Efficient Separation of Single-Stranded and Double-Stranded Deoxyribonucleic Acid in a Dextran–Polyethylene Glycol Two-Phase System*

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ABSTRACT: A dextran–polyethylene glycol, aqueous two-phase system has been refined so that quantitative separation of single-stranded (and partially single stranded) deoxyribonucleic acid (DNA) from double-stranded DNA may be obtained routinely in a single extraction. Partition coefficients for both types of DNA are drastically affected by the exact concentrations of dextran and polyethylene glycol used to form the system, as well as by the ionic environment. These parameters may be quickly adjusted to optimize the partition coefficients during a preliminary calibration, thus compensating for variations that occur in different samples of commercially available dextran. After the desired separation has been accomplished, small amounts of DNA in the top (polyethylene glycol rich) phase may be accurately examined and characterized by direct equilibrium sedimentation in a CsCl density gradient.

The addition of solid CsCl to this phase selectively salts out the polyethylene glycol.

A dextran–polyethylene glycol, aqueous two-phase system was first employed for fractionation of DNA by Albertsson (1962). In a suitably selected mixture of dextran, polyethylene glycol, salt, and water, single-stranded and double-stranded DNA will strongly favor opposite phases. Indeed, in this report conditions are found which allow single-stranded DNA to be *quantitatively* removed from double-stranded DNA in a *single* extraction. Such sharp separations are obtained even at DNA concentrations of 1 mg/ml or more.

Despite its potential usefulness, this separation technique has only rarely been exploited for DNA outside of Sweden where it originated. This is largely due to certain difficulties inherent in the method, *i.e.*, the impossibility of partitioning nucleic acids reproducibly if the exact composition of the phase system is not carefully controlled and the presence of large quantities of dextran or polyethylene glycol in the fractionated material obtained.

The present report briefly describes ways in which these difficulties may be readily surmounted and demonstrates that the dextran–polyethylene glycol system makes possible a simple, reproducible, and quantitative separation of single-stranded (and partially single stranded) DNA from double-stranded DNA. A more general discussion, including other applications of dextran–polyethylene glycol systems, is presented elsewhere (Alberts, 1967).

Experimental Section

Concentrated "phase-system stocks," consisting normally of 16.8% (w/w) Dextran 500 (Pharmacia, Uppsala, Sweden; lot no. 852) and 9.2% polyethylene glycol 6000 (Union Carbide Chemicals, N. Y.) in distilled water, were employed as the source of the polymers. To avoid possible growth of microorganisms, these stocks were kept frozen. Prior to each use, the phase-system stock was extensively agitated, and while still completely emulsified, an aliquot of predetermined weight was pipetted into a tared test tube. A carefully measured volume of DNA solution, exhaustively dialyzed against 0.01 M sodium phosphate buffer (pH 6.8), was then added. The resulting mixture was well emulsified at 4° by inverting the stoppered test tube 40 times, and two sharply defined phases of roughly equal volume were separated by 5 min of low-speed centrifugation at the same temperature. The phases were withdrawn with a Pasteur pipet and, after a tenfold dilution, assayed for DNA either by bacterial transformation or absorbance measurements.

Although the partitioning of DNA is not noticeably affected by either doubling or halving the molarity of sodium phosphate buffer normally used (0.005 M NaH_2PO_4 plus 0.005 M Na_2HPO_4), the presence of small quantities of other ions, in particular chloride, was found to retard the entrance of native DNA into the top phase and thereby distort the fractionation. A profound effect of a variety of salts on the system had been described (Albertsson, 1965); in particular, raising the pH of the sodium phosphate buffer strongly

