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Reductive Microbial Conversion of Anthracycline Antibiotics†

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ABSTRACT: Reductive conversion of several anthracycline glycosides to their 7-deoxyaglycones occurs during their microaerophilic incubation with strains of *Aeromonas hydrophila, Citrobacter freundii,* and *Escherichia coli.* Further, extracts of microaerophilically grown *A. hydrophila* catalyze DPNH-dependent reductive conversion of the same compounds. Anthracycline substrates cleaved by both whole cells and by the cell-free system include steffimycin, steffimycin B, nogalamycin, cinerubin A, and daunomycin. Investigation of glycoside cleavage as a function of both time and anthracycline concentration demonstrated the superiority of *A. hydrophila* over *C. freundii* and *E. coli* in regard to reaction rate and efficiency of conversion. Interestingly, some degree of anaerobicity was required for glycoside cleavage by all three organisms. Evidence supporting 7-deoxyaglycone formation via direct reductive cleavage, as opposed to a multienzyme-catalyzed process involving hydrolysis followed by dehydration and reduction, includes the following. Equilibrium mixtures of glycoside substrate and 7-deoxyaglycone product prepared using both whole cells and their extracts display no anthracycline hydrolysis products. Further, authentic steffimycinone (aglycone), the expected product of hydrolytic sugar cleavage of steffimycin, was shown to be converted to 7-deoxysteffimycinone (7-deoxyaglycone) at a rate slower than steffimycin. These data indicate that, if steffimycinone were present as an unbound metabolic intermediate, it should have been visible in the equilibrium mixture, but none was detected.

Several members (Jacquillat et al., 1967; Boiron et al., 1967) of the anthracycline series of antibiotics (Table I and eq 1) are considered to be effective clinical agents in the treatment of various cancers. One of them, adriamycin, has received considerable attention following its proven efficacy in the treatment of solid tumors (Arcamone et al., 1969). However, the latter drug after prolonged dosage is cardiotoxic. Because of adriamycin's usefulness in the treatment of solid tumors, the research reported here was initiated to generate metabolites of various anthracycline drugs. It was hoped that these compounds, some of them adriamycin analogues, would possess adriamycin's effects on solid tumors but show diminished toxicity toward normal tissue.

Previous studies of anthracycline reductive metabolism deal with the catabolism of daunomycin (Huffman et al., 1972; Bachur and Gee, 1972; Asbell et al., 1972; Felsted et al., 1974) and its hydroxylated derivative, adriamycin (Bachur and Gee, 1972; Asbell et al., 1972; Benjamin et al., 1972). In these previously reported studies, the metabolic systems investigated were of mammalian origin. Recently, we have investigated the microbial metabolism of daunomycin as well as the metabolism of the additional anthracyclines, nogalamycin, steffimycin, steffimycin B, and cinerubin A, using microaerophilically grown *A. hydrophila, C. freundii,* and *E. coli.* Additionally, cell-free extracts of microaerophilically grown *A. hydrophila* were tested. The predominant metabolites isolated from bacterial anthracycline catabolism were 7-deoxyaglycones. Interestingly, these reduced aglycones have been found as anthracycline metabolites in several mammalian systems (Huffman et al., 1972; Bachur and Gee, 1972). Mammalian cell-free, enzyme catalyzed conversion of daunomycin and adriamycin to their 7-deoxyaglycones was demonstrated to be TPNH[1] dependent (Bachur and Gee, 1972; Asbell et al., 1972). The bacterial reductive glycosidase investigated in these studies uses DPNH much more efficiently than it does TPNH.

Materials and Methods

Chemical and Isolation Methods. The following thin-layer chromatography (TLC) solvent systems were used with silica gel plates:

system 1	chloroform–methanol (95:5)
system 2	cyclohexane–ethyl acetate–95% ethanol (50:30:20)
system 3	methyl ethyl ketone–acetone–water (70:20:11)

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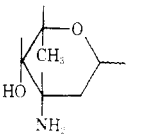
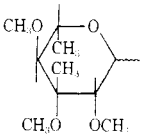
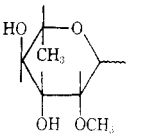
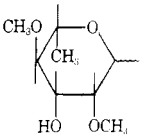
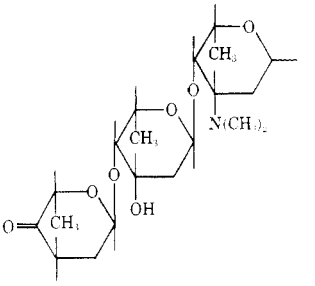
[1] Abbreviations used: TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TLC, thin-layer chromatography; ir, infrared; NMR, nuclear magnetic resonance.

