Journal of Chromatography, 632 (1993) 97-103
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2616

Estimation of the extent of lipid peroxidation in the ischemic and reperfused heart by monitoring lipid metabolic products with the aid of high-performance liquid chromatography

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ABSTRACT

Estimation of lipid peroxidation (LPO) through malonaldehyde (MDA) formation measured by assaying thiobarbituric acid reactive products remains the method of choice to study the development of oxidative stress to assess myocardial ischemic reperfusion injury. However, MDA estimation by this assay is non-specific and often gives erroneous results. In this report, we describe a method to estimate MDA, formaldehyde (FDA), acetaldehyde (ADA), and acetone, the degradation products of oxygen free radicals (OFR) and polyunsaturated fatty acids (PUFA), as presumptive markers for LPO. Isolated rat hearts were made ischemic for 30 min, followed by 60 min of reperfusion. The perfusates were collected, derivatized with 2,4-dinitrophenylhydrazine, and extracted with pentane. Aliquots of 25 μl in acetonitrile were injected on a Beckman Ultrasphere C_{18} (3 μm) column. The products were eluted isocratically with a mobile phase containing acetonitrile-water-acetic acid (40:60:0.1, v/v/v). The peaks were identified by co-chromatography with the hydrazine derivatives of authentic standards. The retention times of MDA, FDA, ADA and acetone were 5.0, 6.3, 9.8 and 15.7 min, respectively. The results of our study indicated progressive increase in all four lipid metabolites with reperfusion time. Thus, our results demonstrate that the release of lipid metabolites from the isolated heart increased in response to oxidative stress. Since MDA, FDA, ADA, and acetone are the products of OFR-PUFA interactions, this method allows proper estimation of LPO to monitor the oxidative stress developed during the reperfusion of ischemic myocardium.

INTRODUCTION

The extent of lipid peroxidation in biological tissue and fluid is considered to be an important parameter for the identification of the oxidative stress developed under the pathophysiologic conditions [1,2]. Formation of malonaldehyde (MDA) is a widely recognized marker for lipid peroxidation [3], and the most commonly used method to estimate MDA formation is the spectrophotometric assay of the MDA following its reaction with thiobarbituric acid (TBA) [4,5]. Although this method is rapid and

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0021-9673/93/$06.00

relatively simple, the significance of the results is often blunted because of the incorrect interpretation of the results. The TBA-reactive products (often referred to as TBAR) as a measure for malonaldehyde formation in non-specific, because TBA not only forms a colored complex with malonaldehyde, but it also reacts with many other compounds including ribose, biliverdin, amino pyrimidines and sialic acid [6].

To overcome this problem a number of methods was developed to estimate MDA-TBA adducts using high-performance liquid chromatography (HPLC) [7,8]. Although these results are more reliable compared to the spectrophotometric methods of TBAR detection, this HPLC technique did not gain popularity because of the extremely complex nature of sample preparation and slowness of the technique.

In this report we describe a highly efficient technique to monitor the lipid peroxidation in biological tissue. It is well known that reperfusion of an ischemic tissue such as heart is associated with the formation of oxygen-derived free radicals which presumably attack the polyunsaturated fatty acids in the membrane phospholipids causing lipid peroxidation [9]. The extent of such lipid peroxidation was measured by estimating MDA and related lipid metabolic products after derivatizing with 2,4-dinitrophenylhydrazine (DNPH) using HPLC. Our results indicated this method to be extremely efficient and reliable to monitor the extent of lipid peroxidation in the ischemic reperfused myocardium as compared to any other available techniques.

EXPERIMENTAL

Materials

TBA and DNPH were obtained from Sigma (St. Louis. MO, USA). Malonaldehyde was purchased from Aldrich (Milwaukee, WI, USA), while formaldehyde, acetaldehyde, and acetone were from Sigma. DNPH standards were generated by derivatization of the pure compounds.

All organic solvents were of HPLC grade (Burdick & Jackson, Muskegon, MI, USA). Water was purified with a Milli-Q system. The mobile phase was filtered through a 0.22-μm nylon-66 solvent filter (Rainin Instrument, Woburn, MA, USA). All other chemicals were of analytical grade.

