Europ. J. Cancer Vol. 15 pp. 923–928

0014-2964/79/0601-0923 $02.00/0

Chemical Interactions of Cardiolipin with Daunorubicin and other Intercalating Agents*

HERBERT S. SCHWARTZ and PETER M. KANTER

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York 14263 *U.S.A.*

Abstract—*Cardiolipin interacts to form complexes with daunorubicin* ($K_d \simeq 1 \times 10^{-5}$ M), *acridine orange* ($K_d \simeq 6 \times 10^{-5}$ M) *and ethidium bromide* ($K_d \simeq 4 \times 10^{-4}$ M), *as determined by difference spectroscopy. The complex with daunorubicin is initially ionic with maximal hypochromicity occurring at* pH 7.8 *and is dissociated by low inorganic phosphate or high salt concentrations. Daunorubicin also interferes with phospholipid peroxidation as it reacts slowly to form a new biologically active addition product* (*Dm* 90), *which is more lipophilic and more highly conjugated than daunorubicin. Reaction conditions suggest a Schiff base addition between the free amino group of the drug and a carbonyl formed during peroxidation of the phospholipid.*

INTRODUCTION

DUARTE-KARIM *et al.* [1] observed that negatively-charged phospholipids increase lipophilicity of adriamycin and daunorubicin, and suggested an ionic interaction between the anthracycline amino-N^+ and PO_4^-. The resultant effect, it was hypothesized, could affect mitochondrial structure and function and might be associated with cardiac toxicity of the anthracycline drugs [2–4]. Because of the importance of this hypothesis with respect to the limiting cardiomyopathies in patients receiving these agents, we measured the affinity of daunorubicin for cardiolipin and described some of the conditions that govern the initial interaction. Besides the anthracyclines [5, 6], other intercalating drugs also bind to and have effects at membranal sites [7–9]; therefore, two standard intercalating agents were included in this study, ethidium and acridine orange. Finally, we report a new addition compound formed non-enzymatically between daunorubicin and a product of phospholipid peroxidation.

Accepted 7 December 1978.
*Supported in part by U.S.P.H.S. (N.C.I.) Core Grant CA-13038 and Research Fellowship Award (P.M.K.) CA-02301.

MATERIALS AND METHODS

Cardiolipin (Na salt of diphosphatidylglycerol), ethidium bromide, and acridine orange were purchased from Sigma Chemical Co. (St. Louis, MO). Anthracycline derivatives were research samples generously provided by Prof. F. Arcamone (Farmitalia, Milan, Italy) and Dr. M. Israel (Sidney Farber Cancer Center, Boston, MA, U.S.A.). All other reagents were best commercial grades.

Spectral absorption assays were carried out with the DW-2 Aminco recording spectrophotometer. Under standard conditions, cardiolipin and drugs were freshly prepared in solutions containing 0.02 M Tris (pH 7.5) and 12–15% ethanol. All spectral studies were carried out in matched, split quartz cuvettes with a 1 cm light path. Binding constants were calculated only from spectra with an unambiguous isosbestic point [10].

Thin layer chromatograms on silica gel G plates were developed in darkness with three solvent systems:

I, $CHCl_3$:CH_3OH:H_2O (80:40:3);
II, $CHCl_3$:CH_3OH:glacial acetic acid (80:20:4);
III, $CHCl_3$:CH_3OH:0.1 M K_2HPO_4 (adjusted to pH 7.0) (80:20:3).

Malondialdehyde was determined by the

thiobarbituric acid colorimetric test according to previously published procedures [11, 12]. Color was read at 553 nm (Varian Techtron 635) and results corrected for blanks (0.02 absorbance) after extraction with $CHCl_3$. Fluorometric recordings were taken from an Aminco–Bowman Spectrophotofluorometer.

Drug toxicity was assayed with a cultured line of CCRF-CEM human leukemia cells maintained in log growth in RPMI 1640 culture media supplemented with 10% heat inactivated (56°C, 30 min) dialyzed fetal calf serum and 1% penicillin–streptomycin solution. The compounds at various concentrations (5–7 at 2-fold increments) were incubated with cells (8×10^5 cells/ml; 10 ml) for 2 hr, washed, and resuspended in drug-free media (1.3×10^5 cells/ml; 6 ml). Cells were counted electronically 50 hr later; control cultures in logarithmic growth increased in number to 4.8×10^5/ml.