TABLE I: Structure of Anthracycline Substrates for Reductive Glycosidase.

Anthracycline	Substitution at Ring Position								R
	A	B	C	D	E	W	X	Y	
Daunomycin	H	H	CH_3O	HO	HO	H, H	CH_3CO	H	
Nogalamycin	$C_6H_{15}NO_4$		HO	H	HO	H, $COOCH_3$	CH_3	H	
Steffimycin	H	CH_3O	HO	H	HO	=O	CH_3	CH_3O	
Steffimycin B	H	CH_3O	HO	H	HO	=O	CH_3	CH_3O	
Steffimycinone	H	CH_3O	HO	H	HO	=O	CH_3	CH_3O	H
Cinerubin A	HO	H	HO	H	HO	H, $COOCH_3$	C_2H_5	H	

system 4 benzene–acetone (7:3)
system 5 chloroform–methanol–water (78:20:2)

Steffimycin, Steffimycin B, and Steffimycinone. Isolation of the products of fermentations converting steffimycin and related products was carried out by a common method (with small individual variations) illustrated by the following procedure. In most cases recovered substrate was identified only by TLC, although steffimycin B and steffimycinone were also identified by ir and melting point in at least one experiment for each.

Four liters of fermentation broth in which *A. hydrophila* had been grown microaerophilically in the presence of 800 mg of steffimycin was filtered. The filter cake including the paper was extracted with four 500-ml portions of methylene chloride filtering each extract. The filtrate was extracted with four 1-l. portions of methylene chloride. All of the extracts were combined and concentrated under reduced pressure leaving 1.24 g of solid residue. The product was chromatographed on 124 g of silica gel packed in $CHCl_3$–MeOH (99:1). The column was eluted with the same solvent mixture until one-hundred and eighty 5-ml fractions had been collected. When the starting materials were steffimycin B and steffimycinone, the entire chromatography was carried out using this solvent mixture. Elution was continued, when steffimycin was the starting material, with a mixture of the same solvents in a ratio of 98:2 until 100 more fractions had been collected followed by elution with a 97:3 composition until a total of 600 fractions were collected. These were combined as shown in Table II principally on the basis of color maxima except for pool 2 which was the fraction between two color maxima. Each pool was concentrated under reduced pressure.

TABLE II: Chromatographic Results from Steffimycin Cleavage.

Pool	Fractions	Weight (mg)
1	146–170	353
2	171–265	71
3	266–406	24
4	407–444	2
5	445–582	263

The material from pool 1 was compared with 7-deoxysteffimycinone produced chemically (Kelly, 1974) by TLC using systems 1 and 2. The R_f values were respectively 0.40 and 0.61. In system 1 the product showed a single strong spot moving with 7-deoxysteffimycinone. In system 2 there was a similar strong spot but also a very weak, slightly faster moving component, mp 190–192 °C [reported 191–194 °C (Kelly, 1974)]. Recrystallization from methanol (subsequently acetone proved to be a better recrystallization solvent) gave 259 mg: mp 191–193 °C; TLC (system 1) single component; R_f 0.40; ir (Nujol) 3310, 1695, 1660, 1605, 1585, 1550, 1475 (sh), 1360, 1295, 1180, 1140, 1090, 950, and 770 cm^{-1}; uv_{max} (CH_3OH)

213 nm (ϵ 24 600), 235 (26 500), 282 (22 200), 435 (15 250), essentially the same as that of 7-deoxysteffimycinone (Kelly, 1974).

Anal. (unrecrystallized material) Calcd for $C_{21}H_{18}O_8$: C, 63.31; H, 4.07. Found: C, 63.09; H, 4.55.

In most experiments 7-deoxysteffimycinone was identified only by TLC (usually in two or three systems) and melting point.

The material from pool 5 was identified as steffimycin by TLC using system 2. It showed a single spot having the same R_f value (0.33) as did steffimycin.

The other three fractions were investigated using systems 1-4. The second fraction showed no colored material in the TLC using system 1. The third fraction was analyzed with systems 1-4 but the fourth fraction only with systems 2-4. In systems 2 and 4 fractions three and four contained a component having the same R_f value as steffimycinone, but in system 3 they had no component moving with steffimycinone.

