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SULPHITE TOXICITY: A CRITICAL REVIEW OF *IN VITRO* AND *IN VIVO* DATA

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Summary—Data on sulphite chemistry and toxicity in *in vitro* systems are reviewed from the perspective of potential mammalian toxicity. The observed toxicity of ingested sulphite in mammals is also summarized and the conclusions reached are compared with the results of the *in vitro* experiments. Information on sulphite metabolism is included to reconcile the different conclusions that may be drawn from these two sets of data. Consideration of data from all sources facilitates the selection of the specific reactions of sulphite most likely to be of toxicological significance in mammals.

Introduction

Sulphur dioxide (SO_2), sulphurous acid (H_2SO_3) and salts of sulphite (SO_3^{--}) and bisulphite (HSO_3^-) exhibit antioxidant and antimicrobial properties which make them logical choices as preservatives for certain foods and beverages. These quadrivalent-sulphur (S^{IV}) substances exist in a pH-sensitive equilibrium as described by the following series of equations:

$$SO_2 + H_2O \rightleftharpoons H_2SO_3 \overset{-H^+}{\rightleftharpoons} HSO_3^- \overset{-H^+}{\rightleftharpoons} SO_3^{--}$$

Which of the above chemical species predominates depends upon the pH and the acid dissociation constants, the latter in turn being somewhat dependent on temperature and ionic strength. For example, at 25°C the pK_2 of HSO_3^- is 6·25 at high ionic strength and 7·2 at low ionic strength (Shapiro, 1977). Under physiological conditions (considered to be pH 7·4 and 37°C) an essentially exclusive mixture of SO_3^{--} and HSO_3^- will result, with the former species predominating, no matter which of the S^{IV} species is initially introduced. For convenience and to avoid confusion, 'sulphite' will be used throughout this paper for referring to any of these readily interconvertible S^{IV} species. However, when specific reference is made to one species only, the chemical formula of that compound or ion (e.g. HSO_3^-) will be used.

Sulphite is used extensively in wine making as a selective inhibitor of yeasts and bacteria and is present in finished wines in concentrations up to approximately 6 mM, although part of this sulphite is in combined form. Foods and other beverages to which sulphite is often added include dehydrated fruits, vegetables and soups, as well as fruit juices and beer (Institute of Food Technologists and Committee on Public Information, 1976). The mean daily intake of sulphite from the diet has been estimated by various sources to be between 0·0014 and 0·14 mmol/kg body weight (in the United States), although the latter figure was reported as a probable over-estimation. Bigwood (1973) estimated that the mean daily intake of sulphite among adults in Belgium was in the range of 0·0011–0·016 mmol/kg. Because of the high concentration of sulphite in many wines and the wide personal variation in wine consumption, it follows that individual consumption of sulphite also varies considerably. Therefore, there is probably a small percentage of the population that takes in amounts of sulphite greatly in excess of these estimated averages.

Sulphite is also taken into the body during the inhalation of air polluted with SO_2. Although the focus of this article is on ingested sulphite, inhaled SO_2 cannot be totally dissociated from this source since it also adds to the body burden of 'exogenous' sulphite, albeit usually to a minor degree. One can calculate, for example, that the daily intake of sulphite due to inhalation of atmospheric SO_2 at the maximum 24-hr average permitted by the EPA (0·14 ppm) would be approximately 25 times less than the sulphite taken in by drinking 250 ml of wine containing sulphite at a level of 5 mM. However, although the subject is outside the scope of this article, it is important to emphasize here that the localized effects of SO_2 on the pulmonary system may be of much greater significance than the absolute amount of SO_2 absorbed.

The subject material of this paper is organized into several areas. The first of these consists of *in vitro* reactions of sulphite with biological compounds under approximately physiological conditions. These data are considered from the perspective of their implications for mammalian toxicity. Next, the *in vitro* modification of enzyme activity by sulphite and the toxicity of sulphite in biologically active *in vitro* assay systems are considered, followed by a review of toxicological data gathered from *in vivo* experiments in mammals. The metabolism of sulphite in mammals is summarized and finally some general observations and conclusions are presented. The literature references, although intended to be sufficiently extensive to be representative, are not necessarily comprehensive. Greater detail in some subject areas can be obtained from two excellent reviews, one by Shapiro (1977) and the other by Hayatsu (1976).

Reactions of sulphite *in vitro*

Addition to aldehydes and ketones

Sulphite reacts reversibly with open-chain aldehydes and ketones to form hydroxysulphonate compounds (Petering & Shih, 1975; Schroeter, 1966). The stability of these adducts varies considerably depending upon the pH and especially upon the reactive species. For example, at pH 7 the apparent dissociation constant (K_d) of glucose hydroxysulphonate is approximately 2·2 M (Vas, 1949), while that of the formaldehyde adduct is 8×10^{-5} M (Dasgupta, DeCesare & Ullrey, 1980). Vas (1949) determined that the velocity constant for the decomposition of glucose hydroxysulphonate at pH 6·1 and 20°C is approximately 1/min (the value at pH 7 was not determined). Extrapolation of these constants to the *in vivo* situation indicates that sulphite would be required to be present continuously if even a small amount of glucose were to be maintained as the hydroxysulphonate adduct.

Other physiological aldehydes and ketones, such as pyruvate, α-ketoglutarate and acetaldehyde, form hydroxysulphonate adducts that are considerably more stable than the glucose adduct. Burroughs & Sparks (1973) give the dissociation constants for pyruvate and acetaldehyde hydroxysulphonates at pH 7 and 20 C as $4·6 \times 10^{-4}$ M and $2·8 \times 10^{-6}$ M, respectively.

Ionic addition to C–C double bonds

The sulphite ion adds to some C–C double bonds forming sulphonic acid compounds. Under suitable conditions this reaction has been shown to occur with several molecules of extreme biological importance.

Addition to pyridine and flavin nucleotides. Sulphite adds reversibly to the 3–4 double bond of the pyridine ring of nicotinamide adenine dinucleotide (NAD), forming a sulphonate group at the active site for reduction by H^-. The stability of this adduct alone at pH 7·5 (25°C) is only moderate, its dissociation constant being approximately 3×10^{-2} M (Shih & Petering, 1973). However, when NAD^+ is associated with certain enzymes, its sulphite adduct is much more stable.

An analogous sulphite adduct is formed with flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) at the N_5 atom of the isoalloxazine ring, the usual site of reduction of the ring by H^-. These adducts are even less stable than the NAD–sulphite adduct (dissociation constants approximately 2 M), but as with the latter, their stability can be extensively enhanced when they are bound to protein (Müller & Massay, 1969). Since the sulphite adducts of NAD^+ and flavin coenzymes cannot accept H^- from the substrate, they cannot function in their usual capacity. Examples of this inhibition of enzyme function will be discussed later.

Addition to menadione. Vitamin K_3 (menadione) is a water-soluble synthetic form of vitamin K. Sulphite, at pH 7·4, adds to the 2–3 double bond of the naphthoquinone ring of menadione to form a sulphonate adduct. Since the natural forms of vitamin K (K_1 and K_2) are fat soluble, the reaction rates of menadione are not necessarily applicable to them. As with other reactions of this type, the addition of sulphite to menadione is reversible. The dissociation constant for the adduct is 10^{-6} M (Shih & Petering, 1973), indicating considerable stability of the adduct in the presence of free sulphite. However, the sulphite adduct is a source of vitamin K when fed to animals (Nir, Kafri & Cohen, 1978), suggesting that the adduct readily dissociates in the body.

Addition to uracil and cytosine. Sulphite adds reversibly to the 5–6 double bonds of uracil, uridine or uridine 5′-phosphate forming the 5,6-dihydro-6-sulphonate adduct (Hayatsu, 1976). The rate of the forward reaction (formation of the adduct) is most rapid at a pH of approximately 7 and the equilibrium shifts in the direction of the reverse reaction above and below that pH. The dependency of the reaction rate on sulphite concentration at pH 7 has been demonstrated in several studies (Hayatsu, Wataya, Kai & Iida, 1970; Pitman & Jain, 1979; Shapiro, Welcher, Nelson & Di Fate, 1976). According to Hayatsu *et al.* (1970), 86% of the uridine reactant at 22°C in 1 M-sulphite (excess) was in the form of the adduct after 0·5 hr, while in 0·1 M-sulphite only 12% of the uridine had reacted in the same period of time. At this pH, the equilibrium of the reaction was decisively in the direction of the adduct and in 1 M-sulphite essentially all of the uridine had been converted to the sulphonate after 1 hr. However, uridine can be regenerated from its sulphonate adduct at pH 7 by removal of free sulphite. The regeneration process (i.e. reversal of adduct formation) approximates first-order kinetics. At physiological pH and in the absence of sulphite, the half-life of 5,6-dihydrouracil-6-sulphonate is several hours. Regeneration occurs more rapidly as the pH rises and at or above pH 11 it is extremely rapid, the half-life of the sulphonate compound being a few seconds (Rork & Pitman, 1974).

