reductase

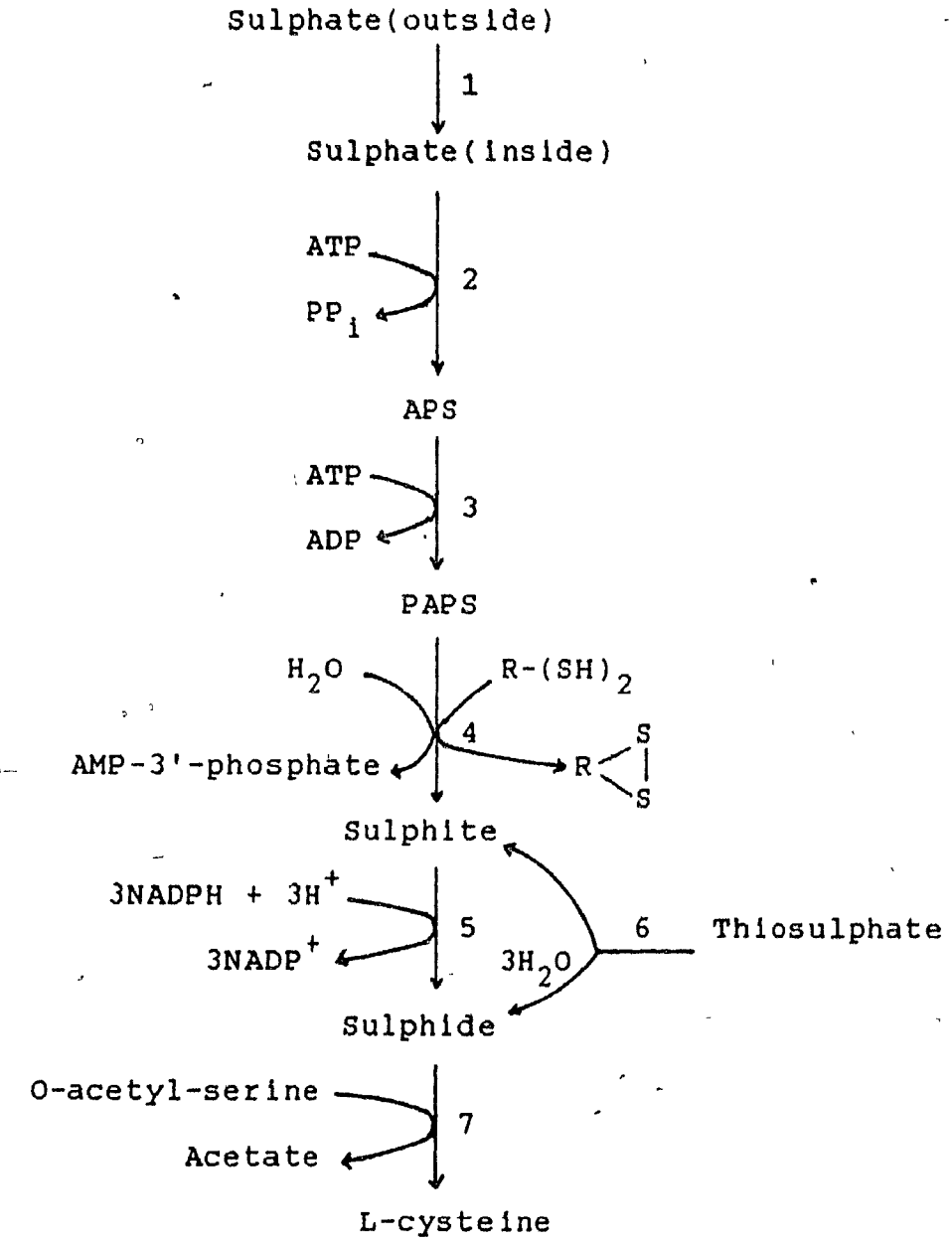
6 = thiosulphate reductase

7 = o-acetylserine sulphydralase

APS = adenosine-5'-phosphosulphate

PAPS = adenosine-3'-phosphate-5'-phosphosulphate

$R-(SH)_2$ = thioredoxin


Sulphate(outside)
1
Sulphate(inside)
ATP
PP i
2
APS
ATP
ADP
3
PAPS
H2O
R-(SH)2
4
AMP-3'-phosphate
R
S
S
Sulphite
3NADPH + 3H+
3NADP+
5
3H2O
6
Thiosulphate
Sulphide
O-acetyl-serine
Acetate
7
L-cysteine

strict anaerobes which use sulphate as a terminal electron acceptor, releasing hydrogen sulphide and producing ATP. These sulphate-reducing bacteria contain high concentrations of b-type or c-type cytochromes as well as menaquinone, several ferredoxins, and flavodoxin. Characteristic to sulphate reducers is siroheme protein, which has sulphite reductase activity as well as the ability to catalyze six-electron transfers (Gottschalk, 1986).

The sulphate reducers are divided into two groups, based on their oxidative capabilities. Group I bacteria (ie., Desulphovibrio spp.), known as incomplete oxidizers, oxidize a number of organic acids and alcohols to acetate while those in Group II (ie., Desulphotomaculum acetoxidans) are able to oxidize acetate to carbon dioxide (Gottschalk, 1986).

An example of incomplete oxidation is the fermentation of lactate and sulphate by Desulphovibrio, summarized as follows:

$$2\ \text{Lactate} + 2H_2O \longrightarrow 2\ \text{Acetate} + 2CO_2 + 8H$$

$$SO_4^{2-} + 8H \longrightarrow S^{2-} + 4H_2O$$

Lactate is oxidized via pyruvate and acetyl-CoA to acetate and CO_2, resulting in the synthesis of ATP. Sulphate is reduced to sulphide by a hydrogen cycling mechanism. The $8H^+$ represent 4 H_2 molecules located in the cytoplasm. These

hydrogens are transferred to a periplasmic hydrogenase, then to cytochrome c_3, channelled through electron carriers in the membrane, and back across the membrane to the APS-reductase and sulphite reductase (Gottschalk, 1986).

Desulphotomaculum acetoxidans (Group II) grows on acetate and elemental sulphur, oxidizing acetate to CO_2 via the tricarboxylic acid cycle and using the resulting reducing power to reduce sulphate to sulphide:

$$\text{Acetate} + 2\ H_2O \longrightarrow 2\ CO_2 + 8H$$
$$4\ S^0 + 8H \longrightarrow 4\ H_2S$$

iii. Microbial Sulphur Oxidation

The oxidation of reduced inorganic sulphur compounds is characteristic of two groups of bacteria, the photosynthetic bacteria in the order Rhodospirillales and two genera of chemolithotrophic bacteria, Thiobacillus and Sulpholobus.

The thiobacilli are small Gram-negative rods which derive their energy from the oxidation of sulphides, elemental sulphur, thiosulphate, polythionates, and sulphite to sulphate (Vishniac, 1974).

Thiobacillus ferrooxidans is frequently found in strongly acidic environments where iron and sulphur are

present in the form of pyrite (FeS_2). Its unique metabolism allows this bacterium to oxidize pyrite to ferrous ions (Fe^{2+}) and sulphate. Under acidic conditions, ferrous ions are further oxidized to ferric (Fe^{3+}) ions, which then react with more pyrite, leading to a propagation cycle of pyrite oxidation via the following reaction:

$$FeS_2 + 3\ 1/2O_2 + H_2O \longrightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$$

$$Fe^{2+} \xrightarrow{O_2} Fe^{3+}$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \longrightarrow 15Fe^{2+} + 2SO_4^{2-} + \underline{16}H^+$$

This chemistry is common in mining regions where sulphide forms highly insoluble minerals with many metals including copper, lead, zinc, and arsenic. Serious environmental problems occur when the byproducts of thiobacilli oxidation leach into the groundwater, lowering the pH and depositing metals which render the water unusable (Brock et al., 1984).

The order Rhodospirillales contains three families which metabolize sulphur in a variety of ways. In the family Rhodospirillaceae, Rhodospirillum and Rhodopseudomonas are microaerophiles which use sulphide and sulphur as the sole photosynthetic electron donor. They are able to oxidize sulphide to elemental sulphur, but cannot further oxidize the S^0 to sulphate. The family Chromatiaceae includes

Chromatium, Thiocystis, Thiosarcina, and Thiospirillum, anaerobes which carry out photosynthesis in the presence of hydrogen sulphide, storing elemental sulphur in globules within the cell. One genus, Ectothiorhodospira, stores its sulphur globules outside the cell. Sulphate is the ultimate oxidation product of the Chromatiaceae. In the family Chlorobiaceae, elemental sulphur is always deposited outside the cell. These genera are strict anaerobes which are capable of photolithotrophic assimilation of carbon dioxide in the presence of sulphide or sulphur which are then photooxidized to sulphate. Genera include Chlorobium, Prosthecochloris, and Pelydictyon (Pfennig and Truper, 1974).

The process by which nutrients enter the cell involves active transport across an impermeable membrane barrier. A discussion of these mechanisms in several different biological systems follows.

II. Active Transport

i. Introduction

The process by which cells remove nutrients from their external environment, move them across the cytoplasmic membrane, and accumulate these nutrients against a

concentration gradient is known as active transport. A carrier protein(s) is involved and the cell must expend metabolic energy in the process (Moat, 1979). It is in this manner that sugars, amino acids, and ions are taken up by cells. The energy for active transport is derived from the coupling of oxidation to phosphorylation and is explained in the chemiosmotic hypothesis of Mitchell (1961). He states that metabolic energy is conserved at the level of the membrane as an electrochemical gradient of hydrogen ions. According to Mitchell, as electrons are transferred along the respiratory chain, protons and hydroxyl ions are separated on opposite sides of the membrane. This results in a chemical gradient of hydrogen ions (difference in pH) as well as a difference in electrical potential across the membrane. The chemiosmotic hypothesis explains phosphorylation as the uptake of extruded protons across the membrane via the ATPase complex, a soluble protein which passes through the membrane. Protons translocated through the ATPase provide the chemical energy to synthesize ATP from ADP and inorganic phosphate, resulting in the conservation of the energy derived from respiration.

ii. Transport Systems

The most well characterized microbial transport systems

are those for sugars (Silhavy et al., 1978; Postma, 1977) and amino acids (Anraku, 1978; Booth and Hamilton, 1980). Far less attention has been given to the study of ion transport.

Potassium transport has been well characterized in Escherichia coli (Rosen, 1986), Streptococcus (Harold and Altendorf, 1974; Kashket and Barker, 1977), and Rhodopseudomonas capsulata (Jasper, 1978). Iron transport mechanisms in a number of microbes have been reviewed by Neilands (1974; 1980). Like potassium, the mechanisms of iron uptake are well characterized and the genetic regulation of the systems is underway. Transport of magnesium, the major intracellular divalent cation in all living cells, has been studied in E. coli (Silver and Clark, 1971; Park et al., 1976) and Bacillus subtilis (Scribner et al., 1977). Uptake of calcium, which accumulates in high concentrations during sporulation in Bacillus, was reviewed by Silver (1977). Manganese transport systems in Bacillus subtilis, Staphylococcus aureus, Rhodopseudomonas capsulata, and E. coli were reviewed by Silver (1978). Since then, Archibald and Duong (1984) showed the presence of a high affinity manganese transport in Lactobacillus plantaraum. Sodium, potassium, chloride, and arsenate transport were the subject of a recent review by Rosen (1986). Ion transport (zinc, cobalt, ammonium, nitrate,

nitrite) in other microbial systems has been reviewed by Silver (1975).

III. Sulphate Transport

Sulphate transport has been studied in a number of organisms ranging from higher plants to cyanobacteria. A brief review of sulphate (and related sulphur compounds) transport in these systems follows.

1. Higher Plants

Sulphate uptake has been studied in intact plants (Nissen, 1973), plant parts (Higinbotham et al., 1967; Holmern et al., 1974; Shargool and Ngo, 1975) and plant cells (Hart and Filner, 1969; Smith, 1975; 1976).

Smith (1975a; 1975b; 1976) has shown that sulphate transport in cultured tobacco cells obeys Michaelis-Menten kinetics (K_m varied between 20 and 40 uM), has a sharp pH optimum (6.5 to 7.5), and is regulated by the intracellular sulphate pool. Sulphate uptake is strongly inhibited by sulphur-containing anions (sulphite, thiosulphite, metabisulphite) and by structural analogues (selenate and chromate). Selenate exhibits competitive inhibition (K_i =

13 uM) while sulphide is a noncompetitive inhibitor. Sulphate uptake is inhibited by respiratory poisons (cyanide and azide), uncouplers (CCCP and DNP) and the ATPase inhibitor DCCD, indicating that energy is required. Sulphydryl reagents inhibit uptake by greater than 90%. Efflux of sulphur-containing metabolites from tobacco cells is linear in the presence of CCCP and the rate of efflux is dependent on the concentration of CCCP.

Vange et al. (1974) and Holmern et al. (1974) have demonstrated that the uptake of sulphate by barley roots is multiphasic. Although phosphate is transported by a different system, the concentration of phosphate as well as pH and sulphate concentration cause shifts in uptake phases. The group VI anions (sulphite, thiosulphate, chromate, molybdate, selenate, and tungstate) are competitive inhibitors which also cause shifts in uptake phases. Calcium or other divalent cations are required for the normal functioning of the uptake site.

ii. Algae

Nearly two decades of study in the laboratory of Jerome Schiff have resulted in the elucidation of the metabolic pathway of sulphate reduction to cysteine formation in the

pathway of sulphate reduction to cysteine formation in the green alga Chlorella (Schiff, 1959; 1964; Hodson et al., 1968a; 1968b; 1971; Hodson and Schiff, 1971a; 1971b; Levinthal and Schiff, 1968; Abrams and Schiff, 1973). Two pathways for sulphate reduction exist in Chlorella and appear to use adenosine 5'-phosphosulphate (APS) rather than adenosine-3'-phosphate-5'-phosphosulphate (PAPS) as do other assimilatory sulphate reducers. In both pathways sulphate is translocated across the membrane and activated to form adenosine 5'-phosphosulphate (APS); in one pathway, the sulpho group is then transferred via APS sulphotransferase to a carrier. A ferrodoxin-dependent thiosulphonate reductase acts on the carrier to yield a bound thiol group which is transferred to O-acetyl-serine to form cysteine. The second pathway uses sulphite reductase and free intermediates to transfer the sulpho group to O-acetyl-serine.

Vallee and Jeanjean (1968a; 1968b) turned their attention to the kinetics of sulphate transport in Chlorella. Uptake of sulphate is an active process, with a K_m of 1.2 uM and is enhanced by sulphur starvation. Group VI anions (selenate, chromate, tungstate, and molybdate) inhibit sulphate uptake; chromate is a competitive inhibitor with a K_i of 0.4 uM. Methionine and cysteine do not repress the uptake of sulphate unless the cells are sulphur-starved.

In contrast to tobacco cells (Smith, 1976), intracellular sulphate does not exchange with sulphate in the medium.

The unicellular red alga, Porphrydium aerueineum, secretes a capsular polysaccharide rich in sulphate (Ramus and Groves, 1972; 1974; Ramus, 1974). This alga grows well on sulphate (K_m for sulphate transport is 2.5 uM) and thiosulphate but does not use organic sulphur compounds. Molybdate ion is a competitive inhibitor and acts by inhibiting the formation of adenosine-5'-phosphosulphate (APS), which in turn cannot form PAPS, the activated sulphate donor, resulting in an inability to transfer sulphate to the polysaccharide.

iii. Cyanobacteria

Utkilen et al. (1976) were the first to study sulphate transport in a cyanobacterium, Anacystis nidulans. This organism shows many of the same characteristics of sulphate uptake seen in other systems. Sulphur starvation enhances uptake, indicating derepression of a sulphate permease. The K_m for sulphate transport is 0.75 uM, with a V_{max} of 0.7 pmol/min/10^6 cells. Transport is temperature- and pH-dependent and thiosulphate and sulphite are competitive inhibitors. Uptake of sulphate does not occur in the dark, even under starvation conditions, suggesting that uptake is

energy-dependent.

Jeanjean and Broda (1977) found that sulphate uptake in A. nidulans is sensitive to CCCP and DCCD as well as darkness. Osmotic shock reduces sulphate uptake suggesting that a periplasmic binding protein is involved in transport. Chromate and selenate ions are inhibitory. In the presence of chloramphenicol, sulphate uptake by sulphur-starved cells is greatly reduced. However, the negative membrane potential increases during sulphate starvation even in the presence of chloramphenicol, indicating that starvation leads to an increase in ATPase activity in this organism.

iv. Fungi

The sulphate transport systems of filamentous fungi have been well studied. Penicillium and Aspergillus (Segel and Johnson, 1961; Yamamoto and Segel, 1966; Tweedie and Segel, 1969) have a single sulphate transport system which is temperature-, pH-, energy-, and concentration-dependent. Thiosulphate, molybdate, and selenate are transported by this permease. The mycelia possess distinct permeases for sulphite and tetrathionate. Equimolar concentrations of L-cysteine and L-cystine suppress sulphate uptake by 65 to 80%. Although sulphate transport is energy-dependent, sulphate-binding to the mycelia is energy- and temperature-insensitive.

Bradfield et al. (1970) have shown that sulphate transport in these same fungi is unidirectional, with no exchange occurring between the intracellular and extracellular sulphate. L-methionine is a metabolic repressor of the sulphate permease, while intracellular sulphate and L-cysteine are feedback inhibitors. The K_m ranges from 60 to 600 uM with a V_{max} of 5 umol/g/min.

Studies with ATP sulphurylase-negative mutants of Penicillium notatum (Cuppoletti and Segel, 1975) have shown that sulphate uptake is promoted by hydrogen and divalent metal cations. Initial velocity studies show that H^+ and Ca^{2+} add to the carrier before the addition of sulphate. While Ca^{2+} and SO_4^{2-} have a 1:1 stoichiometry, the amount of calcium accumulated by the mycelia is only 23% of the sulphate accumulated. This indicates that while calcium plays a role in the translocation of sulphate, it is not used for internal charge balance within the cell. Most of the translocated calcium returns with the unloaded carrier to the external side of the membrane. The authors have suggested that the carrier operates as an anion exchanger, with sulphate translocated inward in exchange for OH^- or HPO_4^{2-}.

George Marzluf has contributed most of the present knowledge on sulphate transport and regulation in Neurospora crassa. Two distinct sulphate permeases, coded for by

separate genetic loci, are present in N. crassa (Marzluf, 1970a; 1970b). Permease I exhibits low affinity (K_m = 0.2 mM) and is present only in the conidia, while a high affinity form, permease II (K_m = 8 uM), predominates in the mycelial stage. Both systems are energy- and temperature-dependent.

Methionine exerts negative control on the sulphate transport systems by repressing a series of enzymes in the sulphate pathway (aryl sulphatase, choline sulphatase, and choline-O-sulphate permease) as well as the sulphate permease. Positive control occurs via a product of the cys-3 locus which is necessary to induce this same family of enzymes (Metzenberg and Parson, 1966; Marzluf and Metzenberg, 1968).

Roberts and Marzluf (1971) showed that chromate is a competitive inhibitor of sulphate (K_i = 30 uM) and is transported by the sulphate permeases in N. crassa. Other group VI anions (sulphite, thiosulphate, tungstate, and molybdate) are inhibitory to these systems. Only permease II is repressed by sulphate. Both permeases are regulated by feedback inhibition, probably by APS, PAPS, or sulphite (McGuire and Marzluf, 1974).

Unlike other fungi, efflux of sulphate from the intracellular pool does occur and is through the sulphate permease in N. crassa (Marzluf, 1974). Efflux occurs via an

exchange reaction requiring external sulphate, chromate, selenate, or thiosulphate; methionine or cysteine do not promote efflux. The sulphydryl inhibitor p-chloromercuribenzoate inhibits both influx and efflux, suggesting that an essential sulphydryl group is part of the permease.

Active sulphate transport was first described in the unicellular yeast Saccharomyces cerevisiae by McCready and Din (1974). They reported one system which is energy-, concentration-, and temperature-dependent and inhibited by structural analogues. In a more detailed study, two sulphate transport systems were identified (Breton and Surdin-Kerjan, 1977). The high affinity permease has a K_m of 4 uM with a V_{max} of 7 nmol/min/mg dry weight while the K_m of the low affinity permease is 0.35 mM with a V_{max} of 1.5 nmol/min/mg dry weight. Synthesis of the permeases is coordinated with synthesis of the four enzymes required for methionine biosnythesis and is under the same genetic regulation.

v. Escherichia coli

The first reports of sulphur metabolism in a heterotrophic bacterium (E. coli) appeared in the 1950's and were the subject of a review by Roberts et al. (1955). More

than 95% of the sulphur in this organism is found as either methionine or cyst(e)ine. The methionine exists only in proteins while the half of the cyst(e)ine is in proteins and half is in the storage tripeptide glutathione. Optimal growth and cellular yields are seen with sulphate, sulphite, sulphide, L-cystine, and lanthionine, while cysteine and glutathione are toxic. Sulphate uptake is inhibited by the same sulphur compounds which allow growth; however, L-cystine transport is not affected by any sulphur compound. When cells pregrown on sulphate are supplied with L-cysteine, the incorporation of radiolabelled sulphate ceases immediately. During periods of sulphur limitation, the cells use glutathione to supply sulphur for protein synthesis. In sulphur-limited cells which are supplied with sulphate, growth is immediate and rapid, indicating that protein synthesis is not dependent on the glutathione pool.

Ellis (1964) found that L-cysteine, the end product of the sulphate reduction pathway, represses the synthesis of the enzymes necessary to reduce sulphate to sulphide. However, the immediate cessation of sulphate transport when L-cysteine is added cannot be accounted for simply by repressive control, and led him to postulate that L-cysteine may be a specific allosteric inhibitor of the sulphate transport system.

Further study showed that the sulphate uptake system in

E. coli is temperature-, pH-, concentration-, and energy-dependent and is inhibited by its structural analogue selenate (Springer and Huber, 1972).

In the late 1970's, Schiff turned his attention from sulphate metabolism in Chlorella to E. coli. Tsang and Schiff (1976) showed that the pathway for sulphate reduction in E. coli , like that of Chlorella, involves bound intermediates. In E. coli, the pathway begins with PAPS rather than APS. The sulpho group of PAPS is transferred via PAPS sulphotransferase to thioredoxin rather than ferrodoxin as in Chlorella (Schmidt et al., 1974). Thiosulphonate reductase further reduces the sulpho group, resulting in a transfer of the thiol group to O-acetyl serine to form cysteine. Further work with mutants lacking thioredoxin activity but able to grow with sulphate as a sole sulphur source has shown that a glutaredoxin is present which acts an alternate cofactor for PAPS reductase (Tsang and Schiff, 1978; Tsang, 1981).

Leive and Davis (1965a,b) described two cystine transport system in E. coli, one which cotransports cystine and diaminopimelic acid and another which is cystine-specific. Heppel and Berger (1972) showed that the common transport system is sensitive to osmotic shock and has a K_m of 0.3 uM for cystine with a K_i of diaminopimelic acid for cystine of 14 uM. The K_m for the cystine-specific transport system is

0.02 uM and is inhibited by selenocysteine.

The kinetics of the transport of sulphate and its structural analogues, selenate and selenite have been studied (Lindblow-Kull et al., 1985). E. coli has a single transport system for the three anions which obeys Michaelis-Menten kinetics. The specificity and the affinity of the transporter are greater for sulphate than for either selenium compound. All three are competitive inhibitors of one another. The kinetic parameters are as follows; the K_m for sulphate is 2.1 uM , for selenate is 17.1 uM, and for selenite is 102.0 uM. The V_{max} values are 0.99, 0.76, and 6.81 umol/min/g cells, respectively.

vi. Salmonella typhimurium

Using a series of mutants, Dreyfuss and Monty (1963) assigned functions to the genes of the cysteine biosynthetic pathway. The first steps in the pathway require a sulphate permease, two activating steps, and two reductions to reduce organic sulphate to sulphide. CysA mutants show a loss of sulphate permease activity. Mutants in cysB lack reductases for PAPS and sulphite. The other loci code for structural genes: cysC for APS kinase; cysD for ATP sulphurylase; cysE for serine transacetylase; cysH

for PAPS reductase; and cysG, cysI, cysJ for sulphite reductase.

Using sulphate transport mutants, Ohta et al. (1971) have determined that three complementation groups exist at the cysA locus. Although cysA mutants cannot transport sulphate, the cysA gene does not code for the binder. Mutants in cysB have neither binding nor uptake activities as well as lower levels of sulphite reductase activity, indicating a regulatory role for this gene.

Leinweber and Monty (1963) showed that thiosulphate is reductively cleaved to yield sulphite and sulphide in S. typhimurium. Sulphide is an obligate intermediate in the assimilation of thiosulphate. This pathway is controlled by the cysB locus. The system is repressible by cysteine, while sulphide is a feedback inhibitor. Once the thiosulphate ion is degraded to sulphite and sulphide, the pathway converges with that for assimilation of sulphate into cysteine.

Dreyfuss (1964) characterized the sulphate- and thiosulphate-transporting systems in S. typhimurium. As in other systems, sulphate uptake is temperature- and energy-dependent and subject to repression by growth on cysteine. Mutations in the cysA gene render the cells unable to transport sulphate or thiosulphate, but growth occurs normally with sulphide, sulphite, or cysteine. Mutants

blocked in the cysC and cysD genes accumulate sulphate but are unable to metabolize intracellular sulphate. Although cysteine represses formation of the sulphate transport system, it does not inhibit transport. Sulphite and thiosulphate are inhibitors of sulphate transport, sulphide is not. The K_m of sulphate uptake is 36 uM with a V_{max} of 6 umol/min/g cell protein. While most other systems show linear uptake of sulphate for several minutes, S. typhimurium has an unusual "overshoot" mechanism. Sulphate uptake is linear for 1 min, followed by a rapid loss of 80% of the accumulated sulphate. This phenomenon is seen when the intracellular sulphate concentration reaches 0.1 mM (Dreyfuss and Pardee, 1966).

A periplasmic sulphate-binding protein from S. typhimurium was purified and crystallized (Pardee, 1966; 1967; Pardee et al., 1966). The 32,000 molecular weight protein is a single polypeptide composed of the typical amino acids, but is unusual in that it lacks sulphur-containing amino acids. About 10,000 copies are present per cell. Thiosulphate does not inhibit sulphate binding but rather affects sulphate uptake. Sulphite, sulphide, group VI anions, and cysteine repress both binding and transport activities.

