Ozone Clinical Study Paper

INTRODUCTION

To date, many studies concerning the clinical evaluation of ozone (O_3) have, unfortunately, been based on assessments of its harmful effects rather than demonstrating any therapeutic benefits that it may offer.[1-4] In the Earth's biosphere, the role of O_3 is controversial. Indeed, it is one of nature's most powerful oxidants and is reactive towards many biomolecules.[5] This 'reactive oxygen species' (ROS) can be produced either by ss ultra-violet rays of the sun, or artificially with an ozone generator. In the former case, O_3 is the major constituent of the atmosphere responsible for shielding off ultra-violet light from the sun and oxidising pollutants in the air. Man for purposes of water purification and sewage treatment can also usefully employ O3.

O_3 was first suggested as a disinfectant for drinking water in the 19th Century in view of its powerful ability to inactivate micro-organisms.[6] Indeed, many researchers have emphasised that the utilisation of this oxidant over limited time periods can effectively disinfect water supplies.[7-9] It acts rapidly and in lower concentrations when compared to chlorine, and has no side effects such as taste and odour which are characteristic of other disinfecting agents.[10] However, high levels of O_3 were found to be insufficient for disinfection in view of the limited solubility of this gas in water and adverse toxicological problems arising from the employment of such high concentrations.[11-13] O_3 is an agent that disinfects by destroying, neutralising or inhibiting the growth of pathogenic micro-organisms, and has been found to be an effective alternative biocidal agent to chlorine.[14] The treatment of drinking water with ozone has been shown to be more efficient against spores of *Bacillus subtilis*, a 0.35 mg/l concentration giving rise to the reduction of at least 5 log values in populations of approximately 1×10^6 cells/ml of *Escherichia coli, Vibrio cholerae, Salmonella typhi, Yersinia enterocolitica, Pseudomonas aeruginosa, Aeromonas hydrophila, Listeria monocytogenes* and *Staphylococcus aureus* when compared with 0.50 mg/l chlorine (with the exception of Vibrio cholerae). Both disinfectants were probably consumed during the treatment period.[12] Indeed, Broadwater *et al.*,[7] agreed with Morris[13] and indicated that O_3 is an effective bactericide against both vegetative cells, and the *B. megaterium* and *B.*

cereus spores of *E. Coli* ATCC 9677. However, on consideration of the level of organic components present in water, they suggested that this ROS can be applied at higher dosages (0.5-10 mg/l) and for longer contact periods (2-10 min.) for practical applications since, having a potent oxidising activity (redox potential ca. +1.6 V) ozone is consumed by such agents, a phenomenon that prevents full utilisation of the applied dose as a disinfectant.[13]

Historically, O_3 has been the agent of choice for the disinfection of public water supplies rather than chlorine in the United States of America. The reason for the preferential application of O_3 here is primarily to remove iron and manganese ion colourations, and to avoid adverse tastes and odours.[9] However, one major disadvantage is that O_3 is stable only for a short period of time and decomposes to form molecular oxygen which is utilised for sustenance by aquatic life,[13] and hence it disappears very quickly from the system.[15] Consequently, it is impossible to maintain a residual level of this ROS in a water distribution system. However, Burleson *et al.*,[16] found the application of a simultaneous treatment of O_3 with sonication to be more effective in a bactericidal treatment process designed for the disinfection of waste-water. Sonication reduced or altered oxidisable organic material as measured by biological oxygen demand and chemical oxygen demand determinations, and therefore the O_3 demand of the secondary effluent was also reduced.[11] This may also enhance the O_3-mediated inactivation of micro-organisms by ozone via dissipation of particulate organic material and clusters of bacteria, a process facilitating their exposure to the oxidant.[14] With pre-treated water, ozone can kill bacteria by rapidly rupturing their cell membranes (within 2 seconds). Chlorine, on the other hand, simply diffuses into the cell and requires 30 min. to achieve bactericidal effects. The rate of lysis is dependent upon the ozone level: higher ozone concentrations are used for highly contaminated systems, whereas lower concentrations are used for maintenance. Following O_3-mediated lysis of the micro-organisms, the cytoplasmic constituents are oxidised by this agent, which is then removed by UV irradiation at a wavelength of 254 nm.[15] O_3 has also been employed commercially to remove trace organic contaminants from water.

