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ELECTROCHEMICAL STUDY OF DNA-ANTHRACYCLINES INTERACTION

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Summary : Binding isotherms of some anthracyclic derivatives with DNA have been determined by modern electrochemical techniques(Voltammetry - ac polarography). These techniques making use of the difference between the diffusion coefficients of DNA-drug complex and of free molecules of drug in solution allow the direct determination of the later ones. No striking differences can be noticed between the tested anthracyclines. No correlation can be established between the affinity of daunorubicin and some of its analogs for DNA and their more or less antitumoral activity.

Studies of interactions between intercalating drugs and DNA has led many authors to try to correlate biological and therapeutic activities with their affinity to DNA. In some cases, this correlation is evident (1,2) but cannot be generalized. The stereochemical structure of the DNA-antibiotic complex seems particularly important, as it has been shown with stereoisomers of doxorubicin (3), of daunorubicin derivatives (4,5) and of a number of heterocyclic compounds (6).

This report describes the results obtained with some daunorubicin derivatives having various biological activities. The techniques classically used such as spectral titration or equilibrium dialysis are not always convenient. For example, spectral titration allows the measurement of bound DNA molecules, but when the ratio of phosphate versus antibiotic (P/D) is low (high concentration of antibiotic) the results are disturbed by the presence, together with the intercalating complex, of a weak ionic binding of the drug.

Equilibrium dialysis requires a relatively high concentration of the drug and sometimes gives imprecise results. In order to obtain accurate and reproducible results, we have developed electrochemical techniques which allowed a direct determination of the number of free molecules. For this purpose, some electrochemical properties of daunorubicin and of some its derivatives are described.

Materials and Methods

Drugs : We are indebted to Rhône Poulenc S.A. for the generous gift of daunorubicin (RP 13057, MW : 527.5) and its derivatives (Table I). Duborimycin or daunorubicinol (RP 20798, MW : 529.5) is a major metabolite of daunorubicin (7). With a lower toxicity than daunorubicin, it has an activity in leu-

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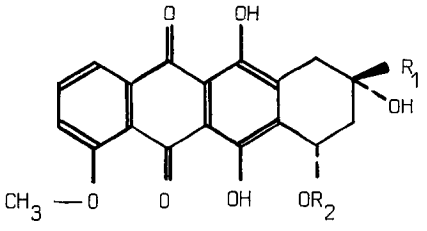
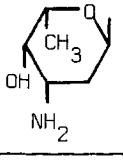
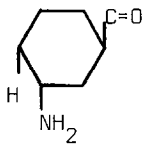
Table I



Compound	R_1	R_2
Daunorubicin (RP 13057)	- $COCH_3$	
Duborimycin (RP 20798)	- $CHOHCH_3$	"
RP 33921	- $COCH_2OCOCH(OC_2H_5)_2$	"
RP 21080	- $COCH_3$	

kemias and experimental tumours at least as good as daunorubicin (8). It has been tested in human chemotherapy (9). Derivative RP 21080 (MW : 523.5) is composed of four stereoisomers which have been isolated : the two trans isomers (RP 33365 and 33366) have a very low chemotherapeutic efficiency ; the two cis isomers (RP 32885 and 32886) have an activity of the same order of magnitude than daunorubicin. They have not been used in human chemotherapy, but have been tested on experimental tumours. Solutions of daunorubicin and of these derivatives are freshly prepared before use at the concentration of 0.5 mg/ml in distilled water. The very active derivative RP 33921 (MW : 673.7) (10) is used in citrate buffer pH 4.6 and the solution is prepared immediately before experiment. All these drugs stabilized the double helical structure of DNA.

All daunorubicin derivatives are semisynthetic. They are kept at 4°C, in the dark.

DNA : Calf thymus DNA, highly deproteinized has been extracted and purified in our laboratory (11). Its molecular weight is 8×10^6 daltons. Sonicated DNA (molecular weight : 0.5×10^6 daltons) was used in order to avoid precipi-

