

*DIRECT EVIDENCE FOR NUCLEAR SYNTHESIS OF  
CYTOPLASMIC RIBOSE NUCLEIC ACID*

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The question of the biosynthetic relationship of nuclear and cytoplasmic ribose nucleic acid (RNA) has received much attention in recent years. Interest has been focused on this problem by current speculation on the transmission of genetic specificity from desoxyribose nucleic acid (DNA) to cytoplasmic components. It has been suggested that RNA could serve as a receptor of a "code" from the DNA in the nucleus and could transmit this specificity to cytoplasmic proteins,<sup>1</sup> with the synthesis of which it may be associated. A general association of RNA with protein synthesis has been postulated by Caspersson<sup>2</sup> and Brachet.<sup>3</sup> The more recent work of Gale and Folkes<sup>4</sup> has helped to place this postulation on a fairly firm basis. The experiments we shall describe were designed to test directly an earlier link in the chain of transmission of specificity: the synthesis of RNA in the nucleus and its transfer to the cytoplasm.

Most of the evidence bearing on this question has been discussed in a recent review article by Brachet.<sup>5</sup> This article should be consulted for references to work bearing on the following discussion.

The bulk of the data which have been interpreted as favoring the hypothesis of the nuclear origin of cytoplasmic RNA fall into several major categories. Caspersson was one of the first to suggest that the accumulation of RNA in the vicinity of the nuclear membrane of many types of cells might indicate the nuclear origin of RNA. More recently, data from Mirsky's laboratory and the work of Hogeboom and Schneider indicate that enzymes concerned with purine and nucleoside metabolism are found in the nucleus in high concentrations. Both types of evidence are circumstantial; they indicate the possibility of nuclear synthesis of RNA but do not show that this is actually the case.

Many workers have shown that radioactive precursors of RNA are incorporated into nuclear RNA at a higher rate, and presumably earlier, than into cytoplasmic RNA. Barnum, Huseby, and Vermund,<sup>6</sup> however, have taken exception to this interpretation of precursor experiments. Brachet<sup>7</sup> has found that living amoebae, whose RNA has been depleted by ribonuclease, show the reappearance of cytochemically demonstrable RNA in the nucleus prior to the cytoplasm. None of this evidence is conclusive since the methods used do not exclude the possibility that RNA is synthesized in the cytoplasm and is shunted into the nucleus as rapidly as it is formed. Moreover, even if one granted that RNA synthesis occurs in the nucleus, this form of evidence could not be used to establish the passage of RNA from nucleus to cytoplasm rather than its synthesis at different rates in nucleus and cytoplasm. Rabinovitch and Plaut<sup>8</sup> have recently demonstrated the total loss of cytochemically demonstrable RNA from the amoeba nucleus at the time of division. Their data, however, are insufficient to establish critically the subsequent location of the nuclear RNA.

Linnet and Brachet, as well as James,<sup>9</sup> have shown that enucleated halves of amoebae lose RNA at a more rapid rate than do nucleated halves. This could be

interpreted as evidence for the nuclear synthesis of cytoplasmic RNA or perhaps for the nuclear control of retention of RNA in the cytoplasm.

The lack of uniformity in biochemical composition of RNA samples derived from nucleus and cytoplasm has been felt by some workers to speak against the simple transfer of nucleus-synthesized RNA to the cytoplasm. Again, this cannot be regarded as critical evidence, since the heterogeneity is subject to alternative explanations.

A more direct approach is necessary to demonstrate the relationship between nuclear and cytoplasmic RNA, and this approach is provided, in our opinion, by the following experimental design. The RNA of the nucleus of a cell may be labeled with a radioactive tracer, and the nucleus may be transferred to a cell whose RNA is unlabeled. Transmission of labeled material from nucleus to cytoplasm may then be traced directly by autoradiographic visualization of the final distribution of the tracer atoms. The methods used were as follows: Amoebae (*A. proteus*) were labeled with  $P^{32}$  by feeding them with *Tetrahymena pyriformis* which had been cultured on a 1 per cent proteose-peptone solution with added  $P^{32}O_4$ . Generally, following two to three days of feeding, the amoebae were assayed at 300–1,000 counts per minute per amoeba (dried amoebae on plastic planchets in windowless *Q*-gas flow counter, Nuclear Instrument and Chemical Corporation Model D-46A), with perhaps 1–2 per cent of the radioactivity within the nucleus. Earlier work has indicated that essentially all the autoradiographically detectable  $P^{32}$  in the nucleus under the above labeling conditions is in RNA. That is, we found that no  $P^{32}$  was present in the nucleus following ribonuclease digestion. The fact that no detectable label could be ascribed to the DNA can be explained by the low concentration of DNA in the amoeba nucleus, as indicated by the faintness of the Feulgen reaction.

