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SOLUTE ENHANCED PARTITION ANALYSIS-A NOVEL METHOD FOR MEASURING THE BINDING OF DRUGS TO DNA.

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ABSTRACT

A solute enhanced phase partition method is described for the measurement of the binding of drugs to nucleic acids. The inclusion of a solute which acts as a phase transfer reagent allows one to enhance the solubility of charged molecules in organic solvents by as much as three orders of magnitude. This development increases the utility of partition analysis as a general method for studying the interaction of small molecules with macromolecules. Solute enhanced partition analysis (SEPA) has been used to measure the DNA binding of positively charged drugs at very low levels of drug binding, where we have observed cooperative binding of daunorubicin to calf thymus DNA.

INTRODUCTION

The method of partition analysis was first proposed by Karush in 1951 (1) as an alternative to equilibrium dialysis for measuring the binding of ions to proteins. Partition analysis consists of the measurement of the equilibrium distribution of a drug or ion between an aqueous buffer phase (in which the macromolecule is dissolved) and an immiscible organic phase. The two solvents are mixed vigorously until the drug (or other small molecule or ion) is equilibrated (i.e., partitioned) between the aqueous and the organic phases. The two solvents are then separated and the drug concentration is measured in both phases. The method of partition analysis has several advantages over equilibrium dialysis techniques(1,4), as will be discussed below. However, except for three papers in the early 1950's (1-3), the method of partition analysis was not utilized until 1975, when Waring and coworkers (4) described the details of partition analysis techniques and discussed the application of partition analysis for the measurement of the equilibrium binding between drugs and nucleic acids. The following year partition analysis methods were used to study the interaction between actinomycin D and deoxynucleotides(5),and the binding of anthracyclines to DNA(6).

The major limitation of the general utilization of partition analysis methods is the necessity of finding a water immiscible organic solvent which simultaneously serves as an acceptable solvent for the large class of positively charged molecules which bind strongly to DNA. Ethidium bromide

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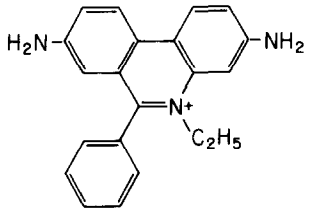

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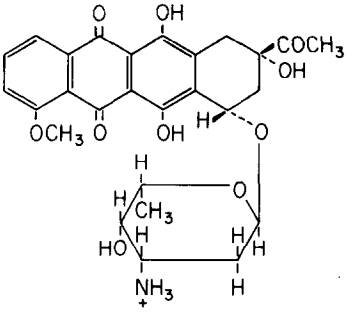

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Figure 1. (A). The chemical structure of the ethidium cation and; (B), the chemical structure of the daunorubicin cation (the predominant form at neutral pH).

and daunorubicin (Figure 1) are examples of important drugs which carry a positive charge and are thus not very soluble in organic solvents. In order to overcome this limitation we propose the addition of an ionic solute which contains an organic anion (such as sodium tetraphenylborate) since this can, in some cases, dramatically enhance the organic phase solubility of the cationic drugs. We have called this approach solute enhanced partition analysis (SEPA), and we have found that SEPA is the method of choice for studying the nucleic acid binding of several positively charged drugs.

Waring et al. (4) noted that i-amyl acetate has an aqueous solubility of 0.17% (w/w), which translates into a concentration of 10 mM of i-amyl acetate in the aqueous buffer; this raised the question as to what effect if any, the i-amyl acetate in the aqueous phase has on the binding equilibrium. Waring et al. (4) found no evidence that the i-amyl acetate altered the structure of DNA. Gabbay et al. (6) used chloroform as the organic solvent to study the binding of anthracyclines to DNA. Although chloroform has a solubility of 60 mM in water (and an inconveniently high vapor pressure), the results from a spectral titration and a partition analysis experiment were not markedly different (6). An advantage of the SEPA method is that it allows for the use of organic solvents with very low aqueous solubility. For example, the use of 1-chloroheptane provides an organic solvent with both a low aqueous solubility and a low vapor pressure. A number of other solvents or solvent mixtures may also be used.