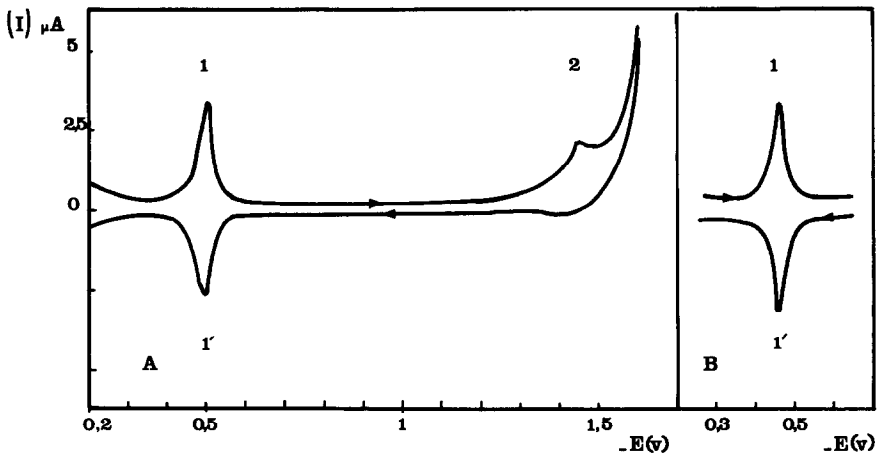


Fig. 1 - Voltammograms of daunorubicin at pH 5 in SSC buffer.
Waiting time : 15 s, sweep rate : 1 Volt/s.
A : daunorubicin 1 x 10^{-5} M , starting potential 200 mV, final potential 1600 mV.
B : daunorubicin 1 x 10^{-5} M, starting potential 300 mV, final potential 650 mV.

tation at high concentration of drug and prepared in conditions previously described (12). To avoid possible contaminations by electrochemically active impurities, DNA solutions were never dialyzed.

Experimental : Electrochemical measurements are made with a polarographic system Solea, including a potentiostat PRT 30-01 connected with a unit PRG 3 for phase sensitive ac polarography and a PRG 4 unit allowing sweep voltammetric measurement. For recording sweep voltammogram, a digital storage memory (Fabri-Tek Ins. 1072) was used. The hanging mercury dropping electrode (HMDE) used in sweep voltammetry was a PAR model 9323 with a surface of 3 mm^2. In ac polarography, the dropping mercury electrode (DME) (flow rate 0.2 mg/s) was used. A three electrodes system was used. The reference electrode was a saturated calomel electrode ; the auxiliary electrode was a platinum coil. Measurements are performed at 25°C. With the RP 33921 compound, unstable at this temperature, measurements are performed at 15°C. The electrochemical cell (constructed in the laboratory) allows experiments to be performed with 5 ml solution. Compartments including the auxiliary and the reference electrodes are separated from the solution by a sintered glass. To avoid adsorption of the products on the cell surface, it is covered with silicone Rhodorsil 240.

Electrochemical signal of anthracyclines

In order to study the binding parameters of the anthracyclines by electrochemical methods, some electrochemical properties of daunorubicin and of its derivatives have been investigated. The characteristics of the reactions at the electrode are widely dependent of the pH of the solution.

1. Behaviour of daunorubicin in neutral and acid solution

Voltammetry (13) : At pH 5, two peaks are observed on the voltammograms of daunorubicin : the first at - 0.5 V and the second at - 1.45 V (Fig. 1).

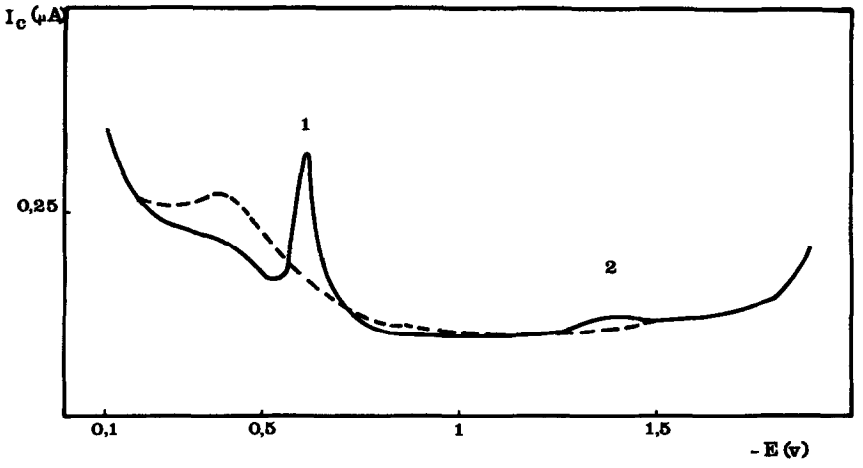


Fig. 2 - ac polarogram of daunorubicin.
------ supporting electrolyte
——— daunorubicin concentration 1 x 10^{-5} M, pH 7, phase angle ϕ=90°, superimposed frequency F = 32 Hz, amplitude of the superimposed tension E = 10 mV (peak to peak), dropping time 3 s.

