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OXIDATIVE REACTIVITY OF THE TRYPTOPHAN METABOLITES 3-HYDROXYANTHRANILATE, CINNABARINATE, QUINOLINATE AND PICOLINATE

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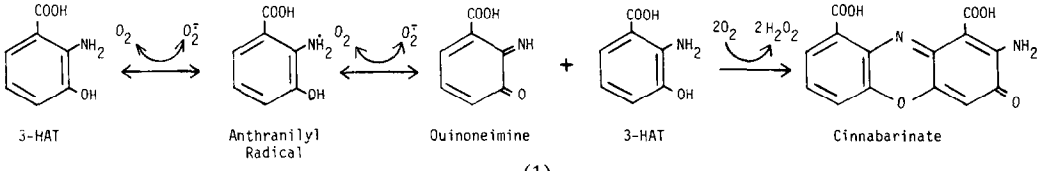
Abstract—The oxidative reactivities of four tryptophan metabolites in the kynurenine pathway were examined as a potential mechanism for their reported neurotoxicities and carcinogenicities. Neither quinolinic acid, a neurotoxin, nor its monocarboxylic analogue, picolinic acid, auto-oxidized over a wide pH range. However, 3-hydroxyanthranilic acid (3-HAT), a carcinogen, readily auto-oxidized and the reaction rate increased exponentially with increasing pH. 3-HAT auto-oxidation likely involves two steps: (1) auto-oxidation of 3-HAT to the semiquinoneimine (anthranilyl radical) which oxidizes to the quinoneimine, followed by (2) condensation and oxidation reactions to yield a second carcinogen, cinnabarinic acid. 3-HAT auto-oxidation to cinnabarinate required molecular oxygen and generated superoxide radicals and H_2O_2. Superoxide dismutase (SOD) accelerated 3-HAT auto-oxidation 4-fold, probably by preventing back reactions between superoxide and either the anthranilyl radical or the quinoneimine formed during the initial step of auto-oxidation. Catalase did not accelerate 3-HAT auto-oxidation, but it did prevent destruction of cinnabarinate by H_2O_2. Interconversion between oxyhemoglobin and methemoglobin occurred during 3-HAT auto-oxidation, although neither form of hemoglobin altered rates of 3-HAT auto-oxidation. Mn^{2+}, Mn^{3+} and Fe^{3+}-EDTA did not directly catalyze cinnabarinate formation in the absence of O_2, but they did accelerate cinnabarinate formation under aerobic conditions.

A number of endogenous metabolites occurring in the kynurenine pathway of tryptophan catabolism are known to be neurotoxic [1] and carcinogenic [2–5]. In this pathway, the ring structure of 3-hydroxyanthranilic acid (3-HAT), which arises at a branch point, is normally opened by the ferro-protein 3-HAT oxygenase to form 2-acroleyl-3-amino fumarate, which then undergoes one of two possible reactions [6]. The opened ring can either spontaneously recyclize to form quinolinic acid which leads to the eventual production of NAD, or the opened ring can be enzymatically decarboxylated, a route which eventually generates two molecules of acetyl-S-CoA. However, this decarboxylated open ring can also spontaneously close, forming picolinic acid in what is considered a metabolic cul-de-sac [7]. Alternatively, if 3-HAT is not opened initially by the oxygenase, it may auto-oxidize in a process that involves the production of organic and oxygen free radicals and which eventually yields the red pigment cinnabarinic acid and hydrogen peroxide [8–12].

Both 3-HAT and cinnabarinic acid are known carcinogens that have been linked to bladder and breast carcinomas [2, 3, 5, 13]. Quinolinic acid has been shown to be a potent neurotoxin [14, 15] that, when injected into rat brain, forms lesions similar to those induced by exogenous excitotoxins such as kainic and ibotenic acids [1]. Although there have been no investigations concerning the potential neurotoxicity of picolinic acid, electron spin resonance (ESR) studies have shown that both quinolinate and picolinate induce alterations in the physical state of red cell membrane proteins [16, 17]. 3-HAT has also been studied in this regard, but does not appear to alter the ESR signal of red cell membrane proteins [17].

Cinnabarinate formation appears to be a two-step process: (1) auto-oxidation of 3-HAT that yields superoxide anion and an anthranilyl radical which, in turn, oxidizes to form the quinoneimine, followed by (2) further oxidation and condensation between the quinoneimine and 3-HAT to yield cinnabarinate and hydrogen peroxide according to the following reaction:



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Given the apparent generation of both superoxide and hydrogen peroxide during 3-HAT auto-oxidation, we have examined the effects of the enzymes superoxide dismutase (SOD) and catalase, as well as transition metals in both free and chelated states, on the process of 3-HAT auto-oxidation. In addition, in an attempt to explain the neurotoxicity of quinolinate and the effects of quinolinate and picolinate on red cell membrane proteins, we have examined the possibility that quinolinate and picolinate might also undergo an auto-oxidative process similar to that seen with 3-HAT.