Methods

Isolated rat heart preparation. Sprague Dawley male rats of about 250 g body weight were anesthetized with intraperitoneal pentobarbital (80 mg per kg). Hearts were removed and quickly mounted on a noncirculating Langendorff perfusion apparatus as described previously [10]. Retrograde perfusion was established at a pressure of 100 cmH_2O ($9.8 \cdot 10^3$ Pa) with oxygenated normothermic Krebs–Henseleit bicarbonate (KHB) buffer containing 3% bovine serum albumin. Hearts were allowed to be equilibrated for 10 min at 37°C with noncirculating KHB buffer. The retrograde aortic flow was then terminated, and the heart was made ischemic for 30 min in physiological saline at 37°C. Reperfusion was then performed with the fresh KHB buffer for 60 min at normothermia. Perfusate samples were collected prior to ischemia (baseline), after ischemia and during the reperfusion for the estimation of MDA.

Assay **for** *MDA as TBA-reactive materials.* TBA-reactive materials were estimated by the well-established technique [11]. In short, 1 ml of the perfusate was mixed with 0.2 ml of 15% trichloroacetic acid. 1 ml of 0.75% TBA in *0.5%* sodium acetate, and the mixture boiled for 15 min [11]. The red color of the TBA-MDA complex was read with a spectrophotometer using 535 nm wavelength.

Derivatization and extraction of ***lipid*** *metabolites.* The lipid metabolites extracted with the perfusate were derivatized using DNPH. For derivatization purpose, 3 10 mg of DNPH was dissolved in 100 ml of 2 *M* HCl, and 0.1 ml of this DNPH reagent (3.13 μmol) was added to 1.5 ml of the perfusate in a 20-ml screw-capped PTFE lined test tube. An aliquot of 0.5 ml of water was added to the tube. the contents were mixed by vortexing and then IO ml of pentane was added to the mixture. The tubes were intermittently shaken for 30 min, and reactions were allowed to occur at room temperature. The organic phase was removed. and the aqueous phase was extracted with an additional 20 ml of pentane. The pentane extracts were combined, evaporated under a stream of nitrogen at 30°C. and reconstituted in 200 μl of acetonitrile. This filtered acetonitrile extract was directly injected onto the HPLC column.

HPLC procedure. A 25-μl volume of the filtered (0.2-μm Nylon-66 membrane filters in Microfilter-

fuge tubes from Rainin, Woburn, MA, USA) sample was injected onto a Beckman Ultrasphere ODS C_{18} (3 μm particle size, 7.5 cm × 4.6 mm I.D.) column (Rainin, Woburn, MA, USA) in a Waters chromatograph (Milford, MA, USA) equipped with a Model 820 full control Maxima computer system, satellite Wisp Model 700 injector, Model 490 programmable multi-wavelength UV detector (4 channels), two Model 510 pumps, and a Bondapak C_{18} Guard-Pak pre-column. The DNPH derivatives were detected at 307, 325 and 356 nm simultaneously with 3 channels of the M-490 detector at a flow-rate of 1 ml/min with an isocratic gradient of acetonitrile-water-acetic acid (40:60:0.1, v/v/v) for a total run time of 18 min. The column was washed with acetonitrile-acetic acid (100:0.1, v/v) before each day's work to remove any bound reagent.

Generation of standard curves. To make hydrazone standards, 30 ml of DNPH stock solution was allowed to react with an excess (1 to 3 nmol) of formaldehyde (FDA), acetaldehyde (ADA), MDA or acetone at room temperature. The precipitated hydrazones were filtered, dried, and recrystallized from methanol. Solutions containing 50 ng/μl of each of the four synthetic hydrazones were prepared and chromatographed as described above. The perfusion samples were spiked with the known amounts of each of the synthetic hydrazones to identify and confirm the presence of hydrazone derivatives of the lipid metabolites.