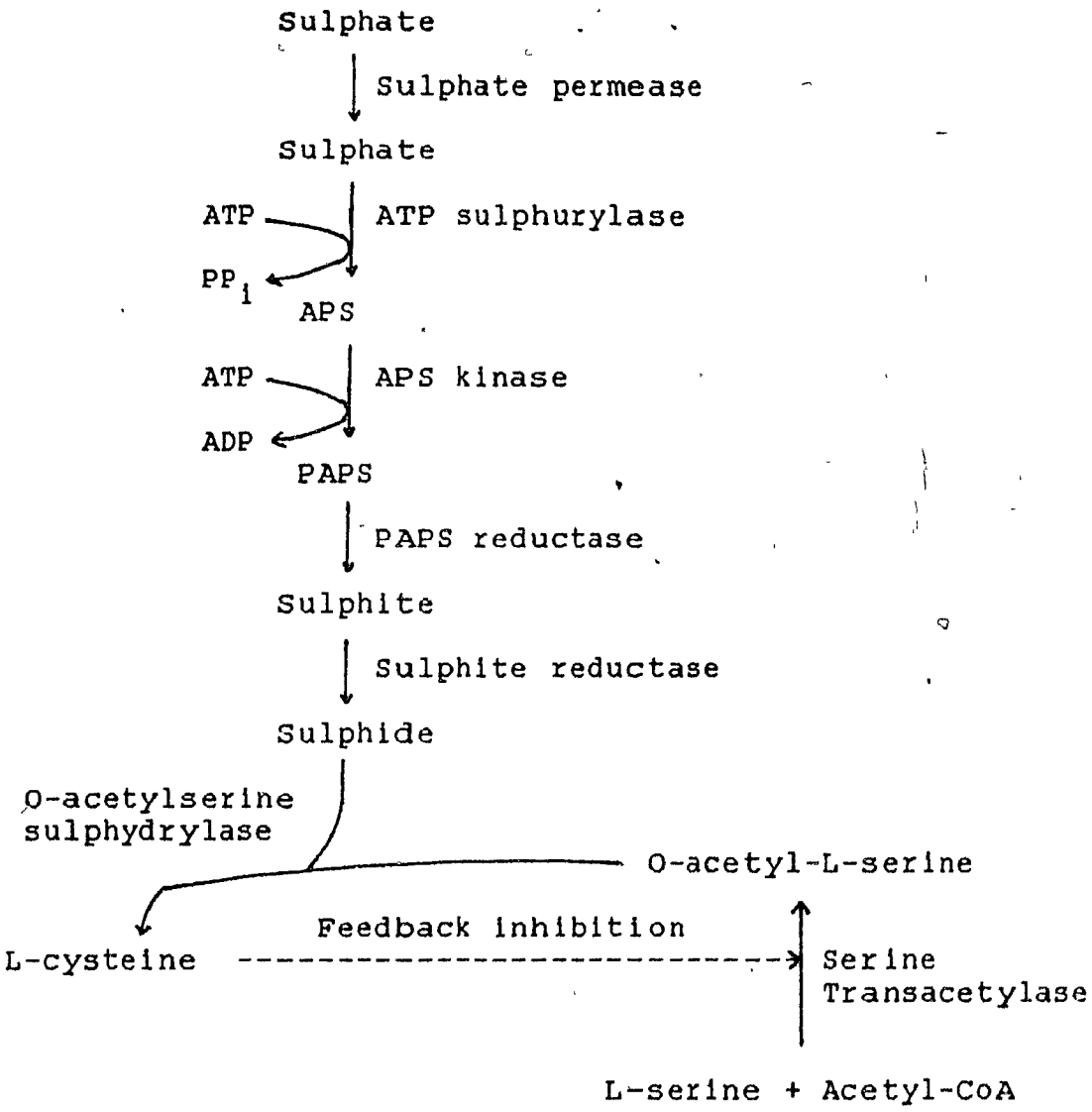
Kredich (1971) showed that under conditions of sulphur starvation, O-acetylserine, a direct precursor of

cysteine, derepresses the sulphate permease, thereby acting as an internal inducer of the cysteine biosynthetic enzymes. A pathway for cysteine biosynthesis in S. typhimurium has been proposed (Figure 2).

Cysteine synthetase, a 309,000 molecular weight multifunctional protein complex has been isolated from S. typhimurium (Kredich et al., 1977). This complex is composed of one molecule of serine transacetylase and two molecules of O-acetylserine sulphydrylase. Concentrations of O-acetylserine ranging from 0.1 to 1 mM cause the complex to dissociate into its components.

Two systems for L-cystine transport exist in S. typhimurium (Baptist and Kredich, 1977). Most of the transport activity is accounted for by CTS-1, which has a K_m of 2.0 uM and a V_{max} of 9.5 nmol/min/mg protein. CTS-2 has a much greater affinity for L-cystine, with a K_m of 0.05 uM and a V_{max} of 0.22 nmol/min/mg protein. CTS-1 is shock-sensitive, indicating that a periplasmic binding protein may be involved in L-cystine transport. Like other enzymes of the cysteine biosynthetic pathway (Kredich, 1971), the expression of CTS-1 is affected by the sulphur source used for growth. O-acetylserine and a gene product of the cysB locus control CTS-1. These same factors regulate the cysteine biosynthetic pathway, leading these authors to propose that both cysteine synthesis and CTS-1 are

Figure 2. Assimilatory reduction of sulphate to form L-cysteine in Salmonella typhimurium, showing L-cysteine as a feedback inhibitor of serine transacetlyase (taken from Kredich, 1971).


Sulphate
Sulphate permease
Sulphate
ATP
ATP sulphurylase
PPi
APS
ATP
APS kinase
ADP
PAPS
PAPS reductase
Sulphite
Sulphite reductase
Sulphide
O-acetylserine sulphydrylase
O-acetyl-L-serine
Feedback inhibition
L-cysteine
Serine Transacetylase
L-serine + Acetyl-CoA

controlled by the same regulon. In contrast to the sulphate permease in E. coli which transports sulphate, selenate, and selenite (Lindblow-Kull et al., 1985) the sulphate transport system in S. typhimurium transports sulphate and selenate but not selenite (Brown and Shrift, 1980). Selenite uptake occurs in both wild-type cells repressed for sulphate transport, in mutants lacking a functional sulphate permease, and in wild-type cells grown on cystine; selenate transport does not. Selenite uptake requires energy and is sensitive to sulphydryl reagents. Transport of this ion is biphasic, with an apparent K_m of 37.8 uM between 10 and 50 uM selenite and an apparent K_m of 2.87 mM at higher concentrations. The K_m and V_{max} for sulphate uptake in this strain (K_m = 0.474 mM, V_{max} = 2.994 umol/min/g) differ somewhat from those reported for S. typhimurium by Dreyfuss (1964). In contrast to E. coli (Lindblow-Kull et al., 1985), selenate is not a competitive inhibitor of sulphate in S. typhimurium.

vii. Marine Bacteria

The assimilatory sulphur metabolism in two marine bacteria was compared by Cuhel and coworkers (1981a; 1981b). Sulphate transport is enhanced after a period of sulphur starvation in Pseudomonas halodurans (K_m = 214 uM, V_{max} =

108 pmol/min/10^9 cells) and Alteromonas luteo-violaceous (K_m = 186 uM, V_{max} = 146 pmol/min/10^9 cells). Thiosulphate transport does occur in P. halodurans (K_m = 14.7 uM, V_{max} = 30.5 pmol/min/10^9 cells) but not in A. luteo-violaceous. Methionine, cystine, and glutathione are poor sulphur sources for P. halodurans but are excellent for growth of A. luteo-violaceous. Transport of sulphate (and thiosulphate in P. halodurans) is inhibited by cyanide, azide, DCCD, and CCCP. Uptake in both isolates is inhibited by the group VI oxyanions. In P. halodurans, sulphate and thiosulphate are competitive inhibitors of each other. Unlike other systems (Roberts et al., 1955; Dreyfuss, 1964), growth on organic sulphur sources stimulated uptake in P. halodurans, eliminating cysteine as a feedback inhibitor of the pathway. Another low molecular weight compound must be responsible for regulation as cells grown on sulphate and thiosulphate show low initial transport rates. The authors have suggested that APS or PAPS plays this role. With A. luteo-violaceous, transport is nonlinear when initial rates are greater than growth demands for sulphur. The rapid inhibition of sulphate uptake in the presence of cysteine or glutathione suggests that feedback inhibition by cysteine regulates sulphate transport.

Further studies by Cuhel et al. (1982a; 1982b) showed that virtually all sulphur metabolism in A. luteo-violaceous

and P. halodurans is directed to the production and utilization of protein precursors. Since sulphate is essentially the only sulphur source in the marine environment, they have proposed that sulphate incorporation into protein be used as a measure of marine bacterial growth.

vii. Other Heterotrophic Bacteria

A few other bacteria have been the subject of isolated reports of sulphur metabolism. A summary of these follows.

Studies by Schook and Berk (1978a; 1978b) have shown that Pseudomonas aeruginosa is able to use a variety of inorganic sulphur sources for growth, including sulphate, sulphite, sulphide, thiosulphate, metabisulphite, tetrathionate, and dithionite. Dithionate, while not toxic to the cells, is unable to support growth. Thiosulphate and metabisulphite oxidases are present in both the soluble and particulate fractions. Rhodanese (thiosulphate sulphur transferase) is constitutively present and is found only in the soluble fraction.

Reversible, pH driven sulphate transport occurs in membrane vesicles of Paracoccus denitrificans (Burnell et al., 1975). Selenate decreases the rate of sulphate uptake as well as loss of sulphate. Artificially formed pH

gradients induce low levels of sulphate uptake with both inside-out and right-side-out vesicles. The authors have postulated that electroneutral symport of $2H^+$ and SO_4^{2-}, driven by pH, is the mechanism by which sulphate enters the cell.

Enterobacter aerogenes possesses a sulphatase, a single protein of 40,700 molecular weight (Rammler et al., 1964; Fowler and Rammler, 1964). In sulphate-limiting media, the concentration of the sulphatase is 600 to 700 times greater than is seen with sufficient sulphate. Thiosulphate, sulphite, and cysteine also repress sulphatase formation, but growth on methionine and its analogues permits its synthesis.

A brief note by De Issaly and Stoppani (1963) on the sulphur metabolism in Pasteurella multocida concluded that sulphate is not used by this organism for amino acid synthesis. Cells grown in sulphide, thiosulphate, or sulphite incorporate the sulphur from these compounds into cysteine, cystathionine, homocysteine, glutathione, and related amino acids; radiolabelled sulphate is not found in any of the amino acids, but rather remains as sulphate.

A comparison of sulphate transport in E. coli and Bacillus subtilis was made by Pasternak (1962). He found APS and PAPS present in both organisms. Sulphate uptake is inhibited by cystine in E. coli, while glutathione and

cysteine decrease sulphate transport in B. subtilis. This inhibition occurs because the enzyme catalyzing the synthesis of PAPS (APS kinase) is repressed by cystine in E. coli and by cyst(e)ine and glutathione in B. subtilis.

Villarejo and Westley (1966) found that sulphate, thiosulphate, or cystine support growth of Bacillus subtilis, while sulphide and cysteine are toxic. The optimum concentration of sulphur for growth is 200 uM, with 10 uM allowing growth with a greatly reduced yield. At 25 uM, sulphide is far less toxic and is a better sulphur source than thiosulphate. This may reflect a regulatory mechanism which favors the use of the most highly reduced sulphur source present. Unlike E. coli, B. subtilis contains rhodanese which can reduce thiosulphate to the oxidation level of sulphide and may account for differences in their metabolism of sulphate.

Although the thiobacilli commonly use thiocyanate as a carbon and energy source (Happold et al., 1954; Katayama and Kuraishi, 1978), thiocyanate utilization in other bacteria is rare. Sources of thiocyanate include the milk, saliva, and urine of mammals and decomposing plant tissue (Wood, 1975). The heterotrophic bacteria which have been shown to degrade thiocyanate are soil isolates. Two pseudomonads which utilize thiocyanate have been reported (Putilina, 1961; Stafford and Callely, 1969). Betts et al. (1979) have

isolated an Arthrobacter species which uses thiocyanate as sources of both nitrogen and sulphur. Usually toxic at much lower levels, 0.1 M thiocyanate causes an increase in lag time but has no effect on cell doubling. These thiocyanate-utilizing bacteria serve as reminders that an organism which is well adapted to its environment has the best chance for survival.

IV. Meningococcal Disease

i. The Bacterium

Neisseria meningitidis is a Gram-negative, nonmotile, nonflagellated diplococcus, 0.6 to 1 uM in diameter (Reyn, 1974). The only natural host of the meningococcus is man, where it resides in the mucous membranes (DeVoe, 1982). Nine serogroups have been identified (A, B, C, D, X, Y, Z, W135, and 29E) based on immunospecifity of capsular polysaccharides (Craven and Frasch, 1979; and Craven et al., 1979). Further subdivision into serotypes is based on immunologically distinct outer membrane proteins and lipopolysaccharides (Frasch, 1979). The serogroup B, serotype 2 meningococcus has been responsible for most of the meningococcal disease outbreaks in the past few years (DeVoe, 1982).

ii. Epidemiology and Immunity

The organism is harbored asymptomatically by 5 to 35% of the healthy population (Griffiss and Artenstein, 1976), while 100% may be carriers during epidemic periods. In high density populations, such as military recruits, the carriage rate may exceed 90% while the disease rate remains less than

1% (Goldschneider et al., 1969a). The meningococcus is disseminated from the nasopharynx through the generation of aerosols (Artenstein et al., 1968).

Children between the ages of 6 months and 2 years are most susceptible to meningococcal disease. The susceptibility increases during the first 6 months of life and correlates with the decrease in passively acquired immunoglobulins (Smith, 1954). Between 6 and 24 months, serum bactericidal activity is at its lowest (Goldschneider et al., 1969), making this group highly susceptible to meningococcal infection. As serum bactericidal activity increases over the age of 2 years, there is a progressive reduction in the incidence of disease (Frasch, 1977).

Within 7 to 10 days after colonization of the oropharynx, antibodies of the IgA, IgM, and IgG classes are elicited by the host (Goldschneider et al., 1969b). These antibodies are formed as a result of either oropharyngeal carriage or infection (Reller et al., 1973), with natural immunity occurring as exposure and carriage continue throughout life (DeVoe, 1982).

iii. The Disease

When host defence mechanisms are overcome, the meningococcus gains entry to the systemic circulation,

manifesting itself in one of several forms of the disease. The most common form is meningococcemia resulting when bacteria enter the blood stream after colonization of the oropharynx. Meningococci may then colonize organs, joints, and tissues during this phase of bacteremia, leading to chronic meningococccemia (Herrick, 1919). In the acute phase, death has been known to occur within hours of the onset of clinical symptoms. In some cases, the bacteremia develops into fulminant meningococcemia or fulminant encephalitis, characterized by hemorrhagic skin lesions (Hill and Kinney, 1947), disseminated intravascular coagulation (Minna et al., 1974), circulatory failure and coma (May, 1960).

Sulphonamides were the drug of choice for treatment of meningococcal disease until sulphadiazine resistance appeared in the 1960's (Artenstein, 1975). While erythromycin and chloramphenicol are usually effective, penicillin and rifampin are most widely recommended for treatment of meningococcal disease. In the absence of treatment, mortality rates may reach 90% while antibiotic therapy reduces mortality to 10 to 15% (CDC, 1981).

iv. Virulence Factors

In order for a pathogen to invade, colonize, and

multiply within the host, it must first overcome the natural host defence mechanisms (Smith, 1977). Factors contributing to virulence may be extracellular, such as toxins or enzymes, or may be surface components of the microbe, such as pili, fimbriae, capsules, or envelopes (Pelczar et al., 1986). The meningococcus has evolved a combination of these virulence factors, permitting it to continue to be a serious human pathogen despite antibiotic therapy.

Colonization of the mucosal surface is a prerequisite to infection. DeVoe and Gilchrist (1975) found that N. meningitidis isolated from the nasopharynx of carriers or from the cerebrospinal fluid of patients with acute disease were piliated while only a small percentage of laboratory strains exhibited pili, indicating the importance of pili for in vivo attachment (DeVoe and Gilchrist, 1974). Stephens and McGee (1981) found greater amounts of surface polysaccharide in disease isolates than in nasopharyngeal strains of meningococci. The loss of polysaccharide biosynthetic capability of mutants of group B meningococcus was directly correlated with a dramatic loss of virulence in mice (Masson et al., 1982). These studies demonstrate the importance of capsules in the production of meningococcal disease.

N. menigitidis produces large numbers of cell wall blebs which release endotoxin to the surroundings during normal in

vitro growth, (DeVoe and Gilchrist, 1973). It is well established that endotoxin plays a role in the pathogenesis of Gram-negative bacterial diseases (Russell, 1976). Meningococci are phagocytized, killed, degraded by human polymorphonuclear leukocytes, PMNs, (DeVoe et al., 1973) and egested from intact PMNs by a specific exocytosis mechanism (DeVoe, 1976). Using a rabbit model, DeVoe and Gilka (1976) showed that small quantities of the leukocyte-egested material acted synergistically with endotoxin or endotoxin-containing cell wall blebs, leading to disseminated intravascular coagulation and hemorrhage. This pathology is the hallmark of fulminant meningococcal disease in man (Vik-Mo et al., 1978).

Nutritional immunity, the ability of a host to sequester vital nutrients from a parasite, is an important factor in preventing infection (Weinberg, 1978). Iron is essential for growth of most organisms and they have evolved various mechanisms to acquire host iron (Neilands, 1980). The meningococcus is able to remove iron from transferrin, a human serum glycoprotein which functions to scavenge free iron, making it unavailable to invading microbes (Archibald and DeVoe, 1978). Under iron-limited conditions, N. meningitidis expresses a high-affinity mechanism for removing iron from transferrin, a phenomenon not seen in the nonpathogenic neisseriae (Simonson et al., 1982).

Brener et al. (1981) found that meningococci grown at low pH under iron-limitation, conditions characteristic of an inflammatory response, showed an 1,150-fold increase in virulence for the mouse. The ability of this pathogen to adapt to potentially adverse environmental conditions presented by the host and use these to its advantage contributes to its frightening success.

V. Sulphur Nutrition and Metabolism in the Neisseria

Although sulphur is a major nutrient, a paucity of reports exists concerning sulphur metabolism in the neisseriae. Using a chemically defined medium, Catlin (1973) determined the nutritional profiles of 146 strains of N. meningitidis, N. gonorrhoeae, and N. lactamica as a basis for differentiation and identification. The only information relating to sulphur and neisseriae from this study is the following: (1) all gonococci have an absolute requirement for cysteine, (2) cyst(e)ine is required by some meningococci; glutathione, but not methionine, can substitute for cyst(e)ine in the medium, (3) some strains of N. lactamica need cysteine to grow, some do not, and (4) sulphadiazine resistance in gonococci is related to a genetic defect which renders the cells unable to synthesize methionine.

Holbein (1981) found that the addition of normal human serum to a chemically defined medium stimulates the growth of N. meningitidis. He found that the major growth-limiting component in human serum is cysteine, and speculated that cysteine may be an important factor for growth during the bacteremic phase which occurs in most forms of meningococcal infection.

Although its function is not understood (Morse, 1979), Tauber and Russell (1962) have shown the presence of a cysteine oxidase in neisseriae which converts cysteine to cystine. Yu and DeVoe demonstrated that the L-cysteine oxidase in N. meningitidis is specific for cysteine and that product of the reaction is cystine. The electrons from the cysteine are carried along the respiratory chain and reduce the major cytochromes. Using inhibitors of the electron transport chain, an alternative cysteine oxidase activity is observed which, unlike the major cysteine oxidase, is insensitive to azide. If the flow of electrons from cysteine along the respiratory chain is inhibited, electrons are shunted to this alternative oxidase. These authors have speculated that the oxidation of cysteine may be involved in translocation across the membrane or may be a mechanism of translocating protons from the cytoplasm to the exterior of the cell, thereby increasing the proton motive force, and driving oxidative phosphorylation.

Le Faou has made the first major attempt to look at sulphur metabolism in the neisseriae. He found that the cysteine requirement of some strains of N. gonorrhoeae can be met by thiosulphate. These strains possess thiosulphate reductase, rhodanese, and tetrathionate and trithionate reductase activities (1981; 1983). Rhodanese (thiosulphate sulphur transferase) is a multifunctional enzyme which exhibits thiosulphate reductase activity (Westley, 1973). The presence of rhodanese and thiosulphate reductase explains the ability of these strains to use thiosulphate as a sole sulphur source. Most N. meningitidis and nonpathogenic neisseriae are able to use sulphate as a sole source of sulphur; those strains which cannot, including all the gonococci, lack sulphite reductase activity (1984).

A recent report suggests that the lipopolysaccharide of the gonococcus is altered when the cells are grown on an organic thiosulphate, sulphocysteine (E.P. Norrod, K. A. Hagstrom, and D. L. Thomsen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D18, p. 75).

VI. Rationale for Thesis Work

There is an increasing awareness that elucidating the mechanisms of bacterial parasitism requires an understanding of the physiology of the parasite. An important facet of this is determining how a parasite acquires vital nutrients from its host. In the case of iron, many such studies have been rewarded with the discovery that the struggle of the parasite to obtain host iron and the ability of the host to sequester it is a critical factor in the course of infection (Weinberg, 1978). The acquisition of the macronutrient sulphur has been studied in a number of eucaryotes and autotrophic bacteria, but little attention has been given to sulphur metabolism in heterotrophic bacteria or pathogens.

Although a variety of reports suggests that iron availability plays a role in gonococcal and meningococcal infections (Payne and Finkelstein, 1975; Holbein et al., 1979), little is known about sulphur acquisition or metabolism by the neisseriae and its potential role in pathogenesis. Therefore, this study of sulphur metabolism in Neisseria meningitidis was undertaken.

VII. Manuscripts and Authorship

Experimental results included in two publications are presented in this thesis. In the paper on thiosulphaate reductase in N. meningitidis (DeVoe, Port, Holbein, and Ingram, 1982), the only experiments which are included in this thesis are those which were done by me, under the supervision of Dr. I. W. DeVoe. The experiments described in the paper on sulphate acquisition by N. meningitidis (Port, DeVoe, and Archibald, 1984) were done solely by me, primarily under the guidance of Dr. Fred Archibald.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism

The serogroup B *Neisseria meningitidis* SD1C used throughout this work was obtained from the *Neisseira* Repository, NAMRU, University of California, Berkeley. Stock cultures were maintained by lyophilization. Working cultures were maintained on Mueller-Hinton (MH: Difco Laboratories, Detroit, MI) agar slants stored at -70°C. *N. meningitidis* SD1C dissociates into rough and smooth colonial forms (DeVoe and Gilchrist, 1978). Only the smooth colonial type was used in these studies.

Media

Unless otherwise noted, the organism was grown in modified *Neisseria* defined medium, MNDM, (Archibald and DeVoe, 1978). The composition of this medium is given in Table 1. When used, the following compounds were added at a concentration of 1 mM (per mole sulphur) unless otherwise noted: Na_2SO_4, Na_2SO_3, $NaHSO_3$, $Na_2S_2O_3$, $Na_2S_2O_4$, $Na_2S_2O_6$, NaSCN, NaSH, L-cysteine, L-cystine, reduced glutathione, oxidized glutathione, L-methionine, mercaptosuccinate, lanthionine, taurine, or carrageenans.

Table 1. Composition of modified Neisseria defined medium.[a]

Component	Final Concentration (mM)
L-glutamic acid	10
D-glucose	10
Uracil	1
L-arginine	1
Tris base[b]	40
NaCl	140
$NaH_2PO_4.H_2O$	1
$CaCl_2.2H_2O$	0.5
NH_4Cl	10
$MgCl_2$	0.2
KCl	2
L-alanine	0.19
L-asparagine	0.21
L-aspartate	0.17
L-glutamine	0.33
glycine	0.21
L-histidine	0.09
L-isoleucine	0.26
L-leucine	0.40
L-lysine	0.26
L-phenylalanine	0.19
L-serine	0.37
L-threonine	0.34
L-tryptophan	0.04
L-tyrosine	0.23
L-valine	0.26
$FeCl_3$.Na-citrate (1:10)	0.05 ug Fe/mL
Sulphur	as appropriate

[a] Original formulation in F.S. Archibald and I.W. DeVoe, 1978. Iron in Neisseria meningitidis: Minimum Requirements, Effects of Limitation, and Characteristics of Uptake. J. Bacteriol. 136:35-48.

[b] Tris hydroxyaminomethane

Cell Growth and Enumeration

Working cultures were thawed and streaked onto Mueller-Hinton agar plates amd incubated for 14 to 16 h at 37°C in 5% CO_2. Cells from these plates were washed in and resuspended in MNDM plus sulphur source. Broth cultures were shaken at 200 rpm in 5% CO_2 at 37°C. Viable and direct counts were determined as previously described (DeVoe and Gilchrist, 1973). Growth was routinely followed turbidimetrically at 600 nm using a Spectronic 20 (Bausch and Lomb, Rochester, NY) spectrophotometer.

Transport Experiments

Unless otherwise noted, uptake studies employed cells grown to late log phase in complete MNDM containing 1 mM sulphur source. Cells were harvested by centrifugation (Sorvall Model RC2-B,) at 4°C , 7,000 x g for 10 min and washed twice in MNDM minus sulphur. These cells were resuspended to an OD_{600} of 0.1 in MNDM containing 1 uM sulphur. Cultures were incubated as before until they reached an OD_{600} of 0.15 to 0.18 (2 to 3 h). These cells were cooled to 4°C, held on ice, and used within 1 h to assay uptake.

Transport activity was usually assayed as follows: 5 mL

of cell suspension was added to a 50 mL flask. Cultures were shaken at 100 rpm in a 37°C water bath shaker (New Brunswick Scientific Co., New Brunswick, NJ) for 5 min prior to adding the appropriate radioisotope (label). Each radioisotope (0.5 to 1 uCi per mL final concentration) was supplemented with its unlabelled form to achieve the desired final concentration (1 to 200 uM). Three radioisotopes were used: $Na_2{}^{35}SO_4$, ^{35}S-L-cysteine, and $Na_2{}^{75}SeO_4$. One-half to one mL samples were withdrawn at intervals from 30 s to 8 h and filtered through Amicon VFM-3 filter cups (0.45 um pore size). The filters were rinsed immediately with 3 mL sulphur-free MNDM (23°C) and placed in scintillation vials. Five mL Formula 947 liquid scintillation cocktail (New England Nuclear, Boston, MA) was added to vials containing either sulphur compound and the ^{35}S present quantitated using a Beckman 8000 liquid scintillation counter. The samples containing radiolabelled selenate were quantitated using a Beckman 8000 gamma radiation counter. Positive controls were done at the beginning and end of each experiment to assure uniformity.

In experiments where competitors or inhibitors were used, the cells were allowed to take up the radioisotope for 5 min, unless otherwise noted, at which time the competitor or inhibitor was added.

In experiments where metabolic poisons were used, all

were dissolved in absolute ethanol except KCN and NaN_3, which were prepared fresh and dissolved in distilled water. An ethanol control was included in each experiment. The cell suspension was shaken at 100 rpm in a 37°C water bath for 5 min prior to adding the metabolic poison. The cells continued shaking for 5 min at which time the radioisotope was added and uptake assayed.

To examine the effect of temperature on sulphate uptake, the cells were allowed to equilibrate at the sampling temperature (21°C to 45°C) for 10 min prior to the addition of radioisotope.

To look at pH effects on sulphate uptake, the sulphur-starved cells were resuspended in MNDM less sulphur at various pH values from 6 to 8. After 10 min the cells were used in the usual transport assay.

In the efflux experiments, cells were exposed to either 10uM $^{35}SO_4^{-2}$ or 10uM ^{35}S-L-cysteine for 5 min at which time unlabelled 10uM L-cysteine or 1mM L-cysteine was added, respectively. Aliquots were removed at 5 min intervals to look for exchange of radioisotope.

Toxicity of Selenate, Molybdate, and Tungstate to Neisseria meningitidis

Mueller-Hinton (MH) plates or MNDM plates were prepared

from broths by adding 1.5% agar. A suspension of N. meningitidis was incubated in MH broth for 4 h and spread on the MH or MNDM agar plates with a sterile swab. Three wells (0.5 cm in diameter) were cut in the agar of each plate. Solutions of Na_2SeO_4, Na_2MoO_4, and $Na_3P[W_3O_{10}]_4$ were each prepared in 4 mM, 200 uM, and 40 uM concentrations. Each well was filled with 100 uL of the concentration of the ion to be tested to give a final concentration of 1 mM, 50 uM, or 10 uM. Plates were incubated for 18-24 h at 37°C, 5% CO_2.

