

Iron Ascorbate-Stimulated Lipid Peroxidation *in vitro*. Why is the Method Controversial?

P. LASZCZYCA, E. KAWKA-SERWECIŃSKA, I. WITAS, B. DOLEŻYCH
and P. MIGULA

*Department of Human and Animal Physiology, University of Silesia,
ul. Bankowa 9, 40-007 Katowice, Poland*

Abstract. *In vitro* generation of thiobarbituric acid reactive substances (TBARS) is frequently used to assess organ susceptibility to lipid peroxidation. The yield of TBARS is severalfold enhanced by an addition of iron ions with reductors or chelators such as ascorbate, NADPH, ADP or pyrophosphate. The process cannot be interpreted in a simple way, since it involves several enzymatic and nonenzymatic reactions. There are no clear interpretations of the ambiguous effects of denaturing factors and chelating agents on TBARS generation. Also controversy arises from the curvilinear relationship between the homogenate concentration and the yield of TBARS. This has been modelled in the present work by combining two functions describing the sequential reaction with two limiting steps. One of them is related to catalytic action of iron and ascorbate, while the other to an enzyme, possibly phospholipase A2, as has been suggested by some investigators. Two models should be considered since it is impossible to decide which kinetic equation should predominate in the model. Nevertheless, the model reflects kinetic properties of the process. The effects of catalyst concentration and some other modification upon the yield of TBARS were also investigated experimentally. The results of experiments and modelling showed that the analytical procedures used by investigators need standardisation as the results obtained under a variety of procedures may reflect quite different properties of the living systems.

Key words: Lipid peroxidation — Thiobarbituric acid — Iron — Ascorbate — Mathematical model

Introduction

Lipid peroxidation is an index frequently determined to assess peroxidative damage to living systems. The principle of the method is the induction of *in vitro* generation of thiobarbituric acid reactive substances (TBARS), mainly malondialdehyde (MDA). For this purpose, an organ homogenate or slice is incubated under aerobic

conditions and products are detected spectrophotometrically (Boehme et al. 1977; Brooksbank and Bazals 1984). However, details of analytical procedures used by various investigators vary dramatically. For instance, factors such as ferric ions and ADP, EDTA, NADPH, pyrophosphate or ascorbate may be added to the system to stimulate peroxidation (Bird and Draper 1984; Halliwell and Gutteridge 1990). The length of sample incubation chosen by different authors vary by a factor of 5 or more (Boehme et al. 1977; Shukla et al. 1987; Barja de Quiroga et al. 1990). Also, significant differences occur with regard to homogenate concentration/dilution, to the kind of buffer used, and to special additives (Table 1), i.e. antioxidative factor – butylated hydroxytoluene (Buckingham 1985; Morehouse and Aust 1988). Moreover, analysing our previous results (Laszczyca et al. 1993) and the data of Boehme et al. (1977) we have found that variations of homogenate dilution produce unpredictable nonlinear changes of TBARS yield.

This promoted us to undertake the present study, which is an attempt at constructing a physico-chemical and mathematical model of the peroxidation process.

Table 1. Major differences between the various methods used to determine lipid peroxidation. Estimated ratios of reagents' concentrations.

Authors	Boehme et al. (1977), Shukla et al. (1987), Manca et al. (1991)	Morehouse & Aust (1988)	Buckingham (1985)	Brooksbank, Bazals (1984), Goran-Anneren, Epstein (1977)	Laszczyca, et al. (1993)
		750 $\mu\text{mol/l}$ ADP			
Peroxidizing agents used	none agent added to homogenate	100 $\mu\text{mol/l}$ FeCl_3 200 μmol NADPH	1 mmol/l ascorbate 1 mmol/l FeSO_4	250 $\mu\text{mol/l}$ ascorbate 10 $\mu\text{mol/l}$ FeSO_4	500 $\mu\text{mol/l}$ ascorbate 20 $\mu\text{mol/l}$ FeSO_4
Sample to TBA weight ratio (g/g)	6.0–24.0	37 nmol lipids	66.6	4.57	3.87
UFAs to TBA quantity ratio (mol/mol)	1.13×10^{-3} 5.40×10^{-3}	–	0.01	0.87×10^{-3}	0.73×10^{-3}
Other differences	addition of Cd^{2+} up to 450 $\mu\text{mol/l}$	artificial system	addition of BHT 10 mmol/l	–	acetate buffer pH 3.5

Abbreviations: TBA – thiobarbituric acid, UFAs – unsaturated fatty acids, BHT – butylated hydroxytoluene (an antioxidative agent)

To do this, we have tried to assess the effects of homogenate dilution and substrate concentration on the yield of TBARS during "lipid peroxidation".