Sulphonate adducts of cytosine and its derivatives, analogous to those of uracil, are formed during incubation with sulphite under the appropriate conditions. The extent of adduct formation is determined primarily by pH and the concentration of sulphite. The cytidine adduct is stable at acid pH, and in high concentrations of sulphite (about 0·5 M) approximately 80% of cytidine is in this form at equilibrium (Shapiro, Di Fate & Welcher, 1974). Compared to uridine adducts, cytidine adducts are relatively unstable at physiological pH, only 10% of the cytidine existing in the combined form at equilibrium under conditions of excess sulphite. Therefore, a high concentration of sulphite is required to maintain the presence of dihydrocytosine 6-sulphonate at physiological pH. Recent data suggest, however, that this adduct may be considerably more stable *in situ* in bacteriophage DNA (Sklyadneva, Chekanovskaya, Nikolaeva & Tikchonenko, 1979a). These data will be discussed later.

Deamination of 5,6-dihydrocytosine-6-sulphonate

Under the appropriate conditions, 5,6-dihydrocytosine-6-sulphonate can be deaminated to the corresponding uracil adduct. This deamination is catalysed by basic substances (including sulphite) and occurs at an optimal rate at pH 5 (Shapiro *et al.* 1974). Thus, *via* the deamination reaction and the addition reactions described above, cytosine can be converted to uracil with obvious toxicological implications. The

optimal pH for the overall conversion is 5, and at physiological pH the rate of conversion declines to approximately 1% of that observed at optimal pH. Further, at low sulphite concentrations compatible with the *in vivo* situation, the rate of conversion can be expected to be extremely low. Slae & Shapiro (1978) have estimated this rate to be $4{\cdot}0 \times 10^{-14}$/sec for a sulphite concentration of 10^{-3} mM.

Transamination of cytosine

Sulphite is capable of catalysing the transamination of cytosine and its derivatives with primary and secondary amines to produce N^4-substituted cytosines. The reactive intermediate for transamination is the 5,6-dihydrocytosine-6-sulphonate adduct discussed previously. Shapiro & Gazit (1977) have carried out transamination reactions at physiological temperature and pH between polylysine and cytidine, polycytidylic acid and lysine, and polylysine and polycytidylic acid. High concentrations of sulphite were used (approximately 1 M) and the reactions were allowed to proceed for hours or days. Crosslinking between monomers and polymers of lysine and cytosine was observed. The significance of these reactions lies in their logical extension to the crosslinking of nucleic acids and proteins. Although attempts to crosslink double-stranded native DNA with polylysine were not successful, evidence for crosslinking was found in a similar experiment involving DNA and histones (Shapiro & Gazit, 1977). Sklyadneva, Shie & Tikchonenko (1979b) were successful in crosslinking lysine with DNA isolated from S_d bacteriophage; 40% of the cytosine residues were transaminated during a 24-hr reaction in the dark at pH 6·25, 0·25 M-sulphite and 25°C.

Sulphitolysis reactions

Sulphitolysis of thiamine. The cleavage of thiamine by sulphite was described by Williams, Waterman, Keresztesy & Buchman (1935). This reaction is irreversible and involves a nucleophilic attack by sulphite on the quaternary nitrogen of the thiazole ring to yield pyrimidine sulphonic acid and 4-methyl-5-β-hydroxyethylthiazole. The kinetics of this reaction have been studied in detail by Leichter (1969) who showed that thiamine sulphitolysis is first order with respect to each reactant. At pH 5 and 25°C, the half-life of 10 μM-thiamine in the presence of 1·0 mM-sulphite is approximately 13 hr. Petering & Shih (1975) have calculated from Leichter's data a second-order rate constant of $6{\cdot}4 \times 10^{-3}$/M. sec for the sulphitolysis of thiamine at pH 7 and 25°C.

Sulphitolysis of disulphide bonds. Sulphite can reversibly lyse disulphide bonds by a nucleophilic displacement mechanism resulting in the formation of thiol and *S*-sulphonate compounds (Cecil, 1963). Under non-denaturing conditions and at physiological pH, the reaction between sulphite and S–S bonds in free cystine goes essentially to completion (cysteine *S*-sulphonate is stable at this pH) while the disulphides of most proteins are unreactive. The unreactive protein disulphide bonds are apparently protected from nucleophilic attack either sterically or by the unfavourable electronic environment of neighbouring amino acids. Quantitative reaction of all disulphide bonds in a specific protein can be accomplished by denaturing the protein and using high concentrations of sulphite reactant.

McArdle (1967) demonstrated the reaction of sulphite with non-mercaptalbumin at physiological pH, producing an *S*-sulphonate compound at the albumin–cysteine (or albumin–glutathione) site. Gregory (1981) has recently confirmed this observation using both purified rabbit-plasma albumin and fresh whole rabbit plasma. Gregory further demonstrated, at physiological pH, the partial sulphitolysis of disulphides present in rabbit-plasma fibronectin protein. Gunnison & Palmes (1978) and Gunnison & Benton (1971) have shown that chemically stable plasma-protein *S*-sulphonate compounds are formed in a matter of minutes or hours during the incubation of sulphite (approximately 0·5 mM) with the plasmas of several species of mammals at physiological pH.

Free-radical reactions

Sulphite can be oxidized to sulphate by free oxygen (autoxidation) *via* a free-radical chain mechanism which is initiated by superoxide-anion ($O_2^{\cdot-}$) or $HSO_3^{\cdot}$ radicals (McCord & Fridovich, 1969; Yang, 1970). Once initiated, chain-propagating reactions generate highly reactive free-radical intermediates such as sulphur–oxygen species (Hayon, Treinin & Wilf, 1972), $O_2^{\cdot-}$, and the peroxide ($HO_2^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radicals. The chain-initiating radical $O_2^{\cdot-}$ can be generated by certain enzymatic reactions and both $O_2^{\cdot-}$ and $HSO_3^{\cdot}$ can be produced by the reaction of transition-metal ions with oxygen (Yang, 1970). Fridovich & Handler (1961) have shown that the catalytic action of several oxidative enzymes (i.e. xanthine oxidase, liver aldehyde oxidase, cytochrome oxidase, lipoxidase and peroxidase) can initiate the aerobic oxidation of sulphite, while diverse other oxidase enzymes cannot. It appears that enzymes capable of initiation are those that effect the univalent reduction of oxygen to produce the superoxide anion.

Free radicals generated by the aerobic oxidation of sulphite initiate several reactions of potential biological significance (Hayatsu, 1976). The rate of these reactions is, in general, enhanced by conditions that favour autoxidation of sulphite, such as the presence of transition-metal ions (e.g. Mn^{++}, Fe^{+++}) and oxygen, and is inhibited by free-radical scavengers (e.g. hydroquinone). Sulphite autoxidation occurs readily at pH 7 and reaction of the free radicals generated with various substrates proceeds more rapidly at sulphite concentrations of approximately 1–20 mM than at higher concentrations. At 1 M, for example, sulphite ions can compete effectively with potential substrates for the free radicals generated by sulphite autoxidation, while at 20 mM this competition by sulphite is relatively ineffective due to low concentration. Conditions favourable for the aerobic oxidation of sulphite (stated above) have been used in investigations of the reactivity of the free-radical intermediates of sulphite autoxidation, and will be referred to in the following paragraphs as a sulphite/free-radical environment.

Significant cleavage of the glycosidic linkages of uridine and cytidine, but not of purine nucleosides or pyrimidine deoxyribonucleosides, occurred in a sulphite/free-radical environment (Kitamura & Hayatsu, 1974). In this same system there was also extensive

fission of the chains of polyuridylic acid [poly(U)] and polycytidylic acid [poly(C)], but not of polyadenylic acid [poly(A)] or poly(U):poly(A). Breaking of DNA phosphodiester linkages was also observed when double-stranded DNA of phage T7 was incubated in a sulphite/free-radical environment and subsequently treated with alkali (Hayatsu & Miller, 1972).

4-Thiouracil and 6-isopentenyladenosine (ipa) are among the minor base constituents of yeast transfer RNAs. Both of these bases are modified as a result of autoxidation of sulphite, probably directly by the sulphite-ion radical ($SO_3^- \cdot$), forming uracil 4-sulphonate and a sulphonated product of ipa, respectively. The site of sulphonate formation in the latter molecule is the unsaturated bond of the isopentenyl side chain and is an example of the addition of the sulphite-ion radical to an olefinic double bond. This class of reaction is described in some detail by Stacey & Harris (1963). The optimum pH for the reaction is in the range 5–7, and the speed of the reaction is highly dependent upon the solubility of the olefin in aqueous medium. Allyl alcohol, for example, readily undergoes addition of sulphite *via* a free-radical mechanism.

Methionine and certain other dialkyl sulphides are oxidized to their respective sulphoxides (R—S—R) in

a sulphite/free-radical environment (Yang, 1970). A reaction scheme has been proposed in which superoxide anion and hydroxyl radicals are responsible for sulphide oxidation. Yang (1973) has also shown that tryptophan is destroyed by free radicals generated during the aerobic oxidation of sulphite.

