Analysis of Binding of Daunorubicin and Doxorubicin to DNA Using Computerized Curve-Fitting Procedures

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Abstract □ The direct and difference spectroscopic methods of determining the binding constant of daunorubicin to DNA were compared. In addition, several other experimental parameters were varied, including the cell path length (1 or 10 cm), solution concentration, and analytical wavelength. The criterion used for judging the conditions and experimental methods was the attainment of a well-defined binding isotherm. Computerized least-squares curve fitting was used to determine the DNA binding constants from the binding isotherms and to compare the fit of a one-site and two-site model to the data. These studies showed that the best technique for determining the daunorubicin–DNA binding constants involved difference spectroscopic analysis of the absorbance change at 480 nm in long path length cells. In addition, overnight equilibration of daunorubicin–DNA complexes produced spectral changes, which indicated that methods involving prolonged equilibration would be inappropriate for daunorubicin–DNA binding studies. Computerized curve fitting of the data obtained from daunorubicin–DNA and doxorubicin–DNA solutions gave DNA binding constants of $1.27 \times 10^6 M^{-1}$ for daunorubicin and $2.04 \times 10^6 M^{-1}$ for doxorubicin and showed that a one-site model fit the data better than a two-site model. These binding constants are consistent with other physical and biological data and provide a very preliminary indication that DNA affinity and biological activity (*e.g.*, antitumor activity) may be related in the anthracyclines. In addition, the value of the number of binding sites obtained from the computerized curve-fitting program was 8/40 or 0.2, which is in agreement with the neighbor exclusion model. The methods and criterion used in this study describe a general approach for determining accurately the binding of drugs to DNA.

Keyphrases □ Daunorubicin—binding to DNA, direct and difference spectroscopic methods compared □ Doxorubicin—binding to DNA, direct and difference spectroscopic methods compared □ DNA—binding to daunorubicin and doxorubicin, direct and difference spectroscopic methods compared □ Spectroscopy—direct and difference methods of determining binding constants for daunorubicin and doxorubicin to DNA □ Binding constants—daunorubicin and doxorubicin to DNA, direct and difference spectroscopic methods compared □ Antineoplastic agents—daunorubicin and doxorubicin, binding to DNA, direct and difference spectroscopic methods compared

The binding of small molecules to nucleic acids is an important and widespread process in biochemistry (1–5), and improvements in the reliability of determining the binding constants of these interactions are needed. In addition, studies of drug–DNA interactions might improve knowledge of drug–receptor binding. Intercalation is a specific kind of DNA binding; it involves insertion of the agent between the base pairs of DNA or RNA (1, 6) and is associated with several simultaneously observed phenomena. These phenomena include UV or visible spectral changes and fluorescence spectral changes of the drug and changes in the hydrodynamic properties and X-ray fiber diffraction patterns of DNA. Individually, any one of these observations does not differentiate between intercalation and other types of nucleic acid binding. However, taken together, these observations provide strong evidence for intercalation.

BACKGROUND

Although several methods, including visible and fluorescence spectroscopy, circular dichroism, and equilibrium dialysis, are available for quantitatively determining the binding parameters of drugs to DNA, methods based on visible spectroscopy are the most widely used. The direct (7) and difference (8) spectroscopic methods are the most popular.

In the direct spectroscopic methods, the drug concentration is held constant and the changes in the drug spectrum are measured directly as the DNA concentration is varied. With the difference spectroscopic method, the DNA concentration is held constant and the changes in the drug spectrum are measured by subtracting the spectrum of the drug from the spectrum of the drug–DNA solution as the drug concentration is varied.

The data from these methods are usually interpreted using Eq. 1 (9, 10):

$$\frac{R}{C} = nK - KR \qquad \text{(Eq. 1)}$$

where R is the fraction of occupied sites, C is the concentration of free ligand, n is the total number of sites, and K is the intrinsic binding constant. According to Eq. 1, a plot of (R/C) *versus* R (Scatchard plot) gives a straight line with a slope of K and an intercept of n.

Comparisons of binding constants measured using spectroscopy and Scatchard plots (Table I) show a surprisingly large variation. This variation is too large to be entirely due to differences in the ionic strength of the solution since, for example, the binding constant of ethidium bromide to DNA is reported to be independent of ionic strength (Table I). An improved understanding of the reason for these variations and the relationship between binding constants measured by direct and difference spectroscopy is one aim of the research reported here.

In addition, the hazards of using reciprocal plots such as the Scatchard plot to derive the parameters K and n were noted previously (10, 11). For cases where the binding constant, K, is small and the data at saturation are incomplete, Klotz (10) showed that linear plots often could lead to an incorrect number of sites through bias or incorrect interpretation of the data. With intercalation, the binding constants are usually large and it is difficult to obtain complete and accurate data at low saturation. Under these circumstances, the bias and unequal weighting of the data inherent with the use of the Scatchard plot lead to errors in determining K. Large errors in the binding constant occur from data that are not measured over at least 75% of the adsorption isotherm (11).

A partial solution to the problems of bias and unequal weighting of the data utilizes computerized least-squares curve fitting of the data to the nonlinear form of Eq. 1:

$$R = \frac{nKC}{1 + KC} \qquad \text{(Eq. 2)}$$

using the programs of Fletcher and coworkers (12–14). These programs are versatile and enable the comparison of several models, including those containing single (Eq. 2) as well as multiple classes of sites:

$$R = \sum_{i=1}^{N} \frac{n_i K_i C}{1 + K_i C} \qquad \text{(Eq. 3)}$$

The anthracycline antibiotics daunorubicin (I) [(8*S*-*cis*)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione] and doxorubicin[1] (II) [(8*S*-*cis*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hex-

[1] Adriamycin.