RESULTS

Separation and identification of lipid metabolites

The DNPH derivatives of authentic standards of FDA, ADA, MDA and acetone were separated using three different wavelengths: 307, 325 and 356 nm. MDA gives an absorption maximum at 307 nm whereas the absorption maximum for FDA, ADA and acetone is 356 nm. The retention times of MDA, FDA, ADA and acetone were 5, 6.3, 9.8 and 15.7 min, respectively, making a total run time of 18 min (Fig. 1).

Construction of calibration curve

With MAXIMA 820 software, calibration curves were produced and response factors were calculated for all the lipid metabolites. Ten different concentrations (10 pmol to 6.25 nmol) of each standard were injected and chromatographed as described previously. Various concentrations of each standard: (A) MDA, (B) FDA, (C) ADA, and (D) acetone were plotted against the peak area obtained. Standard curves were generated for all three wavelengths, 307, 325 and 356 nm.

The response factors were derived from the slope of each curve. Table I shows the values for the response factor, signal-to-noise (S/N) ratio, and the lowest limit of detection for each wavelength.

Quantitative estimation of lipid metabolites in the perfusate

Since reperfusion of ischemic myocardium is as-

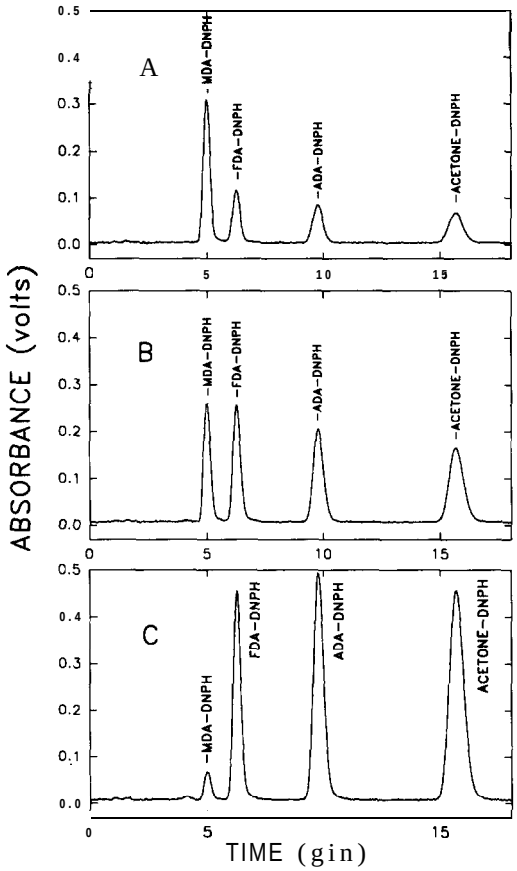

Fig. 1. Separation of MDA-DNPH, FDA-DNPH, ADA–DNPH, and acetone-DNPH standards by reversed-phase HPLC. Aliquots of 25 μl of standard solutions containing 2 nmol of each DNPH standard were injected onto a C_{18} column as described in ***Methods*** and the absorbance measured at three different wavelengths: (A) 307 nm; (B) 325 nm; (C) 356 nm.

TABLE I

RESPONSE FACTOR, r VALUE, LOWEST LIMIT OF DETECTION AND S/N RATIO FOR MDA. FDA, ADA AND ACETONE

Standard	λ (nm)	Response factor[a] ($\cdot 10^{-7}$)	r Value[b]	Lowest limit of detection (pmol)	S/N ratio
MDA-DNPH	307	3.357057	0.9985	10	2.4
MDA-DNPH	325	4.045774	0.998 1	30	2.1
FDA-DNPH	356	2.086545	0.9984	10	2.8
FDA-DNPH	325	3.752380	0.9982	30	2.1
ADA-DNPH	356	I.446131	0.9983	10	3.7
ADA-DNPH	325	3.534486	0.9987	30	4.0
Acetone–DNPH	356	1.125031	0.9984	10	3.9
Acetone–DNPH	325	3.195716	0.9982	30	3.1

[a] Response factor equals the weight of the compound in the calibrating solution (nmol/injection) divided by the integrated peak area of the compound.