MATERIALS AND METHODS

Chemicals and enzymes. 3-HAT is insoluble at pH values near neutrality. Although not directly addressed in the earlier reports [9, 10, 18, 19], this experimental difficulty has been circumvented in recent investigations by dissolving the 3-HAT in acid and then neutralizing this solution prior to use [8, 11, 12]. However, because auto-oxidation of 3-HAT begins immediately upon neutralization (see below), the method and timing of neutralization alter the rate of cinnabarinate formation. Accordingly, we have examined 3-HAT auto-oxidation with particular attention to the timing of neutralization and control of pH in all treatments.

A stock solution of 187.5 mM 3-HAT (Sigma Chemical Co., St. Louis, MO) was prepared daily in 1.0 mM KCl, 0.1 M HCl. Ten molar HCl was added dropwise until all the anthranilate dissolved. The final pH was 2.0, a pH at which auto-oxidation of 3-HAT occurs only very slowly, if at all (see Fig. 1). Both quinolinate (2,3-pyridinedicarboxylic acid) and picolinate (2-pyridinecarboxylic acid), obtained from the Aldrich Chemical Co. (Milwaukee, WI) were dissolved in 100 mM sodium phosphate buffer and the pH was adjusted to 7.4. Catalase (88,823 units/mg) was obtained from Calbiochem-Behring (La Jolla, CA). Superoxide dismutase (SOD), obtained from the Sigma Chemical Co., was diluted in 100 mM sodium phosphate buffer (pH 7.4) to a concentration of 10 units/μl. Oxy- and methemoglobin solutions were prepared from freshly drawn, normal human red blood cells and isolated according to Wind [20]. The concentrations of oxy-, met- and deoxyhemoglobin are expressed as per heme and were calculated using the modified equations of Benesch *et al.* [21]. All other chemicals were of the highest quality commercially available (Fisher Chemical Co.).

Spectrophotometric studies. 3-HAT auto-oxidation was initiated by increasing the pH via addition of 40 μl of stock 3-HAT solution to 3 ml of 100 mM sodium phosphate buffer. The pH of the buffer had been adjusted previously so that addition of the acidic anthranilate stock solution would result in a pH close to the value required for a given experiment (generally either pH 7.4 or 8.7). When necessary, the pH of the reaction mixture was adjusted more precisely, using 0.1 N HCl or NaOH. Dilutions similar to those above were made using stock solutions of quinolinate and picolinate except that pH of the sodium phosphate reaction buffer, previously adjusted to 7.4, did not require further adjustment.

Auto-oxidation of 3-HAT was monitored using a Cary 14 spectrophotometer by following the appearance of cinnabarinic acid, the oxidative dimerization product with a broad absorption maximum at 455–460 nm, either by scanning the absorption spectra of the reaction cuvette between 360 and 660 nm, or by monitoring the rate of change in absorbance at 455 nm. Cinnabarinate concentrations were calculated using a millimolar extinction coefficient of 23 at 455 nm [22]. All data are expressed as concentrations. To convert concentrations to micromoles, multiply data given as mmolar $\times 10^{-2}$ by 3×10^{-3} and data given as μmolar by 3×10^{-3}. The appearance of cinnabarinate typically showed a lag phase of between 10 and 30 sec at pH 8.7 but up to 3 min at pH 7.4. This lag phase was followed by a linear phase of cinnabarinate formation, the duration of which varied depending upon what additions had been made to the reaction mixture, but which persisted for at least 10 min in all treatments. Similar scans were run for both quinolinic and picolinic acids. Temperature was maintained at 25° using a water-jacketed cuvette holder and a circulating water bath. Hypoxic buffer ($P_{O_2} < 2$ mm Hg) was prepared by sparging with N_2 gas prior to transfer to a Thunburg cuvette. Further sparging of the buffer in the cuvette was accomplished via a three-way stopcock, and subsequent additions to the reactions buffer were made by injection through an air-tight rubber port. Reaction mixtures were stirred continually by a magnetic stir bar. In experiments where either oxy- or methemoglobin was added, the change in absorbance due to cinnabarinic acid production was monitored at 475 nm, a wavelength that is an isosbestic point of oxy- and methemoglobin, but that is also on the upper shoulder of the broad peak due to cinnabarinic acid.

Oxygen consumption and H_2O_2 measurements. Oxygen consumption ($\dot{n}_{O_2}$) during 3-HAT auto-oxidation was monitored using a polarographic oxygen electrode (Yellow Springs Instrument Co., model 53 Biological Oxygen Monitor). Auto-oxidation was initiated by adding 40 μl of stock 3-HAT solution to 3 ml of 100 mM sodium phosphate buffer in the sample chamber. The pH of the reaction mixture was adjusted to the desired value as above, and any further additions to the reaction were made via a side port on the electrode. Similar procedures were followed with quinolinate and picolinate. Because catalase in the reaction mixture liberates 1 mole of O_2 for every 2 moles of O_2 incorporated into hydrogen peroxide, the oxygen consumption rates in experiments containing catalase have been multiplied by 2. Temperature (25°) was maintained by a circulating water bath, and the reaction mixture was stirred continuously by a magnetic stir bar. Hypoxic buffer was prepared as above. Accumulation of H_2O_2 in the reaction vessel was estimated by measuring the increase in P_{O_2} upon addition of excess (975 units/ml) catalase. In all cases, the reaction had run for at least 15 min prior to the addition of catalase.

