Biochemical Pharmacology, Vol. 33, No. 14, pp. 2259–2263, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

SERUM LIPID PEROXIDES IN RATS TREATED CHRONICALLY WITH ADRIAMYCIN

WILLIAM S. THAYER
Department of Pathology and Laboratory Medicine, Hahnemann University, School of Medicine, Philadelphia, PA 19102, U.S.A.

(*Received* 9 *September* 1983; *accepted* 7 *December* 1983)

Abstract—Rats treated chronically with the anticancer agent adriamycin exhibited lipid peroxides in the serum and hyperlipidemia. Independent assay methods based on thiobarbituric acid reactivity and iodometric titration confirmed the presence of both lipid endoperoxides and hydroperoxides. Fractionation studies indicated that lipid peroxides were mainly associated with neutral lipids, including triglycerides, cholesterol esters and cholesterol, rather than with phospholipids. The lipid peroxides were distributed throughout the major serum lipoprotein classes. Although the origin of the lipid peroxides has not been established, the dose dependence for elevation of serum lipid peroxides approximately corresponds to that required for the development of cardiomyopathy. These findings support the conclusion that lipid peroxides are formed *in vivo* in rats treated chronically with adriamycin.

Adriamycin is an effective chemotherapeutic agent employed for the treatment of a variety of human cancers. Its clinical use is limited, however, by the occurrence of cardiomyopathy associated with chronic cardiotoxicity [1–3]. Adriamycin-induced heart disease is characterized by the degeneration of heart muscle, and is dose-dependent, cumulative, progressive, and often delayed [4, 5].

The identification of several biochemical activities of adriamycin *in vitro* has suggested several mechanisms for adriamycin cytotoxicity. These include direct interaction with nucleic acids [6], inhibition of coenzyme Q-dependent enzymes [7], direct cell surface interactions [8], and free radical generation as a consequence of redox cycling [9–12]. Myers *et al.* [13] have demonstrated the presence of malondialdehyde, a breakdown product of lipid peroxides, in hearts of mice treated acutely with a single high dose of adriamycin. This finding suggested that free radical reactions and lipid peroxidation may play a role in the cardiotoxicity of adriamycin [13]. However, the possible relationship of lipid peroxidation to the development of cardiomyopathy associated with chronic adriamycin administration has not been ascertained. In this paper, I report that rats treated chronically with adriamycin exhibited elevated levels of lipid peroxides in the serum. The findings provide direct evidence for the occurrence of lipid peroxidation *in vivo*.

MATERIALS AND METHODS

Male, CDF-Fischer rats (150–175 g) were treated with 1.5 mg adriamycin hydrochloride (Adria Laboratories, Columbus, OH) per kg body weight once per week by subcutaneous injection [14]. Control rats received a similar volume of 0.15 M NaCl. Blood was collected from neck vessels at the time rats were killed by decapitation, and it was allowed to clot for preparation of serum. In some experiments, blood was obtained by heart puncture using a heparinized syringe from rats that had been anesthetized with Nembutal. Serum triglycerides and total cholesterol were measured using a Centrifichem Analyzer.

Serum thiobarbituric acid (TBA) reactivity was measured essentially as described by Satoh [15]. Serum (0.25 ml) was mixed with 0.25 ml of 0.15 M NaCl and 2.5 ml of 20% trichloroacetic acid in a glass tube. After 10 min at room temperature, the mixture was centrifuged, and the supernatant fraction was subsequently discarded. The precipitate was dispersed in 2.5 ml of 0.05 N H_2SO_4 and mixed with 3.0 ml of freshly-prepared 0.67% 2-thiobarbituric acid in 2 M Na_2SO_4. Tubes were capped loosely and heated in a boiling water bath for 20 min. After cooling to room temperature, the mixture was extracted with 4.0 ml of *n*-butanol. Spectra of the *n*-butanol extracts exhibited a peak at 532 nm characteristic of the chromogen formed between malondialdehyde (MDA) and TBA. Absorbance at 532 nm was measured relative to a reference at 590 nm and was converted to equivalents of MDA using an absorptivity coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [16].