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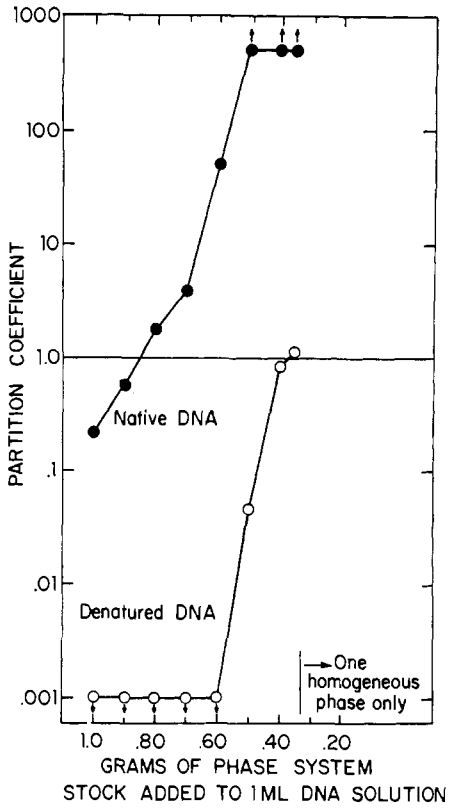

FIGURE 1: The dependence of the partition coefficient for native and denatured DNA on the proportion of phase-system stock added. Partition coefficients are expressed as top-phase absorbance divided by bottom-phase absorbance. Dialyzed calf thymus DNA (Worthington Biochemical) at 300 μg/ml was either partitioned directly or denatured for 5 min at 100° in the 0.01 M sodium phosphate buffer (pH 6.8) used for phase extraction. The concentrated polymer stock here consisted of 16.8% dextran and 9.2% polyethylene glycol.

increases the affinity of all nucleic acids for the top phase.

Essentially indistinguishable results were obtained with phase systems prepared from two different samples of Dextran 500 (Pharmacia lot no. 730: M_w = 375,000 and M_n = 180,000; lot no. 852: M_w = 370,000 and M_n = 185,000) which were combined with either of two lots of polyethylene glycol (Carbowax 6000). However, a third batch of Dextran 500 (lot no. 5406: M_w = 420,000 and M_n = 153,000) produced anomalous behavior in that both native and denatured DNA partitioned completely into the bottom phase at all concentrations of phase-system stock. In this case, if the pH of the 0.01 M sodium phosphate buffer was increased to 9.2, as suggested by the results of Albertsson 1965), a good separation was obtainable (Alberts, 1965). According to Pharmacia, specifications of future batches of Dextran 500 are to be more closely controlled in order to avoid the necessity of such a buffer adjustment.

Bacillus subtilis DNA was prepared by phenol deproteinization and had an average molecular weight of 30–35 × 10^6 Daltons (Alberts, 1965; B. Alberts and P. Doty, 1967, in preparation). Density-labeled, $^{15}N^2H$ DNA was isolated from *B. subtilis* grown in minimal media containing $^{15}NH_4Cl$ and ^2H_2O (Yoshikawa and Sueoka, 1963). CsCl density gradient centrifugation (Meselson *et al.*, 1957) and *B. subtilis* transformation (Anagnostopoulos and Spizizen, 1961) were carried out by customary procedures. Transformation utilized wild-type DNA and was scored for the trp_2 marker of receptor strain SB-1 (his_1^-, trp_2^-, and $sulf^s$); for all assays, the total DNA concentration was kept below 0.1 μg/ml, which is within the linear range of the system's response.

Results

Effect of Total Polymer Concentration. The exact proportion of phase-system stock added to the DNA solution is crucial and must be individually determined for each stock. For this purpose, the phase-system stock is added in increasing amounts to separate tubes containing a fixed volume of either denatured or native DNA, previously dialyzed against the 0.01 M sodium phosphate buffer. The resulting partition coefficients for a typical stock are shown in Figure 1, where partition coefficient (K) indicates the ratio of DNA concentration in top (polyethylene glycol rich) phase to DNA concentration in bottom (dextran rich) phase.

It is evident that with high concentrations of a phase-system stock both native and denatured DNA favor the dextran layer; only at lower concentrations do the two DNAs display strong selective affinities for top and bottom phases, respectively. It is of interest to note that with very small volumes of stock, both DNAs again tend to travel together, but now favor the top phase! This strong dependence of partition coefficient on the relative volume of phase-system stock added is always observed, although it would not be predicted on theoretical grounds (see Discussion).