RESULTS

The rate of 3-HAT auto-oxidation was extremely

sensitive to the pH of the reaction medium, regardless of whether the rate was monitored by following the appearance of cinnabarinic acid (Fig. 1A) or by oxygen consumption (Fig. 1B). Both cinnabarinate production and oxygen utilization increased exponentially as pH increased, and there was a high degree of correlation between the two methods of monitoring the auto-oxidation reaction: analysis of covariance following log transformation of rates of oxygen consumption and cinnabarinate formation demonstrated homogeneity of slopes ($F = 0.65$, $N = 20$, $P < 0.5$). Rates of cinnabarinic acid formation were directly related to the concentration of 3-HAT in solution between the values of 0.0 and 8.0 mM ($y = \mu M$ cinnabarinate/min, $x =$ mM 3-HAT: $y = 0.54x + 0.33$, $r = 0.98$, $N = 11$). This relationship also held when 3-HAT auto-oxidation was monitored as oxygen consumption ($y = \mu M$ O_2/min, $x =$ mM 3-HAT: $y = 6.42x + 1.20$, $r = 0.99$, $N = 11$). The correlation between these two regressions was also excellent ($F = 0.42$, $N = 22$, $P < 0.5$), indicating that both cinnabarinate formation and O_2 consumption increased similarly with increasing 3-HAT concentration.

Initial rates of 3-HAT auto-oxidation (the linear phase of either cinnabarinate formation or oxygen consumption occurring during the first 10 min of the reaction) were altered by the addition of excess amounts of superoxide dismutase (133 units/ml) and/or catalase (975 units/ml) (Table 1). At pH 8.7, the appearance of cinnabarinate was accelerated 4-fold ($P < 0.05$) by the addition of SOD to the reaction mixture, whereas the addition of both SOD and catalase accelerated the reaction 6-fold over control rates (Table 1). Addition of catalase alone did not alter the rate of cinnabarinate appearance. Although the chelator DETAPAC consistently appeared to retard cinnabarinate formation, the effect was significant only when added in combination with SOD (Table 1).

3-HAT auto-oxidation monitored as oxygen consumption was also accelerated significantly by both catalase and SOD, and the fastest rates were

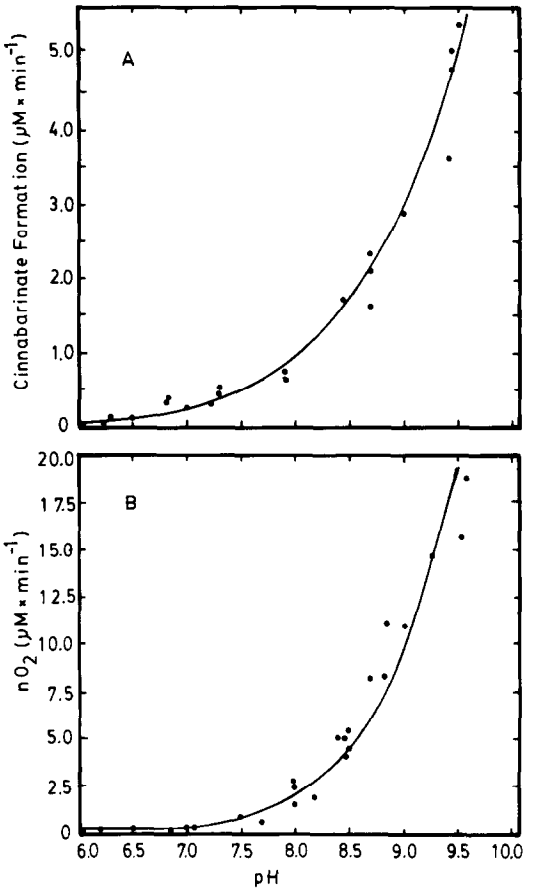

Fig. 1. Effects of pH on the relative rates of 3-HAT auto-oxidation. Auto-oxidation was initiated by adding a small volume (40 μl) of acidic 3-HAT stock solution to 3 ml of phosphate buffer which was then titrated with 1 M HCl or NaOH to the desired pH. Final 3-HAT concentration = 2.5 mM. Temp. = 25°. (A) Formation of cinnabarinate (μM/min) monitored by following change in absorbance at 455 nm ($y = 5.35 \times 10^{-5}e^{1.12x}$, $r^2 = 0.96$, $N = 21$, $P < 0.01$). (B) Oxygen consumption (n_{O_2}; μM/min) ($y = 6.17 \times 10^{-6}e^{1.59x}$, $r^2 = 0.97$, $N = 24$, $P < 0.01$).