[b] r Value is the correlation coefficient for a linear standard curve with a perfect correlation at 1.0000.

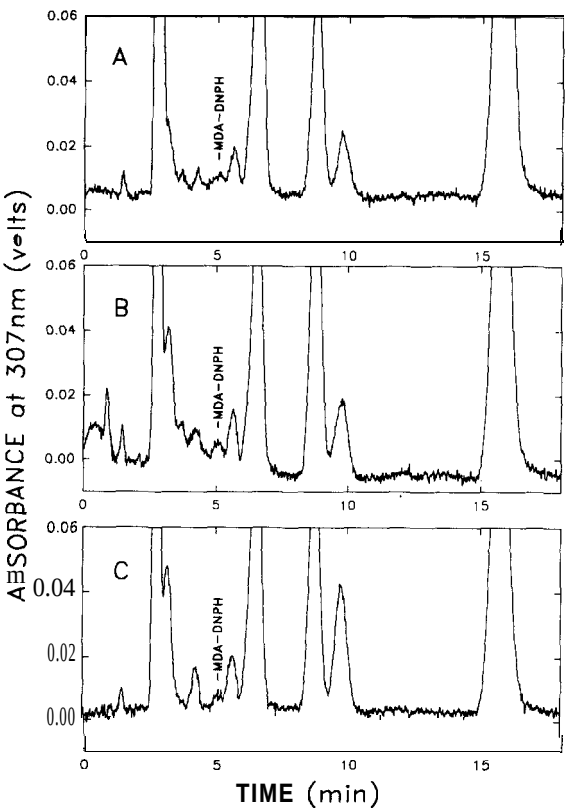

Fig. 2. Separation of MDA-DNPH in rat heart perfusates. Perfusates were collected, derivatized, extracted, and chromatographed as shown in Experimental. Absorbance was measured at 307 nm. (A) Baseline; (B) after 30 min of ischemia and 15 min of reperfusion; (C) after 30 min of ischemia and 60 min of reperfusion.

sociated with the production of lipid peroxidation products from the free radical-lipid interactions, we attempted to measure the lipid metabolites in the ischemic reperfused myocardium. The isolated rat hearts were subjected to 30 min of ischemia followed by 60 min of reperfusion. The perfusates obtained from the heart were processed as described in Methodology and the derivatized extracts were loaded onto the HPLC column equipped with programmable multi-wavelength UV detector. The results from (A) the baseline, (B) 15 min reperfusion, and (C) 60 min reperfusion heart perfusates are shown in Fig. 2 (307 nm), and Fig. 3 (356 nm). As shown in these Figs., the peaks for FDA, ADA, and acetone are already present in the baseline samples, and they increase progressively as the reperfusion progresses. MDA peak is barely present in the baseline sample, and it appears after the reperfusion and like other metabolite peaks, it increases as a function of the duration of reperfusion. The exact values are shown in Table II.

The identify of the peaks were confirmed by comparing the retention times with those of authentic standards. In addition, spiking with the standards was also performed [Fig. 4 (307 nm) and Fig. 5 (356 nm)]. Accuracy of the method was determined by standard addition technique. Addition of 50 pmol

