BioMetals 1996, **9**, 285–290

A hypothesis for the *in vivo* antioxidant action of salicyclic acid

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Received 30 October 1995; accepted for publication 2 January 1996

This paper presents a new hypothesis for the physiological antioxidant action of salicylate. Current theories have focused on the radical scavenging nature of salicylate. This explanation may have limitations because it is unlikely that salicylate reaches the necessary concentrations to effectively prevent damage to cell components. We propose that salicylic acid decreases the flux of hydroxyl radicals through chelation, which causes a redox deactivation mechanism of iron Fenton reaction centers. This is due to voltammetric results which indicate that the iron–salicylate complex does not have the thermodynamic driving force to act as an effective Fenton reagent necessary for the production of damaging oxygen-containing radicals. Furthermore, despite the more facile thermodynamics associated with Fenton-type processes at acidic pH values, the complex maintains Fenton inactivity due to a pH-sensitive redox potential shift that follows as $E_{Fe[Sal]} = 0.793 - (0.059\ pH)$. This is important since inflammation sites are acidic relative to healthy tissue. This redox potential shift is unique to salicylates when compared with other common iron chelation agents such as EDTA. Further evidence for the lack of Fenton-type reactivity of the iron–salicylate complex is offered in the form of oxidation studies of calf thymus (CT) DNA by hydrogen peroxide. Salicylate prevents the iron-catalyzed oxidation of CT-DNA strands as indicated by the detection of the constituent bases by HPLC. However, salicylates were not able to prevent the copper-catalyzed oxidation of CT-DNA. These results are predicted by the cyclic voltammetry of copper–salicylate, which confirms that it is an effective Fenton-type catalyst, further adding to the proof that salicylate acts by redox deactivation of iron, not by hydroxyl radical scavenging. Finally, the iron–salicylate e.m.f. suggests that it may also act as a superoxide dismutase, which indicates another possible important antioxidant feature.

Keywords: antioxidant, *in vivo*, iron, mechanism, salicylic acid

Introduction

It is now well accepted that aspirin (acetylsalicylic acid) acts by the irreversible acetylation of cyclooxygenase which blocks the synthesis of many prostaglandins (Vane 1971, Abramson *et al.* 1985). However, there is some dispute as to whether this forms the basis of the anti-inflammatory effect of this agent, since salicylic acid while lacking an acetyl group altogether, has anti-inflammatory effects equipotent to aspirin (Weissmann 1991). Furthermore, recent literature has indicated that aspirin may have other important medicinal actions which may not be completely explained by the inhibition of cyclooxygenase. These include chemopreventative effects towards cancers, cataracts, inflammation-induced tissue damage and circulatory, Parkinson's and Alzheimer's diseases (Crichton 1991, Florence 1991, Woollard *et al.* 1990, Oberley & Oberley 1993). The pathogenesis of these ailments has been linked to oxidative damage to tissue, which indicates that aspirin has antioxidant properties (Arouma & Halliwell 1988).

Oxidative tissue damage occurs due to the release of hydrogen peroxide, and superoxide ions, which are the by-products of normal respiratory functions and whose concentrations are elevated during the course of the inflammatory response (Lewis 1986, Wallace 1992). The danger posed by hydrogen peroxide is through the generation of $HO^{\bullet}$ ($E^0 = 1.8$ V) via the Fenton reaction through a suitable iron complex (reaction 1).

$$Fe^{III}(complex) + e^- = Fe^{II}(complex) \quad (1a)$$

$$Fe^{II}(complex) + H_2O_2 = Fe^{III}(complex) + HO^- + HO^{\bullet} \quad (1b)$$

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In turn this radical is capable of oxidizing nucleic acids, proteins and lipids with diffusion-limited kinetics which results in the pathogenesis of many diseases (Arouma & Halliwell 1988, Girotti 1990). Superoxide ions act as a precursor to hydrogen peroxide through self-dismutation or as a reducing agent ($E^{0'} = -0.33$ V) for ferric complexes, which in turn promotes the Fenton reaction.

