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Aspects of the degradation kinetics of daunorubicin in aqueous solution

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Summary

The degradation kinetics of daunorubicin has been investigated over the H_o/pH region 0–14 at 50°C. A stability indicating high-performance liquid chromatographic assay with UV-VIS and fluorescence detection was used to separate the degradation products from the parent compound. The influence of buffers, ionic strength and temperature on the degradation was determined. A pH–rate profile was constructed after corrections for buffer and ionic strength effects. The degradation kinetics are modeled using a non-linear curve-fitting computer program.

Introduction

The anthracycline group of antibiotics are among the most potent agents used in cancer chemotherapy (Crooke and Reich, 1980). The best known members of this class of compounds are doxorubicin (Dx) and daunorubicin (Dr) which are characterized by a tetrahydronaphthacenequinone aglycone attached to the amino sugar daunosamine by a glycosidic linkage (Fig. 1).

Dx (Adriblastina) and Dr (Cerubidine) are commercially available as the hydrochloride salt in a freeze-dried formulation containing lactose or mannitol as excipients. After reconstitution with 0.9% sodium chloride solution the compounds are administered intravenously. Although stable in the solid state, Dx and Dr degrade in solution. Apart from the decomposition in acidic solution (pH < 3.5) (Wassermann and Bundgaard, 1983; Beijnen

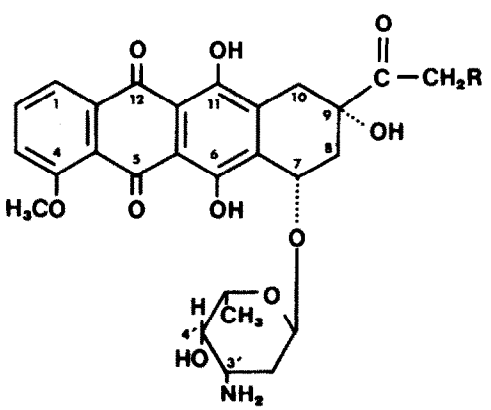

Fig. 1. Structures of daunorubicin (R = H) and doxorubicin (R = OH).

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et al., 1985), no systematic stability studies of these drugs have been reported hitherto. The present study, dealing with Dr, was undertaken in an attempt to obtain insight into the kinetics of the degradation and the influences of external factors (pH, buffers, ionic strength, temperature) on the degradation process. In a subsequent report a systematic study on the degradation kinetics of Dx will be presented.

Experimental

Chemicals

Daunorubicin hydrochloride (Dr) was supplied by Rhône Poulenc Nederland (Amstelveen, The Netherlands). 7-8,9-10-bisanhydrodaunomycinone (III) (a Dr degradation product) was a gift from Dr. S. Penco, Farmitalia (Milan, Italy). All chemicals used were of analytical grade. Deionized water was boiled in order to remove carbon dioxide and oxygen and stored under nitrogen atmosphere.

Buffer solutions

The following aqueous buffer solutions were used for the kinetic studies: pH < 3, perchloric acid; pH 3–6, acetate; pH 6–9, phosphate; pH 9–11.5, carbonate; pH > 11.5, sodium hydroxide. pH values were measured at 50°C using a glass-reference electrode and a pH meter (Metrohm, E520 Titriskop, Herisau, Switzerland) standardized at 50°C. H_o/pH values below 1 were calculated using the Hammett acidity function (Bates, 1973) and pH values over 11.5 were calculated from the equation $pH = pK_w + \log[OH^-]$ where $pK_{w(50°C)} = 13.26$. A constant ionic strength of 0.3 was maintained for each solution by addition of an appropriate amount of sodium chloride, except for the solutions where the concentration of hydrogen or hydroxyl ions exceeded 0.3 M and for the solutions used in the experiments where the ionic strength was varied. To each buffer solution sodium edetate was added to a concentration of 5×10^{-4} M. No significant change of pH was observed throughout the degradations.

Glass equipment

All glass equipment, before contact with Dr, was silanized with dichlorodimethylsilane in toluene (3% v/v) and subsequent rinsing with methanol.