API-Zym® Analytical Strips

Sulphur-starved and sulphur-sufficient cells of N. meningitidis were prepared as described previously. Cells were harvested by centrifugation (4°C) at 7000 x g for 10 min and suspended to a density of 1.5×10^9 cells/mL. Four drops from a sterile Pasteur pipette were added to each well of the API-Zym® test strip (Analytab Products, Plainview,NY).The lids were placed on the tray and trays were incubated at 37°C for 4 h. After incubation, one drop of Reagents A and B was added to each well. Colour was allowed to develop for 5 min and the strip was exposed to bright fluorescent light for 5 min to facilitate negative reactions to become colourless. Each well was then rated

visually for development of colour, with zero corresponding to a negative reaction and five indicating maximum colour development. An approximation of the amount of enzyme present in the inoculum was calculated based on the colour developed.

Effect of Chloramphenicol Treatment on Sulphate Uptake

Sulphur-sufficient cells of N. meningitidis were grown as usual on MNDM agar. The cells were harvested and washed in sulphur-free MNDM broth and suspended to a density of 2 to 4 x 10^8 cells/mL. The cells were divided into three aliquots: Na_2SO_4 was added to one aliquot to achieve a final desired concentration of 1 uM; the second aliquot received Na_2SO_4 (1 uM final concentration) plus 20 ug/mL chloramphenicol; the third aliquot received 1 mM Na_2SO_4 (final concentration) plus 20 ug/mL chloramphenicol. Cultures were shaken at 200 rpm in 5% CO_2 at 37°C. Cells were sampled at 1 and 2 h for ability to transport $Na_2{}^{35}SO_4$, plated on MH agar, and incubated overnight to check viability.

Distribution of ^{35}S in the Meningococcus

To look at the distribution of sulphur in Neisseria

meningitidis, a variation of the method used by Cuhel et al. (1981) was used. Cultures of sulphur-sufficient N. meningitidis were inoculated into MNDM broth containing 100 uM $Na_2{}^{35}SO_4$ and incubated at 200 rpm in 5% CO_2 at 37°C for 4h. Aliquots were removed for processing at 1, 2, 3, and 4h. At 4h, the cells were harvested, washed and resuspended in MNDM broth containing unlabelled 1 uM Na_2SO_4. Incubation was continued for 2h with aliquots removed for processing at 1 and 2h.

The samples were processed as follows to quantitate the form of sulphur in the cells at each time point. Duplicate 1 mL aliquots were centrifuged for 2 min in a microcentrifuge at 12,000 x g. The supernatant fluid was discarded and the cells were suspended and washed in 0.05 M MOPS (morpholinepropanesulphonic acid, Sigma Chemical Co., St. Louis, MO) buffer, pH 7.5. The cell pellet was then resuspended in 0.5 mL 0.05 M MOPS buffer, pH 7.5, and 0.1 mL 1% SDS (sodium dodecyl sulphate) was added and the cells were vortexed briefly. After 15 min incubation at 23°C, 0.6 mL 20% TCA (trichloroacetic acid) was added to each 1.5 mL tube, the contents were mixed briefly, and incubated for 30 min at 4°C. The samples were centrifuged for 2 min (12,000 x g) and the supernatant fluid was removed and saved. The pellet was resuspended in 0.8 mL 10% TCA and centrifuged again for 2 min. This supernatant fluid fraction was

combined with the previous one and saved. The suspended pellet was counted to determine cell breakage. The combined supernatant fluid fractions were treated as follows: 1.75 mL sample was added to 50 uL 5 mM Na_2SO_4 and 250 uL 1M $BaCl_2$, pH 2 and incubated in a microcentrifuge tube for 2h at 4^oC. The samples were centrifuged for 2 min and the supernatant fluids removed with a Pasteur pipette and saved. The pellet was resuspended in 1 M $BaCl_2$, pH 2 and centrifuged for 2 min. The supernatant fluid was removed and added to the first supernatant fluid fraction. This procedure was repeated a third time and all three supernatant fluid fractions were combined. The supernatant fluid fractions constituted the low molecular weight organic sulphur in the cells. The pellet was suspended in water and represented the organic sulphur fraction. Fractions were counted as described previously.

Preparation of Cell Envelope and Cytoplasmic Fractions

Cells were grown on MH plates as described previously, removed with a sterile swab, and suspended in 10 mL broth (MH or MNDM plus sulphur source). This inoculum was added to 1.5 L of the suspension broth (prewarmed to 37^oC) in a 2.8 L Fernbach flask and incubated at 37^oC, 5% CO_2, shaken at 100 rpm until the cell density reached an OD_{600} between

1.0 and 1.2. Cells were harvested by centrifugation at 6000 x g for 10 min at 4°C. Cells from 9 L of broth were suspended in 20 mL of 0.05 M MOPS buffer, pH 7.5 which contained 1 mM $MgCl_2$ and 50 ug/mL DNase. Cells were hydraulically broken using a French pressure cell (American Instrument Co., MD) at 12,000-15,000 psi. Broken cells were centrifuged at 10,000 x g for 10 min at 4°C. The supernatant fluid fraction was removed and ultracentrifuged (Beckman Model L8-70, Ti-65 rotor) at 70,000 x g for 60 min at 4°C. The supernatant fluid fraction was removed, divided into 5 mL aliquots and stored at -70°C. The pellet was resuspended in 20 mL MOPS buffer and centrifuged at 70,000 x g for 60 min at 4°C. The supernatant fluid was removed and discarded. The pellet was resuspended in 5 mL MOPS buffer and stored at -70°C.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the envelope and cytoplasmic fractions was carried out using a modification of the Laemmli(1970) procedure. The following stock solutions were prepared:

1. Monomer solution- 58.4 g acrylamide, 1.6 g Bis, distilled water to 200 mL, stored in dark

2. Running gel buffer- 1.5 M Tris, pH to 8.8 with HCl
3. Stacking gel buffer- 0.5 M Tris, pH to 6.8 with HCl
4. 10% SDS
5. 10% ammonium persulphate
6. Running gel overlay- 0.375 M Tris pH 8.8, 0.1% SDS
7. 2X treatment buffer- 0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol
8. Tank buffer- 0.25 M Tris pH 8.3, 0.192 M glycine, 0.1% SDS

The separating gel was prepared by mixing 20 mL monomer solution, 15 mL running gel buffer, 0.6 mL 10% SDS, and 24.1 mL H_2O. A vacuum was applied for 10 min to deaerate followed by the addition of 300 uL ammonium persulphate and 20 uL TEMED. The solution was carefully pipetted into the slab gel apparatus (Model 600 Vertical Slab Unit, BioRad Laboratories, Richmond,CA), layered with water and allowed to polymerize.

The stacking gel was prepared by mixing 2.66 mL monomer solution, 5.0 mL stacking gel buffer, 0.2 mL SDS,and 12.2 mL H_2O. After deaerating, 100 uL ammonium persulphate and 10 uL TEMED were added. The water was poured off the separating gel and stacking gel solution was added. A comb was inserted into the sandwich and the gel was allowed to harden.

Equal volumes of protein (envelopes, cytoplasmic fractions or standards) were combined with 2X treatment buffer in a microcentrifuge tube and boiled for 90 s. A drop of 1% phenol red was added to each sample to serve as a tracker dye. Samples were held on ice (immediate use) or frozen at -70°C (future use).

Using a 50 uL syringe, samples were underlayered in each well. The upper and lower buffer chambers were filled with tank buffer. A current of 30 mA was applied and electrophoresis was continued until the tracker dye migrated to the bottom of the gel. The gels were stained in a solution of 0.125% Coomassie blue R-250, 50% methanol, and 10% acetic acid for 8 h on a shaker (M. Baines homemade device for staining gels). After destaining in a solution of 50% methanol, 10% acetic acid for 2 h, the gels were placed on Whatman #3 filter paper and placed in a BioRad gel drying apparatus (BioRad Laboratories, Richmond, CA).

Thiosulphate Reductase Assay

N. meningitidis was separated into soluble (cytoplasmic) and particulate (envelope) fractions as described earlier. These cell fractions were assayed for reductase activity (37°C) in the 3 mL chamber of a polarographic oxygen cell (Rank Bros., Boltisham, Cambridge, UK) fitted with gas

ports. Ten uL of 0.1 sodium dithionite (reducing agent) was added to 1.9 mL of the soluble cell fraction 1 min prior to the addition of 10 uL 0.1 M thiosulphate (5 mM, final). Nitrogen gas (N_2) was passed over the reaction at a flow rate of 200 mL/min to a 2 cm diameter sparger in 30 mL 0.1 mM dithionitrobenzoic acid (DTNB) in 0.15 M NaCl, 5 mM K_2HPO_4, pH 6.8. One unit of enzyme activity was defined as the generation of sufficient H_2S to produce an absorbance (412 nm) increase in DTNB of 0.001 O.D. unit/min under the conditions stated above.

RESULTS

RESULTS

I. Sulphur and the Meningococcus

Prior to the beginning of this study, sulphur nutrition in N. meningitidis consisted of the knowledge that while most strains use sulphate for growth, some require cyst(e)ine, and that this requirement can be met by glutathione (Catlin, 1973).

An initial survey was made to ascertain the possible sources of sulphur for the meningococcus. Its minimal sulphur requirements were determined, and the presence of a thiosulphate reductase was confirmed.

i. Sulphur Sources

A chemically defined medium, Neisseria defined medium (NDM), was developed by Archibald and DeVoe (1978) for their studies on iron limitation in the meningococcus. A modification of this medium was made so that it contained no sulphur source which could support growth of N. meningitidis.

The resulting medium, modified Neisseria defined medium (MNDM; Table 1) was used in all of this work. The modifications include substituting chloride salts for

sulphate salts, omitting L-cysteine, and adding a combination of amino acids. The substitution of sulphate salts with chloride salts coupled with the omission of L-cysteine did not allow consistent, reproducible growth of the meningococcus even when a usable sulphur substrate was added. A combination of 15 amino acids was added to the medium. These amino acids are those found in Bacto-Casamino Acids (Difco Laboratories), excluding L-methionine. Bacto-Casamino Acids is a component of Mueller-Hinton medium, an excellent complex medium for the primary isolation and growth of meningococci. The addition of these amino acids allowed excellent, reproducible growth of the meningococcus in MNDM.

Studies were done to ascertain which sulphur compounds could be used by the meningococcus as its sole source of sulphur (Table 2). The chemical structures of the less commonly known sulphur compounds are shown in Appendix A. Although toxic to Bacillus subtilis (Villarejo and Westley, 1966), E. coli (Roberts et al., 1955), and to some strains of meningococci (Catlin, 1973), cysteine was essential for neisserial growth in the original NDM formulation. As the sole source of sulphur in MNDM, L-cysteine permitted excellent growth of N. meningitidis. While not permitting the growth of cells pregrown on 1 mM Na_2SO_4, sodium dithionate produced good growth of cells starved for sulphur

Table 2. Compounds tested as sole sulphur sources for for the growth of N. meningitidis. Each sulphur compound was added to MNDM at 1 mM. Cells were shaken at 200 rpm, 5% CO_2, 37°C and growth was measured spectrophotometrically at 600 nm.

Compound	Growth
Sulphate (Na_2SO_4)	Excellent
Sulphite (Na_2SO_3)	"
Bisulphite ($NaHSO_3$)	"
Thiosulphate ($Na_2S_2O_3$)	"
Dithionite ($Na_2S_2O_4$)	"
Thiocyanate (NaSCN)	"
Hydrosulphide (NaSH)	"
Reduced glutathione (GSH)	"
L-cysteine	"
L-cystine	"
L-lanthionine	Good
L-methionine	Poor
Mercaptosuccinate	"
Taurine	None
Carrageenans(iota,kappa,lambda)	"
Dithionate ($Na_2S_2O_6$)	None/Good[a]

[a] If previously starved for sulphur.

Table 2. Compounds tested as sole sulphur sources for for the growth of *N. meningitidis*. Each sulphur compound was added to MNDM at 1 mM. Cells were shaken at 200 rpm, 5% CO_2, 37°C and growth was measured spectrophotometrically at 600 nm.

Compound	Growth	Generation Time
Sulphate (Na_2SO_4)	Excellent	53 min
Sulphite (Na_2SO_3)	"	55 min
Bisulphite ($NaHSO_3$)	"	"
Thiosulphate ($Na_2S_2O_3$)	"	53 min
Dithionite ($Na_2S_2O_4$)	"	"
Thiocyanate (NaSCN)	"	"
Hydrosulphide (NaSH)	"	60 min
Reduced glutathione (GSH)	"	53 min
L-cysteine	"	"
L-cystine	"	56 min
L-lanthionine	Good	86 min
L-methionine	Poor	111 min
Mercaptosuccinate	"	"
Taurine	None	
Carrageenans(iota,kappa,lambda)	"	
Dithionate ($Na_2S_2O_6$)	None/Good[a]	

[a] If previously starved for sulphur.

for several hours prior to the addition of the dithionate. Taurine and the carrageenans failed to serve as sulphur sources even when the cells were starved for sulphur. Figure 3 shows the growth of N. meningitidis in MNDM when supplemented with the sulphur compounds listed in Table 2. Minimal generation times for N. meningitidis ranged from 53 to 60 min with ten of the compounds tested (curve A). Methionine, mercaptosuccinate, and lanthionine showed significantly longer generation times (86 min for methionine and mercaptosuccinate, 111 min for lanthionine) as well as reduced extents of growth.

Sulphate, a likely in vivo sulphur source, and thiosulphate were chosen to determine the minimal sulphur requirements of N. meningitidis (Figure 4). Concentrations used ranged from 10 uM to 100 mM. Results for both compounds were nearly identical, with maximal growth extent seen at sulphur levels greater than or equal to 500 uM. At concentrations less than 500 uM the growth rate remained maximal but exent of growth was reduced. At 100 uM sulphate or thiosulphate, the cells depleted the sulphur after 5.5 to 6 h growth, and at 10 uM cells became sulphur-limited in 3 to 4 h. Cells grown in sulphur-sufficient MNDM contained 3.4 ug sulphur per mg cell protein while sulphur-limited cells contained 2.6 ug sulphur per mg protein.

The levels of several of the sulphur sources found in

Figure 3. Growth of N. meningitidis at 37C in MNDM supplemented with each of a variety of sulphur compounds at 1 mM (per mole sulphur) as the sole source of sulphur.

A = SO_3^{2-}, SO_4^{2-}, HSO_3^{1-}, $S_2O_4^{2-}$, $S_2O_3^{2-}$, SCN^{1-}, SH^{1-}, GSH, L-cysteine, L-cystine

B = lanthionine

C = methionine, mercaptosuccinate

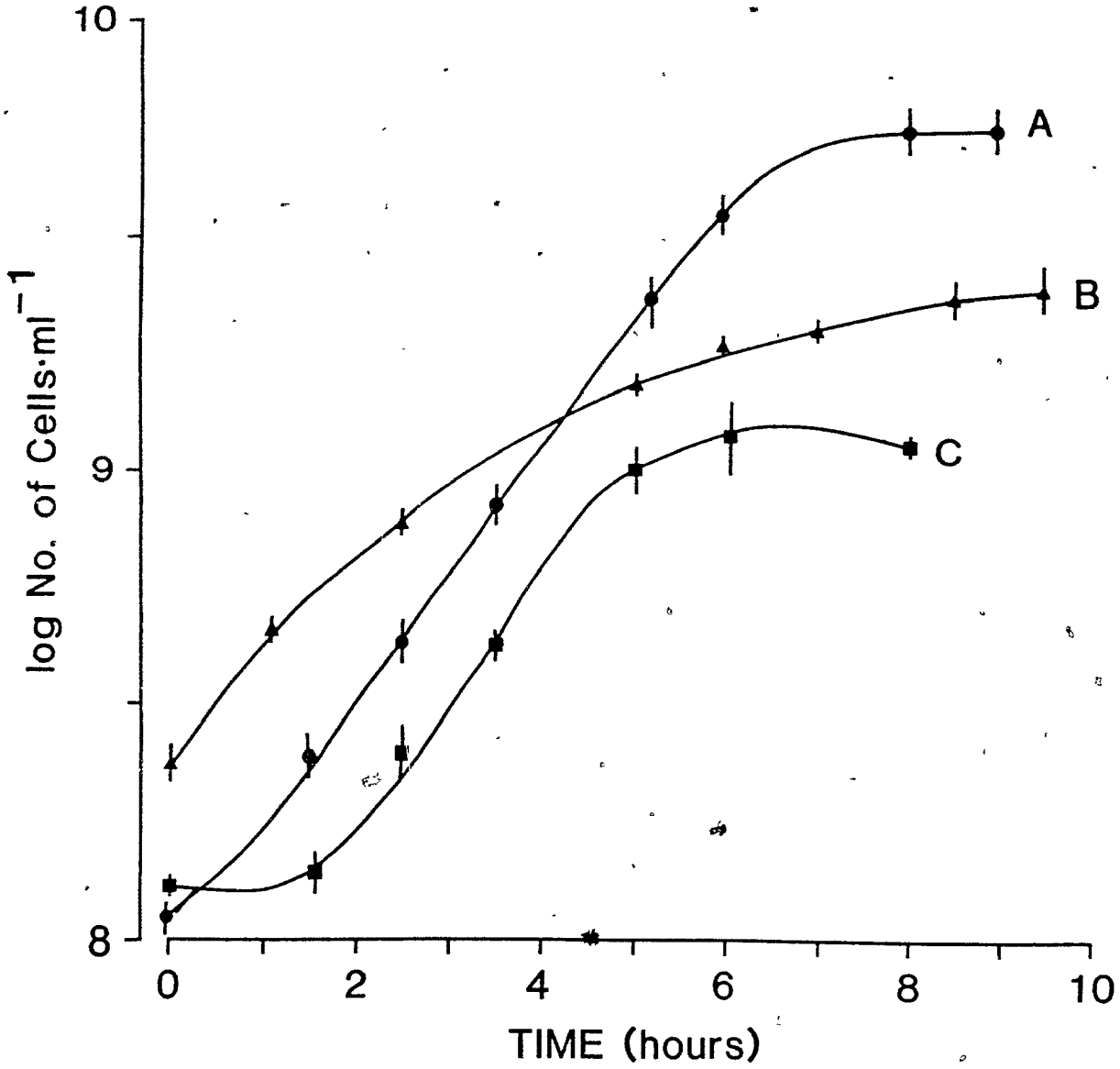

10
9
8
log No. of Cells·ml^{-1}
A
B
C
0
2
4
6
8
10
TIME (hours)

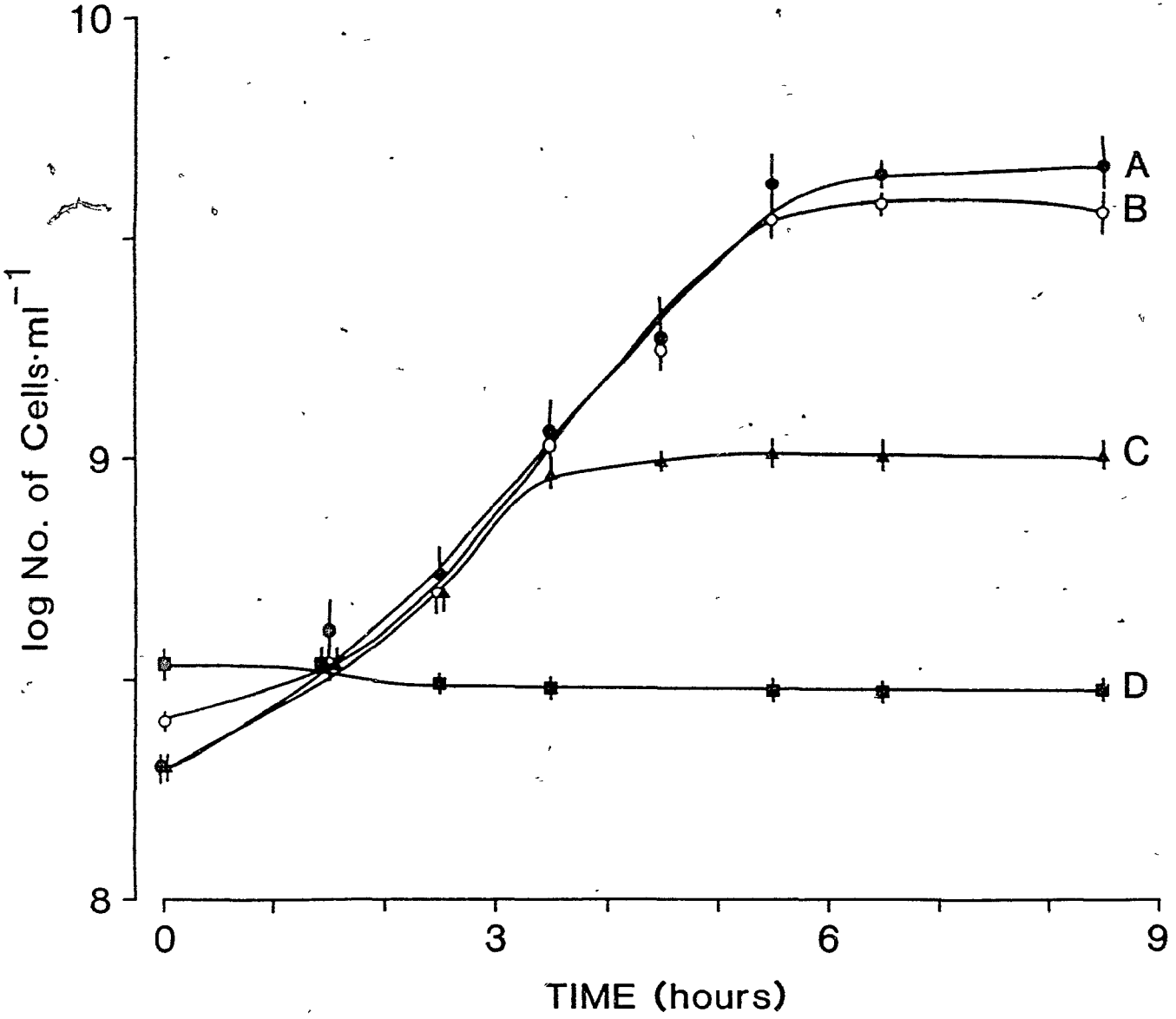
Figure 4. Growth of sulphur-sufficient, washed cells of N. meningitidis at 37C in MNDM supplemented with either Na_2SO_4 or $Na_2S_2O_3$ as the sole source of sulphur at the following concentrations:

A = 500 uM; 1, 10, 50, and 100 mM

B = 100 uM

C = 10 uM

D = no sulphur


10
9
8
log No. of Cells·ml^{-1}
0
3
6
9
TIME (hours)
A
B
C
D

the human host which are available to N. meningitidis are listed in Table 3. There is abundant sulphur in any of the human body fluids from which the meningococcus is usually isolated in meningococcal disease to support good growth of the organism. Growth of N. meningitidis to maximal in vitro cell density (5×10^9 cells per mL) required 16 ug sulphur per mL (0.5 mM sulphate). Levels of available sulphur greater than 0.5 mM sulphur are found in the blood, cerebrospinal fluid, and saliva. Unlike iron availability, sulphur limitation of the meningococcus is unlikely in the human host.

Based on the sulphur availability results shown in Figs. 3 and 4, thiocyanate is the likely in vivo sulphur sources in the oropharynx. Sulphate, L-cysteine, and L-cystine are potential sources of sulphur in the blood, and sulphate and thiocyanate are the most likely sources in the cerebrospinal fluid.

ii. Thiosulphate Reductase Activity

While extensive data have been accumulated on the utilization of inorganic sulphur compounds by the thiobacilli (London and Rittenberg, 1964; Lyric and Suzuki, 1970; and Tuovinen et al., 1976), little is known about the

Table 3. Levels[a] of sulphur sources in the human host available to N. meningitidis.

Sulphur Source	Mean Reported Concentration or Range	Site
Total sulphur	38 mM	Whole blood
Sulphate	310-580 uM	Serum
	6 mM	Cerebrospinal fluid
Glutathione	0.8-1.3 mM	Whole blood
Thiocyanate	0.41-6.6 mM	Saliva
	5.2-50 uM	Cerebrospinal fluid
	13.8 uM	Plasma
Cystine, cysteine	74 uM	Whole blood

[a] From K. Diem and C. Lentner (ed.). 1970. Scientific tables. Ciba-Geigy Ltd., Basel, Switzerland.

mechanisms of sulphur metabolism in heterotrophs. Trudinger (1967) isolated two heterotrophic soil bacteria which oxidize thiosulphate to tetrathionate. Thiosulphate oxidase has also been reported in Alcaligenes (Hall and Berk, 1968), and Schook and Berk (1978) showed Pseudomonas aeruginosa to possess several oxidases including thiosulphate, tetrathionate, and metabisulphite as well as rhodanese. In the enterobacteria, a tetrathionate reductase has been found which yields thiosulphate as a product (Pichinoty and Bigliardi-Rouvier, 1963; Oltmann et al., 1975) Within the neisserias, Le Faou (1981, 1983) has shown thiosulphate reductase, rhodanese, trithionate reductase and tetrathionate reductase activities in Neisseria gonorrhoeae and sulphite reductase in those neisseriae which use sulphate (1984).

Since thiosulphate was an excellent source of sulphur for N. meningitidis, it was possible that the organism contained a thiosulphate reductase. Earlier work in the laboratory of I.W. DeVoe (DeVoe et al., 1982) indicated that the soluble cell fraction of N. meningitidis contained a sulphur-reducing enzyme: When soluble cell fractions subjected to polyacrylamide gel electrophoresis were placed in a solution containing sodium dithionite (reducing agent), thiosulphate, and $FeSO_4$, black FeS deposits appeared in three bands within 30 min. These results indicated that

thiosulphate was being reduced in the presence of sodium dithionite to yield H_2S, which was in turn able to reduce $FeSO_4$ to FeS by three protein components of the soluble fraction. Gas-liquid chromotography had shown H_2S to be the only detectable sulphur-containing gas in the gas phase after 15 min incubation of thiosulphate, dithionite, and the soluble cell fraction.

My contribution to the publication on thiosulphate reductase was to ascertain whether or not thiosulphate was the only substrate for the enzyme(s) and whether or not the enzyme(s) was labile under normal denaturing conditions.

A number of sulphur compounds were tested for their ability to serve as substrate in the reductase reaction (Table 4). Among those tested, only thiosulphate served as a substrate for the enzyme. As seen in the gonococcus (Le Faou, 1981) the soluble cell fraction contained all the thiosulphate reductase activity. When the reaction mixture was exposed to air, thiosulphate reductase activity stopped. Activity could not be recovered for the same extract when N_2 was once again used as the gas to carry the product from the reaction cell to the DTNB cylinder, indicating that the enzyme was poisoned by air only in the presence of the substrate. Exposure of the extract to 121^0C for 5 min destroyed the activity, as did the presence of ethanol (50% final). Digestion of the soluble cell fraction with the

Table 4. Thiosulphate reductase activity in N. meningitidis in the presence of various sulphur compounds.

Substrates[a]	Treatment[b]	Activity[c] Units/mg protein
Thiosulphate ($S_2O_3^{2-}$)	None	42
	Air	3
	N_2 (after air)	<1
	Ethanol (50%)	7
	121°C	<1
	Pronase	10
Metabisulphite ($S_2O_5^{2-}$)	All	<1
Sulphite (SO_3^{2-})	All	<1
Tetrathionate ($S_4O_6^{2-}$)	All	<1
Bisulphite (HSO_3^{1-})	All	<1
Sulphate (SO_4^{2-})	All	<1

[a] All sulphur compounds were used as sodium salts at a final concentration of 5 mM sulphur.

[b] Ethanol added with substrate; aeration of the cell-free extract alone in the absence of substrate had no adverse effects. Pronase data were normalized from separate experiments based on % of activity.