Daunomycin. A microaerophilic fermentation broth using *A. hydrophila* in 4 l. of medium containing 200 mg of daunomycin hydrochloride was filtered. The filter cake was extracted repeatedly with 200-ml portions of $CHCl_3$ until almost no color was being extracted. The extracts were combined and evaporated to dryness under reduced pressure leaving 137 mg of red solid. TLC using systems 1 and 5 showed the absence of daunomycin. The strongest spot had R_f's 0.55 and 0.89, respectively. The residue was chromatographed on 13.7 g of silica gel using a $CHCl_3$-MeOH (98:2) system and collecting twenty-two 5-ml fractions. Fractions 9-13 were combined on the basis of TLC as containing only the fastest moving component of the mixture being chromatographed. The pool thus obtained was evaporated under reduced pressure yielding 85 mg of residue, mp 225-229 °C. Recrystallization from methanol gave 13 mg, mp 229-232 °C [lit. (Arcamone et al., 1970) 229-231 °C]: uv and visible (CH_3OH) max 233 nm (ϵ 30 850), 252 (32 200), 287 (8800), 467 (12 750), 489 (13 780), 525 (8560); NMR δ (DMF-d_7) 2.35 (s, 3 H, CH_3CO), 2-3 (m, 4 H, 2 CH_2), 4.05 (s, 3 H, CH_3O), 7.5-8.0 (aromatic H); mass spectrum *m/e*, low resolution 364 ($-H_2O$), 339 ($-CH_3CO$), 43 ($CH_3C{\equiv}O^+$), high resolution 382.1061 (calcd for 7-deoxydaunomycinone, 382.1057).

Nogalamycin. A microaerophilic fermentation broth using *A. hydrophila* in 16 l. of medium containing 800 mg of nogalamycin was filtered. The filtrate was extracted with three 4-l. portions of ethyl acetate which were then combined and evaporated to dryness under reduced pressure, yield 876 mg. The residue was dissolved in 10 ml of $CHCl_3$ and 50 ml of cyclohexane was added. The precipitate was removed and dissolved in 5 ml of $CHCl_3$. The mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure, yield 273 mg. The residue was chromatographed on 12.5 g of silica gel using the solvent system methyl ethyl ketone-acetone-H_2O (72:20:8) and collecting 5-ml fractions. A weight and TLC analysis of the fractions resulted in combining fractions 6-8 as pool 1 containing a faster moving component, and fractions 9-22 were combined as pool 2 containing a slower moving component. Evaporation of pool 2 under reduced pressure gave 126 mg of residue. TLC in system 5 indicated a single component having the same R_f (0.61) as 7-deoxynogalarol (Bhuyan and Reusser, 1970); NMR ($CDCl_3$) identical with that of 7-deoxynogalarol; mass spectrum *m/e* 569 (M^+) and almost iidentical with that of 7-deoxynogalarol (M^+ 569).

Cinerubin A. A microaerophilic fermentation using *A. hydrophila* in 1 l. of medium containing 50 mg of cinerubin A was filtered. Both the filtrate and the filter cake were extracted with four 250-ml portions of CH_2Cl_2. The extracts were combined and evaporated to dryness under reduced pressure. The residue was mixed with 20 ml of Skellysolve B. After an insoluble material had settled out, the supernatant was decanted. The residue was dried to give 45 mg. The product was chromatographed on 10 g of silica gel using $CHCl_3$-CH_3OH (99:1) and collecting forty-five 5-ml fractions. Fractions 1-20 were combined and evaporated to dryness under reduced pressure, yield 6.9 mg, mp 203-208 °C [lit. (Keller-Schierlein and Richle, 1970) 213-215 °C]; TLC (system 1) single component; R_f 0.75; mass spectrum *m/e* 412 (M^+), 394 ($-H_2O$), 380 ($-CH_3OH$), 365 ($-H_2O$ and $-C_2H_5$), 356 ($-CH_3CH{=}CO$) (calcd for ζ-pyrromycinone, 412).

Analytical Methods. In addition to product isolation, quantitation and characterization discussed in the previous section, a quick method designed for quantitation of the amounts of steffimycin and 7-deoxysteffimycinone present was developed. This method, based on high performance liquid chromatography, allows for 15 min evaluation of fermentations and enzyme-catalyzed reactions. The system employs the Chromatec-2200 chromatographic unit equipped with a Hewlett-Packard Mosely 7128A recorder and Hewlett-Packard 3370B integrator. For separation, a 4-ft column with 2.2-mm internal diameter was used containing Chromasep S silica gel (37-44 μm) developed with $CHCl_3$-CH_3OH-H_2O (94.9:5.0:0.1). The anthracycline, which was monitored at 254 nm (ϵ 16 000), was quantitated on the basis of standard curves made using steffimycin and 7-deoxysteffimycinone in amounts ranging between 0 and 4 μg. The extraction methods employed are discussed elsewhere in this section.

Protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

Microbial Methods. Whole sewage used as a source of facultative bacteria was obtained from the Department of Sewage and Water, Parchment, Michigan. Sewage enrichment cultures containing either steffimycin or nogalamycin at 0.005% were carried out under microaerophilic conditions for 1 to 3 weeks in order to select strains able to metabolize anthracycline drugs. Enrichment media consisted of an inorganic salts base (Conrad et al., 1965) supplemented with 0.1% yeast extract (Difco) with either L-glutamate, succinate, glucose, or nutrient broth (Difco) added at concentrations ranging from 0.1 to 0.3%. To produce the required growth conditions, cultures were made using sealed, glass-stoppered bottles with no air space remaining following inoculation. No reducing or molecular oxygen destroying agents were employed. Incubation temperatures used were between 22 and 42 °C. Microorganisms present following the selection procedure were cloned and identified. Among the cloned organisms, three were able to convert steffimycin and nogalamycin to their 7-deoxyaglycones as did whole sewage. Later, the bacteria were identified as strains of *A. hydrophila, C. freundii,* and *E. coli* (2C, 2A and 2B, respectively).

Microaerophilic culture techniques used during the fermentation experiments were identical with those employed in strain selection. Inoculation rates used throughout the study were between 10 and 20%. Volumes used in the fermentation experiments ranged from 0.13 to 4.0 l. In addition to the culture methods previously discussed, shaken and static flask fermentations were done using the media described, as well as with nutrient broth prepared by the standard method. During the fermentation experiments, the nutritional supplements to the basal media were added at low levels to maintain culture density at a manageable point for product isolation.

TABLE III: Materials Isolated and Yields.

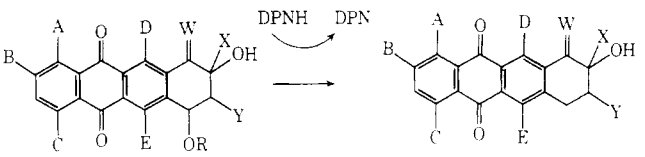
Antibiotic	Conversion Mode	Amount of Substrate (mg)	Recovered Substrate (mg)	Yields of 7-Deoxyaglycone: After Chromatography Wt (mg)	After Chromatography %	Recrystallized Wt (mg)	Recrystallized %
Steffimycin	Mixed sewage organisms	200		86	62	22	16
Steffimycin	*A. hydrophila*	800	263	353	64	259	47
Steffimycin	*C. freundii*	200	122	28	20	10	7.2
Steffimycin	*E. coli*	200	174	26	19	14	10
Steffimycin	*A. hydrophila* cell-free extract	2000	1040	492	35	331	24
Steffimycin B	*A. hydrophila*	200	33	92	68	66	49
Steffimycinone	*A. hydrophila*	200	115	32.6	17	15	7.9
Daunomycin	*A. hydrophila*	200	0	85	57	10	6.7
Nogalamycin	*A. hydrophila*	800	0	126	21.8		
Cinerubin A	*A. hydrophila*	50	0	6.9	27		

The amount of 7-deoxysteffimycinone produced by the fermentations was quantitated using silica gel columns run as described or by the high-performance, liquid chromatographic system discussed earlier in this section.

Biochemical Methods. Cell-free extracts of microaerophilically grown *A. hydrophila* were produced by sonic disruption of 5 g of cell paste in 10-ml of 100 mM potassium phosphate buffer, pH 7.4, with 10 mM β-mercaptoethanol present. Disruption was carried out at 0 °C using a Raytheon 10-kilocycle sonic oscillator. After sonic treatment, the material was centrifuged at $1.5 \times 10^4 g$ for 15 min at 0 °C. The supernatant fluid was used as the cell-free extract. Ten-milliliter volumes of the cell-free extract were dialyzed vs. 1 l. of 50 mM potassium phosphate buffer, pH 7.4, containing 10 mM β-mercaptoethanol changed once during a 24-h period. The dialyzed cell-free extract was used as the crude enzyme source. The standard reaction was run at 37 °C in 2-ml volumes containing 1.5 mM anthracycline substrate, 1.5 mM DPNH, 10 mM β-mercaptoethanol, 100 mM potassium phosphate buffer, pH 8.0 (buffer pH optimum), and dialyzed cell-free extract at levels up to 6 mg/ml. In this range, catalytic activity was directly proportional to the amount of enzyme added. The reaction was initiated by the addition of dialyzed cell-free extract and usually was allowed to run 3 h. All anthracycline substrates tested with the exception of daunomycin hydrochloride were added to the reaction mixtures as dimethylformamide solutions (50 mg/ml). The exception was added as an aqueous solution of identical concentration. Control assays containing all reaction components with the exception of either anthracycline substrate or crude enzyme were performed. All reactions were monitored at 0.5-h intervals with quick freezing in acetone–dry ice mixtures employed to stop the reaction. Experiments were carried out in glass centrifuge tubes suitable for extraction and mixing using 4-ml volumes of $CHCl_3$. The centrifuge tubes were able to withstand a force of $2 \times 10^3 g$ for 5 min employed in phase separation. Aliquots of the $CHCl_3$ layer obtained after centrifugation were taken and used with the previously described pressure chromatographic system in order to determine the amount of 7-deoxysteffimycinone produced.

