ABSORPTION AND FLUORESCENCE PROPERTIES OF AO--DNA COMPLEXES

Thesis by Melvin M. Stephens submitted in partial fulfillment of the requirements for aB. S. degree, April 1965.

ABSTRACT

Experiments on the fluorescence of acridine orange--DNA complexes at a constant concentration of acridine orange show that for a native DNA the highest fluorescence intensity per complexed DNA molecule will occur when the concentration ratio of DNA phosphate groups to acridine orange dye molecules is between 5.5 and 7.2. The corresponding limits for denatured DNA are 6.9 and 8.9. Both absorption and fluorescence studies show that denatured DNA exhibits a greater stacking tendency than does native DNA.

INTRODUCTION

Dr. Norman Davidson and William C. Galley have recently been attempting to develop a new method for rapidly and accurately determining the molecular weights of large DNA molecules (molecular weights of 100 million or more). This method is based on observing and measuring the fluorescence intensity of a small volume of a solution which contains known amounts of both the DNA under investigation and acridine orange dye (henceforth abbreviated AO). The acridine orange forms a complex with the DNA molecules, and it is the fluorescence of this complex which is observed. The success or failure of the proposed procedure depends, among other things, on: (1) the nature of the AO-DNA binding, and (2) the fluorescence characteristics of the AO-DNA complex. For the technique to work the fluorescence intensity coming from a small volume of solution containing a single DNA molecule complexed with acridine orange must be both detectable and substantially greater than the fluorescence intensity coming from an equal volume of solution which does not contain a DNA molecule.[1]

This particular research project was carried out in order to shed more light on the binding characteristics and the fluorescence properties of various acridine orange--DNA complex systems. If there was any one specific goal in mind it was to determine the approximate composition of that AO-DNA system which would be most applicable to the molecular weight determination technique suggested by Davidson and Galley. This turned out to be a somewhat more difficult task than had originally been imagined.

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1) Those interested in a detailed discussion of this proposed molecular weight determination procedure are urged to see the unpublished research report of W. C. Galley of March 1964.

EXPERIMENTAL

A schematic diagram of the apparatus used for studying the fluorescence of substances in solution is shown in Figure 1. The light used for excitation of the sample was produced by an Osram HBO--200 high pressure mercury arc operated at 4.1 amps from a 120 volt D. C. battery.

The exciting wavelength band could be selected by passing the exciting light through either (a) a Bausch and Lomb grating monochromator, or (b) a series of interference and/or absorption filters. A system of filters offered the advantage of a very high intensity of exciting light available for illumination of the sample but to some degree sacrificed the possibility of rapidly changing the wavelength of excitation. In all of the work discussed in this paper a monochromator was used to select the exciting wavelength.

Light coming from the exciting monochromator was focused by a series of lenses onto the fluorescent sample which was contained in a quartz fluorescence cell. This cell was housed in a black box which, within the accuracy of these measurements, was completely light tight. The black box was provided with an inlet slit, and exit slit, and a light trap which reduced the scattering of light within the box. The direction of observation was at right angles to that of excitation. A camera shutter over the inlet slit allowed one to turn the exciting beam on and off without affecting the mercury arc.

Two types of quartz fluorescence cells were used with this apparatus. Usually a cell with a 1 cm. x 1 cm. base was used, in which case the directions of excitation and observation were perpendicular to two adjacent faces (see Figure 2a). With this arrangement the fluorescence observed originated in a small volume of solution located approximately in the center of the cell. Whenever either absorption of the exciting light or reabsorption of the fluores-

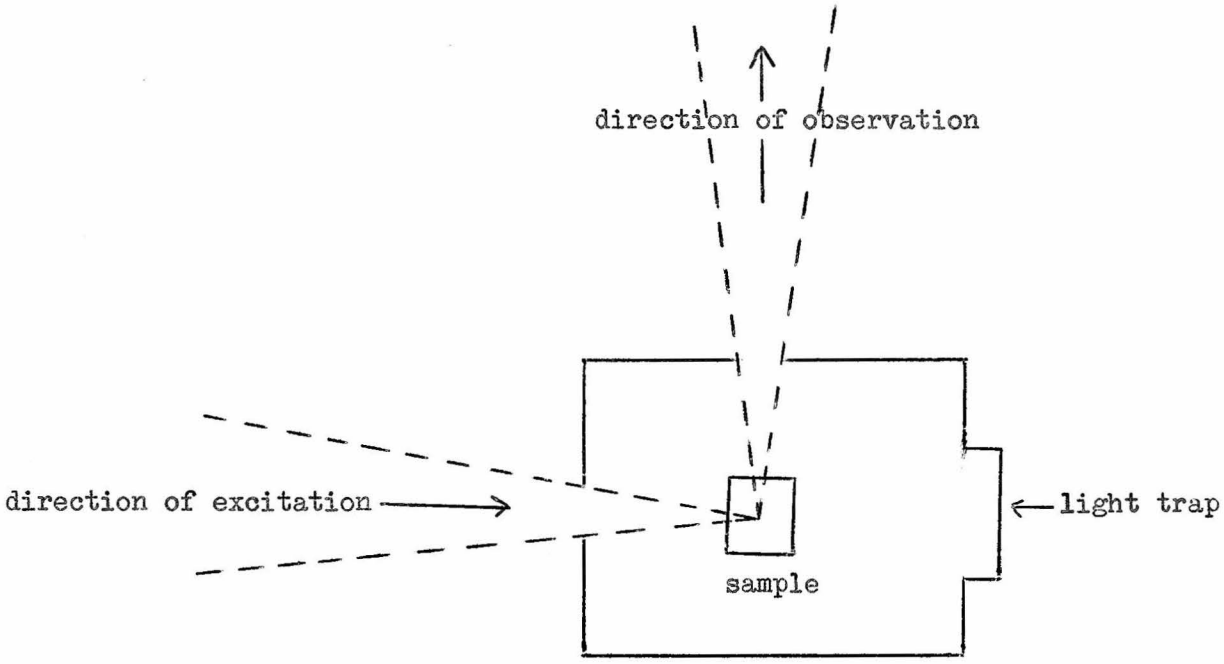


Fig. 2a

1 cm. x 1 cm. fluorescence cell.

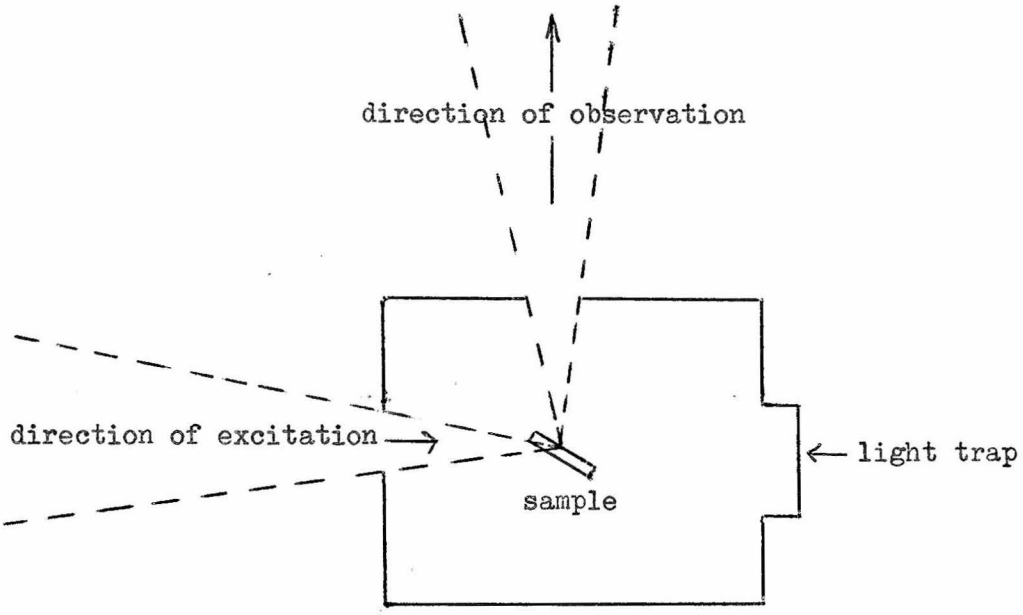


Fig. 2b

Thin fluorescence cell for use with more concentrated solutions.

cent light was significant, however, the solution under investigation was placed in a thin cell with a 1 mm. x 1 cm. cross section (see Figure 2b). By means of a small polyethylene stand this cell was situated so that one of the 1 cm. wide faces made an angle of approximately 20° with the exciting light. The cell was also oriented so that the major portion of the reflected exciting light was directed away from the exit slit. When the optics of the system were such that the volume of solution observed lay just beneath that face of the cell which was closest to the analyzing monochromator, the effects of reabsorption of the fluorescent light (which usually alters the shape of a fluorescence spectrum) could be neglected entirely. When only absorption of the primary light was important, the volume of solution observed should ideally have been immediately beneath that surface of the cell which was closest to the exciting monochromator. In actuality the volume of solution observed was somewhere in between these two limiting cases, however the set up gave us resonably reliable fluorescence spectra for all but the most concentrated of solutions studied. The most serious drawback to the thin cell arrangement was that, because of variations in the exact position in which the polyethylene stand held the cell, the amount of exciting light which was reflected varied from experiment to experiment. This caused a slight variation in the absolute magnitude, but not in the shape, of the fluorescence spectrum. It was found that with sufficient caution with respect to the initial placement of the cell in the polyethylene stand spectra reproducible to within $\pm 5\%$ could be obtained.