Table I—Binding Constants of Agents to Calf Thymus DNA Determined by Visible Spectroscopic Methods

Agent	Method	Solution	K, M^{-1}	n	Reference
Proflavine	Direct	0.1 M Phosphate–ethylenediaminetetraacetic acid, pH 6.8	1.66×10^6	0.14–0.16	39
Proflavine	Direct	Ionic strength 0.1, pH 6.2	8.97×10^5	0.14	—[a]
Ethidium bromide	Direct	0.04 M Tromethamine hydrochloride	1.43×10^6	0.19	4
Ethidium bromide	Direct	Independent of ionic strength	$\sim 6.6 \times 10^6$	—	40
Actinomycin	Direct	0.1 M Phosphate, pH 7	4.2×10^6	0.07	41
Actinomycin	Difference	0.1 M Phosphate, 0.18 M NaCl, pH 6.9	2.3×10^6	0.11	8
Actinomycin	Difference	0.001 M Phosphate, pH 7	8.9×10^6	0.08	42
Daunorubicin	Difference	0.1 M Tromethamine	3.3×10^6	0.18	15
Daunorubicin	Direct	0.15 M NaCl, pH 7.0	$7.2 (\pm 1.2) \times 10^6$	0.158 (0.003)	35

[a] Calculated from the data in Table I of Ref. 7.

opyranosyl)oxy] - 7,8,9,10-tetrahydro-6,8,11-trihydroxy - 8 - (hydroxyacetyl)-1-methoxy-5,12-naphthacenedione] comprise an excellent system for the application of computerized curve-fitting procedures to drug–DNA interactions. Numerous studies indicated that daunorubicin and doxorubicin bind to DNA by intercalation (15–20), and several lines of evidence suggest that DNA intercalation may be the basis of the biological (*e.g.*, anticancer) activity of these antibiotics (21–24).

In addition, many metabolites and derivatives of daunorubicin and doxorubicin are biologically active (25–29). Thus, a knowledge of the binding constants of these derivatives would allow a quantitative comparison of DNA binding and biological activity (29).

This paper reports a study of the binding of doxorubicin and daunorubicin to DNA, using both direct and difference spectroscopy. Computerized curve-fitting procedures were used to determine accurately the binding constant and number of binding sites.

EXPERIMENTAL

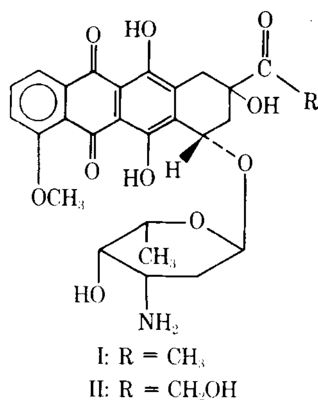
All spectra were recorded on a UV–visible spectrometer with either 1- or 10-cm quartz cells in 0.005 M potassium phosphate buffer, pH 7.4. All solutions were prepared using double-distilled, deionized water. Calf thymus DNA[2] was used for all experiments, and the concentration of the stock solution was determined using an absorptivity of $\epsilon_{260\ nm} = 6600\ M^{-1}\ cm^{-1}$. Doxorubicin hydrochloride, mp 189° [lit. (27) mp 188–190°], and doxorubicin hydrochloride, mp 201° [lit. (30, 31) mp 204–205°], were used as received[3].

Direct Spectral Method—A series of solutions was prepared in 10- or 100-ml volumetric flasks. The anthracycline concentration was constant, and the DNA concentration was varied to obtain the desired DNA to anthracycline concentration ratio. The spectra of the solutions were recorded from 400 to 600 nm with a blank containing the same DNA concentration as the sample. The resulting spectra were analyzed by the published procedure (7). In this analysis, the fraction bound, α_b, the concentration bound, C_b, and R were calculated as shown in Eqs. 4–6:

$$\alpha_b = \frac{A_{obs} - A_{free}}{A_{bound} - A_{free}} \qquad \text{(Eq. 4)}$$

$$C_b = \alpha_b C_{total} \qquad \text{(Eq. 5)}$$

$$R = \frac{C_b}{\text{total macromolecule concentration}} \qquad \text{(Eq. 6)}$$



[2] Sigma Chemical.

[3] Courtesy of Dr. F. Arcamone, Farmitalia, Milan, Italy.

Adsorption isotherms, titration curves, and Scatchard plots were prepared using the R and $C_{free} = C_{total} - C_b$ calculated and the computer programs described.

Difference Spectral Method—A series of solutions in 100-ml volumetric flasks was prepared in which the concentration of DNA was held constant. The drug concentration was varied to obtain the desired DNA to drug concentration ratio. Each solution was recorded against a blank containing an equal concentration of the drug. Thus, the spectra recorded were the differences between the bound and free drug spectra. The absorbance differences at 480 nm, the total drug concentration, and the absorptivity difference, $\Delta\epsilon$, between the bound and free drug were used to calculate R and C_b:

$$C_b = \frac{\text{absorbance difference (observed)}}{\Delta\epsilon} \qquad \text{(Eq. 7)}$$

Adsorption isotherms, titration curves, and Scatchard plots were prepared using the R and $C_{free} = C_{total} - C_b$ calculated and the computer programs described. These data were used to prepare Scatchard and binding plots, which were analyzed using the computer programs. The $\Delta\epsilon$ was taken as the difference between the absorptivities obtained by linear regression (least-squares) analysis of Beer's law plots for free drug and totally bound drug.