Results and Discussion

Reaction and Scope. A group of six compounds of which five were anthracycline antibiotics [steffimycin (Bergy and Reusser, 1967), steffimycin B (Brodasky and Reusser, 1974), nogalamycin (Wiley et al., 1968), cinerubin A (Keller-Schierlein and Richle, 1970), and daunomycin (Arcamone et al., 1970)] and one was an aglycone of an anthracycline antibiotic [steffimycinone (Kelly, 1974)] was used as substrates in biochemical and 1–3-day microbiological reductive conversions. Although complete structural elucidations of this group of compounds have been published only for daunomycin (Arcamone et al., 1970) and cinerubin A (Kelly-Schierlein and Richle, 1970), sufficient structural information has been presented concerning the structures of nogalamycin (Wiley et al., 1968) and the steffimycins (Kelly, 1974; Brodasky and Reusser, 1974; Wiley, 1975) to present generalized structures as shown in eq 1 which indicates their conversion to 7-deoxyaglycones (Table I). Compounds represented by ROH (in most cases presumably sugars) were not isolated.



Steffimycin was converted to 7-deoxysteffimycinone with isolation and identification of the products using mixed sewage organisms, *A. hydrophila, E. coli,* and *C. freundii* and cell-free extracts of *A. hydrophila* (Table III). The other five compounds were investigated similarly using *A. hydrophila,* but of these only steffimycinone and daunomycin were subjected to the action of cell-free extracts. The general procedure for isolation of products was extraction of the fermentation media with an organic solvent followed by separation of the extracted material into its components by chromatography on silica gel. The products were identified by the customary techniques (TLC, melting point, uv, ir, NMR, and mass spectra). Not all criteria were applied in any case, but at least two were used. Identification of recovered starting materials was usually by TLC only, but ir and melting point were used in some cases.

The majority of the experiments were carried out with steffimycin subjecting it to all forms of biological conversion as mentioned above. Yields were substantially better with *A. hydrophila* than with any of the other biological systems. Crude yields of 7-deoxysteffimycinone of 64% were obtained with this organism, and this yield combined with recovered steffimycin accounted for about 97% of the sbustrate. The crude material (i.e., directly off the column after evaporation) as judged by TLC was quite pure and was analytically pure in the only experiment in which elemental analysis was done. Recrystallization decreased the yield to 44–49%. The crude yields were lowest, about 20%, with *C. freundii* and *E. coli* and were intermediate using other agents.

TABLE IV: Bacterial Conversion of Steffimycin Studied as a Function of Aeration.

Culture Method	Organism	Anthracycline Composition at 5 Days: Steffimycin (μM)	7-Deoxy-steffimycinone (μM)
Shaken flask	*A. hydrophila*	83	0
	C. freundii	90	0
	E. coli	77	0
Static flask	*A. hydrophila*	6	80
	C. freundii	96	0
	E. coli	90	0
Sealed bottle	*A. hydrophila*	0	70
	C. freundii	0	65
	E. coli	26	40

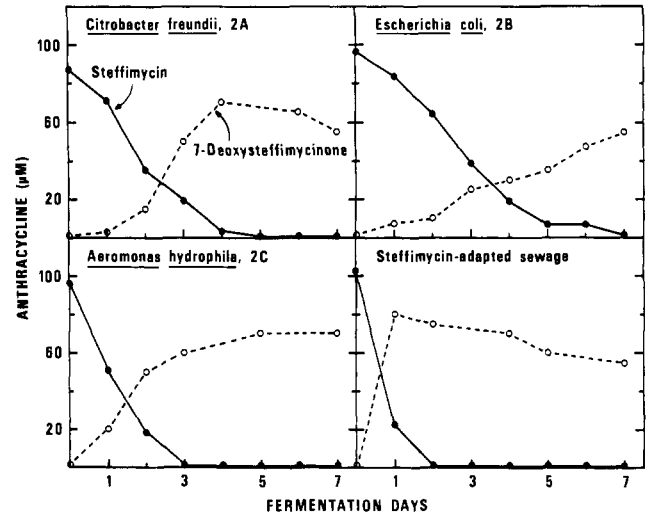


FIGURE 1: Reductive conversion of steffimycin to 7-deoxysteffimycinone by whole cell fermentation. Bacteria were grown microaerophilically at 25 °C using the inorganic salts medium described in the text.