Materials and Methods

The effects of homogenate concentration and reagents concentrations on lipid peroxidation were examined in samples of rat liver, brain and kidney. Fresh organs were homogenised by means of a glass homogenizer in 150 mmol/l KCl with 10 mmol/l phosphate buffer pH 7.4, at 4 °C. The homogenates were diluted to obtain a final ratio of tissue weight to homogenate volume from 1:5 to 1:220. Samples of 0.3 ml diluted homogenates were incubated for 30 min at 37 °C with 0.1 ml of 0.1 mmol/l ferrous sulphate and 0.1 ml of 2.5 mmol/l ascorbate, and subsequently denatured with 1.5 ml of 11% trichloroacetic acid. Equivalent homogenate samples were denatured prior to incubation to serve as blanks. Both kinds of samples were centrifuged (900 × *g*). The supernatants (1.4 ml) were buffered with 1.65 ml of 0.1 mol/l acetate buffer (pH 3.5), 0.25 ml aliquots of 1% thiobarbituric acid solution (TBA) were added and the resulting samples were heated to 100 °C for 15 minutes. The intensity of coloration due to released malondialdehyde was measured spectrophotometrically ($\lambda = 532 \text{ nm}$, $\epsilon = 154,000 \text{ M}^{-1}\text{cm}^{-1}$) (Boehme et al. 1977; Goran-Annereen and Epstein 1977; Brooksbank and Bazals 1984). The yield of TBARS was expressed as micromol per gram wet tissue per 30 min of incubation. Therefore the values of absorbance measured were multiplied by the factor of sample dilution ($h_i = 5 - 220$) and by a coefficient ($k = 0.10204$) which reflects all the quantitative parameters of the analytical procedure.

In another experiment the inhibiting effects of *in vitro* added 1.6; 4.0; or 10 mmol/l Cd^{2+} upon activities of antioxidant enzymes: catalase (Orr 1970), glutathione reductase (Colman 1970), and glutathione transferase (Habig and Jakoby 1981) were assessed. These effects were compared with the effect of cadmium on lipid peroxidation, both unstimulated and stimulated by iron and ascorbate. In an additional experiment the concentrations of TBA or ascorbate were also varied.

The process of lipid peroxidation was modelled mathematically assuming the kinetics of the sequential reaction with two limiting steps (Fig. 3). One of them is a second-order catalytic reaction (with two limiting substrates), while the other one is a first-order enzymatic reaction. Two different models were developed and examined, based on the kinetic equations of both limiting reactions, since the sequence of these reactions has not been predicted. Iterative counting was applied as the models represent integral equations with no algebraic solutions (see Discussion). Computations were conducted using of MathCAD 2.50, according to an own algorithm, and the computation error was estimated.

Results

The experiments suggested that successive dilution of homogenate produces disproportional changes in the yield of the final coloured product of iron and ascorbate-stimulated lipid peroxidation (Fig. 1A, B). The relationship between the quantity of detected TBARS and sample dilution appeared to be nonlinear irrespective of whether linear or logarithmic scale was used on abscissa (Fig. 2A). The yield of

