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Estimation of Product of Lipid Peroxidation (Malonyl Dialdehyde) in Biochemical Systems[1]

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The oxidative degradation of unsaturated fatty acids can be followed by determining the amount of a product of lipid peroxidation or by determining the total consumption of oxygen.

Kohn and Liversedge (1) described the colorimetric reaction with 2-thiobarbituric acid (TBA) of an unknown substance formed during the aerobic incubation of tissue homogenates; this substance was later identified by Bernheim, *et al.* (2) as a three-carbon oxidation product of fats. Patton *et al.* (3) recognized this as malonyl dialdehyde (MDA), a secondary product of lipid peroxidation. The reaction mechanism by which MDA is derived from various unsaturated fatty acids has been described by Dahle *et al.* (4).

As the test for MDA is usually carried out, the heat-induced reaction of 1 mole of MDA with 2 moles of TBA in an acid solution forms a trimethine colored substance (5, 6). However, when the reaction is carried out with tissue homogenates (subcellular particles) or with emulsions of polyunsaturated fatty acids as substrates, interference due to turbidity from protein precipitation and due to absorption of color on the precipitate formed in acid solution (and in the case of lipid emulsions the formation of a milky turbidity) all disturb the photometric measurement. To avoid this interference, filtration or centrifugation is required, which complicates the procedures and also, removes absorbed color from the solution. A modified method employing alkaline pyridine-butanol is proposed that simplifies the procedure and eliminates these problems by producing clear solutions suitable for direct photometric measurement.

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MATERIALS AND METHODS

Tissue Homogenates. Rat tissue homogenates were prepared in a ratio of 1 gm of wet tissue to 19 ml 0.9% NaCl; the supernatant was used after 5 min centrifugation at 400 × *g*.

Mitochondria and microsomes were prepared in the following manner: Tissues were homogenized in 9 vol of 0.16 *M* KCl and centrifuged at 700 × *g* for 10 min, and the supernatant was centrifuged at 5000 × *g* for 10 min. The sediment was resuspended and recentrifuged at 16,000 × *g* for 10 min to yield mitochondria. The 5000 × *g* supernatant was centrifuged at 16,000 × *g* and the supernatant obtained was centrifuged at 105,000 × *g* for 30 min. The sediment obtained was resuspended and recentrifuged at 105,000 × *g* for another 30 min to yield microsomes which were suspended.

MDA. MDA was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (K & K Laboratories, Inc., Plainview, New York, lot No. 33926) with 0.1 *N* HCl (7).

TBA Reagent. An 0.8% TBA solution was prepared by dissolving 2-thiobarbituric acid (TBA) (Sigma Chemical Co., St. Louis, Mo.) in a small amount of NaOH, then neutralized with 7% perchloric acid. The TBA reagent was prepared by mixing 2 vol of this TBA solution with 1 vol of 7.0% perchloric acid.

Procedure. 1.5 ml of solution (containing 0.1–0.2 ml of homogenate, or mitochondrial or microsomal suspension, or emulsion of unsaturated fatty acids, plus inhibitors or activators, all in a 0.2 *M* tris-maleate buffer of pH 5.9 or 0.2 *M* tris-0.16 *M* KCl buffer of pH 7.4, e.g. 0.1 ml of emulsion of 10 m*M* polyunsaturated fatty acid + 0.1 ml of activator or inhibitor + 1.3 ml of buffer) were incubated 30 min at 37°, after which 1.5 ml of TBA reagent was added, the mixture was heated in a boiling water bath for 10 min using a marble as a condenser. After cooling, 3.0 ml pyridine/*n*-butanol (3/1, v/v) and 1.0 ml 1 *N* NaOH were added and mixed by shaking or by aeration. The photometric measurement was carried out at λ_{max} = 548 mμ. A nonincubated blank was used.

Measurements were made on the DB spectrophotometer (Beckman Instruments, Fullerton, Calif.).