RESULTS AND DISCUSSION. The general experimental procedures used in partition analysis have been outlined earlier (1-5), and will be mentioned only briefly here to provide the background for the development and understanding of solute enhanced partition analysis (SEPA). In order to determine the binding isotherm one needs to measure the amount of bound and free drug as a function of the total added drug. Equilibrium dialysis has been extensively utilized for these experiments but the determination of low concentrations of free drug (cf) is usually the limiting factor in extending the measurements to low levels of drug binding. Other possible complicating factors associated

with equilibrium dialysis are the adsorption of substrates to the membrane and the long time periods generally required for the system to reach equilibrium (hours to days). Partition analysis consists of shaking or vortexing a vial which contains both an aqueous phase and an immiscible organic phase until the drug has reached an equilibrium distribution between the phases. The drug molecule may be initially added to either phase. The partition coefficient, P, which is the ratio of the concentration of drug in the organic phase to the concentration of free drug in the aqueous phase, is determined in the absence of polynucleotide. When the binding studies are performed, the two phases are equilibrated and separated. The concentration of drug in the organic phase, [organic phase drug], is measured and this value is used to calculate the concentration of free drug in the aqueous phase, cf, where cf = [organic phase drug]/P.

Partition analysis methods (also referred to as solvent partition (4) and phase partition (5)) are reasonably straight-forward for studying the binding of uncharged drugs to DNA. However, the majority of important DNA binding drugs are positively charged and thus have very low partition coefficients for most organic/aqueous solvent systems. Ethidium bromide, an archetypical intercalating drug, may be used as an example. The data in Table I show that ethidium has a partition coefficient (organic/aqueous) of 0.023 when n-amyl-acetate is used as the organic phase, and 0.018 when 1-chloroheptane is used as the organic phase. The problem is that charged species are generally not very soluble in organic solvents. However, if an organic anion is present then a cationic drug - anionic solute ion pair (eg., the ethidium-tetraphenyl-borate ion pair) will be soluble in the organic phase, as clearly evidenced by the data in Table I.

Sodium benzoate, the first solute tried, did not significantly affect the partition coefficient of ethidium. Sodium p-nitrophenolate was selected next because it has a pK of 7.2. The use of n-amyl acetate saturated with sodium p-nitrophenolate as the organic phase increased the partition coefficient by more than a factor of two when compared to the partition coefficient for the n-amyl acetate/aqueous buffer solution (Table I). This experiment established the principle of solute enhanced partition analysis. The addition of sodium tetraphenylborate (which was suggested by Professor G. McLendon) to the n-amyl acetate/aqueous buffer solution increases the partition coefficient of ethid-ium by a factor 840 (Table I). When 1-chloroheptane is used as the organic solvent, the addition of sodium tetraphenylborate (NaTPB) increases the partition coefficient by a factor of 1000. We have tested other positively charged drugs and we find enhancements in the partition coefficients when NaTPB is added; we anticipate that a variety of suitable solutes for SEPA experiments will be found. The partition coefficient of actinomycin D was not substantially affected by the addition of sodium tetraphenylborate, which is consistent with the observation that actinomycin D is an uncharged molecule at pH 7. A retrospective review of the literature reveals the similarity in the principles of solute enhanced partition analysis with phase transfer catalysis and ion-pairing chromatography (7,8, and references therein).

