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Micro-determination of Lipoperoxide in the Mouse Myocardium by Thiobarbituric Acid Fluorophotometry¹⁾

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The analytical conditions or fluorometric assay of lipoperoxide in the myocardium of mice were investigated in detail. It was found that the reaction of lipoperoxide with thiobarbituric acid (TBA) depended on the acidity of the reaction medium, not on the kind of acid used. The best result was obtained when 2.0 M acetate buffer (pH 3.6) solution was used. In extraction of the fluorescent substance from the reaction medium after the reaction of lipoperoxide with TBA, the addition of one-fifteenth volume of pyridine to *n*-butanol was suitable to remove the turbidity of the reaction medium. Calibration plots gave a good linear relationship in the range of 0.1–1.0 nmol of malondialdehyde. The TBA aqueous solution was found to decompose and therefore we used the solution within 2 weeks after preparation.

This method was used to assay lipoperoxide in the myocardium of mice which had been treated with adriamycin (ADR). Lipoperoxide level in the myocardium was increased from 250.7 nmol/g wet tissue to 674.5 nmol/g wet tissue on the 4th day after ADR administration.

Keywords—fluorophotometry; malondialdehyde; lipoperoxide; thiobarbituric acid; adriamycin; acetate buffer; myocardium

There are several methods to determine lipoperoxide level in organisms. The most widely accepted standard method is Yagi *et al.*'s method,^{2,3)} which is based on the reaction of malondialdehyde (MDA), generated from lipoperoxide, and 2-thiobarbituric acid (TBA). Various modified methods^{4,5)} are known in which the sort of acid in the reaction medium is different.

Recently, Myers *et al.*⁶⁾ reported that an increase of lipoperoxide in the myocardium of mice was caused by adriamycin (ADR), an antitumor antibiotic. However, they used a modified method with trichloroacetic acid, which might be unsuitable because of the influence of biological substances, *e.g.* sialic acid. Therefore, we sought to reexamine the increment of lipoperoxide in the myocardium of ADR-treated mice by Yagi *et al.*'s method with acetic acid.

Fluorometry was recommended by Yagi *et al.* for the microdetermination of lipoperoxide, but optimum conditions for fluorometry have not been discussed in detail. Furthermore, the determination of myocardial lipoperoxide in mice by using fluorometry has not been reported. We first investigated optimum conditions for fluorometry and then applied the method to assay lipoperoxide in the myocardium of ADR-treated mice.

Experimental

Apparatus—Fluorescence spectra and intensities were measured with a Hitachi 204 fluorescence spectrophotometer.

Preparation of Mouse Heart Homogenate—Male CDF₁ mice (6 weeks old, weighing 20–25 g) were killed by cervical dislocation and the heart was removed promptly to determine the wet weight. Then, a 1.0% homogenate solution in physiological saline was prepared in a Potter-type Teflon homogenizer.

Reagents—Malondialdehyde (1,1,3,3-tetramethoxypropane) and 2-thiobarbituric acid were obtained from Tokyo Kasei Kogyo Co., Ltd. Acetic acid and sodium acetate were obtained from Wako Pure Chemical Industries, Ltd.

Malondialdehyde standard solution was prepared by accurate 1/10 dilution of 50.0 μ M malondialdehyde solution. A buffer solution of 2.0 M acetate was prepared from 2.0 M sodium acetate solution and 2.0 M acetic

acid solution. A solution of 0.8% TBA was prepared by adding water to 0.800 g of 2-thiobarbituric acid and heating it until it dissolved. The final volume was made up to 100 ml. The other reagents used in this study were of the highest purity available.

Assay Procedure—Add accurately 0.1—1.0 nmol of MDA standard solution or 1.0% homogenate solution of mice myocardium, 0.5 ml of 3.0% sodium dodecyl sulfate (SDS) solution and 1.5 ml of 2.0 M acetate buffer (pH 3.6) solution into each tube with stirring. After sufficient mixing, add 1.5 ml of TBA solution and make the total volume 4.0 ml with distilled water. Then, heat the mixture at 95°C for one hour in an oil bath. Chill the mixture for five minutes in tap water, add 1.0 ml of 0.2 N HCl and 5.0 ml of *n*-butanol-pyridine (15:1) mixture, and then shake the whole vigorously and transfer the *n*-butanol layer into a cell. Measure the fluorescence intensity at excitation and emission wavelengths of 515 and 553 nm. Prepare a blank solution similarly but without any sample. The procedure is summarized in Chart 1.