Time and temperature of incubation can be changed depending upon the activity of reactants. It is recommended that test tubes be prepared with 1.5 ml of TBA reagent to which are added samples of the incubated mixture; the test tubes are then all placed in the boiling water bath. The final volume of sample is 7 ml; however, the ratio of volumes used can be reduced proportionately to produce a total volume of, e.g., 4.7 ml (1.0 ml of incubate +1.0 ml TBA reagent +2.0 ml pyridine-butanol mixture +0.7 *N* NaOH), etc.

RESULTS

The calibration curve (absorbance) is linear up to a concentration of 30 nanomoles MDA/1.5 ml, i.e., 2.16×10^{-6} gm MDA/1.5 ml or incubated solution (10^{-9} mole = 1 nanomole). Sensitivity of the estimation was found to be 0.1×10^{-9} mole MDA/1.5 ml of incubated solution, i.e., 7.2×10^{-9} gm MDA/1.5 ml.

TABLE 1

MAXIMAL ABSORBANCY (λ_{max}) AND MOLECULAR EXTINCTION COEFFICIENT (ϵ) OF TRIMETHINE COLOR SUBSTANCE

Medium	λ_{max}, $m\mu$	ϵ
Acid[a]	531	1.52×10^5
Pyridine-butanol[b]	536	1.51×10^5
Pyridine-butanol + NaOH[c]	548	1.52×10^5

[a] Estimation made only with 1.5 ml TBA-reagent. *Note:* Maximal absorbancy is at the same wavelength (531 mμ) using trichloroacetic acid instead of perchloric acid.

[b] Estimation made with 1.5 ml TBA reagent +4.0 ml pyridine-butanol mixture.

[c] Described procedure (1.5 ml TBA reagent + 3.0 ml pyridine-butanol + 1.0 ml 1 *N* NaOH).

Table 1 correlates maxima of absorbancy and molar extinction coefficients of the resulting color in various solutions. The ultraviolet region has MDA $\lambda_{max} = 263$ mμ at pH 4.8, $\epsilon = 0.21 \times 10^5$ [compare (7)].

The amount of MDA present in a sample (i.e., amount of MDA in 1.5 ml of incubated solution) is estimated according to the equation:

$$\text{nanomoles MDA} = \frac{V \times OD_{548}}{0.152} = \frac{7 \times OD_{548}}{0.152} = 46 \times OD_{548}$$

where V = final volume of the test solution, OD_{548} = optical density at 548 mμ, and $\epsilon = 1.52 \times 10^5$.

Estimated with MDA solutions, the standard deviation;

$$S = \sqrt{\Sigma x^2/n - \bar{x}^2}$$

(S = standard deviation, x = optical density, $\bar{x}$ = arithmetical mean of determinations), of each determination for different concentrations was not more than $\pm 2\%$ of the means.

The incubated mixture can contain up to about 20 mg of protein and/or 15 mg of lipids (in 1.5 ml) and still yield clear solutions after the described procedure.

The color is stable for 5 to 10 min after addition of alkali (after 5 min 99 to 100% of the developed color remains and after 10 min 98 to 99%)

and then begins to fade. After carrying out the reaction with TBA reagent but before the addition of alkali, the color is stable for at least 7 hr after cooling. The pyridine-butanol mixture and sodium hydroxide can thus be added later, making it possible to measure a group of incubates together.

TABLE 2

DIFFERENCE BETWEEN ESTIMATION OF MDA WITHOUT FILTRATION AND WITH FILTRATION

System	Tissue	Volume, ml	Estimatiun (10^{-9} mole MDA)[a]		Difference, % (100% = estimation without filtration)
			Without filtration	With filtration	
1. Homogenate	Liver	0.00	0.0	0.0	±0
		0.05	4.9	4.5	−7
		0.10	10.6	9.0	−15
		0.20	20.8	16.2	−22
		0.30	23.6	19.5	−18
		0.40	26.3	20.5	−22
		0.50	25.0	20.2	−19
	Testis	0.05	2.2	2.3	+2
		0.10	4.9	4.0	−18
		0.20	9.8	7.7	−21
	Kidney	0.1	5.5	4.7	−15
2. MDA + serum[b]		0.00	14.6	14.6	±0
		0.05	14.6	14.0	−4
		0.10	14.7	13.9	−6
		0.15	14.7	13.6	−7

[a] Incubation at pH 5.9; ratios of reaction system when estimation with filtration was used were the same compared with those described for procedure without filtration. Milligrams of protein in 0.1 ml of homogenates: liver 4.5, testis 4.1, kidney 4.0.

