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PSEUDO MALONALDEHYDE ACTIVITY IN THE THIOBARBITURIC ACID TEST*

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In the thiobarbituric acid test for malonaldeyde, 2-hexenal, 2,4-hexadienal and 13-hydroperoxy-9Z,11E-octadecadienoic acid are found to form the characteristic 532 nm chromogen, at pH = 2.7 and at elevated iron(III) levels, faster than malonaldehyde itself. Under the same conditions, but in the presence of EDTA, the chromogen formation is dramatically suppressed. The findings show that malonaldehyde cannot be generated in situ and are interpreted in terms of chromogen formation via an iron catalysed fragmentation of TBA-aldehyde intermediates.

KEY WORDS: Lipid peroxidation, malonaldehde, TBA activity, iron catalysis.

1. INTRODUCTION

The widely used TBA test for malon(di)aldehyde(MDA) employs thiobarbituric acid (TBA) to convert MDA to the characteristic anionic chromogen (I)[1] (see Scheme). The conditions used to drive the reaction are fairly vigorous and under them many other compounds give positive, albeit proportionately low, responses.[2–5] Thus these measurements of MDA are of little value unless levels of other active species are known to be insignificant. Often of particular concern are the contribtions to (I) of hydroperoxy-dienoic acids[3] and α,β-unsaturated aldehydes.[4,5] The former, which are essential components in the lipid peroxidation "cascade", commonly break down to give the latter as major products.[5] This paper reports findings which indicate how these compounds participate in the TBA test and how their participation may be recognised.

2. EXPERIMENTAL

2.1 Materials

Thiobarbituric acid, 1,1,3,3-tetramethoxypropane(TMP) and 2-hexenal (2-HA) were purchased from the Aldrich Chemical Co. Ltd. 2,4-Hexadienal (2,4-HDA) was obtained from Lancaster Synthesis and linoleic acid (puriss) was from Fluka Chemi A.G. Soybean lipoxygenase (126,500 units/mg protein) was supplied by the Sigma Chemical Co. Ltd. EDTA disodium salt and trichloroacetic acid were from B.D.H. Chemicals Ltd. and Fisons Ltd. respectively.

*Part 2 in the series "Lipid Peroxidation Studies". For Part 1 see Read, G.[1]
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2-HA and 2,4-HDA were redistilled before use and, like the linoleic acid, kept under nitrogen. The aldehydes, linoleic acid and lipoxygenase were stored in the dark at 4°C. Stock solutions of MDA were prepared from TMP and estimated by established methods.[6] The enzymic conversion of linoleic acid with soybean lipoxygenase to 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid(13-HOD) followed the method of Hamberg and Samuelsson.[7] except that CH_2Cl_2 was used to extract the product.

2.2 General Reaction Conditions

For studies on MDA, 2-HA and 2,4-HDA, solutions were added in the following order to 100 cm^2 darkened reaction flasks, immediately prior to heating: (i) thiobarbituric acid, (ii) buffer, (iii) EDTA (where appropriate), (iv) aldehyde, and (v) $FeCl_3$ (where appropriate).

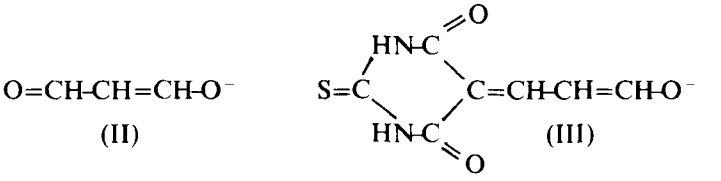
The flasks were lightly stoppered and placed in a water bath (100°C or 95°C). Aliquots (3 cm^3) of the solution were removed at intervals, cooled and transferred to a 1 cm cuvette for measurement of the 532 nm chromogen level. The pHs of the solutions were checked at t = 0 and at the end of the reaction.

For reactions with 13-HOD, the hydroperoxydiene was prepared immediately before use. After washing the CH_2Cl_2 extract with a little water, the solution was evaporated to dryness under reduced pressure at sub-ambient temperatures in the reaction flask to be used in the assay. TBA and buffer solutions were added to the product. The flask was swirled briefly to disperse the 13-HOD before the $FeCl_3$ solution and, where appropriate, EDTA solution were added.

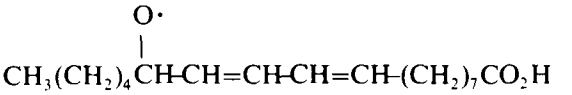
All measurements were carried out in duplicate.

