Isolation of λdg Deoxyribonucleic Acid Halves by Hg(II) Binding and Cs_2SO_4 Density-Gradient Centrifugation*

James C. Wang, Uma Shankar Nandi,† David S. Hogness, and Norman Davidson

ABSTRACT: The application of the Hg(II)-Cs_2SO_4 density-gradient centrifugation technique in the isolation of the λdg deoxyribonucleic acid (DNA) half-molecules is reported. At a molar ratio of Hg(II) to total DNA phosphate of 0.4, the buoyant densities of the i^{λ}-containing half and the gal^{+}-containing half in Cs_2SO_4 differ by 0.04 g/ml, and the two halves can be separated by preparative density-gradient centrifugation.

The biological activities of the half-molecules are retained after addition and subsequent removal of mercury ion. If aggregates containing the two DNA halves are present, a trimodal buoyant pattern is observed with the aggregates being the middle band.

The breakage of λdg DNA molecules into halves and the separation of the half-molecules by an MAK column have been reported by Hogness and Simmons (1964). From the buoyant densities in CsCl of the isolated DNA halves, GC[1] contents of the gal^{+}-containing half and the i^{λ}-containing half were estimated to be 52 and 46%, respectively. Our group has recently reported a new technique for the fractionation of DNA's based on differences in complexing affinity for Hg^{2+} ions by DNA's of different GC content (Davidson *et al.*, 1965; Nandi *et al.*, 1965). The half-molecules of λdg DNA provide an interesting system with which to study the effectiveness of the Hg(II)-Cs_2SO_4 separation method.

Experimental

Materials. The procedures used in the isolation and breakage of the λdg DNA are identical with those reported by Hogness and Simmons (1964) except that the solvent used during breakage was 0.001 M EDTA, 0.01 M Tris-HCl, *p*H 6.7. The size distribution of the sample used for most of our experiments was examined by electron microscopy by Dr. Ross Inman at Stanford University. The maximum of the mass distribution as a function of length occurred at 6.5 μ, which is about one-half the maximum of the distribution for whole molecules (13.5 μ). Approximately 40% by weight of the sample of halves has a length which falls in the range 6–7 μ, with over 90% included within 3–9 μ.

For most of the experiments reported here the DNA was dialyzed into a 0.10 M Na_2SO_4, 0.005 M $Na_2B_4O_7$, *p*H 9, medium by the Stanford group and shipped to Pasadena frozen in solid carbon dioxide. The DNA concentration corresponded to $A_{260} \approx 0.50$. The samples when received were melted, divided into smaller fractions, and refrozen. On remelting for use, each sample was further dialyzed against the same medium (because it was found that there was a small amount of a strong complexing agent for mercury, perhaps EDTA, still present). The remelted samples were stored at 4°. In some early experiments the samples were dialyzed into 0.01 M Na_2SO_4, 0.005 M sodium cacodylate, *p*H 7, by the Stanford group. (For centrifugation, however, the samples were all diluted into a Cs_2SO_4 solution containing 0.005 M $Na_2B_4O_7$, *p*H 9.) It is essential that the solution be free of complexing agents for Hg^{2+} such as EDTA. Chloride ion at concentrations of the order of 0.1 M or Tris buffer at concentrations of the order of 0.001 M do not reverse the Hg(II) binding reaction by DNA at *p*H 9.

Centrifuge Procedure. Analytical and preparative ultracentrifugation experiments were done in the same way as reported previously (Davidson *et al.*, 1965; Nandi *et al.*, 1965). As mentioned above, the Cs_2SO_4 solutions contained 0.005 M $Na_2B_4O_7$, *p*H 9. In the Hg(II)-binding experiments in Cs_2SO_4 density-gradient centrifugation, DNA concentrations at an initial A_{260} of 0.1 were generally used. At lower DNA concentrations the parameter r_f, which is defined as the molar ratio of Hg(II) to DNA-P, is no longer a good approximation for r_b, the molar ratio of Hg(II) bound to DNA-P. It can be shown that, at a given r_b, the difference $r_f - r_b$ is inversely proportional to the total DNA concentration. At a DNA concentration with $A_{260} = 0.02$, the difference between r_f and r_b is estimated to be 0.2 at $r_b = 0.4$ for a DNA with 50% GC.

