MAMMALIAN AND MICROBIAL CELL-FREE CONVERSION OF ANTHRACYCLINE ANTIBIOTICS AND ANALOGS

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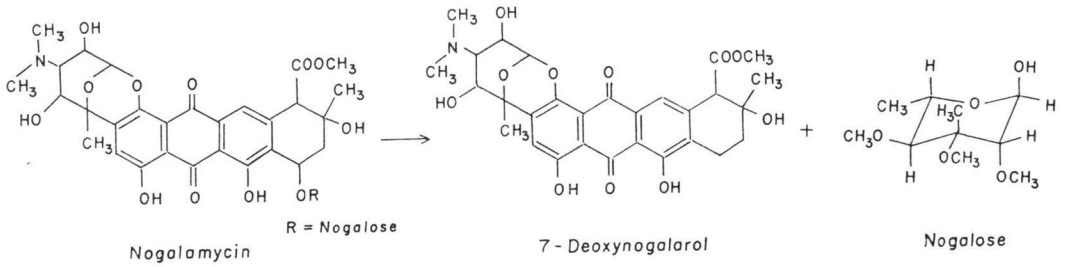
Cell-free preparations of *Streptomyces nogalater* and rat liver catalyze reduced pyridine nucleotide dependent conversion of nogalamycin to 7-deoxynogalarol and nogalose (Scheme 1). The mammalian process requires TPNH and has a specific activity of 85 nmoles of 7-deoxynogalarol formed per hour per mg of protein while the bacterial process prefers DPNH and has a specific activity of 5. The oxygen-sensitive conversions have pH optima of 7.5 (rat) and 9 (*S. nogalater*). Other anthracycline substrates converted to their 7-deoxyaglycones by both systems include nogamycin, 7(R)-O-methylnogarol, 7(R)-O-methylnogalarol, doxorubicin (Adriamycin), steffimycin, and steffimycin B.

Almost all anthracycline antibiotics have antitumor activity and at least two of these, doxorubicin and daunorubicin (Adriamycin and Daunomycin), have been proven effective in the treatment of various neoplasias[1,2]. However, dosage of these drugs must be limited to prevent development of cardiotoxicity[3,4]. The anthracycline antibiotic nogalamycin[5] has been studied extensively for the purpose of making analogs with an improved therapeutic ratio[6,7]. Microbial transformations of nogalamycin may offer a limited means to achieve this. Such microbial transformations of anthracyclines previously reported include keto group reductions[8~12], reductive glycosidic cleavages[10,12~15] and an acylation[16].

Mammalian cells and their enzyme preparations have been shown to catalyze reductive glycosidic cleavage[17~21] and keto-group reductions[17,20,22] of several anthracyclines. However, under aerobic conditions rat liver microsomal preparations convert some anthracyclines to free radical forms which are suspected of having a role in anthracycline mediated cardiotoxicity[23~26].

In addition to our primary purpose of producing analogs of nogalamycin with improved properties, we consider the comparison of the bacterial and mammalian metabolism of nogalamycin to be inherently interesting.

Scheme 1.



Methods

1. Microbiological

S. nogalater, UC®-2783, was maintained on sterile soil and was cultured in a seed medium described by ARCAMONE *et al.*[27]. The organism was aerobically cultured for 72 hours at 28°C in the primary seed stage, transferred (5 ml/100 ml) to fresh seed media, and cultured for 48 hours. The latter cultures were used as inoculum (10 ml/100 ml) for a medium (TYG) composed of tryptone, 5 g; yeast extract, 3 g; and glucose, 20 g; per liter of deionized water. All cultures were grown in volumes of 100 ml in 500-ml wide-mouth Erlenmeyer flasks at 28°C and were shaken at 250 rpm. Following 48 hours of growth in TYG medium, the mycelia were harvested by centrifugation at $5,000 \times g$ (4°C), washed once in saline (0°C) and stored at −20°C.

2. Biochemical

Cell-free extracts of TYG-grown *S. nogalater* were prepared by 5-minute sonic disruption (Raytheon, 10 kc sonic oscillator) of 10 g of cell paste in 10 ml of 33 mM potassium phosphate buffer, pH 7.4, containing 100 μmoles of β-mercaptoethanol. The sonicated material was centrifuged (4°C) at $10^4 \times g$ for 10 minutes and the supernatant fluid was used as the cell-free extract (bacterial crude enzyme preparation).