Determination of Analytical Wavelength and Effect of Overnight Equilibration—The binding of daunorubicin to DNA was studied by the direct spectral method, using a daunorubicin concentration of $3.72 \times 10^{-5}\ M$. The DNA concentration was varied to produce DNA phosphate to daunorubicin ratios between 10:1 and 0.2:1, and 1-cm cells were used. Based on the results of a preliminary study, two spectral regions were chosen for careful study: 450–500 and 530–540 nm. Adsorption isotherms (plots of R *versus* free daunorubicin) were prepared from the absorbances at 480 and 530 nm.

The effect of equilibration of DNA–daunorubicin solutions for extended times was investigated by preparing three solutions having a daunorubicin concentration of $3.72 \times 10^{-5}\ M$ and DNA phosphate to daunorubicin ratios of 0:1, 0.4:1, and 8:1. These solutions were stored in the dark at 3°. Samples were removed every other day for 2 weeks for analysis.

Determination of Optimum Concentration Ranges and DNA to Drug Ratios—Spectra of solutions containing DNA phosphate to daunorubicin ratios of 100:1 to 0.025:1 and a constant daunorubicin concentration of $3.72 \times 10^{-5}\ M$ were recorded in 1-cm cells. Based on these studies, ratios ranging from 10:1 to 0.2:1 were chosen for all further studies in 1-cm cells. Similar experiments showed that reproducible spectral changes could be obtained using DNA phosphate to daunorubicin ratios of 40:1 to 0.4:1 in 10-cm cells with a constant daunorubicin concentration of $3.76 \times 10^{-6}\ M$. These ratios were used in both the direct and difference spectroscopic studies in 10-cm cells.

Beer's Law Evaluations—Beer's law was evaluated for bound and free daunorubicin under various conditions in 1- and 10-cm cells. For 1-cm cells, seven absorbance readings were made over a range of 1.5×10^{-5}–$7.5 \times 10^{-5}\ M$; for 10-cm cells, the same number of measurements were made over a range of 1.5×10^{-6}–$7.5 \times 10^{-6}\ M$. Beer's law also was evaluated for DNA phosphate to daunorubicin ratios of 10:1 and 0.75:1 over a concentration range of 1.7×10^{-6}–$1 \times 10^{-5}\ M$ daunorubicin.

Computer Programs and Methods—Three programs were used; two were for the manipulation and display of binding data, and the third was the computerized least-squares curve-fitting program of Fletcher and Shrager (12).

The first program takes spectral data and converts it to binding data displayed in a convenient form. This program produces a table of C, log C, R, and R/C and Calcomp plots of R *versus* C, R *versus* log C, and (R/C) *versus* R. The second program, a modified version of Fletcher and Shrager's curve-fitting program, allows one to attempt to fit the data to

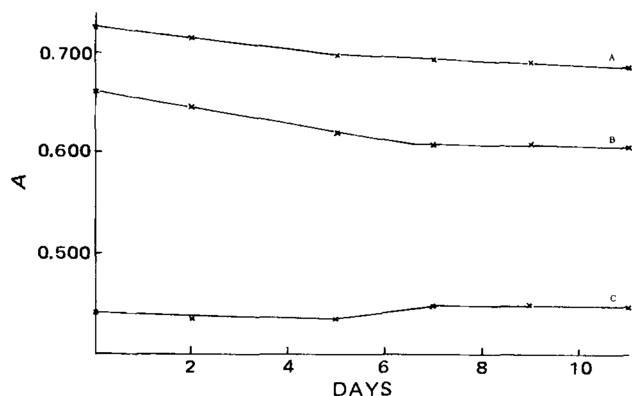


Figure 1—*Change in the absorbance of various daunorubicin solutions with respect to time. The DNA phosphate to daunorubicin ratios were: A, 0:1; B, 0.4:1; and C, 8:1.*

both the single-site (Scatchard) and multiple-site models. This program was modified to express n as a fraction and also as an integer. Arbitrarily, n was chosen to be an integer representing the number of molecules bound per 40 bases. This choice allowed qualitative gauging of the effect of allowing both n and K or just K to vary during the least-squares fitting procedure.

The other program plots the experimental R and C and R and log C values and draws the equation determined by the Fletcher and Shrager program through them. This plot provides a graphical display of how well the model actually fits the data. Details and listings of these programs were described previously (32).

RESULTS AND DISCUSSION

Studies of drug–DNA binding often have been carried out without adequate criteria for determining optimum experimental design. In this study, criteria allowing a systematic approach to the determination of the binding constants of drugs to DNA were used. A wide range of experimental conditions were tested, using the attainment of a well-defined adsorption isotherm (plot of R *versus* C) as a criterion for the selection of the best conditions. Scatchard plots were almost always made, but not extensively used, in these studies because of the work of Klotz (10) and Deranleau (11).

Studies of the effect of overnight equilibration on the visible absorbance of daunorubicin–DNA solutions were carried out to determine whether methods involving long equilibrations (*e.g.*, equilibrium dialysis) were feasible. Figure 1 shows that the absorbance of solutions containing DNA phosphate to daunorubicin ratios of 0:1 and 0.4:1 decreased and then leveled off. This result indicates that methods using overnight equilibration of daunorubicin–DNA solutions are not desirable.

Preliminary examination of the spectra of daunorubicin–DNA solutions (Fig. 2) indicated that several wavelengths could be used to determine the fractions of bound, R, and free, C, drug. The region beyond 550 nm was rejected because the absorbance was very wavelength dependent and a slight error in the wavelength would cause a large error in the absorbance. Two other wavelengths, 480 and 530 nm, were chosen as representative of the other most prominent regions of the spectrum.

