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*A NATURALLY OCCURRING DNA-RNA COMPLEX FROM NEUROSPORA CRASSA**

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Recent progress in the elucidation of the biological role of nucleic acids has led to the postulation of a mechanism for the transfer of information from DNA[1] to RNA. Reports from a few laboratories[2] have indicated that a hybrid molecule containing DNA and RNA may be an intermediate in this process. This report is concerned with the isolation and characterization of a naturally occurring DNA-RNA complex from the fungus *Neurospora crassa* which has some of the properties required of such an intermediate.

Materials and Methods.—Growth of Neurospora crassa strain 74A: Cultures were maintained on agar slants of minimal medium.[3] Freshly inoculated slants were incubated at 30°C for 5 days and then stored at 3–5°C. Experimental material was obtained by suspending the conidia from one agar slant in 10 ml of distilled water and adding this suspension to 1 liter of liquid minimal medium contained in a Fernbach flask. The flasks were then incubated at 30°C with vigorous agitation on a rotary shaker.

Preparation of extracts: When the desired physiological age of the culture was reached, hyphae were collected by filtering the contents of each flask through cheesecloth. The hyphae were washed on the filter with ice-cold distilled water and squeezed dry. The compact mass of tissue was then quickly frozen by submersion in liquid nitrogen. The frozen material was disrupted in a Hughes press,[4] precooled to about −20°C, by subjecting it to a pressure of 24,000 psi. The material which had been forced through the Hughes press was removed and suspended in buffer with the aid of a glass tissue homogenizer with a tight-fitting teflon pestle. These homogenized extracts were the starting material for the analyses based on differential centrifugation.

Analytical methods: DNA was measured by the Burton modification of the

Dische diphenylamine reaction,[5] RNA was measured by the Dische orcinol reaction,[6] and protein was determined by the method of Lowry.[7] DNA and RNA were also measured by their increase in absorption at 260 mμ after digestion with nucleases.

ECTEOLA cellulose chromatography: The ECTEOLA cellulose used was Calbiochem "Cellex-E" which had an exchange capacity of 0.39 milliequivalents per gram. It was exhaustively washed with 2 *M* NaCl followed by 1 *N* NaOH and finally with distilled water. It was packed in chromatographic columns under 30 in. of hydrostatic pressure to a final dimension of 1 × 24 cm. The packed columns were equilibrated with buffer (0.01 *M* Tris; pH 7.1 and 2.5×10^{-3} *M* $MgCl_2$) at 5°C. After application of a sample to the column, about 150 ml of buffer was passed through it. The columns were eluted with linear sodium chloride concentration gradients with a total volume of 500-600 ml and a flow rate of about 5 ml per hour.

Materials: Crystalline pancreatic DNase and RNase were obtained from the Worthington Biochemical Corporation. Their specificity was verified by using purified DNA and RNA as substrates.

Highly polymerized DNA was prepared from calf thymus glands and *Neurospora crassa*. Salmon sperm DNA was obtained from Calbiochem.

The CsCl was an optical grade supplied by the Harshaw Chemical Co.

The buffer used in all the experiments was 0.01 *M* Tris; pH 7 and 2.5×10^{-3} *M* $MgCl_2$.

Experimental Results.—Differential centrifugation was employed to study the distribution of RNA and DNA among the subcellular fractions obtained from extracts of dormant conidia, actively growing young hyphae (10 hr old), and older tissue (48 hr old) which is relatively inactive with respect to growth rate. The results of experiments in which 14 fractions were prepared and analyzed are summarized in Table 1. It can be seen that, with respect to nucleic acid content,

TABLE 1

DISTRIBUTION OF NUCLEIC ACIDS IN FRACTIONS OF *Neurospora crassa* AS PER CENT OF TOTAL EXTRACTED

	Fraction	Conidia		10 hr		48 hr	
		RNA	DNA	RNA	DNA	RNA	DNA
1	to 25,000 × *g*	10	51	8	50	7	53
2	to 65,000 × *g*	12	29	13	25	12	30
3	to 105,000 × *g*	56	4	50	10	61	2
4	Sol	23	17	30	15	21	14