Kaplan, McJilton & Luchtel (1975) have demonstrated that 0·5 to 10 mM-sulphite can induce the oxidation of a heterogeneous mixture of unsaturated fatty acids contained in corn oil in a dose-dependent fashion, presumably by a free-radical mechanism. Although the oxidation products probably consisted mainly of peroxides, the possible presence of other oxidation products could not be excluded by the analytical method used (i.e. reactivity with thiobarbituric acid, TBA). The precise chemistry of the reactions taking place was not investigated. The mechanism of the oxidative reaction(s) was not clear since the formation of TBA-reactive materials was inhibited by the presence of an antioxidant, indicating a free-radical mechanism, and yet Mn^{++} also inhibited rather than enhanced the reaction, suggesting that sulphite autoxidation was unimportant.

Similar results were obtained by Inouye, Ikeda, Ishida, Ogata, Akiyama & Utsumi (1978), who measured lipid peroxidation (TBA-reactive material) in rat-liver homogenate. Addition of 2 mM-sulphite to the homogenate greatly increased the TBA-reactive material while addition of 2 mM-Mn^{++}, even in the presence of sulphite, suppressed it.

In vitro modification of enzyme activity

Sulphite inhibits the *in vitro* activity of several enzymes with either NAD or flavin nucleotide cofactors by adding to the active site of the cofactor, as previously discussed. G. Pfleiderer, D. Jeckel & T. Wieland, in 1956, first postulated an enzymatically inactive complex between lactate dehydrogenase (LDH), NAD^+ and sulphite (from Ciaccio, 1966). Parker, Lodola & Holbrook (1978) estimated the dissociation constant for this complex ($LDH\text{-}NAD^+\text{-}SO_3^- \rightleftharpoons SO_3^- + LDH\text{-}NAD^+$) to be approximately 10^{-7} M (as compared to $1{\cdot}5 \times 10^{-2}$ M for the non-enzymatic adduct) and concluded that the binding of NAD^+ to LDH activated the nicotinamide ring for attack by sulphite by a factor of approximately 10^5. The effect on the activities of LDH and of several other NAD^+-dependent dehydrogenases (malate, alcohol, glutamate and α-glycerophosphate) is apparent from the *in vitro* experiments of Ciaccio (1966) in which 50% inhibition was observed in the presence of 0·03–0·5 mM-sulphite.

The sulphite adducts of flavins bound to enzymes also inactivate the enzymes and exhibit greatly enhanced stabilities relative to adducts of free FAD, FMN and model isoalloxazines. A prime example is the binding of sulphite to FAD–glucose oxidase. The dissociation constant of this complex is approximately 7×10^{-4} M at pH 7 (Swoboda & Massey, 1966) compared to dissociation constants of approximately 2 M for sulphite adducts of free flavin. Although the FAD–sulphite adduct of glucose oxidase exhibits considerable stability in the presence of sulphite, it is, nevertheless, unstable in the absence of sulphite, as was demonstrated by the regeneration of active enzyme by dialysis. This reversibility is an important property with regard to the toxicological significance of the sulphite adducts of flavins (and NAD). Massey, Müller, Feldberg, Schuman, Sullivan, Howell, Mayhew, Matthews & Foust (1969) have catalogued a number of flavoproteins in terms of their reactivity with sulphite, i.e. the formation of flavin nucleotide–sulphite adducts. A series of oxidase enzymes (D- and L-amino acid oxidase, oxynitrilase, lactate oxidase and glycollate oxidase) reacted readily with sulphite, forming complexes with dissociation constants ranging from 10^{-3} to 10^{-7} M. Presumably the complexed enzymes were inactive although this was not measured. It should be noted that the flavoprotein dehydrogenases tested did not form adducts even when incubated in 20 mM-sulphite for several hours.

Incubation of cytochrome oxidase with 0·5 or 5 mM-sulphite at pH 7 for 4 hr inhibited its activity by 37 and 80% respectively (Cooperstein, 1963). The mechanism of inhibition was believed to involve one or more disulphide bonds, since other disulphide bond-reducing agents, such as cysteine and reduced glutathione, were also inhibitory. The inhibition could be reversed by incubation with oxidized glutathione.

α-Glucan phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase; EC 2.4.1.1) from rabbit muscle, which catalyses the reversible formation of glucose 1-phosphate from glycogen, is substantially inhibited at pH 6 by sulphite concentrations in the 10–30 mM range (Kamogawa & Fukui, 1973). This inhibitory effect is highly specific and completely reversible by dialysis. Sulphite acts as a competitive inhibitor (K_i = 7 mM) with respect to glucose 1-phosphate (K_m = 11 mM) in glycogen synthesis and with respect to inorganic phosphate (P_i) in glycogen degradation. The authors speculate that this competition for the phosphate-binding site of the enzyme may be due to the structural similarity of HSO_3^- and phosphate.

Harkness & Roth (1969) have reported a striking

sulphite-induced enhancement of activity of 2,3-diphosphoglyceric acid (2,3-DPG) phosphatase. This enzyme is believed by these authors to be the physiological phosphatase catalysing the conversion of 2,3-DPG to 3-phosphoglyceric acid and P_i. Since 2,3-DPG plays a major role in regulation of oxygen affinity for haemoglobin, any factor that affects its concentration in the red cell could be of physiological importance. In the experiments of Harkness & Roth (1969) incubation of the purified phosphatase enzyme with 20 mM sulphite for 1 hr at pH 7·8 and 37°C produced a 37-fold increase in enzyme activity; at 2·5 mM-sulphite, activity was increased by about 15-fold. Incubations with lower concentrations were not performed.

Sulphite is a potent inhibitor of most sulphatase enzymes (Roy, 1960). For example, the K_i values for sulphite inhibition of aryl sulphatases A, B and C from ox liver are 2 μM, 0·5 mM and 0·1 mM, respectively. If precautions are taken to minimize the autoxidation of sulphite, then K_i for inhibition of sulphatase A decreases to approximately 0·2 μM (Roy, 1976). The early work on the kinetics of aryl sulphatases was performed with nitrocatechol sulphate and other unphysiological substrates without certain knowledge of the true physiological substrates. More recently, the physiological substrates for the sulphatases have been thought to include lipids containing galactosyl 3-sulphate residues (such as cerebroside sulphate), mucopolysaccharides containing *N*-acetylgalactosamine 4-sulphate residues (such as dermatan sulphate), heparin sulphate, chondroitin sulphates and steroid sulphates (Roy, 1976).

Sulphite toxicity in biologically active, *in vitro* test systems

Modification of activities of RNA, DNA and associated proteins

Shapiro & Braverman (1972) have demonstrated that conversion of uracil to the 6-sulphonate adduct interferes with hydrogen binding to adenine and reduces the ability of poly(U) to form a helical complex with poly(A). The uracil–sulphonate adducts were formed in poly(U) by reaction with 1 M-sulphite at pH 7, followed by stabilization at pH 4 and dialysis to remove excess sulphite. The modification of uracil residues of poly(U) also inhibited its ability to code for phenylalanine incorporation into protein in an *Escherichia coli* cell-free protein-synthesizing system operating at approximately physiological pH. A relatively small percentage of adduct formation (2·6%) caused a much larger decrease in incorporation (54%), leading the authors to suggest that a single uracil saturation might be sufficient to block translation at that point. This same research group later showed that sulphite could similarly modify natural messenger RNA (from coliphage MS2) and ribosomal RNA from *E. coli*, leading to decreases in the incorporation of amino acids into protein (Braverman, Shapiro & Szer, 1975).

In related experiments, uracil–sulphonate adducts formed in calf thymus DNA by deamination of cytosine residues, interfered with the DNA polymerase reaction, thus inactivating DNA as a template (Kai, Tsuruo & Hayatsu, 1974). Treatment with alkali, which removed sulphite from the dihydro-6-sulphonate moiety, restored the template activity of DNA. The modified DNA was prepared by heat denaturation and reaction with approximately 1 M-sulphite at pH 6. In experiments in which calf-thymus DNA was not heat-denatured (Shapiro, Braverman, Louis & Servis, 1973), sulphite did not convert residues of cytosine to uracil, implying that single-stranded DNA is a requisite for this reaction.

Sulphite has been shown to inhibit the transforming activity of DNA isolated from a bacterium (strains of *Bacillus subtilis*) under conditions that favour the aerobic oxidation of sulphite, suggesting a free radical-mediated mechanism of DNA alteration (Inoue, Hayatsu & Tanooka, 1972). Inhibition was greatest at approximately 20 mM-sulphite and decreased progressively as the sulphite concentration increased to 1 M. Other factors that decreased the rate of sulphite autoxidation, such as free-radical scavengers and elimination of oxygen, also decreased the sulphite-mediated inhibition of transforming activity. Inactivation of the DNA transforming activity was strongly inhibited by 4-thiouridine which is known to react with the sulphite-ion radical. Thus, this radical was assumed to be the species primarily responsible for the inactivation.

A free-radical mechanism was also implicated in the sulphite-mediated inactivation of bacteriophage lambda (Kudo, Miura & Hayatsu, 1978). Inactivation of phage infectivity of indicator bacteria was observed in sulphite concentrations of 0·1–10 mM in incubations at pH 7 and 37°C. After a 4-hr incubation in 10 mM-sulphite, the infectivity of the phage had decreased to 10^{-5} of its initial value. Phage inactivation was attributed not to DNA damage but to alteration of coat proteins; this affected their adhesion of bacteria and their ability to inject DNA. Several lines of evidence suggested that tryptophan in the coat proteins was modified by reaction with the sulphite-ion radical.