The fluorescent light was analyzed by allowing it to enter a second monochromator located just beyond the exit slit of the box holding the fluorescence cell. This was the analyzing monochromator and the path of the fluorescent light through it was exactly the reverse of the path of the primary light through the exciting monochromator. The light emerged to a photomultiplier

tube located behind the exit slit of the analyzing monochromator.

An RCA model 1P28 photomultiplier was used throughout this research. Initially the load resistor of the photomultiplier was $2.25 \times 10^5 \Omega$ but this was later replaced by a separate variable resistor with a range of from 10^3 to $10^{12} \Omega$. The noise level and frequency response were controlled by connecting a Keithly variable capacitor box in parallel with the load resistor. Power was supplied by a Keithly high voltage power supply.

Two different techniques for amplifying and recording the photomultiplier signals were employed. During the earlier phases of this project the signals from the photomultiplier were fed into a dual beam oscilloscope and displayed on the y axis of the screen. The x axis was made a function of the wavelength through the use of a device which made the wavelength being observed correspond to a voltage which was then fed into the horizontal deflection plates of the oscilloscope. This device worked approximately as follows: A 4.5 volt dry battery was connected across a (Helipot) variable resistor which was geared to the wavelength dial of the analyzing monochromator. As the wavelength being observed was changed (i.e. as the wavelength dial on the analyzing monochromator was turned) the resistance supplied by the variable resistor was altered. This resulted in a changing voltage (which was proportional to the wavelength) being fed into the x axis of the oscilloscope. Thus as the wavelength field of the fluorescent light was scanned, the oscilloscope signal traced out the photomultiplier response over the x--y plane. The spectrum was recorded on photographs taken with a Polaroid camera. For the major part of this work the oscilloscope was replaced by a Mosely x--y plotter--all other features of the apparatus remaining the same. This permitted the fluorescence spectra to be rapidly recorded on ordinary graph paper and was

was substantial improvement in the practical operation of the apparatus.

The design of the above apparatus was mainly a product of the efforts of William C. Galley.

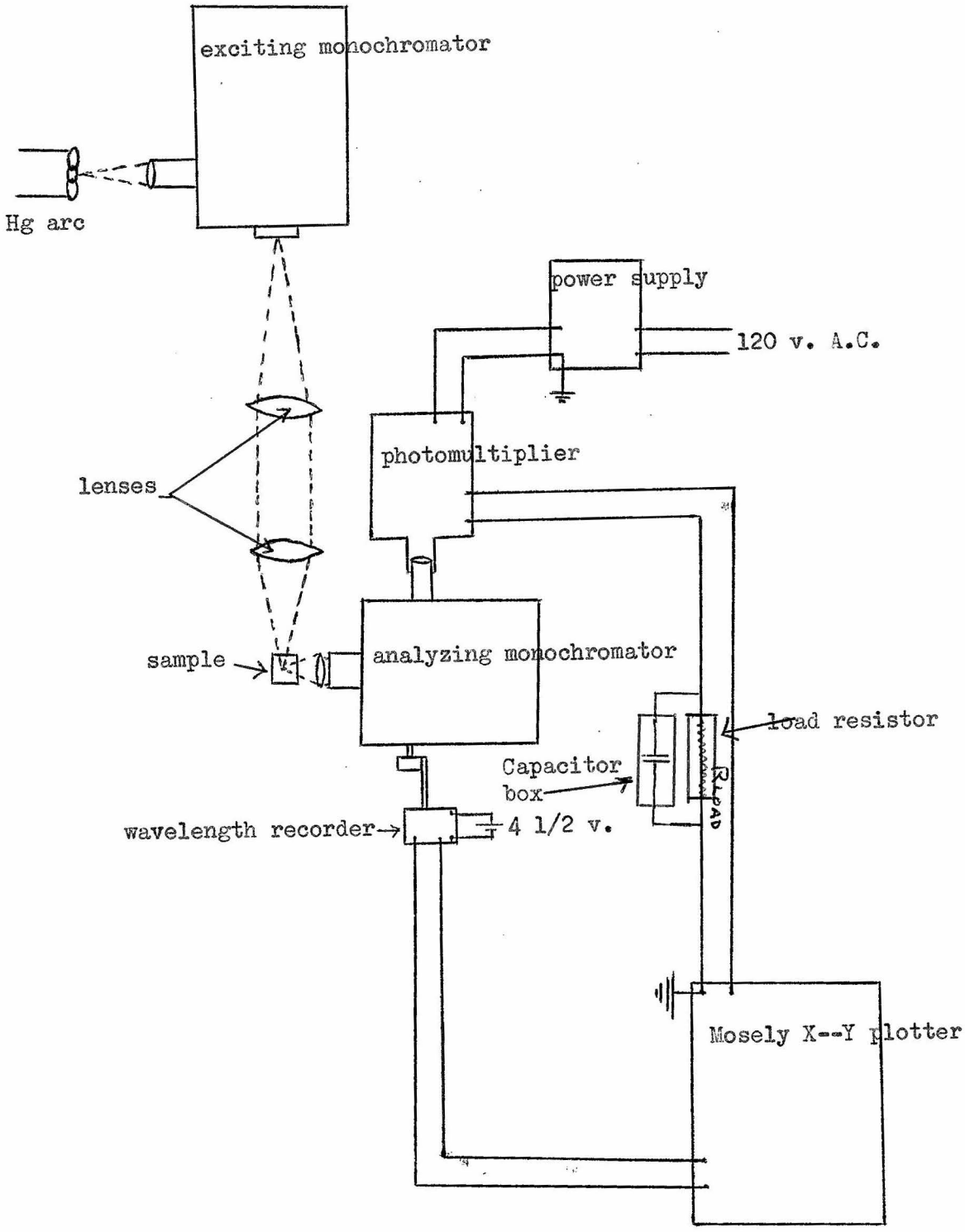


Fig. 1

Schematic diagram of the spectrofluorimeter.

RESULTS

P/D Ratio -- Throughout this report we shall refer to the P/D ratio. This is the ratio of the concentration of DNA phosphate groups to the concentration of acridine orange dye in a given solution. Experiments by Tom Burke and Foch Yew have shown that under the experimental conditions present throughout our research essentially all of the acridine orange in solution was bound to DNA molecules. Thus a P/D ratio of three refers to a complex in which there are three DNA phosphates for every acridine orange dye molecule.

Fluorescence Spectra -- The general shape of the dye--DNA complex fluorescence spectra (from 490 mμ to 610 mμ) remained unchanged over the entire range of P/D ratios investigated in this research. That part of the fluorescence light which we studied was distributed over the range 490 mμ to 610 mμ. The maxima of the fluorescence spectra occured at approximately 526 mμ.