The absorbance change from completely bound to free daunorubicin was only about 0.11 at 530 nm, but this change was 0.195 at 480 nm. In addition, the binding isotherms (Figs. 3 and 4) showed that the 480-nm isotherm plateaued but that the isotherm at 530 nm never reached a clear saturation plateau. These two factors indicated that absorbance changes at 480 nm should be used in all subsequent studies.

Having established the wavelength most suitable for analysis, the optimum concentration of daunorubicin and DNA was determined. Spectral studies of daunorubicin solutions and solutions containing DNA phosphate to daunomycin ratios of 8:1 in 1- and 10-cm cells showed that Beer's law was obeyed over the concentration range of 1.5×10^{-5}–7.5×10^{-6} M daunorubicin. This result is consistent with studies of daunorubicin dimerization, which showed a dimerization constant of 5.7×10^2 M^{-1} (33). In contrast, acridine orange dimerizes even in 10^{-5} M solutions, and this dimerization interferes with studies of DNA binding (see, for example, Ref. 34).

Although Beer's law studies showed that both 1- and 10-cm cells were suitable for analysis, Figs. 3 and 4 show that binding plots of data obtained in 1-cm cells lack points in the region of low drug concentration

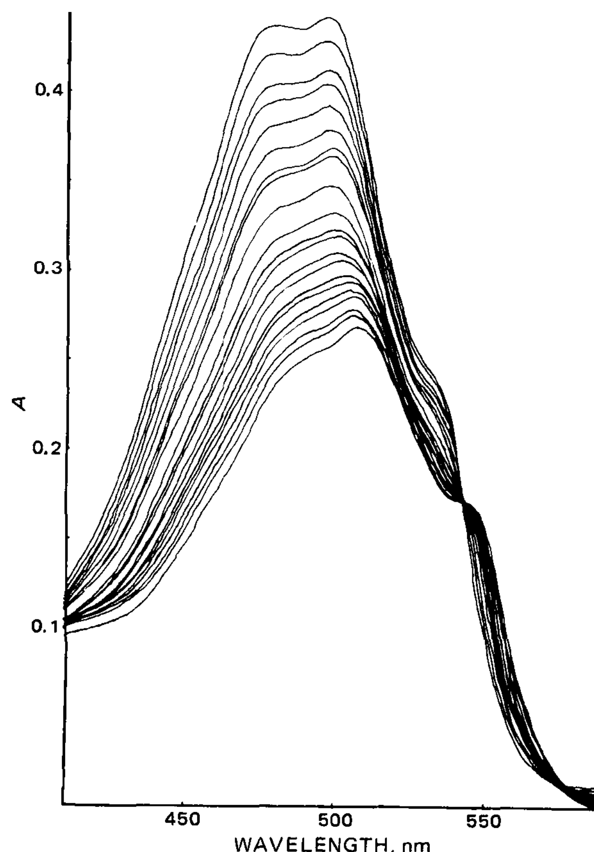


Figure 2—*Change in the absorption spectrum of daunorubicin in the presence of calf thymus DNA using 10-cm cells.*

C. This lack is a particularly serious problem since Deranleau (11) showed that these points were required for the accurate determination of binding constants. Figure 5 shows the adsorption isotherm obtained in 10-cm cells using daunorubicin and DNA concentrations one-tenth those used for the isotherm shown in Fig. 3. Obviously, the 10-cm cells are much more suitable for determining accurate binding constants. In addition, the error in the binding constants determined by computerized curve fitting of the data from the 10-cm cells was 10–15% while that for experiments in the 1-cm cells was 40–50%. The improved accuracy and ease of data analysis for the 10-cm cells were consistent with Waring's studies (4) of DNA–ethidium bromide binding in 4-cm cells.

The binding isotherms obtained from direct and difference spectroscopic studies of daunorubicin–DNA solutions in 10-cm cells were compared. Figure 6 shows the binding isotherm obtained using difference spectroscopy. A comparison of Figs. 5 and 6 clearly shows that difference spectroscopy gives more data points in the critical low C range. Therefore, difference spectroscopy is the method of choice for obtaining accurate binding constants since it gives the data points over a wider range of the binding isotherm (11). On the other hand, the direct spectroscopic method gives more points at the higher C values where the binding iso-

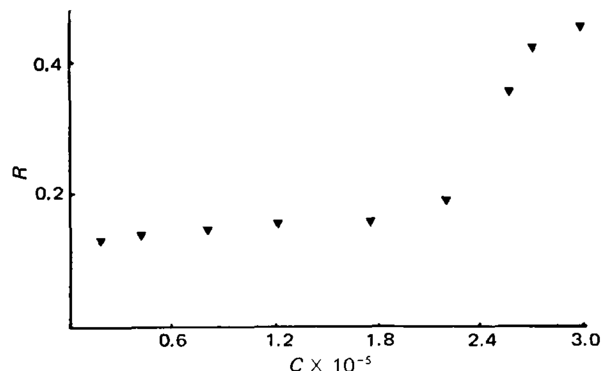


Figure 3—*Binding of daunorubicin to calf thymus DNA using the spectral change at 480 nm in 1-cm cells.*

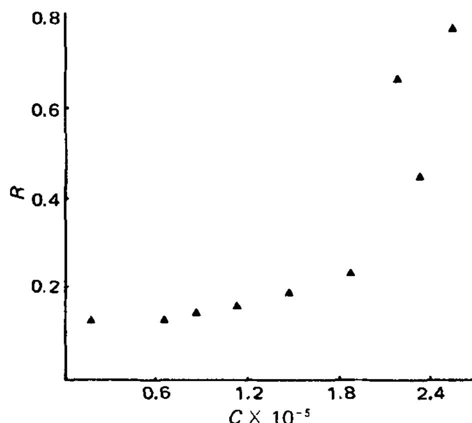


Figure 4—*Binding of daunorubicin to calf thymus DNA using the spectral change at 530 nm in 1-cm cells.*

therm plateaus and should provide a more accurate measure of the number of binding sites.

