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THE EFFECT OF HYDRODYNAMIC SHEAR ON THE DEOXYRIBONUCLEIC ACID FROM T₂ AND T₄ BACTERIOPHAGES*

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In 1956, Levinthal¹ reported autoradiographic studies of T₂ and T₄ bacteriophage deoxyribonucleic acid (DNA). He found that the DNA in each phage particle was present in the form of several chains, one of molecular weight about 45×10^6 and six or more of molecular weight about 12×10^6 . Attempts to differentiate the large and the small pieces of phage DNA by ultracentrifugation have been unsuccessful. For example, in sedimentation velocity experiments Fleischman² found a single peak with a sedimentation coefficient of 30 to 35 S(vedbergs) (the lower value was found on deproteinized preparations); Meselson, Stahl, and Vinograd³ reported the DNA to band with a Gaussian distribution, implying a uniform molecular weight, in equilibrium experiments in a cesium chloride gradient.

With the ultraviolet absorption system in the Spinco Model E ultracentrifuge, DNA solutions below 0.001 per cent concentration can be studied, and sedimentation coefficients ranging from 8–50 S have been reported.^{4, 5} Calculating from the formula which Doty, McGill, and Rice⁶ derived from studies on calf thymus DNA, the large and the small pieces of phage DNA could have sedimentation coefficients about 42 and 26 S. These values may not be accurate since they are deduced from an unwarranted extrapolation of the formula, and, moreover, recent studies have

shown that molecular weights and physical constants for DNA from different sources cannot be correlated,^{7, 8} nevertheless, the failure to observe any complexity in the sedimentation diagrams has raised doubts about the *in vivo* existence of the "large piece," which could be an experimental artifact.

Thomas and Knight⁹ have measured the sedimentation coefficient of the "large piece" using a partition centrifuge cell. They obtained a value of 40–58 S. The disparity between these and Fleischman's results is sufficient to provoke inquiry. The experiments reported below show that the investigations of Fleischman were probably made on DNA which had been inadvertently degraded. They also show that any physical experiment on phage DNA (and possibly DNA from other sources) must be attended by much greater manipulative precautions than have usually been employed.

Experimental.—Four bacteriophage samples were studied, of which two were given by Dr. H. Van Vunakis (T₂ and T₄), one by Dr. J. Tomizawa (T₂), and of which the fourth (T₂) was prepared by the author. Each preparation was finally purified by differential centrifugation until a clean translucent pellet was obtained. The phage were resuspended in neutral phosphate buffer at a suitable concentration (about 2×10^{12} phage per ml). Results from all these preparations were essentially identical.

Experiments were performed in a Spinco Model E ultracentrifuge using a cell with a 30 mm centerpiece. The optical density of the photographs was measured on a Joyce-Loebl microdensitometer. The density tracings were evaluated by the method of Schumaker and Schachman⁵ to give the differential distribution curve. It should be emphasized that no attempt was made to bring the area under the different parts of the curves accurately into accord with the heights in the integral curves. The differential curve was drawn merely to give the best fit through the points derived from at least two of the optical density tracings.

Shear degradation:† Thomas¹⁰ showed that the large piece of T₄ DNA was degraded during deproteinization, presumably by the shaking involved. Flow birefringence experiments on a shockate of T₂ phage demonstrated that some irreversible changes could be effected in the elongated DNA molecules by the hydrodynamic shear imposed by the Couette apparatus.¹¹ Both of these results suggested that hydrodynamic shear gradients of the same magnitude as those commonly imparted to solutions by such simple laboratory manipulation as stirring or pipetting could cause a reduction in the molecular weight of long DNA molecules.

To confirm these observations, T₂ or T₄ phage in 3 M cesium or sodium chloride were osmotically shocked (Anderson)¹² by the rapid addition of 20 volumes of 0.005 M versene pH 8.5, or cold water. Versene was employed in the case of phage preparations which had been treated with pancreatic deoxyribonuclease, to ensure that no enzymatic degradation occurred. Cesium or sodium chloride was then added to the shockate to molar concentration to minimize any association of protein and DNA through salt bonds; later it was found that these salts could be substituted by 0.2 M phosphate buffer. For many studies the DNA was banded in a cesium chloride gradient in a preparative centrifuge to free it from protein.¹³ The DNA recovered from the band was diluted with water to lower the salt concentration and directly examined in the ultracentrifuge. No significant differences were detected between solutions treated in this way and the untreated shockates.