[c] Reductase activity was determined by adding potential substrate and reducing agent to the soluble cell fraction. N_2 gas was passed over the reaction into a cylinder containing DTNB. One unit of enzyme activity was defined as the generation of sufficient H_2S to produce an absorbance (412 nm) increase in DTNB of 0.001 unit/min.

broad-spectrum streptomycete protease pronase (23 µg/mL; 4h) resulted in a 75% loss of activity when compared with controls.

This work clearly demonstrated that the sulphur-reducing enzyme was indeed a thiosulphate reductase, was protein in nature, and was inactivated by air only in the presence of its substrate.

Summary

In order to colonize the host and produce disease, the meningococcus must be able to acquire its vital nutrients from the host. It has been demonstrated in this section that N. meningitidis can readily use each of 13 biologically relevant sulphur compounds as its sole source of sulphur. Several of these compounds occur in the human host at concentrations well above the minimum sulphur requirements of the meningococcus. Sulphur is therefore unlikely to be a nutrient limiting the growth of N. meningitidis in the human host. Unlike the micronutrient iron, it would be impossible for the host to sequester its sulphur from the microorganism.

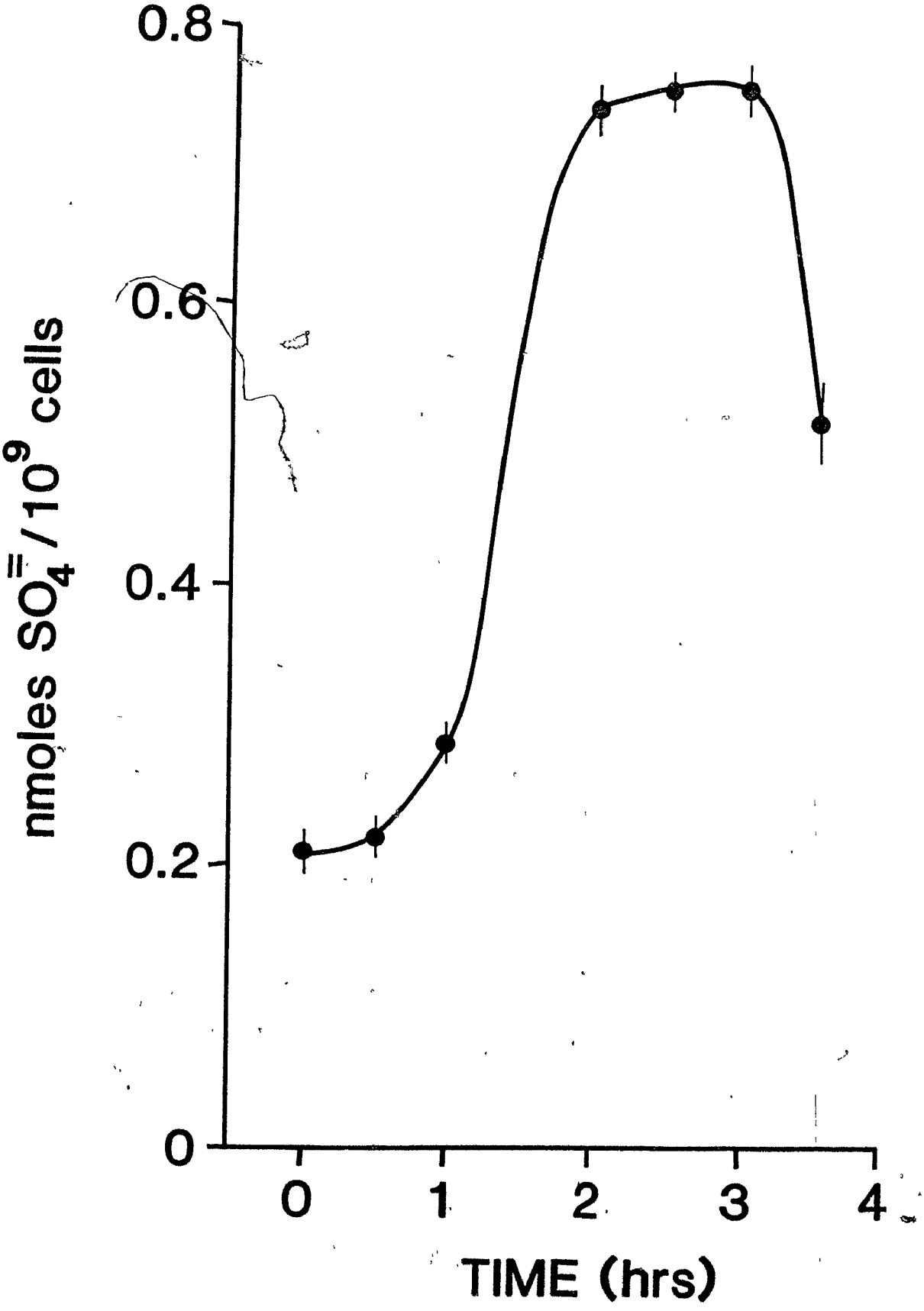
The presence of a thiosulphate reductase in the meningococcus was clearly demonstrated.

II. Sulphate Transport in N. meningitidis

The second phase of this work was a detailed study of the uptake of sulphate by the meningococcus. Sulphate is a likely sulphur source for N. meningitidis in the blood and cerebrospinal fluid of man (see Table 3). Meningococci are usually isolated from these body fluids during the course of meningococcal disease (DeVoe, 1982) and the levels of sulphate present in the blood and cerebrospinal fluid (Table 3) exceed the levels required by N. meningitidis for growth to maximal in vitro cell density.

In order for iron uptake to be observed in N. meningitidis the cells must first be depleted of iron by growing them under iron-limited conditions for several cell doublings (Archibald and DeVoe, 1978). In reports of sulphate uptake in marine bacteria (Cuhel et al., 1981a), sulphate limitation prior to the addition of radiolabelled sulphur was necessary to enhance uptake. Sulphur-sufficient cells of N. meningitidis SD1C grown with 1 mM sulphate as the sole source of sulphur showed very low uptake velocities. After reaching late log phase in 1 mM sulphate, the cells showed maximal sulphate uptake after depletion of intracellular sulphur during a 2 to 3 h incubation in MNDM containing 1 uM sulphate (Figure 5). Longer sulphur deprivation led to loss of cell viability.

Figure 5. Effect of sulphur starvation on the extent of sulphate uptake from 10 uM ^{35}S-labelled sulphate in N. meningitidis. Cells were grown to late log phase in MNDM, harvested, washed, and resuspended in MNDM containing 1 uM sulphate. The effect was followed for 4 h after removal of normal medium sulphur.

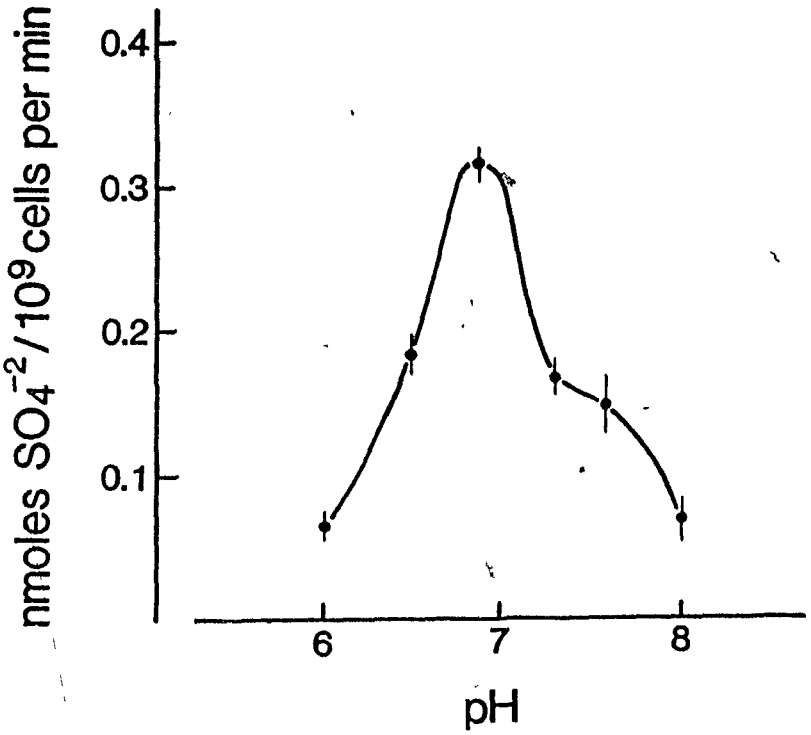

0.8
0.6
0.4
0.2
0
nmoles $SO_4^{=}$ / 10^9 cells
0
1
2
3
4
TIME (hrs)

Sulphate uptake was temperature- and pH-dependent (Figure 6), with maximal uptake at 37^{o}C, pH 6.8. As 37^{o}C is the optimal growth temperature for N. meningitidis it is not surprising that sulphate uptake is maximal at this temperature. Maximum uptake was seen at pH 6.8, which is the pH of MNDM after the cells have grown to late log phase. At pH values slightly above and below the optimum (6.5 and 7.3), sulphate uptake rates fell by nearly 50%. At pH 6 and pH 8, sulphate uptake was reduced by 79% and 76%, respectively. Figure 7 shows the uptake rate after addition of various concentrations of sulphate to sulphur-starved cells. A double reciprocal plot of the uptake rate showed saturation kinetics with a maximal uptake velocity of 1.4 nmol sulphur per 10^{9} cells per min (inset). However, at 30 s the value obtained for the apparent K_{m} was 2.6 uM, while at 1 min a value of 1.4 uM was seen, suggesting allosteric negative feedback in the uptake of sulphate or rapid saturation of binding sites followed by slower active transport.

i. Sulphate Analogues

The group VI anions are known to be analogues of sulphate in other biological systems. These include selenate [SeO_{4}^{2-}] (Brown and Shrift, 1980 and 1982),

Figure 6. Effects of pH and temperature on velocity of 10 uM sulphate uptake by N. meningitidis. Sulphur-starved cells were resuspended in sulphur-free MNDM at various pH values 10 min prior to assaying transport. Sulphur-starved meningococci were allowed to equilibrate at the sampling temperature for 10 min prior to assaying transport.


nmoles SO_4^{-2} / 10^9 cells per min
0.4
0.3
0.2
0.1
6
7
8
pH

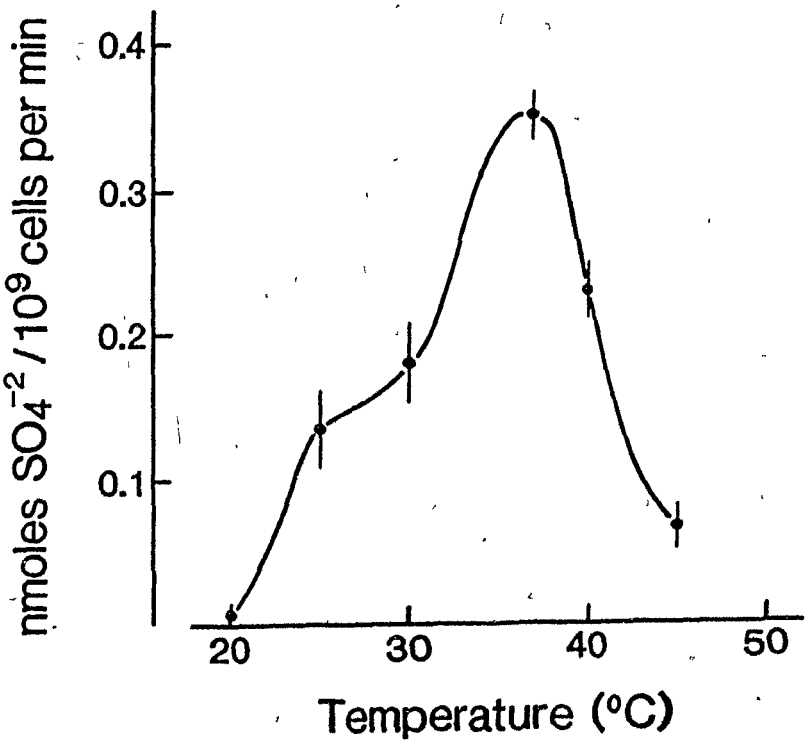
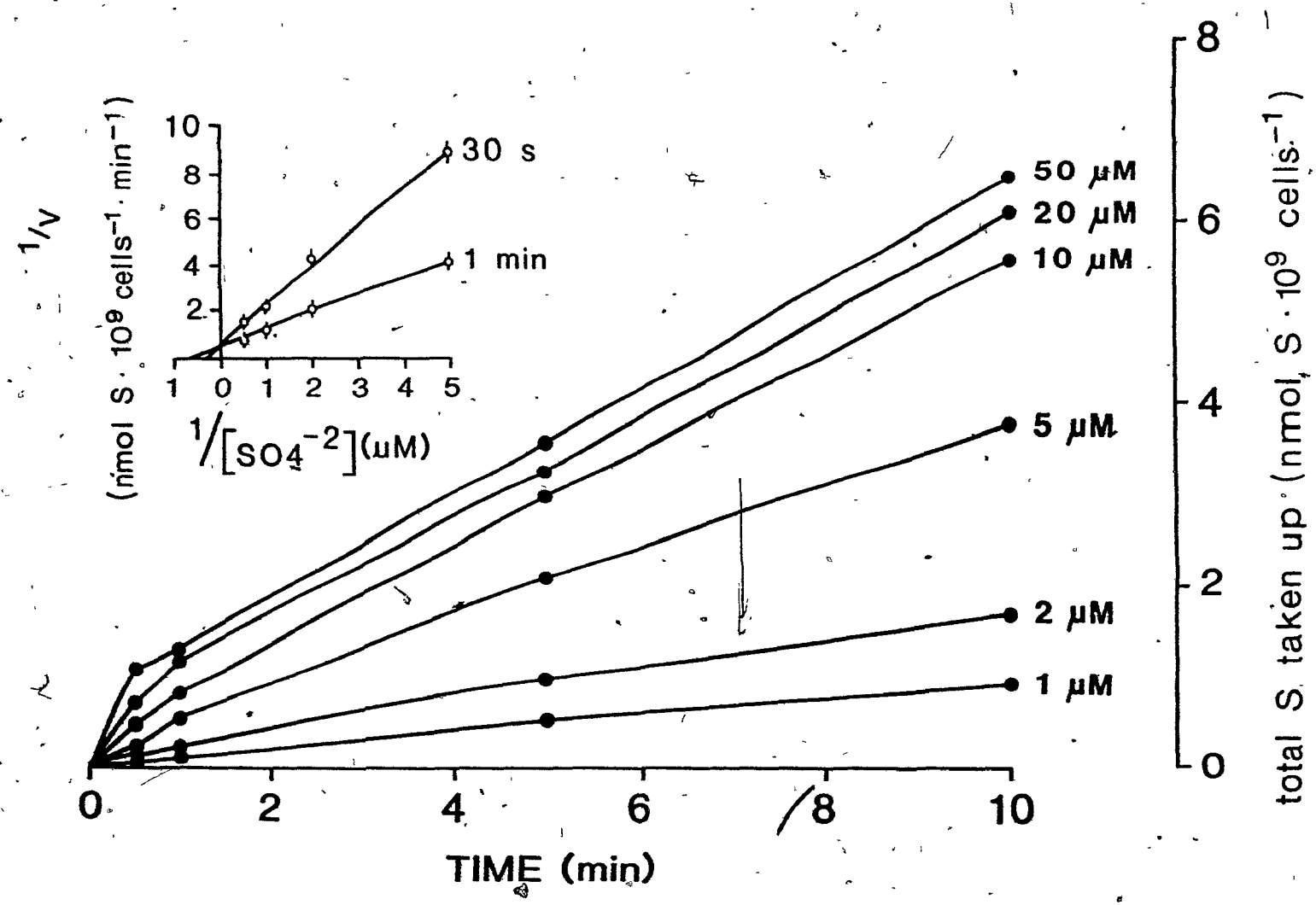

nmoles SO_4^{-2} / 10^9 cells per min
0.4
0.3
0.2
0.1
20
30
40
50
Temperature (°C)

Figure 7. Uptake of various concentrations of sulphate by sulphur-starved N. meningitidis cells. Substrate concentrations added are on the right and the apparent saturation kinetics of sulphate uptake are presented in a double reciprocal plot (inset).


1/V
(nmol S · 10⁹ cells⁻¹ · min⁻¹)
30 s
1 min
1/[SO4⁻²](µM)
50 µM
20 µM
10 µM
5 µM
2 µM
1 µM
TIME (min)
total S taken up (nmol S · 10⁹ cells⁻¹)

molybdate [MoO_4^{2-}] (Tweedie and Segal, 1970), chromate [CrO_4^{2-}] (Marzluf, 1970) and tungstate [$W_3O_{10}^{2-}$] (Vange et al., 1974). Selenate, tungstate, and molybdate were chosen to examine the effect of analogues on the sulphate transport system in N. meningitidis.

Initial experiments were done to look at the potential toxic effects of these ions to N. meningitidis. Results are shown in Table 5. Both MNDM and Mueller-Hinton agar plates were used in this experiment. Meningococcal growth on MH agar plates is consistently very good, and while growth in MNDM broth could be excellent, quite often it was less than optimal. Therefore MH agar plates were added to serve as a control as well as a comparison. Molybdate ion had no inhibitory or enhancement effect on N. meningitidis on either medium. Tungstate ion caused concentration-dependent inhibition of growth on the MNDM plates, completely inhibiting cell growth at 4 mM in an area of 20.6 cm^2. On MH agar plates growth was inhibited less at 4 mM. Selenate ion had no effect on cell growth at 40 or 500 uM on either medium, but was slightly inhibitory on both media at 4 mM.

ii. Competitors and Inhibitors of Sulphate Transport

As many sulphur compounds were shown to be usable by N.

Table 5. Toxicity of selenate, molybdate, and tungstate ions to N. meningitidis. A lawn of meningococci was exposed to the various ions by cutting wells (0.5 cm in diameter) in the agar to which 100 uL of the ion solutions (as sodium salts) were added. Plates were incubated for 18-24 h at 37°C, 5% CO_2.

Agar Plate Medium	Ion Concentration	Ions		
		Selenate	Molybdate	Tungstate
MH	10 uM	0[a]	0	0
MNDM		0	0	2.94
MH	50 uM	0	0	0
MNDM		0	0	6.9
MH	1 mM	4.0	0	3.6
MNDM		7.8	0	20.6

[a] Area of complete inhibition in cm^2.

Values are the average of two experiments run in duplicate.

meningitidis as its sole sulphur source it seemed likely that these compounds were taken up by a common porter and thus would compete with and inhibit the uptake of sulphate. Because they are similar in electronic configuration, the structural analogues of sulphate are often transported by sulphate permeases (Dreyfuss, 1964; Springer and Huber, 1972; and Brown and Shrift, 1980).

In the following experiments, sulphate uptake was allowed to proceed normally for 1 min at which point the appropriate concentration of the alternate sulphur source or analogue was added. A second sample was withdrawn at 5 min and compared to the control to which no second sulphur source or analogue was added. The results are shown in Table 6. Thiosulfate and L-cysteine had a dramatic effect on sulphate uptake; at equimolar concentration (10 uM) thiosulphate completely abolished uptake while L-cysteine reduced uptake by 95%. Selenate was the most effective analogue and was transported preferentially over sulphate, an indication that the two ions may be competing for the same site. Only thiocyanate and tellurite had no effect even at 100 times the concentration of sulphate.

The next experiments were designed to determine the energy requirements of sulphate uptake in N. meningitidis.

Known metabolic poisons and inhibitors were used to determine the energy requirements of sulphate uptake in N.

Table 6. Effects of potential competitors and inhibitors on the uptake of 10 uM sulphate by N. meningitidis. Cells were allowed to transport sulphate for 5 min at which time the inhibitor was added. At 10 min a second sample was withdrawn and assayed.

Addition	Percent of Control[a]
None	100
Sulphate [SO_4^{2-}] (1 mM)	5
(10 uM)	57
L-cysteine (1 mM)	5
(100 uM)	5
(10 uM)	8
Thiosulphate [$S_2O_3^{2-}$] (1 mM)	0
(100 uM)	0
(10 uM)	0
Molybdate [MoO_4^{2-}] (1 mM)	10
(100 uM)	63
(50 uM)	84
(10 uM)	98
Tungstate [$W_3O_{10}^{2-}$] (1 mM)	2
(100 uM)	52
(50 uM)	59
(10 uM)	98
Selenate [SeO_4^{2-}] (1 mM)	2
(100 uM)	2
(50 uM)	11
(10 uM)	16
Reduced glutathione (100 uM)	19
(10 uM)	88
L-methionine (1 mM)	80
(100 uM)	79
(10 uM)	81
Dithionate [$S_2O_6^{2-}$] (100 uM)	60
(10 uM)	99
Thiocyanate [SCN^{1-}] (1 mM)	100
(10 uM)	100
Tellurite [TeO_3^{2-}] (1 mM)	100

[a] Percent of maximal sulphate uptake velocity under starved conditions. 100% actual uptake = 1.84 nmol S/mg protein. Values are the average of three experiments run in duplicate.

meningitidis. Results are shown in Table 7. Cyanide and azide, which inhibit electron transport at the terminal oxidase level, had a dramatic effect, reducing sulphate uptake completely (100%) in the case of cyanide and 97% with azide. Using a large number of cells in this same assay, there was no detectible binding of sulphate to N. meningitidis, although fewer than 20 ions per cell would have been detected (data not shown). Nigericin, a proton ionophore, which acts by exchanging K^+ ions for H^+ ions, thereby eliminating the pH gradient without affecting the electrical component of the membrane (Pressman, 1969; Moore, 1971), reduced sulphate uptake by 94%. Tetrachlorosalicylanilide (TCS) and cyanide-m-chlorophenylhydrazone (CCCP) are proton ionophores, agents which are very specific conductors of H^+ ions across biological membranes, thereby eliminating the proton motive force (Henderson, 1971; Hinkle and McCarty, 1978). Both were extremely effective in reducing sulphate uptake in the meningococcus. N,N'-dicyclodihexylcarbodiimide (DCCD), which reacts covalently with the membrane-bound F_O subunit and inhibits the ATPase (Harold, 1972; Fillingame, 1980) reduced sulphate uptake in the meningococcus by greater than 90%. Valinomycin, a K^+-specific uniporter which conducts K^+ ions across the membrane, neutralizing the electrochemical component but leaving the pH gradient intact (Pressman,

Table 7. Effects of metabolic poisons on 10 uM sulphate uptake in N. meningitidis. Sulphur-starved cells were exposed to each metabolic poison for 5 min prior to assaying uptake.

Addition (final concentration)	Percent of Control[a]
None	100
Ethanol	100
KCN (1 mM)	3
(0.5 mM)	3
(0.2 mM)	3
TCS[b] (1 uM)	5
(0.5 uM)	12
(0.2 uM)	19
Nigericin (5 uM)	6
Valinomycin (25 uM)	100
(2.5 uM)	100
DCCD[c] (50 uM)	6
(25 uM)	20
(10 uM)	27
(2 uM)	51
CCCP[d] (50 uM)	6
(25 uM)	12
(10 uM)	18
(2 uM)	53
NaN_3 (5 mM)	0
(0.5 mM)	0

[a] Percent of maximal uptake velocity under sulphur-starved conditions. 100% uptake = 1.51 nmol S per mg protein. Values are the average of three experiments run in duplicate.

[b] Tetrachlorosalicylanilide

[c] N,N'-dicyclodihexylcarbodiimide

[d] Carbonyl cyanide-m-chlorophenylhydrazone

1965; Harold, 1970) had no effect on sulphate uptake. These results indicate that sulphate uptake by N. meningitidis is energy-dependent and suggest that the transmembrane pH gradient rather than the transmembrane voltage component of the proton motive force drives, or is required, for sulphate transport.

iii. Effect of Chloramphenicol on Sulphate Transport in N. meningitidis

Simonson et al. (1982) found that new protein synthesis is required for the uptake of iron from transferrin by N. meningitidis SD1C. Cells treated with chloramphenicol for 1 h prior to assaying uptake were unable to acquire iron. An attempt was made to ascertain whether or not new protein synthesis was necessary for sulphate transport in this microorganism. Table 8 compares the uptake of sulphate by N. meningitidis in both sulphur-starved and sulphur-sufficient cells exposed to chloramphenicol. Uptake was assayed at both 1 and 2 h starvation as cell viability sharply decreased between 1 and 2 h. At 1 h the sulphate-starved cells took up 70% of the maximal value which was seen at 2 h starvation. When treated with 20 ug/mL chloramphenicol for 1 or 2 h the amount of sulphate transported into the meningococci was greatly reduced (21% at 1 h, 72% at 2 h);

Table 8. Effects of chloramphenicol (CAM) treatment on 10 uM sulphate uptake by N. meningitidis. Sulphur-starved cells were exposed to CAM for 1 or 2 h during the sulphur depletion incubation; sulphur-sufficient cells (1 mM) were exposed to 2 ug/mL CAM for 1 and 2 h. Uptake was assayed as previously described.

Cell Treatment	Sulphate Uptake (%)[a]	Viability[b]
1 uM Sulphate, 1h	70	+
1 uM Sulphate, 2h	100	+
1 uM Sulphate + 20 ug/mL CAM, 1h	49	-
1 uM Sulphate + 20 ug/mL CAM, 2h	28	-
1 mM Sulphate + 2 ug/mL CAM, 1h	0	=
1 mM Sulphate + 2 ug/mL CAM, 2h	0	=

[a] Percent of maximal sulphate uptake under starved conditions. Actual uptake at 100% = 2.63 nmol S /mg protein. Values are the average of two experiments run in duplicate.

[b] +, no loss of cell viability
-, 25-30% loss of cell viability
=, greater than 50% loss of cell viability

however, viability also decreased, with over 50% loss at 2 h. The cells which were sulphate-sufficient and exposed to only 2 ug/mL chloramphenicol showed no uptake of labelled sulphate which was expected. However, these cells showed an even greater loss of viability than the sulphate-starved, chloramphenicol-treated cells, indicating that cells transporting sulphate under normal conditions are more susceptible to the effects of chloramphenicol than are sulphur-starved meningococci which have an altered metabolism. Whether or not new protein synthesis is required for sulphate uptake in the meningococcus is unclear from these data as loss of viability was an important factor in the reduced sulphate uptake. Successful results in the experiments showing new protein synthesis to be necessary for iron uptake from transferrin (Simonson et al., 1982) may have been attainable because iron is a micronutrient rather than a macronutrient. It seems reasonable that sulphur depletion coupled with chloramphenicol exposure is too much physiological stress for the meningococci, resulting in the inconclusive data obtained.

Summary

Like other systems, sulphate transport in the meningococcus was temperature-, pH-, concentration- and

energy-dependent. Binding of sulphate was absent in cells exposed to azide or cyanide. Sulphur starvation greatly enhanced uptake. The system was inhibited by other sulphur sources (except thiocyanate) and structural analogues (except tellurite).