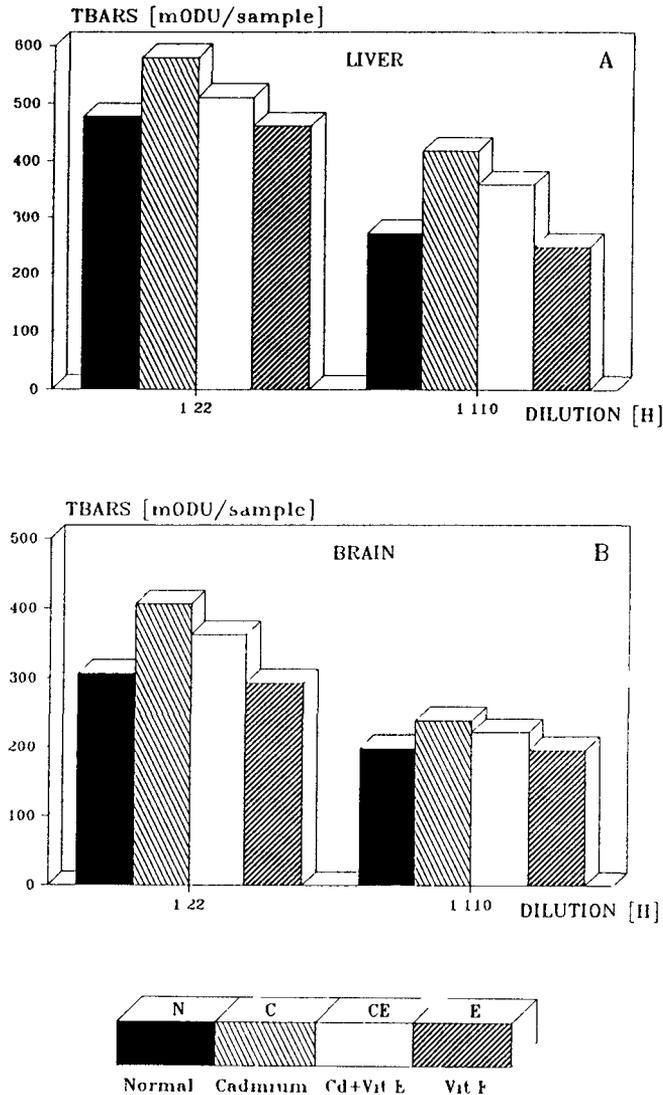


Figure 1. The effects of homogenate dilution on the yield of TBARS in samples of the liver and brain of rats treated orally for 3 months with cadmium acetate and vitamin E. **Abbreviations used in figures 1–8:** H – log H – the factor of dilution and its logarithm respectively; t [s] – time interval taken as the step of iteration in the computed models; [mODO] – one thousandth of the optical density unit; [Z] [Y] [X] – concentrations (in arbitrary relative units) of final product and intermediates of lipid peroxidation recalculated per weight unit of tissue; c U V_0 – parameters of the modelled kinetic equations (Fig 5); CAT GR GST – catalase glutathione reductase glutathione transferase respectively.

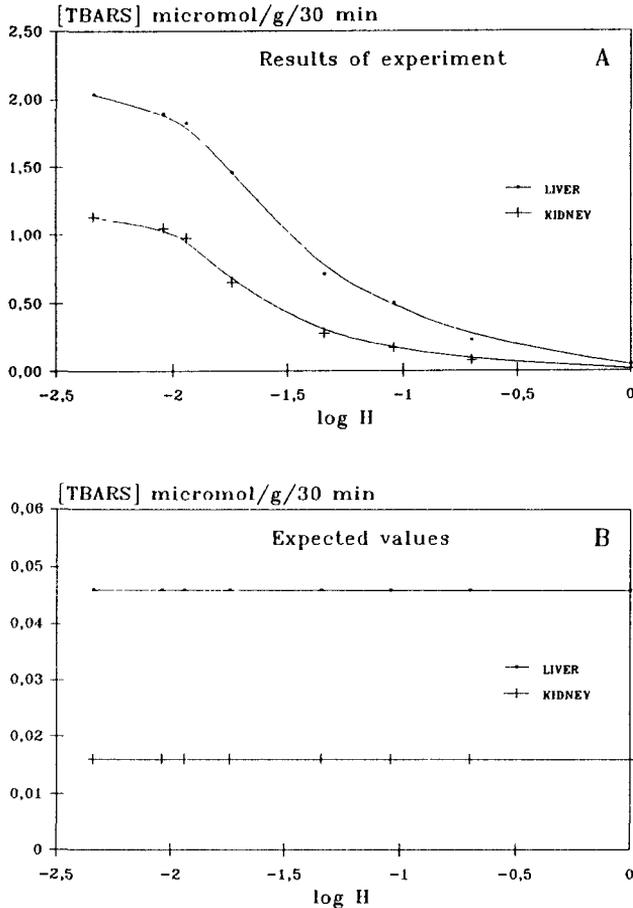


Figure 2. The yield of TBARS per weight unit of fresh rat liver and kidney in relation to homogenate dilution: the results of experiments (A) and values expected if the process of peroxidation were not related to the sample dilution (B).

TBARS reached a maximum value with the increasing dilution, and then decreased again. The effects of varying of ascorbate and TBA concentrations indicated that the detectability of TBARS and the accuracy of the method increased with more diluted homogenate and higher TBA concentrations (Fig. 3A, B). With 0.5% TBA, the coloration intensity decreased 2–3 times in comparison with 1% or 2% TBA, irrespective of sample dilution. Varying ascorbate concentration yielded insignificant results; however, when 2.5 times more concentrated ascorbate reagent was used, values slightly smaller were obtained for TBARS.