All values represent averages of at least three determinations. The values were obtained by measuring the ribose and deoxyribose precipitated by 10 per cent trichloroacetic acid.

fractions 1, 2, and 4 have a relatively constant composition regardless of the physiological age of the starting material. Therefore, our attention was focused on fraction 3, that is, the material which was sedimented at 105,000 × *g* (the microsome fraction), because it showed an interesting direct correlation between DNA content and physiological activity. The data clearly show that while the microsome fractions from conidia and 48-hr-old tissue contain 4 and 2 per cent DNA, respectively, the same fraction from 10-hr-old hyphae may contain as much as 10 per cent of the total extractable DNA. The composition of these microsome fractions remains constant, even after repeated washing by suspension and recentrifugation.

Considering the procedures employed for the preparation of the microsome

fraction, an obvious question arises as to the possibility of these data resulting from some artifact introduced by the fractionation procedure. This may be true, for instance, if young hyphae contained much more DNA than either conidia or older tissue, thereby affording a greater opportunity for DNA to appear in the microsome fraction by some nonspecific adhesion. The gross composition of lyophilized conidia and 12-hr-old hyphae was determined and is expressed as per cent dry weight in Table 2. In contrast to the composition of the microsome fraction, a

TABLE 2

GROSS COMPOSITION OF *Neurospora crassa* AS PER CENT DRY WEIGHT

	DNA	RNA	Protein
Conidia	0.5	6.0	32.4
Hyphae	0.2	6.0	20.0

All values were derived from triplicate determinations of two samples each of lyophilized conidia and hyphae. The values were calculated from ribose, deoxyribose, and protein analyses of material precipitated by 10 per cent trichloroacetic acid.

total extract from conidia contains 2.5 times as much DNA as a total extract from 12-hr-old hyphae.

Another test of the significance of the DNA content of the microsome fraction with respect to nonspecific adhesion would be the effect of added, extraneous DNA on the DNA content of the fraction. DNA from *Neurospora*, calf thymus gland, and salmon sperm was added to crude extracts from 12-hr-old hyphae. The microsome fractions were obtained by centrifugation, washed twice with buffer, and analyzed for DNA content. The data presented in Table 3 show that the addition of DNA from these sources, including *Neurospora* DNA, had no measurable effect on the DNA content of the microsome fraction. These results suggested that the

TABLE 3

EFFECT OF ADDED DNA ON DNA CONTENT OF MICROSOMES FROM 12-HR HYPHAE

Addition	μg DNA in washed microsomes
None	130; 122
280 μg *Neurospora* DNA	127; 132
250 μg calf thymus DNA	130; 131
310 μg salmon sperm DNA	124; 135

The values were calculated from the deoxyribose content of 10 per cent trichloroacetic acid precipitates.

values obtained for the DNA content of the fraction did not arise as the result of a nonspecific adhesion of DNA to the microsomes during the course of their isolation.

The possibility that DNA was an integral component of the microsome fraction led to an examination of the physical state of the DNA in the particles. One approach to this problem was to determine whether or not this DNA was susceptible to digestion by DNase. Table 4 presents some data from experiments in which crystalline pancreatic DNase was added to various microsome suspensions. The effect of DNase treatment was determined by the appearance of acid-soluble deoxyribose and ultraviolet-absorbing material. As can be seen from the data in Table 4, in 40 min at 35°C, the microsomal DNA was completely digested (using the criterion of acid solubility) by the addition of 10 μg of crystalline pancreatic DNase to the particles. In addition to answering the question of the susceptibility of the DNA to DNase digestion, some other interesting information was obtained from these experiments. Incubation of the microsome fraction at 35°C in buffer

TABLE 4

DIGESTION WITH DNASE

Microsomes	DNase	Calf thymus-DNA	°C	μg acid sol. DNA	μg acid insol. DNA
+	—	—	0	0	63.2
+	—	—	35	19.8	47.5
+	+	—	35	65.1	0
—	—	+	35	0	81.4
—	+	+	35	78.3	0
+	—	+	35	72.6	73.1
+	+	+	35	142.7	0

