A C T A B I O C H I M I C A P O L O N I C A

Vol. 34 1987 No. 2

ELŻBIETA BOJARSKA[a], KRZYSZTOF PAWLICKI[b] and BARBARA CZOCHRALSKA[c]

ELECTROCHEMICAL STUDY OF THE REDUCTION OF TOYOCAMYCIN AND SANGIVAMYCIN IN AQUEOUS MEDIA*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warszawa, Poland
[b] *Institute of Biophysics, Silesian Academy of Medicine, 40-952 Katowice, Poland*
[c] *Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 02-089 Warszawa, Poland*

Received 9 December, 1986

The redox behaviour of two antibiotics, toyocamycin and sangivamycin, structurally related pyrrolopyrimidine nucleosides, and their reduction products in buffered aqueous media, have been examined by direct current polarography and cyclic voltammetry.

Both compounds exhibit one 3-electron polarographic wave in the pH range 1 - 6. Macroscale electrolysis at the crest of the polarographic wave was followed electrochemically and by UV spectroscopy. The photochemical transformation of the reduction products on UV irradiation has been examined.

It was found that the reduction of both compounds occurs in the pyrimidine ring, leading to two reduction products. One of these (λ_{max} = 306 nm) is photochemically reversible to the parent compound.

The structurally related pyrrolopyrimidine nucleoside antibiotics toyocamycin and sangivamycin represent a group of nucleoside antibiotics that are highly cytotoxic to mammalian cells in culture, and inhibitory to the growth of bacteria and fungi, as well as to RNA and DNA viruses [1].

Toyocamycin and sangivamycin have been isolated from 13 *Streptomyces* cultures in independent laboratories, and the chemical synthesis of both has been reported by Robins and co-workers [2, 3].

Toyocamycin has been shown to be a significant antitumor agent. It inhibited completely rRNA synthesis [4].

* This investigation was supported by the Polish Ministry of Sciences, Technology and Higher Education (CPBP 01.06).

Sangivamycin is incorporated into RNA and DNA of all tissues except the brain, where it enters only RNA [1].

The close structural relationship of toyocamycin and sangivamycin to adenosine has made these nucleosides extremely valuable tools for studying many cellular and enzyme reactions in which they are substituted for adenosine, ADP and ATP.

In this study the electron-acceptor properties of toyocamycin and sangivamycin in aqueous media have been studied by electrochemical methods, and attempts made to determine the reduction mechanisms.

MATERIALS AND METHODS

Toyocamycin and sangivamycin were synthesized by Z. Kazimierczuk (University of Warsaw) according to the procedure of Tolman *et al.* [2, 3].

Britton-Robinson buffers were used in the pH range between 2 and 6. A Precision Digital pH Meter type OP-208 (Radelkis, Hungary) was employed for pH measurements.

D.c. polarography[1] was carried out with a Radiometer Polariter PO_4 (Copenhagen, Denmark) the characteristics of the dropping mercury electrode being $m = 2.5$ h mg/s, $t = 3.7$ s at 60 mm Hg. Cyclic voltammetry was carried out with Polarographic Analyzer PAR Model 174 (U.S.A.). Curves were recorded at room temperature, and all potentials are relative to the saturated calomel electrode. Solutions were rendered oxygen -- free by flushing with argon.

Preparative electrolysis was performed on a mercury electrode (surface area $\sim 12\ cm^2$) in a three compartment cell, at a constant potential controlled by Potentiostat type OH-404/A (Radelkis, Hungary). The concentration of the depolarizer was 10^{-3} M. For magnetic stirring a Teflon - covered bar was used. Argon was continuously passed through the cell during electrolysis. An integrator type OH-404/C (Radelkis, Hungary) was used to determine the number of electrons involved in the reduction process.

UV absorption spectra were recorded with a Specord UV-VIS instrument using 1- and 10-mm pathlength quartz cuvettes.