Kinetic measurements

Buffer solutions were pre-heated and purged with nitrogen for 5 min at the temperature of study. 7 ml of buffer was then spiked with 70 μl of a Dr solution in water (2 mg/ml) to give an initial drug concentration of 20 μg/ml (3.5×10^{-5} M). The containers (silanized glass flasks) were quickly closed under nitrogen atmosphere with a rubber septum and cap. The reaction solutions were kept in a thermostatically controlled waterbath at 50 ± 0.2°C, protected from light. At regular time intervals 0.5 ml samples were withdrawn through the septum with a polypropylene syringe. These samples were put in polypropylene vessels and the pH was decreased with 10 μl of perchloric acid to give a final pH of about 2.5. These acidified samples were stored at −20°C until HPLC analysis. Under these storage conditions no degradation was observed for at least 2 months.

Apparatus and analytical procedures

HPLC analysis was carried out with a chromatographic system which consisted of a model M-45 pump, a model 440 dual wavelength detector (both from Waters Assoc., Milford, MA, U.S.A.) operating at 280 and 546 nm and a Perkin-Elmer 650 Fluorescence Detector (Norwalk, U.S.A.) with $\lambda_{excitation} = 465$ nm and $\lambda_{emission} = 550$ nm. The analytical column (30 cm × 3.9 mm i.d.) was home-packed with Lichrosorb RP8 (10 μm) material (Merck, Darmstadt, F.R.G.) and used at ambient temperature. The eluent consisted of 0.02 M sodium chloride–acetonitrile (40 : 60 v/v). The pH of the 0.02 M sodium chloride solution was adjusted to 2.0 with perchloric acid (70% w/v) before mixture with the organic modifier. The flow rate was 1.5 ml/min. Samples of 40 μl were injected into the liquid chromatograph with the use of an automatic sample injection device (WISP Model 710, Waters Assoc.). Quantitation of undegraded Dr was based on peak height measurements using a SP 4270 integrator (Spectra Physics, Santa Clara, CA, U.S.A.). Standard solutions of Dr in 0.005 M perchloric acid were chromato-

graphed and calibration curves were constructed. The standard curves exhibited linear responses ($r > 0.999$) for the three detection signals in the concentration range of interest, 3.5×10^{-5} to 3.5×10^{-6} M. Relative standard deviations of 1.9–7.2% were obtained for replicate injections of 3.5×10^{-5} to 3.5×10^{-6} M Dr solutions, respectively. Thin-layer chromatographic analysis of toluene extracts of degraded samples was performed on silicagel plates (60F254, Merck, thickness 0.25 mm). As solvent system chloroform–methanol–6 N ammonia (90 : 10 : 1 v/v/v) was used. The coloured degradation products were detected by viewing under daylight or by irradiation with UV light of 365 nm at which wavelength the compounds fluoresce.

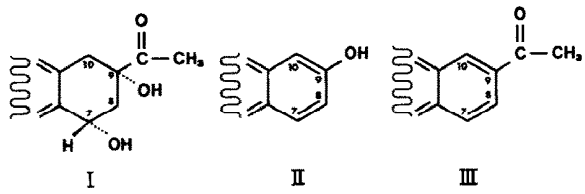
Determination of micro and macro ionization constants

The micro and macro ionization constants of Dr at 25°C and 50°C were determined spectrophotometrically and calculated according to the procedure described by Sturgeon and Schulman (1977). The drug concentration was 20 μg/ml. The second phenolic pK_a value of Dr was measured by recording the change of absorptivity at 635 nm as function of pH. Absorbance measurements were performed using a Shimadzu UV-140 Double Beam Spectrophotometer (Shimadzu, Kyoto, Japan).

Results and Discussion

Degradation products

In acidic medium the glycosidic anthraquinones undergo a proton-catalyzed cleavage of the glycosidic bond yielding an amino sugar (Arcamone et al., 1964a) and a 7-hydroxyaglycone (Arcamone et al., 1964b). From the present study it appears that Dr hydrolyzes to yield daunorubicinone (I) (Fig. 2) quantitatively when the degradation takes place at pH values lower than 3.5, as evidenced with HPLC. The identification of I with mass spectrometry was described earlier (Beijnen et al., 1985). At pH values over 3.5–4 more degradation products were detected. These compounds could effectively be extracted with chloroform or toluene



I II III

Fig. 2. Structures of Dr degradation products.

from the decomposition mixtures. TLC analysis of the extracts revealed that until pH 6 traces of I are present. The two major degradation products (II/III) arising at pH > 4 are both aglycones, the structural formulas of which are illustrated in Fig. 2. Physicochemical and analytical properties of these compounds will be published later. It is important to note that only aglycone degradation products could be traced. Aglycones are devoid of antitumour action for which the presence of the amino sugar moiety is a requisite (Henry, 1979).