Effect of Polymer Composition. In order to determine whether the *relative* amounts of dextran and polyethylene glycol are crucial for the fractionation of DNA, calibration curves of the type shown in Figure 1 were obtained with several different phase-system stocks, each composed of an unique ratio of dextran to polyethylene glycol. A compilation of some of the results is shown in the phase diagram of Figure 2. Increasing the relative quantity of phase-system stock added corresponds on the phase diagram to traveling away from the origin on a straight line of slope equal to the weight ratio of polyethylene glycol to dextran in that particular stock (broken lines). Thus, it may be seen from Figure 2 that regardless of the ratio of dextran to polyethylene glycol tested, reasonable separation of denatured from native DNA could always be obtained at some intermediate concentration of each phase-system stock. Nevertheless, those stocks with a relatively high proportion of dextran (as in Figure 1)

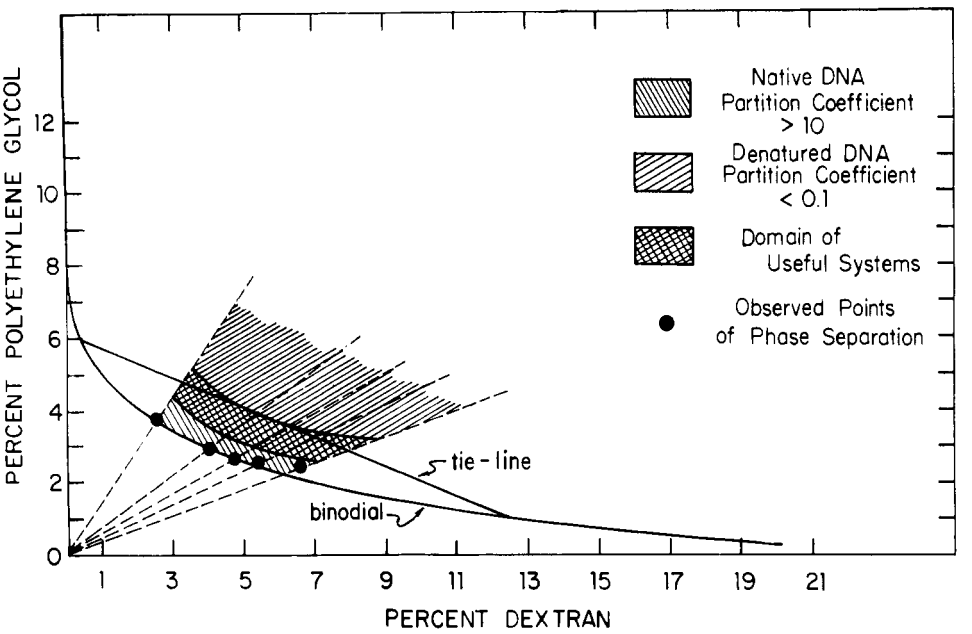

FIGURE 2: The phase diagram of a dextran-polyethylene glycol two-phase system and its relation to the separation of denatured from native DNA. Data such as that presented in Figure 1 has been used to determine the polymer concentrations in the phase diagram yielding partition coefficients greater than 10 for native DNA and less than 0.1 for denatured DNA. The region where both types of behavior are observed has been arbitrarily defined as the domain of useful systems (cross-hatching). The curved "binodial" indicates the minimum concentrations of dextran and polyethylene glycol required for phase separation, with all mixtures below this line consisting of one homogeneous phase. The "tie line" shown is one of a system of almost parallel straight lines, each of which connects mixtures for which the individual compositions of top and bottom phases remain constant. (Along a given tie line, only the relative *volumes* of the two phases change, and the composition of each phase is indicated by the two points of intersection of the tie line with the binodial.) The binodial and tie line shown are approximate, having been derived from the appropriate phase diagrams of Albertsson (1960).

were preferred. At optimal concentration only these stocks reproducibly permitted *complete* removal of single-stranded DNA from the native DNA-containing top phase by means of a *single* extraction (less than 0.02% remaining).