Due to the Fenton reaction, adventitious iron has been implicated in oxidative tissue damage and in the precipitation of many of the aforementioned diseases that salicylates may prevent (Crichton 1991, Florence 1991, Wallace 1992). This has led other investigators to hypothesize that salicylates may exert their chemo-preventative effects through hydroxyl radical scavenging (Woollard *et al.* 1990). Furthermore, chelation of adventitious iron by salicylate may augment radical scavenging since the ligand is in close proximity to the radical generation site (Arouma & Halliwell 1988). However, radical scavenging via iron chelation or otherwise is unlikely to have any pertinence to physiological conditions since the availability of other intrinsic cellular components, i.e. proteins, lipids and nucleic acids, all of which are capable hydroxyl radical scavengers, will always be in much higher concentration than the drug.

For this investigation, we hypothesize that chelation is important, but not for reasons of radical scavenging. An overlooked aspect of the present chelation hypothesis is the importance of the redox potential of the iron–salicylate complex. Under standard state conditions and in neutral pH the Fenton reaction requires that the ferrous complex must have an oxidation threshold of 0.32 V versus the standard hydrogen electrode (SHE) or more negative values. Most *in vivo* ferrous complexes meet this thermodynamic requirement and therefore contribute to hydroxyl radical damage (Winterbourn 1991, Allenthoff *et al.* 1992). Furthermore, the iron storage protein ferritin releases iron upon reaction with superoxide ions (Reif *et al.* 1988). This places iron into a low molecular weight pool which in turn may drive destructive redox cycling processes responsible for aging and oxidative damage due to inflammation (Crichton & Ward 1992, Wallace 1992, Zhao *et al.* 1994). A variety of compounds such as nucleotides, citrates, proteins and phospholipids may act as chelates in the low molecular weight iron pool (Zhao *et al.* 1994). Salicylates may intefere with these processes through a competitive binding mechanism. At physiological pH values, salicylates readily bind iron ($\log \beta_3 = 35.5$) (Kotrlý & Šucha 1985, Gelvan *et al.* 1992). Many investigators have hypothesized that chelation may play an important role in the antioxidant action of NSAIDs by deprivation of available ferric ions (Sedgwick *et al.* 1984, Halliwell & Gutteridge 1989). Similarly, the host organism may also seek to control iron-induced damage by creating a state of localized hypoferremia at the inflammation site (Letendre 1985).

A question remains: if iron chelation is an important mechanism in the action of NSAIDs then why are so few ligands mentioned as useful antioxidant agents? This may be due to the fact that the majority of iron complexes are Fenton active agents. Relatively little attention has been paid to the redox potential of iron complexes when considering the pertinence of chelation therapy as applied to anti-inflammation/antioxidant action. Desferrioxamine B, a microbial siderophore approved for the treatment of acute iron intoxication, is one such chelation agent frequently cited as having possible applications for the control of inflammatory disease ions (Sedgwick *et al.* 1984, Halliwell & Gutteridge 1989). That observation may be due to the ferric-desferrioxamine B reduction potential of -0.9 V versus SHE, which places it beyond the reducing capacity of the superoxide ion and most *in vivo* agents (Helman & Lawrence 1989). Another example of a successful iron redox deactivation agent is the chelating agent 1,10-phenanthroline, which forms a complex whose ferric reduction potential (1.1 V versus SHE) is well positive of the Fenton threshold. The ligand has been observed to reduce the formation of active oxygen species at inflammation centers, possibly due to the redox deactivation mechanism (Halliwell & Gutteridge 1989).

Materials and methods

Instrumentation

Electrochemical investigations were conducted with a Princeton Applied Research Corporation (Princeton, NJ, USA) Model 364 pulse voltammograph in conjunction with a Model 175 potential wave programmer with a Yokogawa 3025 XY recorder. A three-electrode cell design employed a wax impregnated graphite (Fisher Scientific, Pittsburg, PA, USA) disk as a working electrode, a Ag/AgCl/saturated KCl reference electrode (0.197 V versus SHE) and a wax impregnated graphite rod as a counter electrode. Isocratic (50/1 water/methanol) HPLC experiments were performed on a reversed phase (C_{18}) 5.0 cm Zorbex (Wilmington, DE, USA) column with detection performed on a Waters 440 (Milford, MA, USA) detector (254 nm) at 1.2 ml min^{-1}.