The solutions were sucked slowly into a syringe, a hypodermic needle was attached to the syringe, and the solutions were injected into the centrifuge cell at a controlled rate to subject them to a measurable average shear gradient. Figure 1 shows the effects of different shears. The mean shear rate for the passage through the hypodermic needle was calculated, assuming that the flow was streamline, from Kroepelin's formula¹⁴

$$\bar{G} = \frac{8V}{3\pi r^3 t}$$

where V is the volume passing in time t , and r is the radius of the capillary. (For

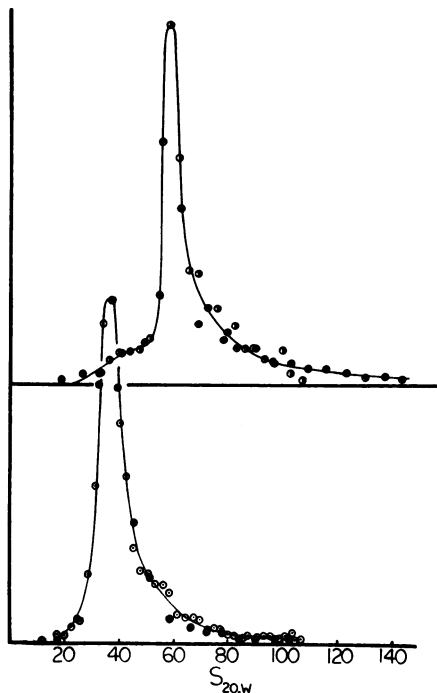


FIG. 1.—Distribution of sedimentation co-efficients in a 0.002% solution of DNA (recovered from banding a T_4 shockate in a cesium chloride gradient) in molar cesium chloride: (top) solution subjected to a shear gradient of 300 sec^{-1} during the filling of the cell; (bottom) solution subjected to 35,000 sec^{-1}

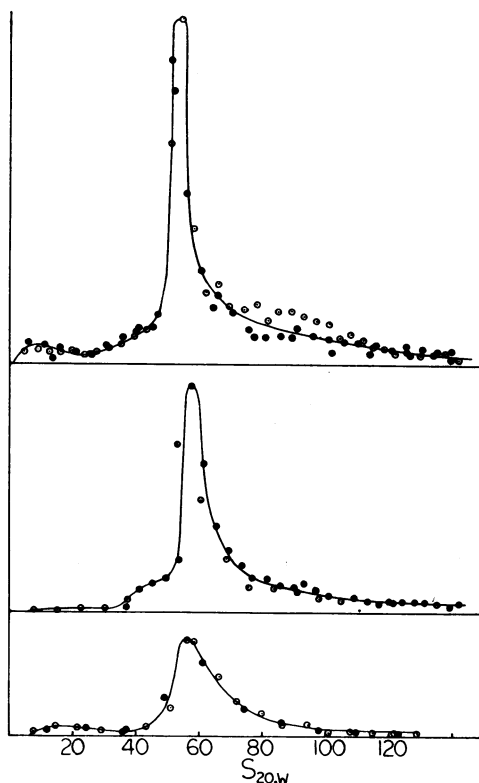


FIG. 2.—Distribution of sedimentation co-efficients in a T_2 shockate in molar sodium chloride at the following DNA concentrations: (top) 0.0021%; (middle) 0.0014%; (bottom) 0.0007%.

example, forcing 1 ml through a No. 27 needle in 15 sec. imposes a mean shear gradient of 55,000 sec^{-1})

The sedimentation diagram of the slowly loaded sample is typical of a large number of shockates examined. The addition of detergent or chymotrypsin to the solutions effected no obvious changes. The sedimentation coefficient of the main peak varied from 50–56 S in sodium chloride solutions, and from 60–70 S in cesium chloride solutions (the upper values in each case corresponded to the most dilute solutions examined). The main peak broadened noticeably as the concentration was lowered (Fig. 2).