Absorption Spectra -- The visible absorption spectrum of an AO--DNA complex exhibits two peaks, one at 473 mμ and one at 500 mμ. The simplest theory of AO--DNA binding assumes that the peak near 473 mμ is the result of the absorption of a polymer form or the complex in which two or more acridine orange molecules are bound to adjacent DNA phosphate groups, and that the 500 mμ peak is caused by the absorption of dye molecules bound to phosphates which are at least one phosphate group removed from the next closest AO molecule. This latter form is called the monomer form of the complex. As the P/D ratio is increased while the concentration of acridine orange is kept constant the peak at 473 mμ gradually decreases while, simultaneously, the peak at

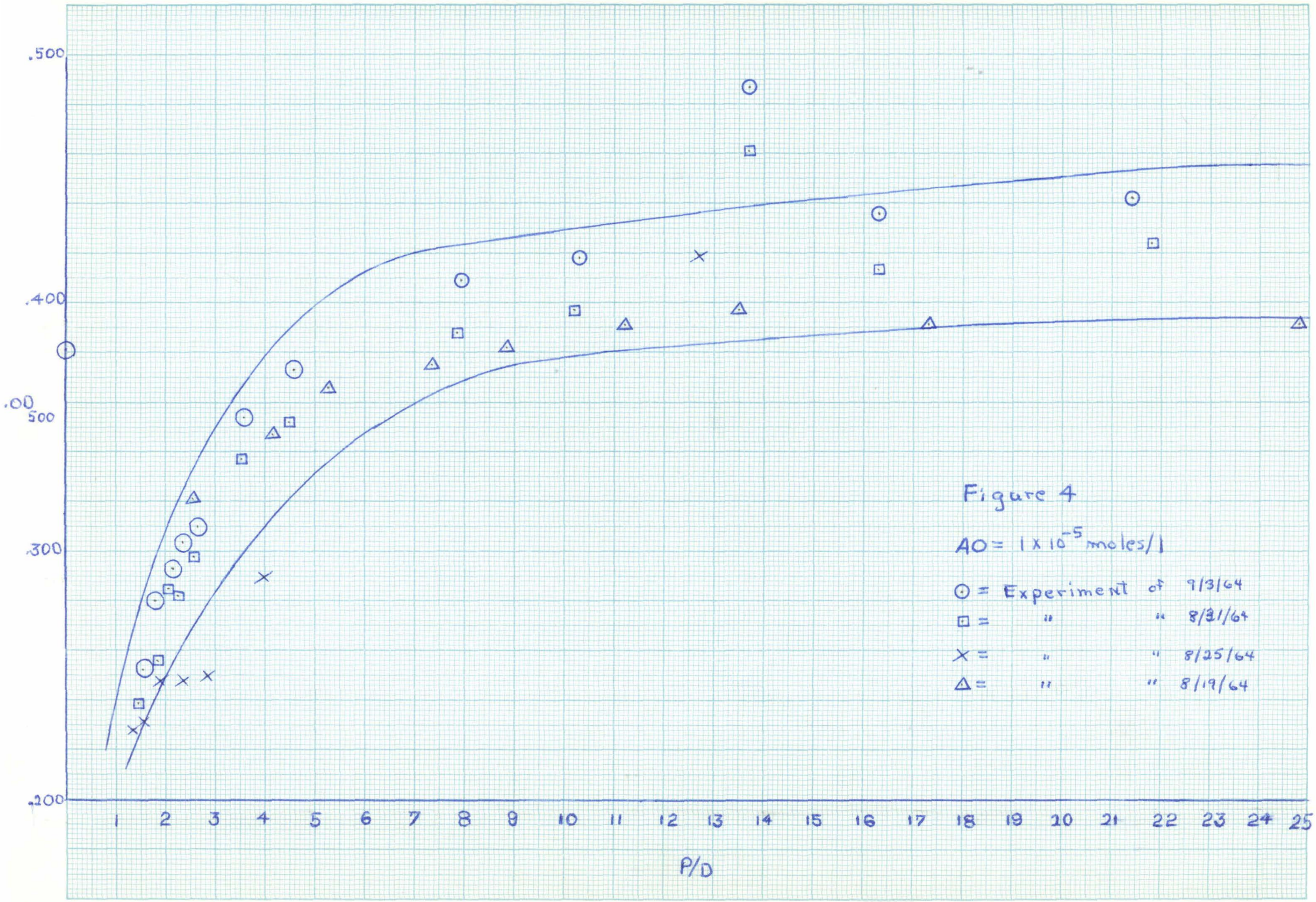
500 mμ increases (see Figure 3). This is in accordance with what would be expected from the theory mentioned above. If the 473 mμ peak is designated the β peak and the 500 mμ peak the α peak, we would expect the α/β ratio to be a measure of the relative percentages of monomer and polymer present. at P/D = 1 the complex should be 100 % polymer while at P/D's of 100 or more we would expect to find only monomer forms present.

During the course of this research it was found that it was very difficult to reproduce the absorption spectrum of an AO--DNA solution of a given composition (constant AO and fixed P/D). Figures 4 and 5 show plots of the optical densities at 500 mμ vs. P/D and at 473 mμ vs. P/D respectively. The AO concentration was held constant at 1 x 10^{-5} moles/l and the DNA was native calf thymus DNA. The points for the O. D.$_{500\ m\mu}$ plot show a very disturbing scatter while those of O. D.$_{473\ m\mu}$ are almost totally random. However a plot of O. D.$_{500}$ ÷ O. D.$_{473}$, which should correspond to the α/β ratio, against P/D yields a smooth and fairly well defined curve (see Figure 6). This behavior seems to indicate that the ratio of monomer to polymer changes in a regular fashion as P/D is increased but that the absolute absorbancies of these two forms do not vary regularly. It is, however, fairly difficult to imagine a physical model of the dye--DNA interaction which would lead to this conclusion. It appears safe to assume that the large variation in the absolute value of the optical density at all wavelengths of the absorption spectrum is caused by some experimental error which we were unable to find and eliminate. A number of experiments by Tom Burke have suggested that significant amounts of acridine orange may be adsorbed onto the surface of glass vessels. Since all of our experiments involved the repeated use of glass vessels, it is possible that the adsorption of varying amounts of free acridine orange could have been an enexpected source of error in our results,

however even the validity of this possibility does not immediately explain the curious behabior of the AO--DNA absorption spectra.

A more plausible explanation would be that during the process of mixing dye and DNA in each experiment a small and non-reproducible amount of the AO--DNA complex precipitated and neither adsorbed or fluoresced. If such a precipitate possessed approximately the same P/D ratio as the bulk of the solution we would expect exactly the behavior noted above. The absolute magnitude of a given point on the absorption spectrum would fluctuate from experiment to experiment because of the non-reproducible amount of precipitate which was present, however the P/D ratio of the solution would not be affected by precipitation and thus for a given P/D ratio the shape of the spectrum would remain constant. This hypothesis of the formation of a precipitate is supported by several observations made during the course of this research. Several of our earlier experiments were stopped after the appearance of a precipitate when we attempted to add a rather concentrated acridine orange solution to a DNA solution. In later experiments there occured several cases of a small amount of precipitate appearing upon the initial mixing of dye and DNA, but because these precipitates dissolved upon sufficient stirring the experiments were continued. If, indeed, these precipitates did <u>not</u> dissolve but merely broke up into particles which could not be seen, then the observed variation in the absorption spectra would be expected. One will note that the variation in the absorption spectra is worst at low P/D values (see Figures 4 & 5). This would be consistent with our observation that precipitates appeared most frequently at low P/D ratios.

<u>Stacking Tendency</u> -- The stacking tendency of a complex is a measure of the degree of non-randomness associated with the selection of a binding site. It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.400
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
P/D
Figure 4
AO = 1 x 10^-5 moles/l
⊙ = Experiment of 9/3/64
⊡ = " " 8/31/64
× = " " 8/25/64
△ = " " 8/19/64