Chemicals

All aqueous solutions were prepared with high-purity water obtained from a Millipore-Q (Bedford, MA, USA) system. Ferric nitrate (99%), cupric sulfate (99%), potassium permanganate (ACS certified), hydrogen peroxide (reagent grade), L-ascorbic acid (reagent grade), sodium oxalate (primary standard), potassium phosphate monobasic (ACS certified) and HPLC grade methanol were purchased from Fisher and used without further purification. Calf thymus (CT) DNA, adenine, thymine, guanine and cytosine were supplied by Sigma (St. Louis, MD, USA) and used without further purification. Hydrogen peroxide solutions were assayed by titration with standardized (by sodium oxalate) aqueous potassium permanganate solutions. CT-DNA solutions were prepared by the method described by Rodriguez *et al.* (1990).

Results and discussion

Electrochemical studies

Cyclic voltammetry of the iron–salicylate (Fe[Sal]$_3$) complex reveals a set of quasi-reversible waves due to the $Fe^{II/III}$ couple with a redox potential of 0.37 V versus SHE in pH 7.2 phosphate buffer (see Figure 1). Attempt at the mediated electrocatalysis of 10 mM H_2O_2 indicates that (Fe[Sal]$_3$) is not capable of driving the Fenton reaction (Figure 1). This result was expected due to the unfavorable thermodynamics of the couple relative to the Fenton threshold ($E^{0'}_{Fenton}$ = 0.307 V versus SHE at pH 7.2). Cyclic voltammetry of the classical Fenton reagent, $Fe^{II/III}$[EDTA], indicates a redox potential of 0.09 V versus SHE (Figure 2). In the presence of 10 mM H_2O_2 the Fe[EDTA] cyclic voltammetric waves are distorted and amplified, which is indicative of an EC′ mechanism and Fenton reactivity (reaction 1) (Bard & Faulkner 1980).

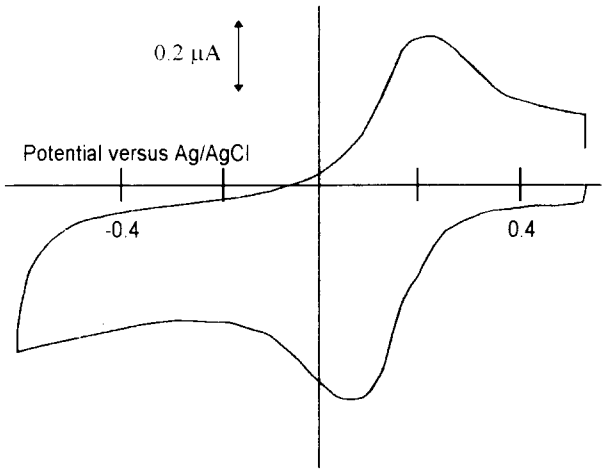

Figure 1. Cyclic voltammogram of iron–salicylate (0.5 mM ferric nitrate with 2.0 mM salicylate) at pH 7.2, 0.05 M phosphate buffer with a potential sweep rate of 5 mV s^{-1}. The cyclic voltammetric waves remain undistorted in the presence of 10 mM H_2O_2. The electrodes consisted of a 0.071 cm^2 wax impregnated graphite disk with a Ag/AgCl saturated KCl reference (0.197 V versus SHE).

pH dependence of $Fe^{II/III}[Sal]_3$ and the impact of this behavior on the thermodynamics of physiological oxidations

In addition to its relatively inert Fenton reaction characteristics, the $Fe^{II/III}[Sal]_3$ couple exhibits an interesting pH-sensitive potential. The redox potential shifts to more positive potentials with increasing pH (Figure 3). This shift is summarized by the following form of the Nernst equation.

$$E_{Fe[Sal]} = 0.793 - (0.059\ pH) \quad (2)$$

The pH dependence of Fe[Sal]$_3$ may indicate that a hydroxide ion is involved in the complexation of the ferric metal center. This redox shift has a very important ramification when considering physiological oxidations. Due to the generation of hydroxide ion the Fenton reaction is also pH dependent. Under standard state conditions the Fenton threshold follows as

$$E_{Fenton} = 0.732 - (0.059\ pH) \quad (3)$$

Inflammation centers are thought to reach acidic pH values, which in turn makes the Fenton process thermodynamically more facile; at pH 5 the Fenton reaction standard state threshold shifts to $E^{0'} = 0.437$ V versus SHE. Salicylate is