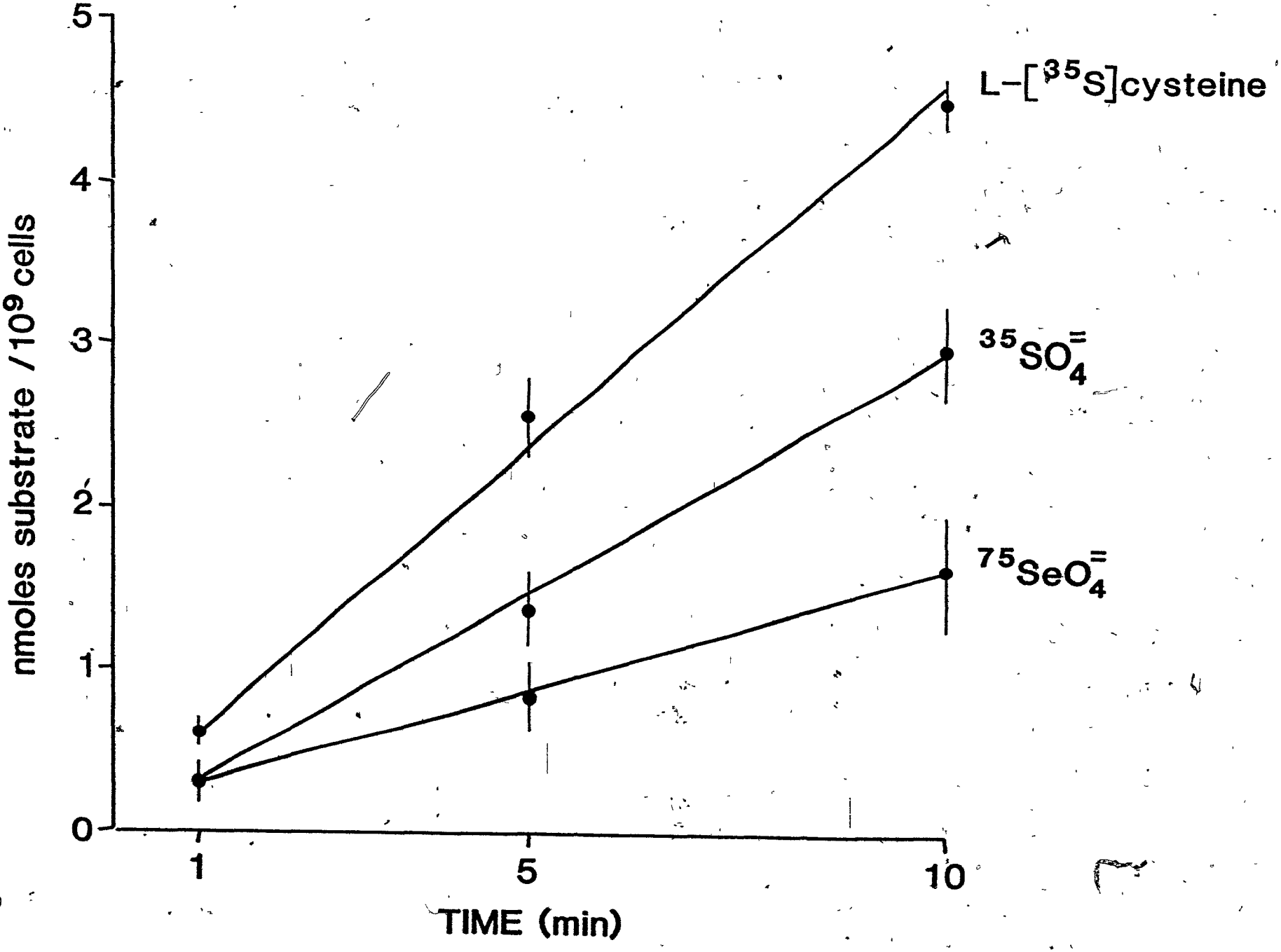
III. Cysteine and Selenate Transport in N. meningitidis

1. Transport Studies

Cysteine and selenate were chosen for further uptake studies with the meningococcus. Cysteine was selected as it is an excellent sulphur source, is present in the human host, and is an amino acid. Selenate, a structural analogue of sulphate, has been shown to employ the same uptake system as sulphate in Escherichia coli (Springer and Huber, 1972; Lindblow-Kull et al., 1985), Salmonella typhimurium (Brown and Shrift, 1980), and Saccharomyces cerevisiae (McCready and Din, 1974).

A comparison of the transport of sulphate, L-cysteine, and selenate is shown in Figure 8. This linear pattern is in sharp contrast to that seen in S. typhimurium, where the cells excrete 80% of the sulphate transported in the first minute (Dreyfuss, 1964).

Figure 8. . Uptake velocities of 10 uM ^{35}S-labelled sulphate, ^{35}S-labealled L-cysteine, and ^{75}Se-labelled selenate labelled substrates by sulphur-starved meningococci.


5
4
3
2
1
0
nmoles substrate /10^9 cells
1
5
10
TIME (min)
L-[35S]cysteine
35SO4=
75SeO4=

The uptake of sulphate and selenate (10 uM) by sulphur-starved cells over 2 h was compared. Results are shown in Figure 9. The cells transporting labelled sulphate became sulphur-limited at 2 h as compared to 3 to 4 h for sulphur limitation seen in Figure 4. However, this difference can be attributed to sulphur-starvation which was shown to clearly enhance uptake. Because it is similar to sulphate in physical, chemical, and metabolic properties, the selenium of the selenate ion is probably incorporated into malfunctioning proteins (Shrift, 1973). This appeared to be true in the meningococcus. Selenate uptake occurred with no change in cell number (data not shown). The cell's inability to metabolize selenate successfully was probably responsible for the sharp drop in the uptake rate after 10 min. Selenate uptake continued for 75 min, suggesting that it is being transported into the cell rather than acting as a surface-competitive inhibitor.

Table 9 shows the effects of potential inhibitors and competitors on L-cysteine uptake in N. meningitidis. The only compound which had a significant effect on L-cysteine transport was reduced glutathione, at 100 times the concentration of L-cysteine. Equimolar L-cysteine and L-cystine caused approximately a 50% reduction of L-cysteine uptake, indicating that the oxidized dimer L-cystine may be transported via the same system as L-cysteine in the

Figure 9. Uptake of substrate by N. meningitidis incubated in 10 uM sulphate or selenate. Cells were grown on 1 mM sulphate to late log phase, harvested, washed, and resuspended in sulphur-free medium for 2 h. These sulphur-starved cells were then inoculated into MNDM containing either 10 uM sulphate or selenate.

▲ = Sulphate

● = Selenate

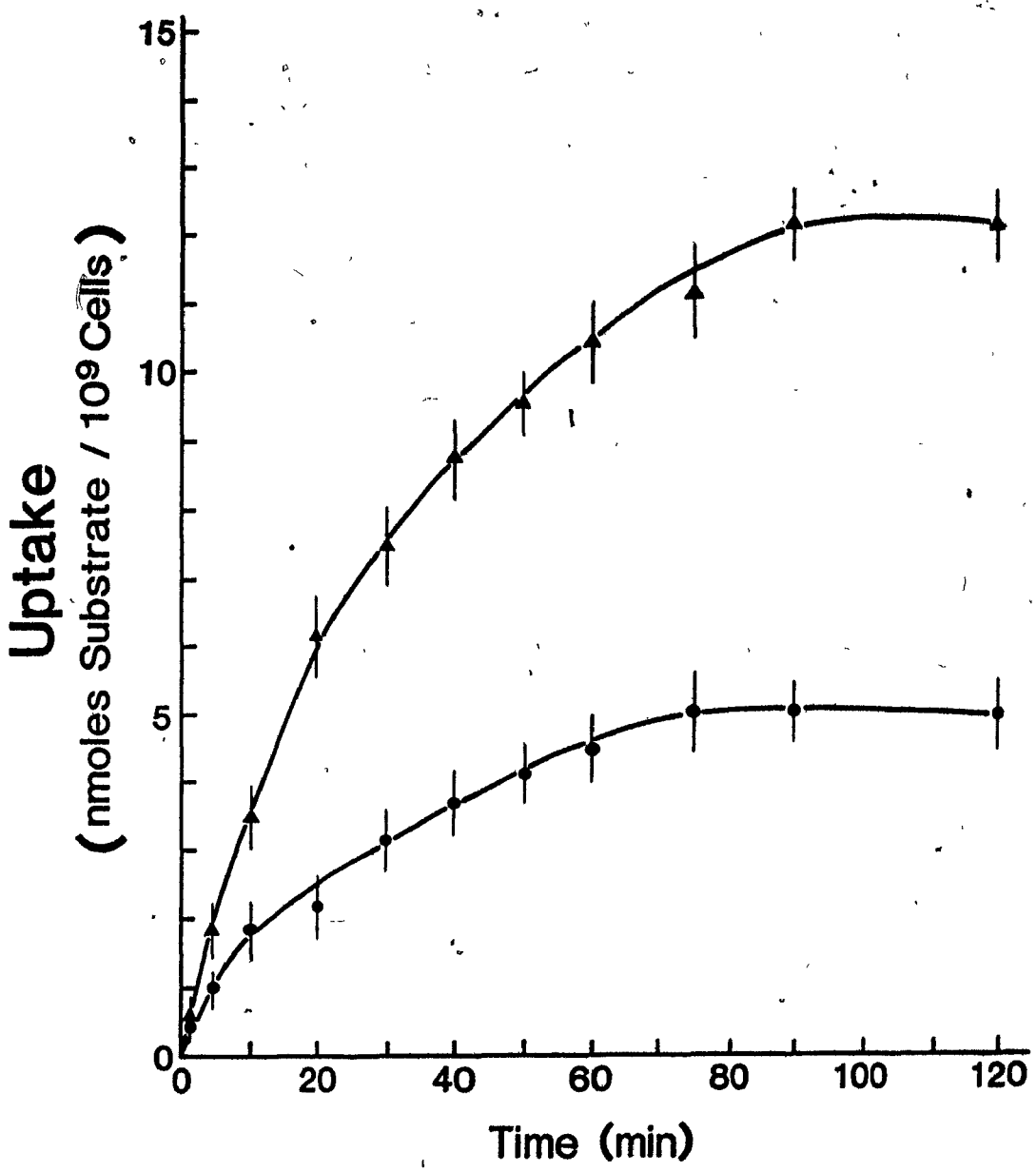

Uptake
(nmoles Substrate / 10^9 Cells)
15
10
5
0
0
20
40
60
80
100
120
Time (min)

Table 9. Effects of potential inhibitors and competitors on 10 uM 35S-labelled L-cysteine uptake by N. meningitidis. After accumulating L-cysteine for 5 min, the cells were exposed to the inhibitor. After 5 min exposure, a second sample was withdrawn and assayed.

Addition	Cysteine in Cells[a]
None	100
L-cysteine (10 uM)	55
L-cystine (1 mM)	12
(10 uM)	50
Sulphate (1 mM)	100
Thiosulphate (1 mM)	100
Molybdate (1 mM)	97
Tungstate (1 mM)	95
Selenate (1 mM)	100
GSSG[b] (1 mM)	100
GSH[c] (1 mM)	60
(10 uM)	92
L-methionine (1 mM)	95
Thiocyanate (1 mM)	100

[a] Percent of maximal L-cysteine uptake velocity under starved conditions. 100% actual uptake = 2.34 nmol S /mg protein. Values are the average of two or more experiments run in duplicate.
[b] Oxidized glutathione
[c] Reduced glutathione

meningococcus. Unlike sulphate transport, other inorganic sulphur sources, the sulphate analogues and oxidized glutathione had no effect on L-cysteine uptake, even at 100 times the concentration.

The effect of metabolic poisons on L-cysteine uptake is seen in Table 10. A substantial reduction of L-cysteine uptake was observed with each type of poison, indicating that, like sulphate transport, L-cysteine transport is energy-dependent.

Selenate uptake is inhibited in other bacterial systems by sulphate, thiosulphate, molybdate, and tellurite in S. typhimurium (Brown and Shrift, 1980) and by sulphate in E. coli (Lindblow-Kull et al., 1985). The effect of competitors and inhibitors on selenate transport is shown in Table 11. If sulphate and selenate employ a common uptake system in N. meningitidis, then similarities should and do exist in the data of Tables 6 and 11. Equimolar concentrations of selenate were much more effective at reducing sulphate uptake (Table 6) than vice versa (Table 11) indicating a preference for selenate over sulphate by N. meningitidis. Thiosulphate and L-cysteine similarly abolished uptake of both ions, while glutathione had a significant effect only on selenate uptake. Thiocyanate, an excellent sulphur source for the meningococcus, did not affect the transport of sulphate, L-cysteine, or selenate.

Table 10. Effects of metabolic poisons on 10 uM L-cysteine uptake in *N. meningitidis*. The amount of accumulated L-cysteine was assayed after 5 min in the cysteine-containing medium, at which time the metabolic poison was added. After 5 min exposure to the poison, a second sample was withdrawn and assayed.

Metabolic Poison	L-cysteine Uptake (%)[a]
None	100
Ethanol	100
NaN_3 (5 mM)	8
DCCD[b] (50 uM)	13
TCS[c] (1 uM)	13
Nigericin (10 uM)	17
CCCP[d] (50 uM)	25

[a] Percent of maximal L-cysteine uptake under starved conditions. 100% actual uptake = 2.24 nmol S/mg protein. Values are the averaage of two experiments run in duplicate.
[b] Dicyclodihexylcarbodiimide
[c] Tetrachlorosalicylanilide
[d] Carbonyl cyanide-m-chlorophenylhydrazone

Table 11. Effects of potential competitors and inhibitors on 10 uM selenate uptake in N. meningitidis. Cells were allowed to transport selenate for 5 min at which time a sample was withdrawn and assayed, followed by addition of the inhibitory compound. After 5 min a second sample was withdrawn and assayed.

Addition	Selenate Uptake (%)[a]
None	100
Sulphate (1 mM)	38
(100 uM)	76
(10 uM)	100
Thiosulphate (10 uM)	0
Reduced glutathione (10 uM)	0
L-cysteine (10 uM)	0
Thiocyanate (1 mM)	97
(100 uM)	100
(10 uM)	100
L-methionine (1 mM)	49
(100 uM)	51
(10 uM)	52
Molybdate (1 mM)	68
(100 uM)	82
(10 uM)	100
Tellurite (1 mM)	76
(100 uM)	93
(10 uM)	98

[a] Percent of maximal selenate uptake under sulphur-starved conditions. 100% actual uptake = 2.25 nmol Se/ mg protein. Values are the average of two or more experiments run in duplicate.

While a separate uptake system for the thiocyanate ion has not been reported in microbial systems, these data suggest that it is a possibility in N. meningitidis.

Azide (1 mM) and cyanide (1 mM) completely blocked the uptake of selenate by the meningococcus, and, as seen with sulphate, there was no dectectable binding of selenate to the cells under these conditions (data not shown). These data indicate that a common transport system for these two ions exists in N. meningitidis SD1C.

The apparent saturation kinetics of L-cysteine uptake are shown in Figure 10. At both 30 s and 1 min, the apparent K_m for L-cysteine uptake in N. meningitidis SD1C is 2 uM. However, at 30 s the value obtained for the maximal uptake velocity was 0.53 nmol sulphur per 10^9 cells per min, while at 1 min the value was 1 nmol sulphur per 10^9 cells per min. While unusual, this same effect was seen in the transport of manganese in Bacillus subtilis (Fisher et al., 1973; Scribner et al., 1975). These authors have proposed that a protein regulator interacts with the manganese transporter, reducing uptake without affecting efflux of the manganese ion, resulting in an increase in V_{max} without an increase in K_m.

Figure 11 shows the apparent saturation kinetics of selenate. The maximal uptake velocity is 1.25 nmol selenium per 10^9 cells per min. As observed with sulphate transport,

Figure 10. Double reciprocal plot of apparent saturation kinetics of L-cysteine uptake by N. meningitidis.

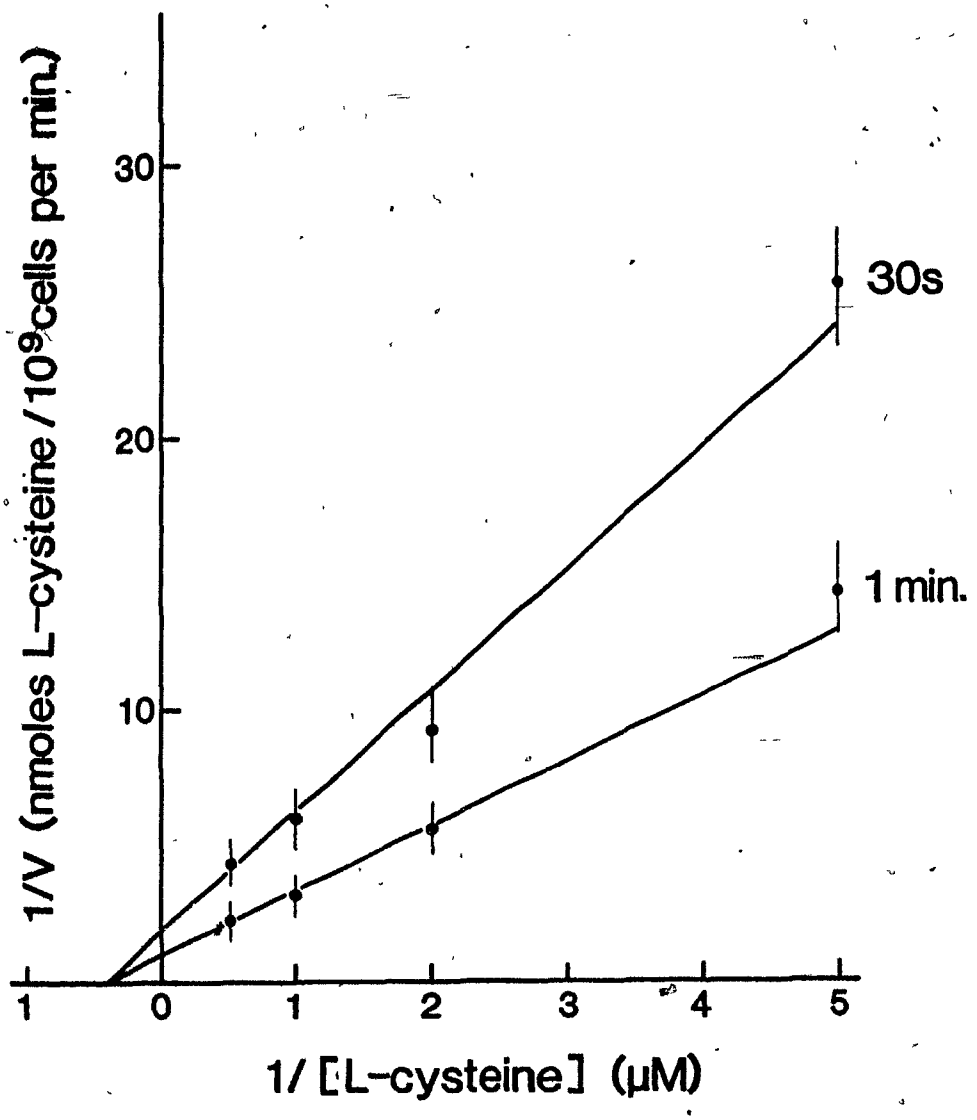
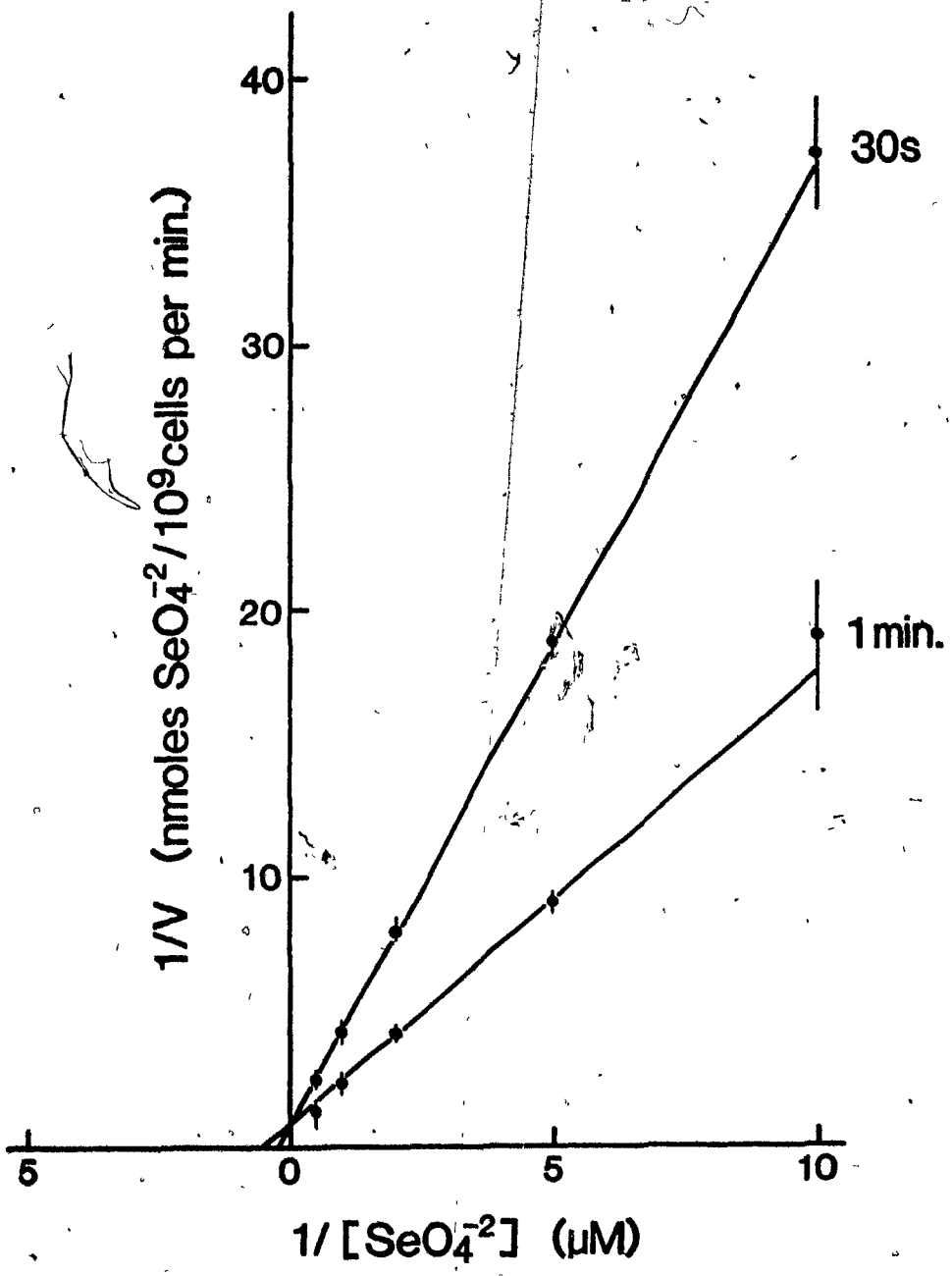

1/V (nmoles L-cysteine /10⁹cells per min.)
30
20
10
30s
1 min.
1
0
1
2
3
4
5
1/ [L-cysteine] (μM)

Figure 11. Double reciprocal plot of apparent saturation kinetics of selenate uptake by N. meningitidis.


40
30
20
10
1/V (nmoles SeO_4^{-2}/10^9cells per min.)
30s
1 min.
5
0
5
10
1/[SeO_4^{-2}] (μM)

the apparent K_m values differ. The 30 s value for the apparent K_m is 5 uM while the 1 min value is 2 uM. These data indicate competitive inhibition, which results when the substrate binds to its receptor on the membrane and effects further substrate binding, resulting in an increase in the apparent K_m with no change in the V_{max} (Segel, 1975).

To further examine the interactions of sulphate and L-cysteine intracellularly, experiments were done in which the cells were exposed to radioisotopes, followed by addition of an unlabelled substrate to see if loss of the isotope occurred. Results are shown in Tables 12 and 13. When unlabelled 1 mM L-cysteine was added to cells labelled with 10 uM L-cysteine, the radioisotope was exchanged, resulting in a 5% reduction after 20 min. When equimolar unlabelled L-cysteine was added to cells which were labelled with ^{35}S-sulphate, an 11% exchange was seen. Exchange of the intracellular sulphur compound required the presence of an extracellular substrate as no loss of label was observed simply by washing the cells in transport buffer (data not shown).

iii. Kinetics of Inhibition

Competitive inhibition of sulphate uptake by selenate has been reported in E. coli (Karbonowska et al., 1977),

Table 12. Efflux of sulphate from N. meningitidis. Cells were exposed to ^{35}S-labelled sulphate for 5 min, at which time 10 uM L-cysteine was added and samples withdrawn for assay.

Time (min)[a]	^{35}S-labelled Sulphate in Cells[b]
0	100
5	97
15	90
25	89

[a] After addition of L-cysteine.
[b] 100% actual uptake = 1.89 nmol S/mg protein. Values are the average of two experiments run in duplicate.

Table 13. Efflux of L-cysteine from N. meningitidis. Cells were exposed to ^{35}S-labelled L-cysteine for 5 min, at which time 1mM unlabelled L-cysteine was added and samples withdrawn for assay.

Time (min)[a]	^{35}S-labelled L-Cysteine in Cells[b]
0	100
5	97
15	95
20	95

[a] After addition of unlabelled L-cysteine.
[b] 100% actual uptake = 1.96 nmol L-cysteine/mg protein. Values are the average of two experiments run in duplicate.

while mixed inhibition of sulphate by selenate has been reported in Salmonella typhimurium (Brown and Shrift, 1980). In E. coli, both selenate and selenite are competitive inhibitors of sulphate (Lindblow-Kull et al., 1985).

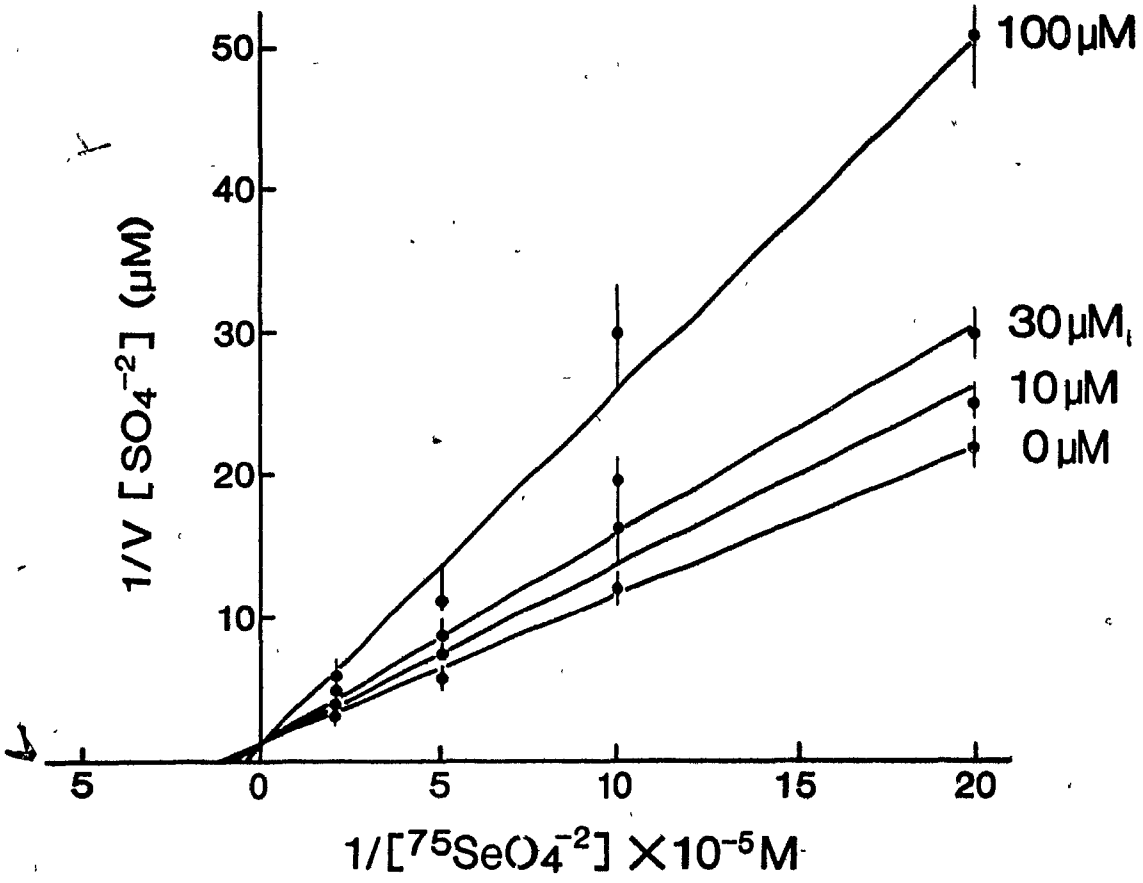
To determine the type of inhibition occurring with sulphate and selenate, uptake was assayed at varying substrate concentrations as the concentration of inhibitor was also varied. The results are seen in Figure 12. As the concentration of sulphate was increased (A), the apparent K_m increased while the V_{max} remained the same. The same effect was seen using radiolabelled sulphate as the substrate and selenate as the inhibitor (B), strongly suggesting that the two ions are competing for uptake by a common system. These same data represented in a Dixon plot (Figure 13) show the K_I values, 80 uM for sulphate inhibition of selenate; 19 uM for selenate inhibition of sulphate, indicating that selenate ion is the preferred substrate for the sulphate-selenate transport system.