It should be noted that, whereas the data presented in Figures 1 and 2 was obtained with commercial calf thymus DNA having a molecular weight of about 10 million Daltons (Worthington Biochemical), indistinguishable results were observed for *B. subtilis* DNA of 30 million Daltons. Neither DNA molecular weight nor base composition strongly affects the partition coefficients observed in this system.

Separation of Fractionated DNA from Phase-System Polymers. Density gradient centrifugation in an analytical ultracentrifuge can be used to examine the DNA partitioned into the top phase by directly adding the proper amount of solid CsCl and centrifuging the resulting solution to equilibrium. Such centrifugation can also serve as a preparative method for removing the polyethylene glycol. This procedure is made possible by the fact that the vast majority of the polyethylene glycol present is salted out and floats harmlessly to the top of the liquid column as a thin immiscible layer. Since very little DNA travels with this layer, one would expect that the molecular species detected in the gradient accurately represent the DNA actually present in the top phase. As a direct test, equilibrium banding patterns were obtained for identical mixtures of native, denatured, and renatured DNAs, with and without premixing with a blank top phase (approximately 6% polyethylene glycol and 0.3% dextran). The results shown in Figure 3 demonstrate that each density species is retained despite the presence of the phase-system polymers from the top phase. In fact, bands representing molecules of denatured or partially denatured conformation show a net gain. This gain is attributed to a reduction in the partial precipitation of denatured DNA otherwise observed in CsCl gradients (Subirana, 1965; Kohn *et al.*, 1966).

It is not possible to examine the DNA which partitions into the bottom phase by directly adding CsCl, since the dextran does not salt out and seriously interferes with the gradient. However, addition of sufficient NaOH to the sodium phosphate buffer to bring the pH to 10 changes the ionic composition and thereby allows any DNA present in the dextran layer to be reextracted nearly quantitatively into a new top phase (see Albertsson, 1965). Gradients prepared after such a reextraction show fractionated bottom-phase DNA to have a density

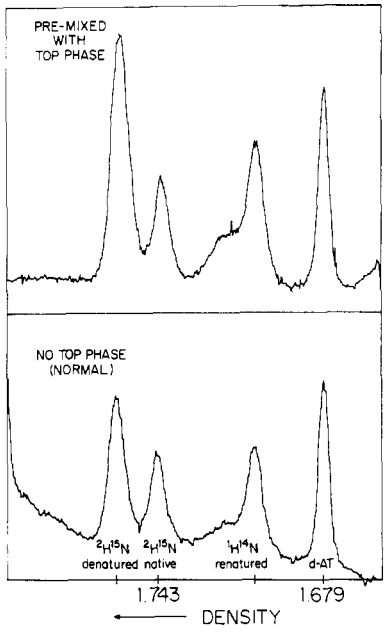

FIGURE 3: The effect of a blank polyethylene glycol rich top phase on the banding of DNA in a CsCl density gradient. Equal aliquots (0.12 ml) of a mixture of $^2H^{15}N$ native, $^2H^{15}N$ denatured, and $^1H^{14}N$ renatured *B. subtilis* DNAs (plus a synthetic d(AT) reference standard) were added to 0.54 ml of 0.01 M sodium phosphate (pH 6.8) and to 0.54 ml of a blank top phase. Solid CsCl (Harshaw optical grade) was then added to a refractive index of 1.4010, and both density gradients were established simultaneously in a Spinco Model E ultracentrifuge with untraviolet optics (44,770 rpm and 25.0°). The actual tracings of films taken at equilibrium are shown, with an identical exposure time and Joyce-Loebl optical wedge used for both gradients. A total of 1 μg of $^2H^{15}N$ native and 2 μg of $^2H^{15}N$ denatured DNA had been present in the initial 0.12-ml aliquot.