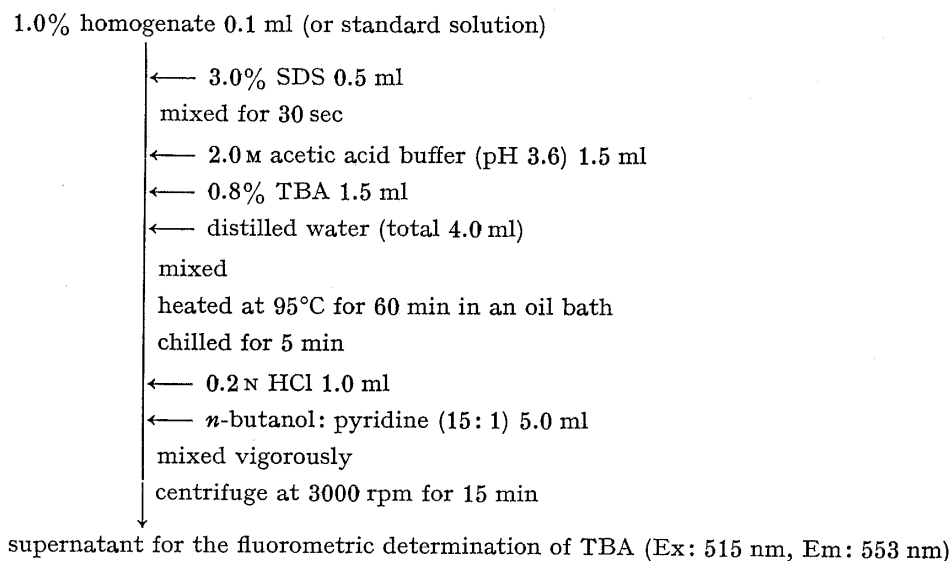


Chart 1. Analytical Method for Lipoperoxide in Mouse Heart

Results and Discussion

Wavelength for Determination

Fig. 1 shows the fluorospectrum of the reaction product of MDA and TBA in *n*-butanol-pyridine (15:1) mixture.

As pointed out by Yagi *et al.*,²⁾ the excitation maximum at 532 nm is linked to the emission maximum at 553 nm. The excitation wavelength of 515 nm was used in this study, as it was by Yagi *et al.*,²⁾ because excitation at 532 nm might affect the measurement of fluorescence. Furthermore, the results at excitation and emission wavelengths of 515 nm and 553 nm were not influenced by the presence of ADR.

Calibration Curve

A linear relationship was obtained between fluorescence intensity and quantity in the range of 0.1—1.0 nmol of MDA.

The precision of the assay procedure was examined on replicate runs for ten samples containing 0.5 nmol of MDA. The coefficient of variation was 2.2%.

Effect of pH of the Reaction Medium

In the reaction of lipoperoxide with TBA, acetic acid is the most suitable acid for acidification of the reaction medium, because it is capable of determining the peroxide of linoleic acid and it is independent of biological substances. Since the optimum conditions with acetic acid have not been fully discussed, the relation between fluorescence intensity and the pH of the reaction medium was investigated first. The pH was adjusted with 2.0 M acetate buffer

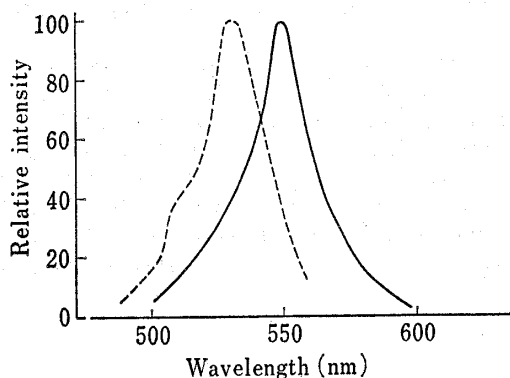


Fig. 1. Excitation and Emission Spectra of the Reaction Product of MDA with TBA

1,1,3,3-Tetramethoxypropane (2.0 nmol/tube) was reacted with TBA solution. After the reaction, the product was extracted with *n*-butanol:pyridine (15:1).

Maximum excitation wavelength=532 nm.

Maximum emission wavelength=553 nm.

---: excitation.

—: emission.