Nonstimulated, control lipid peroxidation was more than 40 times lower when

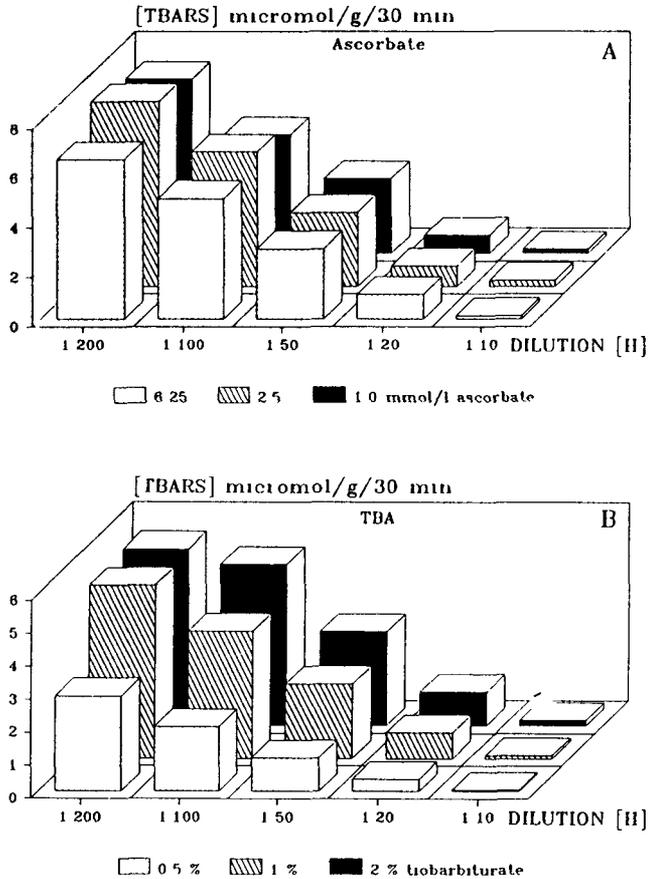


Figure 3. The effect of homogenate dilution and of variations in the concentrations of ascorbate (A) or thiobarbiturate (B) upon the yield of TBARS in rat liver homogenates

compared with the iron-ascorbate-stimulated process. However, in liver homogenate (1:40 w/v) 500 $\mu\text{mol Cd}^{2+}/\text{l}$ added to the system without stimulating agents produced a 6-7-fold increase of the TBARS yield, while no effect of cadmium was observed in iron-ascorbate-stimulated system (Fig. 4B). Similar, but less pronounced relationship was observed for brain homogenate. Cadmium added to liver homogenate (1:22 w/v) *in vitro* in concentrations ranging from 1.6 to 10 mmol/l partially inhibited the activity of catalase (CAT), glutathione S-transferase (GST), and there was a total inhibition of glutathione reductase (GR). Lipid peroxidation was enhanced by 30% with the lowest Cd addition only (1.6 mmol/l).

Discussion

A comparison of recalculated results of the *in vitro* experiment (Fig. 2A) with values expected if the yield of TBARS were independent of sample dilution (Fig. 2B) revealed that the process of lipid peroxidation cannot be interpreted in a simple way. In contrast to the release of volatile hydrocarbons or to the tissue content of MDA (as determined by the method of Ohkawa et al. 1979 or Uhiyama and Mihara 1978) the results reflect neither the content of lipid peroxides nor any particular enzyme activity. The yield of TBARS reached a maximum value with the increasing dilution with a subsequent decrease. The same relationship occurs in the results of Boehme et al. (1977).

Upon varying the concentrations of ascorbate and TBA the detectability of TBARS and the accuracy of the method were shown to increase when using the diluted homogenate and higher concentrations of TBA (Fig. 3A, B). These results seem to be particularly important with regard to the estimated content of lipids in tested samples, and indicate that some modifications of the method may result in shortage of some reagents in the test (lack of stoichiometry).

The addition of Cd^{2+} ions to the *in vitro* system containing Fe and ascorbate did not produce marked alterations of final TBARS yields (Fig. 4B), while in the system lacking Fe and ascorbate, addition of Cd^{2+} enhanced the yield of TBARS. Even if Cd^{2+} has been shown to stimulate *in vitro* lipid peroxidation (Manca et al. 1991), inhibition of lipid peroxidation was observed when up to 10 mmol/l Cd^{2+} were added. This suggests that the process of lipid peroxidation is inhibitable by cadmium in millimolar concentrations, similarly as numerous catalytic reactions (Sheabar and Yannai 1989) as well as the activities of enzymes present in the homogenate (Fig. 4A).

The detailed mechanism of *in vitro* lipid peroxidation has not been known as yet. It has been recognized however that the process involves nonenzymatic and/or enzymatic peroxidation of unsaturated fatty acids in cellular debris followed by hydrolysis of the peroxides to MDA (Aust et al. 1990; Draper and Hadley 1990; Esterbauer and Cheeseman 1990; Halliwell and Gutteridge 1990). The enzymatic generation of lipoperoxides requires reconstitution of the microsomal oxidative system based upon cytochrome P450-related enzymes, in the presence of reduced NADP. Nonenzymatic production of TBARS requires an addition of ferrous (Fe^{2+}) or ferric (Fe^{3+}) ions with reducing agents or chelators such as ascorbate, ADP or NADPH. Both steps, the generation of liperoxides and their decomposition, are considerably enhanced by ferrous ions and ascorbate, while the first step, peroxidation, is inhibited by catalytic amounts of butylated hydroxytoluene (Buckingham 1985; Draper and Hadley 1990; Esterbauer and Cheeseman 1990; Halliwell and Gutteridge 1990) The final reaction of the lipid peroxide decomposition should be preceded by hydrolysis of the ester bond between the peroxidized fatty acid and