Irradiation of electrolyzed solutions, in 10-mm pathlength quartz cuvettes at 254 nm, was done with a Philips TUV 6 - W lamp; radiation below 240 nm was eliminated with the aid of a 5-mm filter of 33% acetic acid. A Philips Osram 125 W HQW Wood's lamp was employed for

---

[1] Abbreviations: D.c. polarography, direct current polarography; C.v. cyclic voltammetry; I_d diffusion current; I_p peak current; $E_{1/2}$ half-wave potential; E_p peak potential; I, diffusion current constant.

irradiation of wavelengths to the red of 320 nm ($\lambda \geq 320$ nm). Cuvettes were maintained at room temperature with the aid of a stream of cooled air.

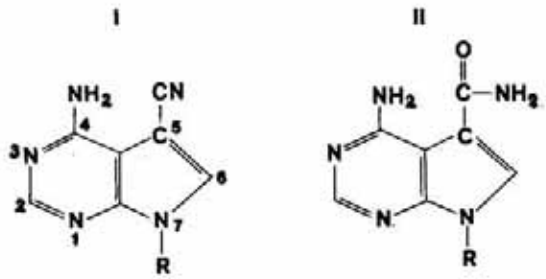
Thin-layer chromatography made use of Merck (Darmstadt, G.F.R.) GF_{254} plates with the following solvent systems: (all proportions v/v) (I) ethanol/1.0 mM ammonium acetate pH 7.5 (7:3), (II) ethanol/chloroform (3:7).

Enzymatic assay with horseradish peroxidase (HRP). To 2 ml of electrolyzed solution of sangivamycin in acetate buffer, pH 4, saturated with O_2, were added H_2O_2 and peroxidase (final concentration of H_2O_2 was 0.5 mM, and peroxidase 2×10^{-6} M respectively).

The resulting decrease in absorbance at 306 nm, and increase at 280 nm (characteristic for sangivamycin) was followed by UV spectroscopy.

RESULTS AND DISCUSSION

Polarography. D.c. and c.v. polarography of toyocamycin and sangivamycin (Scheme 1) were performed in Britton-Robinson buffers at a concentration of 4×10^{-4} M. Both compounds exhibit a single, diffusion-controlled



Scheme 1. Formulae for toyocamycin (I) and sangivamycin (II) (R = Ribose)

cathodic wave or c.v. peak in the pH range 1 - 6 (Fig. 1), both are reduced in protonated state. Above pH 6 the reduction wave (or cathodic peak) disappears, as for adenosine [5] and 4-aminopyrimidine [6]. On the basis of coulometric measurements at pH 4, $n = 3.4$ was found for both compounds. With increasing, pH, half-wave potentials shifted towards more negative potentials, whereas diffusion current I_d decreased (Table 1 and Fig. 2).

The cyclic voltammetric behaviour of toyocamycin and sangivamycin is consistent with d.c. polarography.

The pH-dependence of E_p (peak I_p of sangivamycin) is as follows:

$$E_p = -1.270 - 0.043\ pH;\ (v = 0.1\ V/s)\ (pH\ 2 - 6)$$

No anodic peak complementary to peak I_p appears on the return sweep at pH 1 to 6 at $v = 0.5$ V/s, indicating an irreversible electrode process.