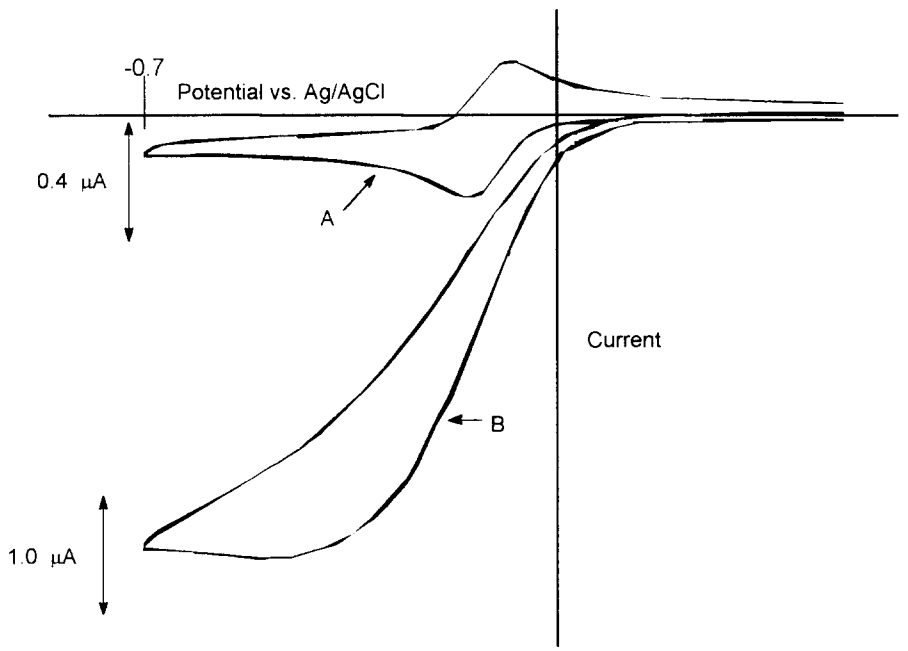

Figure 2. Cyclic voltammograms of (**A**) 0.1 mM Fe(EDTA) (0.1 mM $Fe(NO_3)_3$, 0.4 mM salicylate) and (**B**) with 10 mM H_2O_2. Potential sweep rate was 5 mV s^{-1}, other conditions were identical to Figure 1.

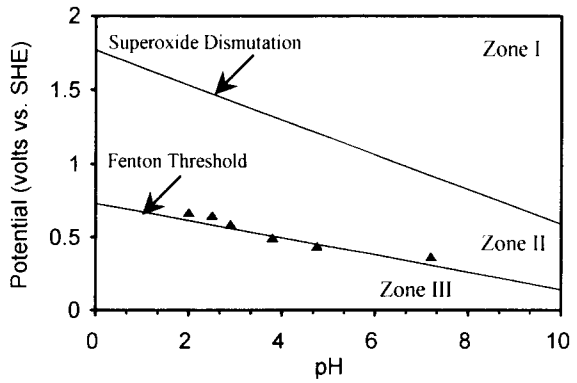

Figure 3. A pH–potential zone diagram for the Fenton reaction and superoxide dismutation. The redox potentials for the $Fe^{II/III}$[salicylate] complexes are outlined with triangles. The buffer systems were as follows: pH 2–2.9, 0.5 M KNO_3, adjusted with HNO_3; pH 3.8 and 4.75, 0.05 M acetate; pH 7.2, 0.05 M phosphate. Note that the complex closely parallels the standard state Fenton threshold and that this line is not a least-squares fit for iron–salicylate. Those species with redox potentials encompassed by Zone I do not have the thermodynamic driving force to act as SOD catalysts under standard state conditions nor as Fenton reaction agents. Zone II encompasses species which can act as SOD catalysts without Fenton reactivity. Zone III contains species which act as both SOD catalysts and Fenton active agents.