Nuclei from  $P^{32}$ -labeled amoebae were transferred by micromanipulation<sup>10</sup> to unlabeled, enucleated, or to normal, unenucleated, amoebae. The success of the operation could be determined from previously established criteria. We had found it possible to predict accurately, from their postoperative appearance and behavior, which of the cells would divide in time and could therefore be considered viable. Only such cells were used for subsequent analysis.

Individual amoebae were fixed at various times following the operation by flattening the cells on a slide with a cover slip carrying a small drop of 45 per cent acetic acid on the underside. After removal of the cover slip and dehydration, the slides were coated with autoradiographic stripping film (Kodak, Ltd., London, England) and stored in the dark for approximately 14 days' exposure. (See Rabinovitch and Plaut<sup>8</sup> for further technical details on the processing of slides.) After photographic development, the preparations were examined with bright-field and phase-contrast microscopy.

We found that in the autoradiographs of amoebae fixed less than 5 hours after the nuclear transfer operation, essentially all the significant radioactivity was still localized within the nucleus (Pl. I, Figs. 1A and 1B). Significant radioactivity denotes a silver grain density in the developed autoradiographic emulsion which is above the low general-background grain density inherent in the emulsion. The very low level of activity in the cytoplasm of these amoebae should be noted; it indicates that the cytoplasm was not significantly contaminated by the transfer oper-

ation. Amoebae fixed 12 or more hours after the operation showed appreciable activity in the cytoplasm (Pl. I, Figs. 2A and 2B). It can be concluded that material, whatever its molecular complexity, is transferred from nucleus to cytoplasm.