Other Methods. Melting profiles of the isolated

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[1] Abbreviations used in this work: G, guanosine; C, cytidine; A, adenosine; T, thymidine; *i*, gal^{+}, genetic markers relating to the immunity specificity and galactose-metabolizing characteristics of the virus.

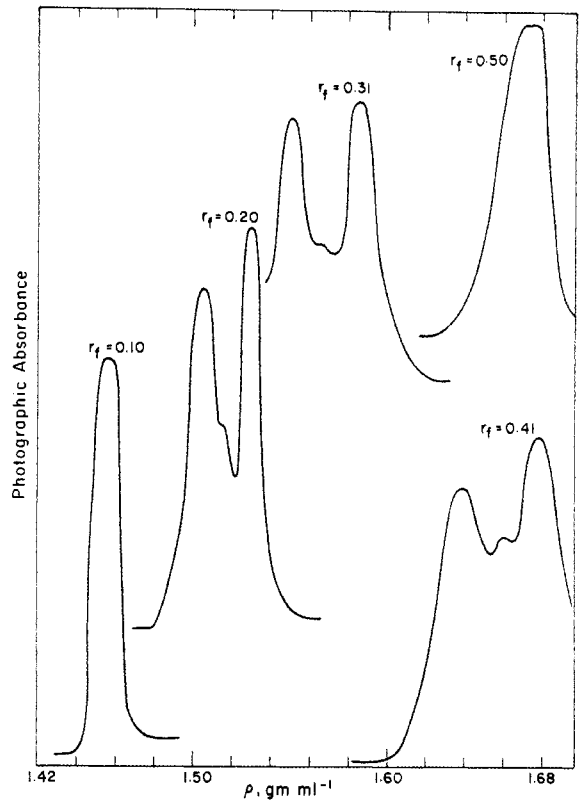

FIGURE 1: Microphotometer trace of distribution of the Hg(II) complexes of λdg DNA halves in Cs_2SO_4 density gradient. DNA concentration: A_{260} = 0.15 before banding. Centrifugation was for 16–18 hours at 44,770 rpm and 25°. The vertical positions for zero photographic absorbance have been arbitrarily displaced for clarity.

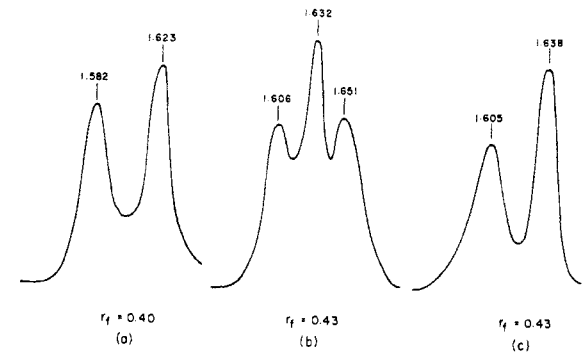

FIGURE 2: Microdensitometer tracings showing the formation of a trimodal banding pattern for sheared λdg DNA after storage. Ultraviolet photographs were taken after 16 hours of Cs_2SO_4 density gradient centrifugation at 44,770 rpm and 25°. (a) Freshly sheared DNA halves, initial DNA concentration at an A_{260} of 0.12, r_f = 0.40; (b) after two months storage at 4° (the DNA stock solution as stored was in 0.01 M Na_2SO_4, 5×10^{-4} M cacodylate buffer, *p*H 7, and A_{260} = 0.5), initial DNA concentration at an A_{260} of 0.19, r_f = 0.43; (c) the stock solution from (b) was heated to 64° for 10 minutes and quickly chilled in ice water before adding Cs_2SO_4 solution and $HgCl_2$ to r_f = 0.43. Numbers indicate buoyant densities in g/ml.

