THE BINDING OF METHYL MERCURY AND DNA

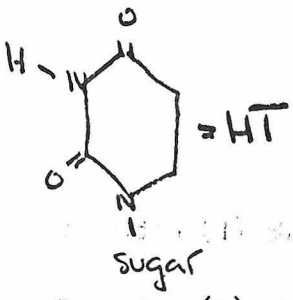
Thesis by Michael M. Rosbash submitted in partial fulfillment of the requirements for a B.S. degree, May, 1965.

ABSTRACT

A method for the quantitative measurement of methyl mercury (CH_3Hg^+) was developed in addition to a method for the quantitative study of the binding of CH_3Hg^+ to DNA. Preliminary results seem to indicate that CH_3Hg^+ binds to DNA; $\frac{[CH_3Hg^+]}{[DNA]}$ bound reaches a maximum as $[CH_3Hg^+]$ is increased. It also appears that more CH_3Hg^+ is bound to denatured DNA than to native DNA.

INTRODUCTION

This research has been prompted by that work already done on the binding of both Hg^{++} and CH_3Hg^+ to the nucleosides. It is known, for instance, that Thymidine (T) binds with CH_3Hg^+.



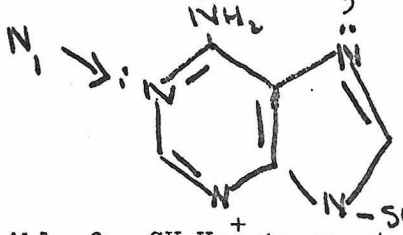
= HT

pK of the acid (arrowed hydrogen) $\simeq$ 9.2 [1]

(1) $HT + CH_3HgOH \rightleftharpoons CH_3HgT \qquad K = 10^{4.2}$ [1]

Reaction (1) should be reasonably stable to pH change (since it is independent of $[H^+]$) providing the pH $\leq$ 9 which ensures the non-ionization of the acidic proton. A similar reaction and pK is known for guanosine.

Crystal structure studies seem to indicate that adenosine is protonated preferentially at the arrowed hydrogen. It seems reasonable to assume, therefore, that CH_3Hg^+ attacks adenosine at the same place.



= As

It is also possible for CH_3Hg^+ to react at the amino N, but this is a slightly less reactive position.

In the former case:

$$H^+ + As + CH_3HgOH \rightleftharpoons CH_3HgAs \qquad K = 10^{7.5}$$ [1]

at pH = 7, As is free and CH_3Hg^+ is mainly CH_3HgOH since

$$H^+ + CH_3HgOH \rightleftharpoons CH_3Hg^+ \qquad K = 10^{4.5}$$

* * * * * * * * * * *

1 R. B. Simpson, J. Am. Chem. Soc., 86, 2059 (1964)

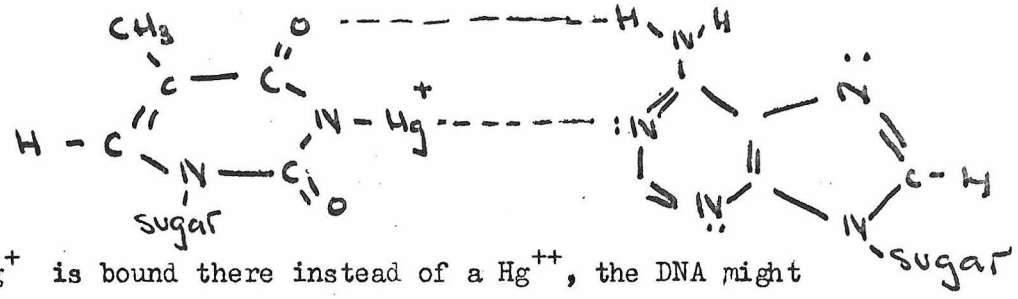
Thus, at pH = 7, $(2)\frac{[CH_3HgAs^+]}{[As]} = 10^{0.5}[CH_3HgOH]_{free}$

In the latter case, when CH_3HgOH reacts with the amino N:

(3) $As + CH_3HgOH \rightleftharpoons AsCH_3Hg$ $\quad K = 10^2$ [1]

This also holds for the amino group of cytidine and guanosine. It is obvious that the amino Nitrogen is less reactive than N_1.

It has been found that Hg^{++} is bound more strongly by DNA than by the monomers. The most plausible explanation is that the Hg^{++} is chelated between the two bases in the DNA chain. It replaces a H^+ as follows.



If a CH_3Hg^+ is bound there instead of a Hg^{++}, the DNA might denature due to the size of CH_3Hg^+ relative to Hg^{++}. Thus, two predictions are made prior to experimentation. CH_3Hg^+ should denature DNA if it binds, and binding be denatured DNA should be comparable to monomer binding and greater than binding by native DNA.