RESULTS

Interactions of cardiolipin with anthracyclines

The initial interaction of the chromophore of daunorubicin with the negatively-charged phospholipid cardiolipin is indicated by hypochromic and bathochromic shifts with a crossover at 537 nm. Isosbesticity at this wavelength is established from difference spectra (inset, Fig. 1) with varying concentrations of cardiolipin. Figure 1 also shows a double reciprocal plot using absorbance changes taken from three wwavelengths (440, 470 and 490 nm) of difference spectra. A dissociation constant (K_d) estimated from these data is approximately 1×10^{-5} M (assuming a mol.wt of 1200 for cardiolipin). The spectral evidence indicates that π-electron interactions occur as the chromophore and phospholipid form a complex of daunorubicin with cardiolipin.

Some of the conditions governing formation of the complex have been investigated by measurements of difference spectra. Absorbance at 470 nm exhibits a maximal hypochromic shift at pH 7.8 with 0.05 M Na Tris–buffer; Na phosphate buffer (0.05 M, pH 6.8 and 7.5) inhibits the interaction (Fig. 2). To further define conditions of complex formation, the effects of ionic strength were investigated. Changes in hypochromicity, as shown in Table 1, are minimal at relatively high salt concentrations and in 95% ethanol but increase at low salt concentrations. The conditions of neutral pH and low ionic strength raise the possibility that an ionic interaction may occur at intramembranal sites in some cells.

Solutions containing approximately equimolar concentrations (2 mM) of daunorubicin and cardiolipin in 0.02 M Tris (pH 7.5) and

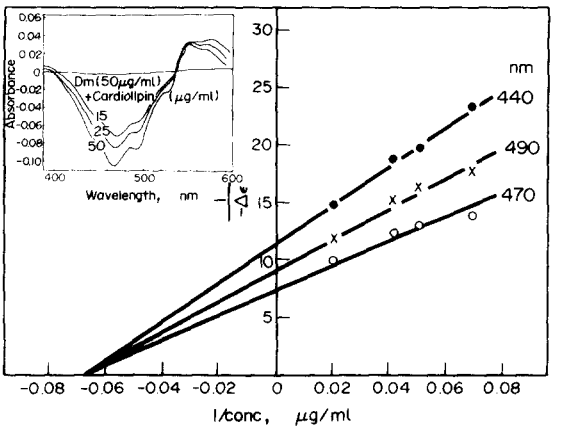

Fig. 1. Difference spectra (inset) and double reciprocal plot of absorbance changes (Δε) of daunorubicin (Dm) after mixing with varying concentrations of cardiolipin. The compartments of experimental and reference split cuvettes contained initially 1 ml each of daunorubicin (100 μg/ml) or cardiolipin, both in 0.02 M Tris (pH 7.4) and 12% ethanol. After recording initial absorbance, contents of the experimental cuvette were mixed and absorbance again recorded. Cardiolipin concentrations (final) were 15,20 (not shown in inset), 25 and 50 μg/ml. The dissociation constant (K_d) is estimated from Δε at 3 wavelengths: 440, 470 and 490 nm.

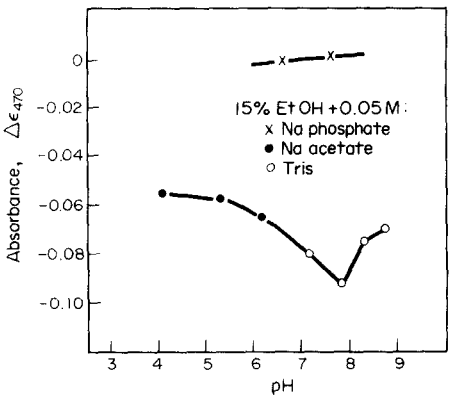

Fig. 2. Change in absorbance (Δε, 470 nm) of daunorubicin (50 μg/ml) and cardiolipin (25 μg/ml) in phosphate, acetate and Tris solutions (0.05 M) containing 15% ethanol. Absorbance was measured by difference spectroscopy described in Materials and Methods and the legend of Fig. 1. Concentrations indicated are final after mixing.