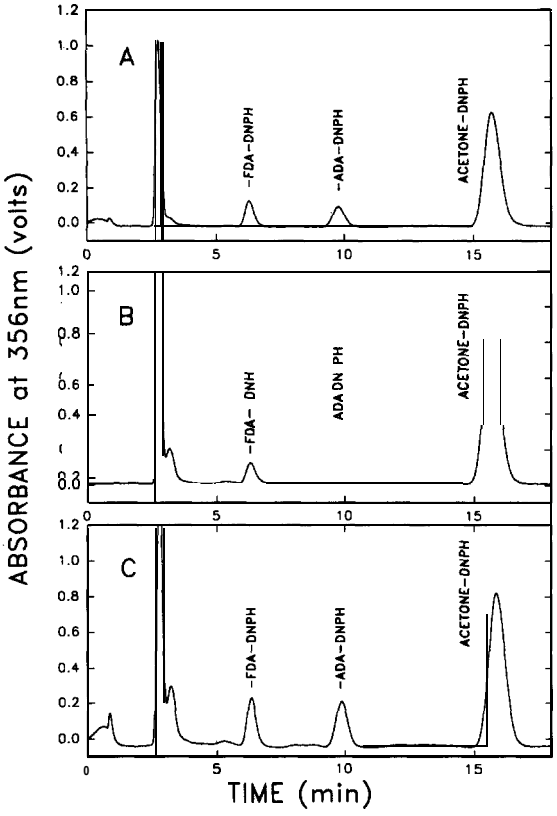

Fig. *3.* Separation of FDA-DNPH, ADA-DNPH, and acetone–DNPH in rat heart perfusates under the same conditions as described in Fig. 2 except that the absorbance was measured at 356 nm. (A) Baseline; (B) after 30 min of ischemia and 15 min of reperfusion; (C) after 30 min of ischemia and 60 min of reperfusion.

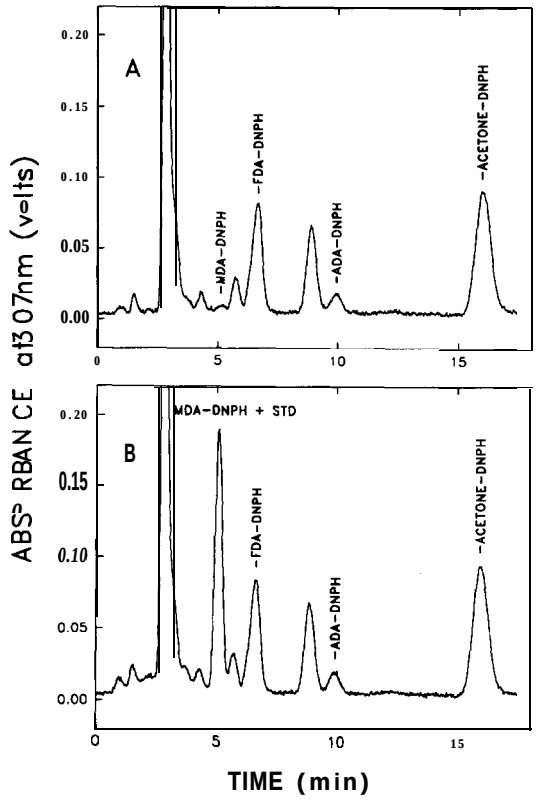

Fig. 4. Peak addition of MDA-DNPH to rat heart perfusate reperfused 15 min after 30 min of ischemia. Amounts of 0.33 nmol of MDA-DNPH standards were added to rat myocardial perfusate and chromatographed as shown in ***Methods*** and the absorbance measured at 307 nm. (A) Rat heart perfusate after 30 min of ischemia and 15 min of reperfusion; (B) same perfusate as in A with the addition of 0.33 nmol of MDA-DNPH standard.

TABLE II

ESTIMATION OF MDA-DNPH, FDA-DNPH, ADA-DNPH AND ACETONE-DNPH IN RAT HEART DURING ISCHEMIA AND REPERFUSION

Time of reperfusion (min)	Amount ± S.D. (n = 6) (nmol/ml perfusate)				
	MDA-TBA	MDA-DNPH (307 nm)	FDA-DNPH (356 nm)	ADA-DNPH (356 nm)	Acetone-DNPH (356 nm)
Baseline	0.23 ± 0.08	0.049 ± 0.016	3.10 ± 0.17	2.84 ± 0.28	21.34 ± 1.16
1	1.15 ± 0.19	0.069 ± 0.009	2.97 ± 0.83	3.14 ± 1.03	26.20 ± 3.57
15	1.58 ± 0.21	0.077 ± 0.014	3.23 ± 0.53	4.15 ± 1.88	19.76 ± 3.80
30	1.61 ± 0.42	0.109 ± 0.033	5.31 ± 2.73	4.23 ± 1.43	25.18 ± 2.17
45	1.78 ± 0.56	0.119 ± 0.009	6.16 ± 1.91	5.70 ± 1.55	22.13 ± 2.0
60	2.41 ± 0.84	0.136 ± 0.024	4.69 ± 1.92	5.24 ± 1.48	26.11 ± 1.82