In view of its powerful oxidising actions, ozone has a very rich chemistry and the oxidation of critical biomolecules undoubtedly accounts for its broad-spectrum biocidal properties.

Indeed, ozone can attack a very wide variety of biomolecules[5,17], for example free or protein-incorporated amino acids such as cysteine, methionine, histidine and tyrosine, carbohydrates, phenolic adducts and, of course, its well-characterised ozonylation of carbon-carbon double bonds, e.g., those of polyunsaturated fatty acids (PUFAs). Oxidation of PUFAs by ozone gives rise to the production of conjugated hydroperoxydiene and subsequently aldehydic species[18], the latter apparently serving as biomarkers of ozonylation[19,20]. Interestingly, reaction of water-soluble, single electron-donors with ozone primarily generates the ozone radical anion, a transient adduct which, on protonation, decomposes to hydroxyl radical and dioxygen[21]. Hence, some of the reaction products which putatively arise from the interactions of ozone with molecules present in tissues and biofluids are, at least in principle, identical to those produced from the attack of hydroxyl radical on such ROS scavengers.

Currently, root caries represents a challenging problem to the dental profession in view of a substantial increase in the population of elderly patients during the late 20th century. This condition is primarily ascribable to tooth demineralisation processes induced by organic acids (e.g., lactic and pyruvic acids) generated by bacteria, predominantly Strptococcus mutans[22], and a recent investigation conducted by Baysan et. al.[23] revealed that ozone exerts a powerful bactericidal action towards this pathogen, together with further micro-organisms associated with primary root carious lesions. Indeed, the application of ozone in the dental practices may serve as a viable, cost-effective and convenient means of treating dental caries, and it is conceivable that it could eventually replace conventional 'drilling-and-filling' procedures currently employed by dental surgeons.

In view of the clear indications for the therapeutic application of ozone in the treatment of periodontal conditions, a clinical trial involving this agent has recently been approved by the US Food and Drug Administration (FDA) (ED-REF!) Therefore, in this investigation we have employed high resolution proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy to achieve a multicomponent evaluation of the oxidising actions of ozone towards biomolecules present in intact human saliva. The therapeutic, aesthetic and

biochemical significance of the results acquired are discussed in detail.

2 MATERIALS AND METHODS

2.1 *Sample Collection and Preparation*

Unstimulated human saliva samples were obtained from a total of 12 healthy volunteers (7 male, 5 female). Subjects were seated comfortably and then asked to collect all saliva into a cup for a period of 10 min. Immediately after collection, all samples were centrifuged at 16,000 × g for 30 min. (4°C) to remove debris.

Aliquots (5.00 ml) of the above salivary supernatant samples were removed and each of them was divided into two equivalent portions (2.50 ml). The first of these was treated with O_3 generated by the HealOzone Unit (CurOzone, USA) for a period of 10 s (equivalent to a delivery of 4.484 mmol. of this oxidant) and then equilibrated at a temperature of 37 C for a 30 min. period prior to NMR analysis ; the second group of portions served as untreated controls.

2.2 *Proton NMR Measurements*

Proton (^{1}H) NMR measurements on the above samples were conducted on a Bruker AMX-600 spectrometer (ULIRS, Queen Mary, University of London facility, U.K.] operating at a frequency of 600.13 MHz and a probe temperature of 298 K. Typically, 0.60 ml of sample was placed in a 5-mm diameter NMR tube, and 0.07 ml of $^2H_20$ was added to provide a field frequency lock.