The small peak or hump before the main peak varied in size and was assumed to indicate a small proportion of molecules ruptured in the turbulent solution at the moment of shocking. It rarely accounted for more than 10 per cent of the DNA and was frequently absent altogether. The long high molecular weight "tail" to the curve occasionally accounted for 25 per cent of the DNA, and, since its sedimentation coefficient was in some cases higher than that of the phage ghosts,⁹ it was assumed that this DNA was associated and possibly crosslinked with protein. Support for this explanation was obtained from flow birefringence experiments indicating the presence of bonds which persisted despite the presence of strong salt in the solution, and which broke and reformed reversibly when the solution was briefly sheared in the apparatus. Further confirmation of the association of DNA and protein was afforded by the observation that from 25 per cent to 40 per cent of the DNA floated above the cesium chloride in the preparative banding experiments. Some of this DNA could be released by treatment with sodium dodecyl sulfate. The cause for the protein association is undetermined, but it is unlikely to be due to incomplete shocking since a plating assay revealed only 0.6 per cent survivors. Because only part of the DNA was free of protein the shockates could not be used to characterize the DNA of the phage, but they served to delimit the conditions under which the molecules were stable.

A series of experiments showed that when the titration of the solution was performed by a machine, a mean shear rate up to 25,000 sec.⁻¹ could be applied without any change in the sedimentation diagram. However, when the solution was injected by hand, even a shear rate as low as 16,000 sec.⁻¹ caused extensive breakdown. The integrity of the molecules could be safely preserved by injecting the solution slowly (1 ml/minute) by hand using a No. 22 needle (the largest the cell orifice will accommodate).

The difference between machine- and hand-pipetting might be explained by the plunger moving in a series of small jerks in the hand; however, a further feature of the sedimentation diagrams required explanation. If the flow in the needle is streamline, then the liquid through the center of the needle should suffer no shear gradient, and hence the sheared solution should show a mixture of degraded and undegraded molecules. In none of the five solutions examined was a bimodal curve detected. This could be understood if the rupture of the molecules occurred also in the turbulence at either end of the needle, where efficient mixing takes place and where high local shears could be present. Confirmation of this idea was obtained from another experiment in which half the contents of a cell was squirted through a fine needle; when this half of the solution was injected rapidly into the remainder of the solution in the cell, no undegraded molecules were detected, but when the sheared solution was added slowly to the unsheared half, a bimodal distribution with maxima at 29 and 53 S (in sodium chloride) was clearly resolved.

With the application of shears of the order of 20,000–40,000 sec.⁻¹ the DNA sedimented as a fairly symmetrical peak with a maximum about 30 S. However, by shearing more rapidly through a syringe needle the maximum could be dropped to 21 S; by 15 seconds' treatment in an Osterizer the maximum was dropped to 17 S.

No increase in the absorption of the DNA solutions at 260 $m\mu$ was detected accompanying the shear degradation.

Since the destructive shear in the case of turbulence cannot be evaluated, it is

difficult accurately to define the conditions which must be avoided to maintain the DNA intact. However, it is likely that any violent agitation of the solution, filtration, pipetting, even chromatography through a fine adsorbent could cause a rupture of the DNA chain.

In an attempt to demonstrate the DNA degradation which Thomas observed in deproteinization experiments,¹⁰ a T_2 shockate was examined before and after gently shaking thirty times in a half-filled test-tube (Fig. 3). The presence of an immis-

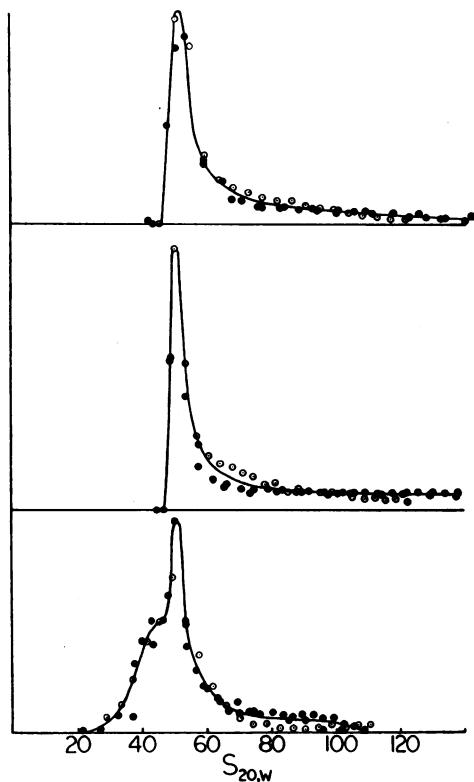


FIG. 3.—Effect of shaking T_2 shockate in 0.2 M pH 8 phosphate buffer (DNA concentration 0.001%): (top) untreated; (middle) shaken 30 times in test-tube; (bottom) shaken 30 times in test-tube in the presence of an equal volume of chloroform.

