FACTORS AFFECTING *IN VITRO* LIPID PEROXIDATION OF RAT BRAIN HOMOGENATE

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Summary: Peroxidation of the unsaturated lipid of tissue homogenates is an established method to assess the antioxidant or vitamin E status of animals. In the present study the spontaneous lipid peroxidation in air of rat brain homogenates is reported. The effects of various factors like pH, time, concentration of tissues, temperature, ferrocompounds and catalysis by added tissues like liver are described. Rat brain homogenates appear to be a suitable preparation for *in vitro* studies of lipid peroxidation.

Key words: rat brain factors affecting lipid peroxidation liver catalysis

INTRODUCTION

Peroxidation of lipids on incubation of certain tissues is a well-recognised occurence in various species of animals depleted of antioxidants (3, 10, 15, 17). The amount of malonic-dialdehyde produced as measured by thiobarbituric acid (TBA) assay has been shown to be a true indication of such endogenous lipid peroxidation (18). Normal rat tissues, however, do peroxise to a small extent (21); we have also observed that a low degree of peroxidation on incubation *in vitro*, is characteristic of tissues, of rats on a balanced diet; brain homogenates showing a markedly high degree of peroxidation (4). The present study is an attempt to define various factors affecting the *in vitro* lipid peroxidation of normal rat brain homogenates. No attempt has been made to express the peroxidation in terms of absolute amounts of malonicdialdehyde formed, and the results are expressed as TBA-index which is the extinction obtained at 535 *mu* in 4 *ml* total volume, using a spectronic 20 spectrophotometer.

MATERIALS AND METHODS

Albino rats of both sexes (body weight 120-150 *gm*) maintained on a commercial diet (Hindustan Lever & Co.) adequate in calories, vitamins and minerals, were sacrificed by heart puncture under light ether anaesthesia and the required tissues were excised out, blotted and stored in the deep freeze (-6 to -10°C). Lipid peroxidation assay, was done as described by Krishnamurthy and Bieri (10). Four to six such experiments were done for each individual peroxidation study and the average value was taken.

RESULTS

Lipid peroxidation as measured by TBA colour developed in brain homogenates, was compared with the amount of oxygen uptake, in aliquots of incubated samples by the iodimetric

method as described by Nakamura (11). Brain homogenate corresponding to 0.25 *gm* tissues after incubation at 37°C, for a given time interval, was mixed with 20 *ml* carbontetrachloride, 30 *ml* glacial acetic acid and 1 *ml* of saturated potassium iodide solution. After shaking well for 1 minute, 100 *ml* of distilled water was added and the free iodine liberated in the solution was titrated against 0.01N sodium thiosulphate solution. 1 *ml* of this thiosulphate solution corresponds to 0.00008 *gm* of oxygen. The results (Table I) show that the increase in TBA colour of incubated brain samples increased with the increase of oxygen up-take by the brain homogenates, however, no absolute co-relation between the two values, was present.

TABLE I : Comparison of TBA index and oxygen consumption during rat-brain lipid peroxidation.

Time mins.	*TBA index at* 535 mμ.	*Titer values* 0.01*N thiosulphate solution required (ml).*	*Corresponding oxygen consumed* (μg).
0	0.03	1.2	—
30	0.58±.03	2.4±0.04	96
60	0.89±.05	5.8±0.82	368

Pooled brain homogenates in acetate buffer, pH 5.0 used.
6 different experiments done and mean with S.D. given.

Effect of pH :

Several investigators (1, 21) have employed the physiological pH (7.4) as an arbitary and suitable hydrogen ion concentration for measuring lipid peroxidation in their studies on the function of vitamin E and other antioxidants. In the present study, rat brain homogenates of concentration 5% W/V prepared in the buffers of range 3.8 to 8.0 were incubated and lipid peroxidation determined. The mean of TBA index at the given time intervels of 6 different rat brain homogenates are given in Fig. 1. It is seen that lipid peroxidation was maximum at pH 5.0 and decreased on both sides of this pH.