Table 1. The effects of NaCl and Tris buffer on absorbance changes ($\Delta\varepsilon_{470}$) of daunorubicin (50 μg/ml) after addition of cardiolipin (25 μg/ml)

Concentrations (M)		
NaCl	Tris	$\Delta\varepsilon_{470}$
—	—*	+0.015
0.2	0.05	+0.020
0.1	0.05	−0.020
0	0.05	−0.075
0	0.02	−0.115

*95% Ethanol; all other solutions contained 15% ethanol, as described in the legend of Fig. 2.

12% ethanol were chromatographed on thin layer plates. In systems I and II, fluorescent spots appeared at R_F 0.82 and 0.78, respectively, moving more like an uncharged aglycone (daunorubicinone, R_F 0.87 in II) than daunorubicin (R_F 0.41 in I; 0.30 in II). Chromatography of the reaction mixture in system I after additions of equal volumes of 0.1 M Na phosphate (pH 7.4) or 95% ethanol results in loss of the rapidly migrating component and reappearance of the fluorescent spot with an R_F of daunorubicin. Similarly, only a spot corresponding to daunorubicin is obtained after chromatography of the reaction mixture in the phosphate-containing system III. The results are consistent with the hypothesized ionic complex [1] which is dissociated at both high and low ionic strength and in competition with phosphate ions.

It is presumed that the ionic interaction involves the daunosamine-N^+ [1] of daunorubicin. This was tested with the uncharged *N*-acetyldaunorubicin; the interaction between its chromophore (50 μg/ml) and cardiolipin is indicated by a small "hyperchromic" shift, maximally at 501 nm. Visible spectra, without a bathochromic shift or an isosbestic point, are not changed by inorganic phosphate (0.1 M) and are thus different from spectra of the ionic complexes described above.

Interactions of cardiolipin with other intercalating agents

Spectral evidence of an interaction between cardiolipin and ethidium is shown in Fig. 3. Difference spectra (inset, Fig. 3) indicate a hypochromic–bathochromic shift from 491 nm with an isosbestic point at 502 nm. The K_d for the interaction is approximately 4×10^{-4} M. Spectral evidence of an interaction between cardiolipin and acridine orange was also obtained: a hypochromic–bathochromic shift accompanied by an isosbestic point at 517 nm, with K_d estimated at 6×10^{-5} M (Fig. 4).

Formation of an addition product of daunorubicin (Dm 90)

Twenty-four hours after formation of the initial ionic complex described above, a second interaction between phospholipid and daunorubicin is evidenced by precipitation of a red water-insoluble product from reaction mixtures. Migration of the washed precipitate in chromatographic systems I, II and III indicates that it is a single component with R_F values that are close to, but always different from, daunorubicinone. The product, whose chemical structure is unknown, is tentatively referred to as Dm 90 because of its R_F (0.90) during preparative chromatography on silica gel plates with $CHCl_3$:CH_3OH (4:1). Dm 90

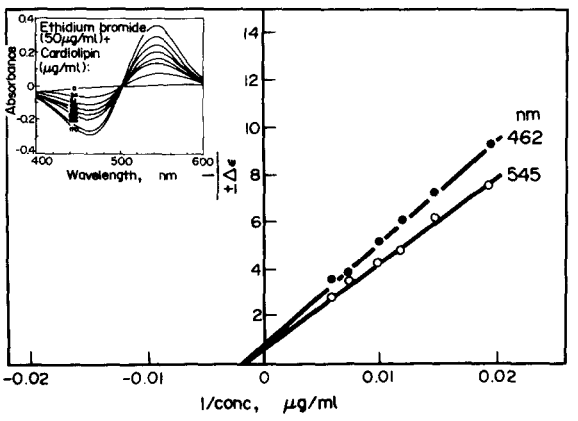

Fig. 3. Difference spectra (inset) and double reciprocal plot of absorbance changes (Δε) of ethidium bromide after mixing with varying concentrations of cardiolipin. Conditions and procedures as described in legend of Fig. 2, except that initial concentrations were 100 μg ethidium bromide/ml and 34, 51, 68, 102, 136 and 170 μg cardiolipin/ml. The K_d is estimated from Δε at 2 wavelengths: 462 and 545 nm.