Turchinsky, Kusova & Budowski (1974) have demonstrated the sulphite-catalysed crosslinking of the maturation and coat proteins with the nucleic acids of the RNA bacteriophage, MS2. The MS2 phages were treated with 1 M-sulphite at pH 7 for 0·5–4 hr, with consequent covalent association of approximately 1% of the protein with RNA. The mechanism of crosslinking was presumably by transamination of 5,6-dihydrocytosine-6-sulphonate as discussed in a previous section.

Sklyadneva *et al.* (1979a) have also presented evidence for the sulphite-catalysed transamination of cytosine bases with protein in bacteriophage DNA. These authors propose that the intermediate, 5,6-dihydrocytosine-6-sulphonate, is stabilized *in situ* by polar groups of protein, especially the amino groups of basic amino acids. This stabilization is effective at refrigerator temperatures for up to 4 months. When phage particles are disintegrated, however, the sulphonate adduct in the DNA becomes unstable and, depending upon the specific conditions, either reverts to a cytosine residue or undergoes transamination. This *in situ* stability of cytosine–sulphonate adducts may have important implications for the *in vivo* rate of deamination of cytosine to uracil.

Chromosome damage and mutagenesis

As anticipated from chemical data, sulphite was shown to cause mutations, presumably by deamination of cytosine to uracil. Cultures of several mutant strains of *E. coli* were treated with 1 M-sulphite at pH 5·2 for 30 min and the frequency of back mutation was determined (Mukai, Hawryluk & Shapiro, 1970). Only those mutants that were cytosine–guanidine (C:G) at the mutant site showed an increase in reversion frequency. When incubations were performed at pH 7 or 8, sulphite had no measurable effect on reversion frequency, a result consistent with chemical data on the pH profile for deamination.

A similar specificity for C:G to A:T (adenine–thymine) transitions was reported by Summers & Drake (1971) using bacteriophage T4rII as a test system (although T4 contains the cytosine analogue 5-hydroxymethylcytosine). At pH 5, inactivation and mutation frequency of the phage showed excellent dose-response relationships with both sulphite concentration (0·2–0·9 M) and treatment time. The reversion frequency resulting from a 4-hr treatment with 0·9 M-sulphite was approximately $110/10^7$. Recent duplication of these experiments, however, revealed the initial findings to be in error. Sulphite was not capable of causing a measurable rate of reversion of T4 phage, although a 10–20-fold lower mutation rate than that initially reported could not be excluded (J. W. Drake, personal communication 1981).

An increase in mutation frequency of phage lambda incubated in 3 M-sulphite at pH 5·6 was observed by Hayatsu & Miura (1970). Maximum mutation frequency was produced after 1·5 hr, while inactivation of the phage continued for the 3 hr duration of the experiment.

In his review of the genetic effects of sulphite, Shapiro (1977) cites two reports in which sulphite apparently caused mutations in *Saccharomyces cerevisiae* and *Micrococcus aureus* at much lower concentrations than are usually required (i.e. 5 and 10 mM, respectively). In the former case, however, a very low pH (3·6) was required.

In the above experiments, sulphite-induced mutagenicity was observed in cells containing double-stranded DNA. This appears to be inconsistent with other experiments in which the cytosine bases in isolated double-stranded DNA were inert to sulphite (Shapiro, 1977; Shapiro *et al.* 1973). In reality, however, DNA, especially in certain growth phases, always exists partially in its reactive single-stranded form (Bjursell, Gussander & Lindahl, 1979).

The proposed mechanism of sulphite-induced mutagenesis, that is conversion of cytosine to uracil, is not consistent with the existence of uracil-DNA glycosidase. This enzyme, discovered initially in *E. coli* (Lindahl, 1974), catalyses the excision from DNA of uracil bases produced by deamination of cytosine, thus apparently preventing C:G to A:T transition. Recent data suggest that sulphite-induced mutations at C:G sites actually involve deamination of 5-methylcytosine to thymine. Since the newly-formed thymine in DNA is not recognized by a glycosidase, it cannot be excised and repaired. Coulondre, Miller, Farabaugh & Gilbert (1978) have demonstrated that 5-methylcytosine residues in *E. coli* are associated with a high rate of spontaneous mutation consisting of C:G to A:T transitions (i.e. hot spots). Furthermore, in a strain of *E. coli* that lacks uracil-DNA glycosidase, the rate of spontaneous transitions at cytosine residues is elevated to the rate observed at 5-methylcytosine residues (Duncan & Miller, 1980).

On the other hand, Wang, Gehrke & Ehrlich (1980) have recently demonstrated that, at pH 5·5 and 3 M-sulphite, under conditions where more than 96% of cytosine residues in single-stranded DNA were converted to uracil, only 2–3% conversion of 5-methylcytosine residues to thymine occurred. Wang & Ehrlich (1980) concluded that it is much more likely that cytosine rather than 5-methylcytosine residues are involved in sulphite-induced mutagenesis. This point is the subject of continuing research and is still open to question. Preliminary experimentation necessary for calculating the rate of 5-methylcytosine deamination under physiological conditions is now underway in the laboratory of Dr R. Shapiro (personal communication, 1980).

Recently, Mallon & Rossman (1981) have demonstrated an enhancement of UV mutagenicity resulting from exposure at physiological pH to much lower sulphite concentrations than are required for measurable conversion of cytosine to uracil. Cells from a Chinese hamster line, V79, exposed to 10 mM-sulphite at pH 7·4, either during or immediately following UV irradiation, showed an approximately twofold increase in mutation frequency over that caused by UV treatment alone. In similar experiments with *E. coli*, 100 mM-sulphite caused an eight-fold increase. In both cases, exposure to sulphite alone had no effect on mutation frequency. Since the co-mutagenic effect of sulphite was essentially of equal potency whether sulphite exposure occurred during or immediately following UV irradiation, Mallon & Rossman (1981) speculated that sulphite might have been affecting a DNA repair process. Subsequent experiments utilizing two *E. coli* strains deficient in the excision repair process demonstrated that the presence of sulphite did not enhance UV mutagenesis, implying that the function of sulphite in the initial experiments was to inhibit excision repair.

Perry & Evans (1975) have demonstrated a positive correlation between mutagenesis and sister chromatid exchange and suggest the latter as a highly sensitive technique for assaying the chromosome mutagenicity of environmental agents. MacRae & Stich (1979) showed that sulphite induces dose-related sister chromatid exchange in Chinese hamster ovary cells at concentrations between approximately 0·03 and 7 mM. The potency of this induction, however, was much lower than that exhibited by the strong mutagenic agents shown to possess this ability. MacRae & Stich (1979) suggested that cleavage of the DNA chain by free radicals, probably hydroxyl radicals, generated by autoxidation of sulphite (Hayatsu & Miller, 1972) might be responsible for this action of sulphite.

In a different type of assay, damage to the chromosomes of mammalian oocytes was observed following *in vitro* exposure to sulphite (Jagiello, Lin & Ducayen, 1975). In the same experiments there was also an inhibition of entry of oocytes into meiosis when they were cultured in the presence of sulphite. Mouse oocytes, in general, were more sensitive than those of the cow or ewe and when exposed to sulphite concentrations of

approximately 0·2 mM or above showed a slight inhibition of entry into meiosis which became complete at a sulphite concentration of 6 mM. Chromosome 'fuzziness' also occurred at 0·2 mM and higher concentrations, but the authors ascribed no genetic significance to this type of aberration. More significant genetically was the fragmentation of chromosomes and the anaphase lagging that occurred sporadically in cow and ewe oocytes at concentrations of sulphite of approximately 3 mM and above.

Effects on mammalian cells in culture

The adhesion of cultured Chinese hamster cells (cell line Don) to the substratum was slightly inhibited by incubation with 10 mM-sulphite and severely inhibited by 30 or 50 mM-sulphite (Kudo, Hayatsu, Yokoiyama & Kuroda, 1980). A possible explanation for this observation has been offered by Gregory (1981) who found that sulphite concentrations at or above 30 mM progressively cleaved disulphide-linked rabbit-plasma fibronectin dimers into monomers. A plasma fibronectin-type protein is also present in disulphide-bonded aggregates in relatively large quantities on the surface of cells (cell surface protein; CSP) where it functions in cell–substratum adhesion and cell–cell interactions. Disruption of CSP by strong disulphide reducing agents has been shown to destroy its functions (Ali & Hynes, 1978).

An observation by Kikugawa & Iizuka (1972) that 7·5 mM-sulphite inhibits ADP- and collagen-induced aggregation of rabbit platelets may well be linked to the above finding of Kudo *et al.* (1980), by similar underlying mechanisms.

Thompson & Pace (1962) measured cell proliferation in mouse fibroblasts, mouse-liver cells and HeLa cells exposed in culture to initial sulphite concentrations ranging from approximately 1 to 20 mM for periods up to 9 days. There was complete inhibition of growth in all cell lines cultured in the high sulphite concentration, while HeLa cells, which were the most sensitive to sulphite, showed marked growth inhibition even at 1 mM-sulphite. Results consistent with these findings were obtained by Das & Runeckles (1974), although synchronous cultures of *Chlorella pyrenoidosa* were used. In these studies, exposure of cells to initial sulphite concentrations of 0·5–2 mM for a period of 48 hr caused a progressive decrease in cell number and in DNA content, expressed as a percentage of dry weight, but not in RNA or protein content. During the 48-hr exposure period, there was a decline in pH from 6·6 to approximately 4 (measured in a culture containing about 2 mM-sulphite) which may have affected entry of sulphite into the cell. The authors concluded that sulphite affected active growth of the cells by impairing DNA synthesis.