All samples were incubated for 40 min. The DNase digested samples were incubated with 10 μg of DNase. The DNA values were calculated from the deoxyribose which was either soluble or insoluble in 10 per cent trichloroacetic acid.

without nuclease treatment results in the digestion of about $^1/_3$ of the DNA initially present in the particles. Moreover, if 8.4 μg of calf thymus DNA is added to the particles in the absence of added nuclease and the mixture is incubated at 35°C for 40 min, about $^2/_3$ of the added DNA is digested. These experiments show, first, that the particles contain an active nuclease capable of digesting both a portion of the DNA originally present in the fraction and DNA which is added to it and, second, that added DNA is more accessible to the action of this nuclease.

The particulate fraction sedimenting at 105,000 × *g* was designated microsomes because examination of this fraction in the electron microscope[8] revealed a typical microsome morphology—that is, membrane structures covered with granules the size of ribonucleoprotein particles (ribosomes).[9] This observation led to an examination of whether the microsomes would be dispersed by treatment with a detergent yielding the free ribosomes, as is the case with microsomes from mammalian tissue.[10] When the microsomes were treated with a final concentration of 0.5 per cent sodium deoxycholate, the resulting particulate material appeared as free ribosomes in the electron microscope. It was of interest to see how the DNA in the microsome fraction would be affected by such treatment. Table 5 presents the

TABLE 5

DEOXYCHOLATE TREATMENT

Final per cent deoxycholate	μg DNA in pellet	μg DNA in supernatant	μg RNA in pellet	μg RNA in supernatant
0	174.5	0	42,200	0
0.1	159.0	20.7	—	0
0.3	—	43.1	—	475
0.5	0	176.9	—	966

Aqueous 2 per cent sodium deoxycholate was added slowly to suspensions of washed microsomes with constant agitation. After 20 min in the cold, the samples were centrifuged at 105,000 × *g* for 180 min. The values for DNA and RNA were calculated from deoxyribose and ribose distributed between pellet and supernatant after the centrifugation.

data concerned with the distribution of DNA and RNA between pellet and supernatant after centrifugation at 105,000 × *g* for 180 min of a microsome suspension treated with various concentrations of sodium deoxycholate. A final concentration of sodium deoxycholate of 0.5 per cent renders soluble all of the DNA originally present in the microsomes as well as a portion of the RNA. Since deoxycholate treatment fractionates the microsome preparation into a particulate component of ribonucleoprotein particles and a soluble fraction containing both DNA and RNA, it was adopted as a step in the purification of the fraction for the purpose of its further characterization.

One of the requirements of an intermediate in the transfer of information from DNA to RNA is that it should contain newly synthesized RNA.[11] The adoption of the above purification procedure (deoxycholate treatment of the microsomes) made it technically feasible to determine the possible biological significance of the nucleic acid fraction in question by testing it for this requirement. Ten-hr-old hyphae were grown in the presence of radioactive phosphorus for 5 min, at which time the hyphal suspension was chilled and fractionated as previously described. The RNA was isolated from untreated microsomes and from the soluble and particulate fractions obtained by sodium deoxycholate treatment. Table 6 presents the specific activities of these RNA fractions. It shows that the deoxycholate-solubilized RNA fraction has a specific activity approximately eight times that of the particulate ribonucleoprotein fraction. We conclude from these data that the deoxycholate solubilized fraction does indeed contain newly synthesized RNA.