Lipids were extracted from 1-ml aliquots of serum or plasma, after addition of butylated hydroxytoluene to a final concentration of 0.01%, by the method of Folch *et al.* [17]. During extraction, the samples were homogenized with a Teflon homogenizer at 1300 rpm for 2 min at 45° [18]. For the TBA assay of lipid extracts, an aliquot was dried under N_2, resuspended in 2.5 ml of 0.05 N H_2SO_4, mixed with TBA reagent, and processed as described above. For determination of lipid hydroperoxides, an aliquot of the lipid extract was dried under N_2, redissolved in 3 ml N_2-purged $CHCl_3$–CH_3COOH (3:2, v/v), and assayed iodometrically [16]. Absorbance at 353 nm was determined from spectra and was converted to hydroperoxide equivalents using an absorptivity coefficient of $1.73 \times 10^4\ M^{-1}\ cm^{-1}$ [16]. For measurement of fluorescent lipid peroxi-

dation products [18], an aliquot of the lipid extract was dried under N_2 and redissolved in 3 ml of cyclohexane–ethanol (1:1). Fluorescence was measured using 368 nm excitation and 440 nm emission wavelengths. Fluorescence intensity was calibrated by measuring an aqueous solution of quinine sulfate (1 μg/ml) as an external standard. Observed intensity values were normalized relative to the quinine standard as 100 units.

Lipid extracts were fractionated by silicic acid (Sigma Chemical Co.) column chromatography [19]. Fractions were concentrated under N_2 and were analyzed by thin-layer chromatography on silica gel H plates (Applied Science Laboratories, Inc.). Neutral lipids were separated using the solvent system petroleum ether (b.p. 35–60°)–diethyl ether–acetic acid (80:20:1, by vol.) [19]. Triolein, cholesterol palmitate and cholesterol, obtained from Sigma, were used as chromatographic standards.

RESULTS

Effect of chronic adriamycin treatment on serum thiobarbituric acid reactivity. Thiobarbituric acid (TBA) reactivity has been widely used as an indicator of lipid peroxidation in both *in vitro* studies [16, 20] and in serum [15, 21]. As shown in Fig. 1, rats treated chronically with adriamycin exhibited elevated serum TBA reactivity compared to saline-treated controls. Serum TBA reactivity (nequiv. MDA/ml serum) increased in a non-linear manner with increasing cumulative doses of adriamycin. After administration of cumulative doses greater than about 13 mg/kg, serum TBA-reactivity values were consistently higher than those of control rats. Although serum TBA-reactivity values for individual rats varied considerably, the overall mean value obtained for rats treated with cumulative doses ranging from 13.5 to 25.5 mg/kg was about 5-fold higher than that for control rats (Table 1). Serum TBA reactivity did not appear to be dependent upon the length of time after administration of the final chronic dose of adriamycin. Rats killed as long as 7 weeks after reaching a cumulative dose of 21 mg/kg had serum TBA-reactivity values as high or higher than those found for rats killed 1 day after reaching the same cumulative dose. Rats treated with a single dose of 1.5 mg/kg, or treated chronically with a relatively low dose (0.25 mg/kg) for a comparable time period, resulting in a low cumulative dose, did not exhibit elevated serum TBA reactivity. Heparinized plasma gave results equivalent to serum.