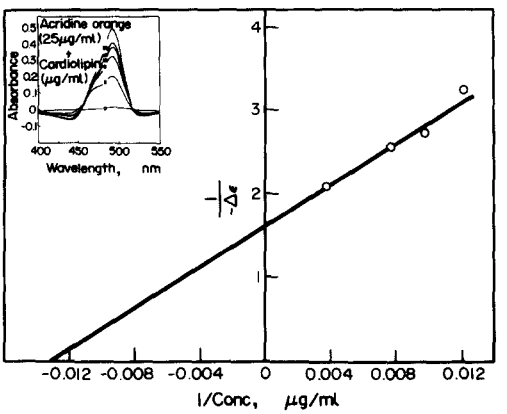

Fig. 4. Difference spectra (inset) and double reciprocal plot of absorbance changes (Δε) of acridine orange after mixing with varying concentrations of cardiolipin. Conditions and procedures as described in legend of Fig. 2, except that initial concentrations were 25 μg acridine orange/ml and 5, 8, 10, 13 and 25 μg cardiolipin/ml. The K_d is estimated from Δε at 492 nm (8–25 μg cardiolipin/ml).

is distinguished on the plates by its intense red color as compared to the orange colors of daunorubicinone (R_F0.95) and daunorubicin (0.41). Absorption–emission fluorescence spectra (Fig. 5) indicate a high degree of conjugation and further distinguish Dm 90 from daunorubicin, the aglycone, and the initial ionic complex. In addition, Dm 90 is not dissociated by either 1 M Na phosphate (pH 7.0) or 6 M urea.

Peroxidative formation of free radicals from phospholipids releases thiobarbituric acid-reactive materials (e.g., malondialdehyde) [12]. Figure 5 shows the time-course of an inhibition by daunorubicin of release of

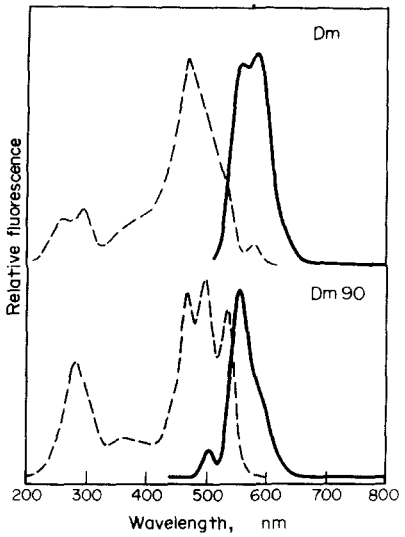

Fig. 5. Fluorescence spectra of daunorubicin (Dm) and Dm 90. Solutions (10 μg/ml) were prepared in ethanol (as described in the text). Absorbance is indicated by dashed lines; emission by solid lines.

thiobarbituric acid-reactive components from cardiolipin. As the drug inhibits neither lipid peroxidation nor the colorimetric reaction, the inhibition may be due to a reaction of lipid peroxidation product(s) with daunorubicin, as suggested below.

Other anthracycline derivatives also form undissociable lipophilic products during aerobic incubations with cardiolipin: adriamycin, 4′-epiadriamycin, and 4-demethoxydaunorubicin. The products were collected as insoluble precipitates and chromatographed (system III). Like Dm 90, each product forms a single red fluorescent zone with an R_F(0.70–0.85) higher than the respective parent compound (0.16–0.22). Comparable reactions do not occur with daunorubicinone, *N*-acetyldaunorubicin, and *N*-trifluoroacetyladriamycin-14-valerate. This series implicates the ionizable daunosamine-N^+ in the formation of Dm 90. Finally, glutaraldehyde also forms a lipophilic product with both adriamycin and daunorubicin, suggesting the possibility that malondialdehyde or other conjugated carbonyls formed from lipid peroxidation may react with positively charged anthracyclines to form the lipophilic addition products.