Optimization of analytical methodology

Initial kinetic runs at pH > 4 were discouraging as the degradation rates were found to be very irreproducible. As a result, the order of the degradation reaction was not definable at this stage of the investigation. These difficulties were not encountered during the studies on the degradation kinetics of anthracyclines in acidic solution (Beijnen et al., 1985). The problems could be overcome by taking precautions in the preparation and handling of the reacting solutions. At first the solutions had to be protected from light. Dr is photolabile (Daugherty et al., 1979; Gray and Phillips, 1981; Williams and Tritton, 1981) and normal room light accelerates the degradation, an effect particularly interfering in kinetic studies at pH > 8. Secondly, in the presence of oxygen at pH > 8 Dr degrades faster than under anaerobic conditions. The initial degradation pattern appears to be similar, though the yield of the products II and III was less in the presence of oxygen, due to subsequent oxidation. Continuation of the oxygen catalyzed degradation led to a complete discolouration of the solutions whereas II and III disappeared. This points to an oxidative destruction of the anthraquinone chromophore. For III it has been established that oxidizing agents can

destroy the chromophore into smaller fragments such as 3-methoxyphthalic acid and trimellitic acid (Arcamone, 1978). Exhaustive deoxygenation by boiling the water and purging the buffers with nitrogen eliminated the oxygen influences. Traces of metal ion impurities, most likely originating from the buffers, also catalyzed the decomposition. This effect could be eliminated by addition of the chelating agent sodium edetate.

Elimination of the aforementioned effects of light, oxygen and traces of metal ions yielded a reproducible degradation pattern.

Anthracyclines have a great tendency to adsorb on glass and it is reported that silanization prevents this behaviour (Tomlinson and Malspeis, 1982). A complicating factor was that the hydrophobic products II and III strongly adsorb on silanized glass, showing a dull pink coating. In non-silanized glass vials the water insoluble aglycones (II and III) precipitate. For studies at pH values over 10 II and III nor adsorb neither precipitate, due to deprotonation of a phenolic function enhancing their solubility. These phenomena explain why in the pH region 4–10 hardly any degradation products appear in the HPLC chromatograms contrary to the results of studies at higher pH values. The adsorbed aglycones can be set free by treating with alkali or apolar solvents such as chloroform, toluene and dimethylsulfoxide. I, the product of the acid degradation, has no affinity towards glass material in acidic solution.

After sampling the test solutions, the samples were acidified to pH 2.5. With this procedure the degradation process is ceased immediately and the samples can be stored at −20°C for at least two months without any further loss. Injection into the chromatograph of acidified Dr samples is more reproducible and accurate than injection of neutral or alkaline solutions. Furthermore, injection with a sample injection device in which the samples are only exposed to stainless steel is preferable.

The proposed HPLC method is stability indicating. The validity of the assay is demonstrated by the fact that the Dr peak disappeared completely during degradation while the ratio between the three detector signals remained constant.

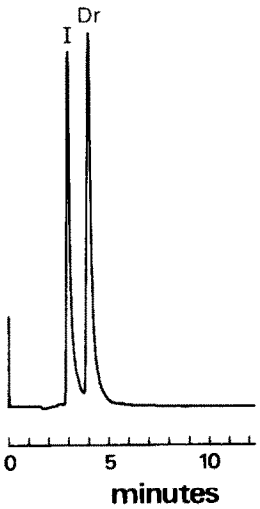


Fig. 3. HPLC chromatogram of a decomposition mixture of Dr at pH 1.5. Fluorescence detection $\lambda_{exc} = 465$ nm, $\lambda_{emiss} = 550$ nm. For chromatographic conditions; see text.

HPLC chromatograms of a decomposition mixture of Dr at pH 1.5 and pH 11.0 are shown in Figs. 3 and 4, respectively. HPLC analysis of II and III is hindered by the occurrence of strongly tailing peaks.