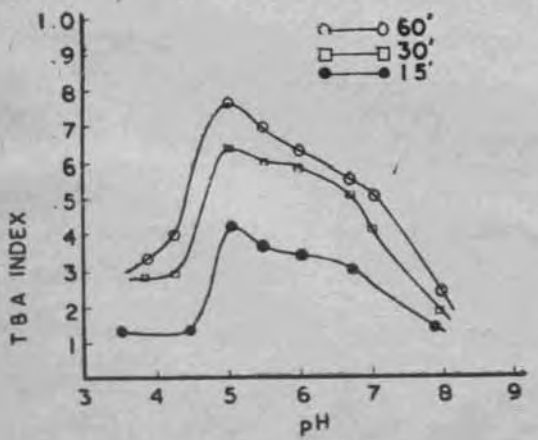

Fig. 1 : *Effect of pH on lipid peroxidation :*

5 *ml* brain homogenate, 5% W/V, in buffers of different pH, incubated at 37°C; at given time intervals. 1 *ml* aliquot added to 1.5 *ml*. 15% well-cooled TCA, and centrifuged, 2 *ml* supernatant added to 2 *ml* 0.67% TBA, kept in a boiling water bath for 10 minutes, cooled and the TBA-index measured.

Effect of concentration of the homogenates :

Several investigators (3, 17, 21) have used 5% W/V tissue homogenates in buffer as a suitable tissue concentration for studying lipid peroxidation, since at this concentration of the homogenates, there occured a proportionate increase in lipid peroxidation with time of incubation. However, one of the peculiarities of the study of lipid peroxidation of tissue homogenates is that the increase in concentration of the tissue homogenates does not always result in increase in lipid peroxidation. Okuma (13) and Bernheim (2) have demonstrated an inhibitory action, of lipid peroxidation by higher concentration of the tissues. It was therefore thought of interest to have a detailed study of the effect of tissue concentration on the rate of lipid peroxidation. Rat brain homogenates of varying strength from 2 to 10% (W/V) in buffer of pH 5.0 were prepared and 5 *ml* portions incubated and lipid peroxidation studied upto 90 minutes incubation and the results are presented in Fig. 2 (Mean of 5 experiments).

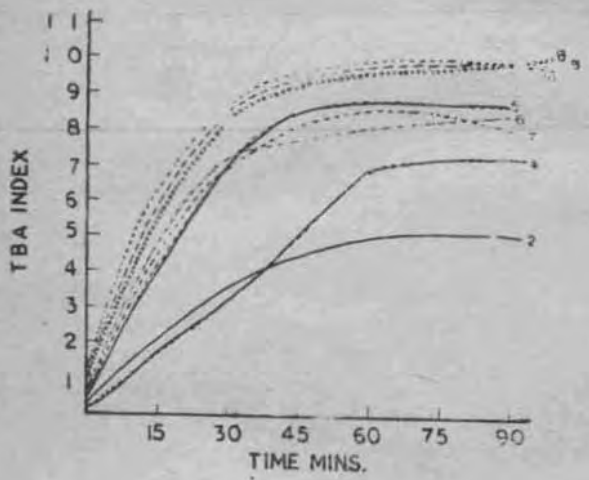

Fig. 2 : *Effect of concentration of the homogenates :* 5 *ml* portions of brain homogenates of varying strength from 2 to 10% W/V, in buffer of pH 5.0 were incubated and TBA-index measured. The numerals in the figure indicate the percentage concentration.

It may be seen that peroxidation at all the time intervels studied showed a linear increase and proportionality with concentration from 2 to 5% W/V, while above 5% W/V there was no such proportionate increase in the amount of malonic dialdehyde formed.

Effect of incubation time and temperature :

The results of incubation of the rat brain homogenate 5% (W/V) at two different temperatures and upto a period of 240 minutes incubation is given in Fig. 3. It may be seen that there was a steep increase in the degree of peroxidation upto 60 minutes at 37°C after which there was little increase in the TBA formed. Incubation at room temperature (24°C) however showed that the peroxidation increased proportionately upto 120 minutes and remained steady later. It is interesting to note that the maximum TBA index at 37°C was obtained after 60 minutes while only after 120 minutes at room temperature.