Inhibition of DNA synthesis (measured by [^{3}H]thymidine incorporation) was also observed by Chin, Bissell & Bassham (1977) in chick-embryo fibroblasts cultured in the presence of sulphite for 18 hr. While 0·05 mM-sulphite caused no measurable inhibition, 0·1 and 1·0 mM-sulphite caused a 14 and 52% reduction in thymidine incorporation, respectively. After incubation for 48 hr, decreased cell viability was observed in 0·5 and 1·0 mM-sulphite. Sulphite did not affect cell-membrane permeability to mannitol (passive diffusion) or to 2-deoxyglucose (carrier-mediated transport). In addition, no discernible effect of sulphite on glucose metabolism was revealed by monitoring selected intermediates of the glycolysis pathway, glycogen synthesis, the pentose shunt and the tricarboxylic acid cycle.

Timson (1973) cultured human lymphocytes in 0·1–10 mM-sulphite and concluded that exposure to 10 mM-sulphite for 72 hr was cytotoxic, while lower concentrations had an antimitotic effect which was possibly due to inhibition of DNA synthesis during the early stages of mitosis. In this system, exposure to 0·1 mM-sulphite over a period of 72 hr caused 43% inhibition of mitosis, and a similar exposure to 1·0 mM-sulphite produced a 63% inhibition.

In a perplexing study which is somewhat difficult to relate to others of its kind, Schneider & Calkins (1970) exposed human lymphocytes in culture to sulphite by bubbling SO_2 through the medium. Although the sulphite concentration was not measured either at the onset of exposure or during the incubation period, which lasted for up to 3 days, it is possible to calculate from the information given that the initial concentration was approximately 0·005 mM, assuming complete absorption of SO_2 by the medium. The pH during the incubation period varied between approximately 6·6 and 7·2. There was a significant decrease both in DNA synthesis, as measured by the percentage of cells that incorporated [^{3}H]thymidine, and in the mitotic index of cells exposed to sulphite in comparison with appropriate control cells. These effects were most apparent in cells exposed before DNA synthesis was initiated. In addition, chromosomal abnormalities, consisting mainly of a reduction in number and in clumping and fuzziness, occurred at a higher incidence in the sulphite-exposed cultures.

These experiments demonstrated detrimental effects at an estimated concentration of sulphite approximately two orders of magnitude below those reported by other investigators as causing similar changes. It is felt, however, that the results of Schneider & Calkins (1970) are open to question because the authors neglected to test other concentrations of sulphite to demonstrate conclusively that the effects observed were truly a function of sulphite exposure. The delivery of the sulphite dose by bubbling SO_2 through the medium introduced the possibility of side effects due to physical damage of cells and/or oxygenation of the culture medium. Although these side effects were supposedly controlled for in cultures that received air only, the possibility of a synergistic effect between sulphite and the bubbling of air through the medium was not considered.

A 50% reduction of the intracellular concentration of 2,3-DPG in human erythrocytes resulted from incubation of the cells for 4 hr with 4 mM-sulphite at pH 7·5 and 37°C (Parker, 1969). Essentially no intracellular 2,3-DPG remained when the concentration of sulphite was increased to 20 mM. These data can be explained by the sulphite-induced activation of 2,3-DPG phosphatase of human erythrocytes discussed previously (Harkness & Roth, 1969). The depletion of 2,3-DPG from erythrocytes was accompanied by a reversible increase in their passive permeability to Na and K ions, a change that was thought to be a direct effect of sulphite and not to be caused indirectly by 2,3-DPG levels.

Mammalian toxicity

Chronic sulphite feeding studies

In studies in which sulphite was administered to animals (usually rats) in the diet or drinking-water, the concentration of sulphite was often expressed in different units, making direct comparisons difficult. Therefore, in this section, an attempt is made to express the sulphite exposure in all experiments in terms of mmol consumed/kg body weight/day. Since some investigators have not given the data on food or water consumption required for these calculations, it has been assumed where necessary that rats ingest 100 ml water and 67 g solid diet/kg body weight/day. Further, the instability of sulphite added to the diet or drinking-water has been noted and taken into account by some investigators in their calculations of intake, and ignored by others. No attempt is made here to adjust intake figures for this variable unless adequate data on stability have been given.

It is well documented that sulphite, when pre-mixed with the diet, can cleave the thiamine molecules contained therein and destroy their activity, thereby causing a deficiency of thiamine in the organism (Bhagat & Lockett, 1964; Fitzhugh, Knudsen & Nelson, 1946). Thiamine deficiency has not resulted, however, when sulphite has been administered in fluids, although there is evidence that thiamine can be destroyed in the stomach when ingested simultaneously with sulphite (Lhuissier, 1966). Furthermore, the possibility that sulphite may inhibit the synthesis of thiamine by the bacterial flora of the intestine cannot be excluded (Cremer & Hötzel, 1966). In spite of this destruction by sulphite, recent data show that thiamine is not destroyed systemically by sulphite (Gunnison, Dulak, Chiang, Zaccardi & Farruggella, 1981a).

One of the earliest attempts to investigate the chronic toxicity of sulphite comprehensively was published in 1946 by Fitzhugh *et al.* These investigators administered sulphite to rats for approximately 1 yr by incorporating it into their diet at several concentrations, resulting in nominal intakes in the treatment groups ranging from 0·08 to 13 mmol/kg/day. Unfortunately, these estimates of sulphite intake are meaningful only as ceiling values, since sulphited food was sometimes left in feeder cups for up to a week between changes, during which time as much as 75% of the sulphite was lost due to chemical reaction. In these experiments the investigators attempted, not entirely successfully, to separate the effects of sulphite-induced thiamine deficiency from those due to the ageing of the sulphited diet and to the direct toxicity of sulphite. They concluded that, in addition to the toxic signs attributable to thiamine deficiency, such as polyneuritis, the stunting of growth and atrophy of organs (the latter two due to inanition), other toxic changes were produced by ingestion of the sulphited diets. At a nominal sulphite intake of 1·6 mmol/kg/day or more, the growth rate and average survival time of rats was decreased and pathological changes including gastric squamous epithelial hyperplasia, bleached incisor teeth, brown uteri, calcified renal tubular casts and atrophy of bone and bone marrow were observed. It was not clear, however, which of these signs could be prevented or mitigated by thiamine therapy, or whether residual toxicity was caused by the interaction of sulphite with constituents of the diet and/or the direct action of sulphite on the organism. In 1960, Lockett & Natoff, administering sulphite chronically to rats in their drinking-water at an intake rate of approximately 1–2 mmol/kg/day, observed none of the toxic signs listed by Fitzhugh *et al.* (1946). Their obvious conclusion, therefore, was that sulphite was not directly responsible for this toxicity.

Bhagat & Lockett (1964) later attempted to clarify the roles of thiamine destruction and storage of diet in the development of toxicity resulting from feeding sulphited diets to rats. In these experiments, the growth rate of young rats over a 5–7-wk period was used as an assay for toxicity. Diets contained approximately 130 μg thiamine/100 g and 0·6% sodium metabisulphite at the time of mixing (equivalent to an intake of approximately 5–9 mmol sulphite/kg/day, assuming no loss of sulphite prior to use). However, subsequent storage at room temperature resulted in the rapid and parallel destruction of both sulphite and thiamine content, and after 8 days only approximately 20% of their initial concentrations remained. Young rats fed this diet within 2 months of its preparation showed a decreased growth rate, which could be corrected by thiamine supplementation in spite of weak antithiamine activity resulting from the exposure of dietary yeast to sulphite. Diets that were stored at room temperature for 75 days or longer caused toxicity in the form of a reduced growth rate and diarrhoea, which could not be completely corrected by thiamine supplementation. Although residual sulphite concentrations in the diets at the time of consumption were not measured, it seems almost certain that the sulphite would have been too low after 75 days of storage to be a factor in the development of the observed toxicity.

The most thorough investigation of chronic sulphite toxicity to date is the work of Til, Feron & de Groot (1972a) and Til, Feron, de Groot & van der Wal (1972b) using rats and pigs. Sulphite was administered in the diet, and losses due to chemical reaction prior to feeding were kept to a minimum by frequent diet preparation and storage at low temperature. The loss of sulphite that did occur was measured and the dietary concentrations were corrected accordingly. In addition, thiamine was added to the diet to compensate for its sulphite-mediated destruction, thus ensuring that any toxicity observed during the experiment would not be due to thiamine deficiency.