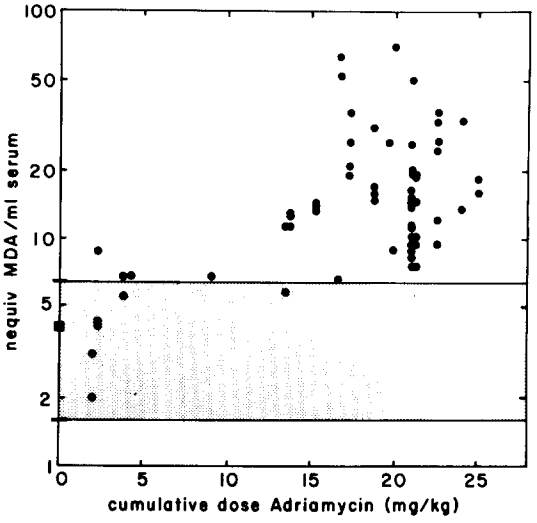

Fig. 1. Dose dependence of serum TBA reactivity in rats treated chronically with adriamycin. Serum TBA reactivity was measured in rats treated chronically with adriamycin for varying lengths of time, resulting in various cumulative doses. Most rats received doses of 1.5 mg per kg per week; however, some received doses of 0.25 mg per kg per week. Most rats were killed 1–3 weeks following the final dosing. The values for control rats, treated with saline for comparable time periods, are shown as mean ± 2 S.D. (square, N = 29) and shaded bar for clarity of presentation (see also Table 1). Note that the scale of TBA-reactivity values is logarithmic.

Table 1. Effect of chronic adriamycin treatment on serum thiobarbituric acid reactivity

	Length of treatment (weeks)	Cumulative dose adriamycin (mg/kg)	Serum TBA reactivity (nequiv. MDA/ml serum)
Saline-treated control rats (29)	9–17	0	4.0 ± 1.2
Adriamycin-treated rats (54)	9–17	19.7 ± 3.0	19.9 ± 13.8
Additional control experiments			
Chronic alcohol-fed rats (4)			3.4 ± 0.6
Spontaneously hypertensive rats (5)			3.1 ± 0.7
Lipemic human serum† (5)			2.8 ± 0.2
Control human serum—elevated lipid‡			3.9
Control human serum—normal§			3.0

* Serum TBA reactivity was measured as described in Materials and Methods. The values tabulated for adriamycin-treated rats were from those that had received cumulative doses ranging from 13.5 to 25.5 mg/kg. Results obtained from analyses of alternative control serum samples are presented in the lower section of the table. Values indicate mean ± S.D.; the number of samples, each assayed in duplicate, is shown in parentheses.

† Randomly selected clinical specimens having triglycerides 150–900 and cholesterol 200–450.

‡ Commercial clinical chemistry preparation from pooled lipemic serum, containing no stabilizers or additives.

§ Commercial clinical chemistry preparation from pooled normal serum, containing no stabilizers or additives.

Table 2. Effect of chronic adriamycin treatment on serum lipids*

	Triglycerides (mg/dl)	Cholesterol (mg/dl)
Saline-treated controls (8)	46 ± 23	41 ± 29
Adriamycin-treated (18)	379 ± 339	244 ± 129

* Serum triglycerides and total cholesterol were measured in rats that had been fasted for 18 hr. Adriamycin-treated rats had received cumulative doses ranging from 16.5 to 25.5 mg/kg (mean ± S.D. dose: 20.6 ± 2.1 mg/kg). Values indicate mean ± S.D.; the number of rats is shown in parentheses.

Spectral characteristics of the TBA adduct formed with serum were consistent with an MDA-derived chromogen (λ_{max} = 532 nm; fluorescence emission λ_{max} = 552 nm), although the spectra also exhibited a second absorbance maximum at 450 nm. The latter did not interfere significantly with measurements at 532 nm because formation of the 450 nm absorbing chromogen was minimized by use of the particular assay method, as reported by Satoh [15].