DNA halves were obtained with a heating cell for a Cary Model 14 spectrophotometer designed by Mr. J. Wetmur. The DNA halves isolated from a preparative run as well as an unseparated sample as a control were dialyzed against four changes of 0.1 M EDTA, *p*H 8, and then four changes of 0.01 M NaCl, 10^{-4} M Tris buffer, *p*H 8. The volume ratio of inner-to-outer solutions was 100. The final DNA solutions had A_{260} values of approximately 0.15. Thus a 0–0.1 absorbance unit slide wire for the Cary Model 14 was used. The hyperchromic increase at 260 mμ for the three samples was approximately 40%. Due to ultraviolet absorbing contaminants from the dialysis bag, the exact per cent increase in hyperchromicity could not be determined at this level of dilution.

Biological assays for the activities of i^λ and gal^+ genes were performed according to method B of Hogness and Simmons (1964). Fractions obtained from a preparative run were diluted fivefold into 0.1 M NaCl, 0.01 M Tris-HCl, *p*H 7.1, and dialyzed against this diluent. The dialysate was then changed to 0.01 M Tris-HCl, 0.001 M EDTA, *p*H 7.1. This is dialysis procedure D1. These samples were then assayed after appropriate dilution of at least 20-fold in 0.01 M Tris-HCl, *p*H 7.1, 0.01 M $CaCl_2$, 0.01 M $MgSO_4$ (solvent 1). Similar results were obtained if fractions were simply diluted twofold into 1.0 M NaCl, 0.01 M Tris-HCl, *p*H 7.1 (procedure D2) and then assayed directly after appropriate dilution of at least 50-fold in solvent 1. It is believed that at *p*H 7.1 and low DNA concentration the chloride ion concentration in the assay medium is sufficiently high to complex all the Hg(II) ions away from the DNA.

Results and Discussion

Distribution of the Hg(II) *Complexes of λdg DNA Halves in a* Cs_2SO_4 *Density Gradient.* Density-gradient centrifugation results obtained with a series of samples of Hg(II)-λdg DNA halves are displayed in Figure 1. In the range r_f = 0.2–0.4, the distribution of macromolecules in the density gradient is essentially bimodal.

Since the AT content of the i^λ half DNA is 6 mole % higher than that of the gal^+ half, it would be expected that the former would bind Hg(II) more strongly than the latter, and therefore would have a higher buoyant density in Cs_2SO_4. As r_f approaches 0.5, however, the two bands reconverge because the complexing of any DNA by Hg(II) is much weaker for $r_b > 0.5$; thus r_b of the denser band would not increase beyond 0.5 before that of the lighter band reaches 0.5.

It is to be noted that, for samples with r_f = 0.2–0.4, there appeared to be a significant amount of material of intermediate buoyant density. The amount of the