The intense water signal (δ = 4.80 ppm) was suppressed by presaturation via gated decoupling during the delay between pulses. Pulsing conditions for one-dimensional (1-D) spectra acquired on salivary supernatant samples were: 64 or 128 free induction decays (FIDS); 16,384 data points; 3-7 μs pulses; 1.0 s pulse repetition rate. Line-broadening functions of 0.30 Hz were utilised for the processing of experimental NMR data. For selected salivary supernatant specimens, the broad protein resonances present in control and dentifrice supernatant-treated salivary supernatant samples were suppressed by the Hahn spin-echo sequence $(D[90°x\text{-}t\text{-}180°y\text{-}t\text{-}collect])^{24}$ which was repeated 128 times (t = 68

ms). Chemical shifts were referenced to external sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TSP; $\delta = 0.00$ ppm). Where present, the methyl group resonances of alanine ($\delta = 1.487$ ppm) and lactate ($\delta = 1.330$ ppm) served as secondary internal references for the saliva samples examined.

The identities of biomolecule resonances present in the salivary ^1H NMR spectra acquired were routinely assigned by a consideration of chemical shift values, coupling patterns and coupling constants. The relative intensities of selected signals therein were determined by electronic integration, and the concentrations of components detectable were determined by comparisons of their resonance areas with that of a 42.0 mM standard solution of TSP located within a coaxial NMR tube insert. This procedure was employed to avoid broadening of the TSP resonance which arises from its binding to salivary proteins or alternative macromolecules. Where required, salivary metabolite concentrations were determined by electronic integration of their ^1H resonances and expressing their intensities relative to that of the 'internal-but-isolated' TSP standard. In view of the protein concentration of human saliva (1.40-6.40 g/l in resting specimens, mean value 2.20 g/l)[25], which is much lower than that of human blood serum (65-83 g/l)[26], the minimal broad macromolecule resonance envelope in single-pulse (one-dimensional) ^1H NMR spectra was found not to interfere with the observation and integration of the sharp pyruvate and actetate signals, and hence all salivary organic acid anion concentrations documented here represent those obtained from such spectra. Of course, the levels given reflect only the non-macromolecule-bound fraction of these biomolecules and therefore are expected to be somewhat lower than the total salivary concentrations. Indeed, it has been established that an 'NMR-invisible' pool of protein-bound metabolites such as lactate can be liberated from macromolecular binding sites by the addition of high levels of ammonium chloride (≥ 0.5 M) to biofluids[27].

Two-dimensional shift-correlated ^1H-^1H NMR (COSY) spectra of human salivary supernatants were acquired on the Bruker AMX-600 facility using the standard sequence of Aue et. al.[28], with 2,048 data points in the t_2 dimension, 256 increments of t_1, a 3.00 s relaxation delay, and 24 transients.

Total correlation spectroscopy (TOCSY) spectra were acquired on these samples using 2,048 data points in the t_2 dimension, 512 increments of t_1, a spin lock of 50 ms, a recycle delay of 1.22 s, and 64 transients (also Bruker AMX-600 facility).

Aqueous solutions containing sodium pyruvate, L-methionine, L-cysteine and B-D-glucose (1.00 or 5.00 mM) (Sigma Chemical Co., Poole, Dorset, UK) were prepared in 40.0 mM phosphate buffer (pH 7.00) which was rigorously deoxygenated with argon gas prior to use (30 min. at ambient temperature). 5.00 ml. aliquots of these solutions were treated with ozone and then equilibrated as described above for salivary supernatant specimens. Matching de-oxygenated solutions of these agents untreated with ozone but equilibrated in the same manner served as controls.

For one-dimensional (1-D) 600 MHz proton NMR spectra acquired on the simple chemical model systems described above (section 2.1), typical pulsing conditions were 64 FIDs using 32,768 data points, 72 degree pulses and a 3s pulse repetition rate to allow full spin-lattice relaxation of the hydrogen nuclei in the samples investigated. Exponential line-broadening functions of 0.30 Hz were routinely employed for purposes of processing.