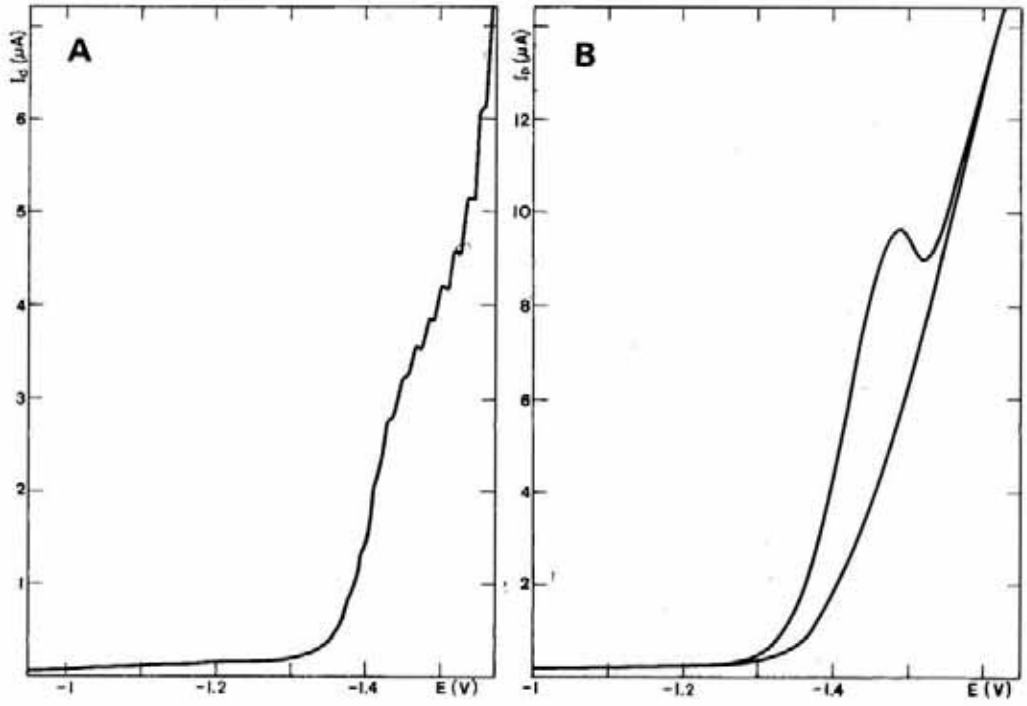


Fig. 1. D.c. polarogram (A) and cyclic voltammogram (B) for 0.4 mM sangivamycin at pH 4.0, at room temperature

Electrolysis at controlled potential. Preparative electrolysis of toyocamycin and sangivamycin, using a large mercury electrode, was carried out at pH 4.0. Following electrolysis at the potential −1.4 V the characteristic absorption maximum at 279 nm ($\varepsilon_{max} = 15400$) for toyocamycin, and 278 nm ($\varepsilon_{max} = 12200$) for sanivamycin, disapeared and a new absorption peak appeared at 306 nm for both compounds (Fig. 3).

After electrolysis the polarographic wave due to the reduction of toyocamycin and sangivamycin disappeared and no other wave appeared.

Paper chromatography of the electrolyzed solutions showed two reduction products, one of which had the absorption maximum at $\lambda_{max} = 306$ nm (40%), the other at $\lambda_{max} = 250$ nm.

After exposure to air the reduction products turned pink, however the electrolyzed solutions stored at pH 4 and 0°C under a nitrogen atmosphere were stable over a period of one week.

Controlled potential electrolysis of toyocamycin and sangivamycin at −1.3 V at pH 1 revealed that the primary reduction product absorbing at $\lambda_{max} = 306$ nm is a transient which, following 2 h electrolysis, was transformed into the product absorbing at $\lambda_{max} = 250$ nm. At the same time the coulometric measurements gave n = 4.1 for toyocamycin and n = 4.3