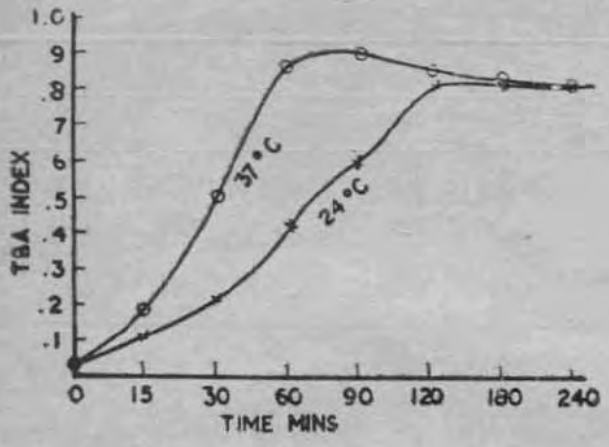

Fig. 3 : *Effect of incubation time and temperature :* 5% (W/V) rat brain homogenate in buffer of pH 5.0 incubated at 37°C and at room temperature (24°C) upto 240 minutes and TBA-index at the given time intervals are shown.

Lipid peroxidation of the various tissues of rat :

In Fig. 4 is given the TBA index obtained by incubation at 37°C of brain, liver, kidney, spleen and heart homogenates; brain showed the greatest degree of peroxidation followed by liver, kidney, spleen and heart in that order. Plasma showed no lipid peroxidation (results not given in the Fig. 4) confirming the earlier reports of Barber (1).

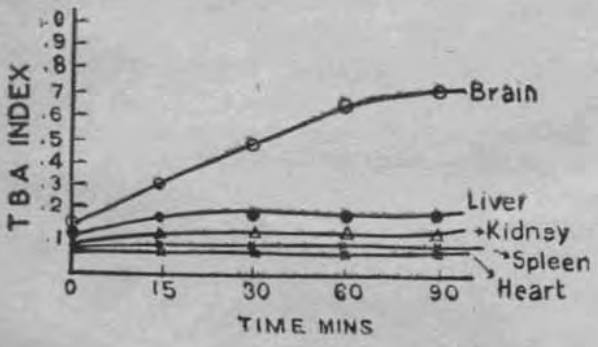

Fig. 4 : *Lipid peroxidation of the various tissues of rat :* Rat brain, liver, kidney, spleen and heart, 5% W/V, in buffer pH 5.0, incubated at 37°C, and TBA-index at different time intervals of the tissues are given.

Effect of heating the brain homogenate and catalysis by liver :

Rat brain suspensions have been reported to undergo lipid peroxidation even after heating in boiling water bath for 5 minutes (12, 14).

Rat brain homogenates (5% W/V) prepared in buffer of pH 5.0 in 50 *ml* Erlenmeyer flasks were kept in a boiling water bath for 15 minutes, one sample under a stream of nitrogen, and another sample in air; cooled, made upto the original volume with the buffer and incubated as described earlier and lipid peroxidation was measured (Table II).

TABLE II : Effect of heating the homogenate on the lipid peroxidation of rat brain.

Treatment	TBA — index (extinction) *Time in minutes* 37°C				
	0′	15′	30′	60′	90′
Brain homogenate, 5% W/V	0.09	0.37	0.70	0.89	0.90
Brain homogenate kept in boiling water bath for 15 minutes under a stream of Nitrogen	0.10	0.31	0.52	0.60	0.61
Brain homogenate heated without N_2.	0.12	0.29	0.53	0.61	0.61

Pooled brain homogenate used and 6 different experiments done.
Mean values given.

A decrease of about 30% in lipid peroxidation value on boiling the homogenate was observed. No difference was observed in the rate and extent of peroxidation when the homogenates were boiled in air or under the stream of nitrogen. It however, may be pointed out that the zero time values for the boiled homogenates were slightly higher compared to the control homogenates.

With the *in vitro* systems of linoleic or linolenic acid emulsions, Wills (21) has studied the effect of catalysis of peroxidation by liver homogenates, to be affected by the concentration of the tissues used and the pH of the reaction medium. Rat liver homogenates of various tissue concentrations (W/V, Wet Weight) were prepared in acetate buffer pH 5.0 and were added to the brain homogenate before the incubation. In separate experiments heated liver homogenate (by keeping in a boiling water bath for 15 minutes) were also used as additives. Both the boiled and unboiled liver preparations at concentration range from 0.2 to 5% W/V, and at pH 5.0, stimulated the brain peroxidation (Table-III). This stimulation, however, was apparent only upto 30 minutes incubation. When the catalysis was done by using 7% liver homogenate at the same pH, there was inhibition instead of stimulation. In another study acetone powder prepared from rat liver, suspended in buffer, pH 5.0, was used instead of the liver homogenate for catalysis and gave the same pattern of results, when expressed on equivalent tissue wet mass (results not shown).