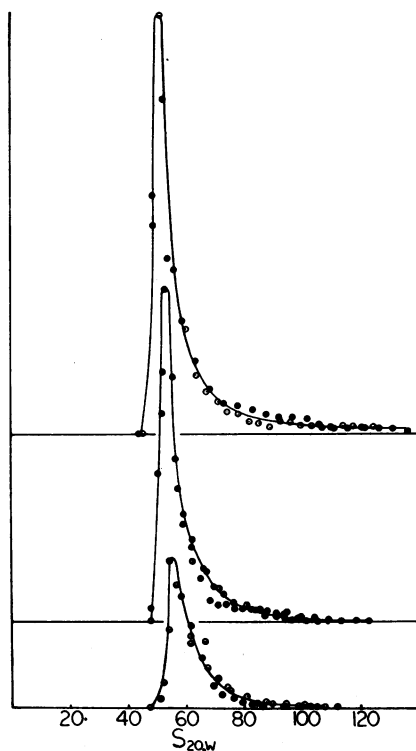


FIG. 4.—Distribution of sedimentation co-efficients in T_2 DNA released by treatment with pH 11 buffer. DNA concentrations: (top) 0.001%; (middle) 0.0007%; (bottom) 0.0004%.

cible liquid appears to be necessary for chain rupture to occur under these mild conditions of shaking.

Characterization of phage DNA: Since some of the DNA in the shockate appeared to be interacting with protein, methods were sought to prevent this interaction. Two methods are obvious: (a) the addition of excess competing molecules, or (b) raising the pH of the solution so that protein and DNA are similarly charged and mutually repel. Both methods were used and gave essentially similar diagrams, but the latter technique was the simpler, particularly when, on the advice of Dr. E. Freese, the osmotic shock treatment was omitted. The centrifuge cell was filled with borate or phosphate buffers of pH 11.0, and the requisite quantity of intact

phage was added from an Agla micrometer syringe. The DNA concentration was kept below 0.002 per cent to lessen the likelihood of concentration-dependent interactions. The solution was mixed by gentle inversion and allowed to stand for 10 minutes at room temperature to allow the phage to burst. The characteristic sedimentation patterns obtained in this manner are shown in Figure 4. The absence of the hump on the low S_{20} side confirmed that the presence of low S_{20} material in the shockates was adventitious.

The high S "tail" on the diagrams, which was much smaller than in the case of the shockates, demonstrated a lower tendency to associate with protein. Sedimentation equilibrium experiments showed that more than 80 per cent of the DNA could be banded at a specific gravity of 1.7; so it may be concluded that these sedimentation diagrams are representative of the DNA in phage.

In a few examples there has been a suggestion of a hump on the high S_{20} side at about 64 S (in sodium chloride). However, since minor irregularities are constantly present in the densitometer traces, it cannot be claimed that there is any reliable indication of heterogeneity in the DNA beyond an undoubted skewness in the peak—and that could be accounted for by a hypersharpening of the boundary.

The DNA released by high pH appeared to be at least as sensitive to shear as in the shockates.

Discussion.—Throughout this paper it has been assumed that the lowering of the sedimentation coefficient corresponds to a lowering of the molecular weight of the DNA. This assumption is reasonable since: (a) it has been shown that for homologous DNA samples the value of $S_{20,w}$ decreases with molecular weight,^{6, 7} and (b) from Thomas's experiments, the treatment which destroys the large piece of DNA¹⁰ also produces material with a lowered sedimentation coefficient. Moreover, it is difficult to imagine that the decrease in $S_{20,w}$ could come about as a result of a change of shape since the shearing of the molecules is accompanied by a decrease in the specific viscosity.

A possibility to be considered is that the changes brought about by shear do not represent a scission of the DNA double helix, but a dissociation of aggregates. If this is the case, the aggregates are probably specific, since they are present in DNA liberated by high pH or shocking, and they do not reform in the solutions after shearing. However, the possibility seems unlikely, because the sedimentation coefficients of the products drop as the violence of the shearing is increased, and there is no evidence for a stable sub-unit. It must be concluded that DNA can be degraded by hydrodynamic shear.