Homogenates were prepared from livers removed from male Sprague-Dawley rats (200～250 g) sacrificed by cervical dislocation. The livers were homogenized (Brinkman-Polytron) in 2 volumes of the previously described buffer–β-mercaptoethanol mixture at 0°C and centrifuged twice at $10^4 \times g$ for 20 minutes. The supernatant fluid was used as the rat liver homogenate (mammalian crude enzyme preparation).

The standard 1-ml assay system used throughout the study contained 0.4 μmoles of anthracycline substrate, 300 μmoles of potassium phosphate, pH 7.5～8.5, and 10 μmoles of β-mercaptoethanol. Microbial reaction mixtures contained cell-free extract at levels up to 25 mg protein and 1.5 μmoles of DPNH. Mammalian reaction mixtures contained rat liver homogenate at levels up to 2 mg protein and 1.5 μmoles of TPNH. Microbial reactions were conducted at 25°C for 6 hours, while mammalian reactions were run at 37°C for 0.33 hours. Reaction mixtures were statically incubated in open glass test tubes (16×125 mm). All reactions were initiated by addition of the crude enzyme and were terminated by quick freezing in a dry ice-chloroform mixture. The anthracycline substrates were added in solution using dimethylformamide as the solvent (15 mg/ml). Control tubes containing all reaction components except either substrate or crude enzyme were employed for each experiment.

3. Analytical

The reaction mixtures were thawed and extracted using 4-ml volumes of $CHCl_3$. Phase separation was attained by centrifugation at $2,000 \times g$ for 5 minutes. Two μl aliquots of the $CHCl_3$ layers were chromatographed on E. Merck silica gel 60 tlc plates and were developed to a distance of 12 cm in solvent system 1. All extraction and separation steps were carried out in the dark to prevent light-dependent decomposition of the nogalamycin analogs. Separated materials were then quantitated by scanning fluorescence densitometry in the manner of WATSON and CHAN[28] using a Schoeffel SD-3000 spectrodensitometer in the reflectance mode. The instrument monochromator was set at 437 nm and the reflected light was passed through a No. 3–69 cutoff filter. Standard curves for each product were prepared daily by spotting known quantities (20～160 ng) of drug standards on the plates and developing and scanning these as described above.

Protein levels were determined according to the method described by LOWRY *et al.*[29], using bovine serum albumin as a standard.

Chemical Methods

Thin-Layer Systems

1. $CHCl_3$ – CH_3OH – H_2O – CH_3COOH (78: 20: 2: 0.025)
2. $CHCl_3$ – CH_3OH – H_2O – CH_3COOH (60: 30: 5: 5)

3. $CH_3CN - CHCl_3 - CH_3OH - H_2O - CH_3COOH$ (20: 55: 15: 5: 5)
4. $CH_3COCH_2CH_3 - CH_3COCH_3 - H_2O - NH_4OH$ (70: 20: 10: 0.5)
5. $C_6H_{12} - CHCl_3 - CH_3COOCH_2CH_3 - CH_3CH_2OH - H_2O - CH_3CN$ (25: 25: 25: 25: 1: 5)
6. $CHCl_3 - CH_3OH$ (925: 75)
7. $CHCl_3 - CH_3OH$ (95: 5)
8. $CHCl_3 - CH_3OH - H_2O$ (78: 20: 2)
9. $CH_3COOCH_2CH_3 - CH_3CH_2OH - H_2O$ (92: 5: 3)
10. $C_6H_{12} - CH_3COOCH_2CH_3 - CH_3CH_2OH$ (5: 3: 2)
11. $CH_3OH - H_2O - CH_3COOH$ (55: 40: 5)
12. $CHCl_3 - CH_3CH_2OH - H_2O$ (4: 5: 1)

Identification of 7-Deoxyaglycones by tlc

All reactions in the substrate specificity experiment were conducted and extracted as previously described. Drug metabolites from the substrates tested were separated in the systems, and the Rf values were compared with those of authentic standards (Table 1). The Rf values for experimentally derived 7-deoxyaglycones were identical or nearly identical to those of the corresponding authentic standards in every instance.