successful at deactivating ferrous ions in acidic media due to the redox shift outlined in equation (2). The $Fe[Sal]_3$ maintains an unfavorable redox potential with respect to the Fenton reaction standard state threshold throughout all pH ranges. These features are demonstrated in the zone diagram shown in Figure 3 (the zone diagram was calculated from data in Koppenol 1987). Another key feature outlined in the zone diagram is the standard state threshold for superoxide dismutase (SOD) activity as written in equation (4)

$$O_2^{\bullet -} + 2H^+ + e^- = H_2O_2 \qquad (4)$$

This is another important antioxidant activity carried out by a variety of enzymes. Metal complexes whose potentials lie negative relative to this threshold have favorable thermodynamics for SOD action. It is clear from Figure 3 that $Fe[Sal]_3$ has this driving force, although at this point of the study this feature has not been explored. However, it is noteworthy to add that copper–salicylates ($E_{\frac{1}{2}} = 0.110$ V versus SHE)($\log \beta_2 = 19.01$) have SOD activity. Finally, one last important consideration can be derived from Figure 3. This is the close proximity of the redox potential of $Fe[Sal]_3$ relative to the Fenton threshold. This feature maximizes the SOD driving force *with a minimum* of Fenton reactivity. Hypothetically many redox species including copper–salicylate have SOD activity; however, this feature comes at the expense of Fenton reaction activity as verified by cyclic voltammetry (Brun & Schrøder 1975). This may be an important consideration in the design of artificial SOD agents.

The $Fe[Sal]_3$ pH-sensitive redox may insure SOD activity with a minimum of Fenton reactivity

To quantify the importance of the relationship described in equation (2), consider the potential driving force for the reaction between the ferrous–salicylate complex and hydrogen peroxide under physiological conditions. At pH 7 the calculated $Fe^{II/III}(Sal)_3$ potential is 0.380 V versus SHE indicating that the cell e.m.f. is spontaneous relative to the Fenton reaction until $\log[HO^\bullet]/[H_2O_2]$ reaches -1.37 as calculated from the Nernst relationship. On the other hand, superoxide dismutation by iron–salicylate will be spontaneous until $\log[O_2^{\bullet -}]/[H_2O_2]$ reaches a value of -9.22. When compared to a species such as ferrous[EDTA] ($E_{\frac{1}{2}} = 0.09$ V versus SHE), $\log[HO^\bullet]/[H_2O_2]$ dramatically increases to 3.88. At pH 5, the ratios for Fenton and SOD activity remain nearly the same for the $Fe(Sal)_3$ complex, wheras the $\log[HO^\bullet]/[H_2O_2]$ increases to 5.9 for Fe(EDTA). It is also important to note that the half-wave potential for the $Fe^{II/III}$[EDTA] complex remained invariant over the pH range of 2.0–7.2 as measured by cyclic voltammetry.

As can be seen from these examples there is a balance between the control of SOD activity while minimizing Fenton reaction activity. This trade-off is illustrated in Figure 4, which describes the extent of Fenton and SOD activities as driven by a hypothetical redox couple whose possible e.m.f. constitutes the x-axis at pH 7. It is interesting to note that the $Fe^{II}(Sal)_3$ e.m.f. is not optimized for a minimum of Fenton reactivity with SOD activity. A hypothetical chelation agent with this property requires that it forms an iron complex whose e.m.f. is about 0.62 V versus SHE. Over the entire extent of pH ranges such species would follow the Nernst relationship

$$E = 1.03 - (0.059\ \mathrm{pH}) \qquad (5)$$

However, factors such as the relative toxicities of superoxide and the hydroxyl radical may play an important role in the selection of the optimal redox potential of an iron complex.