As previously observed [14], rats that had been treated with adriamycin also exhibited serum lipemia, characterized by elevations in both triglycerides and total cholesterol (Table 2). The hyperlipidemia had a dose dependence similar to that of the serum TBA reactivity and likewise showed large variation between individual rats. Linear regression analysis of the relationship between the serum TBA reactivity and serum lipid content for a representative series of samples demonstrated a correlation coefficient of 0.715 (Fig. 2). Thus, we questioned whether the elevated TBA reactivity was simply a consequence of the presence of larger amounts of lipid in the serum. To clarify this, we assayed several other lipemic and nonlipemic rat and human serum specimens (Table 1). The results clearly demonstrated that lipemia alone was not the cause of the elevated serum TBA reactivity found in adriamycin-treated rats and confirmed that peroxidation was not being induced artefactually during this assay.

Characterization of the thiobarbituric acid-reactive material as lipid peroxides derived from neutral lipids. Since MDA is formed readily from lipid peroxides which break down under the conditions of the TBA assay [15, 16, 20], the above data suggested that lipid peroxides were present in serum from adriamycin-treated rats. However, other substances, particularly certain 2-deoxysugars [22] and prostaglandin endoperoxide derivatives [23], can also degrade to form MDA under certain conditions. Thus, we conducted additional studies to ascertain the nature of the TBA-reactive compound in serum of adriamycin-treated rats.

The distribution of the TBA-reactive material in serum components was examined by lipid extraction. Assays conducted with samples derived from the

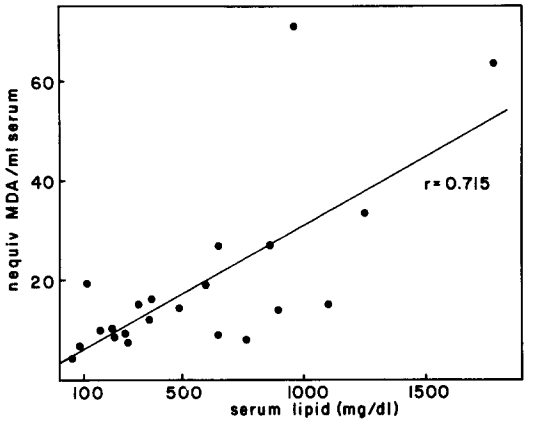

Fig. 2. Relationship between serum TBA reactivity and serum lipemia. Serum lipids (triglycerides plus total cholesterol) were measured for a representative series of samples from adriamycin-treated rats which exhibited TBA-reactivity values spanning the range shown in Fig. 1. The line was calculated by linear regression analysis; correlation coefficient: 0.715.

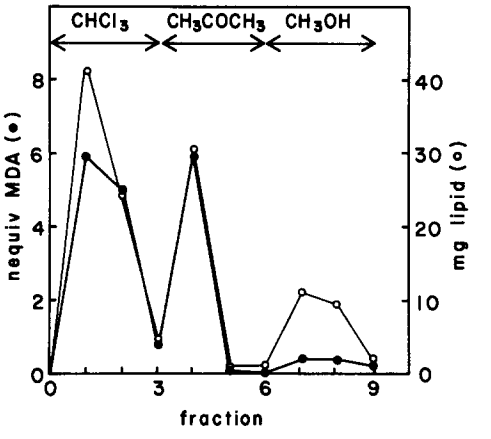

Fig. 3. Silicic acid chromatographic fractionation of serum lipid peroxides from adriamycin-treated rats. Serum lipid extracts obtained from three adriamycin-treated rats (cumulative dose: 21 mg/kg) were pooled, concentrated, and applied to a 1 cm diam. × 7 cm silicic acid column that had been pre-equilibrated in chloroform. The column was eluted sequentially with 50 ml chloroform, 50 ml acetone, and 50 ml methanol while collecting 17-ml fractions. The TBA reactivity [nequiv. MDA, (●)] of each fraction was tested on an aliquot, after evaporation of the solvent under N_2, as described in Materials and Methods. Total lipid (○) in each fraction was estimated gravimetrically. The total recovery of TBA-reactive material applied to the column was 84%.