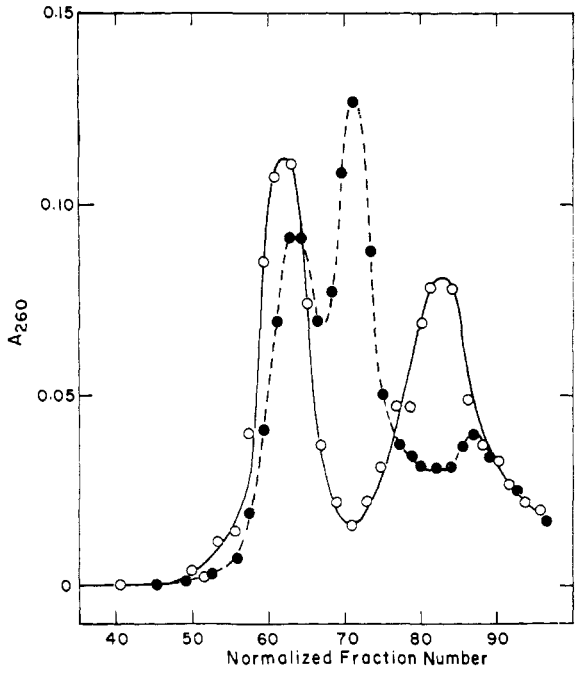

FIGURE 3: Cs_2SO_4 density gradient dripping patterns of two samples of identical composition. (○), DNA solution thermally disaggregated prior to the addition of Hg(II) and Cs_2SO_4. Initial density = 1.651 g/ml. (●), DNA solution not disaggregated. Initial density = 1.649 g/ml. Solution for each sample (2 ml), r_f = 0.35, A_{260} = 0.16, 47 hours at 33,000 rpm, and 25°. DNA recovery: 90%.

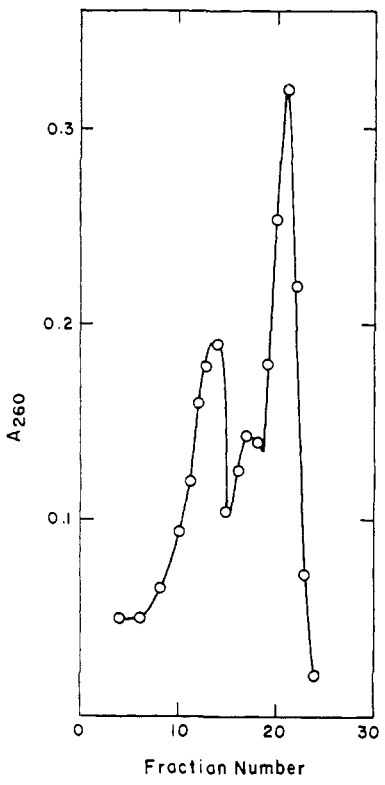

FIGURE 4: Dripping pattern of a preparative Cs_2SO_4 density-gradient run. Solution (2 ml), A_{260} = 0.39 (initial), r_f = 0.4, density of initial solution = 1.616 g/ml, 60 hours at 31,000 rpm, 25°. Each fraction = 2 drops of solution plus 0.2 ml of H_2O. DNA recovery = 95%.

middle band was very small for the freshly melted solution, and increased on standing at 4°. As much as 30-50% was observed for samples which had been stored at about 4° for a few weeks (Figures 2a and b) before adding the $HgCl_2$ and the Cs_2SO_4 and centrifuging.

It is shown below that the light and dense bands are gal^+ and i^λ halves, respectively. The intermediate band arises from a reversible aggregation between the two halves of the λdg molecule. Figure 2c shows that, if solutions of the DNA halves (in 0.01 M Na_2SO_4, 0.005 M borate buffer, *p*H 9) were heated at 64° for 10 minutes and then quickly chilled to 0° prior to the addition of Hg(II) and Cs_2SO_4 density-gradient centrifugation, then no middle band was observed in an analytical run. A similar result for a preparative run is shown in Figure 3. Furthermore, for four fractions of the middle band examined, the buoyant densities in CsCl were found to be 1.709 g/ml, the same as the value obtained when samples of unseparated λdg DNA halves were used. As already remarked, fresh samples (that is, directly after melting the stored frozen material) showed little or no middle band, and the amount of the middle band increased on standing at 4°. From the point of view of the effectiveness of the separation procedure it is important to note that the aggregation is due to storage of the DNA and is not due to cross-linking by Hg^{2+} ions.

We believe that the middle band is a 1:1 aggregate of the two halves. In many analytical runs the light and dense bands were symmetrical and of equal height, as in Figure 2b. Sometimes less symmetrical trimodal distributions were observed (Figure 5).