Table 1. Effects of catalase* (CAT), superoxide dismutase (SOD), and diethylenetriamine penta-acetic acid (DETAPAC) on relative linear rates of cinnabarinic acid formation and oxygen consumption during auto-oxidation of 3-hydroxyanthranilic acid at pH 8.7 (see text for reaction conditions)

Additions	Cinnabarinate formation (μM/min)	Oxygen consumption (n_{O_2}) (μM/min)	Ratio
None	1.42 ± 0.39[a]	12.0 ± 2.9[a]	8.3
DETAPAC (2.5 mM)	1.06 ± 0.16[a]	11.4 ± 3.1[a]	10.9
CAT (975 units/ml)	1.75 ± 0.31[a]	19.5 ± 3.3[b]	10.8
DETAPAC + CAT	1.12 ± 0.16[a]	15.5 ± 3.0[ab]	14.0
SOD (133 units/ml)	6.25 ± 0.93[b]	27.8 ± 3.7[c]	4.4
DETAPAC + SOD	5.10 ± 0.82[c]	26.8 ± 4.3[c]	5.4
CAT + SOD	8.68 ± 1.11[d]	41.0 ± 5.5[d]	4.7
DETAPAC + CAT + SOD	8.48 ± 1.91[d]	37.0 ± 5.5[d]	4.5

Values are mean rates (±SD, N = 4), 25°. Means not significantly different at $P < 0.05$ (one-way ANOVA Student–Newman–Keuls) share superscripts.

* Rates of oxygen consumption in experiments containing catalase have been multiplied by 2 to compensate for generation of O_2 from H_2O_2.

obtained when both enzymes were present (Table 1). The molar ratio of the amount of oxygen consumed to the amount of cinnabarinate formed varied from greater than 10 when catalase was present, to an average of 4.7 in those reactions containing SOD (Table 1), a disparity which suggests that a number of 3-HAT oxidation products other than cinnabarinate are formed. Experiments identical to those presented in Table 1 except done at pH 7.4 yielded data that qualitatively resembled those obtained at pH 8.7 (data not shown). Given the base-catalyzed nature of 3-HAT auto-oxidation, the rates obtained at pH 7.4 were almost an order of magnitude slower than those seen at pH 8.7. However, the ratios of O_2 consumption to cinnabarinate formation were indistinguishable between pH 7.4 and pH 8.7, a reasonable finding considering the significance of the analysis of covariance for the data shown in Fig. 1. Following a lag period that corresponded with the lag observed in cinnabarinate formation, rates of oxygen consumption remained constant in all treatments and never reached a plateau regardless of changes in the rates of cinnabarinate formation.

No cinnabarinic acid, or any other compound with absorbance in the visible region, was detected in the hypoxic treatments for as long as the reaction mixture was held hypoxic (Fig. 2). However, cinnabarinate formation began immediately upon exposure of the reaction buffer to air and proceeded at rates typical of controls (arrow, Fig. 2). Rates of cinnabarinate formation in control reactions lacking enzymic additions began to slow between 10 and 20 min and then reached a plateau. Reactions containing catalase showed rates similar to the controls, but did not slow until after 1 hr and never obtained a true plateau even after 2 hr. When SOD was present, rates of cinnabarinate formation were much faster than either controls or reactions containing catalase (Fig. 2). However, like controls, reactions containing SOD began to slow after about 20 min and cinnabarinate formation reached a plateau at an absorbance typically two to three times higher than the controls. Addition of catalase during this plateau generated rates of cinnabarinate formation identical to those observed when both SOD and catalase were present at the start of the reaction (dashed line labeled +CAT, Fig. 2). When both SOD and catalase were present, initial rates were similar to reactions containing SOD alone, but no plateau was obtained and cinnabarinate formation proceeded in a linear fashion for at least 2 hr. When allowed to continue for 12 hr, 3-HAT spectra from reactions containing both SOD and catalase began to show hypochromatic shifts, but the maximum absorbances, which occurred after 7 hr, indicate that only 30% of the 3-HAT originally in solution undergoes auto-oxidation to cinnabarinate.

Neither the initial nor the long-term rates of cinnabarinate appearance were accelerated significantly by the addition of oxy- or methemoglobin (Fig. 3). However, addition of either oxy- or methemoglobin