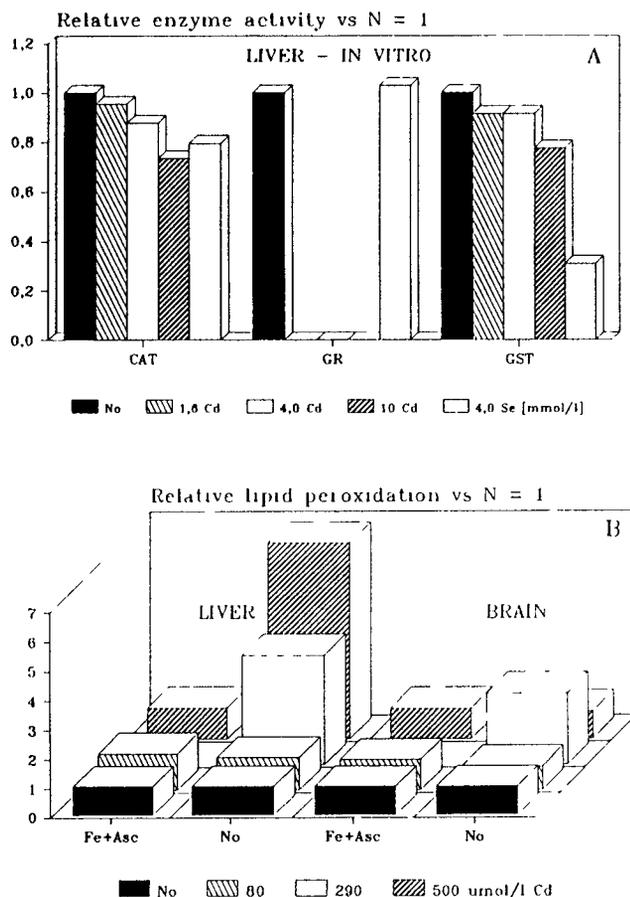


Figure 4. The effects of *in vitro* addition of cadmium or selenite upon the activities of endogenous enzymes (A) and the yield of TBARS (B) in a system with or without iron and ascorbate.

the core compound of the lipid (i.e. glycerol). The activity of phospholipase A2 may play an important role in this reaction (Kuijk et al. 1987 a,b; Yonaha et al. 1992.).

The process of lipid peroxidation is susceptible to thermal denaturation, which increases the yield of TBARS dramatically (Hussain et al. 1987; Shukla et al. 1987). At the same time chemical denaturation (TCA) or trypsin digestion cause TBARS to decrease to a marginal level (Boehme et al. 1977; Goran-Anneren and Epstein 1986; Hussain et al. 1987; Shukla et al. 1987). Similarly, there is no clear interpretation of the chelators action on TBARS generation, for the addition of EDTA (or deferoxamine) to the system inhibits lipoperoxidation. Some authors,

however, reported an increase of TBARS following the addition of EDTA to the system (Bird and Draper 1984).

The mathematical model of lipid peroxidation was based on the assumption that a sequential multistep reaction is involved in the process and that the rate of the process is limited by two critical rate-limiting steps. The product concentrations for the particular steps of a three-stage sequential reaction are given by the following integral equations, which correspond to the respective reaction steps (Substrate $S \rightarrow$ Intermediate $X \rightarrow$ Intermediate $Y \rightarrow$ Final product Z).

$$\begin{aligned} [X] = x &= f_x (s - x) = \int_0^t u \, dt; \\ [Y] = y &= f_y (x - y) = \int_0^t v \, dt; \\ [Z] = z &= f_z (y - z) = \int_0^t w \, dt \end{aligned}$$

Complete formulas presented in Fig. 5, correspond to second-order nonenzymatic catalytic lipid peroxidation mediated by iron and ascorbate ($S \rightarrow X$ step), followed by first-order enzymatic excision of peroxidized fatty acids ($X \rightarrow Y$ step) with final, second-order nonenzymatic, catalytic decomposition of the peroxides ($Y \rightarrow Z$ step), also mediated by iron and ascorbate.