R. B. Simpson, J. Am. Chem. Soc., 86, 2059 (1964)

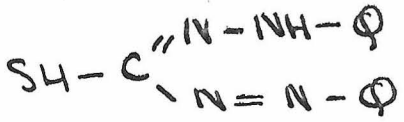
EXPERIMENTAL

The initial problem was to develop a method to measure the extent of binding between CH_3Hg^+ and DNA. The first set of experiments consisted of equilibrium dialysis runs. A known concentration of DNA inside the dialysis tubing was equilibrated with a known concentration of CH_3Hg^+. From basic thermodynamics, it is known that the $[CH_3Hg^+]_{free\ inside}$ (Unbound CH_3Hg^+ inside the tubing) = $[CH_3Hg^+]_{out}$. Thus, by measuring the $[CH_3Hg^+]_{out}$ we know the $[CH_3Hg^+]_{bound}$, for $[CH_3Hg^+]_{bound} = \Sigma CH_3Hg^+ - [CH_3Hg^+]_{out} - [CH_3Hg^+]_{in,\ free}$.

The second and major set of experiments were based on the distribution of CH_3HgAc between a two-phase system. After ensuring the CH_3HgAc was the only species to distribute into the organic layer, the distribution constant of CH_3HgAc between an aqueous and chloroform layer was determined as well as the equilibrium constant of CH_3HgAc. With this information at hand, the extent of binding between CH_3Hg^+ and DNA could be calculated by equilibrating this aqueous layer with Ac^- and a known volume of chloroform. The CH_3HgAc which is abstracted into the organic layer is measured and a value for $[CH_3Hg^+]_{bound}$ can then be calculated.

A first problem was to find a reasonably good method to determine an unknown concentration of CH_3Hg^+. Dithizone (henceforth denoted as Dz) was considered the most likely prospect.

Enolic form:



Dz is highly colored and achieves another color upon complexation with CH_3Hg. It thus seemed acceptable for spectroscopic studies.

When disolved in $CHCl_3$, its spectra, as recorded on a Cary spectrophotometer, exhibited a peak at 6060Å and a shoulder at 5800Å. Since no standard grade Dz was available, an extinction coefficient could not be calculated.

The method finally used was that of disolving Dz in spectral grade $CHCl_3$. Its spectrum had a first peak in the visible at 606 mu. CH_3Hg-Dz absorbed to a very small extent at this or higher wavelengths. A layer of 0.1 N EDTA, pH = 4.5, was placed over the Dz to prevent its complexation with other cations. An unknown amount of CH_3Hg^+, in aqueous solution, was added to this two phase system. The Dz extracted virtually all the CH_3Hg from the aqueous layer. The fractional loss of optical density at 620 mu was considered to be $\frac{[CH_3Hg\text{-}Dz]}{[Dz]} \cdot [Dz]_{initial}$ was recalculated each day by reacting the Dz with a known $[CH_3Hg^+]$. The absorbance of CH_3Hg-Dz at 620 mu could be tested and adjusted for. This method was accurate to within 2%.

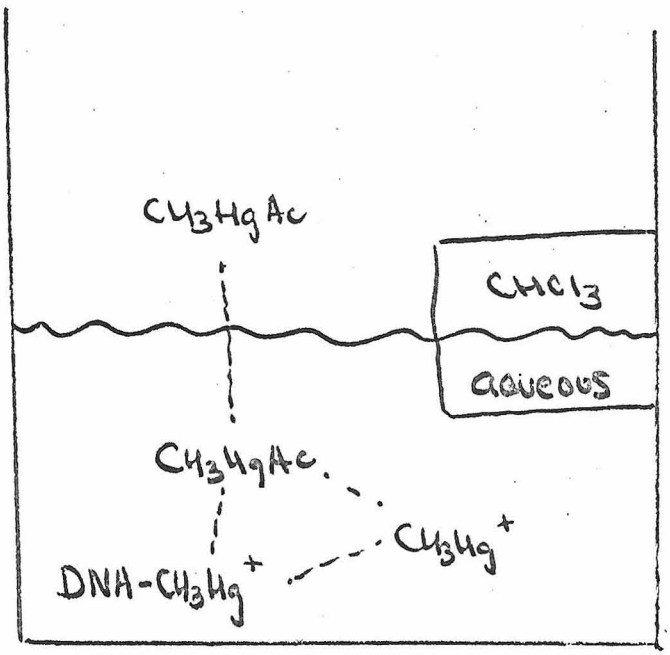
An unknown quantity of CH_3Hg - X, disolved in $CHCl_3$, can also be tested in the same manner. By adding it to the two phases, it will react with the Dz. At the same time, the $CHCl_3$ in which it is disolved will dilute the Dz. By adjusting for this dilution, the CH_3Hg-X can be measured. This, however, is not quite as accurate due to the inherent inaccuracy involved with the mixing of organic solvents.