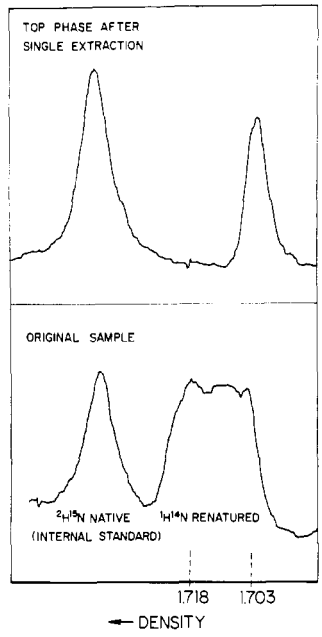

FIGURE 4: Fractionation of a heterogeneous mixture of renatured DNA molecules as judged by buoyant density distribution in a CsCl density gradient. Heat-denatured $^1H^{14}N$ wild-type *B. subtilis* DNA at 20 μg/ml was renatured by annealing for 90 min at 68° in 0.3 M NaCl plus 0.03 M sodium citrate (pH 7). After extensive dialysis into 0.01 M sodium phosphate (pH 6.8) an aliquot of the sample was partitioned in the phase system and the top phase was examined by equilibrium sedimentation in a CsCl density gradient. Densitometer tracings of the film record are shown and compared with identical tracings for the dialyzed unfractionated sample. The densities of fully native and denatured DNA are 1.703 and 1.718 g/cc, respectively; the partition coefficients of these DNAs in the phase system used are 10 and < 0.001, respectively (see Figure 1). As an internal standard, $^2H^{15}N$ native *B. subtilis* (168 trp_2^-) DNA was added prior to the phase extraction. By reference to this standard peak, it was determined that 28% of the DNA in the original renatured sample had partitioned into the top phase.

and apparent band width unchanged from that of the original single-stranded material.

It is frequently desirable to free DNA in the top phase from the polyethylene glycol by methods other than density gradient centrifugation. This may be accomplished by adding solid potassium phosphate (molar ratio dibasic to monobasic of 2:1) to a concentration of 23%. After centrifugation at 4°, the small liquid top layer is discarded and the DNA solution is extensively dialyzed to remove salt. In this manner the absorbance at 260 mμ of a blank top phase could be reduced from the normal value of 0.300 to less than 0.020, while the transforming activity of *B. subtilis* DNA present was unaffected. Addition of potassium phosphate to higher concentrations must be avoided, since this causes the polyethylene glycol to form a fine precipitate which is difficult to separate cleanly from the DNA. Albertsson has recently reported that polyethylene glycol may likewise be removed from DNA in the top phase by chloroform extraction (Rudin and Albertsson, 1967).

The Partition Behavior of Renatured DNA Mixtures. The same phase systems optimal for separation of single- from double-stranded DNA may also be advantageously employed for removing partially single-stranded DNA molecules, created during DNA renaturation (Doty *et al.*, 1960), from the more perfectly renatured, native-like molecules simultaneously present. In the experiment whose results are shown in Figure 4, a very heterogeneous renatured sample of *B. subtilis* DNA was partitioned in the dextran–polyethylene glycol system, and the top phase was then examined in a CsCl gradient. The tracings reveal that a special renatured DNA fraction with a buoyant density indis-

tinguishable from that of normal native DNA was extracted into the top phase during this fractionation procedure. This top-phase fraction, while constituting only 28% of the mass of the renatured DNA sample, contained nine times as much trp_2^+-transforming activity as the renatured DNA in the bottom phase.

The behavior of a renatured sample of *Escherichia coli* DNA during countercurrent distribution in a similar dextran–polyethylene glycol phase system has been studied by Rudin (1967). Renaturation was accomplished by annealing at 250 μg/ml, rather than the 20 μg/ml employed here. The resulting material was therefore extensively aggregated, and 90% was lost at the interface. Nevertheless, in agreement with Figure 4, a substantial proportion of the nonaggregated renatured molecules showed a native-like behavior in the phase system.