The relative fragility of the DNA demonstrated in these experiments is quite understandable on theoretical grounds. Frenkel¹⁵ in 1944 deduced that long polymers might be degraded by shears of the order of a few thousand reciprocal seconds, and Bestul and Belcher,¹⁶ for example, demonstrated the rupture of synthetic polymers of 500,000 molecular weight at shears of 60,000 sec.^{-1} . Since it is only too easy to apply shears (calculated for streamline flow) of 20–30,000 sec.^{-1} in volumetric pipettes or hypodermic syringes, rupture of long-chain molecules is to be expected. Although DNA is a double-strand molecule, it is not easy to predict whether or not the molecule could resist a lengthwise stress twice as great as a single polynucleotide strand. Also it is not possible to calculate accurately the stress applied by the viscous forces to a molecule traversing the streamlines. For example, it is dubious if

Stokes's equation can be applied since the solvent molecules and hydrated ions are by no means small compared with the diameter of the DNA helix. However, Levinthal,¹⁷ employing reasonable values for the bond strengths, calculated the critical shear rates to be about 10,000 sec.⁻¹. The observed threshold of about 25,000 sec.⁻¹ is in reasonable accord, if it can be assumed that the rupture occurs in streamline flow. The degradation of DNA by shear has been briefly mentioned previously by Goldstein and Reichmann.¹⁸

The theoretical calculations show that the rupturing stress applied by the viscous forces is maximal in the center of the chain and increases as the square of the length of the molecules. The molecules should thus break roughly in half when the critical shear is applied, and a very long molecule should be halved repeatedly until a fairly uniform population of chains stable to the shear gradient is obtained.

No demonstration of heterogeneity in the undegraded phage DNA has been achieved, but the sharp skew peak may indicate a complexity which could be resolved at lower concentrations. Unfortunately the optical limitations at present preclude the employment of solutions much below 0.0005 per cent concentration for accurate analyses. It is known that self-sharpening effects persist in normal DNA preparations down to 0.01 per cent, and it may not be unexpected that in the presence of much longer chains the critical concentration should be much lower. However, it is also possible that the DNA population is essentially homogeneous (apart from aggregates). The Doty formula,⁶ if it were applicable, would indicate a molecular weight about 1×10^8 (roughly the DNA content of a phage particle) for a 56 S molecule. If the DNA is normally present as one macromolecule, the autoradiographic experiments must be explained by degradation of this molecule, presumably during the manipulation of the emulsion. This appears unlikely since the application of controlled shear gradients did not suffice to produce a bimodal distribution. However, it is obvious that for an answer to the present difficulties some correlation of molecular weight and sedimentation coefficient is needed for the phage DNA.

Nothing in the experiments reported in this paper has shown that the DNA sedimenting in these sharp peaks is protein-free, although the fact that the material in some experiments has been banded at a specific gravity of 1.7 before study implies that any protein present must be in small proportion. Thus it could be postulated that protein bridges exist along the DNA macromolecule uniting DNA sub-units. It can also be postulated that the shear scission occurs at these or other weak points. If they are present, such bridges must be resistant to chymotrypsin. However, until there is some definite evidence, it seems most reasonable to assume that the hydrodynamic shear is transecting the DNA double helix.

It should perhaps be pointed out that the phage particle itself would suffer no embarrassment from the shear sensitivity of its DNA during the process of injecting these molecules into the bacterium, since the diameter of its tail is too small for the DNA to lie across the streamlines, even if there were a solvent present to impart a viscous drag.

The observed lability of the phage DNA poses the problem of how much the DNAs from other tissues might be degraded in the course of preparation. The relative uniformity of observed molecular weights in sperm and thymus DNA preparations, for example, is no proof that these molecules have not been degraded, since a fairly uniform distribution would be expected from the shearing action. The

vigorous stirring, blending, or filtration to which most preparations of DNA are subjected would certainly degrade phage DNA. If DNA *in vivo* is much longer than is usually recognized, some of the present problems of correlating DNA structure and function would be simplified. Beiser, Pahl, Rosenkranz, and Bendich¹⁹ found that specific pneumococcal transforming activities were chromatographically complex. Assume, for simplicity, that each activity relates to the presence of one enzyme. If the DNA molecule is very long and is ruptured during isolation or deproteinization to give chains of 8×10^6 , average molecular weight (of which 1×10^6 would be needed to code for a protein of 50,000 molecular weight²⁰), the activity might be found on molecules of variable length and composition. Little loss of activity need be envisaged since only one break in eight would interrupt the critical sequence; if the sequence lies close to the end of the gross macromolecule, the sequence would rarely be ruptured.