When final scan potential does not exceed - 1 V (Fig. 1B), the cathodic and anodic parts of the peak 1 are symmetrical in comparison with potential axis. Moreover, the middle height width of the peak is 45 mV. These properties are characteristic of a reversible exchange of two electrons with oxidized and reduced components which are strongly adsorbed on the electrode (14). If the final scan potential exceed the potential of the peak 2, the anodic part of the peak 1 appears at the same potential as the cathodic one, but its intensity is lower. The peak 2 could be the desorption peak of the reduced product, its weak anodic part corresponding to a partial readsorption during the return scale.

Classical polarography (15) : The polarograms of daunorubicin present a well defined wave at the same potential as the peak 1 in voltammetry. The half wave potential varies 60 mV per pH unit. This is indicative of a reversible reaction whose reactional mechanism requires as many protons as electrons (15).

ac polarography (16) : The polarograms show two peaks (Fig. 2), corresponding to those obtained with voltammetry. The peak 1 is located in the adsorption zone of daunorubicin which is extended until - 1.2 V.

With all the techniques, for concentrations higher than 10^{-4} M a second peak (or wave in classical polarography) appears at a potential, 0.15 V more negative which progresses in intensity when the concentration increases while

the peak 1 becomes stable. This could correspond to the reduction of daunorubicin through the film formed by reduced product adsorbed on the electrode at the potential of the peak 1 (14,15).

In acid and neutral solutions, the reaction which takes place at the electrode for the potential of the peak 1 is a reversible reaction in adsorbed state. This reaction requires two protons and two electrons. The oxidized and reduced products are strongly adsorbed at the electrode. One can suggest by analogy with the reduction of quinones, that the electrochemical reaction takes place at the level of the quinone function of the anthracyclic cycle present in daunorubicin and in its derivatives.

2. Behaviour of daunorubicin in alkaline solution

In alkaline solutions, the reaction seems more complex. In particular, the half wave potential variation of the classical polarographic wave is less than 60 mV per pH unit, with a parallel decrease of the intensity of the peak. The reaction seems more limited by the kinetic of protonation rate. At these pH, another irreversible peak appears which suggests that other products of electrochemical reaction could appear.

3. Behaviour of derivative RP 21080 and of its four stereoisomers

In acid medium (pH 2) this compound has the same behaviour as daunorubicin and it is reversibly reduced at adsorbed state. But this reaction rapidly disappears when the pH is increased. The half wave potential variation of the classical polarographic wave is always less than 60 mV per pH unit. At pH 7, the reaction is widely irreversible and appears with difficulty in ac polarography, which is particularly sensitive for adsorption-desorption phenomena and for reversible reactions. The four stereoisomers present similar electrochemical behaviour.

Binding isotherms

Experimental determination

The principle of the electrochemical determination of the binding isotherm of a compound with DNA is the measurement, by mean of an electrochemical reduction of the compound, of the quantity of free compound in solution without interference with bound-DNA compound. This is possible due to the lower diffusion coefficient of the complex DNA-compound (10^{-7} - 10^{-8} cm^2s^{-1}) (17) in comparison with the diffusion coefficient of the free compound (10^{-5} cm^2s^{-1}). Thus, even if the DNA-bound compound is electrochemically active, the recorded electrochemical signal is essentially due to the free compound.

Classical polarography has been previously used to study complexation between DNA and ligands (18,19), but the low sensitivity requires high concentrations of DNA, which is strongly adsorbable on the electrode surface (20). Under these conditions, a large part of the electrode surface is cove-

red with adsorbed DNA, hindering adsorption of the free compound. In order to minimize this problem, we have used modern electrochemical techniques which allow the accurate determination of binding isotherms with concentrations in the range of 10^{-7} M. Then, it is possible to use very low concentrations of DNA (6-7 µg/ml). In this range of concentration and with our experimental conditions, only 1 % of the electrode surface is covered with DNA (20).

According to the electrochemical properties of the studied compounds, we have used two techniques of detection : ac polarography and sweep voltammetry. In all cases, peak 1 (Fig. 1 and 2) was recorded. The DNA concentration in the solutions is kept constant and increments of compounds (few microliters) are added and mixed in the solution. The concentration of free compound in presence of DNA is determined by comparison with a standard curve obtained without DNA. The dependence of the height of the peak with concentration is linear between 10^{-5} and 10^{-7} M. The data are analyzed by Scatchard's method (21). Binding parameters were obtained from the slope (K_{app}, apparent binding constant) and from the intercept (n, apparent number of binding sites per nucleotide) of the linear region of the binding curve with the horizontal axis.