[b] 1.5 ml of incubated mixture contained 14.6×10^{-9} mole MDA + given amount of rat serum (5.3 mg protein in 0.1 ml).

In Table 2 are given the results obtained by this procedure compared to the acid procedure combined with filtration (this step is needed to remove cloudiness). Results in which centrifugation instead of filtration was used were the same.

DISCUSSION

The detailed mechanism of lipid peroxidation in biological systems is not clear. However, considerable information has been obtained, with the aid of the MDA estimation, about the biochemical oxidation of unsaturated fatty acids (8–14). These studies have included the influence of pH (12) and of serum inhibitor (14), lipid peroxidation in microsomes (10, 11), studies on the TBA reaction, etc.

The proposed method of MDA estimation using an alkaline solution simplifies the procedure, and at the same time yields better values, since no MDA or trimethine colored compounds will be absorbed by precipitated proteins or filter paper. While the color obtained in alkali has its maximum at 548 mμ rather than the 532 mμ maximum found in acid solution, the molecular extinction coefficient is essentially the same. This value is 1.52×10^5 in alkali at 548 mμ; Sinhuber and Yu (15) found molecular absorbancy = 1.56×10^5 at 535 mμ for a similar estimation in acid solution and Sawicki *et al.* (16) found $\epsilon = 1.58 \times 10^5$ at 530 mμ and $\epsilon = 1.53 \times 10^5$ at 532 mμ.

TABLE 3
ACTIVITY OF LIPID PEROXIDATION/10^{-9} MOLE MDA IN MITOCHONDRIA AND MICROSOMES

System[a]	Mitochondria	Microsomes
1. [a]	0.1(1.3)[b]	0.1(2.6)
2. Glutathione reduced 3 *mM*	2.5(9.2)	3.6(14.4)
3. Glutathione reduced 3 *mM* + amytal 7 *mM*	14.1	16.8
4. NADPH 0.6 *mM*	2.1(3.7)	3.8(3.2)
5. NADPH 0.6 *mM* + amytal 7 *mM*	0.3	0.5
6. Fe^{2+} 0.02 *mM*	9.1(9.4)	12.2(11.9)
7. Ascorbic acid 0.07 *mM*	11.3(12.4)	11.1(11.2)

[a] Systems contain 0.1 ml of rat liver mitochondria or microsomes (500–800 μg protein) + 0.1 ml of activator or inhibitor (given in final concentration in incubated solutions) + 1.3 ml of buffer pH 7.4 (tris-KCl).

[b] Results in parentheses are for aged fractions (48 hr at 0–5°C).

Table 3 gives representative data on lipid peroxidation in liver mitochondria and microsomes under various conditions as estimated by the described procedure.

Potentially the estimation could be interfered with by certain organic compounds that produce a pink color (e.g., 2-aminopyrimidine and other compounds which, after oxidation, produce MDA, e.g., desoxysugars) and by compounds that give a yellow to light brown color during the reaction; however, the latter compounds are paler in alkali than in acid (17, 18) while the former do not normally occur in biological systems. In biochemical estimations, the interference caused by high concentrations (*mM*) of NADPH, ATP, etc., is corrected for by proper blanks. Such blanks also account for possible autoxidation, which could occur during the boiling of incubated samples in acid solution.

Under the conditions used, the only pink colored substance found was the one due to formation of MDA complex, identified as such in all cases by paper or thin-layer chromatography.

SUMMARY

A new thiobarbituric acid method for the estimation of the malonyl dialdehyde produced as a result of lipid peroxidation is described in which the trimethine colored substance is estimated in alkaline solution.

Alkaline pyridine-butanol mixture dissolves both proteins and lipids that are precipitated or opalescent during the reaction, yielding a more accurate MDA estimation as well as simplifying the procedure.

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