Studies using the direct spectral method gave a few points at low free drug concentration C because of the experimental procedure normally used (7). Data are measured at fixed drug and varying DNA concentrations; as the DNA concentration becomes large (and the free drug concentration becomes small), the spectra change very little when the binding constant K is large because essentially all drug in solution is completely bound. Thus, the steep changes in R occurring at low free drug concentrations (small C) where most drug is bound are associated with small spectral changes, and it is difficult to obtain data in this region. With difference spectroscopy, the DNA concentration is held constant and spectral changes are measured with increasing drug concentration as the difference between the spectrum of the free drug solution and the DNA–drug solution. Hence, small spectral changes start to occur only when further increases in the drug concentration produce very little additional change in the spectral difference between the free drug and the DNA–drug solution. This situation occurs when saturation and high drug concentrations are reached. Thus, in contrast to the direct spectral method, numerous points in the range of low free drug concentration can be obtained with the difference spectroscopic method.

The effect of the choice of limiting spectra on binding constants determined by difference and direct spectroscopic methods also was studied. The spectra of the bound and free drug were used to determine the $\Delta\epsilon$ in the difference spectroscopic method. Small random changes in $\Delta\epsilon$ had little effect on the binding constant. In contrast, in the direct spectral method, the spectrum taken as that of the completely bound drug had a substantial effect on the binding constant. For example, the spectra corresponding to DNA phosphate to daunorubicin ratios of 10:1, 20:1, 40:1, and 60:1 were chosen as those of totally bound daunorubicin and the data were analyzed by Scatchard plots. The binding constants varied from $3.7 \times 10^6 M^{-1}$ for the 60:1 limit to $1.5 \times 10^7 M^{-1}$ for the 10:1 limit.

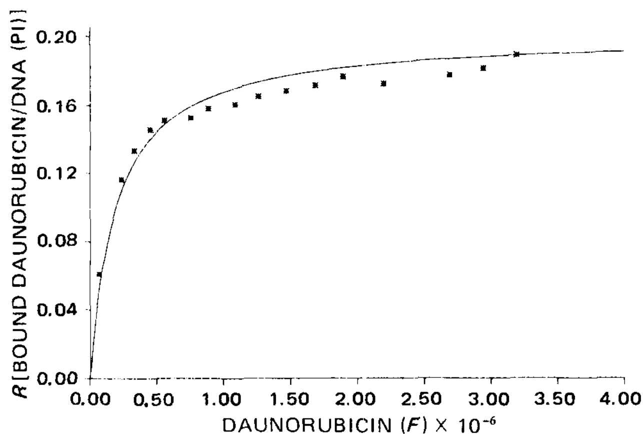


Figure 5—*Binding of daunorubicin to calf thymus DNA using direct spectroscopy [F refers to free daunorubicin and DNA (PI) refers to DNA phosphate]. Stars represent experimental data from the spectral change at 480 nm in 10-cm cells. The solid curve represents the adsorption isotherm generated from the computer-fitted model.*

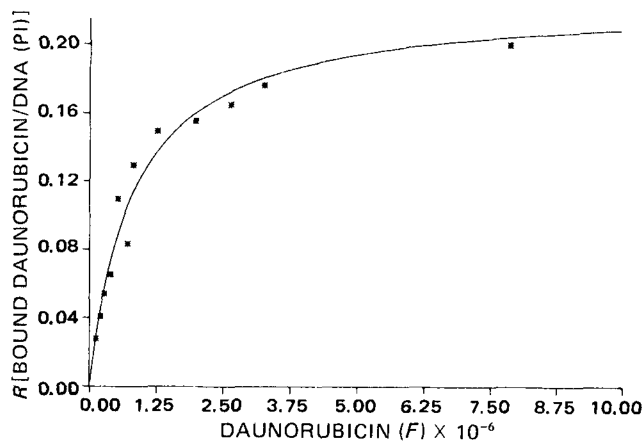


Figure 6—*Binding of daunorubicin to calf thymus DNA using difference spectroscopy. Stars represent experimental data from the difference spectra at 480 nm in 10-cm cells. The solid curve represents the adsorption isotherm generated from the computer-fitted model.*

This result is consistent with previous studies (11) since the choice of the limiting spectrum determines the number of points on the binding isotherm between saturation and zero and thus causes changes in the accuracy of the determination of K. In this example, the binding plot using the 10:1 ratio had only two points between saturation and zero, while the 60:1 limit gave a binding plot with five points in this region. The number of points between saturation and zero can thus have an important effect on the magnitude of the binding constant.

In this study, as the number of points between saturation and zero decreased, the binding constant increased. It is tempting to speculate that this trend explains the differences between daunorubicin–DNA binding constants determined by direct and difference spectroscopy, which are $7.2 \times 10^6 M^{-1}$ (35) and $3.3 \times 10^6 M^{-1}$ (21), respectively. In the present studies, the binding constants for both daunorubicin and doxorubicin to DNA were consistently higher when measured by direct spectroscopy. One study of actinomycin–DNA binding by difference spectroscopy gave a higher binding constant than that determined by direct spectroscopy (Table I), in disagreement with the trend noted previously.