Table 3. Comparison of various assays for lipid peroxide analysis of serum*

Assay method	Control	Adriamycin-treated
Direct serum TBA (nequiv. MDA/ml serum)	4.3 ± 1.2	15.7 ± 9.3†
Lipid extract TBA (nequiv. MDA/ml serum)	2.8 ± 1.6	14.6 ± 7.7†
Lipid hydroperoxides (nequiv./ml serum)	40.9 ± 13.6	94.6 ± 37.9†
Fluorescent lipid peroxidation products (relative units/ml serum)	7.4 ± 6.3	4.5 ± 4.0‡

* Lipid peroxides were measured by three different methods in serum lipid extracts as described in Materials and Methods. For comparison, the serum TBA-reactivity values measured directly with these particular samples are also presented. Serum was obtained from rats treated for 14 weeks (cumulative adriamycin dose: 21.0 mg/kg). Values indicate mean ± S.D. for eight to eleven determinations.

† Significantly different from control value at $P < 0.01$.

‡ Not significantly different from control value.

organic and aqueous phases, as well as protein residue, obtained during lipid extractions showed that the percent of TBA-reactive material associated with the various fractions was: lipid extract, 93.5 ± 4.3; protein residue, 4.3 ± 1.9; and aqueous, 0.4 ± 1.1 (mean ± S.D., N = 6). These data confirmed that the reactive substance was a lipid peroxide.

To characterize further the TBA-reactive lipid peroxide, serum lipid extract was fractionated by silicic acid column chromatography [19]. The elution profile for the TBA-reactive material corresponded to that of the neutral lipids (Fig. 3). The bulk of the TBA-reactive material was recovered in two major fractions, eluted by chloroform and acetone respectively. By contrast, only a relatively small amount of TBA-reactive material was found in the phospholipid fraction eluted by methanol. Thin-layer chromatographic analysis of these fractions indicated that the chloroform eluate contained triglycerides and cholesterol esters, and it showed that free cholesterol was the major component of the acetone eluate. When thin-layer chromatography plates containing aliquots of these fractions were sprayed with TBA reagent and then heated at 120° for 5 min and subsequently viewed under long wavelength u.v. illumination, orange fluorescent spots were observed on the deep purple background of the plate. The location of these spots corresponded in migration to triglyceride, cholesterol ester, and free cholesterol. Under the same experimental conditions, non-peroxidized chromatographic standard lipids were not detectable by this technique. These results suggested that lipid peroxides were present in the major neutral lipids of serum from adriamycin-treated rats.

Correlation with alternative products of lipid peroxidation. Since the TBA reaction detects primarily lipid endoperoxides [16, 20], we used an independent assay method to test for the possible presence of lipid hydroperoxides. Measurements with an established iodometric procedure revealed a substantial increase (2.5-fold) also in lipid hydroperoxides in serum from adriamycin-treated rats as compared to controls (Table 3). The possible presence of a third type of lipid peroxidation product was investigated by measuring specific fluorescence of lipid extracts [18]. With this assay, only very low, nearly background, levels of fluorescence were detected with samples from both treated and control rats (Table 3), suggesting the absence of such compounds. However, since fluorescent lipid peroxidation products arise primarily from phospholipids [18, 24], this result is consistent with the silicic acid chromatography data showing little association of lipid peroxides with serum phospholipids.

Lipoprotein distribution of lipid peroxides. To examine the association of lipid peroxides with lipoproteins, serum from adriamycin-treated rats was fractionated into major lipoprotein classes by ultracentrifugation [25]. Assays conducted with fractions enriched in high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) showed that the TBA-reactive lipid peroxides were distributed in all fractions, rather than exclusively associated with any specific class of lipoproteins.