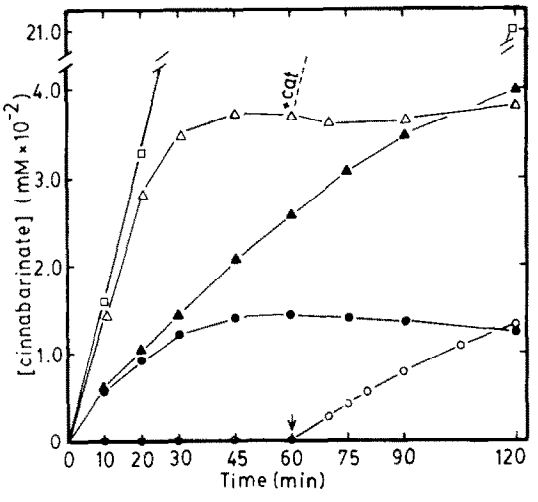

Fig. 2. Effects of superoxide dismutase (SOD), catalase, and anoxia on the relative rates of 3-HAT auto-oxidation at pH 7.4. Auto-oxidation was monitored by following change in absorbance due to cinnabarinate at 455 nm. Rate of cinnabarinate formation with no additions (●), and upon the addition of excess SOD (133 units/ml; △), catalase (975 units/ml; ▲), and both SOD and catalase (□). rate of cinnabarinate formation in anoxic buffer ($P_{O_2} < 2$ mm Hg; ○) and after the reaction mixture was exposed to air (indicated by arrow). Broken line labeled + CAT indicates addition of 975 units/ml catalase to the SOD treatment.

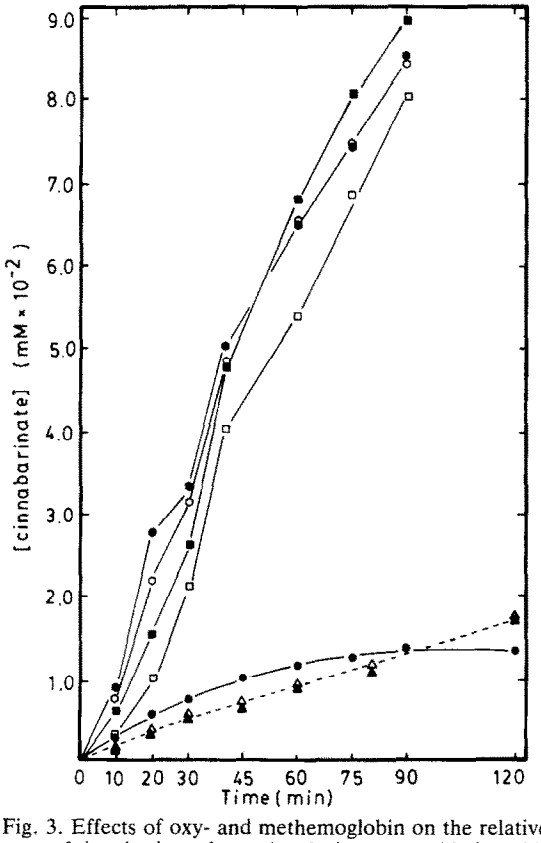

Fig. 3. Effects of oxy- and methemoglobin on the relative rates of cinnabarinate formation during auto-oxidation of 3-HAT at pH 7.4. Cinnabarinate formation with no additions (●), and in the presence of a 35 μM concentration of either oxyhemoglobin (closed symbols) or methemoglobin (open symbols) with no enzymic additions (triangles), in the presence of hemoglobin plus excess (660 units/ml) SOD (squares), and both SOD and catalase (hexagons).

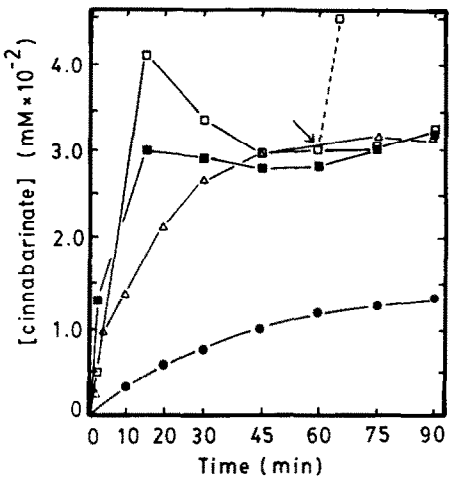

Fig. 4. Cinnabarinate formation with no additions (●) and in the presence of 2.5 mM $FeCl_3$ chelated by EDTA (△), $MnCl_2$ (□), or Mn_3O_4 (■). Addition of excess catalase (975 units/ml) to the Mn^{2+} treatment after 60 min indicated by an arrow.

in combination with SOD produced rates of cinnabarinate formation indistinguishable from those seen when both SOD and catalase were present. Moreover, as in the presence of SOD plus catalase, there was no plateau in cinnabarinate formation when SOD plus either form of hemoglobin were present (Fig. 3). The addition of Fe^{3+} chelated by EDTA accelerated both cinnabarinate formation and oxygen consumption during 3-HAT auto-oxidation almost 4-fold (Fig. 4), whereas EDTA alone had no effect on either measurement (data not shown). Addition of manganese ions either as Mn^{2+} or as Mn^{3+} also greatly accelerated initial cinnabarinate formation (Fig. 4), and production reached a plateau within 15 min. Catalase abolished this plateau, and initial rates of cinnabarinate formation were again observed (arrow, Fig. 4). No cinnabarinate formation was detected in anoxic buffer upon addition of either form of manganese or EDTA-chelated iron.