3 *RESULTS*

3.1 High-field, high resolution proton NMR analysis of human saliva

The 600 MHz single-pulse ^{1}H NMR spectra of typical control (untreated) unstimulated human salivary supernatant sample are shown in Figure 1. The high- and low-field regions of the spectrum of the control (untreated) specimen contains an abundance of prominent, sharp resonances ascribable to a wide range of low-molecular-mass components. Indeed, signals assignable to short-chain organic acid anions, amino acids, and carbohydrates are readily observable. In addition to considerations of chemical shift values, coupling patterns and coupling constants, together with comparisons with established literature values (making allowances, where appropriate, for the analytical samples' pH values and the pH dependence of selected resonances in spectra acquired therefrom), the identities of many of

these resonances were confirmed or supported by the employment of further procedures.

Firstly, two-dimensional (2D) proton-proton COSY and TOCSY NMR spectra of several salivary supernatant specimens were acquired and data obtained confirmed the presence of many biomolecules with coupled hydrogen nuclei. Indeed, connectivities in these 2D spectra were concordant with the presence of the organic acid anions , n-butyrate, lactate and propionate, the amino acids alanine, leucine, lysine, ornithine, phenylalanine, proline and tyrosine, carbohydrates such as glucose, galactose and N-acetylsugars, and ethanol.

Secondly, for components with singlet resonances, or those with multiplets not readily detectable in the above 2-D spectra in view of their low concentrations in human salivary supernatant samples were treated with standard additions of (i.e., 'spiked' with) a range of authentic, pure components (addition of microlitre aliquots of ca. 10 mM aqueous solutions). In this manner, further evidence supporting the identities of acetate, choline, dimethylamine, formate, glycine, methanol, methylamine, pyruvate, succinate, trimethylamine and trimethylamine oxide (species with a single class of uncoupled hydrogen nuclei under the conditions employed in our studies, i.e., one singlet present in the spectrum of each component), and 3-D-hydroxybutyrate, iso-butyrate, histidine, methionine, sarcosine and n-valerate (compounds with >1 class of coupled and/or uncoupled nuclei) resonances was obtained.

The organic acid anions are mainly derived from microbial metabolism and hence these agents (either individually or two or more in concert) conceivably serve as chemotaxonomic markers of microbial activity in the oral environment. For example, n-butyrate is generated by the pathogenic micro-organism P.gingivalis[29].

Also notable is a broad resonance at 2.04 ppm which, in view of previous investigations conducted on a series of further biofluids (human or otherwise)[30,31], is assignable to the acetamido methyl group protons of N-acetylsugars in the highly mobile side-chains of 'acute-phase' glycoproteins (i.e. salivary mucins). This signal underlies several sharp acetamido-methyl group resonances ascribable to either free N-acetylsugars such as N-

acetylglucosamine and N-acetylneuraminate, or those present in low-molecular-mass saccharide fragments, which may arise from the actions of bacterial hyaluronidase and/or neuraminidase, respectively. Furthermore, both methanol and ethanol were detected in many of the samples subjected to NMR analysis; the former is probably derived from the passive or direct inhalation of cigarette smoke (which results from the combustion of tobacco lignin which contains many methoxy aromatic substituents), and, in the absence of alcoholic beverage consumption, the latter represents a microbial-derived fermentation product.

3.2 Multicomponent proton NMR investigations of the oxidation of salivary biomolecules by ozone

A series of typical 600 MHz single-pulse proton NMR spectra of human saliva specimens acquired prior and subsequent to in vivo treatment with ozone in the manner described in section 2 are displayed in Figure 2. Clearly, this powerful oxidant gave rise to marked reductions in the intensity of the pyruvate methyl group resonance (singlet at 2.388 ppm), an observation reproducible in all saliva specimens tested in this manner. These data are fully consistent with the oxidative consumption of salivary pyruvate by this ROS in accordance with equation (1). Indeed, previous

$$CH_3COCO_2^- + O_3 \text{ ---------- } CH_3CO_2^- + CO_2 + O_2 \quad (1)$$

investigations have shown that biofluid pyruvate acts as a powerful endogenous electron donor (i.e., a water-soluble antioxidant) and is oxidatively decarboxylated to acetate and carbon dioxide on reaction with hydrogen peroxide[32]. As expected, the ozone-dependent reductions observed in the intensities of the pyruvate methyl group resonance were accompanied by quantitative increases in that of the acetate methyl group signal (although the latter were relatively small in view of the knowledge that salivary pyruvate concentrations are much lower than those of acetate).