In comparing sulphate to L-cysteine, only radiolabelled sulphate was used as a substrate with unlabelled L-cysteine as the inhibitor because 1 mM sulphate had no inhibitory effect on L-cysteine uptake in N. meningitidis SD1C. The results are presented in Figure 14. As the concentration of L-cysteine was increased, the apparent K_m remained the same but the V_{max} decreased, indicating that L-cysteine is a non-

Figure 12. Kinetics of sulphate uptake and selenate uptake in N. meningitidis.

(A) Uptake of ^{75}Se-selenate alone and in the presence of varying concentrations of sulphate.

(B) Uptake of ^{35}S-sulphate alone and in the presence of varying concentrations of selenate.


100 µM
30 µM
10 µM
0 µM
50
40
30
20
10
5
0
5
10
15
20
1/V [SO4-2] (µM)
1/[75SeO4-2] ×10-5M

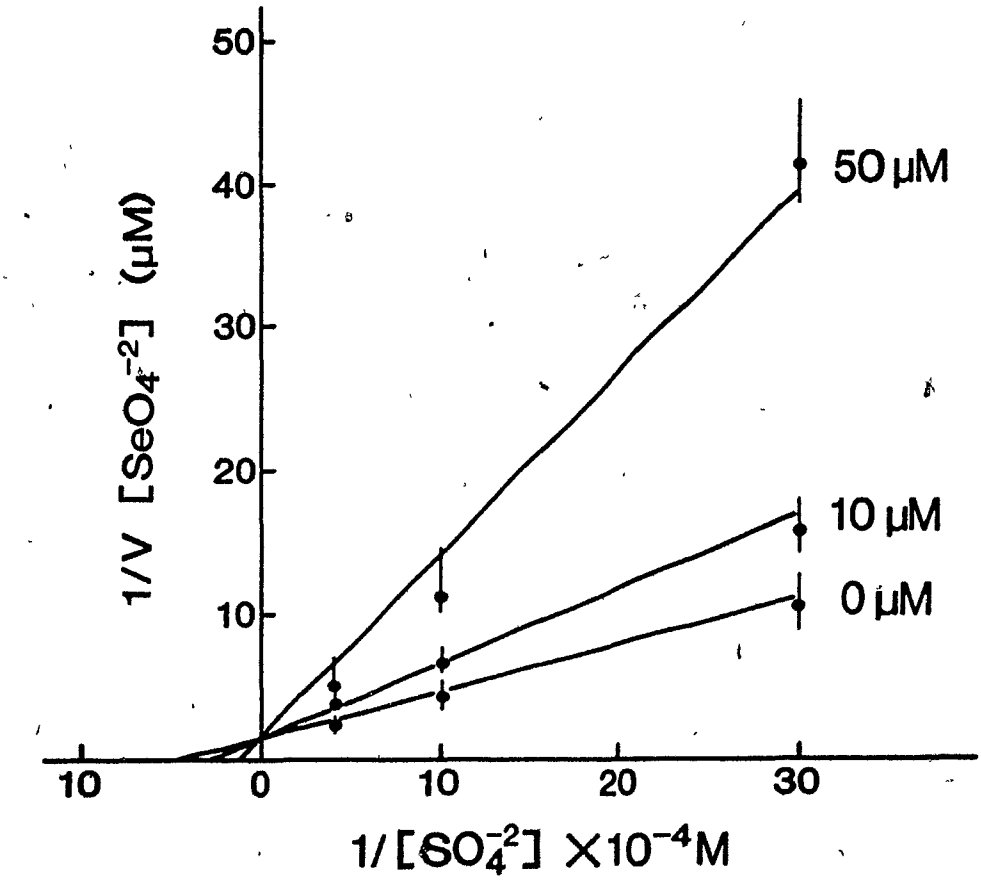
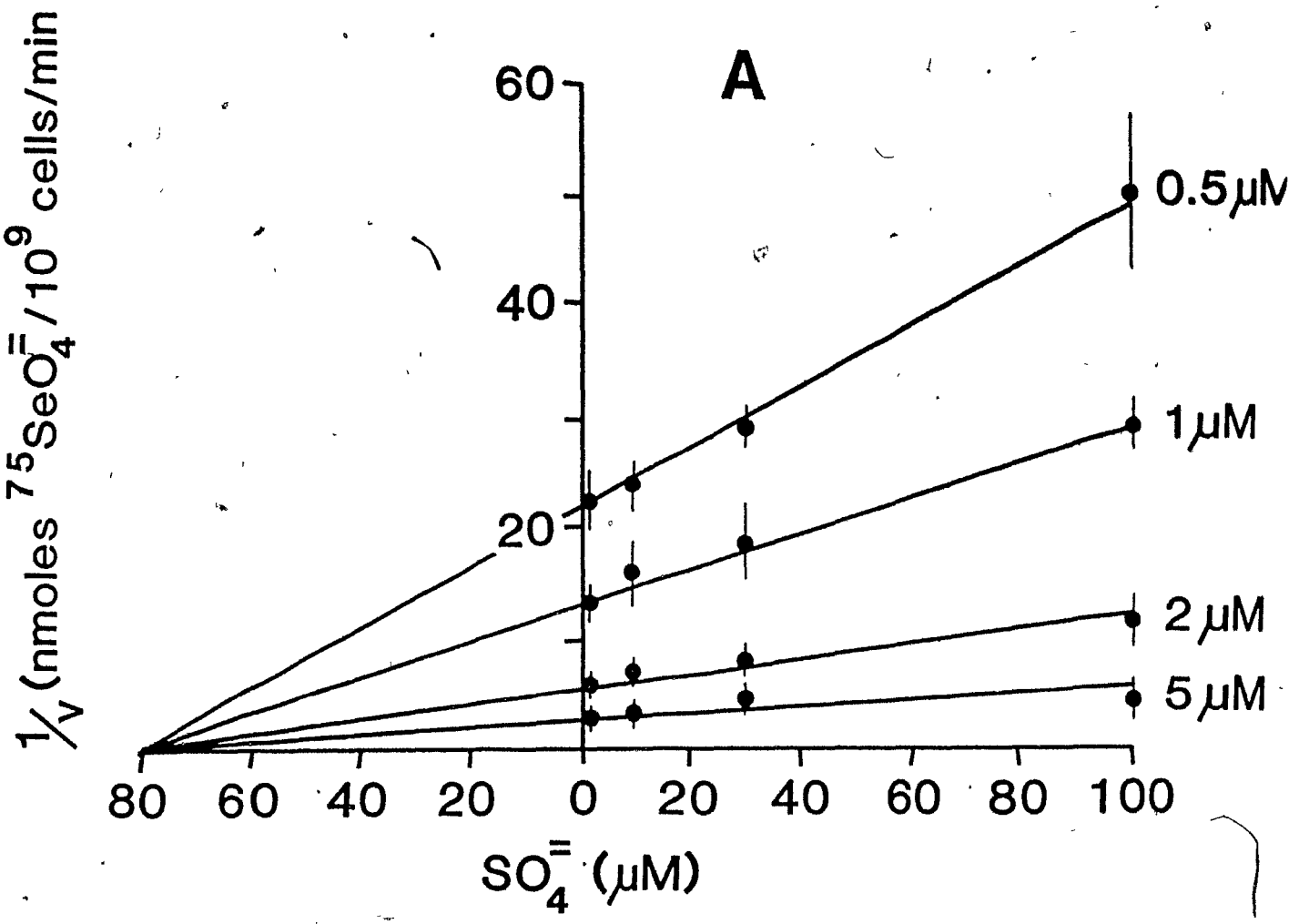

50 µM
10 µM
0 µM
50
40
30
20
10
10
0
10
20
30
1/V [SeO4-2] (µM)
1/[SO4-2] ×10-4M

Figure 13. Determination of the K_i of sulphate for ^{75}Se-labelled selenate uptake (A) and the K_i of selenate for ^{35}S-labelled sulphate uptake (B) by sulphur-starved cells of N. meningitidis.

$V_{initial}$ values were determined on duplicate 0.5 mL portions of cells 1 min after the simultaneous addition of radiolabelled and unlabelled ions to the assay mixture.


A
60
40
20
0.5μM
1μM
2μM
5μM
80 60 40 20 0 20 40 60 80 100
$SO_4^=$ (μM)
$1/v$ (nmoles $^{75}SeO_4^=/10^9$ cells/min

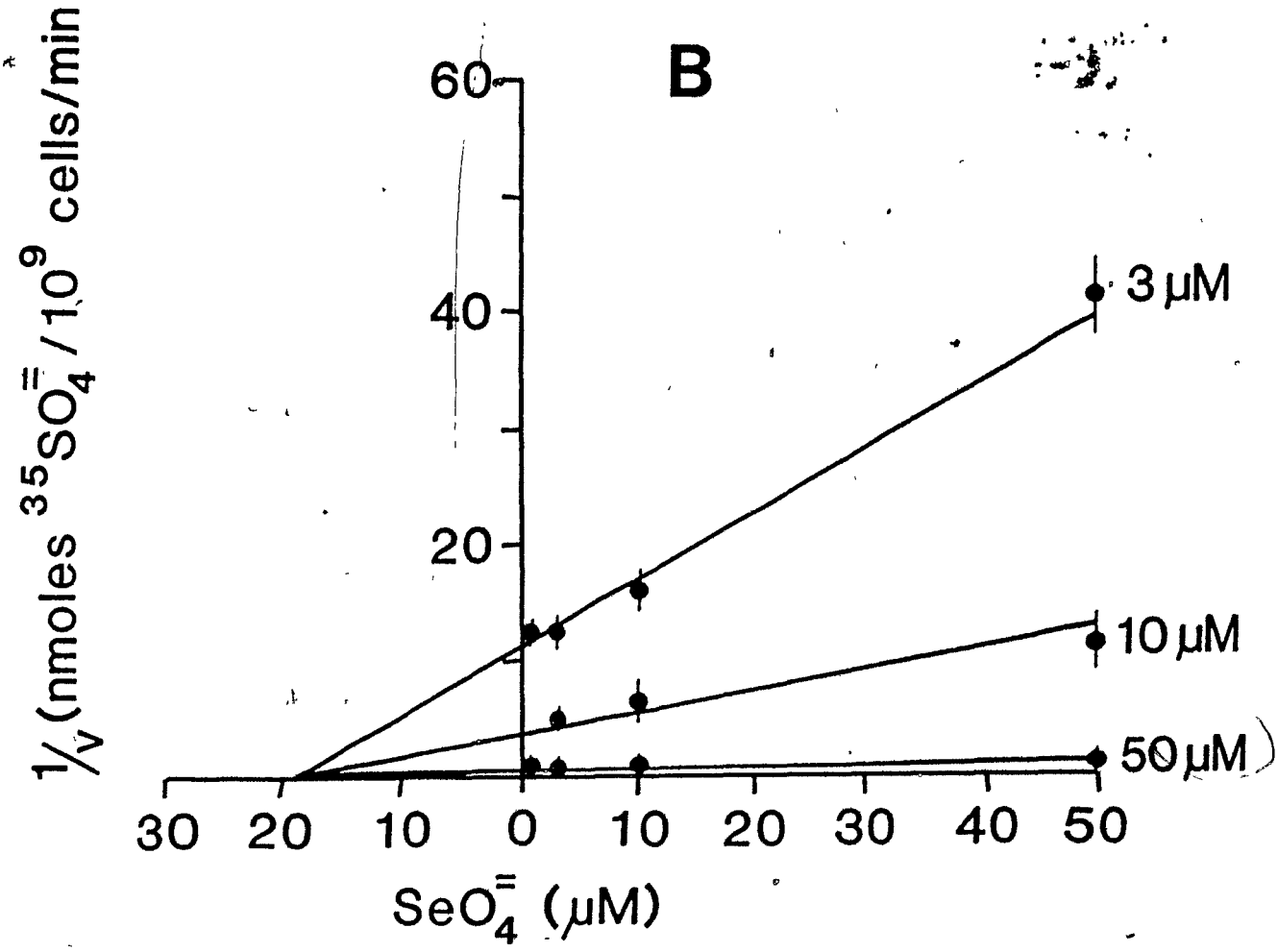
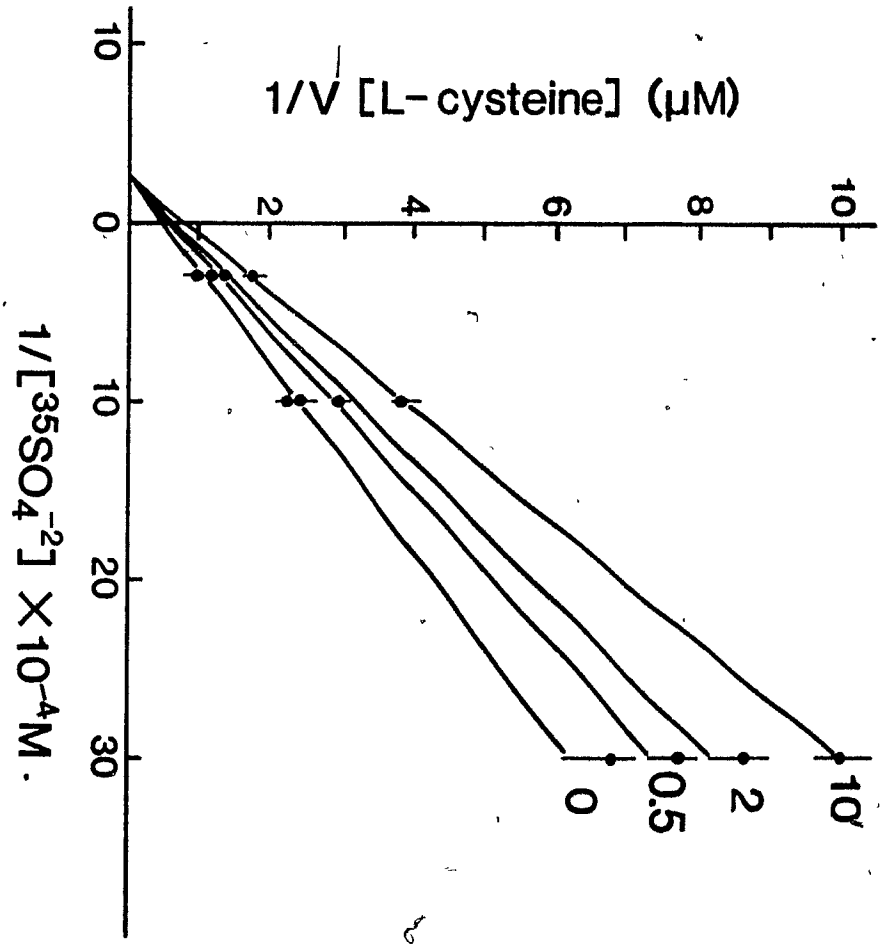

B
60
40
20
3μM
10μM
50μM
30 20 10 0 10 20 30 40 50
$SeO_4^=$ (μM)
$1/v$ (nmoles $^{35}SO_4^=/10^9$ cells/min

Figure 14. Kinetics of L-cysteine uptake by N. meningitidis.

Uptake of ^{35}S-sulphate alone and in the presence of varying concentrations of L-cysteine. V = uM per mg protein per min.

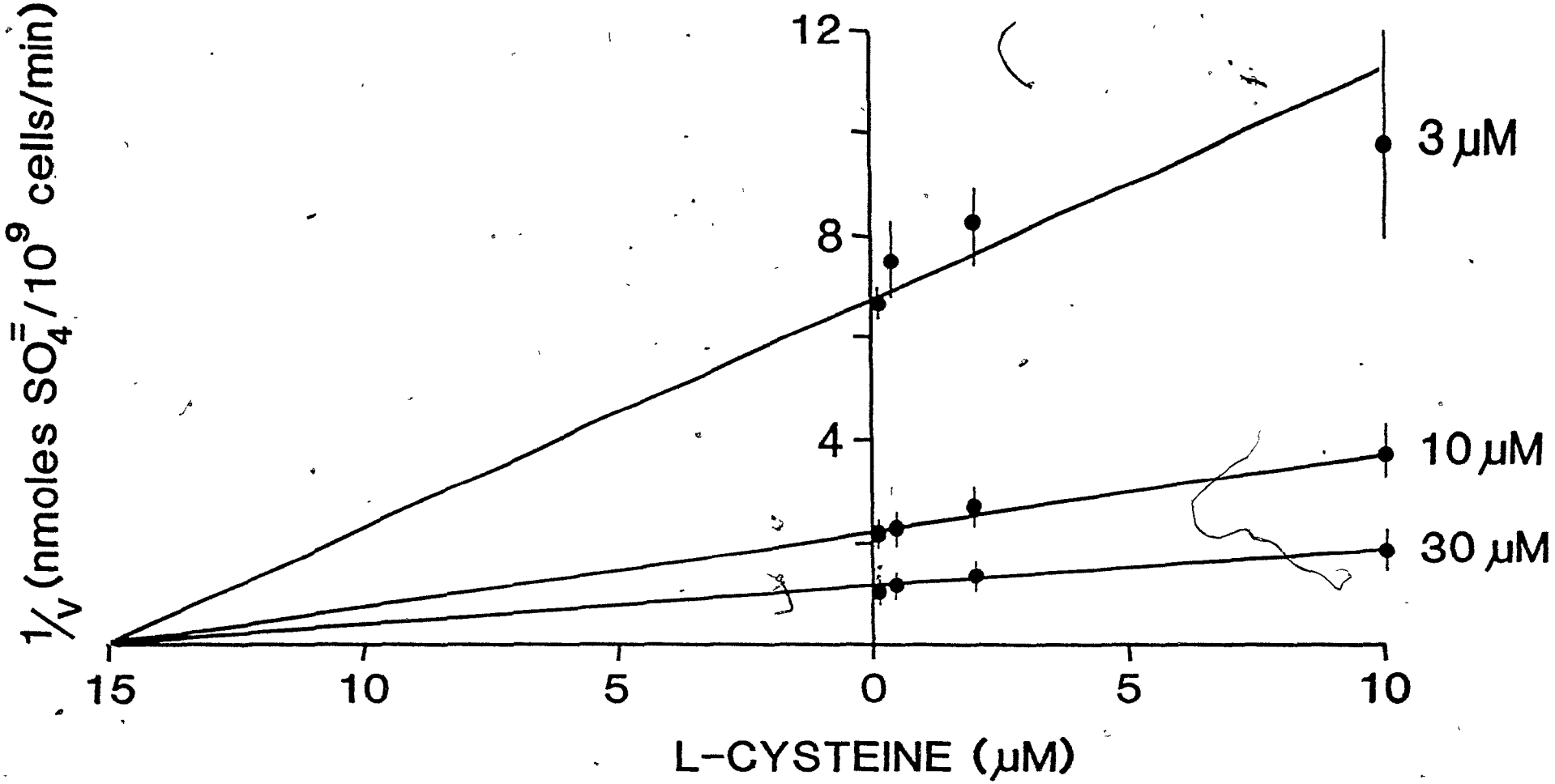

1/V [L-cysteine] (μM)
1/[35SO4-2] X10-4M.
10
0
10
20
30
2
4
6
8
10
0
0.5
2
10

competitive inhibitor of sulphate in the meningococcus. A Dixon plot of these data (Figure 15) shows the K_i of L-cysteine inhibition of sulphate was 15 uM.

Summary

Selenate and L-cysteine were actively transported by the meningococcus. Like sulphate, binding of selenate to the cells was not detected in the presence of metabolic poisons. Inhibition of selenate uptake with competing substrates and analogues was similar to that seen in sulphate; L-cysteine transport was unaffected by other inorganic sulphur substrates or analogues. The saturation kinetics of selenate uptake was like that of sulphate, showing a decrease in apparent K_m with no change in V_{max} between 30 s and 1 min, suggestive of competitive inhibition. L-cysteine uptake exhibited an unusual pattern, an increase in V_{max} without an increase in the apparent K_m, which has been noted in manganese transport in B. subtilis Loss of intracellular sulphate and L-cysteine to the medium occurred in the presence of L-cysteine. Selenate and sulphate were mutually competitive inhibitors and appeared to be transported by a common system. L-cysteine uptake appeared to occur by a separate system.

Figure 15. Determination of the K_i of L-cysteine for ^{35}S-labelled sulphate uptake by sulphur-starved cells of N meningitidis. Assay performed as described in legend of Figure 13.


12
8
4
3 µM
10 µM
30 µM
15
10
5
0
5
10
1/v (nmoles SO4= / 10^9 cells/min)
L-CYSTEINE (µM)

IV. Effects of Sulphur Starvation on N. meningitidis

Deprivation of a substrate is often necessary to derepress the transport system for that substrate. This phenomenon is observed in sulphate transport in other organisms, including algae (Vallee and Jeanjean, 1968a, b), cyanobacteria (Utkilen et al., 1976), and in the present study of the meningococcus. In E. coli (Roberts et al., 1955) and S.typhimurium (Dreyfuss, 1964), growth on organic sulphur sources represses sulphate uptake while in P. halodurans (Cuhel et al., 1981a) sulphate transport is stimulated when the cells are pregrown on sulphur-containing amino acids. These characteristics reflect the regulatory processes within the cell which assure a steady supply of nutrients.

Since sulphur starvation promoted maximal sulphate uptake in the meningococcus, a closer look at the effects of starvation was taken in an effort to understand the regulation of sulphur metabolism. The first experiment set out to determine two things: (1) will cells grown on other sulphur sources take up labelled sulphate? and (2) is sulphur starvation necessary in order for them to take up labelled sulphate?

Meningococci were grown to late log phase on one of five sulphur sources. The cells were then either exposed to ^{35}S-

labelled sulphate while sulphur-sufficient, or starved for 2 h to deplete intracellular sulphur before exposure to the labelled sulphate. Results are shown in Table 14 and Figure 16. As seen previously (Figure 5), sulphate starvation enhances the amount of sulphate taken up by N. meningitidis by about 6-fold. Meningococci grown on other sulphur sources took up appreciable amounts of sulphate only when they were starved for sulphur. When inorganic sulphur sources were used for initial growth, the rate of sulphate transport by sulphur-starved cells was equal to that of sulphate-grown cells; the extent of sulphate transport at 5 min was equal to or slightly greater than the extent seen with those grown in and starved for sulphate. With organic sulphur in the growth medium, sulphur-sufficient cells took up none of the labelled sulphate; however, when cells were grown in L-cysteine or glutathione and sulphur-starved they took up 32% and 52% of the amount of labelled sulphate transported by the sulphate grown, sulphate-starved cells, respectively. This is analogous to the system in S. typhimurium in which growth on L-cystine represses sulphate and selenate transport (Brown and Shrift, 1980).

A comparison of sulphate and L-cysteine transport was made, using each as the sole source of sulphur for growth with the other as the ^{35}-S-labelled form. Results are shown in Table 15. As seen in the previous experiment, sulphur-

Table 14. Extent of uptake at 5 min of 10 uM sulphate by sulphur-sufficient and sulphur-starved N. meningitidis grown on a variety of sulphur sources. Meningococci were grown to late log phase in 1 mM sulphur and harvested and washed. Cells were used immediately to assay sulphate uptake or starved for 2 h prior to assaying uptake.

Cell Growth Sulphur Source	Sulphate in Cells (%)[a]	
	Sulphur-sufficient Cells	Sulphur-starved Cells
Sulphate	16	100
Thiocyanate	8	>100
Thiosulphate	8	>100
Reduced glutathione	0	52
L-cysteine	0	32

[a] The value obtained for maximal sulphate uptake by sulphate-starved cells was defined as 100% Actual sulphate uptake at 100% = 1.91 nmol S/mg protein. Values are the average of two or more experiments run in duplicate.

Figure 16. Rates and extents of sulphate uptake by sulphur-starved and sulphur-sufficient cells of N. meningitidis. Cells were grown in MNDM to late log phase, harvested, washed, and either resuspended in sulphur-free medium for 2 h or used immediately in the transport assay.

▲ = sulphur-starved cells

● = sulphur sufficient cells

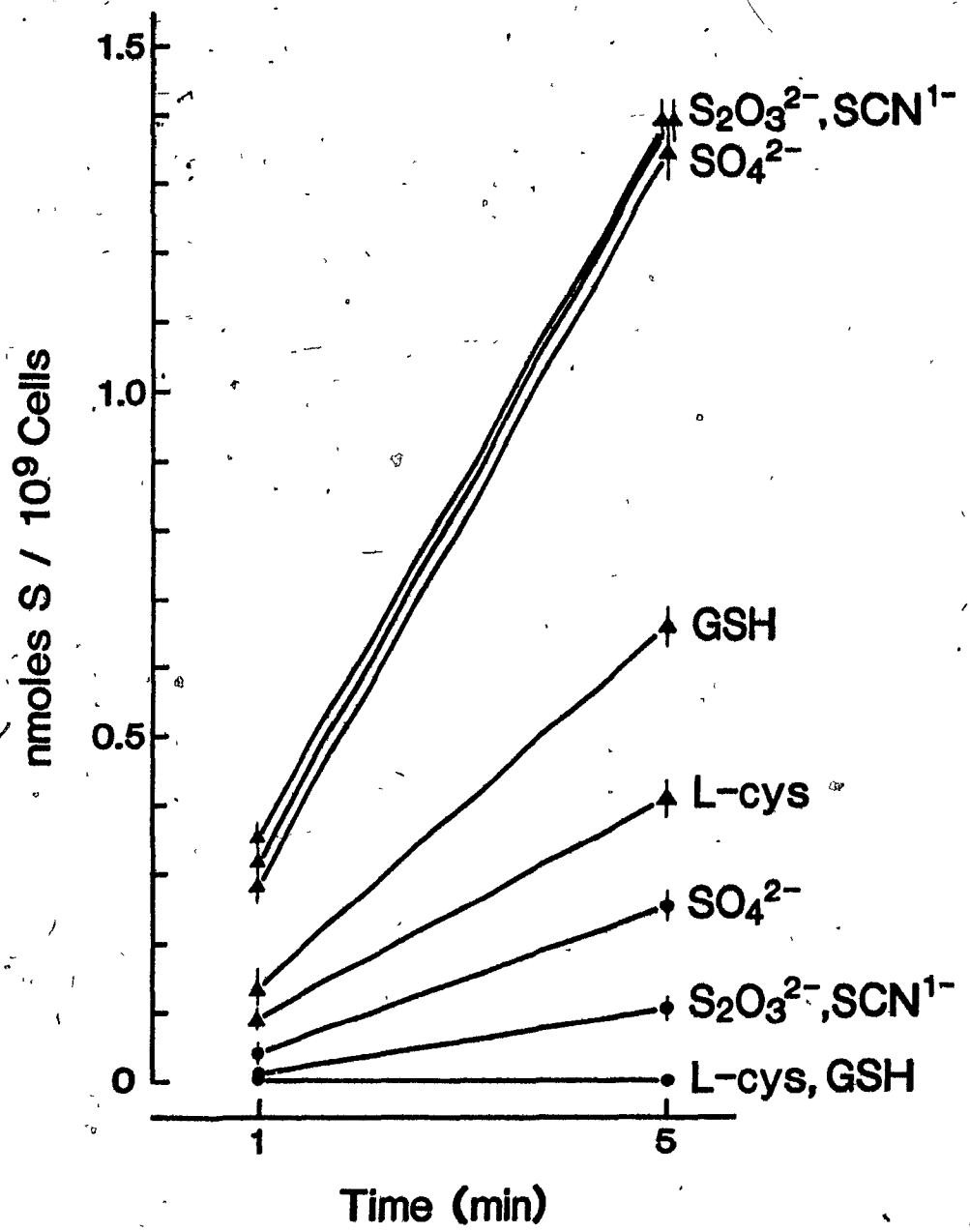

1.5
1.0
0.5
0
nmoles S / 10^9 Cells
$S_2O_3^{2-}$, SCN^{1-}
SO_4^{2-}
GSH
L-cys
SO_4^{2-}
$S_2O_3^{2-}$, SCN^{1-}
L-cys, GSH
1
5
Time (min)

Table 15. Extent of uptake of 10 uM sulphate or L-cysteine by N. meningitidis pre-grown on and subsequently starved for either sulphate or L-cysteine. Cells were grown to late log phase, harvested, washed, and resuspended in sulphur-free medium for 2 h. The rates of uptake of either ^{35}S-labelled sulphate or ^{35}S-labelled L-cysteine were measured over 5 min.