The absorbance spectra of oxyhemoglobin and methemoglobin between 430 and 660 nm changed during 3-HAT auto-oxidation (Fig. 5), indicating an interconversion between the two forms. Half of the oxyhemoglobin was converted to methemoglobin during 1 hr of incubation with 3-HAT (Fig. 5A), but the spectra began to lose resolution and the isosbestic points disappeared, indicating the generation of non-intact hemoglobins (Fig. 5A). Methemoglobin was also converted to oxyhemoglobin during 3-HAT auto-oxidation (Fig. 5B), although at twice the rate: 50% of the methemoglobin became oxyhemoglobin within 30 min. After 30 min, however, these spectra also began to degenerate and non-intact hemoglobins were apparent (Fig. 5B). Increasing the rate of 3-HAT auto-oxidation with SOD accelerated the conversion of oxyhemoglobin to methemoglobin and, within 45 min, fully 75% of the hemoglobin remaining intact had become methemoglobin. However,

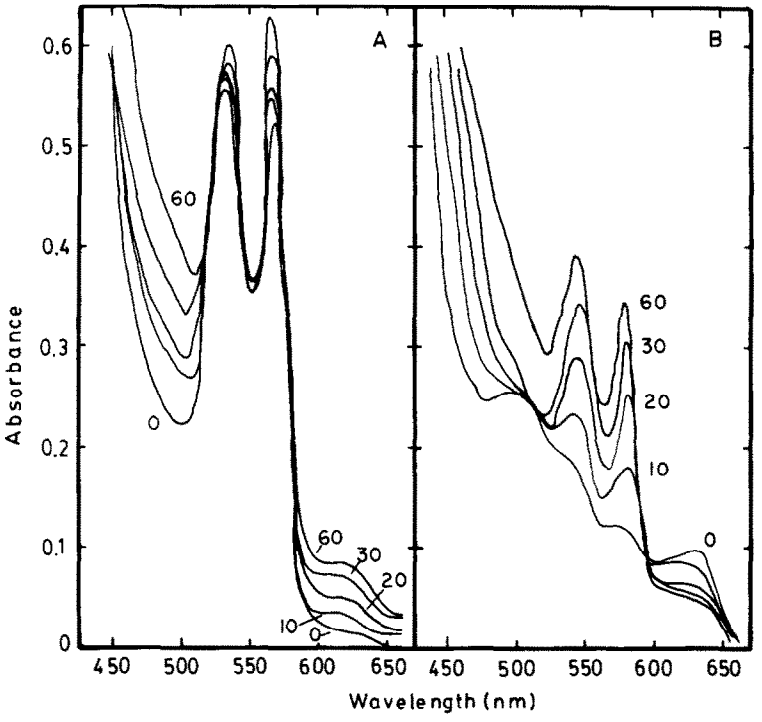

Fig. 5. Changes in the absorbance spectra of hemoglobin between 660 and 430 nm in the presence of 2.5 mM 3-HAT at pH 7.4. Number of minutes following addition of HAT and initiation of auto-oxidations indicated. In addition to HAT and buffer, at the start of the reaction the cuvette contained a 35 μM concentration of either (A) oxyhemoglobin or (B) methemoglobin.

SOD did not accelerate the conversion of methemoglobin to oxyhemoglobin above control rates.

Neither quinolinate nor picolinate showed any changes in their visible spectra or oxygen consumption above controls in the range of pH values between 6.0 and 9.5 during the 2-hr period that the reactions were monitored.

DISCUSSION

An examination of the oxidative reactivity of several naturally-occurring metabolites of tryptophan known to be either neurotoxic (quinolinate), carcinogenic (3-hydroxyanthranilate, cinnabarinate) or capable of altering the conformation of erythrocyte membrane proteins (quinolinate, picolinate) has shown that neither quinolinate nor picolinate autooxidized over a wide pH range. This supports the suggestion [1] that the neurotoxicity of quinolinate is due to its structural similarity to other dicarboxylic excitotoxins, and not to oxidative reactivity. However, 3-HAT spontaneously auto-oxidized in a process that requires molecular oxygen and yields superoxide, H_2O_2, and cinnabarinate.

3-HAT auto-oxidation to cinnabarinic acid was a base-catalyzed process: The rates of both cinnabarinate formation and oxygen consumption were equally sensitive to the pH of the medium and both increased exponentially between pH 6.5 and 9.5. If cinnabarinic formation was the only process sensitive to pH, it could be argued that the reaction would not be true base-catalysis, but either a suppression of product formation or an enhanced product decomposition due to decreased pH. However, not only was oxygen consumption during 3-HAT auto-oxidation as sensitive to pH as cinnabarinate formation, but cinnabarinate was stable at acidic pH values.