Furthermore, at present most speculations on chromosome structure assume that the DNA is of relatively uniform molecular weight (about $4-8 \times 10^6$). The evidence presented in this paper shows that the experimental grounds for this assumption are questionable. It is obvious that some of the earlier physical work on DNA must be repeated with suitable precautions to avoid the scission of any very long molecule which may be present.

Summary.—(1) Turbulence and high shear gradients have been shown to cause a decrease in the sedimentation coefficients of T₂ and T₄ bacteriophage deoxyribonucleic acid molecules in solution. (2) In sedimentation velocity experiments, carefully treated DNA solutions have shown a sharp peak, skew on the high S_{20,w} side, with a maximum about 60 S.

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† Throughout this paper the word "degradation" is used to denote a reduction in molecular weight—presumably by transverse scission of the DNA double helix.

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THE PHOTOCHEMICAL OXIDATION OF DPNH WITH RIBOFLAVIN PHOSPHATE

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Recently this laboratory has reported studies on the non-enzymatic catalysis of oxidation by flavin coenzymes of various nitrogen compounds in light.^{1,2} In an extension of these studies to other coenzymes, we have found that reduced diphosphopyridine nucleotide (DPNH) can also be efficiently oxidized photochemically by

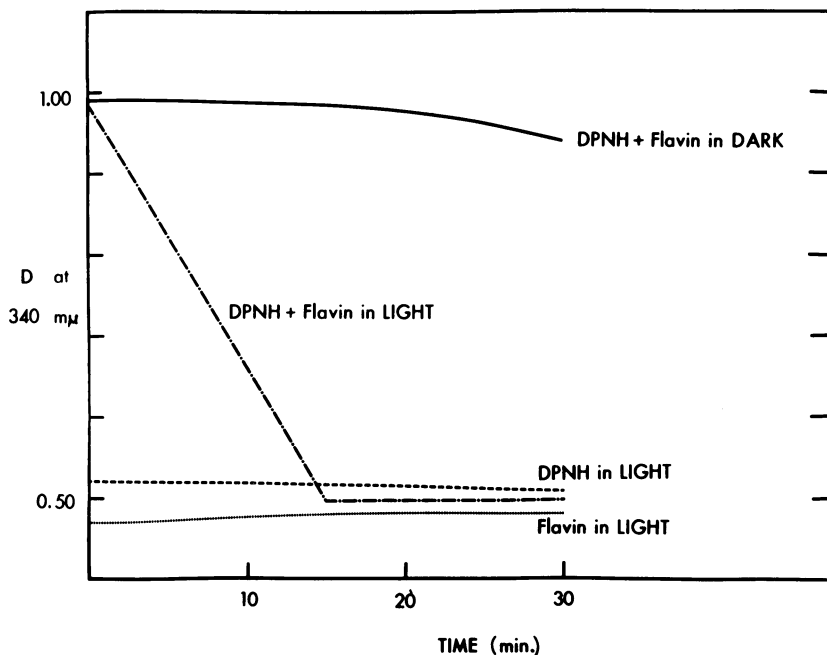


FIG. 1.—Oxidation of DPNH by riboflavin phosphate in light under aerobic conditions. Quartz cuvettes contained 0.27 μ mole of DPNH (Sigma Chemical Co.) and/or 0.25 μ mole of riboflavin phosphate (Sigma Chemical Co.) in 3.0 ml of 0.075 *M* potassium phosphate buffer (0.0001 *M* in Mg^{++}), pH 7.8. For illumination, cuvettes were placed 2 cm from 36-in. General Electric "black light" fluorescent lamp, and "dark" controls covered with black paper. Optical densities at 340 $m\mu$ measured with Beckman DU spectrophotometer.

riboflavin phosphate in the absence of apoenzymes or any other organic compounds. Typical results are presented in Figure 1. Thus, it will be seen that when a solution