The crucial problem was to develop a method to measure the binding of CH_3Hg^+ to DNA. In the equilibrium dialysis experiments, the sausage tubing absorbed CH_3Hg^+. Saturation of the tubing could not be achieved to any reproducible degree, so no quantitative measurements could be taken.

A preperative centrifugation experiment was performed. In this experiment; DNA, CH_3Hg^+, 10^{-2} $NaClO_4$, and 10^{-3} Cacadylate buffer at pH = 7 were placed in a centrifuge tube. It appears as if the Calf Thymus DNA was heterogeneous, for it did not distribute as expected upon centrigugation. Also, a great deal of precipitate was found making spectrophotometric analysis extremely difficult.

The major experiments included the distribution of $CH_3HgAcetate$ between a two phase system of $CHCl_3$ and aqueous layers. It was first shown that under the experimental CH_3Hg^+ concentrations(10^{-3} or less) no CH_3Hg^+ distributed into the $CHCl_3$ within our normal experimental error. Then, a distribution constant for CH_3HgAc was determined.

$$K_d = \frac{CH_3HgAc_{org}}{CH_3HgAc_{Inorg}} = 2.12$$



Thus, by measuring the $[CH_3HgAc]_{org}$ one could find the $[CH_3HgAc]_{inorg}$. With this information, the equilibrium constant for the following reaction was determined.

$$(4)\ CH_3HgOH + Ac^- \rightleftharpoons CH_3HgAc + OH^- \qquad K = 6.4 \times 10^{-7}$$

A solution containing a known $[CH_3Hg]$, known $[DNA]$, $10^{-1}Ac^-$, $10^{-2}NaClO_4$, 10^{-3} Cacadylate buffer is equilibrated with an equal volume of $CHCl_3$. These two phases are shaken for about 15 minutes and allowed to settle. A known volume is extracted from each phase and the CH_3Hg in each is found using the dithizone method previousely discussed. From these results, one finds the ΣCH_3Hg_{inorg} (total CH_3Hg concentration in the inorganic phase) and the ΣCH_3Hg_{org}. The DNA was found to not interfere with the dithizone analysis.

Since neither CH_3Hg^+ nor CH_3Hg-DNA distributes into the $CHCl_3$ layer, all CH_3Hg in this layer is in the form of CH_3HgAc. By knowing the distribution constant of CH_3HgAc, we thus know the $[CH_3HgAc]_{inorg}$. From (4) and its corresponding equilibrium constant, we can calculate the $[CH_3HgOH]_{free}$ (i.e., unbound) in the aqueous layer. The $\Sigma CH_3HgOH_{bound} = \Sigma CH_3Hg - CH_3HgOH_{free} - CH_3HgAc_{inorg}$. By knowing the DNA we can then get a value for $X = \frac{[CH_3Hg]_{bound}}{[DNA]}$.

If one defines $R = \frac{\Sigma[CH_3Hg]}{[DNA]}$ before $CHCl_3$ extraction, it is obvious that this does not equal the effective $R = R'$. As the CH_3HgAc is abstacted into the organic layer, the total $[CH_3Hg^+]$ which is available to the DNA for binding is lowered and thus the effective $R = R' < R$.

$R' = \frac{[CH_3HgOH]}{[DNA]} = .8\ \frac{\Sigma CH_3Hg}{[DNA]}$ depending on the pH and the $[Ac^-]$.

RESULTS

Ultracentrifugation Experiment -- Although inconclusive, this experiment did seem to indicate that there was more CH_3Hg in the regions of higher DNA . One would expect this small molocule to distribute evenly throughout the tube if it exhibited no affinity for DNA. This implies that some binding exists, but it is a tenuous observation.