TABLE 6

DISTRIBUTION OF P^{32} IN MICROSOME FRACTIONS

	Specific activity
Untreated microsomes	3.5
Deoxycholate supernatant	24.6
Deoxycholate pellet	3.1

Ten-hr-old hyphae in 6 liters of medium were collected, washed, and incubated in 500 ml of a $^1/_{10}$ dilution of medium for 30 min with agitation at 30°C. Potassium phosphate, containing P^{32}, was added to the hyphal suspension and growth conditions were maintained for 5 min, at which time the culture was quickly chilled by the addition of crushed ice. The tissue was fractionated as described previously. Samples were prepared for analysis by precipitating the fractions with 10 per cent trichloroacetic acid, extracting lipids from the precipitates, and dissolving the precipitates in 1 *N* NaOH. After hydrolyzing the RNA at 37°C for 20 hr, the samples were acidified and centrifuged. The O. D. at 260 mμ of aliquots of the supernatants was determined and portions were dried on stainless steel planchets and radioactivity determined with a gas flow detector equipped with a micromil window (Nuclear Chicago Corporation).

Having established a possible biological role for this nucleic acid fraction, attention was focused on its further purification so as to enable us to examine some of its chemical and physical properties.

The deoxycholate-solubilized fraction was chromatographed on ECTEOLA cellulose columns.[12] Figure 1 shows a typical elution pattern employing a linear sodium chloride concentration gradient. A major symmetrical peak (fractions 31–42) which elutes at about 0.75 *M* NaCl contains over 90 per cent of the DNA and a fraction of the RNA applied to the column. Analysis of the fractions within this peak revealed that they contain deoxyribose and ribose in a molar proportion of about 1.5 to 1.0. If a portion of the eluant in this major peak is dialyzed to remove sodium chloride and rechromatographed on ECTEOLA cellulose under similar conditions, the result is an elution pattern which is depicted in Figure 2. A major symmetrical peak is eluted at approximately 0.75 *M* NaCl, the same salt concentration which eluted the material originally. It contains about 90 per cent of the ultraviolet absorbing material applied to the column. Therefore, with respect to chromatography on ECTEOLA cellulose, this purified fraction which contains both DNA and RNA exhibits a constant behavior and composition.

The original elution pattern (Fig. 1) shows a minor peak (fractions 15–23) eluted at about 0.3 *M* NaCl. This peak is composed almost entirely of protein with the property of being able to digest both DNA and RNA and is indifferent to the source of the nucleic acid it digests. This protein peak can be eliminated from the elution pattern by repeated extraction of the deoxycholate supernatant fraction

