# APPLICATION OF THE CHANGE IN PARTITION COEFFICIENT WITH pH TO THE STRUCTURE DETERMINATION OF ALKYL SUBSTITUTED GUANOSINES

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Summary: A versatile method for the determination of the ionization of guanosine is described. Suitably derivatized alkyl-guanosines are partitioned between organic solvents and aqueous buffer solutions at various pH values. Ionization is revealed by a change in partition coefficient with pH. The method is ideally suited for application to micro samples since any quantitative method can be used to determine the partition coefficient. A procedure for distinguishing $N^1$ or $O^6$, $N^2$, and $C^8$ alkylation of guanosine is described.

Introduction: Recently, a series of covalently bound products derived from *in vitro* and *in vivo* reactions between chemical mutagens and nucleic acids have been described (1). While in several cases the mutagen was known to be substituted on guanosine, the precise position of substitution, an important point in view of the difference in mutation efficiency for substitution of guanine sites (2), could not be established. These substituted guanosines were obtained either in such minute quantities as to make physical measurements impractical or with physical characteristics, particularly strong u.v. absorbance of the attached mutagen, which prevented application of the classical u.v. methods of structure elucidation in purines (3).

While there exists a sensitive method for determination of $N^7$ or $C^8$ alkylation via tritium release of 8-[$H^3$]-guanosine (4), no comparable method exists for determination of $N^1$, $N^2$, $N^3$, or $O^6$ alkylation. Detection of the acidic $pK_a$ characteristic of guanosine residues is diagnostic for the presence of a proton at $N^1$ and requires that both $N^1$ and $O^6$ be unsubstituted. We describe here the application of the change in partition coefficient with pH (5) (partition titration) to the structural assignment of substituted guanosines

Materials and Methods: All reagents were at least reagent grade. Buffer solutions were prepared by mixing stock solutions of 0.05M citric acid (pH 1.8),

0.05M $K_2HPO_4$ (pH 7.0), and 0.05M $NaHCO_3$ (pH 9.0), to provide intermediate buffer solutions from pH 2.0 to pH 9.0, whereas 1N HCl or 1N KOH were added to obtain buffer solutions not within this range. PH was measured using a glass electrode vs. S.C.E. with a Corning Model 10 pH meter. U.V. spectra were recorded using a Beckman Model 25 spectrophotometer. Radioactivity was determined with a Packard B 3450 liquid scintillation counter.

Nucleosides: Triacetylguanosine was purchased from Aldrich Chemical Co. Xanthosine, $N^1$-, and $N^2$-methylguanosines were purchased from Sigma Chemical Co. Triacetyl derivatives of xanthosine, $N^2$-methylguanosine, and $O^6$-benzylguanosine were prepared by acetylation at either steam bath or room temperature with pyridine/acetic anhydride (2/1), 1 mg of tetraethylammonium chloride was added to obtain solution in some cases. The products were isolated by removing volatile solvents under high vacuum. The dry residues were dissolved in n-butanol and extracted with three equal volumes of water. The n-butanol was easily removed under reduced pressure by addition of an equal volume of water. The product purity was checked by silica gel TLC using 10% methanol in dichloromethane: triacetylguanosine, Rf = 0.34; triacetylxanthosine, Rf = 0.1; triacetyl-$N^2$-methylguanosine, Rf = 0.40; $N^1$-methylguanosine, Rf = 0.08; $O^6$-benzylguanosine (6), Rf = 0.25; triacetyl-$O^6$-benzylguanosine, Rf = 0.95. $N^2$-$H^3$-BP-guanosine[a] was prepared by treatment of polyguanylic acid at pH 7.0 with 7β,8α-dihydroxy-9α,10α-epoxy-7,8-dihydro-9,10-ditritiobenzo[a]pyrene (2.36 μCi/μmole) kindly provided by Drs. D. M. Jerina and H. Yagi of NIAMDD, National Institutes of Health. The polymer was hydrolyzed and nucleoside product isolated as described in reference (7).

Partition Titration: Stock solutions of nucleosides in methanol or water ≈ 2.0 O.D. at 260 nm per 10 μl were prepared. Aliquots (10 μl each) were added to 1.0 ml of buffer solutions and partitioned with 1.0 ml of organic solvent. Since the polarity of each nucleoside varied it was necessary to adjust the polarity of the organic phase accordingly. Convenient mixtures of n-butanol and ethyl ether or ethyl acetate were made to obtain a partition coefficient of ≈ 1.0 between the organic phase and pH 4.0 or pH 7.0 buffer solution. After vigorous shaking the phases were allowed to separate. Occasionally, use of a centrifuse was necessary to separate emulsions. The aqueous phase was removed by pipette and diluted to 4.0 ml with saturated $K_2HPO_4$ at pH 7.0. The amount of nucleoside present was then determined spectrophotometrically. For radiochemical analysis an aliquot of the organic phase was added to 7.0 ml of aquasol liquid scintillation fluid and counted twice. All experiments were performed in duplicate or triplicate.