Isolation of the DNA Halves. The dripping pattern of a preparative ultracentrifugation run is shown in Figure 4. Fractions 11, 12, 13, and 14 were pooled (solution A) and so were fractions number 20, 21, and 22 (solution B). The buoyant density of the former in CsCl was determined to be 1.707 g/ml [the buoyant density of the marker *Micrococcus lysodeikticus* DNA was taken as 1.731 g/ml (Schildkraut *et al.*, (1962)], and that of the latter was determined to be 1.712 g/ml. These values are in agreement with data reported by Hogness and Simmons for the i^λ-half DNA and the gal^+-half DNA, respectively.

This suggests that for the more or less bimodal pattern shown in Figure 1 and the multimodal banding patterns shown in Figures 2 and 3, the band of lowest buoyant density in each sample represents the gal^+ half while that of the highest buoyant density represents the i^λ half. This is confirmed by the biological assays as reported below. The melting temperatures of the DNA's also agree with this assignment. In a medium

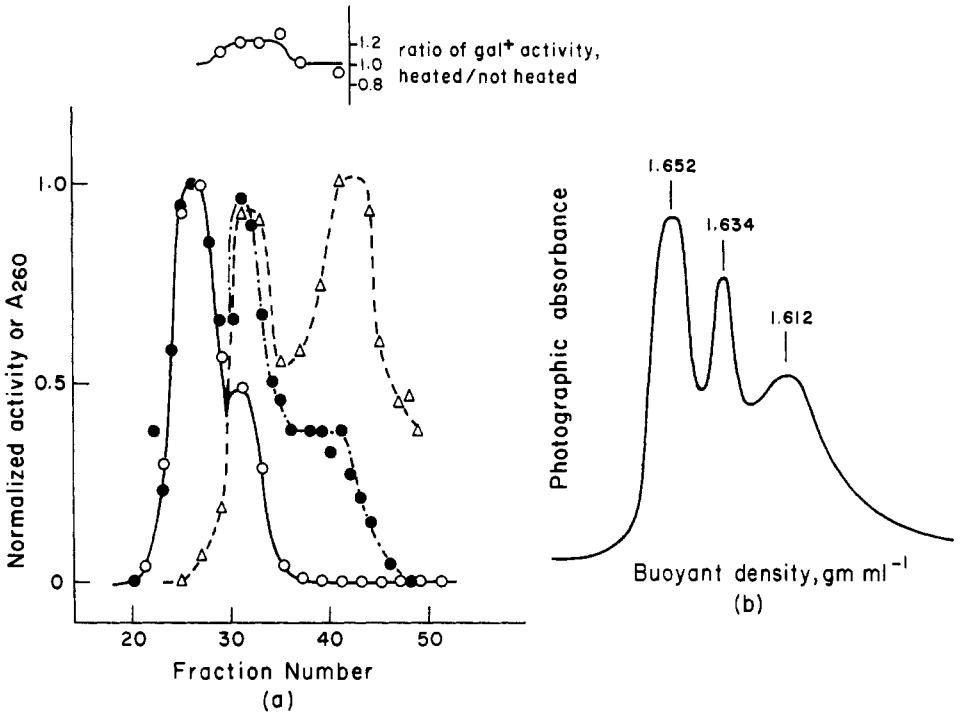

FIGURE 5: *Gal*$^+$ and i^λ activities of fractions from a preparative run. Sample dripped after 45 hours at 31,000 rpm. (○), i^λ activity; (△), *gal*$^+$ activity; (●), A_{260}. The effect of heating the DNA in 0.01 M Tris-HCl, 0.001 M EDTA, *p*H 7.1, at 65° for 10 minutes on the *gal*$^+$ activity is shown in the figure. An analytical run on an aliquot of the same solution is shown in (b).

of 0.01 M NaCl and 10^{-4} M Tris-HCl, *p*H 8, the DNA obtained from solution A had a melting temperature of 68.0° and that from solution B had a melting temperature of 70.6°. The unseparated halves showed a melting temperature of 69.4°. From the dependence of melting temperatures on GC content it is estimated that the two DNA's differ in GC content by 5–6 mole% (Dove and Davidson, 1962; Marmur and Doty, 1959).