TABLE I. EFFECTS OF SOLUTE ADDITION ON DRUG PARTITION COEFFICIENTS

DRUG	ORGANIC PHASE	AQUEOUS PHASE	P(ORGANIC/AQUEOUS)
Ethidium Bromide	n-amyl acetate	Buffer	0.023
	n-amyl acetate (saturated with sodium p-nitrophenolate)	Buffer	0.052
	n-amyl acetate (plus 1mM sodium tetraphenylborate)	Buffer (plus 0.5mM sodium tetraphenylborate)	18.1
	1-chloroheptane	Buffer	0.018
	1-chloroheptane (saturated with sodium tetraphenyl-borate)	Buffer (plus 0.5mM sodium tetraphenylborate)	18.2
Daunorubicin	n-amyl acetate	Buffer	0.566
	n-amyl acetate-hexanes (1:1)	Buffer	0.253
	n-amyl acetate-hexanes (1:1) (plus 0.2mM sodium tetraphenyl-borate)	Buffer	6.22
	n-amyl acetate-hexanes (1:1) (plus 1mM sodium tetraphenyl-borate)	Buffer	66.4
	1-chloroheptane	Buffer	0.026
	1-chloroheptane (saturated with sodium tetraphenyl-borate)	Buffer (plus 0.2mM sodium tetraphenylborate)	1.52
	1-chloroheptane (saturated with sodium tetraphenyl-borate)	Buffer (plus 1mM sodium tetraphenylborate)	5.95

For studying the phase partitioning of ethidium bromide, 100 µl samples of [^{3}H]-ethidium bromide (provided by B. Baser, University of Rochester) with concentrations ranging from 0.5 µM to 0.1 mM were prepared in phosphate buffer (0.01 M sodium phosphate, pH 7.0, 0.1 M sodium chloride, 0.001 M sodium EDTA). The aqueous samples were mixed with 100 µl of the appropriate organic solution and shaken overnight on a mechanical shaker (New Brunswick). After seperation of the phases (by either clinical or Eppendorf centrifuge), 20 µl aliquots of the aqueous and organic phases were measured (liquid scintillation counter, Beckman) for determination of drug concentrations in each phase. The specific activity of the ethidium bromide stock solution was determined by measurement of the radioactivity of a solution of known concentration (determined optically, $\epsilon_{480} = 5680\ M^{-1}cm^{-1}$).

It should be noted that solute enhanced partition analysis methods introduce additional equilibria which must be considered. For example, it is important that the added solute be present in large excess over the amount of drug which is transferred to the organic phase in order to ensure that the concentration of the drug in the organic phase is linearly related to the concentration of the free drug in the aqueous buffer solution. The data for the solute enhanced partitioning of daunorubicin (Figure 1) in two different

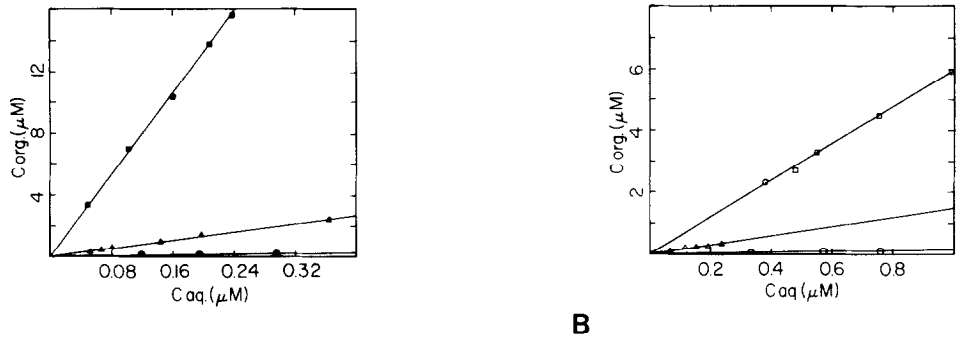

Figure 2. (A). Plot of the concentration of daunorubicin in a 1:1 mixture of n-amyl acetate:hexanes versus the concentration in the aqueous phase (0.01 M sodium phosphate buffer, pH 7.0, 0.1 M sodium chloride, 0.001 M sodium EDTA). The daunorubicin (0.2 μM to 5 μM) was originally in the aqueous phase and an equal volume (0.6 ml) of the organic phase was added to the plastic vial. The two phases were vigorously mixed for five 60-second intervals at 22°C. Separation of the phases was accomplished with a 10 minute centrifugation at 1000 rpm, 22°C. The concentration of daunorubicin in each phase was measured by fluorescence spectroscopy. (B). 1-chloroheptane was used as the organic phase with the same experimental conditions described above.