DISCUSSION

Results of these studies indicate that the serum of rats treated chronically with cumulative doses of adriamycin greater than approximately 13 mg/kg contains peroxidized lipids. Chemical assays confirmed the presence of both lipid endoperoxides and hydroperoxides. Fractionation studies demonstrated that several distinct types of lipid peroxides were present within the major neutral lipids, including triglycerides, cholesterol esters and free cholesterol. Interestingly, the serum phospholipids did not appear to contain comparable amounts of peroxidized lipids. The data also indicated that the TBA-reactive material detected with whole serum did not arise from circulating MDA or a deoxy sugar, and that prostaglandin endoperoxides were not a major contributor to the observed reaction. Rats treated chronically with adriamycin also exhibited elevated levels of serum lipids. However, results of control experiments, particularly those with control lipemic

serum, verified that the mere presence of high amounts of lipids was not sufficient for detecting peroxidation. This confirms that peroxidation was not being induced artefactually under assay conditions and suggests that lipid peroxides arose as a consequence of adriamycin treatment. Thus, the data support the conclusion that lipid peroxides are present *in vivo* in rats treated chronically with adriamycin.

Although the pathophysiological mechanisms underlying adriamycin cytotoxicity *in vivo* are clearly complex and multifaceted, the present findings support the hypothesis that adriamycin may promote the generation of free radicals and that free radical-initiated reactions, such as lipid peroxidation, may be a component of its cytotoxicity [13]. However, several physiological manifestations of cytotoxicity accompany adriamycin administration [2, 3, 14, 26, 27]. For this reason, the presence of lipid peroxides in the serum cannot be ascribed to a particular form of adriamycin-induced injury. Nevertheless, it is interesting to note that the dose dependence for appearance of elevated serum lipid peroxides approximately paralleled that for the development of cardiomyopathy. Previous investigations [14, 26], confirmed by ourselves with the rats used in these studies, have shown that administration of a cumulative dose of adriamycin corresponding to approximately 20 mg/kg is required to demonstrate histologic evidence of cardiomyopathy in rats, although ultrastructural abnormalities can be detected at lesser doses by electron microscopy [5, 14, 26].

The hyperlipidemia produced by adriamycin treatment is notable, and it is indicative of a disturbance in serum lipid metabolism. Lipemia may arise from either enhanced synthesis or, more commonly, decreased utilization of plasma lipoproteins [28]. Elevated serum lipids, particularly cholesterol, are regarded as having a role in the development of cardiovascular disease [29, 30], and they may, therefore, contribute to the cardiac disease induced by adriamycin treatment.

The cellular origin of serum lipid peroxides remains to be determined. Since the liver is the major site of anthracycline metabolism [31] as well as lipid and lipoprotein biosynthesis, and because metabolism of adriamycin is obligatorily linked to free radical generation [32], lipids may be exposed to initiators of peroxidation within the liver. Alternatively, lipids may be peroxidized at other sites and subsequently released to the circulation. The association of serum lipid peroxides primarily with neutral rather than phospholipids is particularly intriguing and may provide a novel insight to cellular biology. Since neutral lipids, particularly triglycerides and cholesterol esters, tend to be a 'storage' form of lipid, whereas phospholipids play a more structural role, repair mechanisms may operate so as to cleave peroxidized fatty acyl chains from membranes. Should the latter not be degraded readily, owing to abnormal structures, or be detoxified by enzymes such as glutathione peroxidase, they might instead be packaged into storage lipid forms. These, in turn, might then be resistant toward normal metabolic processes and accumulate *in vivo*. They may also act as toxins in a manner analogous to that proposed for xenobiotic-lipid hybrids [33].

Acknowledgements—This work was supported by Grant HL27929 from the National Heart, Lung and Blood Institute. I thank Dan Minczak, Susan DeLeeuw, and Mark Hoy for technical assistance.