Results

Fig. 3 shows the binding isotherm obtained for the complexation of daunorubicin with native and sonicated DNA. The systematic error of experimental points is represented. The curves display the two kinds of complex described in a previous work (22) : the intercalation complex observed for the low values of r and the ionic complex for higher values of r. The same pattern of curves is obtained for all the studied derivatives. The reproducibility of the results is good (≃ 10 %, Fig. 3). The values of the constants are independent of the molecular weight of the DNA and are not influenced by DNA concentration up to 30 µg/ml. The binding parameters of daunorubicin and of its derivatives are given in Table II. When the electrochemical properties of the anthracycline derivatives allow the use of the two different methods, there is good agreement between the results ; therefore, it is possible to compare the values of the constant determined by one or the other method. We cannot notice any striking difference in the binding parameters of all the tested derivatives. It is not possible to establish a correlation between the affinity of the different drugs for DNA and their more or less antitumoral activity. It has to be emphasized that the two derivatives with a very low activity (RP 33365 and 33366) have similar affinity constants than the highly active compounds. If the results are compared with those obtained by spectral absorption, the values of K_{app} and n are lower with daunorubicin, duborimycin and RP 21080 (23). This can be explained by the intervention of the ionic complex for high values of r, which perturbs calculation in the spectral titration

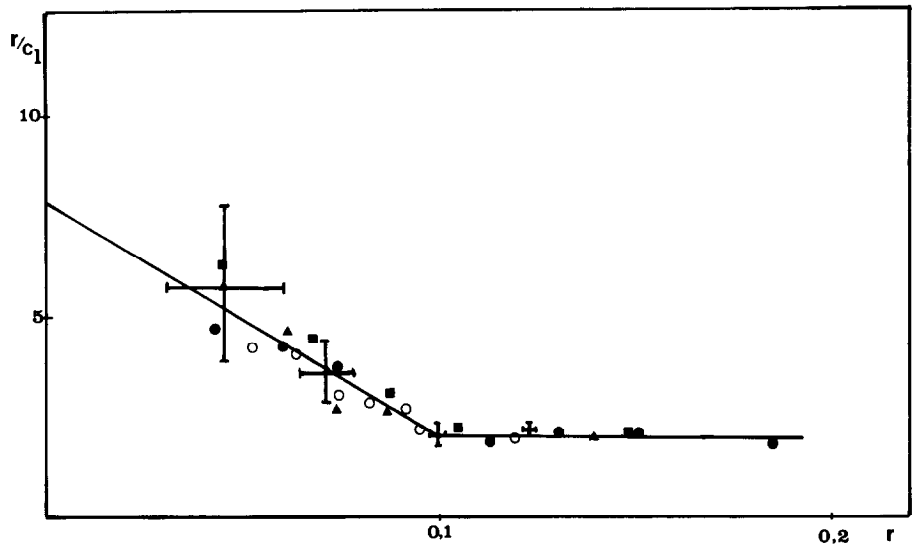


Fig. 3 - Scatchard's plot of the complexation of daunorubicin with calf thymus DNA in SSC buffer
This study was carried out in ac polarography. Superimposed frequency : 78 Hz, superimposed tension amplitude E = 10 mV pp, phase angle ϕ 50°, dropping time 2 s.
Δ, ●, ■, are the results of three successive experiments with native DNA.
o experiment made with sonicated DNA.

technique. There is a better agreement with the results obtained by equilibrium dialysis (unpublished data) which involves also the measurement of free moleculesin solution. With electrochemical techniques, one can avoid most of the disadvantages of equilibrium dialysis (use of large quantity of product, adsorption on the dialysis membrane, time consuming experiments). It is possible to obtain rapid and reproducible results with small quantities compound.

Discussion

From the results obtained with different derivatives of daunorubicin, it has not been possible to establish a correlation between their affinity for DNA and their antitumoral activity. Similar conclusion has been obtained in studying the thermal stabilisation of the double stranded DNA in presence of antibiotics. But, *in vivo*, the mode of action of these drugs is far from being elucidated and other interactions such as regulation systems or specific action of enzymes can interfere with the ability of the drug to give intercalating complex with DNA.

We have developed a new method for the determination of binding isotherms with DNA. This rapid, reproducible and accurate method is applicable to the

Table II

Binding parameters for the interaction of daunorubicin and some of its derivatives in SSC buffer, according to Scatchard representation with calf thymus sonicated DNA.