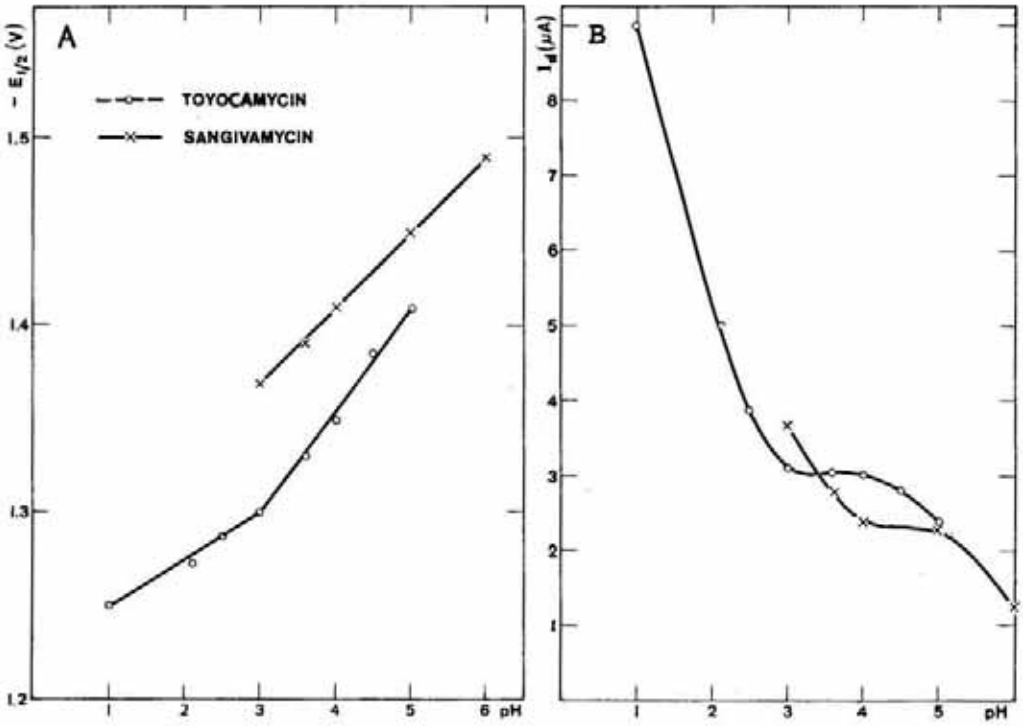


Fig. 2. Variation with pH of $E_{1/2}$ (A) and I_d (B) of polarographic waves of toyocamycin and sangivamycin (0.4 mM) in aqueous buffered solution, at room temperature

for sangivamycin for the reduction process similarly as it was found for 4e reduction of adenosine [5].

A positive Nessler test [7] for NH_3 was found for solutions of toyocamycin and sangivamycin electrolyzed at pH 1 for 2-3 h. This is in accord with results of Kwee & Lund [8] pointing to deamination of the reduced adenosine in 2 M HCl.

On the basis of the above results it is obvious that the reduction of toyocamycin and sangivamycin occurs in the pyrimidine ring. This is further supported by the polarographic non-reducibility of pyrrol in aqueous media [9].

Oxidation of the Electrolyzed Solutions

A. Photooxidation. Photochemical transformation of the reduction products was attempted in order to see whether they are photochemically oxidized to the parent compounds, as found for the reduction products of 4-aminopyrimidine [6] and 2-oxopyrimidine and other pyrimidine hydrodimers [10].

Table 1

D.c. polarographic behaviour of 0.4 mM toyocamycin and sangivamycin in aqueous media (0.04 M Britton-Robinson buffers)

pH	$E_{1/2}$ (V)	Wave slope (mV) $E_{1/4}$-$E_{3/4}$	I_d (μA)	I^a
		toyocamycin		
1.00 (0.1 M HCl)	−1.250	35	9.0	9.78
2.12	−1.265	35	5.0	5.43
2.50	−1.285	40	3.9	4.24
2.98	−1.295	45	3.1	3.37
3.59	−1.330	48	3.0	3.32
4.05	−1.350	50	2.9	3.26
4.52	−1.385	55	2.8	3.04
4.97	−1.410	60	2.4	2.61
		sangivamycin		
3.05	−1.370	40	3.7	4.02
3.59	−1.390	45	2.9	3.10
4.05	−1.415	45	2.4	2.61
4.97	−1.450	50	2.3	2.55
6.05	−1.495	55	1.3	1.36

[a] $I = (6/7)\ I_d\ (\max)/Cm^{2/3}t^{1/6}$

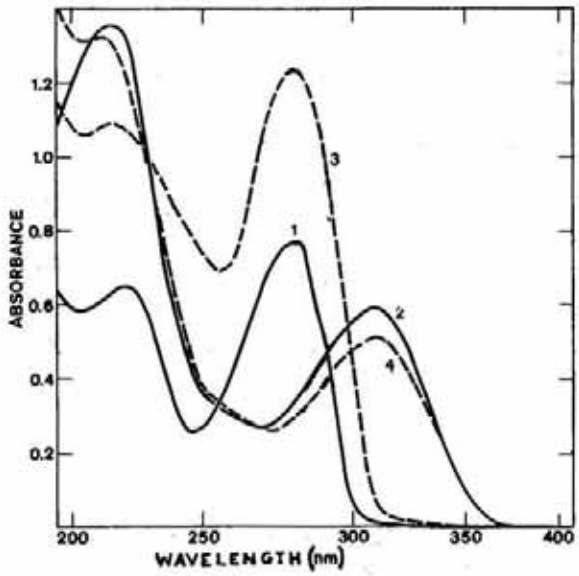


Fig. 3. Ultraviolet absorption spectra of toyocamycin (solid line) and sangivamycin (dashed line) before electrolysis (1, 3) and after electrolysis (2, 4) at pH 4.0