Biological Assays. These experiments were done before the method of eliminating the middle band was known and with preparations which showed a significant amount of middle band. Two series of experiments were done (I and II), but only II will be described in detail. Both experiments indicate quite clearly that the dense band carries exclusively the i^λ marker and the light band exclusive the *gal*$^+$ marker. The nature of the middle band is less certain.

The question of possible biological inactivation of the DNA by the Hg^{2+}, Cs_2SO_4 medium was examined. The controls studied as a part of experiment I provide the best evidence on this point.

The sheared λdg was originally in a medium of 0.001 M EDTA, 0.01 M Tris-HCl, *p*H 6.7. This is solution C1. Some of C1 is dialyzed in 0.01 M Na_2SO_4, 0.001 M sodium cacodylate, *p*H 7, by the Stanford group and shipped to Pasadena. This DNA solution was made 1.7 M in Cs_2SO_4 (ρ = 1.50) and 0.005 M in $Na_2B_4O_7$ (*p*H 9), with DNA-P = 2.9×10^{-5} M. This is solution C2. To a portion of C2, $HgCl_2$ was added to r_f = 0.40. This is C3. The relative specific activities in C1, C2, and C3 for the *gal*$^+$ marker were: 1.00, 0.54, and 0.58; for i^λ, 1.00, 0.56, and 0.61. Thus there was some inactivation in preparing the Cs_2SO_4 solution, but no additional inactivation by the addition of $HgCl_2$. We suspect that, under ideal conditions, there will be no inactivation due to Cs_2SO_4 or any of the other reagents used.

Figure 5 displays the results of biological assays of the fractions obtained from a preparative density gradient fractionation (experiment II). It is clear from Figure 5 that there is a fractionation of the i^λ and *gal*$^+$ activities. The left-hand peak consists of almost purely the i^λ-half DNA and the right-hand peak almost purely the *gal*$^+$-half DNA. The middle peak, however, contains both the i^λ activity and the *gal*$^+$ activity.

The distribution of *gal*$^+$ activity in Figure 5 is obviously bimodal. This is not the case for i^λ activity. It appears to peak in the dense band and to tail over into the middle band. Nevertheless we have drawn a curve through the i^λ activity points in such a way as to suggest a bimodal distribution. Our reasons for taking this point of view are explained below.

An alternative hypothesis is that the dense band is i^λ DNA and both the middle and light bands are essentially *gal*$^+$ DNA, with some tailing of the i^λ activity into the middle band because of poor resolution.

In the preceding paper, we report that pure, homogeneous dAT does give two bands in Hg^{2+}, Cs_2SO_4 due

TABLE I: Specific *Gal*$^+$ Activities of Light and Middle Bands.

	Fraction No.								
	45	44	41	39	37	35	33	31	29
g = normalized *gal*$^+$ activity[a]	0.60	0.93	1.00	0.74	0.57	0.73	1.09	1.10	0.20
D = normalized A_{260}[b]		0.15	0.38	0.38	0.38	0.47	0.67	0.96	0.65
(g/D)		~6	2.62	2.06	1.50	1.55	1.63	1.13	0.31

[a] For DI samples heated to 65° and cooled, then diluted into solvent 1. [b] A_{260} = 0.024 for fraction 31.

to kinetic reasons, if the solution is not carefully stirred. Furthermore, it is remarked there that it is theoretically possible for a homogeneous DNA to give a bimodal distribution because of a disproportionation reaction in the centrifugal field. Neither of these phenomena are responsible for the bimodal *gal*$^+$ distribution. This was shown by an experiment in which the isolated *gal*$^+$-half DNA from the light band was readjusted to r_f values of 0.3, 0.35, 0.4, and 0.45 and rebanded in Cs_2SO_4. None of the samples exhibited a bimodal distribution. Furthermore, λ DNA has the same base composition as *E. coli* DNA and should behave similarly. It was shown in the previous paper that kinetic effects are not important for the addition of Hg^{2+} to a mixture of *E. coli* and T4 DNA's, but that the Hg^{2+} comes to its equilibrium distribution. The same result is expected for the λ DNA halves.