Growth inhibition of CCRF-CEM cells by Dm 90

The biological activities of Dm 90 and other anthracycline derivatives were tested with a cultured line of human leukemia cells (CCRF-CEM). As described in Materials and Methods, the agents were incubated at varying concentrations with the CCRF-CEM cells for 2 hr. Cells were then washed and incubated for 50 hr in drug-free medium. The results of the assay of Dm 90 (Table 2) indicate that it is less potent than either adriamycin or the parent compound daunorubicin. Because the studies described above indicate that Dm 90 may be an *N*-substituted product, it was also compared with two other *N*-substituted derivatives. These results show that the potency of Dm 90 is about twice that of *N*-trifluoroacetyladriamycin-14-valerate and 10 times that of *N*-acetyldaunorubicin.

DISCUSSION

Daunorubicin and cardiolipin from a reversible ionic complex ($K_d \simeq 1 \times 10^{-5}$ M) in solutions of low ionic strength. The complex is

Table 2. Concentrations of compounds producing 50% inhibition of growth (ID_{50}) of CCRF-CEM cells

Compound	ID_{50}* (μg/ml)
Dm 90	0.70
Daunorubicin	0.045
Adriamycin	0.25
N-acetyldaunorubicin	10
N-trifluoroacetyladriamycin-14-valerate	1.7

*Cells were incubated for 2 hr with each compound, washed, and reincubated for 50 hr in drug-free medium, as described in Materials and Methods.

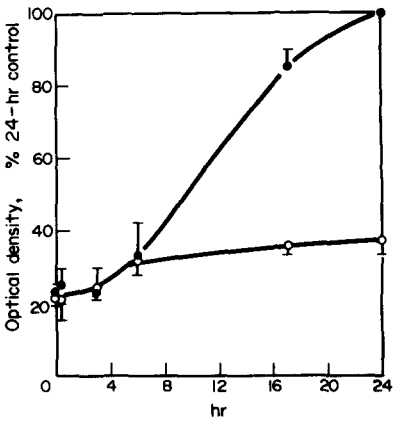

Fig. 6. Colorimetric assay of thiobarbituric acid-reactive materials during aerobic incubation of cardiolipin with (○) *and without* (●) *daunorubicin. Incubation-solutions* (6 ml) *contained* 1.7 mg *cardiolipin, and daunorubicin* (1 mg) *in* 0.02 M *Tris* (pH 7.4) *and* 12% *ethanol; daunorubicin was added at* 0 *time and* 0.5 ml *aliquots were withdrawn from a shaking water bath* (37°C) *at indicated times for assay. Vertical lines indicated standard deviations.*

dissociated in the presence of low concentrations of inorganic phosphate and by raising ionic strength with NaCl. Spectral studies with acridine orange and ethidium indicate that they also form complexes with cardiolipin but with estimated K_d values that are 6 and 40 times higher, respectively, than that of the daunorubicin complex. Isosbestic points in visible spectra of the three intercalating agents suggest the possibility of specific orientations of the chromophores with respect to the phospholipid. Hypochromic–bathochromic shifts further suggest π-electron interactions between the chromophores and cardiolipin. It is this interaction that seems likely to explain the affected thiobarbituric acid reaction and the delayed formation of Dm 90. It also seems likely that formation of Dm 90 involves malondialdehyde or other conjugated carbonyl products of lipid peroxidation [12] which may react with the amino group of daunosamine to yield a Schiff base [13].

In other studies, Handa and Sato [14] and Bachur *et al.* [15] demonstrated anthracycline-associated radicals formed during oxidation–reduction reactions with microsomal preparations. It is noteworthy that the conditions of formation of Dm 90 (low ionic strength, neutral pH) are similar to those that may exist within plasma and microsomal or other intracellular membranes where negatively charged phospholipids may be relatively abundant. Results of the present communication suggest that membranal phospholipids may be both sites of binding and nonenzymic transformation of the anthracyclines. Since the transformation apparently occurs in conjunction with peroxidation, the newly formed compounds (e.g., Dm 90) may trap radicals and thereby become activated within membranes. This hypothesis seems to be consistent with observations that cardiotoxicity due to adriamycin and daunorubicin appear to be ameliorated by membrane-bound ubiquinone [16] and by radical scavenging agents such as α-tocopherol [17]. It seems reasonable at the present time to postulate that formation of Dm 90 (or similar products with other anthracyclines) may occur in heart tissue. The amount and rate of formation, however, might depend upon endogenous lipids or quenching systems or both. This speculation suggests that depletion or inactivation of the radical quenching systems (as by irradiation) may have a role in the formation of Dm 90. Whether Dm 90 contributes to cardiac toxicity, however, is not yet known.