The micro and macro ionization constants of Dr were determined and calculated according to Sturgeon and Schulman (1977). The pK_a value of the deprotonation of the second phenolic function

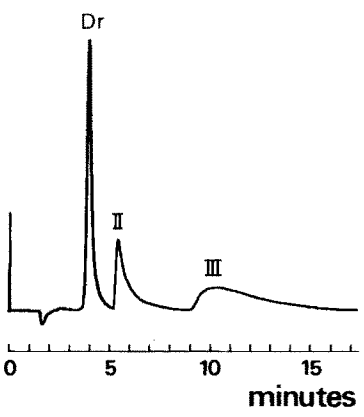


Fig. 4. HPLC chromatogram of a decomposition mixture of Dr at pH 11.0. Fluorescence detection $\lambda_{exc} = 465$ nm, $\lambda_{emiss} = 550$ nm. For chromatographic conditions; see text.

was determined by monitoring the absorbance at 635 nm as a function of pH. Degradation interferes with these experiments at pH values over 12.5 and 50°C, which is shown by a rapid decrease in absorbance as time elapses. Consequently, the absorbance–pH curve could not be completed, whereas the absorbance of the dianionic species could not be obtained. However, graphical treatment of the reliable part of the curve yields the value of the dissociation constant. In terms of absorbance, K_a can be expressed as:

$$K_a = \frac{(A^- - A)[H^+]}{(A - A^{2-})} \quad (1)$$

where A^- is the absorbance of the monoanion, A^{2-} is the absorbance of the dianion and A the measured absorbance at a given pH. Eqn. 1 can be rearranged to:

$$A = A^{2-} + \frac{[H^+](A^- - A)}{K_a} \quad (2)$$

A plot of A versus $[H^+](A^- - A)$, up to the region where degradation is negligible, yields a straight line with slope $1/K_a$, thus leading to the pK_a. Micro and macro ionization constants at 25°C and 50°C are given in Table 1.

TABLE 1

THE NEGATIVE LOGARITHMS OF MICRO AND MACRO IONIZATION CONSTANTS OF DOXORUBICIN (Dx) AND DAUNORUBICIN (Dr)

	Dx [a] 25°C	Dr	
		25°C	50°C
pk_1	8.22	8.00	7.44
pk_2	9.01	8.67	8.31
pk_3	10.10	10.01	9.44
pk_4	9.36	9.33	8.57
pK_1	8.15	7.92	7.38
pK_2	10.16	10.09	9.50
pK_3	13.20	13.30	12.80
k_5 [b]	0.159	0.21	0.136

[a] Data from Sturgeon and Schulman (1977).

[b] The ratio of the concentrations of zwitterionic and neutral anthracycline species.

Degradation kinetics

Order of reactions. Under the experimental conditions, the degradation reaction could be unambiguously established as (pseudo)-first-order with respect to the Dr concentration.

Standard deviation in k_{obs}. The standard deviation in the observed rate constant k_{obs}, obtained from log concentration–time curves, was determined at pH 8.0 and buffer concentration 0.01 M total phosphate ($\mu = 0.3$). The value of k_{obs} and the standard deviation, calculated from 6 observations, is $1.5 \pm 0.1 \times 10^{-6}$ s^{-1}. Other rate constants are mean values of duplicate determinations.

Influence of buffers. The degradation of Dr is accelerated by the presence of buffers, which has also been noticed for Dx (Janssen et al., 1985). This catalytic effect manifests itself from experiments where k_{obs} is measured at constant pH, ionic strength and temperature but at different buffer concentrations. At each pH, where buffers were used ($3 < pH < 11.5$), this catalysis could be demonstrated. The relationship between k_{obs} with increasing buffer concentration was found to be non-linear for acetate, phosphate and carbonate buffers. An example is given in Fig. 5.

Influence of ionic strength. At each pH studied the influence of the ionic strength (μ) on the degradation rate was checked by adding various amounts of sodium chloride to the buffers while keeping pH, buffer concentration and temperature constant. At $pH < 4$ rate constants, in terms of log k_{obs}, increase linearly with increasing ionic strength, in terms of $\sqrt{\mu}$. At $pH > 4$ none or only minor influences of the ionic strength could be observed in the μ region 0.1–0.4.