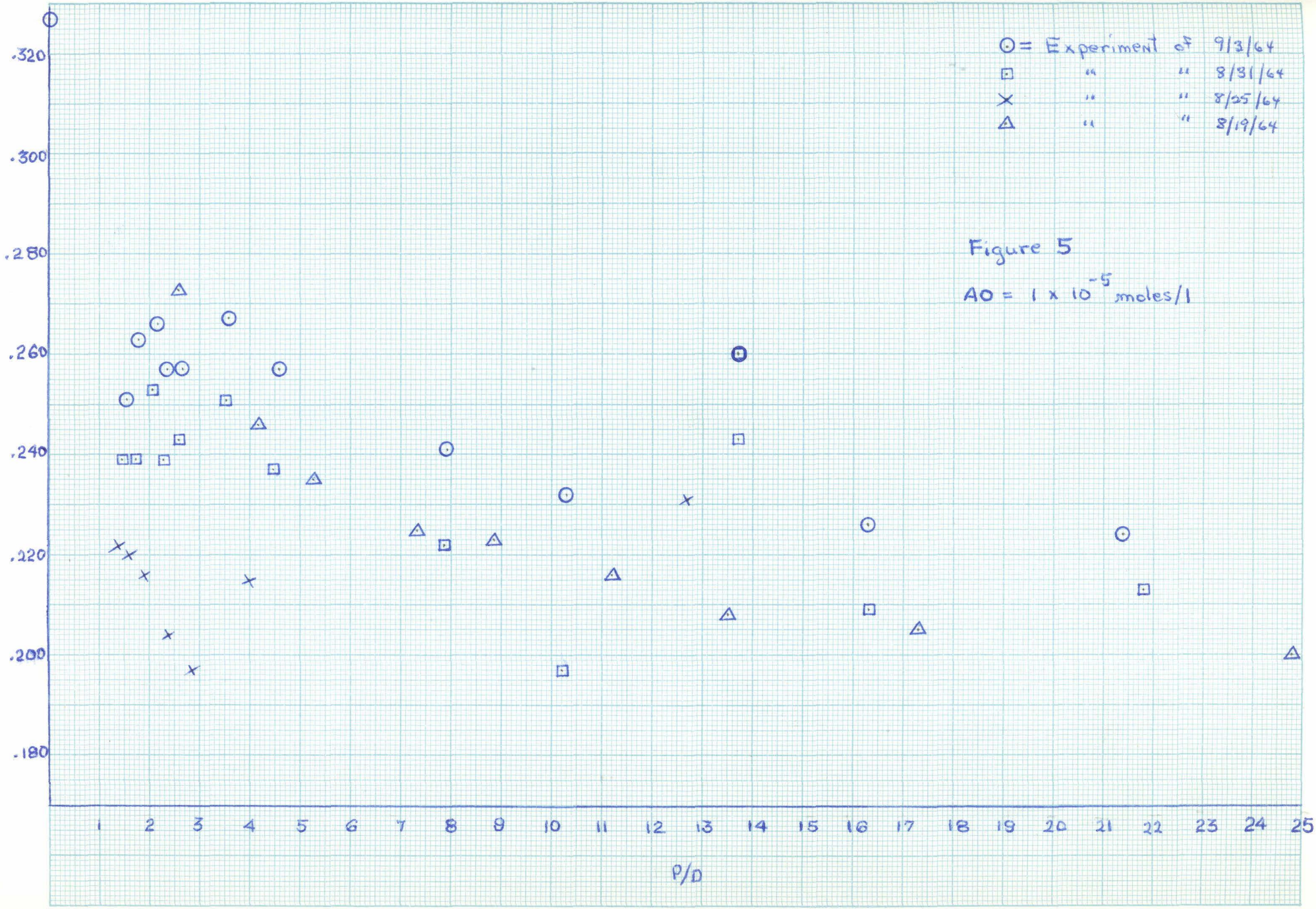


Figure 5

$A_0 = 1 \times 10^{-5}$ moles/l

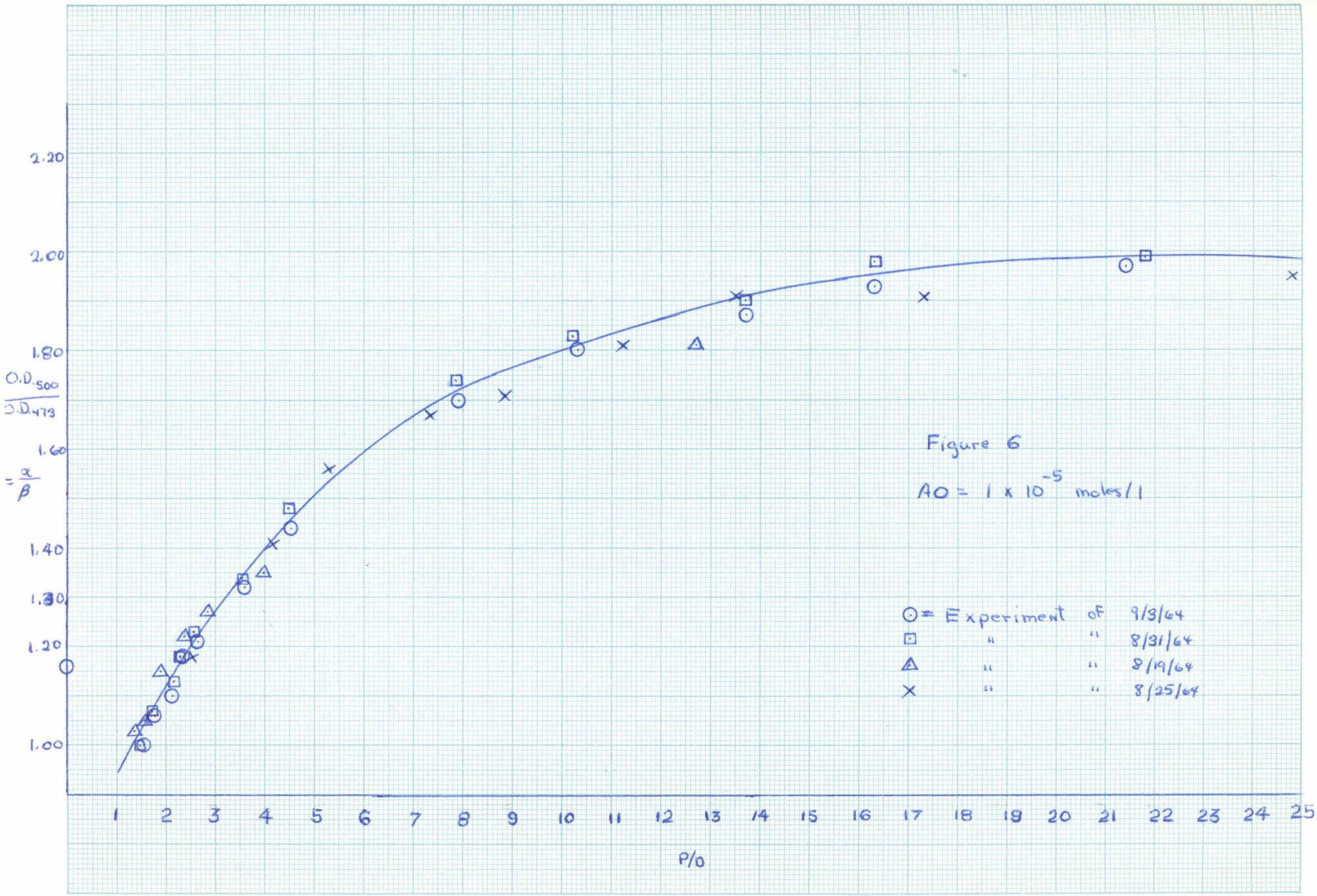

2.20
2.00
1.80
O.D.500 / O.D.473 = α/β
1.60
1.40
1.30
1.20
1.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
P/O
Figure 6
AO = 1 x 10^-5 moles/l
⊙ = Experiment of 9/3/64
⊡ " " 8/31/64
△ " " 8/19/64
× " " 8/25/64