Sulphite was administered to three generations of rats for periods up to 2 yr at intake rates of approximately 0·7, 1·5, 3, 6, and 13 mmol/kg/day for the five treatment groups, and the health of these animals was compared with that of a matched control group receiving the same diet with no added sulphite (Til *et al.* 1972a). Slight growth retardation was observed in the F_1- and F_2-generation rats of the high-dose group and marginally reduced haematocrit, haemoglobin and erythrocyte counts also occurred in F_0 rats at this treatment level. Occult blood was present in the faeces of approximately 20–50% of rats ingesting 6 mmol/kg/day. Abnormal morphology of the forestomach was observed in some F_2-generation rats ingesting 3 mmol/kg/day, and in rats of the two highest treatment groups more severe hyperplasia and inflammation of both the fore- and glandular stomach

was observed. There were no dose-related trends in tumour frequencies among F_0- and F_1-generation rats that died during the experiment or were killed at 2 yr of age. Likewise, sulphite had no effect on the indices of reproduction and early development investigated, i.e. on fertility (% of females with litters), mean number of pups per litter, birth weight and mortality prior to weaning. In F_2 generation females, kidney weight relative to body weight was increased in the high dosage group, although kidney function, as measured by phenol red excretion, urine specific gravity and glutamic–oxalacetic transaminase activity in the urine, was not adversely affected by sulphite feeding. Increased relative weights of kidneys have also been observed in other chronic and subchronic sulphite toxicity studies. The no-effect level determined from the experiments of Til *et al.* (1972a) was equivalent to an intake of 1·5 mmol/kg/day. This level of consumption, adjusted by a 100-fold safety factor, has been adopted by WHO as its maximum acceptable daily intake (ADI) level, i.e. 0·70 mg (as SO_2)/kg body weight (Joint FAO/WHO Expert Committee on Food Additives, 1974).

In a companion experiment to the one described above, Dutch Landrace pigs were fed sulphite from weaning for periods of up to 48 continuous weeks (Til *et al.* 1972b). The same nominal concentrations of dietary sulphite were used for these animals as for the rats, but actual intakes were considerably less, approximately 0·1, 0·3, 0·6, 1·6 and 3·6 mmol/kg/day in the five treatment groups. The thiamine added to the diet was sufficient to prevent deficiency in all groups except that on the highest dose, in which a slight reduction in hepatic thiamine level occurred. Growth and food consumption were significantly decreased only in the highest dose group (3·6 mmol/kg/day). Paired-feeding studies showed that this decreased rate of growth was due solely to decreased food consumption and was not a direct cause of ingested sulphite. Increases in the relative weights of liver and kidney in this same treatment group, however, were attributable to sulphite consumption, but were not accompanied by histological changes. As in the experiment with rats, the most damaging effects of sulphite were the inflammatory and hyperplastic changes in the stomach mucosa of animals in the two highest dosage groups, although no occult blood was found in the faeces of these animals.

Several other chronic feeding studies of merit conducted in rats will not be reviewed here in any depth (Cluzan, Causeret & Hugot, 1965; Lanteaume, Ramel, Girard, Jaulmes, Gasq & Ranau, 1965; Lockett & Natoff, 1960). In all of these studies sulphite was either added to the drinking-water or given in solution by gastric intubation, resulting in daily intakes of between 0·05 and 2 mmol/kg. Usually several generations of rats were treated and all experiments lasted for at least 1 yr. In these studies, investigators measured the effect of sulphite on such parameters as fertility, the general health and growth rate of offspring, organ weights, haematology, food intake and growth rate of adults, histological appearance of major organs and tumour incidence. In general, no consistent trends attributable to sulphite exposure were apparent in any of these parameters.

Short-term studies

Investigations by Til *et al.* (1972a) of the toxicity of high doses of sulphite ingested with the diet over relatively short periods, showed that the food intake, food efficiency and growth rate were drastically reduced in young male rats ingesting approximately 50 mmol sulphite/kg/day for 8 wk. In addition, severe anaemia, increased spleen weight and a slightly increased leucocyte count were observed after only 3 wk. Gunnison *et al.* (1981a) have confirmed these observations and have shown that the anaemia results not from the systemic activity of sulphite following ingestion but from the interaction of sulphite with a constituent(s) of the diet, possibly cyanocobalamine.

In other short-term studies of up to 4 months duration, daily sulphite intakes ranging from approximately 0·5 to 6 mmol/kg caused a slight increase in the excretion of calcium and had no effect on the hepatic stores of vitamin A (Joint FAO/WHO Expert Committee on Food Additives, 1974; Lanteaume, Morin, Palluel & Pallaget, 1978). The effect of sulphite on calcium excretion is probably of little or no physiological significance.

In thiamine-deficient rats, small amounts of sulphite administered separately from the diet depressed weight gain and survival, while in rats not deficient in thiamine, sulphite intake up to 6·2 mmol/kg/day had no effect on weight gain over a 3-month period (Cremer & Hötzel, 1966). This observation may help to explain the toxicity of sulphite reported by Fitzhugh *et al.* (1946) and discussed earlier. In contrast to the rat data, approximately 0·1 mmol sulphite/kg/day administered in imbibed fluids for 25 consecutive days to human volunteers with thiamine deficiency (determined by biochemical signs) caused no clinical, neurophysiological or biochemical alterations compared with controls (Cremer & Hötzel, 1970). The bulk of evidence accumulated from both long- and short-term studies supports the view that sulphite administered in fluids separately from the solid diet does not measurably reduce the thiamine status of the animal.

In spite of evidence of sulphite-induced chromosome aberrations resulting from *in vitro* exposure (see previous section), sulphite did not induce a detectable increase in dominant-lethal mutations in either male or female germ cells of mice receiving repeated daily intraperitoneal injections of 2·9–4·8 mmol/kg (Generoso, Huff & Cain, 1978). Nor were Jagiello *et al.* (1975) successful in inducing chromosome aberrations in mouse oocytes cultured *in vitro* following a single intravenous injection of up to approximately 2 mmol sulphite/kg.

Reviewing the data from experiments designed to evaluate the mammalian toxicity of ingested sulphites leads to the conclusion that apart from the indirect toxicity resulting from destruction of dietary thiamine or other changes in the diet, and the direct irritant effect on the gastro-intestinal tract at relatively high intake levels, no serious adverse effects were observed as a result of chronically administered sulphite. This conclusion is surprising in the light of the reactivity of sulphite with many biologically important molecules and the toxicity of sulphite observed in biologically active *in vitro* test systems. The difference between the toxic potential of sulphite perceived from *in vitro* data

and the toxicity observed in *in vivo* experiments has been noted previously (Shapiro, 1977); the probable explanation becomes apparent with an understanding of sulphite metabolism.

Mammalian sulphite metabolism

The primary route of sulphite metabolism in mammals is its enzymatically mediated oxidation to sulphate. The enzyme involved, sulphite:cytochrome *c* oxidoreductase (EC 1.8.3.1), termed sulphite oxidase, is apparently ubiquitous among mammalian species and is present at high levels in the liver and in lower concentrations in most of the other tissues of the body. Sulphite oxidase, located in the mitochondrial intermembranous space, exists as a dimer of identical subunits, each consisting of a molybdenum ion (Mo^{6+}) and a haem molecule in addition to the apoenzyme. The *in vivo* oxidation of sulphite involves the transfer of a pair of electrons from sulphite to the Mo^{6+} ions and then to the haems associated with the enzyme molecule. The electron pair is then passed to cytochrome *c* of the respiratory chain, eventually reducing 0·5 O_2 to H_2O and producing 1 molecule of ATP in the process. Although sulphite can be autoxidized by a free-radical chain mechanism under appropriate conditions, it is thought that this reaction sequence does not proceed readily in mammalian tissues for a variety of reasons (Cohen & Fridovich, 1971).

In addition to being the major metabolic pathway of exogenous sulphite (ingested sulphite and inhaled SO_2), enzymatically mediated oxidation of sulphite to sulphate is the terminal step in the catabolism of sulphur-containing amino acids. Thus, an enzyme that apparently evolved to protect the tissues of the body from the insult of endogenously-produced sulphite also functions in the metabolism (and presumably the detoxification) of this same substance originating from exogenous sources. Assuming that the sulphate excreted by animals in 'sulphur balance' originates primarily from the catabolism of sulphur-containing amino acids, it can be estimated from data on sulphate excretion that the daily quantity of endogenous sulphite generated by humans is approximately 0·3–0·4 mmol/kg (Institute of Food Technologists and Committee on Public Information, 1976). Under normal circumstances this is considerably greater than the estimated intake of exogenous sulphite (see Introduction).

The capacity of mammalian sulphite oxidase for sulphite oxidation is extremely high compared with the normal sulphite load from endogenous and exogenous sources. For example, Cohen, Drew, Johnson & Rajagopalan (1973) have estimated by *in vitro* assay that the sulphite oxidase contained in the tissues of the rat is theoretically capable of oxidizing sulphite at the rate of approximately 750 mmol/kg/day. Also, Oshino & Chance (1975) and Wilkins, Greene & Weller (1968) demonstrated that the perfused livers of rats and dogs can oxidize sulphite for short periods of time at rates of at least 58 and 18 mmol/kg/day, respectively. Using established pharmacokinetic techniques, Gunnison, Bresnahan & Palmes (1977) investigated the rate of sulphite oxidation in intact rats following rapid intravenous delivery. Elimination of sulphite, which occurred predominantly by metabolism to sulphate, was characterized by first-order rate constants of the order of 0·7–1/min which is equivalent to a half-life for sulphite of approximately 1 min. Further, Gibson & Strong (1973) were unable to detect sulphite in the urine of rats following administration of approximately 6 mmol/kg by gastric intubation. Since sulphite is readily absorbed from the gastro-intestinal tract (Bhagat & Lockett, 1960), this observation attests to the capacity of rats to metabolize systemic sulphite rapidly.