Results and Discussion: In theory any physical property which changes upon ionization can be used to determine $pK_a$. We found that monitoring the change in partition coefficient with pH is particularly useful for guanosines since unionized substituted guanosines are much more soluble in organic solvents than either their cations or anions. However, due to the extreme water solubility of simple guanosines it was necessary to use a less polar derivative for systems with a free $N^1$ proton. Acetylation of these model compounds provided

[a] Abbreviation used is $N^2$-$H^3$-BP-guanosine, $N^2$-[7β,8α,9α-trihydroxy-7,8-dihydro-9,10-ditritio-10β-benzo[a]pyrenyl]-guanosine.

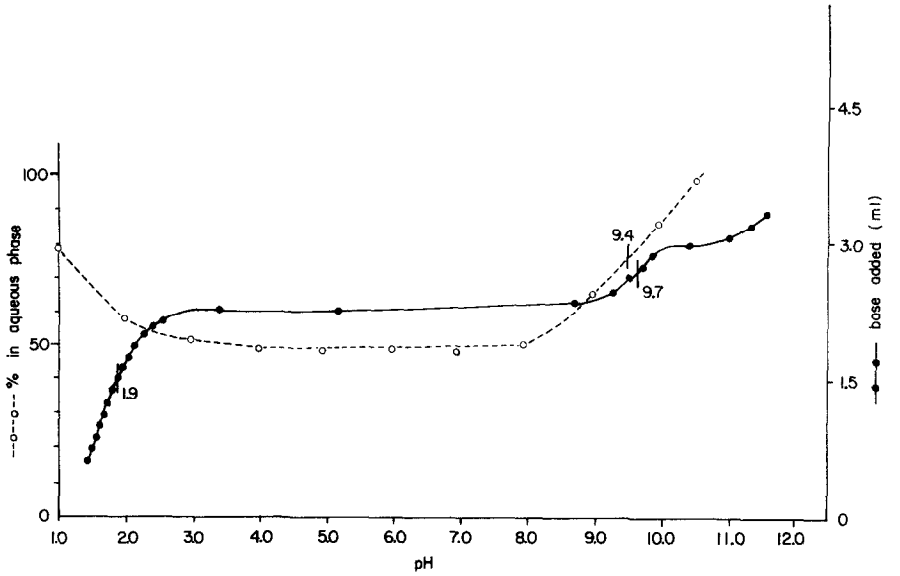


Figure 1. Comparison of the change in partition coefficient with pH of guanosine triacetate to the potentiometric titration of guanosine. --o--o--: guanosine triacetate partitioned with 50% n-butanol in ethyl ether; —●—●— : guanosine titrated with 0.5N KOH.