Isolation of Cross-Linked DNA Molecules. The above dextran–polyethylene glycol two-phase system has been used to separate in a single step a small fraction of cross-linked, reversibly denaturable DNA molecules from the single-stranded products predominating in normal denatured DNA samples (Alberts, 1965; B. Alberts and P. Doty, 1967, in preparation). This separation is made possible by the fact that a cross-linked molecule is unique in retaining a partition coefficient characteristic of native DNA after exposure to denaturing conditions. Cross-linked molecules remain fully reversibly denaturable after their purification, and it can thereby be inferred that no appreciable double- or single-stranded breaks are introduced into DNA during the fractionation procedure. The additional observation that the transforming activity of biologically active *B. subtilis* DNA is recovered in high yield (>80%) further demonstrates that there are no deleterious effects on partitioned DNA molecules.

Discussion

The dextran–polyethylene glycol two-phase system provides a powerful yet simple method for separating DNAs differing in their extent of double-helical structure. The concentration of the phase-system polymers and the ionic environment both have a profound effect on the partition coefficient of DNA in the system, but routine care to standardize these parameters suffices to ensure reproducible fractionations. Prior to a given application, it is recommended that a concentrated phase-system stock, consisting of approximately 17% dextran and 9% polyethylene glycol, be added in increasing proportions to separate solutions of native and denatured DNA in sodium phosphate buffer. Trial extractions are then performed. If both DNAs favor the bottom phase, even at low proportions of phase-system stock, then partition coefficients are raised by elevating the pH to change the distribution of phosphate anions. In the converse situation, either the pH is decreased or a low concentration of KCl is added. The optimal conditions determined in such a calibration will remain valid for all subsequent work with the particular phase-system stock tested.

In some cases, it may be advantageous to attempt fractionations in the presence of extraneous salts, such as $MgCl_2$, in order to avoid lengthy dialyses of the DNA samples to be analyzed. In this event, trial extractions should be carried out with the exact ionic environment to be used. Partition coefficients, if unsatisfactory, may often be adjusted to desired values by further addition of an appropriate ionic species (Albertsson, 1965; Öberg *et al.*, 1965).

A point of theoretical interest arising from this study is the unexpected strong change in *sign* of the partition coefficient for native DNA as the concentration of phase-system polymers is increased by serial additions of phase-system stock (Figure 1). With such additions, the composition of the original two phases becomes more extreme, the bottom phase becoming richer in dextran and the top phase richer in polyethylene glycol. Therefore, one would expect partition coefficients to increase for substances with $K > 1.0$ and decrease for substances with $K < 1.0$, as in fact is observed for the distribution of proteins in this system (Albertsson, 1960).

The unknown interactions which determine the behavior of DNA in the two-phase system might be revealed by sensitive physical studies of concentrated solutions of dextran and of polyethylene glycol, as both the concentration and the ionic composition are varied. However, it should be realized that partition coefficients of large polymers are inherently very sensitive to extremely small effects. For example, a partition coefficient of 10^4 would reflect a difference in the chemical potential of a DNA molecule of 5100 cal/mole in favor of the top phase. Even for relatively "small" DNA molecules of 2×10^6 Daltons, this is less than 1 cal/*mole of nucleotide residues*. Thus, a total energy change of only 2 cal/*mole of nucleotide residues* is more than sufficient to explain the entire range of shifts observed in Figure 1. Indeed, the sensitivity with which DNA reacts to subtle changes in the quality of the polymeric phases suggests the intriguing possibility that similar phenomena might underlie observed changes in the distribution and degree of condensation of the genetic material within the living cell.

Acknowledgments

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References

Alberts, B. M. (1965), Ph.D. Dissertation, Harvard University, Cambridge, Mass.

Alberts, B. M. (1967), *Methods Enzymol. 12A* (in press).

Albertsson, P. A. (1960), Partition of Cell Particles and Macromolecules, New York, N. Y., Wiley.