The apparent high lipophilicity of Dm 90 suggests that it may have a variety of effects within membranes [5, 6], possibly contributing to the selectivity of the parent compound [18]. Although the present observations are concerned primarily with cardiolipin, the reaction may not be limited to this phospholipid but may have implications with other unsaturated lipids from which carbonyl radicals are liberated. Thus, the reactions may not be confined to mitochondrial membranes. Also, because other anthracycline derivatives having free amino groups may form the addition compounds, the proposed Schiff base mechanisms is not limited to daunorubicin alone. These observations, within this broader context, may contribute to a better understanding of the mechanisms of toxicity and selectivity of the antitumor anthracycline drugs, extending, perhaps, to other agents with free amino groups that intercalate DNA.

Acknowledgements—Competent assistance by M. J. Berrigan and I. W. Harper is gratefully acknowledged.

REFERENCES

1. M. Duarte-Karim, J. M. Ruysschaert and J. Hildebrand, Affinity of adriamycin to phospholipids, a possible explanation for cardiac mitochondrial lesions. *Biochem. biophys. Res. Commun.* **71,** 658 (1976).

2. E. A. Lefrak, J. Pitha, S. Rosenheim and J. A. Gottlieb, A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer (Philad.)* **32,** 302 (1973).
3. R. S. Jaenke, An anthracycline antibiotic-induced cardiomyopathy in rabbits. *Lab. Invest.* **30,** 292 (1974).
4. H. M. Olson and C. C. Capen, Subacute cardiotoxicity of adriamycin in the rat. *Lab. Invest.* **37,** 386 (1977).
5. S. A. Murphree, L. S. Cunningham, K. M. Hwang and A. C. Sartorelli, Effects of adriamycin on surface properties of sarcoma 180 ascites cells. *Biochem. Pharmacol.* **25,** 1227 (1976).
6. G. Schioppacassi and H. S. Schwartz, Membrane actions of daunorubicin in mammalian erythrocytes. *Res. Commun. Chem. Pathol. Pharmacol.* **18,** 519 (1977).
7. C. Gitler, B. Rubalcava and A. Coswell, Fluorescence changes of ethidium bromide on binding to erythrocytes and mitochondrial membranes. *Biochem. biophys. Acta (Amst.)* **197,** 479 (1969).
8. A. Azzi and M. Santato, Interaction of ethidium with the mitochondrial membrane. *Biochem. Res. Commun.* **44,** 211 (1971).
9. R. M. Fico, T. K. Chen and E. S. Canellakis, Bifunctional intercalators: relationship of antitumor activity of diacridines to the cell membrane. *Science* **198,** 53 (1977).
10. A. Blake and A. R. Peacocke, The interaction of aminoacridines with nucleic acids. *Biopolymers* **6,** 1225 (1968).
11. L. K. Dahle, E. G. Hill and R. T. Holman, The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. *Arch. Biochem.* **98,** 253 (1962).
12. T. W. Kwon and H. S. Olcott, Malondialdehyde from the autoxidation of methyl linoleate. *Nature (Lond.)* **210,** 214 (1966).
13. K. S. Chio and A. L. Tappel, Synthesis and characterization of the fluorescent products derived from malondialdehyde and amino acids. *Biochemistry* **8,** 2821 (1969).
14. K. Handa and S. Sato, Generation of free radicals in NADPH-microsome system. *Gann* **66,** 43 (1975).
15. N. R. Bachur, S. L. Gordon and M. V. Gee, Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Molec. Pharmacol.* **13,** 901 (1977).
16. C. Bertazzoli, L. Saba, E. Solcia and M. Ghione, Experimental adriamycin cardiotoxicity prevented by ubiquinone *in vivo* in rabbits. *IRCS med. Sci.* **3,** 468 (1975).
17. C. E. Meyers, W. P. McGuire, R. H. Liss, I. Ifrim, K. Grotzinger and R. C. Young, Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science* **197,** 165 (1977).
18. H. S. Schwartz, G. Schioppacassi and P. M. Kanter, *Antibiot. and Chemother.* **23,** 247 (1978).