Our data are the first to implicate pH as a major determinant of 3-HAT auto-oxidation. Our findings support earlier reports [8, 19] which conclude that cinnabarinic acid is the major product of 3-HAT auto-oxidation and that its formation involves two processes: auto-oxidation of 3-HAT to form the quinoneimine, followed by condensation and oxidation reactions between two 3-HAT-derived monomers to form cinnabarinate (see Equation 1). Both cinnabarinate formation and oxygen consumption increased similarly with increasing pH (Fig. 1) and with increasing 3-HAT concentration, affirming that cinnabarinate formation is a reasonable indicator of overall 3-HAT auto-oxidation. If cinnabarinate were the sole product of 3-HAT auto-oxidation, a ratio of 3 moles of oxygen consumed per mole of cinnabarinate formed would be expected (Equation 1). In experiments lacking SOD, the observed ratio was 9.0 (Table 1), indicating that in these experiments only one-third of 3-HAT auto-oxidation could be accounted for by cinnabarinate formation alone. In the presence of SOD, the observed ratio decreased to 4.5, indicating that, in this case, fully two-thirds of 3-HAT auto-oxidation involved cinnabarinate formation.

The other products of 3-HAT auto-oxidation implied by the above ratios of oxygen consumption to cinnabarinate formation are as yet uncharacterized. In spite of exponential increases in reaction rates with increasing pH, the ratios between initial rates of oxygen consumption and cinnabarinate formation were constant over a wide pH range. This suggests that the rate of 3-HAT auto-oxidation to the anthranilyl radical was both the base-catalyzed component in cinnabarinate formation and the rate-determining step for subsequent oxidations and condensations.

The mechanism whereby SOD both accelerated overall 3-HAT auto-oxidation and increased cinnabarinate yield cannot be firmly established based upon the available data. It is reasonable that a decrease in the concentration of superoxide in the medium should retard the reverse reaction between anthranilyl radical or quinoneimine and superoxide (see Ref. 23). If the availability of the anthranilyl radical or quinoneimine were rate-limiting for subsequent oxidations and condensations, then prevention of the reverse reaction and resulting increase in anthranilyl radical or quinoneimine concentrations would account for the observed acceleration of cinnabarinate formation.

Given its apparent lack of effect on the rate of cinnabarinate formation, it would appear that catalase acts by removing H_2O_2, thereby preventing bleaching of cinnabarinate [8, 11]. The observed persistence of oxygen consumption long after cinnabarinate formation had reached a plateau also supports the contention that H_2O_2 bleaches the condensation product and does not affect the initial step of 3-HAT auto-oxidation. In addition to direct reaction with H_2O_2, cinnabarinate could also be bleached by hydroxyl radicals formed via reaction of H_2O_2 with transition metal cations present as contaminants, a mechanism known to operate during auto-oxidation of 6-hydroxydopamine [24]. Direct evidence for H_2O_2 consumption during cinnabarinate bleaching is provided by the observation that, regardless of pH, the recovery of H_2O_2 from O_2 consumed during 3-HAT auto-oxidation was never greater than 38% (50% recovery would indicate that all O_2 consumed went into the formation of recoverable H_2O_2) (data not shown).

Besides providing a mechanism for hydroxyl radical production, transition metal cation contaminants can function as catalysts of auto-oxidations [24]. However, the lack of cinnabarinate formation in anoxic buffer upon addition of either Mn^{2+}, Mn^{3+}, or EDTA-chelated iron argues that direct transition metal catalysis is not an important factor in the initial steps of 3-HAT auto-oxidation. It therefore appears reasonable that EDTA-chelated iron, as well as both forms of manganese, accelerate 3-HAT auto-oxidation by removing superoxide radicals, a suggestion made previously for the action of Mn^{2+} [8]. In the presence of non-chelated Mn^{2+}, Mn^{3+}, or EDTA-Fe^{3+}, both the acceleration of cinnabarinate formation and the maximum absorbances obtained were similar to those seen in the presence of SOD (see Figs. 2 and 4). Moreover, addition of catalase during these plateaus accelerated cinnabarinate formation to rates indistinguishable from those observed in the presence of both SOD and catalase.

Although it has been reported that cinnabarinate formation is accelerated by methemoglobin compared to experiments containing oxyhemoglobin

[12], rates of cinnabarinate formation in the absence of hemoglobin are not presented and, without such information, interpretation of differences between samples containing either form of hemoglobin becomes tenuous. Our data indicate that neither 3-HAT auto-oxidation nor cinnabarinate formation was accelerated above control rates by the addition of either oxy- or methemoglobin even though direct interaction between 3-HAT undergoing auto-oxidation and hemoglobin in the reaction medium was evident. Although these iron proteins did not catalyze 3-HAT auto-oxidation, i.e. accelerated neither initial rates of cinnabarinate formation (Fig. 3) nor oxygen consumption, they were capable of scavenging H_2O_2 which would otherwise bleach cinnabarinate. Like catalase, both met- and oxyhemoglobin prolonged cinnabarinate formation after controls had obtained a plateau (Fig. 3). Moreover, addition of SOD plus either form of hemoglobin resulted in rates of cinnabarinate formation identical to rates observed in the presence of SOD plus catalase (Fig. 3).