Albertsson, P. A. (1962), *Arch. Biochem. Biophys.*,

Suppl. 1, 264.
Albertsson, P. A. (1965), *Biochim. Biophys. Acta 103*, 1.
Anagnostopoulos, C., and Spizizen, J. (1961), *J. Bacteriol. 81*, 741.
Doty, P., Marmur, J., Eigner, J., and Schildkraut, C. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 461.
Kohn, K. W., Spears, C. L., and Doty, P. (1966), *J. Mol. Biol. 19*, 266.
Meselson, M., Stahl, F. W., and Vinograd, J. (1957), *Proc. Natl. Acad. Sci. U. S. 43*, 581.
Öberg, B., Albertsson, P. A., and Philipson, L. (1965), *Biochim. Biophys. Acta 108*, 173.
Rudin, L. (1967), *Biochim. Biophys. Acta 134*, 199.
Rudin, L., and Albertsson, P. A. (1967), *Biochim. Biophys. Acta 134*, 37.
Subirana, J. A. (1965), *Biochim. Biophys. Acta 103*, 13.
Yoshikawa, H., and Sueoka, N. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 806.

Nucleic Acid Synthesis during the Hormone-Stimulated Growth of Excised Oat Coleoptiles*

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ABSTRACT: The effect of the plant hormone, indoleacetic acid, on nucleic acid synthesis during the hormone-stimulated elongation of oat coleoptile sections was studied by incubating the sections with and without the hormone in the presence of $H_3{}^{32}PO_4$ for 6 hr, following which time the total nucleic acid complement of the sections was extracted and fractionated on MAK columns, and the extent of isotope incorporation into the various nucleic acid species was determined. The contribution to the radioactivity profiles made by contaminating bacteria in the incubation media was visualized and then made negligible by the inclusion of gramicidin in the medium. The hormone was found to stimulate twofold the incorporation of isotope into soluble ribonucleic acid and ribosomal ribonucleic acid, although there was no net increase in either species of nucleic acid during incubation. The hormone stimulated fourfold the isotope incorporation into a ribonucleic acid fraction that is tenaciously bound to methylated albumin kieselguhr columns and not elutable with a salt gradient at neutral pH. In addition, the base composition of this tenaciously bound ribonucleic acid from hormone-treated tissue was found to resemble that of oat deoxyribonucleic acid when the composition was based on the radioactivity of the ribonucleotides.

Many reports have linked mammalian hormone action with DNA-directed RNA synthesis. More recently the action of several plant hormones has been related to RNA synthesis. The effects of IAA,[1] 2,4-D, and gibberellic acid on excised plant tissues have been shown to be actinomycin D sensitive (Nooden and Thimann, 1963; Venis, 1964; Hamilton *et al.*, 1965; Roychoudhury and Sen, 1964; Key and Shannon, 1964; Chandra and Varner, 1965). In several of these studies an enhanced incorporation of radioactive precursors into RNA has been shown to accompany the hormone-stimulated growth of these tissues (Key and Shannon, 1964; Roychoudhury and Sen, 1964; Hamilton *et al.*, 1965).

In this report we present data that show an enhanced incorporation of radioactive precursors into RNA in excised oat coleoptiles incubated with the hormone IAA at a concentration that stimulates markedly the elongation of this excised tissue. This corroborates the findings of Hamilton *et al.* (1965), who have also reported such an enhancement in this tissue. In addition, we have attempted to effect a quantitative extraction of the tissue nucleic acid, to characterize by MAK chromatography the species of RNA whose synthesis is stimulated by the hormone, and to characterize the artifactual contribution by contaminating bacterial nucleic acid synthesis to the radioactivity profiles of the tissue nucleic acids.

Materials and Methods

Preparation and Incubation of Tissue. Seeds of *Avena sativa* L. (Svalöf's Original Victory I: Allmänna Svenska

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[1] Abbreviations used: IAA, indoleacetic acid; TB-RNA, tenaciously bound RNA; 2,4-D, 2,4-dichlorophenoxyacetic acid; SDS, sodium dodecyl sulfate; MAK, methylated albumin kieselguhr; SSC, standard saline citrate (0.015 M NaCl and 0.0015 M trisodium citrate, pH 7); AMP, CMP, GMP, TMP, and UMP, adenosine, cytosine, guanosine, thymidine, and uridine monophosphates.