It is apparent on inspection of Figure 5 that the specific *gal*$^+$ activity (relative transforming activity divided by A_{260}) is higher in the light band than in the middle band. The quantitative data are presented in Table I. They are not very accurate, probably because of the low A_{260} values and a high background, but they indicate that the specific *gal*$^+$ activity of the middle band is about one-half of that of the light band. If one-half of the DNA of the middle band is *gal*$^+$, then one-half is i^λ DNA. The A_{260} values of the middle and heavy bands are equal. The i^λ activity of fraction 31 (middle band) is indeed one-half that of fraction 27 (dense band). Thus the results are consistent with the interpretation that the middle band is an approximately 1:1 aggregate of the two halves formed upon standing at 4° in 0.01 M Na_2SO_4, 0.005 M $Na_2B_4O_7$ for several weeks. The reason why the i^λ activity is not itself more clearly bimodal may be that the maximum of the middle band lies closer to the i^λ peak than to the *gal*$^+$ peak.

It is plausible to assume that two half-molecules of such an aggregate are held together by the cohesive ends which are responsible for the formation of the closed or circular monomers of whole λ DNA (Hershey *et al.*, 1963; Hershey and Burgi, 1965; Kaiser and Inman, 1965). It is known that the λdg DNA molecules used here contain such cohesive ends. Thus after standing for several days at 5° in 2 M ammonium acetate electron micrographs reveal that 72% of whole λdg DNA monomers are in the closed form (R. Inman, personal communication). These conditions have been shown to cause the two halves of λ DNA to join to each other in an end-to-end manner (Kaiser and Inman, 1965). Furthermore, the loss of the middle-band aggregate by heating to temperatures somewhat below the melting temperature of the DNA is consistent with the behavior of such end-to-end aggregates which are also destroyed by this type of heating (Hershey *et al.*, 1963; Kaiser and Inman, 1965).

If the middle-band aggregates are of the end-to-end type, one might expect their biological activity to be increased by heating. Thus storage of halves of λ DNA in 2 M ammonium acetate not only causes the formation of the end-to-end aggregates but also results in loss of as much as 70% of the biological activity (Kaiser and Inman, 1965). Heating destroys the aggregates and reverses the inactivation. The same heat-reversible inactivation of λdg halves has been observed, though its magnitude is generally only one-half that given above (D. S. Hogness, unpublished observations). Heating of the dialyzed fractions of Figure 5 to 65° for 10 minutes results in an increase in the *gal*$^+$ activity specific to the middle band which is barely significant (20%, Figure 5).

If it were clear that end-to-end aggregates are completely inactive this result would indicate that either the middle band does not consist of such aggregates or that such aggregates are broken apart during subsequent dialysis and assay. However, it is not clear that end-to-end aggregates are not active since the proportion of free halves in the preparations stored in 2 M ammonium acetate is not known. Consequently the end-to-end aggregates could be active, but less active than the free halves. Indeed, as pointed out by Kaiser and Inman (1965), certain end-to-end aggregates (e.g., those having lengths equal to or larger than whole DNA) might be active while the others were not. In this case the amount of inactivation could depend upon the distribution of sizes of the half molecules; the narrower the distribution, the less the degree of inactivation.

These uncertainties leave the nature of the middle-band aggregates unresolved. However we suspect that they are the usual end-to-end type which have been largely broken apart by subsequent treatment of the fractions.

It is to be recalled that denatured DNA binds Hg^{2+} more strongly than native. Indeed, at equilibrium at room temperature, the native DNA-mercury complex

may be metastable so that it should at equilibrium decompose into the denatured DNA-mercury complex. We propose that on adding mercury ion to the end-to-end aggregate mercury cross-links are formed between the two complementary single strands that are the cohesive ends. However, when this DNA is diluted into the high chloride ion medium used in the transformation assay the mercury is removed in two steps. First the end-to-end aggregate dissociates, leaving a mercury-denatured DNA complex for each single strand end; then the mercury is complexed away from each end leaving the dissociated half molecules.