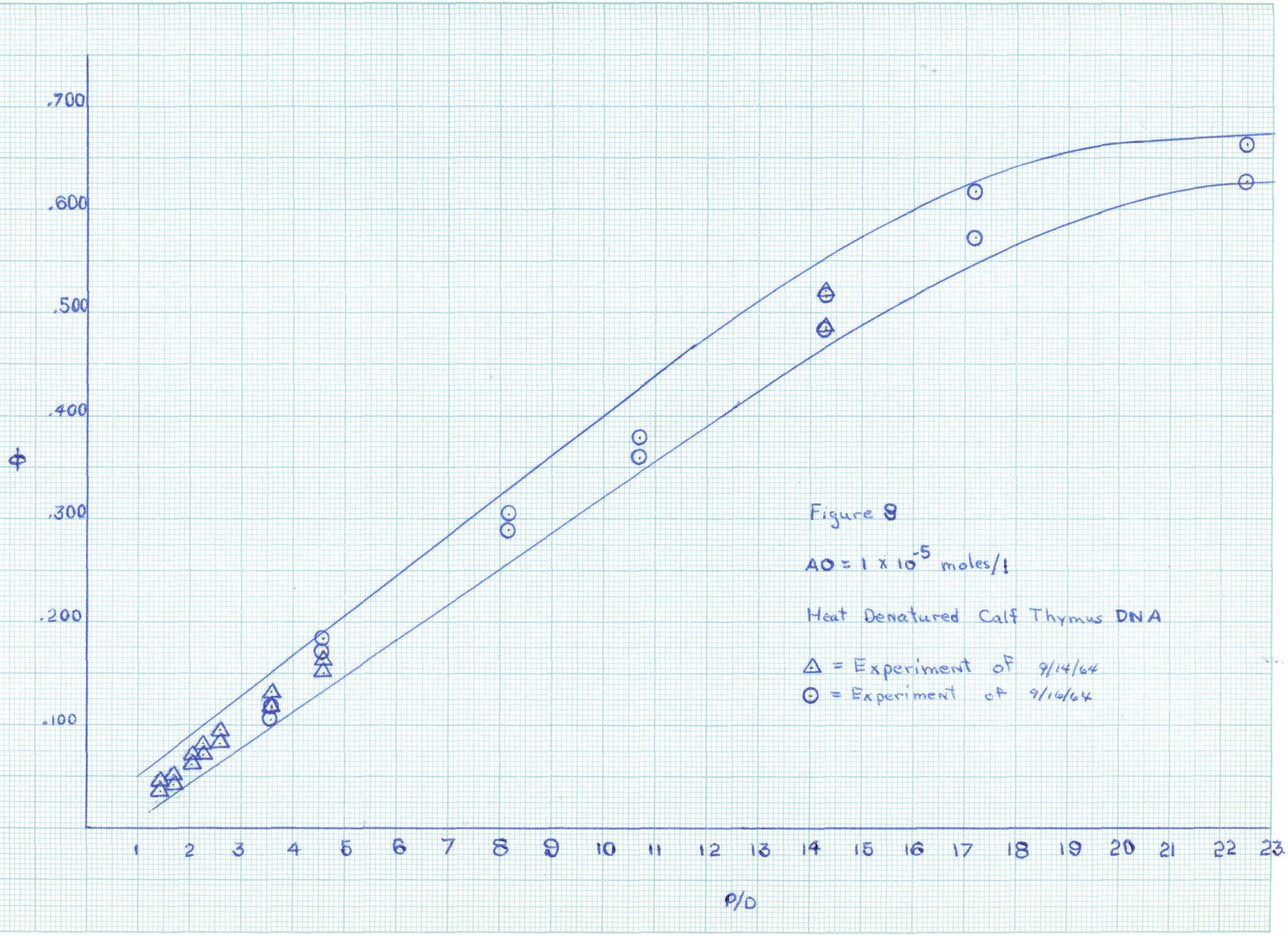
Table 2 -- Native Calf Thymus DNA

$AO = 1 \times 10^{-5}$ moles/l

P/D	$O.D._{436}$ maximum	$O.D._{436}$ minimum	Φ max.	Φ min.
1.5	.108	.070	.0305	.0220
1.7	.095	.066	.0485	.0337
2.1	.084	.063	.0493	.0370
2.3	.080	.061	.0612	.0467
2.6	.075	.059	.0689	.0542
3.5	.066	.055	.149	.124
4.5	.060	.054	.243	.219
7.8	.054	.047	.413	.359
10.2	.053	.047	.461	.418
13.7	.053	.047	.490	.435
16.3	.052	.047	.498	.451
21.8	.051	.047	.610	.562

Table 1

P/D	$O.D._{500}$	$O.D._{473}$	$\frac{O.D._{500}}{O.D._{473}} = \frac{\alpha}{\beta}$
Native DNA			
1.6	.252	.251	1.00
1.8	.280	.263	1.06
2.1	.293	.266	1.10
2.3	.304	.253	1.18
2.7	.310	.257	1.21
3.6	.354	.267	1.32
4.6	.371	.257	1.44
5.3	.366	.235	1.56
7.3	.375	.225	1.67
7.9	.409	.241	1.70
8.9	.382	.223	1.71
10.3	.418	.232	1.80
13.5	.397	.208	1.91
16.3	.436	.226	1.93
21.4	.442	.224	1.97
69	.426	.211	2.02
135	.511	.248	2.06
Denatured DNA			
1.4	.199--.202	.230--.233	.865--.867
1.7	.208	.220	.945
2.0	.234	.235	.996
2.3	.243	.233	1.04
2.6	.256	.239	1.07
3.6	.294	.255	1.15
4.6	.317	.259	1.22
8.2	.352	.241	1.46
10.7	.377	.238	1.58
14.3	.463	.268	1.73
17.2	.415	.227	1.83
22.5	.425	.227	1.87
72	.455	.226	2.01
143	.505	.245	2.06


.700
.600
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Φ
.300
.200
.100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
P/D
Figure 8
AO = 1 x 10^-5 moles/l
Heat Denatured Calf Thymus DNA
△ = Experiment of 9/14/64
⊙ = Experiment of 9/16/64

is perhaps best explained through an example. Imagine a segment of a DNA molecule in which only one binding site is occupied by an acridine orange dye molecule. A second dye molecule now has the choice of binding either at one of the two sites immediately adjacent to the one already occupied or at one of the other unoccupied sites on the molecule. If the probability of the second dye molecule attacking a binding site next to one which is already occupied is greater than the probability of its attacking an isolated site, we say that stacking occurs. A high stacking tendency corresponds to a high probability of a dye molecule binding to a site immediately next to an occupied site.

Bradley and Stone[2] have investigated the stacking tendencies of a number of native and denatured DNA's and have given a formula for evaluating a stacking coefficient which relates the change of the absorption spectrum (from the spectrum of the pure monomer form to that of the pure polymer) with the P/D ratio. We are unable to calculate stacking coefficients for our experiments because we can not reliably estimate the extinction coefficients of the monomeric and the polymeric forms of the AO--DNA complex, two quantities which Bradley and Stone used in their formula. In spite of this drawback we can still make at least one statement concerning the relative stacking tendencies of native and denatured DNA. Table 1 illustrates how the α/β ratio changed with P/D for both native and denatured calf thymus DNA. It shows that for a given P/D ratio the α/β ratio was lower for denatured than for native DNA (greater relative percentage of polymer form present). This indicates more stacking for denatured than for native DNA and is concistent with the findings of Bradley and Stone.

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2) Bradley and Stone, J. A. C. S. 83, 3627 (1959).

A Model of AO--DNA Binding -- The model of AO--DNA binding which assumes that only two different types of absorbing species are present -- a monomer form and a polymer form -- is pleasing because of its simplicity and offers an immediate explanation of the α and β peaks of the absorption spectrum but may not be entirely accurate. A close examination of the absorption spectrum of the dye--native DNA complex (Figure 3) shows that there are two approximate isoesbestic points as we progress from P/D = 1.5 to P/D = 21.4. A model of the dye--DNA binding in which there were three rather than two different species would be consistent with these two isoesbestic points. The intersection at 466 mμ represents that point at which the extinction coefficients of species 1 and species 2 are equal while the point at 490 mμ corresponds to the point at which species 2 and species 3 have equal extinction coefficients (assuming that all of species 1 disappears before any of species 3 appears). It is probable that species 1 is a form of the complex in which the AO dye molecule is bound to a site which has at least one occupied site on each side of it. Species 2 would then be that form of the complex in which the AO molecule was bound to a site which had an occupied site on one side and an unoccupied site on the other, while species 3 would be the monomer form of the complex in which the AO dye molecule is bound to an isolated site. Work by L. S. Lerman[3] suggests that in the monomer species (species 3) the AO dye molecules are actually intercalated between the strands of the DNA helix. Both species 2 and species 1 bind to sites on the outside of the DNA helix. A diagram of these three possible types of binding is given in Figure 7. We would, of course, expect to find the monomer species at very high P/D ratios, the completely surrounded species at very low P/D's, and the half-surrounded species at intermediate P/D's. Just where we found these last two forms would

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3) L. S. Lerman, J. Cell. & Comp. Physiology 64, Supp. 1, 1 (1964).