7-Deoxynogalarol

(**1**) From *S. nogalater* Cell-free Extract. A 250-ml *S. nogalater* cell-free extract conversion of 75 mg of nogalamycin was mixed with 12.5 mg of filter-aid and filtered. The filter cake was washed with 50 ml of water. The combined filtrate and washings were extracted with four 125-ml portions of $CHCl_3$, and the filter cake was extracted with five 125-ml portions of $CHCl_3$. All of the extracts were combined and evaporated to dryness *in vacuo*. The residue was triturated with a few ml of mixed hexanes. After the insoluble material had settled, the supernatant was decanted. The dried residue weighed 194 mg. The residue was chromatographed on 20 g of silica gel using $CH_2Cl_2 - CH_3OH$ (9: 1) collecting one hundred and fifty 5-ml fractions. Those fractions (34~75) containing 7-deoxynogalarol as determined by tlc in system 8 were combined and evaporated *in vacuo*, yield 21 mg (40%). The residue had the same Rf as authentic 7-deoxynogalarol in system 8, system 11 (Rf 0.39), and system 12 (Rf 0.34). Mass spectrum, *m/e* 569.

(**2**) From Cell-free Homogenized Rat Liver. A 250-ml cell-free homogenized rat liver conversion of 75 mg of nogalamycin was adjusted to pH 2.3 with conc. HCl. Filter-aid (12.5 g) was added, and the mixture was filtered followed by washing with 50 ml of water. The combined filtrate and washings was extracted with three 62.5-ml portions of *n*-BuOH. The combined extracts were evaporated *in vacuo* to a thick oil. The residue was dissolved in 30 ml of water, and the solution was adjusted to pH 7.4 with 1 N NaOH and extracted with three 25-ml portions of CH_2Cl_2. The extracts were combined and evaporated to dryness *in vacuo*, yield 85 mg. The product was chromatographed on 10 g of silica using $CH_2Cl_2 - CH_3OH$ (9: 1), collecting fifty-two 5-ml fractions. Those fractions (19~30) containing 7-deoxynogalarol on the basis of tlc in system 8 were combined and evaporated to dryness *in vacuo*. The residue was chromatographed by preparative tlc using solvent system 8. The 7-deoxynogalarol band was extracted to give 19 mg which had the same Rf value as 7-deoxynogalarol by tlc in systems 8, 11, and 12. Mass spectrum, *m/e* 569.

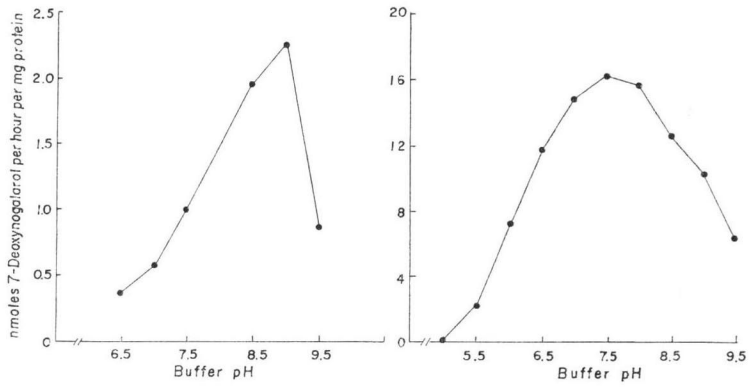
Results and Discussion

Cell-free preparations of *S. nogalater* and of rat liver were found to convert nogalamycin and several additional anthracycline compounds by reduction to their 7-deoxyaglycones. In the cases of nogalamycin conversion by both enzyme preparations, 7-deoxynogalarol was isolated by chromatography on silica. The purified material was characterized on the basis of chromatographic mobility in three solvent systems (systems 8, 11, and 12), as well as by a mass spectrum. Nogalose was not isolated, but it was assumed to have been formed.

The rates of conversion of nogalamycin to 7-deoxynogalarol by both systems were investigated as functions of buffer pH using potassium phosphate added at 0.5 pH unit increments over a range of pH 5~10. All reactions were conducted as described in the "Methods" section. Fig. 1 presents the

data for reductive conversion of nogalamycin catalyzed by crude enzyme preparations of *S. nogalater*. The resulting curve is roughly triangular in shape; the optimal buffer pH is between pH 8.5 and 9.0. Data for reductive cleavage of nogalamycin by rat liver homogenate studied as a function of pH are also presented in Fig. 1. This curve is roughly bell-shaped with a buffer pH optimum between pH 7.0 and 8.0.

Fig. 1. Effect of buffer pH on bacterial (left panel) and mammalian (right panel) reductive conversion of nogalamycin.
Reaction conditions are described in the text.