Influence of temperature. The effect of temperature on the degradation rate of Dr was determined in 0.01 M buffer at pH 8.0 and at pH 1.5 over the range 40–60°C. The Arrhenius equation is obeyed. From the slope and the intercept of a plot of ln k_{obs} versus the reciprocal of absolute temperature the activation energy ($\Delta H^{\neq}$) and frequency factor (A) were calculated. The results are: pH 8.0, $\Delta H^{\neq} = 79$ kJ · mol^{-1}, $A = 5.5 \times 10^6$ s^{-1}; pH 1.5, $\Delta H^{\neq} = 114$ kJ · mol^{-1}, $A = 5.5 \times 10^{13}$ s^{-1}.

Influence of pH. At each pH the degradation rate was studied as function of the buffer con-

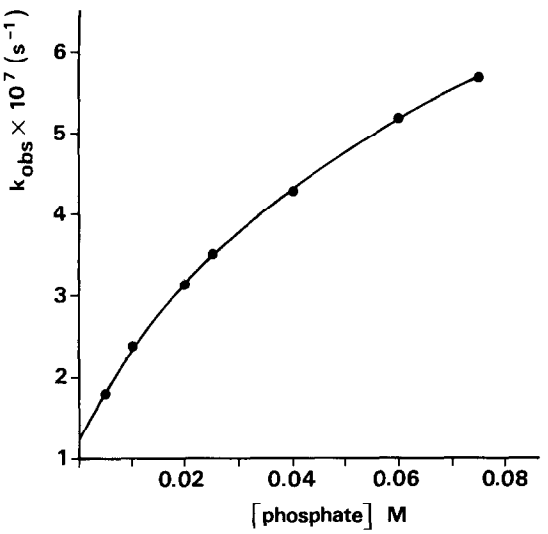


Fig. 5. The effect of phosphate concentration on k_{obs} for the degradation of Dr at pH 7.0. Temperature 50°C, $\mu = 0.3$.

centration at constant ionic strength ($\mu = 0.3$) and as a function of the ionic strength at constant buffer concentration. If an ionic strength effect was observed, the data of the experiments were corrected for this influence. After these corrections the obtained rate constants were extrapolated to zero buffer concentration. These mathematical procedures provide rate constants (k′) including only H_2O, H^+ and OH^- as catalysts. The general rate equation for such a combination of catalytic processes can be written as:

$$v = k'[R] = (k_o + k_H[H^+] + k_{OH}[OH^-])[R] \quad (3)$$

where k_o is the first-order rate constant for degradation in water only and k_H and k_{OH} represent the specific second-order rate constants for proton and hydroxyl catalyzed degradation, respectively. [R] refers to the total reactant concentration. In the pH region studied Dr is involved in three prototropic equilibria from which two overlap, attributing to the 3′-amino group and a phenolic function (Table 1). This suggests that in the pH range 0–14 Dr exists in five different forms: as a monocation, Dr^+; as a zwitterion, $Dr^{\pm}$; as a neutral molecule, Dr^o; as a monoanion, Dr^- and as a dianion Dr^{2-} (Fig. 6), whereas the total Dr concentration is:

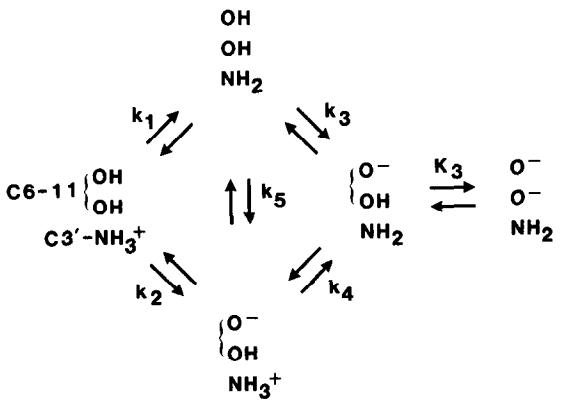


Fig. 6. Protolytic equilibria of Dr in aqueous solution.