Relatively few experiments were run on the other antibiotics (Table III). In all cases in which product was isolated *A. hydrophila* was used as the microorganism. In those experiments on daunomycin and steffimycinone using cell-free extracts of *A. hydrophila,* the formation of 7-deoxyaglycones was established only by TLC.

The question of the formation of steffimycinone as an unbound intermediate in the conversion of steffimycin to 7-deoxysteffimycinone was investigated. In two experiments, one using *A. hydrophila* and one using cell-free extracts of this organism, the fractions obtained from chromatography were compared in three solvent systems with steffimycinone. No component of any of the column fractions had the same R_f value as did steffimycinone in every solvent system. It was shown in experiments involving conversion of steffimycinone that it was readily separable from 7-dexoysteffimycinone by column chromatography. It is possible that a trace of steffimycinone undetectable visually or with uv was present, but it could have been no more than a trace. In one experiment using *A. hydrophila* as the organism and 800 mg of steffimycin as the substrate there were obtained a yield of 353 mg (64%) of 7-deoxysteffimycinone and a recovery of 263 mg (33%) of steffimycin (Table III). A TLC analysis of each gave no indication of the presence of steffimycinone. Combined yield and recovery was 97% establishing that steffimycinone formation (if any) was less than 3%.

Operational Microorganisms and Their Extracts. Figure 1 demonstrates the rates and efficiencies of steffimycin reductive conversion using whole sewage and isolated sewage organisms. Of the cloned organisms investigated, *A. hydrophila* effects the most rapid steffimycin cleavage with 50% of the steffimycin converted in about 1 day. Following *A. hydrophila* in efficiency of conversion are *C. freundii* (50% conversion at approximately 2 days) and *E coli* (50% conversion at 2.5 days), as compared with whole sewage which causes 50% conversion of steffimycin in less than 1 day. Interestingly, all of the organisms tested caused the complete disappearance of steffimycin following 1 week of incubation. However, in no instance was there complete conversion to 7-deoxysteffimycinone indicating the formation of other reaction products, possibly lower catabolic intermediates (Figure 1). These data, as well as the disappearance of 7-deoxysteffimycinone observed following maximal conversion in the cases of *C. freundii* and whole sewage, suggest further catabolism of steffimycin beyond reductive conversion (Figure 1).

Each microorganism tested required some degree of anaerobicity for steffimycin's reductive cleavage (Table IV). In the instances of shaken flask and sealed reagent bottle cultures, the results were uniform among the different organisms. Shaken flask cultures produced no 7-deoxysteffimycinone, while the sealed reagent bottle cultures were uniformly able to catalyze reductive conversion. However, in the case of static culture, using closures permeable to air, only *A. hydrophila* produced 7-deoxysteffimycinone. These data indicate possible inducer or repressor roles associated with O_2 tension and E_0', or conversely an enzyme sensitivity to various degrees of these factors. However, the crude enzyme preparations are stable to aerobic conditions, when held at 5 °C for 1 week. The latter observation supports the argument for an inducer or repressor mechanism, as opposed to enzyme sensitivity.

In addition to the anaerobic or microaerophilic requirement displayed by whole cell conversion, data presented in Figure 2 demonstrate the analogous requirement in regard to conversion by extracts of *A. hydrophila.* Extracts of microaerophilically grown *A. hydrophila* assayed in the presence of DPNH and steffimycin are three- to fivefold more active than those made from aerobically grown cells assayed under identical conditions. These data also support the contention that O_2 tension and E_0' may play a role in the induction or repression of reductive glycosidase.

Interestingly, media composition does not appear to affect the rate of steffimycin conversion. In addition to the standard inorganic salts medium (Conrad et al., 1965) supplemented with yeast extract and nutrient broth, enrichments were made to the basal medium using compounds known to act as catabolite repressors (i.e., glucose and succinate), as well as L-glutamate which generally has no repressor role. Under these conditions of growth there was no apparent difference in the levels of steffimycin conversion at 36 h irrespective of growth substrates employed. Conversion of steffimycin by *A. hydrophila* at 36 h is close to its half maximum (Figure 1); therefore, the rates of conversion differing from those observed should be visible at this point; however, at 36 h all conversion levels were essentially identical.