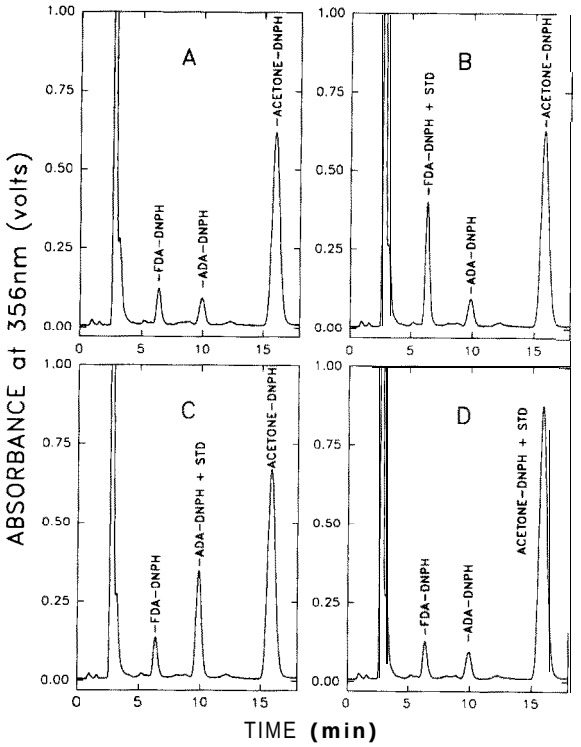

Fig. 5. Peak addition of DNPH standards to rat heart perfusate. Conditions were the same as described in Fig. 4, except that the absorbance was measured at 356 nm. (A) Rat heart perfusate after 15 min of reperfusion; (B) perfusate with the addition of 0.33 nmol of FDA-DNPH standard; (C) perfusate with the addition of 0.33 nmol of ADA-DNPH standard; and (D) perfusate with the addition of 0.33 nmol of acetone-DNPH standard.

TABLE III

ESTIMATION OF MDA AS TBA-REACTIVE PRODUCTS IN RAT HEART DURING ISCHEMIA AND REPERFUSION

Time of reperfusion (min)	Amount of MDA ± S.D. (n = 6) (nmol/ml perfusate)
Baseline	0.23 ± 0.08
1	*1.15 ± 0.19*
15	1.58 ± 0.21
30	1.61 ± 0.42
45	1.78 ± 0.56
60	2.41 ± 0.84

of each of the standards were accurately reflected in the peak heights. Within-run and inter-run variations were 1 and 5%, respectively.

TRA-reactive materials

The same perfusate samples were also estimated by assaying the TBA-reactive materials. The values for TBA-reactive materials are shown in Table III. As expected these values were much higher compared to those obtained for MDAs from the corresponding myocardial perfusate samples.

DISCUSSION

Lipid peroxidation is a complex process in which polyunsaturated fatty acids are subjected to attack by the oxygen-derived free radicals resulting the formation of lipid hydroperoxides. In biological tissues these lipid hydroperoxides are broken down into variety of products including aldehydes and ketones [1 1]. A large variety of methods are available for the detection of lipid peroxidation products which include determination of diene conjugation [123, lipid hydroperoxides [13], chemiluminescence [14], hydroxy acids [15], ethane [16] and TBA-reactive materials [4,5]. Among these methods estimation of TBA reactive products is most popular and widely used to study the lipid peroxidation in biological samples. The success of the TBAR method depends on the accuracy of the determination of MDA content. However, as mentioned earlier, TBA reacts with many other compounds besides MDA, and as a result it often overestimates the MDA content and yields erronious results. This method was subsequently modified to determine MDA-TBA complex by HPLC, but the slowness as well as the complexity of the procedure could not make it easily adaptable for the routine assay of MDA.