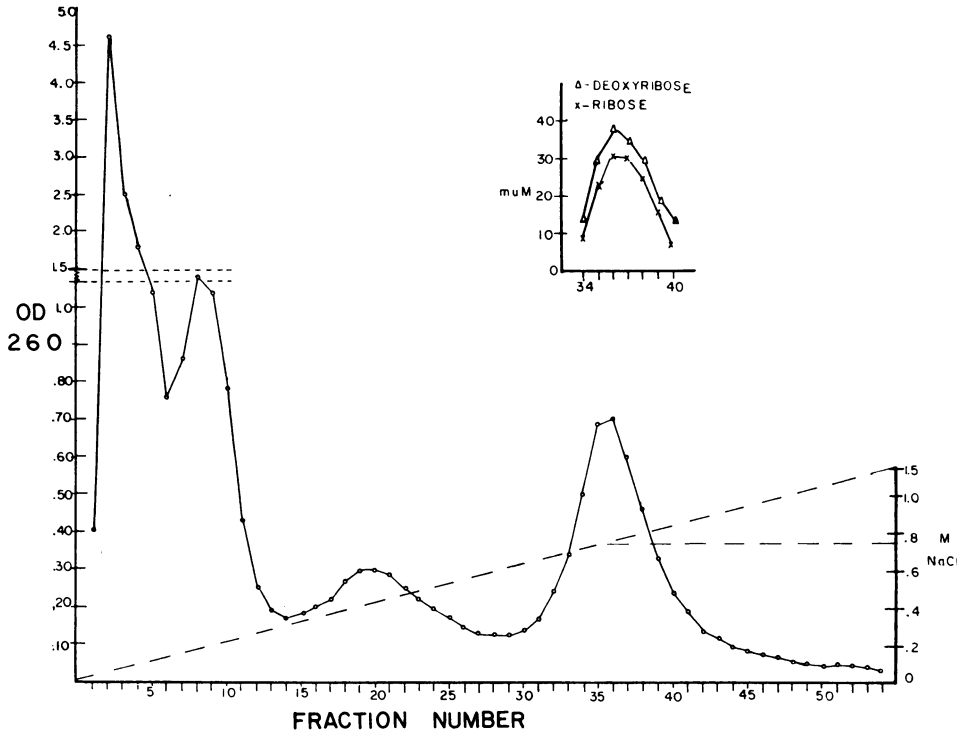

FIG. 1.—Elution of deoxycholate supernatant from ECTEOLA cellulose.

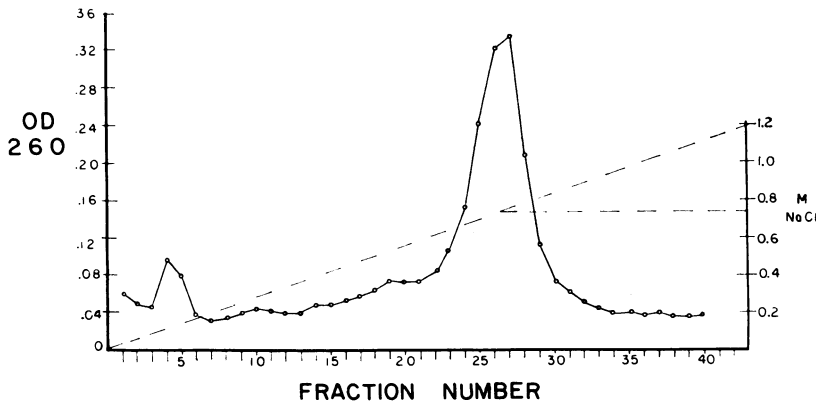

FIG. 2.—Rechromatography of dialyzed DNA-RNA containing fraction from Figure 1 on ECTEOLA cellulose.

with chloroform-octanol (10:1) before it is applied to the ECTEOLA cellulose column.

Evidence based on the digestion of the DNA-RNA fractions with crystalline pancreatic nucleases suggests that the DNA-RNA fraction prepared without chloroform-octanol extraction is partially degraded but nevertheless a complex of DNA and RNA. Table 7 shows the results of an experiment in which chromato-

TABLE 7

NUCLEASE DIGESTION OF PARTIALLY DEGRADED COMPLEX

	Per cent increase O. D. 260 mμ
Complex + DNase	31
Complex + RNase	29
Complex + DNase, RNase	31

graphically purified complex, prepared without chloroform-octanol extraction, was digested with crystalline pancreatic nucleases. The progress of the reactions was followed by measuring the increase in the optical density at 260 mμ of the reaction mixture. Digestion by DNase and RNase, either separately or simultaneously, resulted in the same increase in absorption. These data indicate that the nature of the complex is such that complete degradation, as measured by the resulting hyperchromicity, can be achieved by either DNase or RNase digestion. From the results of subsequent experiments with purified complex prepared from chloroform-octanol extracted deoxycholate supernatant, we concluded that the material just described was partially degraded complex.

If the previous experiment is repeated using chromatographically purified complex prepared from chloroform-octanol extracted deoxycholate supernatant, the effects of nuclease digestion are nearly additive as shown in Table 8, and therefore, this

TABLE 8

NUCLEASE DIGESTION OF UNDEGRADED COMPLEX

	Per cent increase O. D. 260 mμ
Complex + DNase	25
Complex + RNase	9
Complex + DNase, RNase	32

material we believe is undegraded complex.

The kinetics of digestion of undegraded complex with purified DNase and RNase (Fig. 3) show that when the untreated complex is digested with DNase, there is a lag of 6–8 min before the onset of an increase in optical density. However, if the complex is pretreated with RNase, this lag in the DNase digestion is eliminated. Since this is not observed with a control mixture of DNA and RNA, it is taken as evidence of some physical interaction between the DNA and RNA of the sample in question.