3. RESULTS AND DISCUSSION

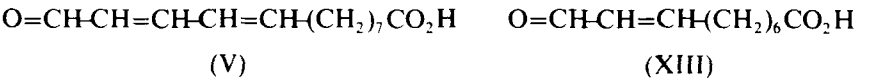
To establish conditions under which the rate of formation from MDA of the 532 nm chromogen could be readily monitored we examined the TBA reaction at 100°C, with 7×10^{-3} M TBA and 4.5×10^{-6} M MDA, over a range of pH using the reciprocal of time to half completion ($t_{1/2}^{-1}$) as a crude indicator of rate. The results (Figure 1) confirm that the rate falls off rapidly as the pH increases from 1 to 4, reflecting the influence, at the lower acidities, of higher concentrations of the unreactive MDA anion (II) and intermediate anion (III).



(II) (III)



(IV)



(V) (XIII)

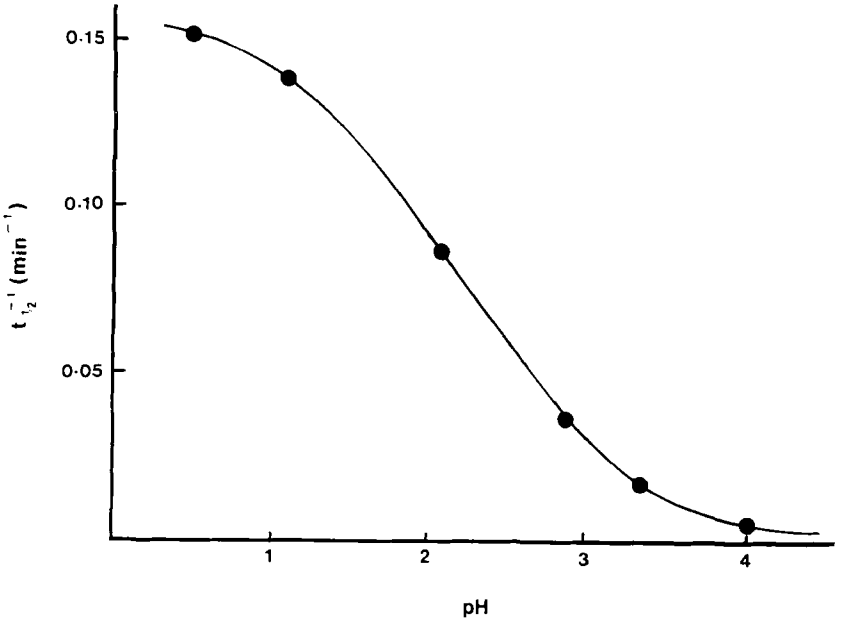


FIGURE 1 Influence of pH on the rate of chromogen formation. See text for conditions.

For further investigations, pH = 2.7 was chosen as the standard acidity. The bath temperature was reduced to 95°C and the TBA concentration was 1×10^{-2} M. Preliminary experiments had shown that chromogen formation from 2-HA, 2,4-HDA and 13-HOD was sensitive to variations in iron(III) concentration and this was therefore held at the abnormally high level of 4×10^{4} M. Under these conditions MDA generated the expected concentration of 532 nm chromogen. Working concentrations of 2-HA, 2,4-HDA and 13-HOD were taken to produce final absorbances which were relatively close to the final figure from the MDA. A subsequent normalisation of these optical densities was carried out so that the final values corresponded to the final MDA figure. This is shown in Figure 2. The concentrations given are slightly adjusted to take this normalisation into account.

The marked sensitivity of these pseudo-MDA activities to "free" iron(III) was apparent when the assays were repeated in the presence of EDTA (1.4×10^{-4} M). As Figure 2 shows, chromogen formation in the case of 2,4-HDA was negligible. Somewhat higher levels were found for 2-HA and 13-HOD but these still represent 90 and 75% suppressions respectively of chromogen formation.