The conversion of nogalamycin to 7-deoxynogalarol catalyzed by *S. nogalater* crude enzyme preparations was investigated as a function of crude enzyme protein addition. Fig. 2 presents these data as a linear function from 0 to 26 mg protein added per ml. Similarly, Fig. 2 presents the corresponding data for 7-deoxynogalarol formation catalyzed by the rat liver homogenate. The reaction rate increases linearly from 0 to 5 mg protein added per ml. These results indicate that the reaction rates in each instance are directly proportional to the amount of protein added within concentration ranges tested.

Fig. 2. Conversion of nogalamycin to 7-deoxynogalarol by bacterial (left panel) and mammalian (right panel) enzyme preparations studied as a function of crude enzyme addition.
Experimental conditions are described in the text.

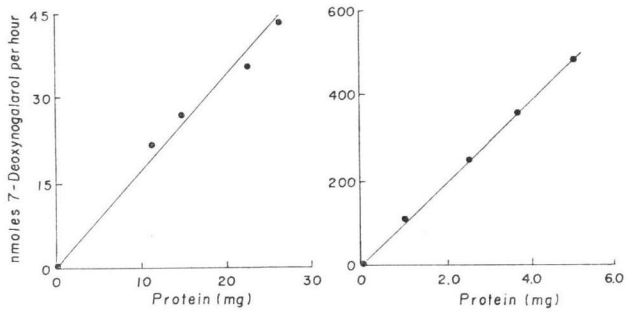


Fig. 3. Reduced pyridine nucleotide dependent metabolism of nogalamycin by bacterial (left panel) and mammalian (right panel) enzyme preparations.

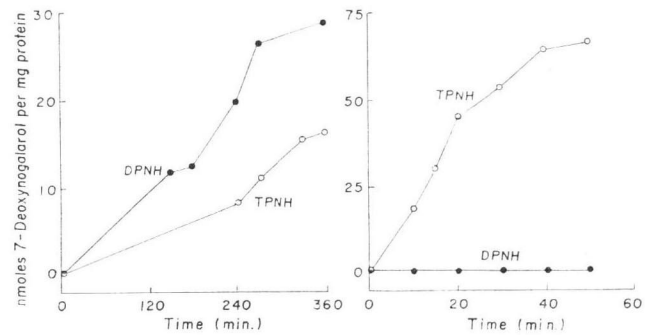


Fig. 3 presents the reduced pyridine nucleotide requirement for the reductive cleavage of nogalamycin by the *S. nogalater* cell-free extracts. The reactions were allowed to proceed 6 hours and were assayed at 0.5-hour intervals. Although activity is present with either cofactor, DPNH is superior to TPNH as an assay component. No product is formed in the absence of added reduced pyridine nucleotide. Under standard assay conditions and in the presence of added DPNH, the rate of product formation is ap-

proximately linear through 4.5 hours. Previous studies on the reductive cleavage of nogalamycin and other anthracyclines by cell-free extracts of *Aeromonas hydrophila* have also demonstrated a higher rate of conversion with DPNH than with TPNH[13,14]. Reductive cleavage of daunorubicin by crude enzyme preparations of *Streptomyces steffisburgensis* has an absolute requirement for DPNH[11].

Fig. 3 additionally presents the reduced pyridine nucleotide requirement for reductive conversion by rat liver homogenate. The reaction was allowed to proceed 50 minutes under standard conditions and was assayed at 10-minute intervals. No product was formed after the addition of DPNH or in the absence of reduced pyridine nucleotide. In the presence of TPNH, the reaction rate appeared diphasic, with a considerable reduction in rate occurring at 20 minutes. Maximum levels of 7-deoxynogalarol were obtained by 40 minutes. The TPNH requirement for reductive conversion by rat liver homogenate is consistent with previous studies which demonstrated TPNH-linked reductive cleavage of daunorubicin and doxorubicin in mammalian systems[18,19]. OKI *et al.*[24] have shown that the enzyme in rat liver microsomes which catalyzes the reductive cleavage of anthracycline antibiotics is identical with the microsomal TPNH-cytochrome c reductase. It would be of interest to more clearly define the nature and role of the DPNH-linked reductive glycosidase in microbial cells.