REFERENCES

1. E. A. LeFrak, J. Pitha, S. Rosenheim and J. A. Gottlieb, *Cancer, N.Y.* **32**, 302 (1973).
2. L. Lenaz and J. A. Page, *Cancer Treat. Rev.* **3**, 111 (1976).
3. R. C. Young, R. F. Ozols and C. E. Myers, *New Engl. J. Med.* **305**, 139 (1981).
4. V. J. Ferrans, *Cancer Treat. Rep.* **62**, 955 (1978).
5. R. S. Jaenke and L. F. Fajardo, *Am. J. surg. Path.* **1**, 55 (1977).
6. E. Calendi, A. DiMarco, M. Reggiani, B. Scarpinato and L. Valentini, *Biochim. biophys. Acta* **103**, 25 (1965).
7. Y. Iwamoto, I. L. Hansen, T. H. Porter and K. Folkers, *Biochem. biophys. Res. Commun.* **58**, 633 (1974).
8. T. R. Tritton, G. Yee and L. B. Wingard, *Fedn. Proc.* **42**, 284 (1983).
9. K. Handa and S. Sato, *Gann* **67**, 523 (1976).
10. N. R. Bachur, S. L. Gordon and M. V. Gee, *Molec. Pharmac.* **13**, 901 (1977).
11. J. Goodman and P. Hochstein, *Biochem. biophys. Res. Commun.* **77**, 797 (1977).
12. W. S. Thayer, *Chem. Biol. Interact.* **19**, 265 (1977).
13. C. E. Myers, W. P. McGuire, R. H. Liss, I. Infrim, K. Grotzinger and R. C. Young, *Science* **197**, 165 (1977).
14. P. Mettler, D. M. Young and J. M. Ward, *Cancer Res.* **37**, 2705 (1977).
15. K. Satoh, *Clinica chim. Acta* **90**, 37 (1978).
16. J. A. Buege and S. D. Aust, *Meth. Enzym.* **52**, 302 (1978).
17. J. Folch, M. Lees and G. H. Sloane-Stanley, *J. biol. Chem.* **226**, 497 (1957).
18. B. L. Fletcher, C. J. Dillard and A. L. Tappel, *Analyt. Biochem.* **52**, 1 (1973).
19. M. Kates, *Techniques of Lipidology* (Eds. T. S. Work and E. Work), pp. 397–401. Elsevier, New York (1972).
20. G. L. Plaa and H. Witschi, *A. Rev. Pharmac. Toxic.* **16**, 125 (1976).
21. K. Yagi, *Biochem. Med.* **15**, 212 (1976).
22. L. D. Saslaw and V. S. Waravdekar, *Meth. Enzym.* **12A**, 108 (1967).
23. T. Shimizu, K. Kondo and O. Hayashi, *Archs Biochem. Biophys.* **206**, 271 (1981).
24. W. R. Bidlack and A. L. Tappel, *Lipids* **8**, 203 (1973).
25. T. J. Bronzert and H. B. Brewer, *Clin. Chem.* **23**, 2089 (1977).
26. H. M. Olson and C. C. Capen, *Toxic. appl. Pharmac.* **44**, 605 (1978).
27. T. Bertani, A. Poggi, R. Pozzoni, F. Delaini, G. Sacchi, Y. Thoua, G. Mecca, G. Remuzzi and M. B. Donati, *Lab. Invest.* **46**, 16 (1982).
28. P. Nilsson-Ehle, A. S. Garfinkel and M. C. Schotz, *A. Rev. Biochem.* **49**, 667 (1980).
29. G. J. Miller, *A. Rev. Med.* **31**, 97 (1980).
30. P. Samuel, D. J. McNamara and J. Shapiro, *A. Rev. Med.* **34**, 179 (1983).
31. N. R. Bachur, *Cancer Treat. Rep.* **63**, 817 (1979).
32. P. L. Gutierrez, M. V. Gee and N. R. Bachur, *Archs Biochem. Biophys.* **223**, 68 (1983).
33. J. Caldwell and M. V. Marsh, *Biochem. Pharmac.* **32**, 1667 (1983).