Catalytic effect of hematin compounds :

In Fig. 5 the potentiating effect of haemoglobin, cytochrome "c" (horse) and ferrous sulphate on brain homogenate lipid peroxidation is shown. It may be seen that haemoglobin, cytochrome C, at 2 x 10^{-5}M, stimulated the peroxidation, although, haemoglobin is a better catalyst than cytochrome C. In the case of inorganic iron, the zero time values of the sample gave an extinction of 0.65, as compared to a value of 0.1 in the control and also in the samples where cytochrome C and haemoglobin were used. Substracting this initial values from the higher values obtained at the different times of incubation it would show that ferrous ion is least effective as a catalyst for *in vitro* peroxidation of brain homogenate. It may be pointed out that ferrous ion by itself, has been reported to markedly potentiate the intensity of the TBA, malonic dialdehyde complex, at 535 $m\mu$ (22).

TABLE III: Brain peroxidation and liver catalysis.

Experiments		*TBA — index. Time in minutes*			
		0′	15′	30′	60′
Brain homogenate		0.09	0.37	0.70	0.89
Brain homogenate + 0.2% liver (W/V)	unboiled	0.14	0.49	0.88	0.85
	boiled	0.18	0.51	0.87	0.83
Brain homogenate + 1.0% liver (W/V)	unboiled	0.12	0.65	0.95	0.90
	boiled	0.20	0.63	0.94	0.88
Brain homogenate + 3% liver (W/V)	unboiled	0.15	0.69	0.98	0.93
	boiled	0.20	0.70	0.99	0.90
Brain homogenate + 5.0% liver (W/V)	unboiled	0.12	0.75	1.2	0.95
	boiled	0.22	0.78	1.0	0.93
Brain homogenate + 7.0% liver (W/V)	unboiled	0.18	0.30	0.45	0.43
	boiled	0.23	0.33	0.43	0.42

1 *ml* of liver homogenates, containing required tissue (wet weight) in acetate buffer, pH 5.0 (both boiled and unboiled, as described in text) were added to 5 *ml* of 5% W/V brain (pooled) homogenates, incubated, and aliquots at given time intervals were used for estimating the TBA index. Mean of 5 different expts. given.

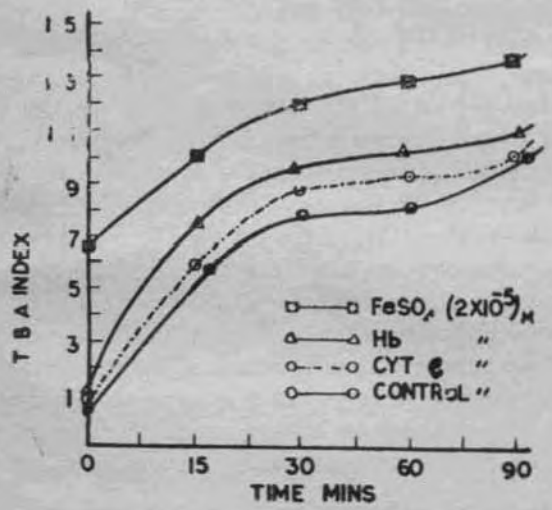

Fig. 5 : *Catalytic effect of haematin compounds :*

5% brain homogenate in acetate buffer of pH 5.0, incubated each with ferrous sulphate, haemoglobin and cytochrome C, at a concentration of (2×10^{-5}M) and TBA-index obtained at various time intervals are given.