It is apparent from the present results and earlier work [12] that products of 3-HAT auto-oxidation can convert not only oxyhemoglobin to methemoglobin but also methemoglobin to oxyhemoglobin. Interconversion between oxyhemoglobin and methemoglobin indicates the presence of one-electron donors and acceptors such as 3-HAT, anthranilyl radical, quinoneimine, superoxide, and oxidized and reduced transition metal cations, which can react with the iron proteins without denaturing the protein (at least not initially). We also observed that non-functional hemoglobin derivatives were formed which implies irreversible oxidative attack by intermediates such as H_2O_2, hydroxyl radical, and possibly by organic radicals such as anthranilyl. Since it is apparent that the overall thermodynamic drive is oxidative, the conversion of methemoglobin to oxyhemoglobin is a transient process: methemoglobin initially gives rise to oxyhemoglobin which is converted back to methemoglobin prior to the final conversion into non-functional hemoglobin.

In summary, neither the neurotoxic quinolinate, nor its decarboxylated analog picolinate undergoes auto-oxidation. However, 3-HAT is most likely to exert its potentially toxic effects through its auto-oxidation products such as superoxide, H_2O_2, hydroxyl and anthranilyl radicals. SOD and catalase removed products of oxygen reduction generated during 3-HAT auto-oxidation, but in so doing SOD also accelerated overall 3-HAT auto-oxidation rates and cinnabarinate production, perhaps by preventing back reactions between superoxide anion and the anthranilyl radical or the quinoneimine formed during the initial steps of 3-HAT auto-oxidation. Catalase prevented cinnabarinate bleaching by H_2O_2 directly or indirectly by hydroxyl radicals formed by reaction of H_2O_2 with transition metal cations. Transition metal cations accelerated 3-HAT auto-oxidation, not by oxidizing 3-HAT, but apparently by removing superoxide anions, thereby increasing the availability of anthranilyl radicals necessary for subsequent condensation reactions. Oxyhemoglobin and methemoglobin scavenged reactive intermediates of 3-HAT auto-oxidation, but in so doing they were degraded to biologically inactive products.

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REFERENCES

1. R. Schwarcz, W. Whetsell and M. Mangano, *Science* **219**, 317 (1983).
2. M. J. Allen, E. Boyland, C. E. Dukes, E. S. Horning and J. G. Watson, *Br. J. Cancer* **11**, 212 (1957).
3. G. T. Bryan, R. R. Brown and J. M. Price, *Cancer Res.* **24**, 596 (1964).
4. H. Ehrhart, W. Ried, W. Benoit and H. Dorfler, *Z. Krebsforsch.* **71**, 1 (1968).
5. K. Fujii and M. Watanabe, *J. Cancer Res. clin. Oncol.* **96**, 163 (1980).
6. A. L. Lehninger, *Biochemistry*, 2nd Edn. Worth, New York (1975).
7. D. Bender, *Amino Acid Metabolism.* Wiley-Interscience, London (1975).
8. H. Ogawa, Y. Nagamura and I. Ishiguro, *Hoppe-Seyler's Z. physiol. Chem.* **364**, 1507 (1983).
9. G. Ehrensvard, J. Liljekvist and R. G. Heath, *Acta chem. scand.* **14**, 2081 (1960).
10. P. V. Subba Rao and C. S. Vaidyanathan, *Biochem. J.* **99**, 317 (1966).
11. H. Ogawa, Y. Nagamura and I. Ishiguro, *Hoppe-Seyler's Z. physiol. Chem.* **364**, 1059 (1983).
12. A. Tomoda, E. Shirasawa, S. Nagao, M. Minami and Y. Yoneyama, *Biochem. J.* **222**, 755 (1984).
13. E. Boyland, *Acta. Un. int. Cancr.* **16**, 273 (1960).
14. I. P. Lapin, *J. neural Transm.* **42**, 37 (1978).
15. T. W. Stone and M. N. Perkins, *Eur. J. Pharmac.* **72**, 411 (1981).
16. B. T. Farmer and D. A. Butterfield, *Life Sci.* **35**, 501 (1984).
17. B. T. Farmer, M. N. Nicholas and D. A. Butterfield, *Biochim. biophys. Acta.* **778**, 260 (1984).
18. N. Savage and W. Prinze, *Biochem. J.* **161**, 551 (1977).
19. R. N. Feinstein, *Proc. Soc. exp. Biol. Med.* **158**, 398 (1978).
20. M. M. Wind, *Ph.D. Thesis.* New York University Medical Center, New York (1973).
21. R. E. Benesch, R. Benesch and S. Yung, *Analyt. Biochem.* **55**, 245 (1973).
22. I. Ishiguro, Y. Nagamura and A. Hara, *J. pharm. Soc. Japan*, **91**, 760 (1971).
23. S. G. Sullivan, C. C. Winterbourn and A. Stern, in *Oxy Radicals and Their Scavenger Systems* (Eds. G. Cohen and R. A. Greenwald), Vol. 1, p. 364. Elsevier, Amsterdam (1983).
24. S. G. Sullivan and A. Stern, *Biochem. Pharmac.* **30**, 2279 (1981).