In a typical linear Scatchard plot of (R/C) *versus* R, the number of points between saturation and zero is not apparent. For this reason and those discussed earlier, binding plots are preferred over Scatchard plots for the determination of binding constants.

In summary, the present studies show that, for the determination of daunorubicin–DNA and, presumably, anthracycline–DNA binding constants, the following conditions are best: 480 nm for absorbance measurements, 10-cm absorption cells, difference spectroscopic method, and binding plots instead of Scatchard plots.

Having determined the optimum experimental conditions and the best method of plotting the data, it was desirable to analyze these data by the least biased and most efficient method, computerized curve fitting. The computerized curve-fitting programs of Fletcher and Shrager (14) were modified for use with DNA–drug binding data. This modification allowed the determination of the binding constant that best fits the data with the number of sites free to vary as a fractional value per base. Comparison of two typical experiments, using data obtained from direct spectral studies of daunorubicin–DNA binding, gave the following parameters if n was allowed to vary: $K = 6.94 \times 10^6 M^{-1} \pm 0.42$, $n = 0.188$, and $K = 5.26 \times 10^6 M^{-1} \pm 0.40$, $n = 0.196$. However, the statistical data (14) indicated a dependence of one parameter on another because the condition codes were orders of magnitude larger than unity and had values of 78 and 98.

In an attempt to overcome this difficulty, n was also set at $[(n \times 40) - 1]/40 = 8/40$ and $[(n \times 40) + 1]/40 = 9/40$, and the data were refitted allowing only K to vary. Evaluation of the results for K fitted with each value of n revealed that the results obtained for n fixed at 8/40 provided the best model. An $n = 8/40 = 0.200$, as obtained using computerized curve-fitting procedures, is expected for the neighbor exclusion model (36). In addition, the values of K were in much better internal agreement with $K = 5.21 \times 10^6 M^{-1} \pm 0.52$ and $4.75 \times 10^6 M^{-1} \pm 0.33$ and condition codes = 2 and 3. The best qualitative explanation of these observations is that the program varies n and K to produce the best fit to the data; however, since n and K are interdependent, there is a series of values for n and K that gives nearly the same agreement with the data. Fixing n

Table II—Comparison of DNA Binding Ability and Biological Effects of Daunorubicin and Doxorubicin

Antibiotic	Binding Constant, $\times 10^6 M^{-1}$	Increase in DNA Melting Temperature[a]	Inhibition of Cell Free Polymerase Reactions, ID_{50}[b], $\times 10^{-5} M$		Specific Inhibition of Cellular Nucleic[c] Acid Synthesis		Cell Killing Potential
			DNA	RNA	DNA[d]	RNA[e]	
Daunorubicin	1.27	13.4°	1.6	1.0	119	147	1×
Doxorubicin	2.04	14.8°	1.2	0.6	152	215	2×

[a] Reference 15. [b] Reference 43. [c] Specific inhibitory activity = percent inhibition/cellular concentration. [d] Reference 44. [e] Reference 45.

removes this interdependence and gives K values that are the best fit of the data with little increase in the error in K.

Attempts were also made to fit the direct spectral data to a two-site model (see Eq. 3). However, the error and condition code were much larger than for the one-site model, and it was concluded that a two-site model did not improve the fit to the data.

In addition to the computerized curve-fitting program, two other programs were written to assist in data analysis and display. The binding plots in this paper were produced using these programs and the Calcomp plotter.

Having established the optimum methods for the determination of the daunorubicin–DNA binding constants, identical methods were used to determine doxorubicin–DNA binding constants. Use of the identical methods was justified since the daunorubicin–DNA spectra were very similar to the doxorubicin–DNA spectra.

The binding constant for daunorubicin obtained using this procedure ($1.27 \times 10^6 M^{-1}$) was significantly lower than that obtained by direct spectroscopy, which was $4.98 \times 10^6 M^{-1}$ (the average of 5.21×10^6 and 4.75×10^6). This result agrees with a trend noted earlier that the small number of points between saturation and zero with binding plots obtained from the direct spectral method is associated with abnormally large binding constants.

Some reports (36, 37) showed that vagrant binding to a linear array of sites can cause exclusion of some sites, which leads to curved Scatchard plots and an overestimation of the binding constant. To circumvent this problem in studying binding to DNA, it was suggested (8, 36–38) that the y-intercept of the Scatchard plot should be used because $R/C = Kn$ at this point. The K obtained using the y-intercept of a Scatchard plot based on difference spectroscopic data was also $1.27 \times 10^6 M^{-1}$, in exact agreement with the value determined by computerized curve fitting. Although exact agreement is probably fortuitous, the good agreement obtained substantiates the value of computerized curve fitting for DNA binding studies.

Doxorubicin has a significantly greater affinity for DNA than daunorubicin. The binding constants and n values were $1.27 \times 10^6 M^{-1}$ (±0.10) and $n = 8/40$ for daunorubicin and $2.04 \times 10^6 M^{-1}$ (±0.18) and $n = 8/40$ for doxorubicin. This result was true even if n was allowed to vary completely (32). This order of affinities is consistent with comparisons of several properties of doxorubicin and daunorubicin. Doxorubicin increased the melting temperature of DNA by 14.8° while daunorubicin only increased it by 13.4° (15). In addition, doxorubicin had a greater inhibitory effect on both cell free polymerase activity and cellular nucleic acid synthesis than daunorubicin (Table II).