Cell Growth Sulphur Source	^{35}S-labelled Sulphur Substrate	nmol labelled S/mg Protein
Sulphate	Sulphate	1.44
L-cysteine	Sulphate	0.64
Sulphate	L-cysteine	2.48
L-cysteine	L-cysteine	3.00

Values are the average of two or more experiments run in duplicate.

starved cells grown on L-cysteine take up less than half as much labelled sulphate as sulphur-starved cells grown on sulphate. However, sulphur-starved cells exposed to ^{35}S-labelled L-cysteine took up more label than did the cells exposed to labelled sulphate, regardless of sulphur source used for initial growth. These data are consistent with previous data which showed L-cysteine to be transported at a faster rate than sulphate (Figure 8) by sulphur-starved cells. These results suggest that sulphate starvation not only derepressed the sulphate permease but was highly effective in derepressing the L-cysteine transport system as well.

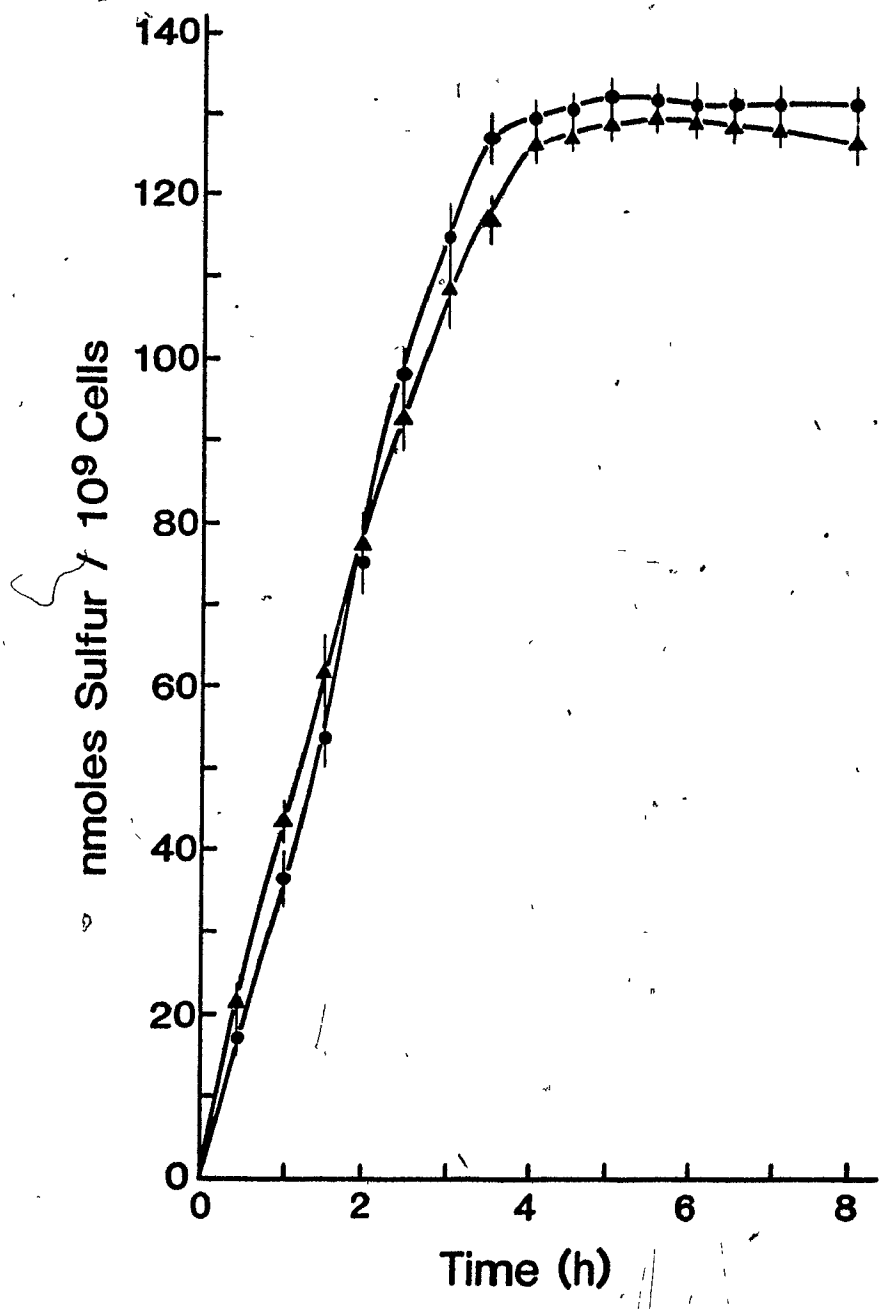
Although sulphate and L-cysteine are transported at different initial rates and appear to be transported by different mechanisms in N. meningitidis SD1C, either compound was an excellent sole source of sulphur and no difference was seen in the rate or extent of growth (Figure 3). These results were confirmed when meningococci were grown with either ^{35}-S-labelled sulphate or ^{35}S-labelled L-cysteine as the sole source of sulphur (Figure 17). There was no lag phase as the cells were sulphur-starved at the time of inoculation and sulphur transport as well as growth was maximal. Growth was exponential for over 4 h, followed by a typical stationary phase with a maximum of 130 nmol sulphur per 10^9 cells.

Figure 17. Uptake of ^{35}S-labelled sulphate and ^{35}S-labelled L-cysteine (500 uM) by N. meningitidis.

Cells were pre-grown to late log phase in MNDM with either 1 mM sulphate or 1 mM L-cysteine, harvested, washed, and resuspended in MNDM containing the radiolabelled sulphur source. Growth was followed for 8 h.

● = L-cysteine

▲ = Sulphate


140
120
100
80
60
40
20
0
nmoles Sulfur / 10^9 Cells
0
2
4
6
8
Time (h)

The effects of sulphur starvation on certain enzyme levels in N. meningitidis SD1C were determined using API-ZYM^{R}, a semi-quantitative micromethod system designed for the detection of enzyme activities. Results are shown in Table 16. Of 19 enzymes assayed, 13 were not detectable in the meningococcus. Sulphur-starved cells had slightly reduced levels of cystine aminopeptidase and acid phosphatase. Alkaline phosphatase levels were halved while esterase (C4) and esterase-lipase (C8) were reduced four-fold in sulphur-starved meningococci. Of particular interest was leucine aminopeptidase which was increased two to three-fold in the sulphur-starved meningococci. The results of this simple assay show that deprivation of a major nutrient had an effect on the level of all the enzymes detectable in this assay.

To look at the fate of the sulphur atom inside the meningococcus, a variation of the method used by Cuhel et al., (1981a) was used. The distribution of low molecular weight sulphur compounds in the soluble pool was determined during normal growth. These same cells were then put under sulphur-limited conditions and changes in the soluble sulphur pool were monitored. Results are shown in Figure 18. The low molecular weight organic sulphur pool in N. meningitidis increased in a linear fashion during the 4 h growth period, reaching a maximum of 38.1 nmol sulphur per

Table 16. Effects of sulphur starvation on certain enzyme levels in N. meningitidis cells. Sulphur-sufficient and sulphur-starved meningococci were assayed using API-Zym analytical enzyme assay strips.

Enzyme	Sulphur-sufficient Cells	Sulphur-starved Cells
Alkaline phosphatase	3-5[a]	1-2
Esterase (C4)	10	2-3
Esterase-lipase (C8)	10	2-3
Leucine aminopeptidase	15-20	35-45
Cystine aminopeptidase	5	2-3
Acid phosphatase	5	2-3
Lipase (C14)	0	0
Valine aminopeptidase	0	0
Trypsin	0	0
Chymotrypsin	0	0
Phosphoamidase	0	0
alpha-galactosidase	0	0
beta-galactosidase	0	0
beta-glucuronidase	0	0
alpha-glucosidase	0	0
beta-glucosidase	0	0
N-acetyl-b-glucosaminidase	0	0
alpha-mannosidase	0	0
alpha-fucosidase	0	0

[a] Approximation of nmoles of enzyme present per 5.5×10^8 cells based on the visual determination of the amount of colour development.

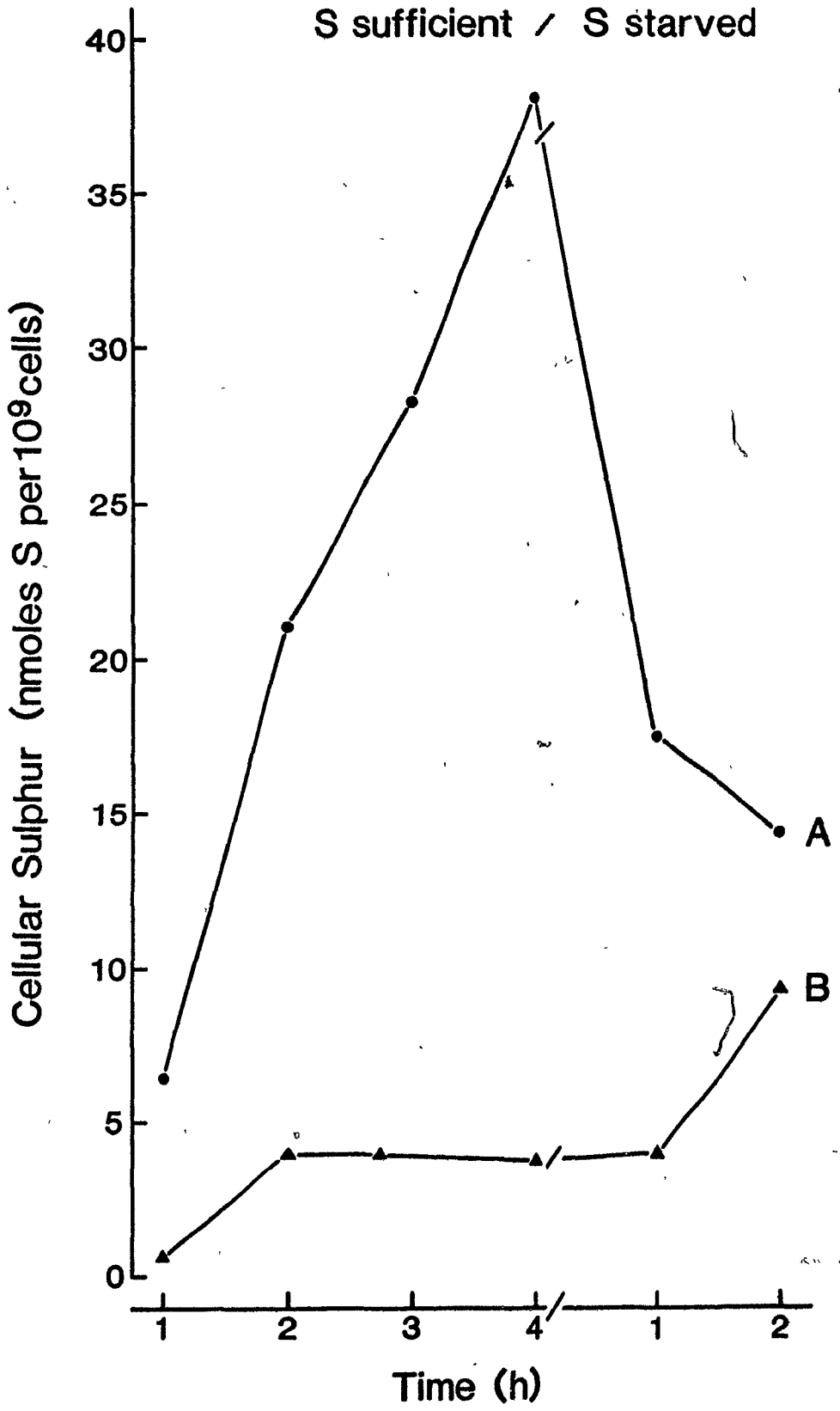
Values are the average of two experiments.

Figure 18. Distribution of low molecular weight sulphur compounds in soluble pools in N. meningitidis.

Meningococci were grown in MNDM containing 100 uM ^{35}S-sulphate for 4 h. Samples were removed each h and assayed to determine the levels of sulphur in the soluble pools under sulphur-sufficient conditions. At 4 h, the remaining cells were harvested, washed, and resuspended in MNDM containing 1 uM unlabelled sulphate. Incubation was continued at 37^{0}C and samples were removed and assayed at 1 and 2 h sulphur starvation.

A = soluble low molecular weight sulphur pool

B = inorganic sulphate


S sufficient / S starved
Cellular Sulphur (nmoles S per 10^9 cells)
40
35
30
25
20
15
10
5
0
A
B
1
2
3
4
1
2
Time (h)

10^9 cells. The inorganic sulphate pool levelled off after 1 h and remained fairly constant at a level of 3.7 nmol per 10^9 cells. After 4 h incubation, the cells were rinsed free of sulphate-containing medium and resuspended in sulphur-free MNDM. The immediate loss of radioactivity from the soluble sulphur pool is similar to what is reported for sulphur starvation in E. coli (Roberts et al., 1955). Half of the soluble low molecular weight sulphur pool (predominantly glutathione) was depleted within the first hour, with little change seen in the inorganic sulphate pool. However, at 2 h the low moleccular weight pool had been reduced further accompanied by an increase in the sulphate pool. It was at this point in time (2 h) that maximal sulphate was observed in these cells. These data suggest that one or more organic sulphur compounds represses the synthesis of the sulphate transport system. When the levels of this compound(s) are sufficiently depleted, the system is derepressed and sulphate uptake proceeds. The increase in the intracellular sulphate pool between 1 and 2 h may indicate that the conversion of organic sulphur to sulphate ion is necessary for the biosynthesis of the transport system.

Summary

Sulphur starvation is an important factor in preparing the meningococcus to transport sulphur-containing substrates. Sulphate starvation enhanced the rate and extent of sulphate and L-cysteine uptake. Six enzymes which could be detected in the meningococcus were affected by sulphur starvation. Analysis of the soluble sulphur pool showed that organic sulphur depletion accompanied by an increase in sulphate is necessary for synthesis of the sulphate transport system.

V. Nutrition and Membrane Composition in N. meningitidis

Changes in the composition of cell membranes in response to a changing nutritional or physical environment is well documented in Gram-negative bacteria (Ellwood and Tempest, 1972; Robinson and Tempest, 1973). When E. coli is limited for glycerol, it becomes highly virulent whereas sulphur limitation results in an avirulent population (Ellwood, 1974). The surface protein profiles on polyacrylamide gels of E. coli change in response to carbon and sulphur sources, temperature, and availability of iron (Lutgenberg et al., 1976; McIntosh and Earhart, 1976). When Enterobacter aerogenes is limited for glucose or sulphate, no differences

in the outer membrane are observed when examined in thin section using electron microscopy; however, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) reveals major differences in the outer membrane proteins (Robinson and Tempest, 1973).

The chemical composition of the pathogenic neisseriae are also affected by environmental conditions (Norqvist et al., 1978; Hoff and Grasch, 1979). It was therefore likely that growth of N. meningitidis on different single sulphur sources would result in differences in cell wall composition detectable by SDS-PAGE. These differences would reflect protein induction or repression, lending evidence to the idea of more than one transport system for sulphur in the meningococcus.

Cells were grown on different single sulphur sources, separated into two fractions, an envelope fraction containing both inner and outer membranes, and a cytoplasmic fraction and subjected to SDS-PAGE. The results of the SDS-PAGE are shown in Figures 19 and 20. Table 17 summarizes the changes seen in protein composition as the sulphur sources varied. While several bands showed increases in the envelope fraction, the only difference in the cytosol fraction was a lack of 21-23 Kd protein in the sulphate-grown cells. This may reflect conditions during which a sulphate-transporting protein is derepressed in the presence

Figure 19. SDS-PAGE of membrane fractions of N. meningitidis grown in a variety of sulphur sources as follows:

1 = Molecular weight standards

2 = Sulphate

3 = Sulphite

4 = Thiocyanate

5 = Thiosulphate

6 = Bisulphite

7 = L-cysteine

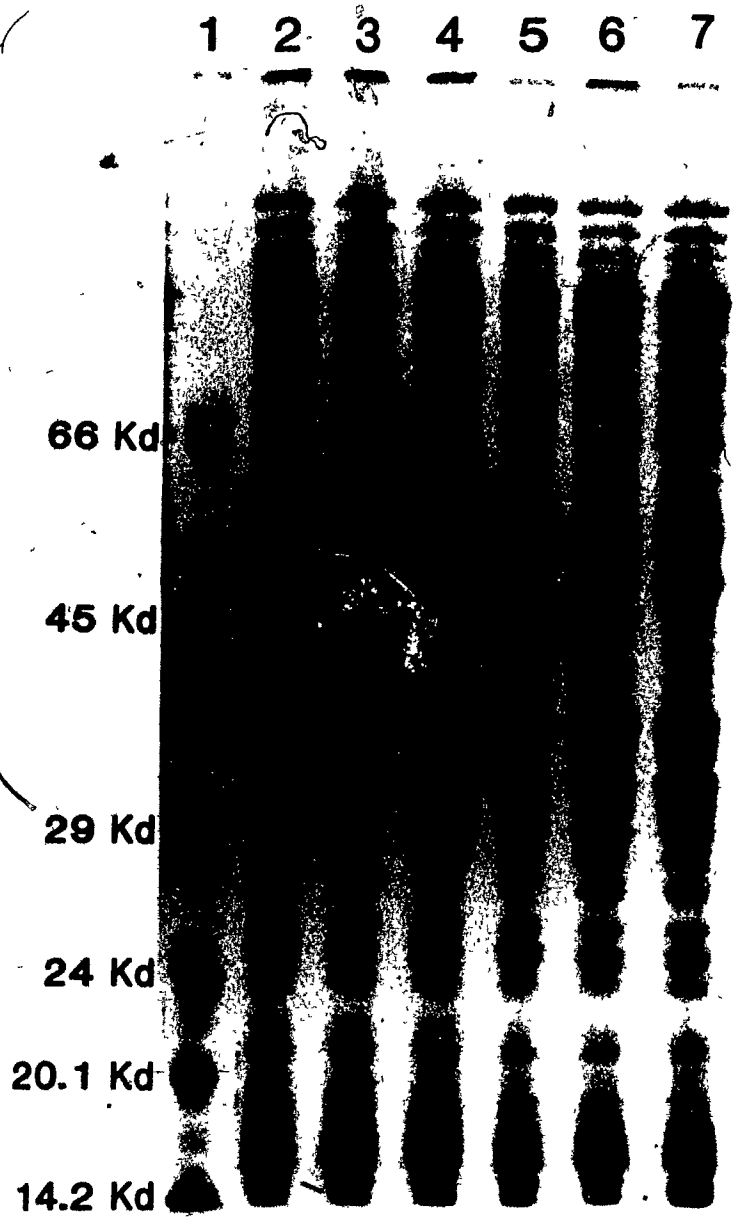

1
2
3
4
5
6
7
66 Kd
45 Kd
29 Kd
24 Kd
20.1 Kd
14.2 Kd

Figure 20. SDS-PAGE of cytosol fractions of N. meningitidis grown in a variety of sulphur sources as follows:

1 = L-cysteine

2 = Mueller-Hinton broth

3 = Thiosulphate

4 = Bisulphite

5 = Thiocyanate

6 = Sulphate

7 = Sulphite

8 = Molecular weight standards

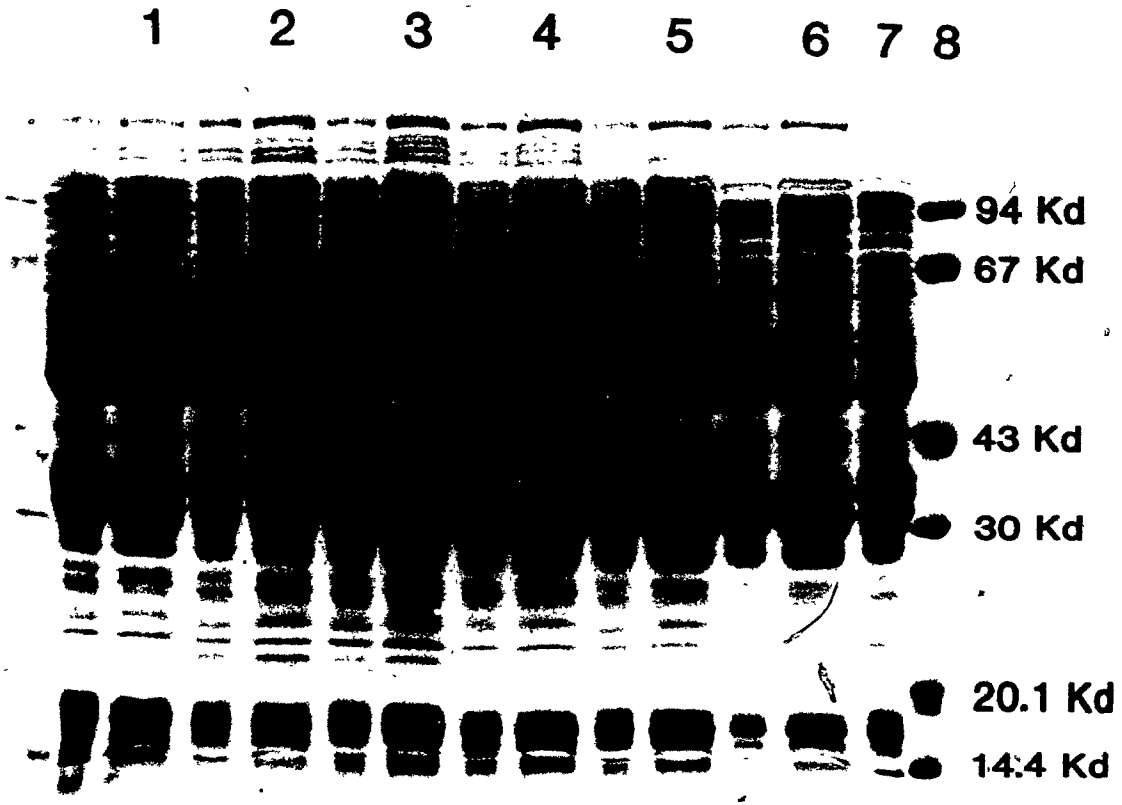

1
2
3
4
5
6
7
8
94 Kd
67 Kd
43 Kd
30 Kd
20.1 Kd
14.4 Kd

Table 17. Changes in protein bands in SDS-PAGE of membrane and cytosol fractions of N. meningitidis grown under sulphur-sufficient conditions.

Cell Growth Sulphur Source	Membrane Protein Band	Cytosol Protein Band
L-cysteine	60 Kd 14 Kd	50 Kd
Thiocyanate	60 Kd 34 Kd	
Sulphate	34 Kd 26 Kd	21-23 Kd
Sulphite	34 Kd 29 Kd	
Bisulphite	34 Kd 29 Kd	
Thiosulphate	34 Kd 26 Kd	

[a] Numbers are the approximate molecular weights as estimated against protein standards.

of sulphate and may explain the reason that sulphur starvation was necessary for sulphur uptake in *N. meningitidis*.

DISCUSSION

DISCUSSION

I. Introduction

Despite the fact that N. meningitidis is the etiological agent of widespread epidemics of meningococcal disease (DeVoe, 1982), the pathogenesis of this organism is still poorly understood. Elucidating the mechanisms of bacterial parasitism requires an understanding of the physiology of the parasite. An important facet of this is determining how the parasite acquires vital nutrients from the host, while the key to survival of the host is sequestering nutrients from the invading pathogen (Weinberg, 1978).

In the case of iron, the host attempts to sequester this micronutrient on the serum glycoprotein transferrin, reducing the concentration of circulating free iron to a level which will not support growth of the meningococcus. However, iron limitation by the host induces the meningococcus to synthesize a high-affinity system which specifically recognizes transferrin and removes iron from it, thereby successfully overcoming this host defence mechanism (Archibald and DeVoe, 1979; 1980; Simonson et al., 1982). Since availability of iron plays a critical role in the virulence of the meningococcus, an initial goal of this

work was to determine if the acquisition of sulphur is also important in meningococcal disease.

II. Sulphur Acquisition in the Meningococcus

To look at the role of sulphur availability in relation to meningococcal disease it was necessary to start at the most basic level since there was essentially nothing known about sulphur metabolism in the neisseriae. A list of potential sulphur sources was compiled. Sulphate and L-cyst(e)ine are sulphur sources for the meningococcus (Catlin, 1973) and were obviously included. However, there are other sulphur-containing compounds in the human host which, depending on the metabolic capabilities of the meningococcus, were potential sulphur sources (Diem and Lentner, 1970) and were included. Other compounds were selected as they are interesting sulphur sources for other bacteria.

While the gonococcus has limited sulphur metabolic capabilities, requiring L-cysteine (or thiosulphate in some strains) for growth (Le Faou, 1984), the meningococcus was much more versatile. N. meningitidis used a variety of organic and inorganic sulphur compounds for growth. At 10 uM sulphate or thiosulphate, meningococci grew to a concentration of 10^9 cells per mL in 3 h while maximal

growth was achieved with 100 uM sulphur. This particular strain produces a fatal infection in 50% of mice inoculated with as few as 4.6×10^3 cells (Brener et al., 1981). In keeping with its status as a macronutrient, the sulphur requirement of N. meningitidis was considerably higher than its iron requirement. Archibald and DeVoe (1978) found that this organism requires 50 mg per mL (0.9 uM) iron for minimal doubling times in a defined medium. With sulphur, growth became limited when there was less than 2.1 ug sulphur per ng cell protein. Growth to maximal in vitro cell density (5×10^9 cells per mL) required 16 ug (0.5 mM sulphur) per mL.

Schook and Berk (1978) found that Pseudomonas aeruginosa pregrown in 0.5% sodium sulphate is able to use each of a variety of inorganic sulphur compounds as sole sulphur sources for growth, including sulphate, sulphite, dithionite, and thiosulphate, but not dithionate. This same pattern was seen with the meningococcus. Dithionate did support growth of N. meningitidis after a period of sulphur starvation. Dithionite, while an excellent sulphur source for the meningococcus, decomposes in aqueous solution to bisulphite and thiosulphate (Burlamacchi et al., 1969). While the decomposition rate depends on temperature and pH, the half-life of dithionite under the conditions used in these experiments is probably minutes. The two products of

dithionite breakdown were shown to be good meningococcal sulphur sources and are likely the species present when dithionite is the added sulphur substrate.