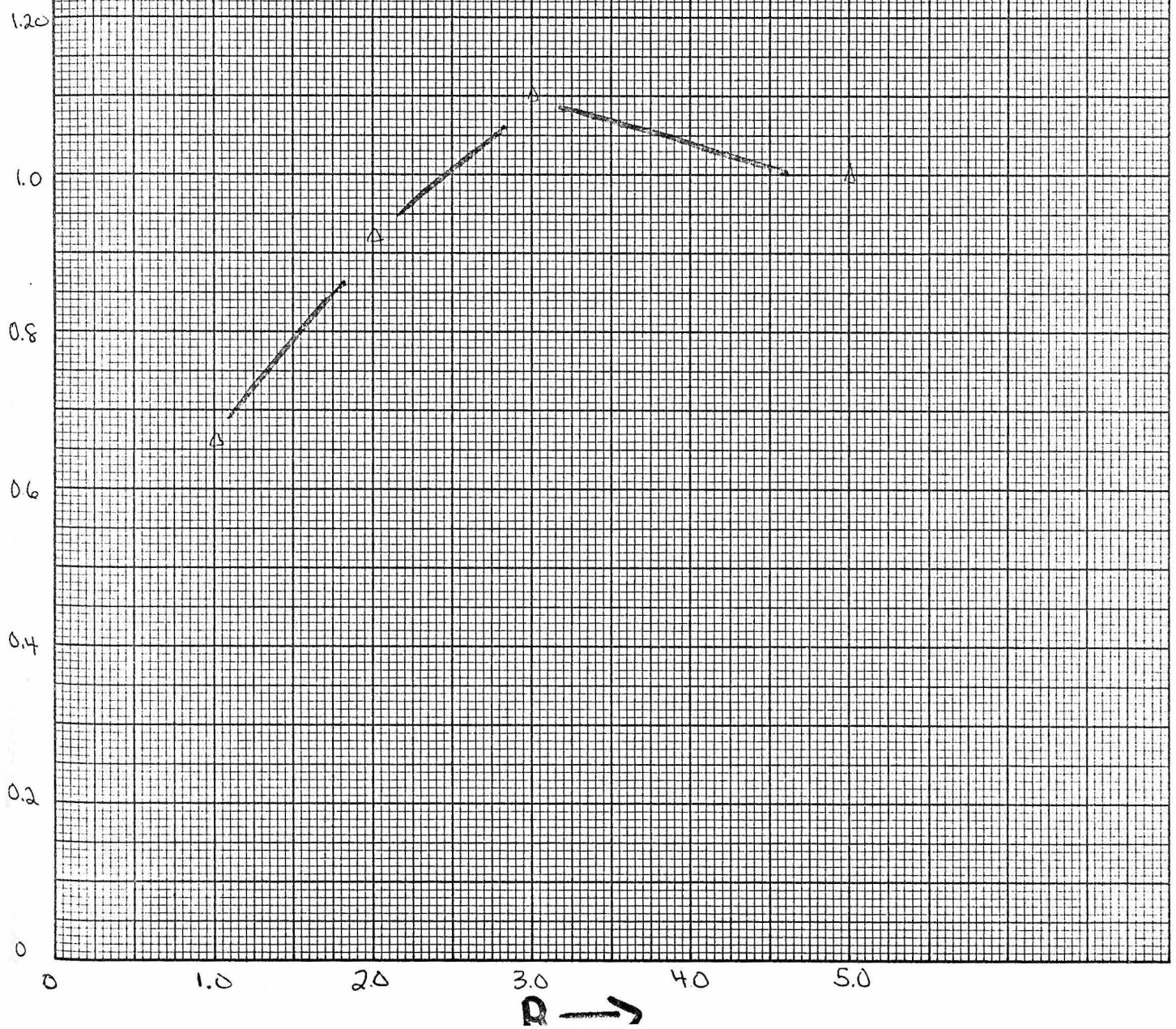
CH_3HgAc Abstraction Procedure -- Quantitative results were obtained for R = 1,2,3, and 5 using denatured DNA. They are charted and graphed on the following page. Values were also taken at R = 1,2, and 3 for native DNA; but since the necessity of taking the pH of the aqueous layer was not realized until well after these experiments were completed, no X's could be calculated. As preliminary results, however, these figures seem to indicate that there is definite binding between the CH_3Hg and DNA. It also appears that X levels off to some figure in the vicinity of 1 as the $[CH_3Hg]$ is increased. This coincides with our preliminary hypothesis; that the CH_3Hg is bound between the base pairs in the DNA.

Z = pH of the aqueous phase after equilibrium is reached

$X = \frac{[CH_3Hg]_{bound}}{DNA}$

$[DNA] = 1 \times 10^{-4}$

R	R'	Z	X
1.0	0.80	7.09	0.63
2.0	1.60	7.44	0.86
3.0	2.40	7.68	1.10
5.0	4.00	7.88	1.0



ACKNOWLEDGEMENTS

I am grateful to the National Science Foundation for its financial aid, given in the form of a summer fellowship. I am especially endebted to Dr. N. R. Davidson for his calm and patient guidance, scientifically and otherwise. I would also like to thank Dr. Davidson's research group, in particular Dr. Janis Vasilevskis, for their helpful advice.