Irradiation at pH 0.4 at $\lambda = 254$ or $\lambda \geq 320$ nm, in the presence of a mercury drop, of toyocamycin and sangivamycin, immediately after electrolysis resulted in a decrease of the 306 nm peak and simultaneous growth of an absorption peak at 279 nm with all intermediate absorption curves passing through an isosbestic point (Fig. 4a).

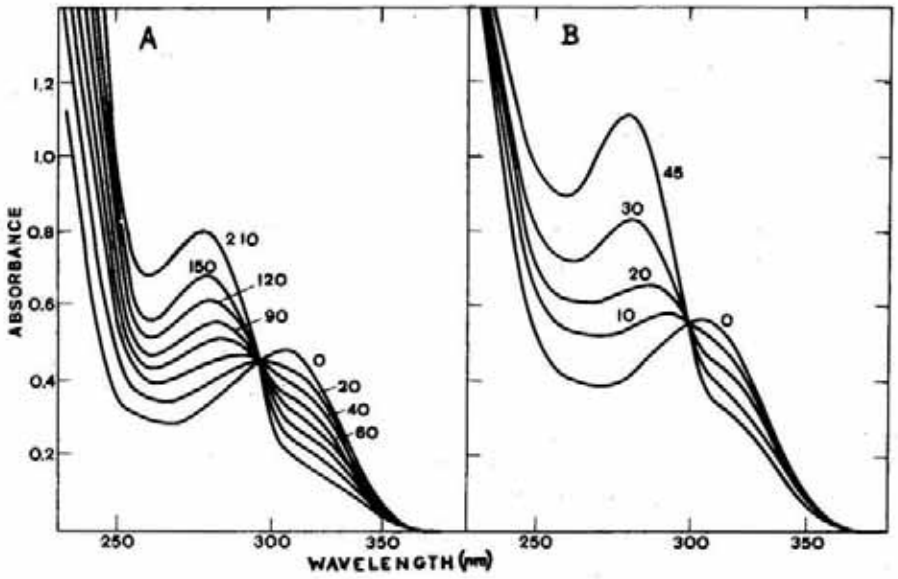


Fig. 4. (A) Photochemical transformation on irradiation at 254 nm of the reduction product of sangivamycin in the presence of mercury. The product prepared by electrolysis of sangivamycin at pH 4.0 (0.2 M Britton-Robinson buffer) and diluted with 5 vol. of 0.4 M HCl. (B) Enzymatic oxidation of the reduction product of sangivamycin by horseradish peroxidase at pH 5.0 (acetate buffer). Numbers besides each curve correspond to time of irradiation (A) or enzymatic reaction (B) in minutes

Photochemical exposure in the presence of metallic mercury was examined because the presence of the mercury significantly increased the efficiency of the photochemical transformation, found for 4-aminopyrimidine [6]. UV spectrophotometry, polarographic analysis and paper chromatography all demonstrated unequivocally that the photochemically transformed product is the oxidized form of toyocamycin (sangivamycin). The amount of the dimeric reduction product estimated polarographically by its photooxidation to the parent compounds was found to be $\sim 40\%$.

The photooxidation of the reduction products of toyocamycin and sangivamycin occurs only at pH < 1. At higher pH (1 - 6), rearrangment and hydrolysis of the products occurs in the same way as for 4-aminopyrimidine.

B. Enzymatic oxidation by HRP. It is of some interest that enzymatic oxidation of the electrolyzed solutions of toyocamycin and sangivamycin occurs in a manner resembling photooxidation process, leading to oxidized forms of toyocamycin and sangivamycin (Fig. 4b), the enzymatic process being 7-fold faster than photooxidation. The products of enzymic oxidation were identical, as found polarographically and by chromatography (Methods).