Gunnison *et al.* (1977) compared the activity of sulphite oxidase in rats with that in rabbits and rhesus monkeys using *in vivo* kinetic methods, and demonstrated that rats exhibit approximately three and five times greater activity, respectively, than the latter two species. In addition, Johnson & Rajagopalan (1976a,b) have shown by *in vitro* assay that rat liver possesses approximately 10–20 times more sulphite oxidase activity than does human liver. These comparisons suggest that the rat may be a poor species for the evaluation of sulphite toxicity in humans since the opportunity for potentially damaging reactions of sulphite is comparatively less in the rat due to more rapid metabolism of sulphite to sulphate.

Although the rapid rate of oxidation of sulphite by sulphite oxidase certainly minimizes the quantitative importance of other metabolic pathways, rats injected intraperitoneally with approximately 3 mmol/kg/day and rabbits and rhesus monkeys ingesting approximately 2 mmol/kg/day nevertheless metabolized a portion of this exogenous sulphite to *S*-sulphonate compounds in the plasma (Gunnison & Palmes, 1978). Indeed, the rats and rabbits possessed detectable concentrations of plasma *S*-sulphonate compounds prior to exposure to exogenous sulphite, indicating that the sulphite generated endogenously from sulphur-containing amino acid catabolism was also partially metabolized *via* this route. Because of their relative biological stability, these *S*-sulphonate metabolites were evident in plasma, which does not usually contain detectable free sulphite.

When sulphite is present in the tissues in sufficiently high concentration, it reacts with β-mercaptopyruvate (a normal intermediate in sulphur-amino acid catabolism) forming inorganic thiosulphate ($S_2O_3^{2-}$). This metabolite is, at most, marginally detectable in the urine of normal humans and rats, while in both these species large quantities are excreted into the urine of individuals that are deficient in sulphite oxidase and have, therefore, relatively high systemic levels of sulphite.

Throughout the literature on the investigation of mammalian sulphite toxicity there is an almost total absence of any attempt to determine tissue sulphite concentrations. This seems inexplicable, particularly since concentration is the most logical and appropriate basis for the correlation of findings from *in vivo* experiments with data gathered *in vitro*. The limited data existing on *in vivo* sulphite concentration comes from studies of sulphite metabolism in normal and sulphite oxidase-deficient mammals. Gunnison & Palmes (1973 & 1978) determined free sulphite in the plasma of several species, both prior to and during administration of sulphite. Sulphite originating from endogenous sources was not detectable (i.e. was less than 3 μM) and when exogenous sulphite was adminis-

tered in the drinking-water of rats, rabbits and rhesus monkeys at a rate of approximately 2 mmol/kg/day and of mice at approximately 6 mmol/kg/day, plasma sulphite was detected only in the rhesus monkeys. Of the plasma samples collected from six rhesus monkeys at various times during the light cycle (i.e. primary drinking time), sulphite ranging in concentration from 8 to 67 μM was detected in 10 of 23. Gunnison, Farruggella, Chiang, Dulak, Zaccardi & Birkner (1981b) administered sulphite to rats (2–9 mmol/kg) by gastric intubation and measured plasma-sulphite concentration with respect to time following intubation. Peak concentrations ranged from 70 to 800 μM and detectable concentrations persisted for 1–3 hr depending on the dose.

Free sulphite has been reported in the plasma of a child diagnosed as deficient in sulphite oxidase (Shih, Abroms, Johnson, Carney, Mandell, Robb, Cloherty & Rajagopalan, 1977). The plasma concentration increased from 14 μM to about 130 μM and then decreased to 2 μM when the child's diet was first enriched with cysteine and then restricted in sulphur-amino acid content. In sulphite oxidase-deficient rats possessing approximately 1% of the enzyme activity of normal adults, plasma-sulphite concentrations ranged from undetectable to 60 μM with a mean of 18 μM (Gunnison, *et al.* 1981b).

Gunnison & Farruggella (1979) maintained approximately steady-state plasma-sulphite concentrations in the range of 400–650 μM for up to 6 hr by zero order intravenous infusion of rabbits at a rate of approximately 0·9 mmol/kg/hr. The purpose of these infusions was to investigate the kinetics of *S*-sulphonate formation in the aorta and lung. However, during the course of performing the experiments, it was also learned that rabbits could not survive for longer than 2 or 3 hr when plasma-sulphite levels were maintained in the range of 700–1000 μM (unpublished data).

It is worthy of note that in the majority of the *in vitro* experiments discussed earlier, sulphite concentrations were either close to or exceeded the concentrations shown to be lethal to rabbits, and were, in addition, almost always at least one order of magnitude greater than those observed in animals and humans severely deficient in sulphite oxidase. This fact does not diminish the value of the *in vitro* data since the primary purpose of such experiments is usually to identify, or define more accurately under optimal conditions for their development, potentially toxic changes which may also appear *in vivo* under more prolonged but less severe exposure conditions. Nevertheless, the relationship of sulphite concentrations used *in vitro* to those attainable *in vivo* should be considered in predictions of the likelihood of the occurrence of a particular toxic effect *in vivo*.

Alternative mammalian models for evaluation of sulphite toxicity

A case has been made against the use of the rat for the evaluation of sulphite toxicity in humans because of the lower activity of sulphite oxidase in the latter species. In addition, it is known that a genetic deficiency of this enzyme in humans can lower its activity still further. These cases of severe deficiency result in grave health effects which can lead to death (Irreverre, Mudd, Heizer & Laster, 1967). Although occurrences of extreme deficiency are apparently rare, they do illustrate the crucial role of this enzyme in human health and raise questions concerning the pattern of normal variation in sulphite oxidase activity within the human population, as well as the significance of possible minor (i.e. subclinical) deficiencies of sulphite oxidase on the metabolism of sulphite and ultimately on the development of chronic toxic effects. It is clear that the sulphite oxidase-competent rat cannot be used to investigate these questions.

In the course of their thorough investigations into the nature and functioning of sulphite oxidase, Johnson, Rajagopalan & Cohen (1974) found that rats deficient in this enzyme could be produced by manipulation of the tungsten (W) and Mo content of the diet. In an internal environment of relatively high W and low Mo, W either replaces Mo or prevents its incorporation into newly synthesized apoenzyme molecules, causing these molecules to be inactive (Johnson, Cohen & Rajagopalan, 1974). This results in the progressive loss of sulphite-oxidase activity to a lower steady-state level, the magnitude of which is dependent upon the W:Mo intake ratio. By manipulation of this ratio, steady-state sulphite-oxidase activities can be attained over a wide range (Gunnison *et al.* 1981b). This phenomenon has been exploited in our laboratory where groups of sulphite oxidase-deficient female rats, possessing approximately 1–2% of the activity exhibited by normal female adults, have been characterized metabolically and used to investigate sulphite toxicity (Gunnison *et al.* 1981a,b). These deficient rats are considered to be models for humans having approximately 10% of the sulphite-oxidase activity ascribed to normal individuals. The model has helped to answer some questions regarding the primary toxicity of sulphite, specifically concerning the systemic destruction of thiamine and the development of anaemia, as mentioned previously. More importantly, using this model a serious concern has been raised regarding the possible involvement of sulphite in the aetiology of early breast cancer. A low incidence of mammary adenocarcinoma (4/149) was observed in sulphite oxidase-deficient rats of less than 5 months of age which had been treated with a high W/low Mo regime for only 40–65 days. The tissues of these rats were, of course, exposed to elevated concencentrations of endogenously-generated sulphite. No tumours were found in age-matched controls. Although the difference between the sulphite oxidase-deficient and control groups was not statistically significant, the authors believed that because of the early age at which the tumours developed, they were very likely to be treatment related. If this is true, then the role of excess W and a deficiency of Mo (apart from its effect on sulphite-oxidase activity) in the production of early mammary tumours must be considered in addition to that of systemic sulphite. These factors are germane to the evaluation of any toxic effect observed using this model. The direct toxicity of excess W and of Mo deficiency can be at least partially determined by the use of controls, but the possibility of synergism with sulphite must also be considered and is more difficult to evaluate. In spite of these drawbacks, the sulphite oxidase-deficient rat shows

promise as a model for the evaluation of human sulphite toxicity.