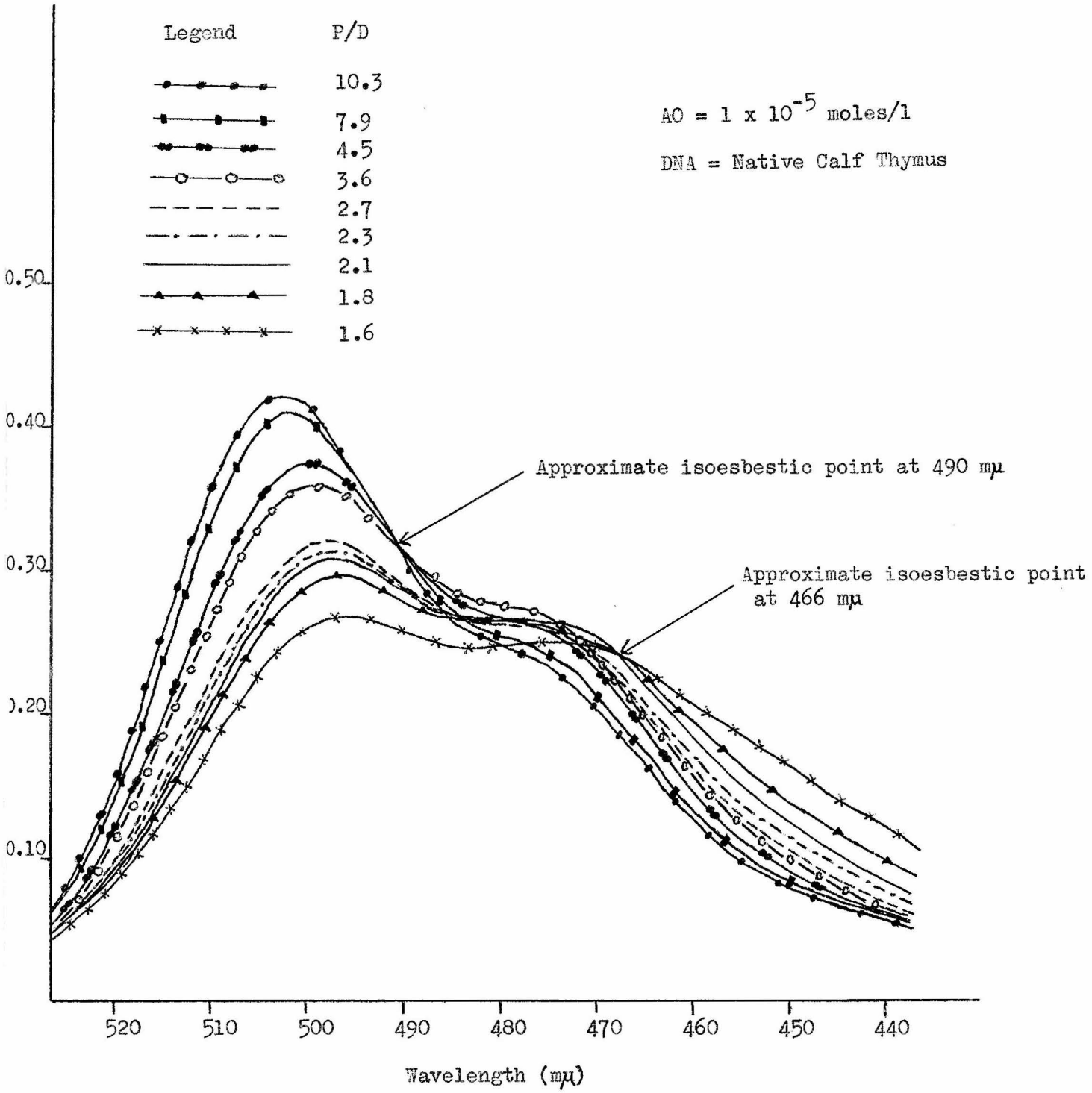

Figure 3. Absorption spectra of AO--DNA complexes.

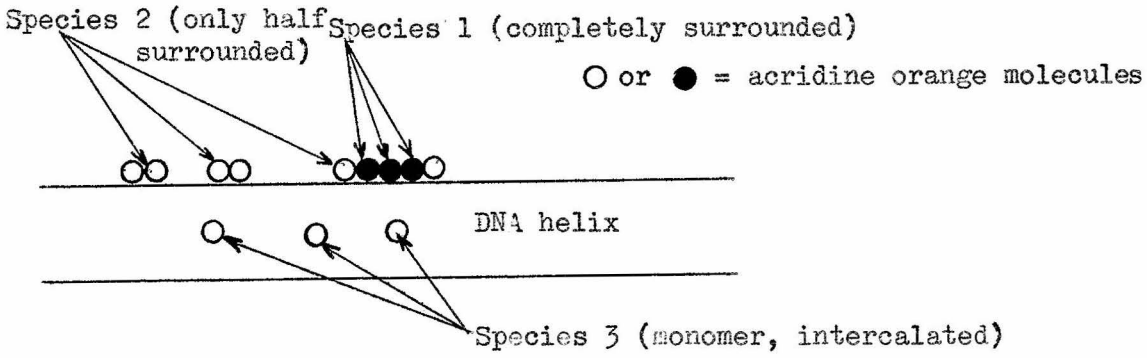

Figure 7 -- Three possible AO--DNA complex species.

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depend on the stacking tendency of the particular DNA being studied.

It should be pointed out that even this three species model may be a bit too simplified. It is quite possible that a dye molecule binding to a site surrounded on either side by two occupied sites absorbs and fluoresces differently than does a dye molecule bound to a site surrounded by only one bound site on each side and that both of these molecules act differently than one found in the middle of a long stack of bound dye molecules. Also it may be the case that two dye molecules attached to sites that are separated by an unoccupied site exhibit some sort of interaction which affects their absorption and fluorescence properties. Nevertheless there is a lot to be said in support of the three species model. It represents the binding process simply enough to allow mathematical calculations of binding constants to be carried out and yet offers a bit more variety than does the two species model.

As explained earlier the optical density at a given wavelength for a solution of fixed composition varied widely from experiment to experiment, and we were therefore unable to evaluate the extinction coefficients of those forms of the AO--DNA complex corresponding to $P = D$ and $P \gg D$. Without this information it was extremely difficult to evaluate the different equilibrium

constants for complex formation and it was likewise hard to estimate the relative concentrations of monomer, half-surrounded, and fully surrounded forms of the complex at a given P/D and a fixed dye concentration. It might be possible to carry out these calculations through the use of a computer program but since it was not immediately obvious exactly how this could be done no such analysis was attempted.

Quantum Yields -- The quantum yields of all acridine orange or AO--DNA complex solutions were estimated by comparing the absorption and fluorescence properties of the solution in question with the absorption and fluorescence properties of a standard solution of 9-aminoacridine. The Hg 436 mμ line was used for excitation of all solutions containing acridine orange while Hg 406 mμ light was used to excite all 9-aminoacridine solutions. We assumed that the quantum yield of 9-aminoacridine (henceforth abbreviated 9-AA) was unity and computed the quantum yields of acridine orange or AO--DNA complex solutions from Equation 1:

$$\text{(Eqn. 1)} \quad \Phi = \frac{\text{Max. fluorescence signal of AO solution}}{\text{Max. fluorescence signal of 9-AA solution}} \times \frac{\text{O.D. 9-AA solution at 406 m}\mu}{\text{O.D. AO solution at 436 m}\mu}$$

The derivation of Eqn. 1 is given in Appendix 1. The maximum fluorescence signals of all solutions under investigation were taken from fluorescence spectra run in the spectrofluorimeter described earlier. The pertinent optical densities were obtained from absorption spectra recorded with a Cary Model 14 Spectrophotomator.

By far the greatest source of error in the calculation of quantum yields came from the measurement of the optical density at 436 mμ of the solution containing the acridine orange dye. It was found that the absolute value of the O. D. at 436 mμ was disturbingly unreproducible for a solution of fixed AO and

DNA concentration. This was particularly true of solutions with a low (1.5--3.0) P/D ratio. The variation of the O.D. at 436 mμ was significant enough that we have calculated a maximum and a minimum quantum yield for each solution studied. This was done by drawing error bars on a plot of O.D. at 436 mμ vs. P/D and then using the limiting values of the first quantity to calculate $\Phi_{max.}$ and $\Phi_{min.}$ for a given P/D (see Table 2). In order to give a clear picture of the reliability of our results we have plotted <u>both</u> $\Phi_{max.}$ <u>and</u> $\Phi_{min.}$ on every graph involving, in any way, the quantity Φ.