$$[Dr]_{tot} = [Dr^+] + [Dr^{\pm}] + [Dr^o] + [Dr^-] + [Dr^{2-}] \quad (4)$$

The two phenolic functions are assumed to be equivalent with respect to deprotonation. In principle each species can undergo a proton catalyzed, a hydroxyl-catalyzed and a solvent-catalyzed reaction. The contributions of these reactions to k′ for each species is the product of the ($k_o + k_H[H^+] + k_{OH}[OH^-]$) term, applying to the species, multiplied by the fraction of that species. The fractions can be calculated from the expressions of the macro and micro ionization constants of Dr. The macro ionization constants are defined by:

$$K_1 = \frac{[H^+]([Dr^o] + [Dr^{\pm}])}{[Dr^+]} \quad (5)$$

$$K_2 = \frac{[H^+][Dr^-]}{([Dr^o] + [Dr^{\pm}])} \quad (6)$$

$$K_3 = \frac{[H^+][Dr^{2-}]}{[Dr^-]} \quad (7)$$

and the micro ionization constants are defined by:

$$k_1 = \frac{[H^+][Dr^o]}{[Dr^+]} \quad (8)$$

$$k_2 = \frac{[H^+][Dr^{\pm}]}{[Dr^+]} \quad (9)$$

$$k_3 = \frac{[H^+][Dr^-]}{[Dr^\circ]} \quad (10)$$

$$k_4 = \frac{[H^+][Dr^-]}{[Dr^\pm]} \quad (11)$$

whereas k_5 is defined as the pH independent ratio between the zwitterions and neutral Dr species: $[Dr^\pm]/[Dr^\circ]$. The relationships between the macroscopic and microscopic ionization constants are:

$K_1 = k_1 + k_2$; $1/K_2 = 1/k_3 + 1/k_4$ and

$K_1 \times K_2 = k_2 \times k_4 = k_1 \times k_3$

With the use of Eqns. 3–11 the expression for k′ over the entire pH region studied, should be written as:

$$k' = \{k_o^+ + k_H^+[H^+] + k_{OH}^+[OH^-]\} \times \frac{1}{1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2} + \frac{K_1K_2K_3}{[H^+]^3}}$$

$$+ \{k_o^\pm + k_H^\pm[H^+] + k_{OH}^\pm[OH^-]\} \times \frac{1}{1 + \frac{1}{k_5} + \frac{[H^+]^3 + K_1K_2[H^+] + K_1K_2K_3}{k_2[H^+]^2}}$$

$$+ \{k_o^\circ + k_H^\circ[H^+] + k_{OH}^\circ[OH^-]\} \times \frac{1}{1 + k_5 + \frac{[H^+]^3 + K_1K_2[H^+] + K_1K_2K_3}{k_1[H^+]^2}}$$

$$+ \{k_o^- + k_H^-[H^+] + k_{OH}^-[OH^-]\} \times \frac{1}{1 + \frac{[H^+]^2}{K_1K_2} + \frac{[H^+]}{K_2} + \frac{K_3}{[H^+]}}$$

$$+ \{k_o^{2-} + k_H^{2-}[H^+] + k_{OH}^{2-}[OH^-]\} \times \frac{1}{1 + \frac{[H^+]}{K_3} + \frac{[H^+]^2}{K_2K_3} + \frac{[H^+]^3}{K_1K_2K_3}} \quad (12)$$

The superscripts of the specific reaction constants refer to the appropriate Dr species.

Most of the suggested reactions are kinetically indistinguishable, except for the reactions between H^+ and Dr^+ and between OH^- and Dr^{2-}. The kinetic equivalent reactions are:

(1) $H^+ + Dr^\pm$, $H^+ + Dr^\circ$, $H_2O + Dr^+$,

(2) $H^+ + Dr^-$, $H_2O + Dr^\circ$, $H_2O + Dr^\pm$, $OH^- + Dr^+$,

(3) $H^+ + Dr^{2-}$, $H_2O + Dr^-$, $OH^- + Dr^\circ$, $OH^- + Dr^\pm$ and

(4) $H_2O + Dr^{2-}$, $OH^- + Dr^-$.