Substantial reductions in the intensity of the lactate methyl and -CH group signals

[located at 1.33(d) and 4.13 ppm(q) respectively] were also observed following ozone treatment, indicating that this salivary biomolecule is oxidised to pyruvate which, in turn, is further consumed by ozone to yield acetate and carbon dioxide as described above. Indeed, we have previously reported that hydroxyl radical (OH) readily oxidises lactate to acetate and carbon dioxide via pyruvate[32].

In specimens in which low-molecular-mass methionine was NMR-detectable (n=5) , the spectra acquired provided evidence for its ozone-mediated oxidation, i.e., significant reductions in the intensity of its thioether methyl group singlet resonance at 2.13 ppm.and the generation of signals attributable to the side-chain methyl groups of its oxidation products methionine sulphoxide and sulphone (singlets located at 2.725 and 3.157 ppm respectively). This observation is of much clinical significance in view of the fact that methionine, present as a residue in many salivary proteins, is liberated as the free amino acid via bacterially-mediated proteolysis and subsequently serves as a precursor to volatile sulphur compounds (VSCs) which are predominantly which are responsible for oral malodour (e.g., methyl mercaptan which accounts for approximately 60% of the VSCs detectable)[33].

The well-known ketone body product, 3-D-hydroxybutyrate, a further salivary electron-donor, was also found to be consumed by ozone , i.e., significant reductions in the intensity of its terminal methyl group doublet resonance (1.20 ppm), indicating oxidation to acetoacetate which spontaneously decomposes to acetone and carbon dioxide. Consistent with these findings, a singlet signal at 2.245 ppm ascribable to acetone was generated (or proportionately increased in intensity if already present in the corresponding control spectra) in spectra of the ozone-treated specimens.

The results acquired also revealed the generation of a resonance located at 5.40 ppm (doublet, J= 1.2 Hz) ascribable to allantoin which was present in spectra of all the ozone-treated specimens but absent their corresponding control specimens. Hence, allantoin appears to represent a very clear 'marker' of ozone treatment in human saliva, and arises from the oxidation of the antioxidant urate which is present at a mean level of ca. 70 micromoles per litre in this biofluid. This observation

corresponds to that of Grootveld et. al. (1993)[34] who found that radiolytically-generated hydroxyl radical also generates allantoin from urate in inflammatory knee-joint synovial fluid.

Further ozone-induced modifications to spectra which, although of some biochemical and clinical significance, can only be considered as minor in view of their observation in <6 of the 12 saliva specimens examined were (1) the oxidation of malodorous trimethylamine, presumably to trimethylamine oxide (i.e., decreases in the intensity of its characteristic methyl group singlet resonance located at 2.91 ppm), (2) elevations in the salivary concentrations of formate (singlet, 8.46 ppm) which is known to arise as a product derived from oxidative damage to carbohydrates such as glucose, sucrose and fructose[35], (3) increases in the intensities of one or more sharp signals in the 2.05-2.10 ppm regions of spectra which are ascribable to the acetamido methyl group protons of free N-acetylsugars (N-acetylglucosamine or N-acetylneuraminate) and/or those present in low-molecular-mass saccharide fragments derived from the oxidative depolymerisation of salivary or tissue glycosaminoglycans, in accordance with results obtained from previous investigations conducted on the fragmentation of hyaluronate by oxidants such as hydroxyl radical[34,35] and hypochlorite[36], (4) reductions in the intensity of the vinylic proton resonance of polyunsaturated fatty acids (either free or glycerol-bound), a phenomenon consistent with their well-known oxidation to ozonides[18-20], (5) the oxidative consumption of both ethanol and methanol (decreases in the intensities of the methyl group signals of these alcohols), presumably to acetaldehyde and formaldehyde, and then acetate and formate respectively which conceivably also partially account for increases in the levels of the latter two agents observed, (6) elevations in the salivary concentration of succinate (ethylenic group proton singlet at 2.405 ppm) which presumably arise from the oxidation of 2-oxoglutarate (i.e., an oxidative decarboxylation process as delineated above for pyruvate, also an alpha-keto acid anion), (7) oxidation of glucose and additional low-molecular-mass carbohydrate species as evidenced by reductions in the intensities of their alpha-anomer H-1 proton resonances (5.30 ppm for glucose). Moreover, one of the samples investigated contained glycerol , a component which presumably arises from the subject's use of a toothpaste preparation shortly before sample collection, and its