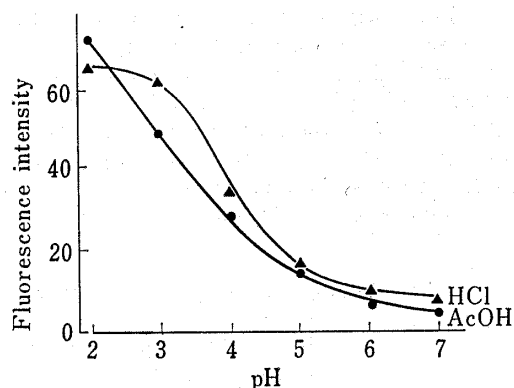


Fig. 2. Effect of pH on the Fluorescence Intensity of the Reaction Product with TBA

The pH of the reaction solution was changed by adding acetic acid or HCl, then MDA (1.0 nmol) was reacted with TBA in the solution.

solution. As the control, reaction media adjusted to pH 1—7 with 0.2 *N* HCl and 0.1 *N* NaOH solution were used. The results obtained are shown in Fig. 2.

The fluorescence intensity was stronger at lower pH and could not be observed at all in alkaline solution. These findings differ slightly from the results of the colorimetric study performed by Ohkawa *et al.*⁷⁾ Ohkawa *et al.* found that the optimum pH was 3.5. However, we did not observe such an optimum pH in acetate buffer or control medium. In the control medium, no further increase of fluorescence intensity was observed at pH 2 or below because precipitation of TBA was observed at these pHs. As similar results were obtained with acetate buffer medium and control medium, the pH of the reaction medium is clearly important. Therefore, we used acetate buffer solution in order to obtain a stable pH. The pH of acetate buffer solution used was 3.6, which was the minimum pH obtainable with acetate buffer solution.

Effect of Molar Concentration of Acetate Buffer Solution

We carried out an experiment to determine the influence of the molar concentration of acetate buffer solution. Fig. 3 shows the results in the reaction of MDA with TBA at pH 3.6, performed at various acetate molar concentrations between 1.0 and 7.0.

The acetate molar concentration did not influence the results much. However, the strongest fluorescence was obtained with 2.0 *M* acetate buffer solution. Therefore, 2.0 *M* solution was used in our experiments.

Extraction Solvent

In extraction of the fluorescent substance from the reaction medium into *n*-butanol after the reaction of lipoperoxide with TBA, turbidity was observed sometimes in the reaction medium after centrifugation. As Yagi *et al.* used a solution of *n*-butanol and pyridine in the ratio of 15:1 (volume) instead of *n*-butanol, we investigated the optimum ratio of pyridine addition (Table I).

In the solution of *n*-butanol and pyridine in the ratio of 1:1, the organic layer mixed with the aqueous layer to form a homogeneous solution. At ratios of 5:1—5:4, turbidity appeared in the organic layer. Consequently, we also used the ratio of 15:1.

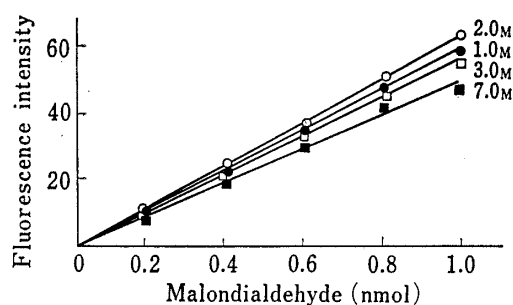


Fig. 3. Relation between Acetic Acid Concentration and Fluorescence Intensity of the Reaction Product with TBA

Acetic acid concentration was changed from 1.0 M to 7.0 M. MDA was reacted with TBA in the solution.

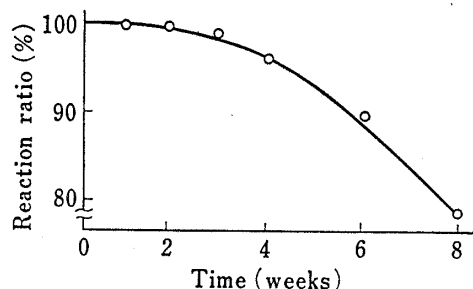


Fig. 4. Stability of TBA Solution

TBA solution (0.8%) was stored in a light-resistant bottle and left to stand for up to 8 weeks at room temperature.