By investigating the fluorescence of a series of AO--DNA complexes, all of which had a constant dye concentration of 1×10^{-5} moles/l, it was possible to obtain information concerning how the quantum yield of the AO--DNA complexes varied as the P/D ratio was changed. A plot of Φ vs. P/D for native DNA is shown in Figure 8. The general shape of this plot agrees with the observations of G. Weill and M. Calvin for their experiments on acridine orange bound to native DNA[4]. The quantum yield rises rather rapidly as the P/D ratio is increased until P/D = 10 to 20. It then levels off and remains essentially constant as the P/D ratio is raised indefinitely. A plot of Φ vs. P/D for heat denatured DNA (see Figure 9) indicates that, with respect to the plot for the AO--native DNA complex, the quantum yield rises more slowly with increasing P/D and levels off initially at a higher value of this ratio. As it is thought that the fluorescence of a stacked species is less than that of a monomer this behavior is to be expected of a DNA for which the stacking tendency is relatively high. A denatured DNA, having a greater stacking tendency than a native DNA, will exhibit a relatively greater percentage of the low yield stacked species and will thus have a lower overall quantum efficiency. Our results of this

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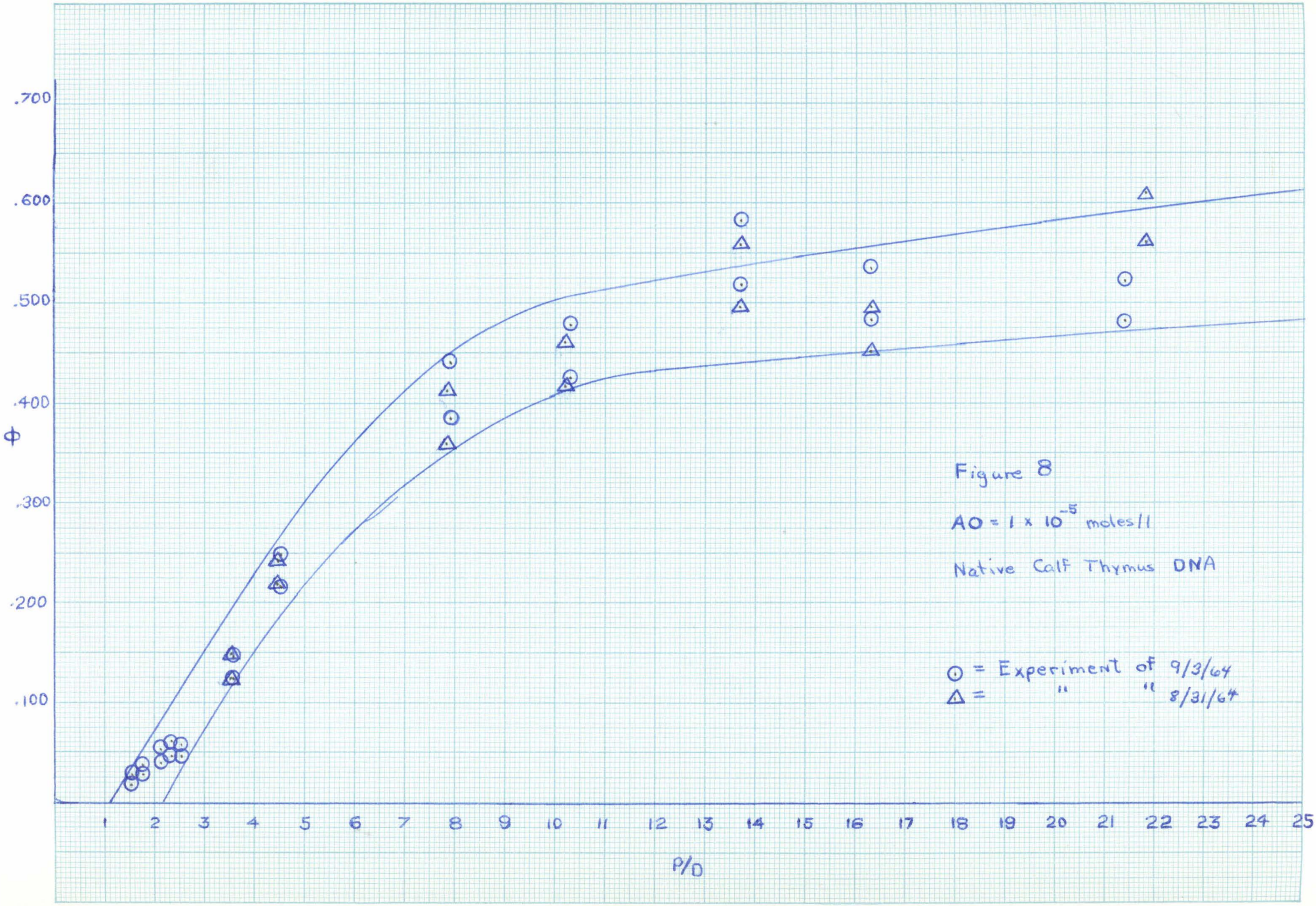
4) G. Weill & M. Calvin, <u>Biopolymers 1</u>, 401--417 (1963).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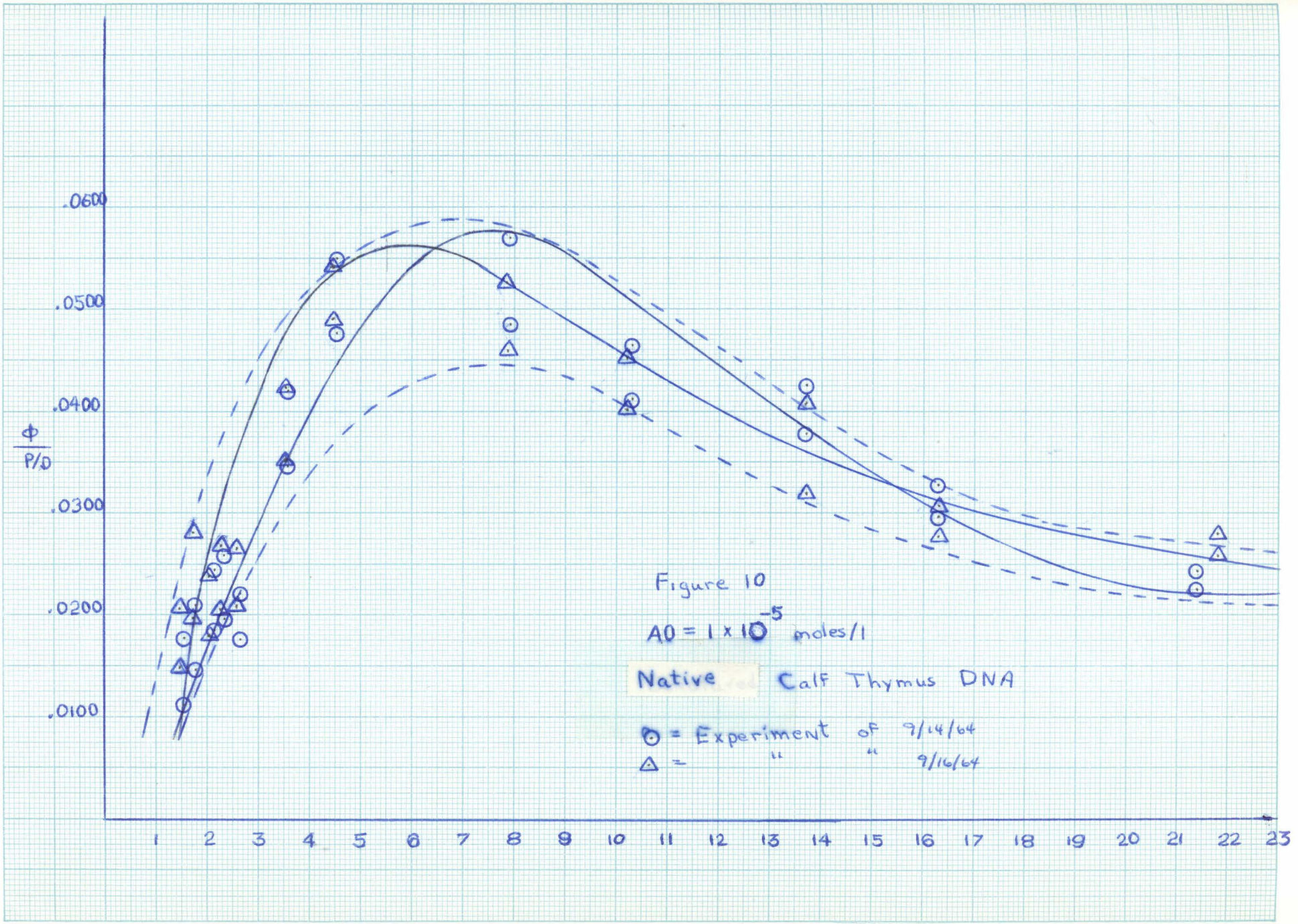
P/D
Figure 8
AO = 1 x 10^-5 moles/l
Native Calf Thymus DNA
⊙ = Experiment of 9/3/64
△ = " " 8/31/64

experiment therefore support our earlier conclusions that denatured DNA has a greater stacking tendency than does native DNA.