Reaction Sequence. Several reaction sequences appear plausible in explaining the mechanism of steffimycin conversion to 7-deoxysteffimycinone. One of them is direct reductive cleavage previously proposed as the mechanism for reductive conversion of daunomycin and adriamycin to their 7-deoxyaglycones as catalyzed by mammalian tissue preparations

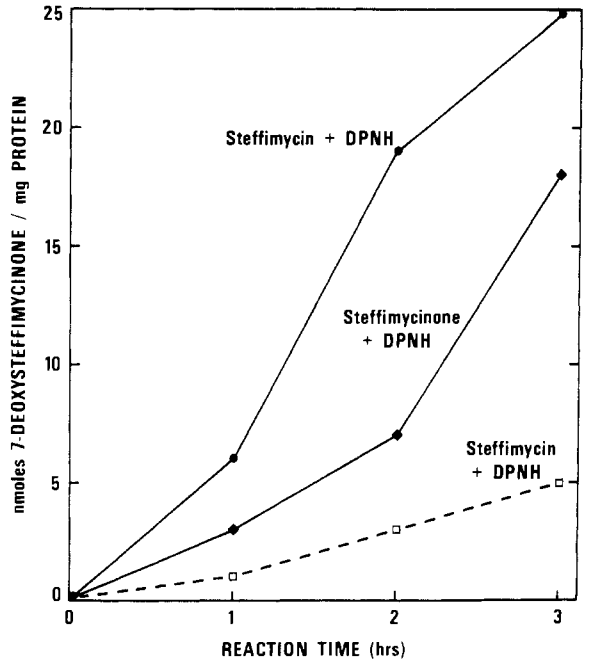


FIGURE 2: Reductive glycosidase activity as a function of anthracycline substrate and anaerobicity of culture. Dialyzed cell-free extracts assayed at 37 °C in the presence of DPNH, 1.5 mM; anthracycline, 1.5 mM; β-mercaptoethanol, 10 mM; and potassium phosphate buffer, pH 8.0, 100 mM. (- - -) Extracts of microaerophilically grown *A. hydrophila*. (—) Extracts of aerobically grown *A. hydrophila*.

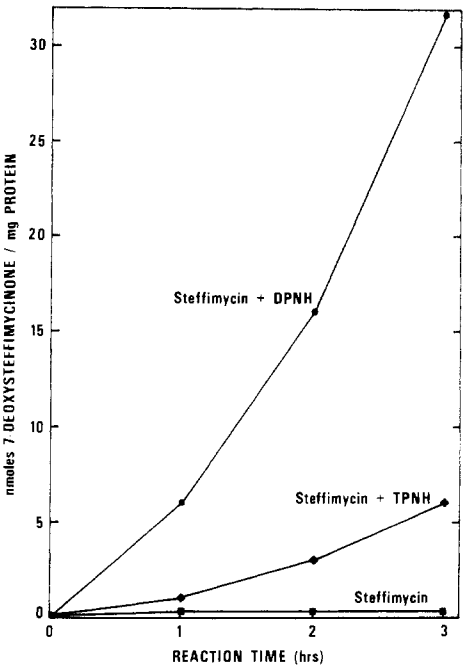


FIGURE 4: Reductive glycosidase activity as a function of reduced pyridine nucleotide addition. Dialyzed cell-free extract of microaerophilically grown *A. hydrophila* assayed at 37 °C in the presence of steffimycin, 1.5 mM; and reduced pyridine nucleotide (●) DPNH, 1.5 mM; (♦) TPNH, 1.5 mM; and (■) no pyridine nucleotide added.

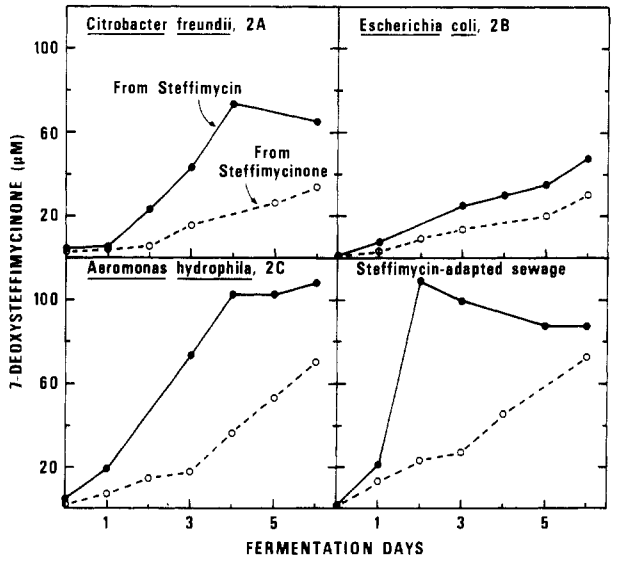


FIGURE 3: Reductive conversion of anthracycline to 7-deoxysteffimycinone by whole cell fermentation. Bacteria were grown microaerophilically at 25 °C in inorganic salts medium containing 125 μM anthracycline.