In looking for an algebraic solution for the simplified sequential model, with a nonenzymatic, first-order reaction ($S \rightarrow X$) as the primary process and an one-substrate, enzymatic reaction ($X \rightarrow Y$) as the secondary process we obtained the following complex integral equation.

$$[K + s(1 - e^{-kt})]dy/dt - Vs(1 - e^{-kt}) + (V - dy/dt) \int_0^t (dy/dt) dy = 0$$

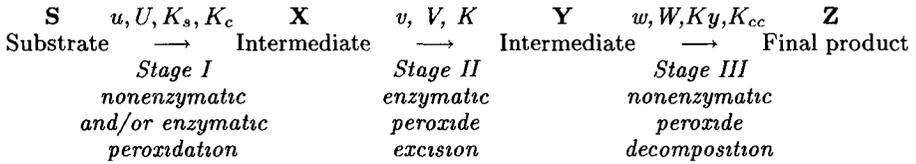
This equation has no algebraic solution, but satisfactory approximation may be obtained by substituting a finite sum by time instead of the unsolvable integer. Iteration has to be applied in this case assuming that the product quantity is a function of the reaction rate and time.

$$y_i = \sum (v_i \cdot \Delta t)$$

A curvilinear time dependence of the instantaneous reaction rate, which in turn is a function of the instantaneous substrate concentration, requires the introduction into the iterative equations of a concentration dependent rate member. A correction for a change in reaction rate during the period equivalent to the interval of iteration was also introduced. The method of the integer value estimation may be summarised in a set of general equations which should be substituted with exact

Figure 5. Postulated kinetics of lipid peroxidation

THE MODEL OF THE RATE LIMITING STAGES OF LIPOPEROXIDATION:



The rate equation for stage I nonenzymatic catalytic reaction of two substrates:

$$u = \frac{U(s_j - x)(c - x)}{(s_j - x)(c - x) + K_s(c - x) + K_c(s_j - x) + K'_s K_c}$$

$$x' = x - y$$

The rate equation of the stage II enzymatic excision of intermediate X:

$$v = \frac{V_j(x - y)}{K + (x - y)}$$

$$y' = y - z$$

The rate equation of the stage III catalytic decomposition of intermediate Y:

$$w = \frac{W(y - z)(c - z)}{(y - z)(c - z) + K_y(c - z) + K_{cc}(y - z) + K'_y K_{cc}}$$

where:

- s_j – initial concentration of substrate S (lipids) for stage I reaction: $s_j = s_0/h_j$
- h_j – homogenate dilution factor
- c – initial concentration of cosubstrate C (ascorbate) for stage I catalytic reaction
- U – rate constant of the catalytic reaction dependent on the concentration of the catalyst (Fe^{2+})
- K_s, K'_s, K_c – substrate constants for S & C
- u – reaction rate for stage I nonenzymatic catalytic process: $u = -ds/dt = dx/dt$
- x, x' – total production and instantaneous concentration of intermediate X, which has not been transformed in stage II
- y, y' – total production and instantaneous concentration of intermediate Y
- v – rate of stage II enzymatic reaction: $v = -dx/dt = dy/dt$
- V_j – rate constant of stage II enzymatic reaction dependent on the enzyme concentration/activity in the reaction environment; $V_j = V_0/h_j$
- K – substrate constant for stage II enzymatic reaction
- W – rate constant of the catalytic reaction dependent on the concentration of the catalyst (Fe^{2+})
- K_y, K'_y, K_{cc} – substrate constants for Y & C
- w – reaction rate for stage III nonenzymatic catalytic process: $w = -dy/dt = dz/dt$
- z – concentration of the final product Z

formulas (Fig. 5) to model the relationships of a second-order catalytic reaction and a one-substrate enzymatic reaction. Homogenate dilution/concentration (h_j) was included into these formulas. Upon increasing homogenate dilution the quantity of peroxidizable lipid in the sample decreases, as do the activities of endogenous enzymes; both can be expected to reduce the final yield of TBARS.

$$\begin{aligned}
 i &= 0, \dots, n; \quad \Delta t = T/n; \quad j = 1, \dots, m; \quad h_j = 5, \dots, 220; \\
 s_j &= s_0/h_j; \quad x_0 = 0; \quad y_0 = 0; \quad z_0 = 0 \\
 u'_i &= f_x(s_j - x_i); \quad \Delta x'_i = u'_i \Delta t; \\
 u_i &= f_x(s_j - x_i - 0.5 \Delta x'_i) = f_x(s_j - x_i - 0.5 \Delta t u'_i) = \\
 &= f_x[s_j - x_i - 0.5 \Delta t f_x(s_j - x_i)] \\
 \Delta x_i &= u_i \Delta t; \quad x_{i+1} = x_i + \Delta x_i \\
 v'_i &= f_y[h_j'(x_i - y_i)]; \quad \Delta y'_i = v'_i \Delta t; \\
 &\dots \text{ etc. for } y \text{ and for } z.
 \end{aligned}$$

where apostrophe indicates non corrected values; Δ stands for a change of the initial value; h_j is the factor of homogenate dilution, which affects the values of s_j and V_j .