Reduction mechanism

D.c. and c.v. polarographic analysis revealed that both toyocamycin and sangivamycin undergo single step electrochemical reduction in pH range 1 - 6 as found for adenine [5], adenosine [5], and 4-aminopyrimidine [6].

The reduction of adenine and its derivatives occurs in the pyrimidine ring [9] and pyrrol itself is not polarographically reducible [9]. The $E_{1/2}$ and I_d dependence on pH for toyocamycin and sangivamycin similar to that found for adenosine [5] and 4-aminopyrimidine [6], point to reduction of pyrimidine ring.

This was further supported by the UV spectra of the two reduction products ($\lambda_{max} = 250$ nm and $\lambda_{max} = 306$ nm) of toyocamycin and sangivamycin, similar to the electroreduction products of 4-aminopyrimidine.

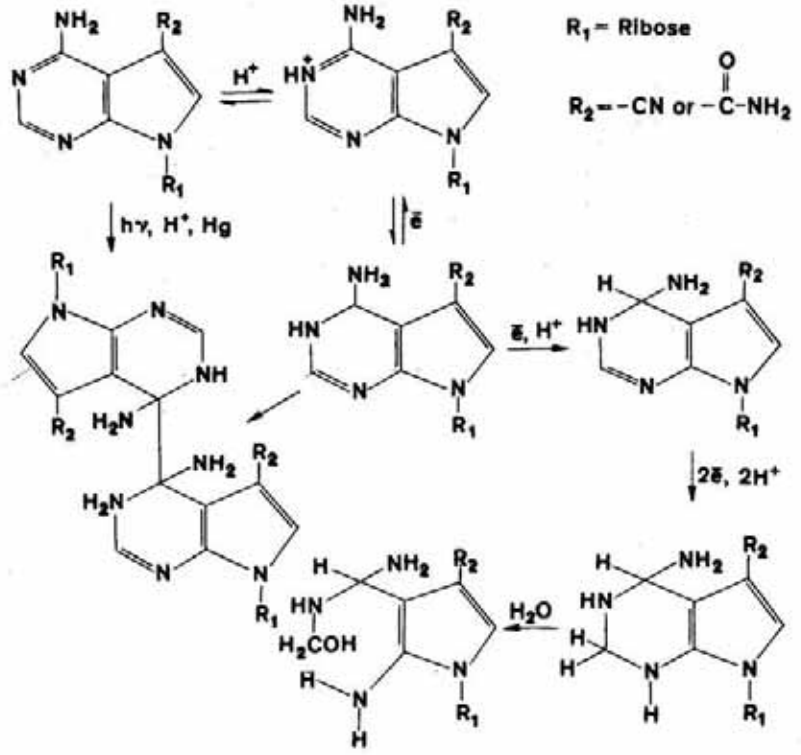
Although identification of the primary electrolysis products of toyocamycin and sangivamycin is difficult because of their instability, comparison of the electrochemical behaviour of toyocamycin and sangivamycin with those of related compounds (4-aminopyrimidine, adenine and purine) suggests the reduction mechanism shown in Scheme 2.

The initial 1e nucleophilic attack on the pyrimidine ring results in reduction of the N(1)-C(6) bond and formation of 6,6′-hydrodimers, by the free radical produced. Since reduced pyrimidines are more basic than the non-reduced species [11], and since the hydrodimers produced in the present study are generally protonated, protonation may be responsible for the shift to longer wavelength in the ultraviolet absorption spectrum. A similar shift is seen for cationic forms of 2,2′-bipirydyl [12] and reduced 4-aminopyrimidine [6].

If the proton activity is sufficiently high the carboanion path is favored (Scheme 2). Since the carboanion is rapidely protonated to the dihydropyrimidine at $pH < 1$, the reduction involving the fourth electron addition is accompanied by catalytic hydrogen discharge. The reduction products of pyrimidine can undergo isomerization, hydrolysis and rearrangement [6, 13].