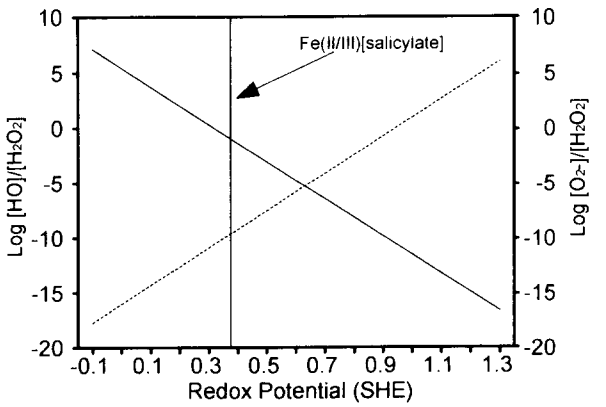

Figure 4. Equilibrium concentrations of $[HO^\bullet]/[H_2O_2]$ (solid line) and $[O_2^{\bullet -}]/[H_2O_2]$ (dashed line) versus the e.m.f. of a hypothetical redox species. Note that $Fe^{II/III}$[salicylate] potential (solid vertical line) intersects $\log [HO^\bullet]/[H_2O_2]$ at -1.37 V and $\log[O_2^{\bullet -}]/[H_2O_2]$ at -9.22 V (see text).

Verification of the salicylate antioxidant mechanism through DNA oxidation studies

CT-DNA oxidation studies were conducted to verify the redox deactivation hypothesis. Ferric ions readily complex to DNA strands via the phosphate residues. The Fe–DNA complex was formed at pH 5 (unbuffered) to guard against ferric hydrolysis. The final incubation mixture consisted of 0.1 mM $Fe(NO_3)_3$, 0.2 mM CT-DNA (in base pairs), 0.05 M phosphate buffer at pH 7.2, 7.8 mM H_2O_2 and 1.0 mM ascorbate as a reducing agent. Phosphate buffer was selected for use in this study since organic buffers such as TRIS or HEPES are efficient radical scavengers (Hicks & Gebicki 1986). Pulse voltammetric studies indicate that the Fe–DNA complex acts a Fenton reaction agent. Under the outlined conditions the incubation was found to reach completion after 120 min at 22°C. Hydroxyl radical oxidation of the DNA strands releases the constituent bases, of which adenine, guanine and thymine are detectable by HPLC (see Figure 5) (Zhao *et al.* 1994). Cytosine peaks were obscured by other incubation components. Addition of 0.4 mM salicylate to the incubation mixture greatly reduced the release of the three detectable bases, which indicates that hydroxyl radicals were available for the oxidation of DNA strands (Figure 5).

To insure that redox deactivation of iron rather than radical scavenging was responsible for the antioxidant behavior of salicylate, incubation studies with copper salicylates were carried out. As indicated by the $Cu^{I/II}[salicylate]_2$ redox potential and through verification by cyclic voltammetry in the presence of H_2O_2, this complex acts as a Fenton reaction center. Salicylate was not able to hinder the copper-induced release of bases from the DNA strand during the incubation conditions outlined in Figure 5. This is a clear indication that redox potential of the

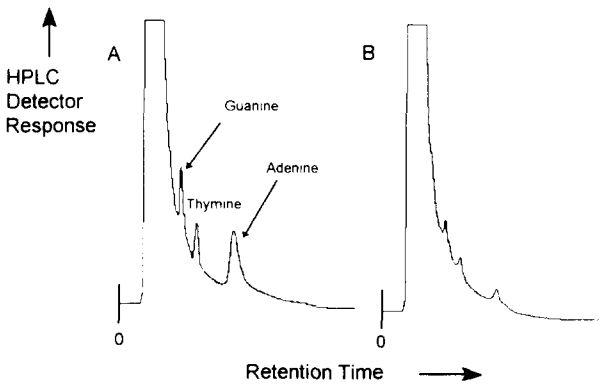

Figure 5. HPLC chromatogram following incubation of CT-DNA (0.2 mM in base pairs) with 0.1 mM $Fe(NO_3)_3$, 1.0 mM ascorbate and 7.8 mM H_2O_2 after 120 min at 22°C. Trace **A** no salicylate present. Trace **B** 0.4 mM salicylate present. Under conditions where 0.1 mM $Cu(SO_4)$ was substituted for $Fe(NO_3)_3$ bases were released regardless of the presence of salicylate (see trace **A**). Separation conditions: 50/1 water/methanol mobile phase, C_{18} reversed-phase Zorbex cartridge column, absorbance detection at 254 nm. Retention times: guanine, 1.09 min; thymine, 1.44 min; adenine, 2.35 min.