It is believed by this author that sulphite oxidase-competent mammals are adequate models for evaluation of the localized effects of inhaled sulphur dioxide on the upper respiratory tract of humans, in terms of accurately reflecting tissue sulphite concentration. There is recent evidence that the upper airways of the respiratory tract directly exposed to inhaled SO_2 (including the nasal passages, trachea and major bronchi) can build up considerable localized concentrations of sulphite without measurably affecting the overall systemic concentration of sulphite (Gunnison, Zaccardi, Dulak & Chiang, 1981). It is probable that the capacity of the animal to oxidize sulphite enzymatically has little bearing on the concentrations of sulphite that develop in the upper respiratory tract of animals inhaling SO_2. Although the voluminous literature on the potential toxicology of inhaled SO_2 is, in general, not within the scope of this review, an important chronic study involving SO_2 will be discussed here because it suggests an active role for sulphite in the origin of bronchogenic tumours. Without citing particular references, it is accurate to state that numerous investigations in a variety of mammals exposed chronically to concentrations of SO_2 several times greater than those ordinarily observed in urban environments have revealed no irreversible toxic response in the respiratory tract. In contrast, however, lifetime intermittent exposure of rats to benzo[*a*]pyrene (BP), a known carcinogen, in conjunction with SO_2 suggests that SO_2 may be acting as a cocarcinogen (Laskin, Kuschner, Sellakumar & Katz, 1976). The data from this study are summarized in Table 1. Although the significance of the temporal aspect of SO_2 exposure relative to that of BP is difficult to assess, consideration of the data *in toto* strongly suggests a role for SO_2 in the aetiology of these bronchogenic squamous-cell carcinomas.

Discussion

Although *in vitro* research has been extremely useful in increasing our knowledge of sulphite chemistry and of the potentially toxic reactions of sulphite, *in vitro* systems have, in general, been poor models for predicting mammalian toxicity, largely because *in vivo* mammalian sulphite concentrations have been greatly overestimated. Extrapolations of *in vitro* data to the *in vivo* situation have often been erroneous (Inouye *et al.* 1978; Kaplan *et al.* 1975; Schneider & Calkins, 1970), primarily because of ignorance of sulphite metabolism. Now, however, quantitative data relating exogenous sulphite exposure to *in vivo* sulphite concentrations in mammals are available, and provide a basis for evaluation of the *in vivo* implications of *in vitro* data. Thus, whereas plasma-sulphite concentrations of up to approximately 100 μM were observed in a rhesus monkey ingesting 2 mmol sulphite/kg/day, most of the sulphite toxicity demonstrated *in vitro* resulted from concentrations that were considerably higher.

Further, because of its rapid metabolic clearance by sulphite oxidase, chronically ingested sulphite does not accumulate in the tissues and reach an elevated steady-state concentration but is rapidly eliminated after absorption, giving sporadic brief episodes of elevated tissue-sulphite levels; these will not sustain sulphite adducts which are unstable in the absence of free sulphite. Therefore, the toxicological significance of the reversible reactions of sulphite described in the earlier sections is probably slight or nil under conditions of intermittent exposure of body tissues to sulphite—the expected pattern of human exposure.

Compared to reversible reactions, the irreversible reactions of sulphite are of potentially greater toxicological significance. The sulphitolysis of thiamine, for example, although a relatively slow reaction, results in significant destruction of the vitamin in stored diets as well as in the gut.

The sulphite-catalysed deamination of cytosine and 5-methylcytosine residues in DNA is an irreversible reaction of possible toxicological significance. Shapiro (1977) has calculated, on the basis of the *in vitro* rate of conversion of cytosine to uracil under physiological conditions, that a sulphite concentration of only 0·3 μM is required to double the spontaneous mutation rate in humans. The toxicological implications of this calculation are now unclear, however, in the light of the apparent lack of involvement of cytosine residues in C:G to A:T transitions. As pointed out earlier, this is an area of current investigation.

Another group of reactions with toxic potential are the reactions of free radicals, produced by the autoxidation of sulphite, with tryptophan, methionine (and certain other sulphides), DNA and olefins. Again, reactions with the first three substrates are apparently

Table 1. *Squamous-cell carcinomas in rats chronically exposed to benzo[a]pyrene and SO_2, alone and in combination**

Exposure conditions (on 5 days/wk)	No. of rats		Incidence (%)
	Per group	With carcinomas	
Filtered air	15	0	0
10 ppm SO_2 (6 hr)	15	0	0
BP† (1 hr)	30	1	3
10 ppm SO_2 (6 hr) followed by BP† (1 hr)	30	2	7
BP† with 4 ppm SO_2 (1 hr)	45	4	9
10 ppm SO_2 (6 hr) followed by BP† with 4 ppm SO_2 (1 hr)	46	9	20

*Data from Laskin *et al.* 1976.
†Exposure to benzo[*a*]pyrene at 10 mg/m³.

irreversible, while information on the reversibility of the latter is not available. The implications of these reactions include damage of membranes due to free-radical attack on their unsaturated lipids, and chromosome aberrations resulting from sulphite-induced DNA chain breaks. Although free-radical reactions proceed at lower concentrations than many of the ionic reactions of sulphite, it is difficult to speculate on their occurrence *in vivo* because of the presence of numerous free-radical scavengers which prevent significant sulphite autoxidation, even at favourable sulphite concentrations, and because superoxide dismutase may inhibit the initiation of autoxidation by O_2^-.

Most of our knowledge of mammalian sulphite toxicity originates from experiments in which sulphite oxidase-competent rats were fed large quantities of sulphite. From these data most toxicologists would legitimately conclude that the hazard to humans of sulphite consumption at present levels is very low. However, the role of sulphite-oxidase activity in sulphite toxicity has not as yet been adequately investigated. We have pointed out previously that, with respect to sulphite-oxidase capacity, the rat is a poor model for humans. There are, in addition, essentially no data on the variability of sulphite-oxidase activity in the human population. Further, little is known of the quantitative relationships between intake of sulphur-containing amino acids, sulphite-oxidase activity and endogenous sulphite concentration.

Given the relatively low mean sulphite-oxidase capacity of humans, the possibility of significant genetic variation in sulphite-oxidase activity among individuals, the uncharacterized variable of dietary sulphur-amino acid content and the generally low human consumption of sulphites, it appears that the importance of endogenous sulphite generation relative to exogenous sulphite intake may have been underestimated in most previous investigations of sulphite toxicity. Further, endogenous sulphite is produced intracellularly in relatively close proximity to DNA and other potential target molecules, while ingested sulphite must traverse several barriers before reaching these sites.

In human sulphite oxidase-deficiency disease as well as in sulphite oxidase-deficient rats, steady-state sulphite concentrations in the micromolar range have resulted from purely endogenous sources. Under these conditions, reversible reactions of sulphite can be of physiological significance provided the sulphite adduct is reasonably stable. As an example, it was previously stated that sulphite reacts readily, although reversibly, with NAD when associated with LDH, forming an enzymatically inactive adduct with a pH-independent dissociation constant of approximately 10^{-3} M (Parker *et al.* 1978). Using this K_d, one can calculate that in the presence of 50 μM SO_3^{--}, more than 99% of the NAD bound to LDH would be in the form of the sulphite adduct and, therefore, presumably inactive. There is some discrepancy, however, between this calculated value and the experimentally determined inhibition of LDH *in vitro*. Ciaccio (1966) and Oshino & Chance (1975) demonstrated 50% inhibition of the enzyme by 350 and 200 μM sulphite, respectively. Similar data for other enzyme–cofactor–sulphite adducts and other types of sulphite addition products suggest that metabolic disturbances may result from steady-state sulphite concentrations in the micromolar range, as can be expected to occur in animals sufficiently deficient in sulphite oxidase.

A series of enzymes that are inhibited by micromolar concentrations of sulphite are the sulphatases, most notably sulphatase A. Cerebroside sulphatase, which is thought to be identical to sulphatase A, is deficient in cases of metachromatic leucodystrophy (MLD), a genetically determined disease in which myelin degeneration is associated with accumulation of cerebroside sulphate (Moser, 1972). There is also a series of genetically determined diseases (mucopolysaccharidoses) many of which are caused by, or associated with, functional deficiencies of one or more other sulphatase enzymes (Roy, 1976). Nearly all of these diseases are characterized by mental retardation as well as by physical defects, especially those of bone. The possibility of inhibition of sulphatase enzymes *in vivo* by sulphite was recognized by the investigators who first identified sulphite oxidase-deficiency disease. Analysis of the urine and tissues from a patient who died of this disease revealed no significant increase in tissue or urinary sulphate esters as would be expected if sulphatases had been inhibited (Percy, Mudd, Irreverre & Laster, 1968). However, these results are difficult to interpret since the severely decreased availability of sulphate in this patient would have resulted in a reduced rate of sulphate ester synthesis.

Several experiments reviewed in this paper have suggested, or are consistent with, a role for sulphite as a cocarcinogen. Certainly the development of bronchogenic squamous-cell carcinomas following inhalation exposures to BP and SO_2 (Laskin *et al.* 1976) and the *in vitro* demonstration of sulphite enhancement of UV mutagenicity (Mallon & Rossman, 1981) support this hypothesis. In addition, the appearance of mammary adenocarcinomas in young sulphite oxidase-deficient rats might result from an interaction of sulphite and tungsten. Although this is admittedly speculative, the cocarcinogenesis hypothesis should continue to be investigated.

The *in vitro* investigations of sulphite reactivity and toxicity reviewed in this paper have suggested an array of mechanisms that could produce toxicity in mammals. Consideration of this information and the data from *in vivo* evaluations of sulphite toxicity in the light of mammalian sulphite metabolism, makes possible the elimination of some of these mechanisms as unlikely and the selection of others as being of greater potential and as deserving further investigation.

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