As P/D increases the quantum yield of each individual complexed dye molecule increases, but at the same time the total number of AO molecules bound to an individual DNA molecule decreases. Thus a complex with P/D = 50 will show a high quantum efficiency but will give a very low total fluorescence intensity per DNA molecule because only one out os every fifty binding sites are occupied. If we are interested in maximizing the intensity of fluorescence coming from a single DNA molecule we will have to find that P/D ratio for which the increase in quantum yield is least compensated for by the decrease in the absolute numbers of dye molecules. Since the quantity $\Phi \div P/D$ is proportional to the total fluorescence intensity per DNA molecule, a plot of $\Phi \div P/D$ ($= \Phi \times D/P = \Phi$ x percentage of occupied binding sites) vs. P/D should go through a maximum which corresponds to that P/D ratio which, at a given intensity of excitation and a given concentration of AO dye, will give the maximum fluorescence intensity per complexed DNA molecule. Such a plot for native DNA is shown in Figure 10 and a similar plot for denatured DNA is given by Figure 11. On both of these graphs the points corresponding to $\Phi_{max.} \times D/P$ and $\Phi_{min.} \times D/P$ were plotted first and then two smooth curves were drawn within the limits set by these points. One of these curves gives the maximum value of P/D at which the maximum can occur and the other gives the corresponding minimum value of this ratio. Our results indicate that a P/D value between 5.5 and 7.2 will permit the highest fluorescence intensity per complexed DNA for a native DNA. These limits for denatured DNA are 6.9 and 8.9 respectively. The larger limits for denatured DNA are, of course, a direct direct result of the shape of the Φ vs. P/D curve. As discussed above this indicates greater stacking for denatured DNA relative to native DNA.


.0600
.0500
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.0300
.0200
.0100
Φ/P/D
1
2
3
4
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8
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Figure 10
A0 = 1 x 10^-5 moles/l
Native Calf Thymus DNA
⊙ = Experiment of 9/14/64
△ = " " 9/16/64

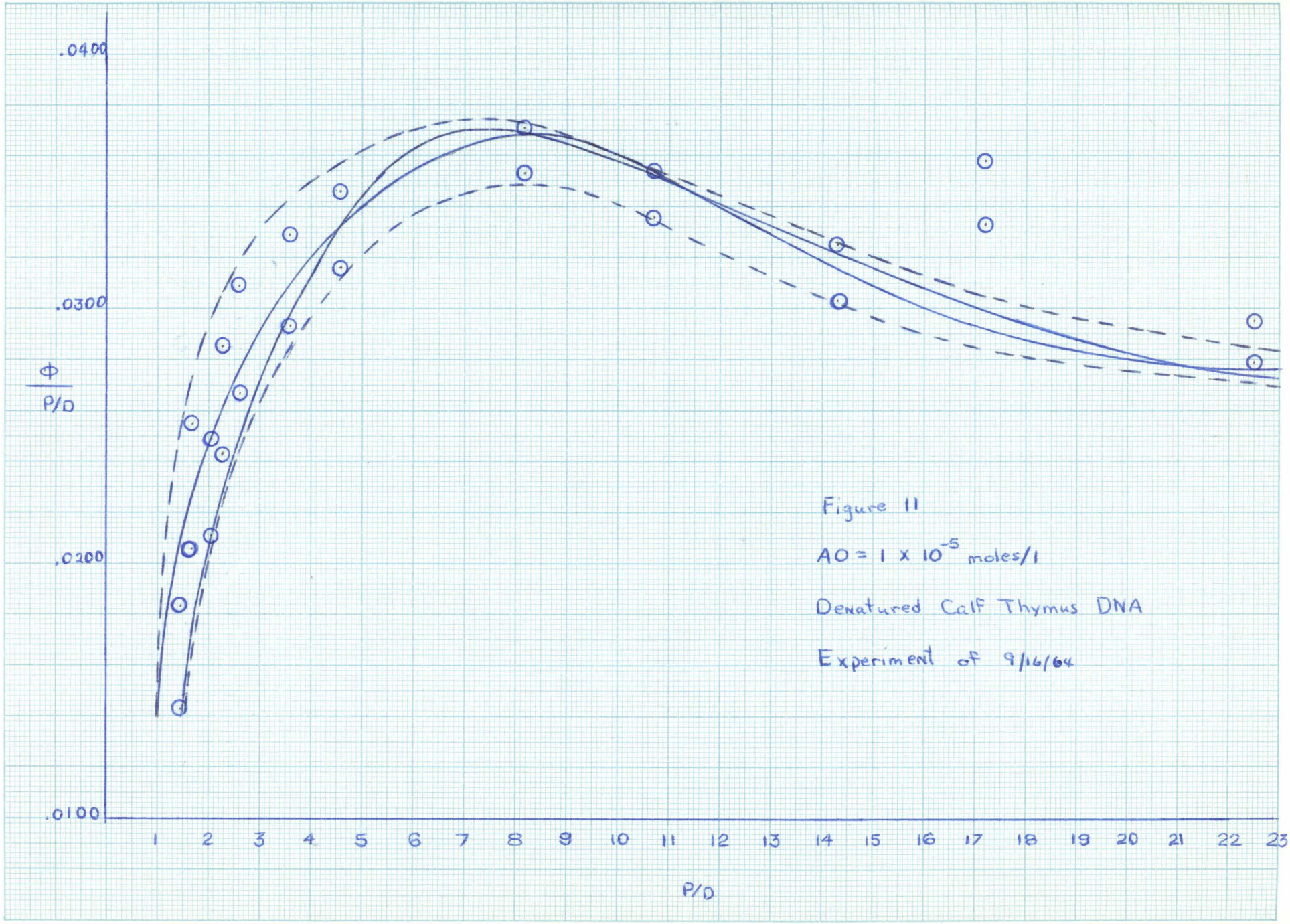

Figure 11

$AO = 1 \times 10^{-5}$ moles/l

Denatured Calf Thymus DNA

Experiment of 9/16/64

APPENDIX 1

Derivation of Equation 1:

$$\phi = \frac{\text{Max. fluorescence signal of AO solution}}{\text{Max. fluorescence signal of 9--AA solution}} \times \frac{\text{O.D.}_{406} \text{ of 9--AA solution}}{\text{O.D.}_{436} \text{ of AO solution}} .$$

Quantum yield is defined as,

$$\Phi = \frac{\text{number of quanta fluoresced}}{\text{number of quanta absorbed}} .$$

But for the steady state conditions of our experiments,

$$\Phi = \frac{F \text{ (in quanta per second)}}{A \text{ (in quanta per second)}} .$$

But $F = \int F \, d\nu$

and $A = \iint - dI \, d\nu$.

From Beer's law,

$$-dI = kcI \, dl \quad \text{where } k = 2.3\epsilon .$$

Therefore $$\phi = \frac{\int F \, d\nu}{\iint - dI \, d\nu} = \frac{\int F \, d\nu}{\iint - kcl \, dl \, d\nu} = \frac{\int F \, d\nu}{\int - kcll \, d\nu} .$$

For our experiments the absorption of the exciting light was relatively unimportant, so $I \cong I^0$, and

$$\phi = \frac{\int F \, d\nu}{-kclI^0 \int d\nu} = \frac{\int F \, d\nu}{-2.3 \text{ O.D. } I^0 \int d\nu} .$$

The second equality in the equation immediately above is true because

$O.D. = \epsilon cl.$

Now we assume that the fluorescence can be approximated by $F_{max.}$ instead of $\int F \, d\nu$. This is not too strained of an approximation for our experiments.

Therefore:

$$\phi = \frac{F_{max.}}{-2.3 \; O.D. \; I^0 \int d\nu} \quad .$$

But considering the ratio of two quantum yields,

$$\frac{\phi_1}{\phi_2} = \frac{F_{1,\,max.}}{F_{2,\,max.}} \times \frac{O.D._2}{O.D._1} \times \frac{I^0_2 \int d\nu}{I^0_1 \int d\nu}$$

or

$$\phi_1 = \frac{F_{1,\,max.}}{F_{2,\,max.}} = \frac{O.D._2}{O.D._1} = \frac{I^0_2}{I^0_1} \times \phi_2 \quad .$$

Our last assumption is that the intensity of irradiation using the Hg 406 mμ line is the same as that obtained using the Hg 436 mμ line. According to the maunfacturer's calibration curve for the Osram HBO--200 mercury lamp which was used, this is not far from the actual case.

Therefore,

$$I^0_1 = I^0_2 \; .$$

Finally we let $\phi_1 = \phi$ of the AO solution and $\phi_2 = \phi$ of the 9-AA solution = 1, and we obtain the equation given as Equation 1:

$$\phi_{AO} = \frac{F_{AO,\,max.}}{F_{9\text{-}AA\;max.}} \times \frac{O.D._{9\text{-}AA}}{O.D._{AO}} \quad .$$

The O.D.'s are naturally evaluated at the wavelengths of the corresponding I^0's.

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