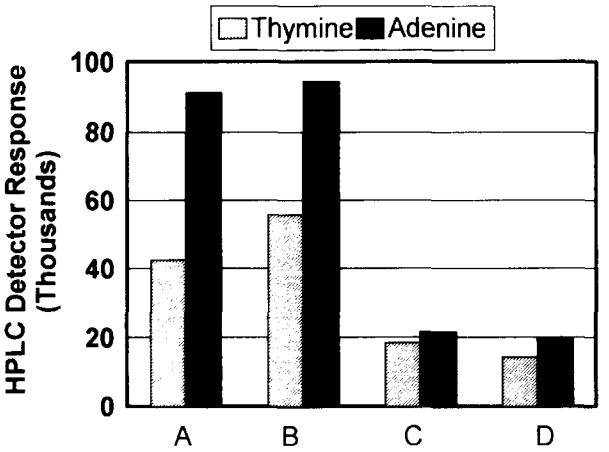

Figure 6. HPLC incubation results (absorbance detection at 254 nm) following CT-DNA (0.2 mM base pair) with 1.0 mM ascorbate and 7.8 mM, H_2O_2 after 120 min at 22°C. (**A**) 0.1 mM $Fe(NO_3)_3$, (**B**) 0.1 mM $Fe(NO_3)_3$ and 0.15 mM EDTA, (**C**) 0.1 mM $Fe(NO_3)_3$ and 0.4 mM salicylate, (**D**) 0.1 mM $Fe(NO_3)_3$, 0.15 mM EDTA and 0.4 mM salicylate.

metal–salicylate complex, and not radical scavenging, is the primary consideration for the salicylate antioxidant activity. Presumably if radical scavenging were the primary mechanism for the antioxidant action of salicylate, then base release should be hindered regardless of the identity of catalytic metal center.

Incubation of CT-DNA with 0.1 mM $Fe^{III}[EDTA]$ releases DNA bases as expected of this classical Fenton reagent. Introduction of 0.4 mM salicylate surprisingly hinders base release. Cyclic voltammetric studies indicate that salicylates are not able to displace EDTA from ferric ions ($\log \beta_1 = 25.1$) at pH 7.2. This may be due to the effective complexation of ferric ion by salicylate at this pH ($K = 8.5$) which takes into account the pH-dependent equilibria of the ligand (Gelvan *et al.* 1992). Given this, it is remarkable that salicylate is able to deactivate the $Fe^{III}[EDTA]$ complex. This feature may be due to the release of iron ions by EDTA upon the oxidation of the ligand followed by sequestration by salicylate or by a mixed salicylate–EDTA iron complex. This deactivation route for $Fe^{II}[EDTA]$ may have much significance in the *in vivo* control of Fenton activity of low molecular weight iron complexes by salicylate since the majority of conceivable physiological ligands will have chelation sites similar in nature to EDTA. Figure 6 summarizes the results of the various incubation studies for adenine and cytosine release.

Summary

The results of the studies presented in this paper may provide the basis for a new approach for the physiological mechanism of salicylate action. The salient point is that salicylate acts as an iron chelation agent and that the resulting complex has a pH-sensitive ferric–ferrous redox potential that keeps the Fenton reactivity of the iron ion in check. This indicates

that the salicylate moiety of aspirin may be responsible for the latter's antioxidant behavior, which indicates that aspirin may act as a prodrug for salicylate, a hypothesis that was first presented by Dreser with further evidence provided by Higgs and coworkers (Dreser 1899, Higgs *et al.* 1987). Furthermore, it is important to add that aspirin is rapidly hydrolyzed to salicylic acid under *in vivo* conditions (Higgs *et al.* 1987). In this investigation we have focused on the antioxidant characteristics of salicylate. However, it is conceivable that some of the anti-inflammatory characteristics of salicylates are due to its antioxidant properties since so much of the damage and pain associated with inflammation are caused by activated oxygen (Andrews *et al.* 1987). Future studies will focus on the SOD nature of the iron–salicylate complex. Furthermore, the chelation hypothesis may provide a rational basis for the observed empirical structure–activity relationships of the salicylates since non-active compounds may either be unable to chelate iron or lack redox potential modifying characteristics (Borne 1986).

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