The effect of the time interval (Δt), chosen as the step of iterative computing, was evaluated for such a model. The efficacy of the model was compared with that of a simpler model lacking the rate correcting members and computed for a two times smaller time interval than the corrected model. Moreover, the effect of a third reaction, intercalated between the two decisive rate limiting steps was tested.

As could have been expected, a shorter time interval (Δt) resulted in an improvement of the approximation obtained (Fig. 6). At the point of iteration corresponding to the reaction progress of 50% or more than 90%, for the satisfactory small step of iteration (Δt), a twofold decrease of Δt produced less than 0.1% increase of estimated product yield, while at the point corresponding to less than 10% progress the change did not exceed several percent. The computation error increased dramatically for small values of h_j (concentrated homogenate) and large values of time interval (Δt); the estimated concentration of product Z rapidly decreased for the smallest dilution ($H = 1:1$) when the time interval exceeded 2 seconds (Fig. 6A). However, the computation error of the model was marginal for factors of dilution between 5 and 220. Also, large differences in the values were observed between basal, non-corrected, and corrected equations for the dilution $H = 1:1$ (Fig. 6B). The values obtained using the corrected model were underestimated, while the non-corrected model provided overestimated values. The third reaction did not significantly influence the results of computations when its rate was 50 or more times greater than the rates of the two other reaction steps.

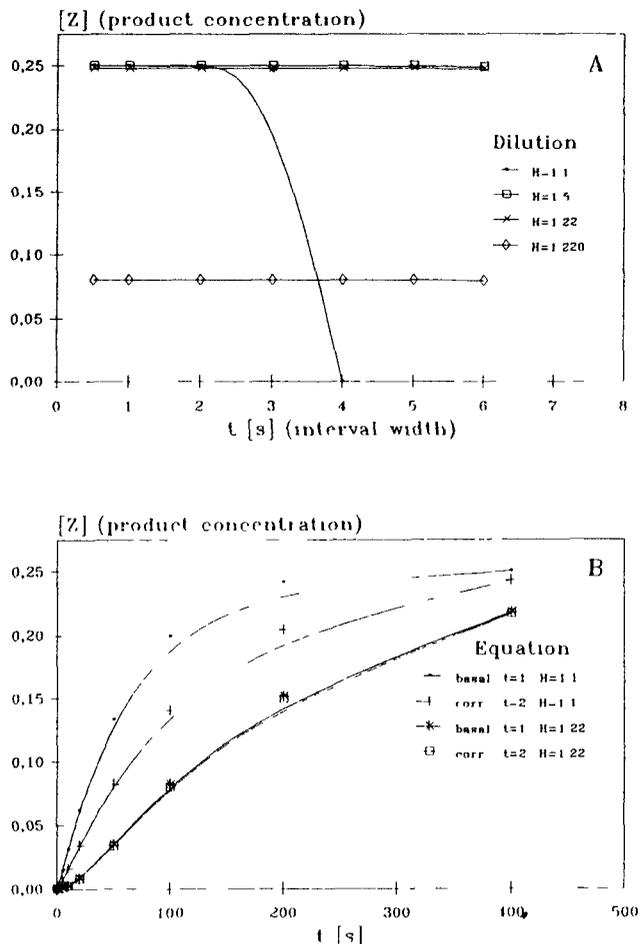


Figure 6. An assessment of the computation error in relation to the time interval (t) of iteration (A) and to the formula of iteration tested. basal – non-corrected or corrected (B); for details see the text.

At least two alternative models should be considered, since at present it is impossible to predict whether the nonenzymatic, rate limiting step precedes the enzymatic one, or the other way round. Each of the mathematical models tested provided a set of curves (Fig. 7, 8) which corresponded to those obtained experimentally (Fig. 2, 3). However, it is impossible to conclude at the present stage which of the two models is adequate, and to determine the values of empirical coefficients.