treatment with ozone gave rise to the consumption of this agent [i.e., a clear removal of its two methylene (3.58 and 3.67 ppm) and single methine group (3.78 ppm) multiplet proton signals], an observation consistent with its oxidation to corresponding aldehydic and/or carboxylate anion adducts.

A further striking difference between the between the pre- and post-ozone treated specimens were clear ozone-induced increases in the normalised intensities of selected low-molecular-mass salivary components which would not normally be considered as oxidation products derived from alternative biomolecules, especially glycine, and less commonly alanine, n-butyrate, lysine and propionate. These modifications indicate that ozone, hydroxyl radical derived from its single electron reduction product ($O_3^{\bullet-}$), or a combination of these highly reactive oxidants have the ability to release these low-molecular-mass metabolites from protein or alternative macromolecule binding-sites [slow molecular motion of these components when bound to salivary proteins gives rise to short spin-spin relaxation times (T_2) and, consequently, broad undetectable NMR resonances]. For example, if low-molecular-mass anionic biomolecules are electrostatically bound to positively-charged lysine or arginine residues of proteins, and the one or more of the above oxidants has the ability to attack and degrade such residues (or more specifically, the charged portion of the molecule which acts as a binding-site), then such chemical modifications could result in the mobilisation of these anionic biomolecules from their binding-sites. However, it should be noted that the nature and extent of these resonance intensity increases were highly sample dependent.

3.3 Chemical model studies of the reactions of ozone with salivary electron donors

1.00 or 5.00 mM aqueous solutions of pyruvate were treated with ozone as described in section 2 in order to further investigate the reaction occuring between these redox-active species, and proton NMR analysis of these solutions demonstrated the complete transformation of the alpha-keto acid anion to acetate and carbon dioxide, an observation consistent with the reaction depicted in equation 1. Indeed, these results are not unexpected

in view of the large excess of ozone added, although its effective concentration in the system employed here is limited by its solubility in water, together with its rate and level of its consumption by the scavenger employed, and its catalytically-promoted dissociation to dioxygen during the 30s delivery period. Consistent with this observation, a singlet resonance at 1.50 ppm ascribable to pyruvate hydrate (the enol form of this alpha-keto acid anion), of much lower intensity than that of the keto form at 2.388 ppm, was also removed from spectra after ozone treatment.

As expected, treatment of aqueous solutions of L-methionine with ozone confirmed oxidation to its corresponding methionine sulphoxide and sulphone species under our experimental conditions (i.e., the generation of singlet resonances at 2.725 and 3.157 ppm attributable to the side-chain terminal methyl groups of the sulphoxide and sulphone respectively, accompanied by marked decreases in the methionine thiomethyl group signal at 2.13 ppm).