The specific base-pairing of a double-stranded helix of DNA may be disrupted by heat, and the progress of the helix-coil transition can be followed by measuring the hyperchromicity resulting from the denaturation while increasing the temperature of the sample.[13] We thought that information concerning the structure of the undegraded DNA-RNA complex from *Neurospora* might be obtained by determining its ability to undergo a helix-coil transition. Figure 4 shows the results of such an experiment, in which untreated complex underwent a sharp transition with a *Tm* of 60°C. RNase-treated material had a still sharper transition with a *Tm* elevated to 71°C, and treatment with DNase eliminated the sharp transition entirely. This behavior is evidence of a specific combination between the DNA and RNA fractions of the complex. Furthermore, the sharp transition and elevated *Tm* resulting from RNase treatment indicates that after the RNA is digested a double-stranded helical form of DNA remains, which suggests that a reasonable

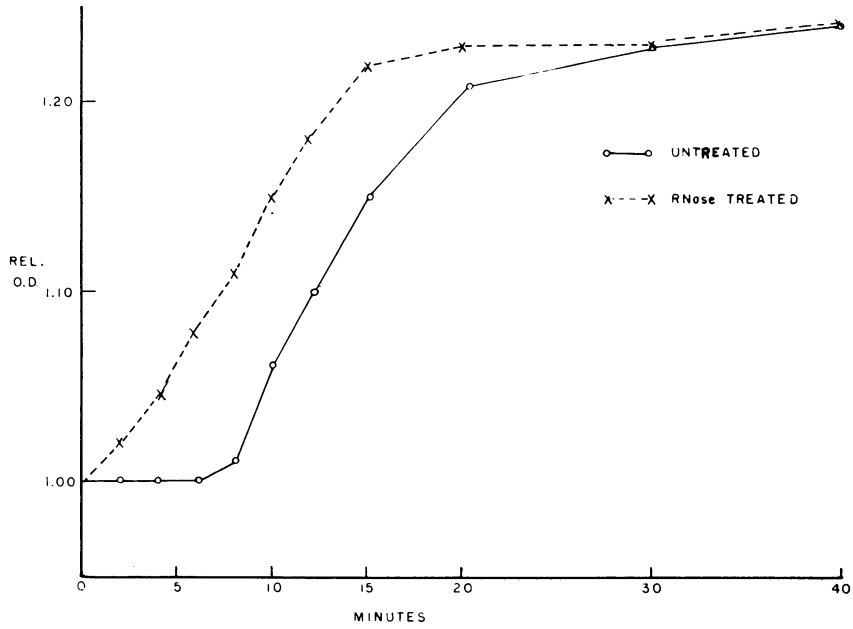

FIG. 3.—DNase digestion of DNA-RNA complex prepared from chloroform-octanol extracted deoxycholate supernatant. 10 μg each of DNase and RNase were used.