Nevertheless, the tested models allow to anticipate the effects of variations of

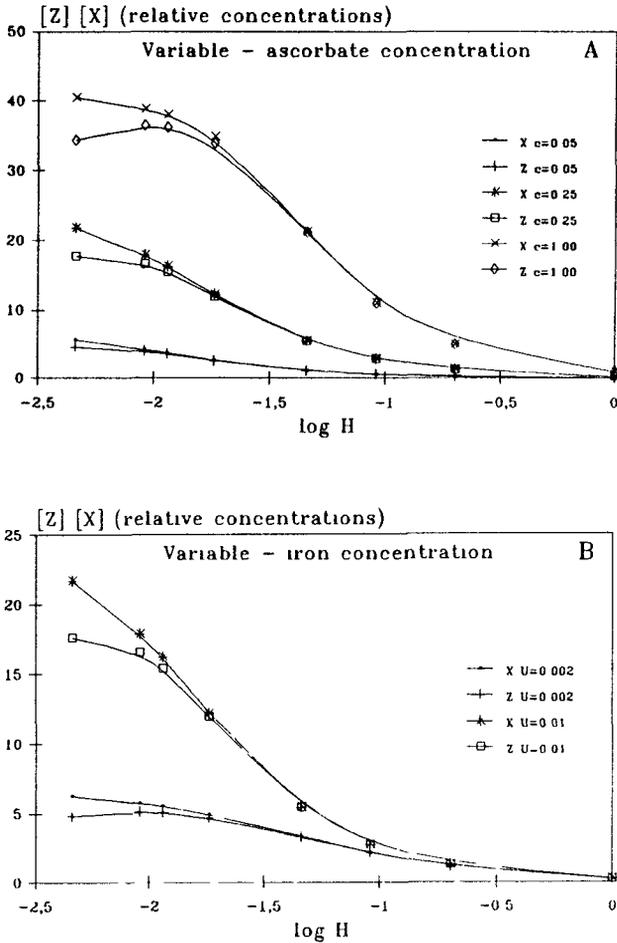


Figure 7. The modelled effects of variation in ascorbate (A) and iron (B) concentrations upon the yield of TBARS. An enzymatic reaction follows nonenzymatic catalysis.

the method and suggest that measurements of lipid peroxidation for experimental purposes should be assessed very carefully. The results of the present work point to the fact that the method reflects the enzymatic activity of the analyzed biological sample.

Conclusion

1. An analysis of the method used to estimate lipid peroxidation shows that this parameter characterises the enzymatic activity of the biological sample, and is

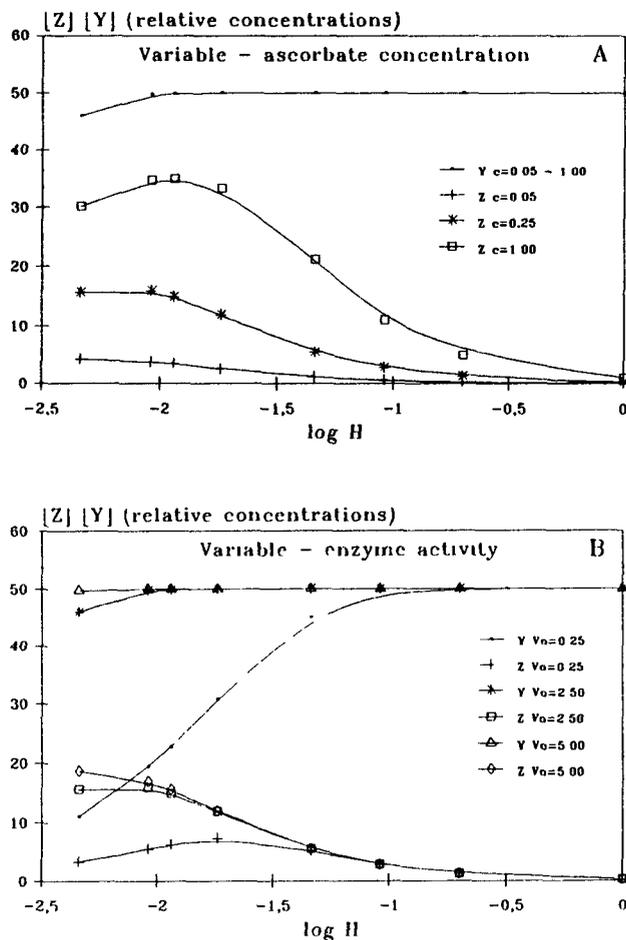


Figure 8. The modelled effects of ascorbate concentration (A) and endogenous enzyme activity (B) upon the yield of TBARS. Nonenzymatic catalysis follows enzymatic reaction.

limited by the composition of the reaction environment as well as by the rates of the accompanying nonenzymatic reactions.

2. The presented mathematical models imply that the process of lipid peroxidation can be interpreted as a sequential catalytic reaction with at least two limiting steps/reactions.

3. The approach to mathematical modelling presented in this work seems to be helpful in kinetic analyses and for predicting kinetic mechanisms of biological processes.

This paper was presented at the 2nd Symposium "Free Radicals in Biology and Medicine" in Łódź (Poland), May 30 – June 1, 1994.

Acknowledgements. The authors wish to express their gratitude to Professor Tadeusz Dłotko (University of Silesia) and MSc Renata Drobny for helpful advice on mathematical problems.

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Final version accepted February 2, 1995