Rammler et al. (1964) found that when Enterobacter aerogenes is grown on methionine or lanthionine, longer generation times and less extent of growth is seen than when cells are grown on sulphate, sulphite, or thiosulphate. This was true with N. meningitidis as well. However, cystine-grown cells of E. aerogenes also have diminished growth rates, while cysteine-grown cells show maximal growth rates and extents. The meningococcus showed maximal growth on either L-cystine or L-cysteine.

The compounds available in the human host which support meningococcal growth are listed in Table 3. Thiocyanate, at the level it is found in the plasma, supported the growth of the meningococcus to 9 x 10^8 cells per mL. The other sulphur sources which are present in man are available at concentrations well above that required for excellent, unlimited growth of the meningococcus. Taurine, although present in the plasma at a concentration of 136 uM (Diem and Lentner, 1970), was not a sulphur source for N. meningitidis. Carrageenans were tested to see if the meningococcus was capable of removing the sulpho group at the cell surface without transporting the entire molecule. The structure of carrageenan is similar to that of the

sulphomucpolysaccharides, found in high concentrations in mucous secretions and cartilaginous tissues (Diem and Lentner, 1970), and the ability to cleave extracellularly the sulpho group from this molecule might provide an additional mechanism for obtaining sulphur. However, no growth occurred with carrageenans.

Thiocyanate utilization is of particular interest not only because it is readily assimilated by the meningococcus, but heretofore has been rarely reported as a substrate for heterotrophic bacteria and then only in organisms much different than the neisseriae, namely Pseudomonas (Putilina, 1961; Stafford and Callely, 1969) and Arthrobacter (Betts et al., 1979). The relatively high concentration of thiocyanate in the saliva (0.41 to 6.6 mM), coupled with the absence of other known sulphur sources in this fluid, makes it the likely sulphur compound for the establishment of the meningococcus in the throat. Once the infection progresses from the carrier state to a bacteremia or meningitis, other sulphur sources, sulphate, glutathione, and cyst(e)ine are present at sufficient levels to ensure a constant sulphur supply. Unlike iron, the concentrations of sulphate and L-cysteine in host serum far exceed the apparent K_m values for transport of these compounds. Holbein (1981) demonstrated that the stimulation of meningococcal growth in serum is due to cysteine. Based on

these results, it is tempting to postulate that thiocyanate may serve as a sulphur source for other microbes which colonize the oropharynx.

The optimal and growth-limiting concentrations of sulphate for the meningococccus are close to those reported in other bacteria. Like B. subtilis (Springer and Huber, 1972) and P. aeruginosa (Schook and Berk, 1978), extent of growth is severely limited at 10 uM sulphate. Growth is optimal in P. aeruginosa at 100 uM sulphate while 200 uM is best for B. subtilis.

Although N. gonorrhoeae is said to have an absolute requirement for cyst(e)ine (Catlin, 1973), some strains can use thiosulphate as an alternate sulphur source due to the presence of a thiosulphate reductase (Le Faou, 1981). Therefore, finding this enzyme in N. meningitidis was not unexpected. The role of thiosulphate reductase activity in the neisseriae is unknown. It seems unlikely that the meningococcus could encounter a situation in the host where it would be wholly dependent upon thiosulphate as a sole source of sulphur. However, as microcolonies of meningococci are often found in deep purpura, areas where oxygen is extremely limited, the possibility exists that thiosulphate may act as a terminal respiratory acceptor for electrons during anaerobic growth (DeVoe et al., 1982). Although dissimilatory reduction of inorganic sulphur

compounds is generally assumed to be carried out only by obligate anaerobes, thiosulphate is used as a terminal electron acceptor for growth of a marine pseudomonad (Tuttle and Jannasch, 1973). Several members of the Enterobacteriaceae contain tetrathionate reductase which allows them to use this compound as a terminal electron acceptor for anaerobic respiration during carbohydrate metabolism (Oltmann et al., 1975; Papavassiliou et al., 1969; Le Minor and Pichinoty, 1963). The gonococcus grows anaerobically using nitrate as an alternate electron acceptor (E. P. Norrod, Letter, ASM News 53: 465,1987). Adding strength to the proposal that thiosulphate may serve as an alternate electron acceptor during growth in deep tissue, Clark et al. (1987) have recently reported in vivo anaerobic growth of N gonorrhoeae. Another possibility is that thiosulphate plays a role in the cysteine biosynthetic pathway in the meningococcus. In Aspergillus nidulans thiosulphate is an intermediate in a pathway involving cysteine-S-sulphonate as a direct precursor of cysteine (Nakamura and Sato, 1963).

Based on the information presented, the meningococcus appears to be versatile in its ability to acquire sulphur. It is unlikely that sulphur is a nutrient which can be limited in the host. On the other hand, the inability of the gonococcus to use sulphur sources other than cysteine

may explain its more limited ability to cause disseminated disease.

III. Transport Studies

i. Sulphate

As seen in many other systems including tobacco cells (Smith, 1975a,b; 1976), filamentous fungi (Segel and Johnson, 1961; Tweedie and Segel, 1969), Anacystis (Utkilen et al., 1976), and E. coli (Springer and Huber, 1972), sulphate transport in N. meningitidis was dependent on temperature, pH, concentration and energy.

The narrow pH range observed for sulphate transport may be a reflection of the general metabolism of this organism. At pH values below 6.6 or above 8.0, the rate of cell division in this strain is extremely slow (D. Brener, Ph.D. thesis, McGill University, Montreal, Quebec, 1981).

The apparent K_m values for sulphate were extremely close in many of the systems studied, with Chlorella (Vallee and Jeanjean, 1968a), Porphyridium (Ramus and Groves, 1972), Anacystis (Utkilen et al., 1976), Neurospora (Marzluf, 1970a), E. coli (Springer and Huber, 1972), and the meningococcus having values between 0.75 uM and 2.5 uM. The

100-fold greater apparent K_m values seen in marine bacteria (Cuhel et al., 1981a) probably reflect the high and constant concentration of sulphate (25 mM) in seawater.

The energy-dependence of sulphate transport in N. meningitidis seemed to be a function of the transmembrane pH gradient rather than the transmembrane electrical component. This finding agrees with the limited information available on the mechanism of entry of sulphate into the cell. In Paracoccus denitrificans sulphate transport is via electroneutral symport with hydrogen ions, driven by the pH gradient (Burnell et al., 1975). In Penicillium notatum, divalent cations and hydrogen ions are necesssary for translocation of sulphate across the membrane (Cuppoletti and Segel, 1975).

The effect of structural analogues and other sulphur substrates was similar to that reported in other systems. Thiosulphate inhibition of sulphate transport occurs in S. typhimurium as these compounds share the sulphate permease (Dreyfuss, 1964). A common transport system for sulphate and thiosulphate operates in P. chrysogenum, although mutants lacking the sulphate permease are able to transport thiosulphate at 1-5% of the wild-type (or revertant) rate, as well as sulphite and tetrathionate, indicating that a second sulphur anion permease is present in these mutants (Tweedie and Segel, 1970). The effectiveness of

thiosulphate in abolishing sulphate uptake at equimolar concentrations in the meningococcus may reflect a preference for the reduced (sulphane) moiety relative to sulphate. Incorporation of the sulphane moiety of thiosulphate prior to the sulphite moiety or sulphate into amino acids is well documented (Dreyfuss and Monty, 1963; Hodson et al., 1968).

L-cysteine, a noncompetitive inhibitor of sulphate transport in N. meningitidis, caused an efflux of sulphate from cells preloaded with radiolabelled sulphate. This is in contrast to sulphate efflux in fungal systems or in S. typhimurium. Sulphate efflux in Neurospora is mediated by external sulphate or related ions but not by cysteine (Marzluf, 1974), whereas sulphate and cysteine transport in Penicillium and Aspergillus is unidirectional (Bradfield et al., 1970). In S. typhimurium, sulphate efflux is regulated internally. When net uptake increases the internal sulphate concentration to 100 uM, sulphate is rapidly excreted back into the medium.

Thiocyanate and tellurite are of interest as they were the only compounds which had no effect on sulphate transport, even at a concentration of 1 mM. Although tellurite is an effective inhibitor of sulphate uptake in S. typhimurium (Brown and Shrift, 1980), this ion is larger than sulphate or selenate and its uptake may be restricted by size. Size selectivity is a factor in the inhibition of

sulphate transport by group VI anions in marine bacteria; those closer in size to sulphate are better inhibitors (Cuhel et al., 1981a). As an excellent and biologically relevant source of sulphur for the meningococcus, the inability of thiocyanate to affect sulphate uptake indicates that is is not taken up via the sulphate permease. Based on the impermeability of the membrane and the fluctuating environmental conditions experienced by bacteria, Silver (1978) feels that it is highly unlikely that any ionic nutrient passively diffuses into the cell. It is very likely, therefore, that thiocyanate is transported by a mechanism other than the sulphate permease.

ii. Selenate

Several studies suggest that microorganisms transport selenate and sulphate by the same system (Tweedie and Segel, 1970; Shrift, 1973; Brown and Shrift, 1980; Lindblow-Kull et al., 1985). This appears to be true in the meningococcus. Binding and transport of both ions were energy-dependent. The apparent K_m values were similar for both substrates and the same increase in apparent K_m was seen when the values were measured at 30 s and 1 min, suggesting allosteric negative feedback in the uptake of substrate or rapid saturation of the binding sites followed by slower active

transport for both substrates. As seen in E. coli (Lindblow-Kull et al, 1985), sulphate and selenate were mutually competitive inhibitors. This is in contrast to the mixed inhibition of sulphate by selenate seen in S. typhimurium (Brown and Shrift, 1980). While sulphate is the preferred substrate in E. coli (Lindblow-Kull et al., 1985), selenate was the preferred ion for the sulphate transport system in the meningococcus. The preference of a high-affinity transport system for an ion which is toxic to the cell has been reported for the manganese-cadmium transport systems in L. plantarum (Duong and Archibald, 1984) and S. aureus (Perry and Silver, 1982). Meningococcal preference for selenate may be the mechanism used to acquire low levels of this compound for the synthesis of selenium-dependent enzymes. Although none have been identified in N. meningitidis, several selenium-containing proteins exist as essential components in other biological systems, including the formate dehydrogenase of E. coli and anaerobes, glycine reductase in clostridia, and glutathione peroxidase in mammals and birds (Stadtman, 1980). Selenocysteine is found at the active sites of bacterial selenium-dependent enzymes (Cone et al., 1976; Jones et al., 1979), and the presence of selenocysteine lyase has been demonstrated in a variety of bacteria (Chocat et al., 1983). A preference for selenate by the sulphate transport system would assure that the cell

acquires the trace amounts of selenium it requires without the need for a separate transport system for this ion. Since selenate occurs at very low concentrations in most environments (Stadtman, 1974), it is unlikely that competition with sulphate resulting in toxicity would occur.

iii. L-Cysteine

A striking feature of L-cysteine transport in the meningococcus was the inability of other sulphur compounds to inhibit uptake. Cystine and reduced glutathione were the only effective inhibitors of L-cysteine transport. Uptake of L-cysteine displayed Michaelis-Menten kinetics and was energy-dependent, strongly suggesting that a separate transport system for this amino acid exists in the meningococcus. Specific uptake systems for L-cystine exist in S. typhimurium (Kredich and Baptist, 1977) and E. coli (Leive and Davis, 1965a,b; Heppel and Berger, 1972). L-cysteine is an absolute requirement for gonococci and for strains of meningococci lacking sulphite reductase (Le Faou, 1984) as well as an important sulphur source for the meningococcus in the serum (Holbein, 1981). The results of this study coupled with this pertinent information strongly support the existence of a cysteine transport system in N. meningitidis.

Efflux of L-cysteine from the meningococcus may be partly responsible for the unusual kinetics displayed by this organism. The increase in V_{max} with no change in the apparent K_m for L-cysteine transport between 30 s and 1 min may be due to a regulatory mechanism in which the regulator interacts with the transporter to reduce uptake of L-cysteine without affecting efflux. This mechanism has been proposed as the model to explain this same kinetic pattern seen in manganese transport in B. subtilis (Fisher et al., 1973; Scribner et al., 1975).

IV. Internal Sulphur Regulation

Under conditions of sulphur limitation, changes occur in the sulphur-containing molecules as stored sulphur is released for use by the cell. How these storage compounds are transformed yields information about the metabolic and regulatory mechanisms operating within the cell. In most living cells, sulphur is stored as glutathione (L-glutamyl-L-cysteinyl-glycine), reaching concentrations of 10 mM (Kosower, 1976). This nonprotein tripeptide is the major component of the molecular weight thiol fraction in cells (Meister, 1975).

In E. coli, the size of the glutathione pool is regulated by the available amino acid pool (Apontoweil and

Berends, 1975), and specifically by the level of cysteine in the cells (Loewen, 1979).

The effects of sulphur sources used for growth and the response to sulphur limitation are manifested quite differently in S. typhimurium and P. halodurans. In S. typhimurium, both binding and transport activities are repressed by growth on L-cysteine (Pardee et al., 1966). The enzymes of the L-cysteine biosynthetic pathway in S. typhimurium (except serine transacetylase) are repressed by sulphide and cysteine and derepressed by sulphur starvation (Kredich, 1971). O-acetyl-L-serine, a direct precursor of L-cysteine, exerts internal control as it is also required for derepression.

Growth on sulphur-containing amino acids has the opposite effect in P. halodurans, resulting in a stimulation of sulphate transport (Cuhel et al., 1981a). This represents a unique system in that growth on endproducts of the biosynthetic pathway fails to exert a regulatory effect on sulphate transport. A regulator does exist as starvation is necessary to stimulate uptake in sulphate- and thiosulphate-grown cells. Enhancement of transport is coupled to a decrease in the low molecular weight pool in these cells, indicating that the regulator is a component of this pool. These authors have postulated that either APS or PAPS regulates sulphate transport in P. halodurans.

Sulphur starvation was clearly a prerequisite to the transport of sulphate in the meningococcus whether the cells were pregrown on organic or inorganic sulphur sources. During the sulphur depletion incubation period approximately a doubling (1.6 to 2.1 doublings) of the cells occurred, a far smaller biomass increase than that required to induce maximal uptake of other ions. Approximately 12 doublings are necessary for maximal manganese depletion and uptake stimulation in Lactobacillus plantarum (Archibald and Duong, 1984). N. meningitidis SD1C requires at least four doublings to achieve maximal iron uptake (Archibald et al., 1981). Thus it appears that there was relatively little storage of nonessential sulphur in the meningococcus.

A depletion of the soluble low molecular weight (LMW) sulphur pool during the 2 h incubation in the absence of sulphur began as soon as the meningococci were transferred to the sulphur-free medium (Figure 18). No change was seen in the inorganic sulphate pool until 1 h incubation, at which time a sharp rise between 1 and 2 h was noted in the amount of inorganic sulphate. This is in contrast what is seen in P.halodurans. In this bacterium, the (LMW) sulphur pool shows a steady decline while the sulphate pool remains constant (Cuhel et al., 1981a). In E. coli the radiolabelled LMW sulphur pool, which is 95% glutathione, begins to disappear as soon as the cells are transferred to

a sulphur-deficient medium, with a concomitant increase in radiolabelled cellular protein (Roberts et al., 1955). The size of the meningococcal LMW sulphur pool is in close agreement with that of Roberts et al. (1955). The glutathione pool comprises 25% of the cellular sulphur in E. coli and was 28% of the total sulphur in N. meningitidis. I believe that the meningococcal LMW sulphur pools regulate sulphate transport in the following manner. The LMW sulphur pool (glutathione and amino acids) is used immediately for protein synthesis and these components of the pool supply the cell's sulphur needs during the first hour of incubation. At this point, the level of a regulatory component in the pool is decreased to a concentration where the cell is or soon will be limited for sulphur. To survive, the cell must synthesize more sulphur-containing amino acids. In E. coli (Roberts et al., 1955), glutathione is depleted from the LMW sulphur pool prior to methionine. The slow growth rate and lower yield of meningococci seen with methionine as the sole source of sulphur relative to cysteine or glutathione indicates that conversion of methionine to cysteine is unfavourable. Therefore, intracellular cysteine and glutathione are more likely synthesized from sulphate. To accomplish this the meningococcus must oxidize components of the LMW sulphur pool to form sulphate, explaining the increase in the

inorganic sulphate pool seen between 1 and 2 h. This is well coordinated with the time noted for the maximal expression of sulphate transport in N. meningitidis, suggesting that the depletion of the LMW sulphur pool, the increase in the inorganic sulphate pool, or both are regulators of derepression of the sulphate permease in the meningococcus.

L-cysteine or glutathione are likely candidates as the internal regulator for derepression and synthesis of the sulphate permease. Pregrowth on these compounds repressed sulphate transport in the meningococcus and subsequent starvation resulted in only slight enhancement of sulphate transport. This leads me to believe that the inorganic sulphate pool does play a regulatory role in sulphate permease synthesis. It is reasonable to assume that the inorganic sulphate pool is negligible or absent in cells growing on amino acids or glutathione. When the cells are subjected to sulphur limitation they are able to use the LMW sulphur pool. As the pool is depleted, they have no readily available inorganic sulphate pool from which to synthesize new amino acids. This inorganic pool presumably can be created from the remaining LMW sulphur pool. The partial stimulation of sulphate transport seen when meningococci are pregrown on and subsequently starved for organic compounds may reflect this lag in the synthesis of

the inorganic sulphate pool.

L-cysteine was a potent inhibitor of sulphate transport in N. meningitidis. Growth of E. coli on L-cyst(e)ine represses synthesis of PAPS (Pasternak, 1962), but control by repression alone cannot explain the immediate cessation of radiolabelled sulphate incorporated into protein (Roberts et al., 1955). It is more plausible that L-cyst(e)ine is acting as an allosteric inhibitor of the sulphate transport mechanism in E. coli (Ellis, 1964). Based on the data presented, I believe that L-cysteine acts in this same manner as an allosteric inhibitor of sulphate transport in the meningococcus.

There is some evidence to support the proposal of a third transport system in the meningococcus by which thiocyanate enters the cell. This compound is an excellent sulphur source for the meningococcus and appears to be the primary sulphur source for establishment of this organism in the oropharynx. High concentrations of thiocyanate had no effect on the transport of either sulphate or L-cysteine. And according to the current theories on bacterial transport, it is unlikely that it is passively acquired by the cell, leading me to propose that it has its own as yet undefined, transport mechanism.

V. Nutrition and Cell Wall Composition

There are over 15 antigenically distinct serotypes of group B meningococcus (Frasch, 1979) which display distinct outer membrane protein profiles on SDS polyacrylamide gels. Frasch et al. (1976) have demonstrated that the protein and lipopolysaccharide of the meningococcal outer membrane varies with cultural conditions. A drop in the pH of the growth medium causes a reduced amount of the major 41,000-dalton protein with an increase in the 28,000-dalton protein in type 2 strain M986.

The pH of growth and iron availability affect outer membrane profiles of the gonococcus (Magnusson et al., 1979a,b) and the meningococcus (Brener et al., 1981; Simonson et al., 1981). N. meningitidis SD1C grown under iron-limited conditions at low pH expresses a 69,000-dalton protein thought to be the transferrin receptor (Simonson et al., 1982). A reduction in the dissolved oxygen concentration in gonococcal cultures results in a decrease in a series of outer membrane proteins (Leith and Morse, 1980; Clark et al., 1987).

Like other neisseriae, N. meningitidis showed differences in cell wall composition when grown on different nonlimiting sulphur sources. The absence of protein in the 21,000-23,000 dalton range in the cytosol fraction of

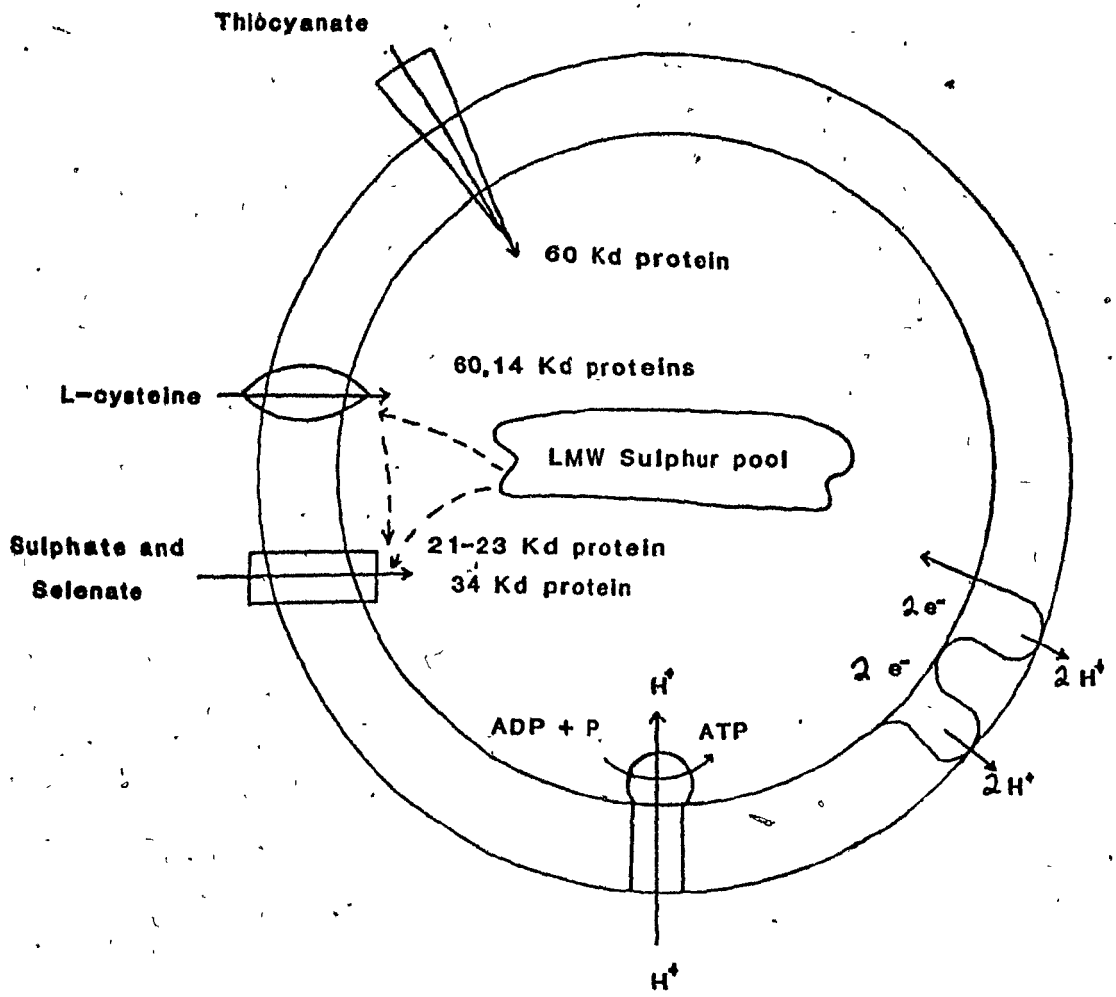
sulphate-grown cells may reflect the repression of the sulphate permease or some component of it. However, there are bands seen in this region for other inorganic sulphur sources which are likely transported by the sulphate permease. Growth on L-cysteine resulted in expression of different proteins than did growth on inorganic sulphur, although protein differences were noted even between compounds which are chemically quite similar.

Figure 21 is a proposed model for the mechanism of sulphur acquisition in the meningococcus. Sulphate and selenate share a transport system while thiocyanate and L-cysteine each have a separate transport mechanism. A transmembrane pH gradient is necessary for the uptake of sulphate and L-cysteine. L-cysteine is an allosteric inhibitor of the sulphate/selenate transporter. One or more components of the soluble sulphate pool regulate sulphate and L-cysteine transport. Growth of the meningococcus on different sulphur sources results in changes in envelope and cytosol protein profiles. These proteins may be components of the sulphur transport mechanism.

VI. Closing Statement

This dissertation has presented the first comprehensive study of sulphur acquisition in

Figure 21. Model of sulphur transport and regulation in N. meningitidis. Three transport systems are postulated: sulphate/selenate, L-cysteine, and thiocyanate. Growth on sulphate results in expression of a 34 Kd envelope protein and absence of 21-23 Kd proteins in the cytoplasm. Growth on L-cysteine results in expression of 93 Kd, 60 Kd, and 14 Kd envelope proteins, while thiocyanate-grown cells express a 60 Kd protein. Transport is energy-dependent, requiring the transmembrane pH gradient. A low molecular weight sulphur pool regulates uptake of sulphur-containing compounds and was shown to exert its effects on sulphate and L-cysteine transport. L-cysteine appears to be an allosteric inhibitor of the sulphate transport system.

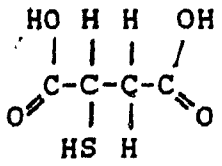

Thiocyanate
60 Kd protein
60,14 Kd proteins
L-cysteine
LMW Sulphur pool
Sulphate and
Selenate
21-23 Kd protein
34 Kd protein
2e⁻
2 e⁻
2 H⁺
H⁺
ADP + P
ATP
2 H⁺
H⁺

N. meningitidis. It is also the first detailed study of sulphur metabolism in a serious human pathogen. As is the nature of exploratory work, new areas of meningococcal metabolism have been opened and require further study. Much more needs to be done to unravel the complexities surrounding meningococcal sulphur metabolism. For example, the meningococcus grows on thiocyanate, a unique but readily available source of sulphur in the oropharynx of the host. Whether or not this is a significant factor in colonization of the throat by the meningococcus or other parasites needs to be examined further. The presence of a thiosulphate reductase in N. meningitidis and the recent report of the in vivo anaerobic growth of the gonococcus is quite exciting. Whether or not the meningococcus uses inorganic sulphur compounds as terminal electron acceptors when growing under anaerobic conditions during fulminant meningococcemia would shed some light on the complex pathology of this disease. N. meningitidis uses a common transport system for sulphate and selenate, as do many other biological systems. The documentation of selenium-dependent enzymes in bacteria is just beginning. It would be interesting to know whether the meningococcus actually transports selenate for synthesis of these enzymes or if this common transporter is an artifact which exists due to the physical and chemical similarity of the two ions. The

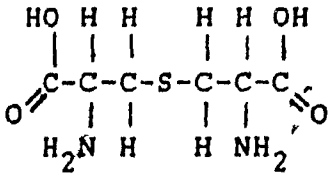
change seen in cell wall proteins when the meningococcus is grown on different sulphur sources needs to be addressed as modifications in surface proteins contribute to the virulence properties of the gonococcus and the meningococcus. As well, an understanding of the regulation of the internal sulphur pools would add to the knowledge of the physiology of this parasite. An understanding of the physiology of any organism is a prerequisite to understanding the host-parasite relationship and the pathogenesis of disease.

APPENDIX

STRUCTURES



Mercaptosuccinate



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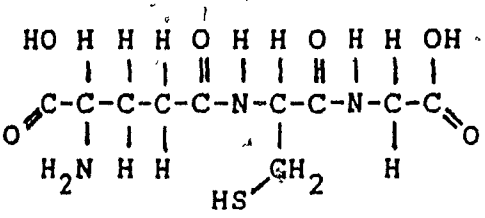
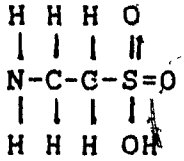
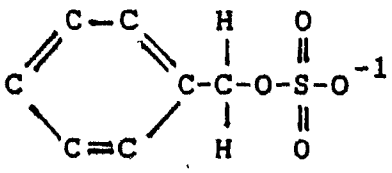


Reduced Glutathione



Taurine



Carrageenan

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