In this report we have described a method which can accurately estimate at least four different lipid breakdown products, MDA, FDA, ADA and acetone. In this method the carbonyls present in the myocardial tissue are converted into hydrazone derivatives by reacting with DNPH. This method was previously used to examine the lipid metabolites in the urine samples [17]. However, no attempt has ever been made to use this method to estimate the extent of lipid peroxidation under the pathophysi-

ological conditions in biological tissues such as heart. It has long been known that reperfusion of ischemic tissue produces oxygen free radicals, which subsequently attack the PUFA of the membrane lipids causing lipid peroxidation. Accurate estimation of the extent of lipid peroxidation is of utmost importance to monitor the development of oxidative stress in the ischemic reperfused tissue and to understand the pathophysiology of reperfusion injury. Our results indicate that DNPH method can be successfully used to estimate the lipid peroxidation in ischemic and reperfused heart.

Our results indicate that the values for TBA-reactive products are much higher compared to actual malonaldehyde values. This is not surprising, because TBA-reactive products estimates many other compounds besides MDA as mentioned earlier, thus overestimating the actual MDA values. MDA formation indeed occurs during the reperfusion of ischemic heart, but the amounts of MDA formed are very low.

ACKNOWLEDGEMENTS

This study was supported by Grants HL 22559 and HL 33889 from the US Public Health Service (National Institute of Health). We greatly appreciate the excellent secretarial assistance of Mrs. Laurie Amara.

REFERENCES

1 D. K. Das, R. M. Engelman, D. Flansaas, H. Otani, J. Rousou and R. H. Breyer, ***Basic Res. Cardiol., 82 (1987) 36.***
2 J. M. Petruska, S. H. Y. Wong, W. Sanderman and B. T. Mossman, ***Free Rad. Biol.*** Med., 9 (1990) 51.
3 H. S. Lee and A. S. Csallany, Lipids, 22 (1987) 104.
4 T. F. Slater, ***Methods Enzymol.,*** 105 (1984) 283.
5 R. P. Bird and H. H. Draper, ***Methods Enzymol., 105 (1984) 299.***
6 J. M. C. Gutterdge and T. R. Tickner, Anal. ***Biochem., 91 (1978) 250.***
7 H. Esterbauer, J. Lang, S. Zadravec and T. F. Slater, ***Methods*** Enzymol., 105 (1984) 319.
8 L. W. Yu, L. L. Latriano, S. Duncan, R. A. Hartwick and G. Witz, *Anal.* ***Biochem., 156 (1986) 326.***
9 ***X.*** Liu, R. Prasad, R. M. Engelman, R. M. Jones and D. K. Das, ***Am. J. Physiol., 259 (1990)*** H1101.
10 H. Otani, R. Prasad, R. M. Jones and D. K. Das, ***Am. J.*** Physiol., 257 (1989) H252.
11 H. Esterbauer, in D. C. H. Brien and T. F. Slater (Editors), ***Free Radicals, Lipid Peroxidation and Cancer,*** Academic Press, London, 1982, p. 101.
12 R. 0. Recknagel and A. K. Ghosal, ***Exp. Mol. Pathol., 5 (1966) 413.***
13 W. A. Pryor and L. Castle, ***Methods Enzymol., 105 (1984) 293.***
14 J. R. Wright, R. C. Rumbaugh, H. D. Colby and P. R. Miles, ***Arch. Biochem. Biophys., 192 (1979) 344.***
15 J. Capdevila, L. J. Mamett, N. Chacos, R. A. Prough and R. W. Estabrook, *Proc.* ***Natl.*** *Acad.* ***Sci. USA, 79 (1982) 767.***
16 ***C.*** Riely, G. Cohen and M. Lieberman, ***Science,*** *(Washington, D.C.),* ***183 (1974) 208.***
17 M. A. Shara, P. H. Dickson, D. Bagchi and S. J. Stohs, ***J. Chromatogr., 576 (1992) 221.***