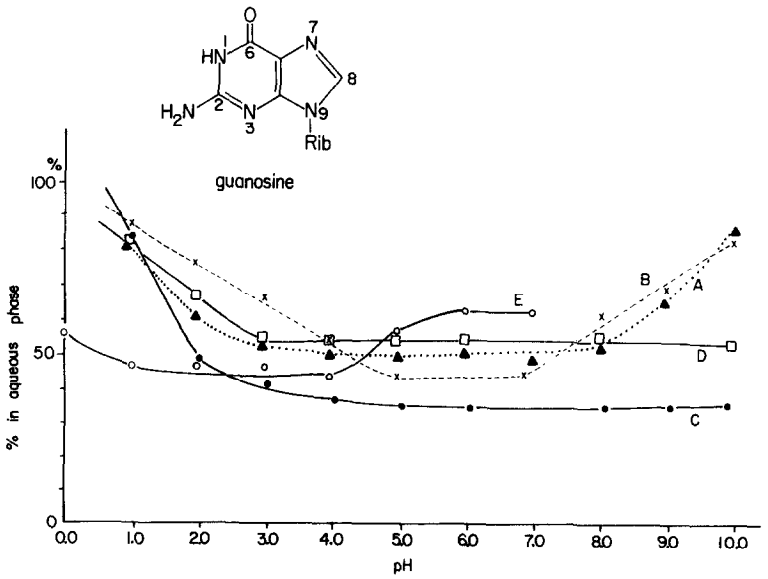


Figure 2. Partition titration of guanosine derivatives. Nucleic acids were partitioned (see Materials and Methods) with the following organic solvents; A, ··▲··▲··, guanosine triacetate: 50% n-butanol in ethyl ether; B, --x--x--, $N^2$-methylguanosine triacetate: 50% n-butanol in ethyl ether; C, —●—●—, $O^6$-benzylguanosine: 15% n-butanol in ethyl ether; D, —□—□—, $N^1$-methylguanosine: 2 volumes of 100% n-butanol; E, —o—o—, xanthosine triacetate: 70% n-butanol in ethyl acetate.

acceptable solubility, while no derivatization was required for the $O^6$-benzyl-, $N^1$-methyl-, or benzo[a]pyrenylguanosines.

Figure 1 compares potentiometric titration of guanosine with partition titration of guanosine triacetate. The acidic $pK_a$'s observed are remarkably similar considering the inherent inaccuracies of partition titration. Since the neutral form is preferentially extracted, the partition process will tend to shift the acid-base equilibrium away from the ionized form. The amount of shift depends on the partition coefficient ($K_p$) but for values of $K_p \approx 1.0$ it is generally less than 0.5 $pK_a$ unit. Correction for this known inaccuracy is not required since various other factors such as the effect of dissolved organic solvents apparently tend to counteract this extraction distortion. What is more, the value of partition titration for structure determinations does not depend on an accurate $pK_a$ value, but rather on the detection of an acidic ionization. $PK_a$ values of $\pm$ 1 unit are quite acceptable for this purpose.

The utility of the process for the detection of the presence or absence of the acidic ionization in substituted guanosines is demonstrated in Figure 2. $N^1$-Methylguanosine and $O^6$-benzylguanosine which lack a free $N^1$-proton do not show any change in partition coefficient near pH 9.0.[b] $N^2$-Methylguanosine triacetate and guanosine triacetate, on the other hand, possess both a free $N^1$-proton and a change in their partition coefficient near pH 9.0. Thus, partition titration dramatically reveals whether guanosine derivatives are substituted at $N^1$ or $O^6$. In addition, large deviations from the normal acidic guanosine $pK_a$, e.g., triacetylxanthosine (see Figure 2), are easily distinguished. Note that while the basic $pK_a$ near 1.0 does not readily

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[b] $O^6$-Benzylguanosine triacetate shows essentially the same response to pH change as the unacetylated compound. However, the use of borate for basic buffer solutions led to an apparant $pK_a$ of 8.6 for $O^6$-benzylguanosine but did not affect the response of its triacetate derivative. Thus, complex formation between the cis-2',3'-hydroxyls and borate necessitate either the protection of the free cis-2',3'-hydroxyls of guanosines or the exclusion of borate buffer for meaningful results with this method.

provide useful structural information in this case, it is a convenient second check on the validity of the extraction method.

Since the physical property being measured is the amount of sample in one phase or the other, there is no fundamental restriction on the quantitative method of detection, e.g., u.v. absorbance, fluorescence, radioactivity, or bioassay could all be used. The minimum sample size required is determined only by the sensitivity of the detection method. Partition titration of $N^2$-$H^3$-BP-guanosine (≈ 1,000 cpm/pH point) allowed detection of a $pK_a$ at 9.7 using less than 5 nmoles total sample.

Using the extraction method described here coupled with nitrous acid treatment, alkyl substituted guanosines at $N^1$ or $O^6$, $N^2$, and $C^8$ can be easily distinguished as follows. The detection of an acidic ionization around 9.5 eliminates $N^1$ and $O^6$ derivatives. Conversion of unsubstituted 2-amino groups to ketones by nitrous acid treatment results in a drastic increase in the acidity of the $N^1$-proton (see Figure 2) (8). On the other hand, $N^2$-alkylguanines react with nitrous acid to form $N^2$-nitroso derivatives,[c] which are readily converted to the starting compounds by a variety of chemical means (9). Thus, guanosines substituted at $N^1$ or $O^6$ do not show a $pK_a$ near 9.5. In contrast, guanosines alkylated at $N^2$ or $C^8$ show a characteristic $pK_a$ around 9.5 (10) with the $C^8$ substituted guanosines being converted to the relatively acidic 2-keto componds by nitrous acid treatment.

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[c] $N^2$-Methylguanosine undergoes identical reactions (unpublished results).

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