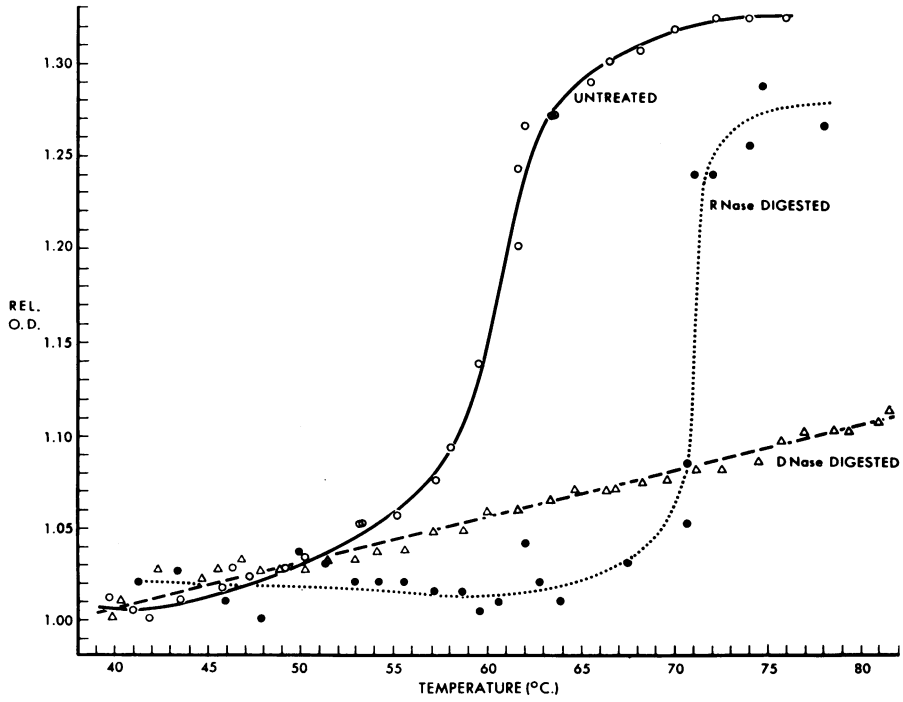

FIG. 4.—Helix-coil transition of undegraded, RNase treated, and DNase treated DNA-RNA complex.

model for the original material may be one in which three polynucleotide strands (2 DNA and 1 RNA) are held together by specific hydrogen bonds.

Another test of the physical union between the DNA and RNA portions of the complex would be its ability to maintain its integrity after centrifugation in as cesium chloride density gradient.[14] A sample of undegraded complex was centrifuged in 8.2 Molal CsCl in an SW39L rotor in a Model L centrifuge at 32,000 rpm for 110 hr. At the conclusion of the run, holes were punctured in the bottoms of the tubes, and fractions were collected from each by drop-counting.[15] The optical density at 260 mμ of each fraction was determined. The results presented in Figure 5 show that untreated material contains a major component which bands

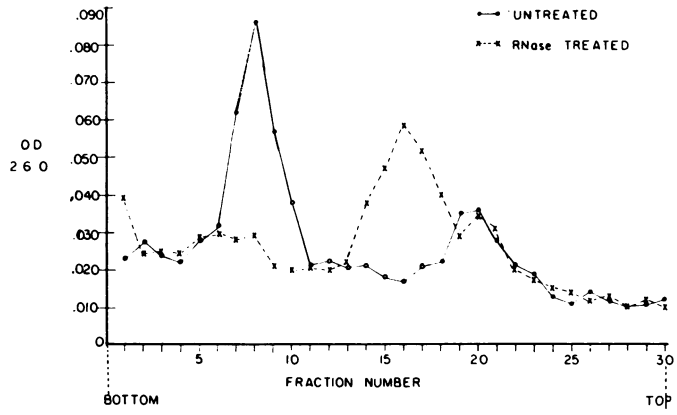

FIG. 5.—CsCl density gradient centrifugation of DNA-RNA complex prepared from chloroform-octanol extracted deoxycholate supernatant.

toward the bottom of the tube, while RNase treatment of the starting material substantially decreases the density of this fraction. The fact that the untreated material bands in a cesium chloride density gradient speaks against it being a simple mixture of DNA and RNA—for two species of polyanions having markedly different densities would not stay together. The shift in density caused by RNase treatment is consistent with what would be expected of a specific complex of DNA and RNA.

Discussion and Summary.—The general acceptance of the postulated biological role of the nucleic acids prompted us to search for an intermediate in the transfer of information from DNA to RNA. The isolation of a molecular complex containing DNA and RNA is consistent with the logical requirement for the existence of such an intermediate. This requirement has arisen as the biological functions of the parent nucleic acid molecules of the complex have been elucidated with greater and greater experimental detail. Indeed, recent reports from many laboratories[1] utilizing different organisms suggest that DNA-RNA hybrids as intermediates in the expression of genetic information may be widespread in nature and may take different forms possessing different properties depending on their source.

The experiments described in this paper present some preliminary observations of a naturally occurring DNA-RNA hybrid isolated from actively growing *Neurospora crassa*. With respect to its biological source, chemical composition, short-

time P^{32} labeling, and its behavior during chromatography, CsCl density gradient centrifugation, and thermal denaturation, it fulfils some of the requirements of an intermediate in the transfer of genetic information from DNA to RNA.