Compound	Technique	K_{app} $M^{-1} \times 10^{-5}$	n	Kn x M^{-1} 10^{-4}
Daunorubicin	ac polarography	6	0.13	7.8
Duborimycin	ac polarography	3.1	0.127	3.9
Daunorubicin	voltammetry	6	0.125	7.5
RP 33921	voltammetry	5.6	0.197	11.03
RP 21080	voltammetry	4.4	0.12	5.3
RP 32885	voltammetry	7	0.143	10
RP 32886	voltammetry	5.8	0.105	6.1
RP.33365	voltammetry	6.5	0.094	6.1
RP 33366	voltammetry	7.2	0.088	6.3

K_{app} and n were determined from Scatchard's plots as described in text. For daunorubicin and duborimycin, ac polarography was used in experimental conditions described in the legend of the Fig. 2. For the other derivatives, voltammetry was used in the following conditions : starting potential - 0.2 volt, extent of potential 0.5 volt, scan speed 10 volts/s, time of initial deposit 5 s. The detection limit is then 5×10^{-7} M.

compounds giving electrochemical signal (reduction, oxidation, adsorption/desorption). Some electrochemical properties of daunorubicin and of some of its derivatives were investigated and it has to be noticed that the quinone group could play a preponderant part in the electrochemical reactions. This mode of study could be able to bring informations on the mode of action *in vivo* of these drugs : for example, on the possibility of insertion of daunorubicin in one of the redox system of the organism. In this way, recent work postulates a redox cycle of daunorubicin and doxorubicin (24), when associated with a reducing agent. Generated free radicals could initiate endogenous lipid peroxidation which could be responsible for the cardiotoxicity of these

drugs. It seems to us that the determination of the standard potential of daunorubicin could be able to provide quantitative answers to these questions.

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References

1. Le Pecq, J.B., N'guyen-dat Xuong, Gosse, C. and Paoletti, C. (1974) Proc. Nat. Acad. Sci. USA 71, 5078-5082.
2. Müller, W. and Crothers, D.M. (1968) J. Mol. Biol. 35, 251-290.
3. Zunino, F., Gambetta, R., Di Marco, A., Velcich, A., Zaccara, A., Quadrifoglio, F. and Crescenzi, V. (1977) Biochim. Biophys. Acta 476, 38-46.
4. Zunino, F., Gambetta, R., Di Marco, A., Luoni, G. and Zaccara, A. (1976) Biochem. Biophys. Res. Comm. 69, 744-750.
5. Penco, S., Angelucci, F., Vigevani, A., Arlandini, E. and Arcamone, F. (1977) J. Antibiotics 30, 764-766.
6. Sharples, D. and Brown, J.R. (1976) FEBS Lett. 69, 37-40.
7. Bachur, N.R. and Gee, M. (1971) J. Pharm. Exp. Ther. 177, 567-572.
8. Maral, R., personal communication.
9. Chauvergne, J., Carton, M., Berlie, J., Brulé, G., Clavel, B., Gary-Bobo, J., Guerrin, J., Klein, T. and Pommatau, E. (1976) Bull. Canc. 63, 41-58.
10. Maral, R., Ducep, J.B., Farge, D., Ponsinet, G. and Reisdorf, D. (1978) C.R. Acad. Sc. Paris 286, 443-446.
11. Sicard, P.J., Obrénovitch, A. and Aubel-Sadron, G. (1972) Biochim. Biophys. Acta 268, 468-479.
12. Obrénovitch, A. and Aubel-Sadron, G. (1971) J. Chim. Phys. 68, 521-526.
13. Delahay, P. (1966) New Instrumental Method in Polarography, Interscience Publ., London.
14. Laviron, E. (1974) J. Electroanal. Chem. 52, 355-395.
15. Heyrosky, J. and Kuta, J. (1966) Principles in Polarography, Academic Press, New-York.
16. Breyer, E. and Bauer, H.H. (1963) Alternative Current Polarography and Tensammetry, Interscience Publ., London.
17. Lang, D. and Coates, P. (1968) J. Mol. Biol. 36, 137-151.
18. Prasal, Z. (1976) Roczu. Chem. 50, 13-31.
19. Berg, H. and Eckardt, K. (1972) Z. Naturf. 25, 362-367.
20. Malfoy, B., Sequaris, J.M., Valenta, P. and Nürnberg, H.W. (1976) Bioelectrochem. Bioenerg. 3, 440-460.
21. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
22. Barthelemy-Clavey, V., Maurizot, J.C. and Sicard, P.J. (1973) Biochimie 859-868.
23. Barthelemy-Clavey, V., Thèse Doctorat ès-Sciences, Orléans, 1976.
24. Goodman, J. and Hochstein, P. (1977) Biochem. Biophys. Res. Comm. 77, 797-803.