(Bachur and Gee, 1972). Another reasonable possibility is hydrolytic sugar cleavage followed by dehydration and reduction. A third possibility is a direct reductive conversion of steffimycinone arising from hydrolysis without prior dehydration. Evidence obtained in this study appears to support direct reductive cleavage of steffimycin, primarily because no steffimycinone has been detected in equilibrium mixtures of steffimycin and 7-deoxysteffimycinone prepared by whole cell fermentations and by cell-free enzymic conversion. Further, whole cells (Figure 3) and cell-free extracts (Figure 2) catalyze the conversion of steffimycin to 7-deoxysteffimycinone at a rate equal to or greater than that obtained using steffimycinone as the reaction substrate. These data strongly limit the possibility that unbound steffimycinone is present in equilibrium mixtures only in trace amounts because of its extremely rapid conversion to 7-deoxysteffimycinone. The same mechanism would presumably apply to other anthracyclines.

Direct reductive cleavage by the proposed mechanism apparently derives reducing potential preferentially from DPNH (Figure 4). Dialyzed cell-free preparations of *A. hydrophila* were incubated in the presence of steffimycin either alone or in the presence of DPNH or TPNH. Data presented in Figure 4 demonstrate the absolute requirement for reduced pyridine nucleotide in the reductive conversion of steffimycin, as well as the superiority of DPNH over TPNH as an assay component. Because of the endogenous levels of reduced pyridine and nucleotide and the transhydrogenase activity present in cell-free preparations of *A. hydrophila*, it was necessary to dialyze the cell-free extract as previously described in order to determine the pyridine nucleotide requirement of reductive glycosidase. Interestingly, the mammalian reductive glycosidase requires TPNH (Bachur and Gee, 1972).

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Annular Lipids Determine the ATPase Activity of a Calcium Transport Protein Complexed with Dipalmitoyllecithin†

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ABSTRACT: Pure complexes of dipalmitoyllecithin (DPL, 16:0, 16:0) with Ca^{2+}, Mg^{2+} dependent ATPase from sarcoplasmic reticulum are unusual in retaining significant ATPase activity down to about 30 °C, well below the transition temperature of the pure lipid at 41 °C. A minimum of about 35 lipid molecules per ATPase is required to maintain maximal ATPase activity, but the complexes are progressively and irreversibly inactivated at lower lipid to protein ratios. Complexes containing more than the minimum lipid requirement show very similar temperature profiles of activity above 30 °C over a wide range of lipid to protein ratios, up to 1500:1. Spin-label studies indicate that, at lipid to protein ratios of less than about 30 lipids per ATPase, no DPL phase transition can be detected, but at all higher ratios, a phase transition occurs at about 41 °C. In all of these complexes there are breaks in the Arrhenius plots of ATPase activity at 27–32 °C and at 37.5–38.5 °C. Experiments with perturbing agents, such as cholesterol and benzyl alcohol which have well-defined effects on the DPL phase transition, indicate that these breaks in the Arrhenius plots of ATPase activity cannot be attributed to a depressed and broadened phase transition in the lipids near the protein molecules. These results are interpreted as evidence for a phospholipid annulus of at least 30 lipid molecules which interact directly with the ATPase and cannot undergo a phase transition at 41 °C. This structural interaction of the ATPase with the annular DPL molecules has a predominant effect in determining the form of the temperature–activity profiles. *However, the perturbation of the DPL phase transition does not extend significantly beyond the annulus* since a phase transition which starts at 41 °C can be detected as soon as extraannular lipid is present in the complexes. We suggest that it may be a general feature of membrane structure that penetrant membrane proteins interact with their immediate lipid environment so as to cause only a minimal perturbation of the lipid bilayer.

The sharp breaks, which occur in the temperature profiles of the activities of many transport proteins, have usually been attributed to lateral phase separations (Overath et al., 1970) or the formation of clusters in the lipid bilayer (Lee et al., 1974; Wunderlich et al., 1975). In bacterial membranes with high proportions of a single phospholipid, the breaks coincide quite closely with the phase separations which can be detected in the isolated lipids, implying that the membrane proteins sense a transition which is unaffected by the presence of the protein. Studies of the phase transitions in these membranes by calorimetry and x-ray diffraction have shown that most of the lipids are able to undergo a normal phase transition. Since the penetrant protein molecules in many membranes are only separated laterally by small numbers of lipid molecules, a membrane protein presumably cannot have an extensive effect on phase separations beyond the lipid in its immediate environment. In contrast, in model systems of pure lipid bilayers it is well-established that extraneous small molecules and various polypeptides and proteins (including cytochrome *c*, basic myelin (A1) protein, and polylysine) can either raise or lower the phase transition temperature of an extensive lipid bilayer, depending on the structure of the added component (Hui and Barton, 1973; Papahadjopoulos et al., 1975). This suggests the

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