The profiles in Figure 2 show clearly that the formation of (I) from 2-HA, 2.4-HDA and 13-HOD in the presence of "free" iron is considerably faster than from MDA under the conditions used. The maximum rates obtained are very similar and are approximately 2.5 times faster than that obtained for MDA. It follows that in these cases MDA cannot be an intermediate in chromogen formation. It is also apparent from the results that iron plays an intimate role in the fragmentation of the substrates. Under the experimental conditions decomposition of the 13-HOD by iron catalysis and thermal homolysis is expected, giving the radical intermediate (IV) which fragments further into the pentyl radical and 13-oxo-9,11-tridecadienoic acid(OTDA) (V).[8] The latter, like 2,4-HDA, carries a conjugated dienal grouping. In seeking to

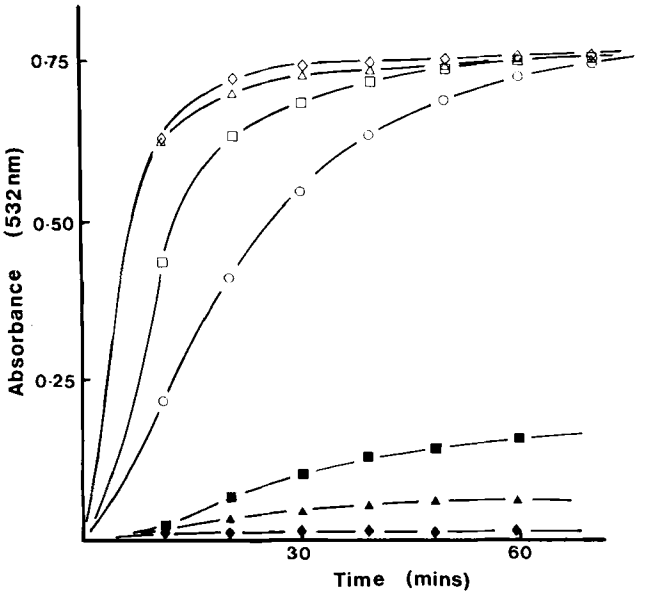
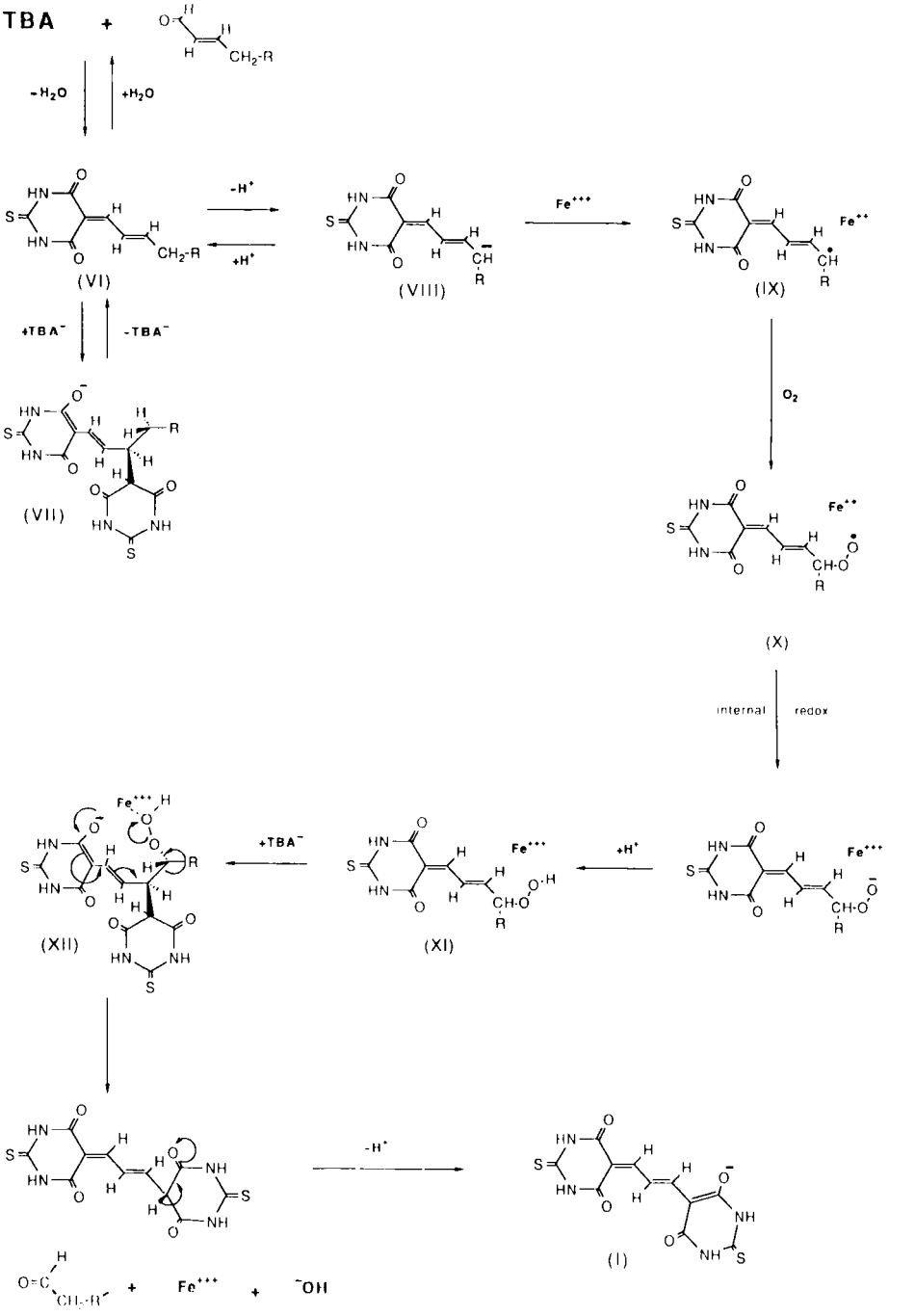