One would expect that the melting-temperature increase and the binding constant would parallel each other regardless of the mechanism of action of the drug. If the mechanism involves DNA binding, then the inhibition of cell free polymerase activity and nucleic acid synthesis should also parallel DNA binding. Table II shows that this effect is indeed observed; these data, taken together, provide a preliminary indication that there may be a correlation between the DNA binding constant and biological activity in the anthracyclines. One recent report (29) also led to a similar suggestion that DNA binding and biological activity are related.

The binding constants determined in this study and the pains taken to ensure their accurate and unbiased determination suggest that the earlier order determined for the DNA binding constants of daunorubicin, $3.3 \times 10^6 M^{-1}$, and doxorubicin, $2.3 \times 10^6 M^{-1}$ (15), should be revised. Recent studies (29) using fluorescence spectroscopy gave DNA binding constants of $4.8 \times 10^6 M^{-1}$ and $3.3 \times 10^6 M^{-1}$ for doxorubicin and daunorubicin, respectively, in agreement with the order determined in this report.

REFERENCES

(1) V. A. Bloomfield, D. M. Crothers, and I. Tinoco, "Physical Chemistry of Nucleic Acids," Harper and Row, New York, N.Y., 1974.

(2) R. J. Douthart, J. P. Burnett, F. W. Beasley, and B. H. Frank, *Biochemistry*, **12**, 214 (1973).

(3) R. W. Armstrong and N. M. Panzer, *J. Am. Chem. Soc.*, **94**, 7650 (1972).

(4) M. J. Waring, *J. Mol. Biol.*, **13**, 269 (1965).

(5) E. J. Gabbay, K. Sanford, and S. Baxter, *J. Am. Chem. Soc.*, **94**, 2876 (1972).

(6) H. M. Sobell, S. C. Jain, T. D. Sakore, and C. E. Nordman, *Nature*, **231**, 200 (1971).

(7) A. R. Peacocke and J. N. H. Skerett, *Trans. Faraday Soc.*, **52**, 261 (1956).

(8) W. Muller and D. M. Crothers, *J. Mol. Biol.*, **35**, 251 (1968).

(9) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).

(10) I. M. Klotz, *Acc. Chem. Res.*, **7**, 162 (1974).

(11) D. A. Deranleau, *J. Am. Chem. Soc.*, **91**, 4044 (1969).

(12) J. E. Fletcher and R. I. Shrager, "A Users Guide to Least Squares Model Fitting," DCRT Technical Report No. 1, 1968.

(13) J. E. Fletcher and A. A. Spector, *Comput. Biomed. Res.*, **2**, 164 (1968).

(14) J. E. Fletcher, A. A. Spector, and J. D. Ashbrook, *Biochemistry*, **9**, 4580 (1970).

(15) F. Zunio, R. Gambetta, A. DiMarco, and A. Zaccara, *Biochim. Biophys. Acta*, **277**, 489 (1972).

(16) E. Calendi, A. DiMarco, M. Reggiani, B. Scarpinato, and L. Valenti, *ibid.*, **103**, 25 (1965).

(17) M. M. Fishman and I. Schwartz, *Biochem. Pharmacol.*, **23**, 2147 (1973).

(18) F. Quadrifoglio and V. Crescenzi, *Biophys. Chem.*, **2**, 64 (1974).

(19) E. J. Gabbay, D. Grier, R. F. Fingerle, R. Reimer, R. Levy, S. W. Pearce, and W. D. Wilson, *Biochemistry*, **15**, 2062 (1976).

(20) W. J. Pigram, W. Fuller, and L. D. Hamilton, *Nature New Biol.*, **235**, 17 (1972).

(21) F. Zunio, A. DiMarco, A. Zaccara, and G. Luoni, *Chem.-Biol. Interact.*, **9**, 25 (1974).

(22) P. Chandra, A. DiMarco, F. Zunio, A. M. Casazza, D. Gericke, F. Guiliana, C. Soranzo, R. Thorbeck, A. Gotz, F. Arcamone, and M. Ghione, *Naturwissenschaften*, **59**, 448 (1972).

(23) V. Barthelemy-Clavey, G. Serros, and G. Aubel-Sadron, *Mol. Pharmacol.*, **11**, 640 (1975).

(24) S. Aktipis and N. Panayotatos, *Biochem. Biophys. Res. Commun.*, **68**, 465 (1976).

(25) S. Takanashi and N. R. Bachur, *Drug Metab. Disp.*, **4**, 79 (1976).

(26) D. W. Wilson, D. Grier, R. Reimer, J. D. Bauman, J. F. Preston, and E. J. Gabbay, *J. Med. Chem.*, **19**, 381 (1976).

(27) *Ibid.*, **19**, 381 (1976).

(28) L. Lenaz, A. Necco, T. Dasdia, and A. DiMarco, *Cancer Chemother. Rep., Part 1*, **58**, 769 (1974).

(29) A. DiMarco, A. M. Casazza, R. Gambetta, R. Supio, and F. Zunio, *Cancer Res.*, **36**, 1962 (1976).

(30) F. Arcamone, G. Francheschi, S. Penoco, and A. Selva, *Tetrahedron Lett.*, **1969**, 1003.

(31) A. DiMarco, M. Gaetani, and B. Scarpinato, *Cancer Chemother. Rep., Part 1*, **53**, 33 (1969).

(32) G. D. Dolch, Ph.D. thesis, Purdue University, 1976, Appendix I.

(33) V. Barthelemy-Clavey, J. C. Maurizot, J. L. Dimicoli, and P. Sicard, *FEBS Lett.*, **46**, 5 (1974).

(34) M. E. Lamm and D. M. Neville, Jr., *J. Phys. Chem.*, **69**, 3872 (1965).

(35) P. J. Gray and D. R. Phillips, *Eur. J. Cancer*, **12**, 237 (1976).