The separate contributions to k′ of the kinetically indistinguishable reactions cannot be assigned from the experimental data. Neglecting certain reactions may be attractive but such assumptions cannot be verified. Therefore all reactions were included. For the proper use of the non-linear curve-fitting computer program the reaction constants of the kinetic equivalent reactions are combined to new constants c_2–c_5. Inserting these con-

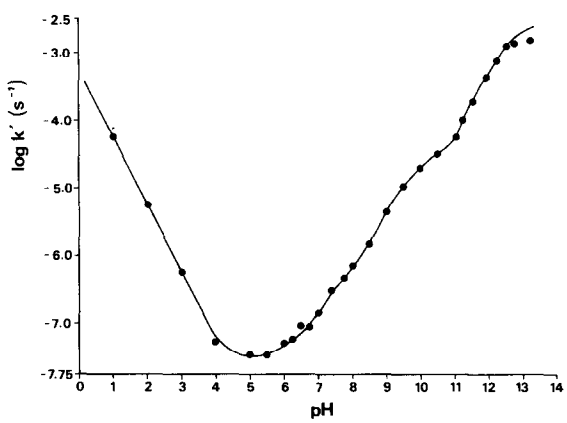


Fig. 7. Log k′–pH profile for Dr degradation at 50°C. The line has been simulated from Eqn. 13 using the constants (c_1–c_6) mentioned in the text and the $pK_{a(50°C)}$ values from Table 1.

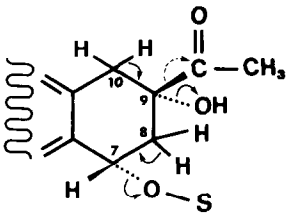


Fig. 8. Degradation scheme of Dr at pH > 4. S refers to the daunosamine sugar moiety.

stants and rearrangement of Eqn. 13 yields:

$$k' = \left\{c_1[H^+]^4 + c_2[H^+]^3 + c_3[H^+]^2 + c_4[H^+] + c_5 + c_6[H^+]^{-1}\right\}\left\{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3\right\}^{-1} \quad (13)$$

where $c_1 = k_H^+$; $c_2 = k_o^+ + k_1k_H^o + k_2k_H^\pm$; $c_3 = k_{OH}^+K_w + k_o^ok_1 + k_o^\pm k_2 + k_H^-K_1K_2$; $c_4 = k_{OH}^ok_1K_w + k_{OH}^\pm k_2K_w + k_o^-K_1K_2 + k_H^{2-}K_1K_2K_3$; $c_5 = k_{OH}^-K_wK_1K_2 + k_o^{2-}K_1K_2K_3$; $c_6 = k_{OH}^{2-}K_wK_1K_2K_3$. With the use of the non-linear curve-fitting program c_1–c_6 can be calculated from the experimental data. The results are: $c_1 = 5.6 \times 10^{-4}$ mol$^{-1}\cdot$s^{-1}; $c_2 = 1.7 \times 10^{-14}$ s^{-1}; $c_3 = 2.7 \times 10^{-8}$ mol$\cdot$s^{-1}; $c_4 = 2 \times 10^{-22}$ mol$^2\cdot$s^{-1} $c_5 = 6.6 \times 10^{-33}$ mol$^3\cdot$s^{-1}. The micro and macro ionization constants were fixed at the values obtained from the spectrophotometric experiments. From computer experiments where c_1–c_5 were kept constant and c_6 was varied it appeared that the $c_6/[H^+]$ term does not contribute to k′ and, consequently, $c_6 = k_{OH}^{2-} \sim 0$. The other terms contribute significantly to k′. In Fig. 7 the points are experimental values and the solid line calculated theoretically by employing Eqn. 13, the ionization constants from Table 1 and the reported c_1–c_6 values. The experimental data show a good agreement with the calculation of Eqn. 13 indicating an adequate description by this equation of the kinetics of the Dr degradation in the pH region studied. The section $0 \leqq pH \leqq 3.5$ of the log k′–pH graph has a slope −1 indicating specific proton catalysis for the conversion of the Dr monocation into I. The degradation rate of Dr between pH 4.5 and 5.5 shows pH independence. The shape of the pH profile at higher pH values implies that the overall degradation rate is strongly influenced by dissociation equilibria. No parts of the curve have a slope of +1. The degradation of Dr at pH > 4 yields principally II and III. A proposal of the degradation scheme is illustrated in Fig. 8. Probably, the reaction is initiated by removal of a C10 benzylic proton (Arcamone, 1978). Subsequent splitting of the C9 acetyl group, or the C9 hydroxyl function, and the sugar moiety results in the formation of II, or III, respectively.

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