FIGURE 2 Relative rates of chromogen formation. MDA (2.6×10^{-6} M) ○; 4-HA (6.6×10^{-4} M) Δ; 2,4-HDA (8.4×10^{-5} M)◇; 13-HOD (3.0×10^{-4} M)□; open symbols without EDTA closed symbols with EDTA (1.4×10^{-4} M). See text for remaining conditions.

explain the above observations we focus therefore on the chemistry of the unsaturated aldehydes.

In the case of alkenals, the initial condensation with TBA can be expected to proceed smoothly giving products of the general formula (VI) (Scheme). These will be particularly susceptable to nucleophilic attack at the α- and γ-positions of the side chain and, when TBA anion is the nucleophile, the products from such an attack at the γ-position will have the general structure (VII). In competition with this nucleophilic addition will be side chain deprotonation of (VI) at the δ-position to produce the resonance stabilised anion (VIII). These early reactions must be regarded as reversible. However, in the presence of iron(III) an irreversible oxidation of the anion (VIII) to the radical (IX) may be anticipated. The resonance stabilised radical will react rapidly with molecular oxygen giving amongst other species, the alkylperoxy radical(X) which, if iron remains complexed, could convert to the complex (XI) by an internal redox process and protonation. Nucleophilic addition of the TBA anion to (XI) will give the anionic species (XII) which, with the aid of the co-ordinated metal ion, can be expected to fragment in the manner depicted, producing the bis-TBA precursor of the 532 nm chromogen (I) normally associated with MDA. If this proposal is correct, the residual fragment from 2-HA will be propanal. By the same mechnism 2,4-HDA will produce propenal and OTDA the 10-oxo-8-decenoic acid (XIII). In principal both these α,β-unsaturated aldehydic products could generate a further molecule of (I) which may account partly for the greater chromogen yield in the case of 2,4-HDA. In the cases of 2-HA and 2,4-HDA in particular it is clear that other irreversible processes must complete with this iron mediated transformation.



SCHEME 1 Proposed mechanism for pseudo-MDA activity in the case of α,β-unsaturated aldehydes.

Several may be anticipated including attack by oxygen at the β-position of the side chain in (IX). Evidence for such an attack is presented in a later paper.

These observations have an important bearing on the interpretations of many published MDA assays. With reagent concentrations and reaction times commonly employed, quantitative convertion of MDA to the chromogen (I) can only be assumed with some certainty if acidities are relatively high (pH $<$ 1.0). Furthermore, either the levels of other substrates capable of generating (I) must be shown to be insignificant or a powerful iron(III) chelator, such as EDTA or desferrioxamine, must be introduced into the assay immediately prior to the introduction of TBA (see Ref.[10]). It is unfortunate that these precautions are rarely taken for not only are enals and dienals produced in lipid peroxidation but elevated levels of iron(III), derived from added iron(II), are often present in systems studied thereby increasing the contribution such aldehydes and hydroperoxydienes make to the final MDA value. In practice some measure of this contribution can be obtained by running parallel TBA assays with one solution containing iron chelator. However, the sensitivity of the psuedo-TBA reaction to substrate type and iron concentration prevents quantitative exploitation of the difference in readings.

Acknowledgement

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