L-cysteine was also chosen for these chemical model system experiments since, (1) like methionine, it serves as a precursor to malodorous VSCs[33] and (2) our inability to detect this thiol in human saliva by NMR analysis in view of its low concentration in the 'free' (non-protein-incorporated) state and also its complex ABX coupling pattern (i.e., no clearly visible sharp resonances of low multiplicity). Proton NMR analysis demonstrated that exposure of aqueous solutions of L-cysteine to ozone (section 2) generated its corresponding disulphide, cystine, as a major product. Indeed, a reference spectrum acquired on an authentic sample of L-cystine confirmed its identity [clear multiplets located at 3.20 and 3.41 ppm (AB protons) and 4.14 ppm (X proton)].

Proton NMR analysis also showed that ozonolysis of alpha-D-glucose in the manner described in section 2 generated formate as a major reaction product (singlet resonance at 8.46 ppm), an observation consistent with previous studies conducted on the interactions of ROS (e.g., radiolytically-generated hydroxyl radical) with carbohydrates in general.

4 *DISCUSSION*

High resolution, high field ^{1}H NMR spectroscopy is a technique which offers many advantages over alternative time-consuming, labour-intensive analytical methods since (1) it permits the rapid, non-invasive and simultaneous examination of a very wide range of components present in biofluids (e.g., human saliva as outlined in this study) and (2) it has little or no requirement for knowledge of sample composition prior to analysis. Furthermore, chemical shift values, coupling patterns and coupling constants of resonances present in ^{1}H NMR spectra of such multicomponent systems provides an inexhaustive supply of information regarding the molecular nature of both endogenous and exogenous chemical species therein.

As demonstrated here, the technique is of much value concerning multicomponent assessments of the interactions of O_3 with human salivary biomolecules, and the oxidative decarboxylation of salivary pyruvate by O_3 serves as an important example of this which may be of some relevance to its cariostatic properties.

Indeed, pyruvic acid is a very powerful proton donor (K_a = 3.20 mM) being much stronger in this capacity than lactic acid (K_a = 0.14 mM)[37], and hence may play an important role in promoting tooth demineralisation processes. Therefore, the removal of salivary pyruvate by O_3 may suppress the development and progression of primary root caries lesions since organic acids detectable in human saliva readily diffuse into enamel (in their unionised form). In view of these considerations, O_3 may offer caries-preventative actions and experiments to investigate this further are currently in progress.

Consumption of salivary methionine and cysteine by ozone is of great importance to oral hygeine and periodontology since both methyl mercaptan and hydrogen sulphide are generated from these amino acids via metabolic pathways operational in gram-negative micro-organisms[38]. Hence, our data indicate that ozone has the capacity to clinically alleviate oral malodour via the direct oxidative inactivation of VSCs and their amino acid precursors.

Moreover, the oxidative consumption of trimethylamine by ozone is also of some aesthetic and clinical interest in view of the fact that it is extremely malodorous, and also that it may represent one of the bacterially-generated toxic amines implicated in the aetiology of periodontal diseases.

Data acquired here are similar to those obtained in previous NMR investigations conducted to explore the attack of •OH radical and superoxide anion ($O_2^{\cdot -}$) on biomolecules present in intact human blood serum and inflammatory knee-joint synovial fluid[34,35]. For example, the oxidation of pyruvate to acetate and CO_2, elevations in the concentration of formate arising from oxidative damage to carbohydrates in general, the production of allantoin from urate, and the release of low-molecular-mass biomolecules from protein binding-sites were also observed in these studies, results which lend support to the postulate that some of the observations made in this work are conceivably attributable to the actions of $^{\cdot}OH$ radical derived from $O_3^{\cdot -}$ which, in turn, is generated from the single electron reduction of O_3. Single electron-transfer agents present in saliva which conceivably offer a contribution to this process include L-cysteine, thiocyanate anion and any dietary Fe(II) (giving rise to corresponding thiyl radical, thiocyanatyl radical and Fe(III) respectively), and further investigations to determine their involvement are currently underway in our laboratory.

In conclusion, the applications of high resolution NMR spectroscopy to determine the nature and extent of salivary biomolecule consumption by ozone administered in the modern dental clinic provide much valuable multicomponent information regarding its potential therapeutic, aesthetic and, indirectly, microbicidal actions in the oral environment.

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