(36) D. M. Crothers, *Biopolymers*, **6**, 575 (1968).

(37) J. L. Bresloff and D. M. Crothers, *J. Mol. Biol.*, **95**, 103 (1975).

(38) D. E. V. Schmechel and D. M. Crothers, *Biopolymers*, **10**, 465 (1971).

(39) S. Das Gupta, D. N. Misra, and N. N. Das Gupta, *Biochim. Biophys. Acta*, **294**, 38 (1973).
(40) I. O. Walker, *ibid.*, **109**, 585 (1965).
(41) R. Bittman and L. Blau, *Biochemistry*, **14**, 2138 (1975).
(42) B. A. K. Chibber, A. Balasubramanian, and T. VisWanatha, *Can. J. Biochem.*, **51**, 204 (1973).
(43) M. F. Goodman, M. J. Bessman, and N. R. Bachur, *Proc. Natl. Acad. Sci., USA*, **71**, 11931 (1974).
(44) W. D. Meriwether and N. R. Bachur, *Cancer Res.*, **32**, 1137 (1972).
(45) N. R. Bachur, M. Steele, W. D. Meriwether, and R. C. Hildebrand, *J. Med. Chem.*, **19**, 651 (1976).

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Improved Stability of Proteins Immobilized in Microparticles Prepared by a Modified Emulsion Polymerization Technique

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Abstract □ Proteins can be immobilized in spherical microparticles of polyacrylamide gel (having a diameter of about 1 μm) by an emulsion-polymerization technique. Highly cross-linked gels have a structure consisting of relatively large pores. This structure is advantageous when dealing with biologically active proteins acting on molecules dissolved in the surrounding medium. A rapid equilibrium is established between the interior of the particles and the medium, and rate-limiting diffusion is not observed. A suspension of carbonic anhydrase immobilized in microparticles will thus have kinetic properties very much like the free enzyme. In addition to the entrapment of the protein molecules in the three-dimensional network formed by the polyacrylamide threads, protein molecules are also fixed in the cross-linked threads of polyacrylamide. This fixation is probably responsible for the improved stability of the protein molecules against heat denaturation. Not even autoclaving at 110° for 30 min denatured the immobilized enzyme completely (more than 25% of the enzyme activity was left). The higher resistance of molecules in microparticles against proteolytic degradation also is documented.

Keyphrases □ Proteins—immobilized in polyacrylamide microparticles, effect on stability □ Microparticles, polyacrylamide—containing immobilized proteins, effect on stability □ Polyacrylamide microparticles—containing immobilized proteins, effect on stability □ Stability—proteins immobilized in polyacrylamide microparticles

The immobilization of proteins in polyacrylamide microparticles with a high degree of cross-linking (T-C = 8-25[1]) by emulsion polymerization was studied recently (2–4). The macromolecules apparently are immobilized by two mechanisms: fixation in the highly cross-linked threads of polyacrylamide and, if sufficiently large, entrapment in the network formed by the polyacrylamide threads (4). Consequently, the immobilized macromolecules will be localized partly on the microparticle surface, enabling them to react also with substrates or cells that cannot penetrate into the particles (2, 5). The highly cross-linked gels (C = 25%) also produce a network with relatively large pores, which is advantageous with biologically active proteins interacting with molecules dissolved in the surrounding medium because the macromolecules retain most of their activity when bound in the microparticles.

The present work was undertaken to investigate the consequences of the immobilization in highly cross-linked microparticles of polyacrylamide on the activity and stability of proteins, especially carbonic anhydrase. A fraction of the enzyme is remarkably stable, even against heat denaturation, while some enzymatic activity is lost in a normal way. A modified procedure is described for the large-scale microparticle preparation.

EXPERIMENTAL

Materials—Human serum albumin[2], immunoglobulin G[2], bovine β-lactoglobulin[3] (as a mixture of the A and B forms), protease[3] Type VI from *Streptomyces griseus*, and bovine carbonic anhydrase[3] (EC 4.2.1.1) were used without further purification. Acrylamide[3], *N,N'*-methylenebisacrylamide[3], *p*-nitrophenyl acetate[3], *N,N,N',N'*-tetramethylethylenediamine[3], tris(hydroxymethyl)aminomethane[3], and other chemicals were analytical grade.

Determination of Carbonic Anhydrase Activity—The esterase activity of carbonic anhydrase was determined with *p*-nitrophenyl acetate as the substrate (6). The enzyme (50 μg in free solution or in microparticles) was added to 5 ml of 0.05 *M* tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.5. The formation of the hydrolysis products was followed spectrophotometrically at 400 nm. The velocity of the reaction is expressed as an increase in absorbance during 1 min.

Appropriate correction was made for the spontaneous hydrolysis of the substrate in samples not containing any enzyme. Microparticles of polyacrylamide not containing enzyme did not affect the reaction, as found in control experiments. The particles did not cause any light scattering at the wavelength used, and no sedimentation was detectable during the time of the measurement.

Protein Determination—The concentrations of human serum albumin, immunoglobulin G, and β-lactoglobulin were determined from the optical density at 280 nm. The $E_{1\,cm}^{1\%}$ values were 5.3 (7) for albumin, 13.6 for immunoglobulin, and 7.90 for lactoglobulin (8). The spectra were recorded with an automatic recording spectrophotometer at 400 nm to correct for the light-scattering effect (9). The amount of protein immobilized in the microparticles was determined after hydrolysis in 6 *M* HCl

[1] The nomenclature suggested by Hjertén (1) is used.

[2] KABI AB, Stockholm, Sweden.
[3] Sigma Chemical Co.