The open and filled circles represent the partition of daunorubicin in the absence of sodium tetraphenylborate (○ and ●). The open and filled triangles (△ and ▲) are for a concentration of 0.2 mM sodium tetraphenylborate, while the open and filled squares (□ and ■) are for a concentration of 1 mM sodium tetraphenylborate. In all of these experiments, the drug concentrations in the two phases were linearly related to the total drug concentration.

solvent systems are given in Table I and in Figure 2. The linearity observed in Figure 2 is a necessary condition for the use of partition analysis for the measurement of equilibrium binding. If the NaTPB concentration is much larger than the daunorubicin concentration then it is easy to show that the partition coefficient will be a function of the NaTPB concentration in the aqueous phase, as illustrated by the data in Figure 2 and Table I. This provides some freedom in the selection of the partition coefficient.

The SEPA method greatly extends the range of molecules which may be studied by partition analysis. Furthermore, the SEPA method allows for the use of organic solvents with low aqueous solubility (e.g., 1-chloroheptane) which minimizes the concern that the small amount of organic solute dissolved in the aqueous phase may be perturbing the binding equilibrium. It should be noted that sodium tetraphenylborate also partitions between the aqueous phase and the organic phase;this partitioning will not affect the results as long as the total amount of drug to be transferred is small compared to the total amount of NaTPB present. However, the solute used for transferring the positively charged drugs to the organic phase (as an ion pair) will also form an ion pair with the drug in the aqueous phase. It is important that the aqueous phase ion pair formation constant between the drug and the solute (such as NaTPB) be less than 1000 L/M so that there is no substantial amount of the ion pair in the aqueous solution. It is also preferable that the ion pair formation constant be at least two orders of magnitude less than the

equilibrium binding constant of the drug to the nucleic acid, in order that the binding isotherm obtained with SEPA techniques reflects the true binding isotherm. The magnitude of this ion pair formation constant may be obtained from a titration of an aqueous phase drug solution with a concentrated NaTPB solution. The aqueous phase ion pair formation constant between the positively charged daunorubicin and the tetraphenylborate anion is approximately 100 L/M. This value is more than three orders of magnitude lower than the equilibrium binding constant between daunorubicin and calf thymus DNA (9,10), and does not present a problem in terms of data analysis. The ethidium ion pair formation constant with tetraphenylborate is substantially larger than 1000. A large ion pair formation constant complicates the determination of the partition coefficient because one may not assume that the concentration of the aqueous phase ion pair is much less than the concentration of free ethidium in the aqueous phase. Although the SEPA method is, in principle, still valid under these conditions the best approach is to find a different solute with acceptable values for both the partition coefficient and the aqueous phase ion pair formation constant.

The development of the SEPA techniques has allowed us to measure the binding of daunorubicin to calf thymus DNA to very low levels of bound drug (i.e., r = bound drug/base pair = 0.001). A Scatchard plot of the binding data shows that daunorubicin binds in a cooperative manner at very low levels of bound drug ($r < 0.04$), while for r values above 0.06 the binding data is well represented by neighbor exclusion binding with an apparent equilibrium binding constant which is consistent with previously reported values (eg., references 6,9,10). The agreement at high values ($r > 0.06$) with previously published data (all of which was limited to the $r > 0.06$ range) supports the validity of the SEPA techniques. The important role of cooperativity and allosteric effects in the binding of daunorubicin and other drugs to various DNA's will be discussed in detail in a subsequent paper.

In summary, the use of solute enhanced partition analysis for the study of the binding of positively charged drugs to nucleic acids:(a),eliminates the membrane adsorption problems associated with equilibrium dialysis;(b), provides for rapid equilibration, which should allow for the measurement of the equilibrium binding constants of reactive and/or unstable molecules (eg., carcinogens);(c),minimizes Donnan effects;and (d), extends the molecules which may be studied by partition analysis(1,4) as well as allowing for the use of organic solvents with extremely low aqueous solubility.

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