Reductive conversion of nogalamycin by both microbial and mammalian crude enzyme preparations was limited in reaction mixtures carried out under highly aerobic conditions. Open tubes containing the standard assay components with either bacterial cell-free extract or rat liver homogenate were incubated (shaken at 250 rpm) 6 hours and 1 hour, respectively. No 7-deoxynogalarol was detected. Static reaction mixtures incubated for the corresponding time intervals resulted in the formation of 7-deoxynogalarol at normal levels. Previous studies have demonstrated a similar inhibitory effect by oxygen on the reductive conversion of anthracycline substrates by whole bacterial cells[13], by microbial cell-free extracts[13,14], and by rat liver microsomal preparations[19,24], including a purified preparation of microsomal TPNH-cytochrome c reductase[24].

Substrate specificities of the microbial and mammalian crude enzyme preparations were determined (Table 2). The substrates tested were nogalamycin, 7-(R)-O-methylnogalarol, nogamycin, 7-(R)-O-methylnogarol, steffimycin, steffimycin B, and doxorubicin. (The Rf values for the conversion products are presented in Table 1.) In both instances, steffimycin and steffimycin B were poor sub-

Table 1. Rf values for 7-deoxyaglycone standards in five solvent systems.

	Rf Value				
Solvent system	1	2	3	4	5
7-Deoxynogalarol	0.34	0.68	0.29	0.30	0.36
7-Deoxynogarol	0.26	0.66	0.27	0.30	0.36
Solvent system	6	7	8	9	10
7-Deoxysteffimycinone	0.65	0.58	0.80	0.61	0.52
7-Deoxyadriamycinone	0.36	0.24	0.66	0.52	0.42

Table 2. Specific activities of bacterial and mammalian crude enzyme preparations on anthracycline substrates.

Reaction conditions are described in the text.

Drug	Specific activity (nmol/hr/mg protein)	
	Bacterial	Mammalian
Nogalamycin	5.2	84.1
7(R)-O-Methylnogalarol	4.2	148.2
Nogamycin	3.2	73.0
7(R)-O-Methylnogarol	2.0	57.2
Steffimycin	1.0	26.5
Steffimycin B	0.3	30.6
Doxorubicin (Adriamycin)	3.8	1,055.2

strates for reductive conversion. The bacterial cell-free extracts utilized each of the other antibiotics to roughly the same extent. In contrast, the rat liver homogenate catalyzed the reductive cleavage of doxorubicin far more effectively than the other substrates tested. Interestingly, microsomal preparations tested previously[21] were reported to be unable to catalyze the reductive conversion of nogalamycin and steffimycin to their 7-deoxyaglycones. This discrepancy could be due to differences in the catalytic properties of liver microsomal preparations and crude liver homogenate.