The simplest model for this hybrid, suggested by its chemical composition and behavior during thermal denaturation is a hydrogen-bonded helix consisting of two strands of DNA and one strand of RNA. Its behavior is consistent with what might be expected of the triple-stranded hybrid molecules which have been suggested.[16]

The isolation procedures which were used do not permit any conclusions as to the *in vivo* location of the hybrid. Its isolation in the microsome fraction is probably fortuitous and does not necessarily suggest that it is of cytoplasmic origin. The *in vivo* experiments of Zalokar,[17] in which rapidly labeled RNA is localized in the nucleus indicate that the hybrid described in this report may be of nuclear origin.

Historically the methods of nucleic acid chemistry precluded the characterization of a cellular fraction containing both DNA and RNA, for purity was of the utmost importance—and a major criterion of purity in a preparation of one of the nucleic acids was the complete absence of the other kind of nucleic acid. It is interesting to note that in spite of the voluminous nucleic acid chemistry literature produced in recent years, the documented isolation of hybrids came only after there was a place for them in contemporary biological concepts.

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[1] The abbreviations used are DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase, deoxyribonuclease; RNase, ribonuclease; Tris, tris(hydroxymethyl) aminomethane; ECTEOLA, anion exchanger prepared by the reaction of epichlorohydrin and triethanolamine with cellulose; *Tm*, the temperature at the midpoint of the helix-coil transition.

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*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE, II**

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Previous studies[1] on the incorporation of amino acids into acid-insoluble products by an *Escherchia coli* system, in the presence of synthetic polyribonucleotides, have been continued. This paper presents additional results with previously used and new copolymers containing uridylic[2] and guanylic acid (UG); uridylic, adenylic, and cytidylic acid (UAC); uridylic, cytidylic, and guanylic acid (UCG); and uridylic, adenylic, and guanylic acid (UAG). Assuming a triplet code, code letters (although in an as yet unspecified sequence) can now be assigned to the following eleven amino acids: cysteine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

Preparations and Methods.—These were as previously described.[1] The following new polymers were prepared with polynucleotide phosphorylase. Poly UC (3:1), from a mixture of UDP and CDP in molar ratio 3:1; poly UG (5:1), from UDP and GDP in molar ratio 5:1; poly UAC, from UDP, ADP, and CDP in molar ratio 6:1:1; poly UCG and poly UAG, from mixtures of the appropriate nucleoside 5′-diphosphates in molar ratio of 6 of UDP to 1 of each of the other two. Their sedimentation coefficients ($s_{20,w}$) were as follows. UC (3:1), 11.9; UG, 3.4; UAC, 2.4; UCG, 5.0; UAG, 4.8 S.

Results.—Experiments with poly UG: In the previous work, the following amino acids were found to be incorporated into acid-insoluble products with use of various polynucleotides: phenylalanine with poly U (cf. also footnote 3); phenylalanine, leucine, serine, and proline with poly UC; and phenylalanine, isoleucine, and tyrosine with poly UA.

As shown in Table 1, poly UG promoted, as expected, the incorporation of phenylalanine and added two more amino acids, valine and cysteine, to the list. In contrast to poly UA, poly UG had no effect on the incorporation of isoleucine and tyrosine and, contrary to poly UC, it had no influence on the incorporation of serine and proline (cf. previous paper[1] and Table 2). Poly U, poly UC, and poly UA had no effect on the incorporation of valine and cysteine.

As further shown in Table 1, poly UC (3:1) promoted the incorporation of serine and leucine to about the same extent (an average of 0.75 mμmole/mg ribosomal protein). The incorporation ratio phenylalanine/serine (or leucine) was 2.2/0.75 = 2.9. On the assumption of a triplet code, the ratio of UUU to UUC (or UCU, or CUU) triplets in this polymer would be 3. This result is in good agreement with the results reported in this and the previous paper with poly UC (5:1).