TABLE I. Effect of Pyridine on Extraction of the Reaction Product of TBA with MDA

BuOH: pyridine	Fluorescence intensity		Note
	Organic layer	Aqueous layer	
BuOH only	52.3±0.5	15.7±0.8	
15:1	67.0±1.7	8.5±0.2	
5:1	67.2±2.3	6.6±0.4	Turbidity was seen.
5:2	67.5±1.0	6.4±0.7	Turbidity was seen.
5:3	64.6±1.4	7.7±0.5	Turbidity was seen.
5:4	49.2±3.4	11.5±0.4	
1:1	Organic layer was inseparable. (mixed layer: 41.4±0.5)		

Stability of the TBA-aqueous Solution

As a 0.8% aqueous stock solution had a slight smell of H_2S , indicating decomposition of TBA, we investigated the stability of the TBA solution. Aliquots of MDA solution were tested using 0.8% TBA aqueous solutions which had been prepared immediately before or up to eight weeks before the experiment. The relationship between fluorescence intensity and stocking time is shown in Fig. 4.

It is clearly desirable to use a 0.8% TBA aqueous solution prepared freshly, but a long time is required for its preparation because TBA is only slightly soluble in water. Thus, if a fresh preparation can't be obtained, a solution less than two weeks old should be used.

Application to Homogenate of Mouse Myocardium

An experiment was carried out on the myocardium homogenate from CDF_1 mice by applying the experimental results described above. We determined the recovery percentage of MDA added to the myocardium homogenate from CDF_1 mice. The average recovery of MDA (0.5 nmol) was $90.2 \pm 3.7\%$ ($n=10$); this high recovery was similar to the recovery (89%) in an experiment by Naito *et al.*⁵⁾ using serum. Next, the relation of fluorescence intensity to the volume of the homogenate was investigated. Fig. 5 shows the results.

A linear relation was observed in the range of 0.05–0.50 ml of the 1.0% homogenate solution.

Augmentation of Lipoperoxide in the Myocardium by ADR

According to the procedures described above, lipoperoxide was determined in the myocardium of normal CDF_1 mice and the concentration was found to be 250.7 ± 45.9 nmol/g wet

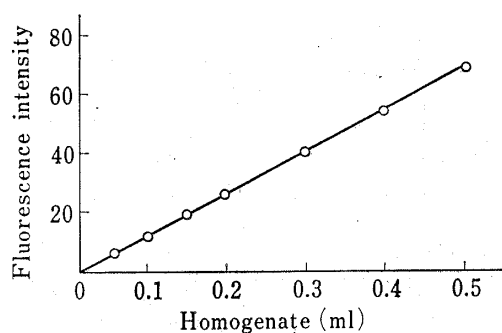


Fig. 5. Relation between the Amount of Tissue Homogenates and Fluorescence Intensity of the Reaction Product of Tissue Lipoperoxide with TBA

One percent homogenate solution of mouse heart (0.05—0.50 ml) was reacted with TBA solution. After the reaction, the product was extracted with *n*-butanol: pyridine (15: 1).

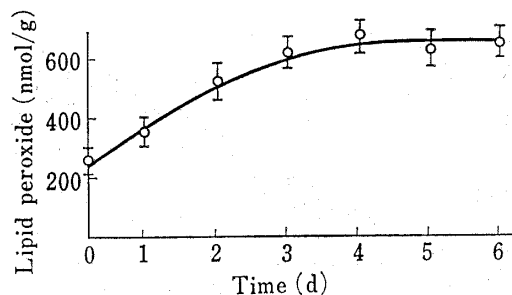


Fig. 6. Time Course of Lipoperoxide in Mouse Heart after ADR Administration

Male mice (CDF₁, 6W, 20—25 g) received an intraperitoneal injection of ADR (15 mg/kg). The mice were killed by cervical dislocation. The hearts were rapidly removed, and weighed. One percent homogenate heart was prepared, and a 0.1 ml aliquot was used for determination of lipoperoxide.

tissue. However, Myers *et al.* reported that no lipoperoxide could be detected by their method. On the other hand, our result is almost the same as the level in the myocardium of rats, 206.4 ± 27.5 nmol/g wet tissue, which was obtained in colorimetric analysis carried out by Ohkawa *et al.*⁶⁾ In male CDF₁ mice, myocardial lipoperoxide was determined on the 1st—3th day after intraperitoneal injection of ADR at a dose of 15 mg/kg. The results obtained are shown in Fig. 6.

The lipoperoxide level in the myocardium was increased to 674.5 ± 49.6 nmol/g wet tissue after ADR injection, in apparent agreement with the result of Myers *et al.*,⁶⁾ however, our result can't be directly compared with theirs, since it is expressed differently. These results confirm that the procedure in Chart 1 can be applied satisfactory to the myocardium of mice.

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References and Notes

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