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References

1) BOIRON, M.; C. JACQUILLAT, M. WEIL, M. THOMAS & J. BERNARD: Treatment of acute granulocytic leukemia with rubidomycin. Pathol. Biol. 15: 921～924, 1967
2) JACQUILLAT, C.; Y. NAJEAN, R. FANZER, M. WEIL, M. BOIRON & J. BERNARD: Treatment of acute lymphoblastic leukemia with rubidomycin. Pathol. Biol. 15: 913～918, 1967
3) LEFRAK, E. A.; J. PITHA, S. ROSENHEIM & J. A. GOTTLIEB: A clinicopathologic analysis of adriamycin cardiotoxicity. Cancer 32: 302～314, 1973
4) BONODONNA, G. & S. MONFARDINI: Cardiac toxicity of daunorubicin. Lancet 1969-1: 837, 1969
5) BHUYAN, B. K. & A. DIETZ: Fermentation, taxonomic, and biological studies of nogalamycin. Antimicr. Agents & Chemoth. 1965: 836～844, 1966
6) WILEY, P. F.; R. B. KELLY, E. L. CARON, V. H. WILEY, J. H. JOHNSON, F. A. MACKELLAR & S. A. MIZSAK: Structure of nogalamycin. J. Am. Chem. Soc. 99: 542～549, 1977
7) WILEY, P. F.; J. L. JOHNSON & D. J. HOUSER: Nogalamycin analogs having improved antitumor activity. J. Antibiotics 30: 628～629, 1977
8) FELSTED, R. G.; M. GEE & N. R. BACHUR: Rat liver daunorubicin reductase. J. Biol. Chem. 249: 3672～3679, 1974
9) KARNETOVA, J.; J. MATEJU, P. SEDMERA, J. VAKOUN & Z. VANELC: Microbial transformation of daunomycin by *Streptomyces aureofaciens* B-96. J. Antibiotics 29: 1199～1202, 1976
10) MARSHALL, V. P.; E. A. REISENDER & P. F. WILEY: Bacterial metabolism of daunomycin. J. Antibiotics 29: 966～968, 1976
11) ASZALOS, A. A.; N. R. BACHUR, B. K. HAMILTON, A. F. LANGLYKKE, P. P. ROLLER, M. Y. SHEIKH, M. S. SUTPHIN, M. C. THOMAS, D. A. WAREHEIM & L. H. WRIGHT: Microbial reduction of the side-chain carbonyl of daunorubicin and N-acetyldaunorubicin. J. Antibiotics 30: 50～58, 1977
12) MARSHALL, V. P.; J. P. MCGOVREN, F. A. RICHARD, R. E. RICHARD & P. F. WILEY: Microbial metabolism of anthracycline antibiotics daunomycin and adriamycin. J. Antibiotics 31: 336～342, 1978
13) WILEY, P. F. & V. P. MARSHALL: Microbial conversions of anthracycline antibiotics. J. Antibiotics 28: 838～840, 1975
14) MARSHALL, V. P.; E. A. REISENDER, L. M. REINEKE, J. H. JOHNSON & P. F. WILEY: Reductive microbial conversion of anthracycline antibiotics. Biochemistry 15: 4139～4145, 1976
15) WILEY, P. F.; J. M. KOERT, D. W. ELROD, E. A. REISENDER & V. P. MARSHALL: Bacterial metabolism of anthracycline antibiotics: steffimycinone and steffimycinol conversions. J. Antibiotics 30: 649～654, 1977
16) HAMILTON, B. K.; M. S. SUTPHIN, M. C. THOMAS, D. A. WAREHEIM & A. A. ASZALOS: Microbial N-acetylation of daunorubicin and daunorubicinol. J. Antibiotics 30: 425～426, 1977
17) BACHUR, N. R. & M. GEE: Daunorubicin metabolism by rat tissue preparations. J. Pharmacol. Exp. Ther. 177: 567～572, 1971
18) ASBELL, M. A.; E. SCHWARTZBACH, F. J. BULLOCK & D. W. YESAIR: Daunomycin and adriamycin metabolism via reductive glycosidic cleavage. J. Pharmacol. Exp. Ther. 182: 63～69, 1972
19) BACHUR, N. R. & M. GEE: Oxygen sensitive reductive cleavage of daunorubicin and adriamycin. Fed.

Proc. 31: 835, 1972
20) TAKANASHI, S. & N. R. BACHUR: Daunorubicin metabolites in human urine. J. Pharmacol. Exp. Ther. 195: 41~49, 1975
21) BACHUR, N. R. & M. GEE: Microsomal reductive glycosidase. J. Pharmacol. Exp. Ther. 197: 681~686, 1976
22) BACHUR, N. R.: Cytoplasmic aldo-keto reductases: A class of drug metabolizing enzymes. Science 193: 595~597, 1976
23) HANDA, K. & S. SATO: Stimulation of microsomal NADPH oxidation by quinone group containing anticancer chemicals. Gann 67: 523~528, 1976
24) OKI, T.; T. KOMIYAMA, H. TONE, T. INUI, T. TAKEUCHI & H. UMEZAWA: Reductive cleavage of anthracycline glycosides by microsomal NADPH-cytochrome C reductase. J. Antibiotics 30: 613~615, 1977
25) SATO, S.; M. IWAISUMI, K. HANDA & Y. TAMURA: Electron spin resonance study on the mode of generation of free radicals of daunomycin, adriamycin, and carboquone in NAD(P)H-microsome system. Gann 68: 603~608, 1977
26) BACHUR, N. R.; S. GORDON & M. GEE: Enzymatic activation of quinone anticancer agents to free radicals. Fed. Proc. 37: 561, 1978
27) ARCAMONE, F.; G. CASSINELLI, G. FANTINI, A. GREIN, P. OREZZI, C. POL & C. SPALLA: Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius* var. *caesius*. Biotech. Bioeng. 11: 1101~1110, 1969
28) WATSON, E. & K. K. CHAN: Rapid analytical method for adriamycin and metabolites in human plasma by a thin-film fluorescence scanner. Cancer Treat. Rep. 60: 1611~1618, 1976
29) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193: 265~275, 1951