DISCUSSION

Lipid peroxidation by incubated tissue homogenates, as well as by unsaturated fatty acids or fat emulsions in the presence of a suitable catalyst, have been extensively studied in relation to vitamin E and selinium function (5, 20). More recently free radical damage of cellular (6), mitochondrial, microsomal or lysosomal membranes by lipid peroxidation have also been studied in various experimental conditions like radiation (8, 19), carbontetrachloride (16) or ethanol insult (7). There are two kinds of lipid peroxidation in tissues, one spontaneous and non-enzymic (9) and the other catalysed by Nicotinic acid Adenine dinucleotide (reduced) and localised in the microsomal part of the cells (16). Although direct uptake of oxygen will give a true nature of lipid peroxidation, the more commonly employed technique is by estimating the production of malonic dialdehyde, (one of the end products of unsaturated fat peroxidation) by the pink pigment formed with 2-thiobarbituric acid. Various investigators have used different systems for such study. Purified polyenoic acids as model systems were used by Tappel (20), and Wills (21) while isolated mitochondria or microsomes from liver homogenates were used by Slater and Swayer (16); Robinson (14) and Barber (1) have used normal rat brain homogenates as the test system in their studies on lipid peroxidation. However the use of various systems have only tended to give a confusing idea of the mechanism of lipid peroxidation since the conditions for optimum lipid peroxidation vary with the source or nature of the fatty acids, the catalyst and the aeration of the samples. The TBA pigment estimation at 535 $m\mu$ is itself reported to be intensified in the presence of ferrous ions (22). In the present study we have attempted an evaluation of the TBA method with that of the corresponding oxygen up-take during the peroxidation, by iodometry. Our results show that although the TBA colour of incubated samples increased with the increase of oxygen up-take by the brain homogenates, there is no algebraic correlation with the oxygen consumption and the TBA pigment formation during the incubation, indicating that the TBA method of measuring the extent of lipid peroxidation in tissue homogenates, is a qualitatively reliable indicator, but may not be considered as a true reflection of the amount of oxygen consumption. Of the different tissues from normal rat, the brain showed a considerably high degree of peroxidation, while liver, kidney, spleen and heart homogenates showed comparatively low peroxidation. The malonaldehyde production by brain homogenates was found to be both pH and tissue concentration dependent, the maximum peroxidation was obtained at pH 5.0 and the peroxidation increased with tissue concentration from 2 to 5% W/V, but above this, the malonaldehyde production was not proportional to the increase in tissue concentration. This may be due to the increased viscosity of proteins at these concentrations as was suggested by Robinson (14). Brain homogenate were found to peroxidise linearly with time at 37°C upto 60 minutes, after which there was no further increase in malonaldehyde production. Interestingly, on incubation at room temperature there was progressive peroxidation even upto 120 minutes. However, the amount of malonaldehyde production at this period was of the same value as obtained for 60 minutes at 37°C, indicating an equilibrium with the maximum production of malonaldehyde for a given sample irrespective of the temperature of incubation. Boiled homogenates showed approximately 30% decrease of peroxidation. This may be due to the destruction of the haemoprotein catalysts during heating. The bulk of the peroxidation system of rat brain, however, is non-enzymatic since it is unaffected by protein denaturation.

Among the catalysts tested haemoglobin appeared to be markedly superior than cyto-

chrome C, or ferrous sulphate. The effect of added other tissue homogenates, in low concentrations to a model system consisting of linoleic acid emulsions was studied by Wills (21), who has reported that heart, liver, spleen and kidney homogenates at (0.5% W/V) actively catalyse the peroxidation at acidic pH 5.0. In the present study it is found that both boiled and unboiled liver homogenate, and the fat-free acetone powder did actively catalyse the peroxidation of brain homogenates when added tissue concentration were upto 5% W/V at pH 5.0. Higher concentrations of the added tissues however, inhibited peroxidation. Wills (21) has reported that in acidic pH, liver tissue concentrations upto 4% W/V, gives a proportionate increase in catalysis, however, in our study it may be seen that concentrations above 5% W/V, at pH 5.0 decreased the catalysis, similar to the inhibition noted by Wills (21), in alkaline medium for concentrations of 1% and above.

Our studies indicate that rat brain homogenates (5% W/V concentration at pH 5.0) may be readily adopted as a convenient model system for investigations of *in vitro* lipid peroxidation, in place of unsaturated fatty acid emulsions which usually produce turbidity in the final malonaldehyde-TBA colour production and which also require added catalyst like haemocompounds. The brain lipid peroxidation as studied here is non-enzymatic and represents the endogenous peroxidation uncomplicated by the NADH-dependent enzymic peroxidation, of liver which according to Slater and Swayer (16) is the one stimulated by hepato-toxins like carbontetrachloride. The brain homogenate has apparently the necessary unsaturated fatty acids and the catalyst for peroxidations in the architecture of the cell itself which are readily available for reaction with molecular oxygen to undergo lipid peroxidation.

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