On the basis of the present results, it is obvious that polarographic reduction waves to toyocamycin and sangivamycin could be used for analytical identification of both antibiotics (concentration range: 5×10^{-4} M - 2×10^{-5} M at pH 4.0). Especially d.p.p. (pulse polarographic method) could be useful for detection of very small amounts (10^{-6} M) [14].

It is of some interest that UV irradiation at 254 nm and 320 nm and oxidation by HRP in presence of H_2O_2 of the reduction products regenerates toyocamycin and sangivamycin, but only on freshly electrolyzed solutions for 4-aminopyrimidine. Thus, either enzymatic assays with HRP,



Scheme 2. Proposed reaction pathway for the electrochemical reduction of toyocamycin and sangivamycin in aqueous media

or UV irradiation, could be used for identification of the freshly reduced toyocamycin and sangivamycin at concentration of 5×10^{-5} - 1×10^{-6} M in acidic medium.

The authors wish to thank Dr. Z. Kazimierczuk for the synthesis of toyocamycin and sangivamycin and P. Przybora for technical assistance. We are grateful to Professor D. Shugar for reading the manuscript and for discussion.

REFERENCES

1. Suhadolnik, R. J. (1970) *Nucleosides Antibiotics*; pp. 298 - 353. Wiley Interescience, New York.
2. Tolman, R. L., Robins, R. K. & Townsend, L. B. (1968) Pyrrolo 2,3-d pyrimidine Nucleoside Antibiotics. Total synthesis and Structure of Toyocamycin, Unamycin B, Vengicide, Antibiotic E-212, and Sangivamycin (BA - 90912) *J. Am. Chem. Soc.*, **90**, 524 - 526.
3. Tolman, R. L., Robins, R. K. & Townsend, L. B. (1969) Pyrrolopyrimidine Nucleosides. III. The Total Synthesis of Toyocamycin, Sangivamycin, Tubercidin, and Related Derivatives. *J. Am. Chem. Soc.*, **91**, 2102 - 2108.
4. Tavitian, A., Uretsky, S. C. & Acs, G. (1968) Selective Inhibition of Ribosomal RNA Synthesis in Mammalian Cells. *Biochim. Biophys. Acta*, **157**, 33 - 42.
5. Janik, B. & Elving, P. J. (1970) Correlation of Electrochemical Reduction of Adenine Nucleosides and Nucleotides with Structure and Orientation in Solution. *J. Am. Chem. Soc.*, **92**, 235 - 243.
6. Czochralska, B. & Elving, P. J. (1981) Electrochemical Reduction of 4-Aminopyrimidine in Aqueous Media. *Electrochim. Acta*, **26**, 1755 - 1769.
7. Taras, M. J. (1958) *Colorimetric Determination of Nonmètals* (Boltz, D. F., ed.) pp. 75 - 160, Interscience, New York.
8. Kwee, S. & Lund, H. (1972) Electroorganic Preparations. XXXIV. Electrolytic Reduction of some Substituted Purines. *Acta Chem. Scand.*; **26**, 1195 - 1200.
9. Dryhurst, G. (1977) Electrochemistry of Biological Molecules; pp. 88 - 93 and 397, Academic Press, New York.
10. Czochralska, B., Wrona, M. & Shugar, D. (1986) Electrochemically Reduced Photoreversible products of Pyrimidine and Purine Analoges. *Topics in Current Chemistry*, **30**, 133 - 181.
11. Brown, D. J. (1970) The Pyrimidines: Supplement I, pp. 355 - 356 and 370, John Wiley, New York.
12. DMS UV Atlas of Organic Compounds, Vol. III (1967) Butterworth and Verlag Chemie, London and Weinheim.
13. Kashima, C., Shimizu, M. & Omote, Y. (1985) The Tautomeric Study in 2-Substituted 1,6-Dihydro-4,6,6-Trimethyl-Pyrimidine Systems. *Tetrahedron Letters*, **26**, 5057 - 5060.
14. Bojarska, E., Pawlicki, K., Smyth, M. R. & Czochralska, B. (1986) *Electrochemistry, Sensors and Analysis*. (Smyth, M. R. & Vos, J. G., eds) pp. 221 - 228, Elsevier Science Publishers B. V., Amsterdam.