Acknowledgments

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References

Davidson, N., Widholm, J., Nandi, U. S., Jensen, R., Olivera, B. M., and Wang, J. C. (1965), *Proc. Natl. Acad. Sci. U.S. 53*, 111.

Dove, W. F., and Davidson, N. (1962), *J. Mol. Biol. 5*, 467.

Hershey, A. D., and Burgi, E. (1965), *Proc. Natl. Acad. Sci. U.S. 53*, 325.

Hershey, A. D., Burgi, E., and Ingraham, L. (1963), *Proc. Natl. Acad. Sci. U.S. 49*, 748.

Hogness, D. S., and Simmons, J. R. (1964), *J. Mol. Biol. 9*, 411.

Kaiser, A. D., and Inman, R. B. (1965), *J. Mol. Biol.* (in press).

Marmur, J., and Doty, P. (1959), *Nature 183*, 1427.

Nandi, U. S., Wang, J. C., and Davidson, N. (1965), *Biochemistry 4*, 1687 (this issue; preceding paper).

Schildkraut, C. L., Marmur, J., and Doty P. (1962), *J. Mol. Biol. 4*, 430.

Characterization of the Polynucleotide-dependent Transfer Reaction in Protein Biosynthesis Employing a Cell-free System from the Yeast *Saccharomyces fragilis**

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ABSTRACT: The transfer of radioactive phenylalanine and lysine from soluble ribonucleic acid to peptide linkage in washed yeast ribosomes, in the presence of polyuridylic acid and polyadenylic acid, respectively, has been studied. For optimal activity, both reactions require guanosine-5′-triphosphate (2–4 mM), Mg^{2+} (6 mM), NH_4Cl (100 mM), spermine (1 mM), polynucleotide (100 μg/ml), aminoacyl-soluble ribonucleic acid, and ribosomes. A supernatant fraction was not required even with ribosomes that were washed with buffer for as many as five times. Ribosomes that were washed with 0.5 M NH_4Cl or 0.5% deoxycholate solutions, however, occasionally showed a significant stimulation by supernatant, but the total activity recovered in these preparations was 10% or less of that present prior to treatment. Observations presented in this report were obtained with buffer-washed ribosomes in the absence of supernatant. The transfer of both amino acids has a *p*H optimum of 7.0. Both reactions are inhibited by preincubation of the ribosomes with *p*-mercuribenzoate. Chloramphenicol, griseofulvin, amphotericin B, and mycostatin have little or no effect on either reaction; puromycin inhibits the incorporation of lysine and phenylalanine about 50%.

Studies by density-gradient sedimentation indicate that the radioactive polyphenylalanine is associated with a monosomal component. Thus far, no evidence has been obtained which would suggest that separate enzymes are involved in the transfer of phenylalanine and lysine.

The final stages in protein biosynthesis involve the transfer of amino acids from aminoacyl-s-RNA into polypeptides bound to ribosomes. In addition to ribosomes, a requirement for at least two soluble enzymes, guanosine triphosphate, messenger RNA, Mg^{2+}, K^+ or NH_4^+, and a sulfhydryl compound have been established in this reaction (Hülsmann and Lipmann, 1960; Nathans and Lipmann, 1960, 1961; Von Ehrenstein and Lipmann, 1961; Nathans *et al.*, 1962; Bishop and Schweet, 1961b; Fessenden and Moldave, 1961; Takanami, 1961; Allende *et al.*, 1962; Lamfrom and Squires, 1962; Nakamoto *et al.*, 1963; Arlinghaus *et al.*, 1963). The present paper describes the characteristics of the transfer reaction with phenylalanyl- and lysyl-s-RNA in the presence of poly-U and poly-A,

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