Although fifteen cases of successful transfers of nuclei to enucleated amoebae

## PLATE I

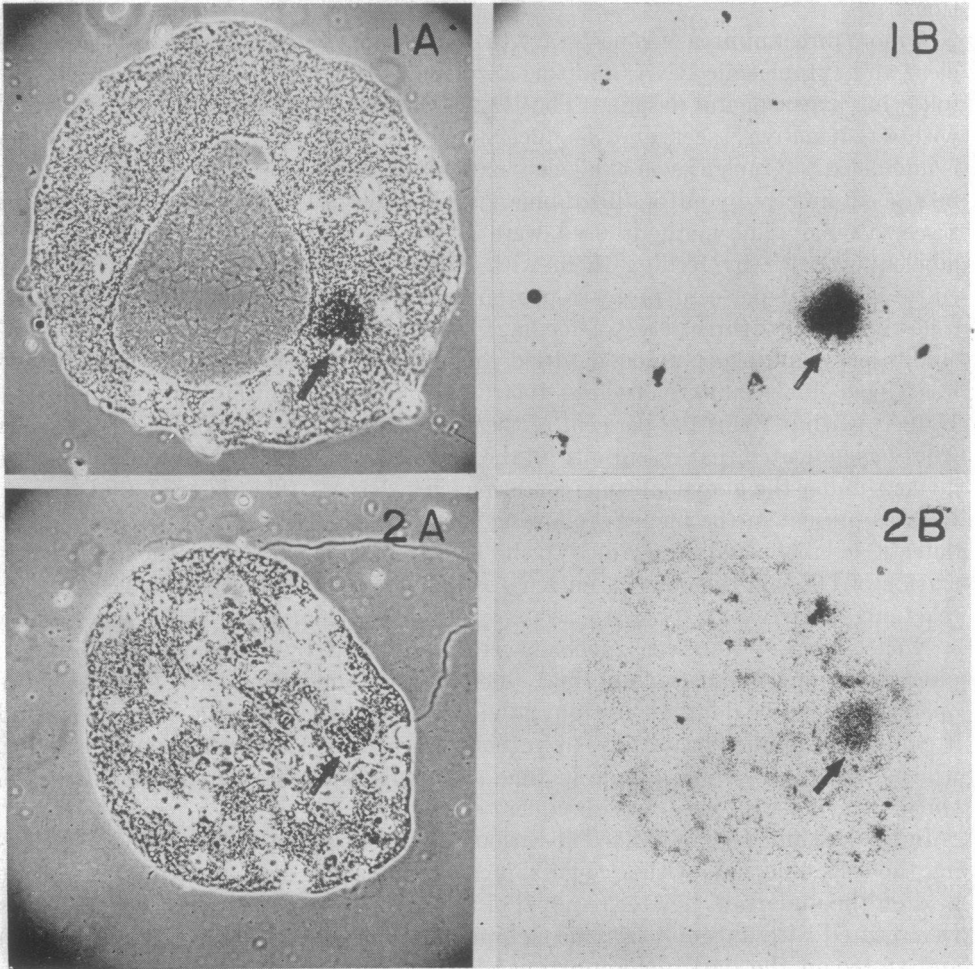


FIG. 1A.—Photomicrograph of phase-contrast view of an amoeba with a  $P^{32}$ -labeled nucleus grafted 6 minutes before fixation ( $\times 117$ ). FIG. 1B.—Photomicrograph of autoradiograph of same amoeba as in Fig. 1A. ( $\times 117$ ). FIG. 2A.—Photomicrograph of phase-contrast view of an amoeba with a  $P^{32}$ -labeled nucleus grafted 62 hours before fixation ( $\times 117$ ). FIG. 2B.—Photomicrograph of autoradiograph of same amoeba as in Fig. 2A ( $\times 117$ ). (Arrows indicate the location of the nuclei in each case.)

have been studied, the rate of transfer has not been estimated because the physiological state, i.e., the stage of cellular growth in the interphase of the mitotic cycle, of the "donor" and "host" amoebae involved in the transfers has not been controlled. We have not been able to see any consistent pattern in the change of the ratio of cytoplasmic to nuclear radioactivity with time. On the whole, of course,

this ratio increases with time elapsed following the transfer. Nonetheless, we have observed instances in which a relatively short-time postoperative amoeba exhibited a higher ratio than an amoeba fixed considerably longer after the transfer operation. This circumstance suggests that the mechanism responsible for the transfer of the labeled nuclear material to the cytoplasm is not simple diffusion.

Experiments with ribonuclease (0.04 per cent Worthington Biochemical Laboratory ribonuclease in distilled water adjusted to pH 6.7 with  $\text{Na}_2\text{HPO}_4$ , at 40° C. for 2-3 hours) have indicated that all the detectable radioactive label in nucleus and cytoplasm of these "renucleated" amoebae is in RNA. Amoebae treated with this enzyme prior to autoradiography failed to show any significant radioactivity. Had the label left the nucleus as  $\text{P}^{32}\text{O}_4$  and not as part of a more complex molecule, we would have expected some residual cytoplasmic label after ribonuclease digestion, since other phosphorus-containing compounds, such as phosphoproteins, are synthesized in significant quantities in the cytoplasm<sup>11</sup> and would not be removed during our processing.

Further evidence that the radioactive label leaves the nucleus as part of an entity more complex than the  $\text{PO}_4$  ion is furnished by observations on twelve artificially binucleate amoebae in which a  $\text{P}^{32}$ -labeled nucleus had been transferred to an unlabeled cell already containing a nucleus. After 12-90 hours of existence as binucleates, the cells were fixed and autoradiographed. Examination of the photographically developed preparations showed that, whereas the labeled nucleus gradually lost its activity to the cytoplasm, the originally unlabeled nucleus did not acquire any significant amount of radioactivity (Pl. II, Figs. 3A and 3B). Of the twelve cells studied, only two could be regarded as possible exceptions. There appears, therefore, to be no transfer to the unlabeled nucleus of the labeled material the cytoplasm has received from the initially labeled nucleus.

One of the essential conditions to satisfy the hypothesis of RNA mediation between gene and cytoplasm is that the nucleus modifies the RNA that becomes localized in the cytoplasm. It can do this either by synthesizing RNA and supplying it to the cytoplasm or by transferring to the cytoplasm a modified RNA precursor. The evidence presented in this report suggests strongly that this required relationship between nuclear and cytoplasmic RNA exists. We have shown that the labeled material leaves the nucleus and appears in the cytoplasm. The labeled material in the nucleus initially, and in both nucleus and cytoplasm after a period of time, is in RNA, since all radioactivity is removed by digestion with ribonuclease. The label is therefore in RNA at both the initial and the terminal points of its migration. Moreover, the fact that the label demonstrated in the cytoplasmic RNA does not enter the second, initially unlabeled, nucleus of the binucleate cells leads to the conclusion that the cytoplasm does not supply RNA to the nucleus and that the nucleus, therefore, synthesizes its own. If such a transfer were taking place, the autoradiograph of the originally unlabeled nucleus in the binucleates should show a higher level of radioactivity than that in the cytoplasm, since cytochemical evidence shows that the concentration of RNA in the nucleus of *A. proteus* is substantially higher than that in the cytoplasm.<sup>8</sup> In ten of the twelve experimental binucleates studied there was no evidence for nuclear labeling in the initially unlabeled nucleus. The other two cases (e.g., Pl. II, Fig. 4) indicated the possibility of some label in the second nucleus. However, in view of the sharp

labeling contrast obtained between nucleus and cytoplasm when a labeled precursor is fed to amoebae (Pl. II, Fig. 5), such slight labeling, if significant at all, is more readily explicable as the result of a partial breakdown of labeled cytoplasmic RNA

## PLATE II

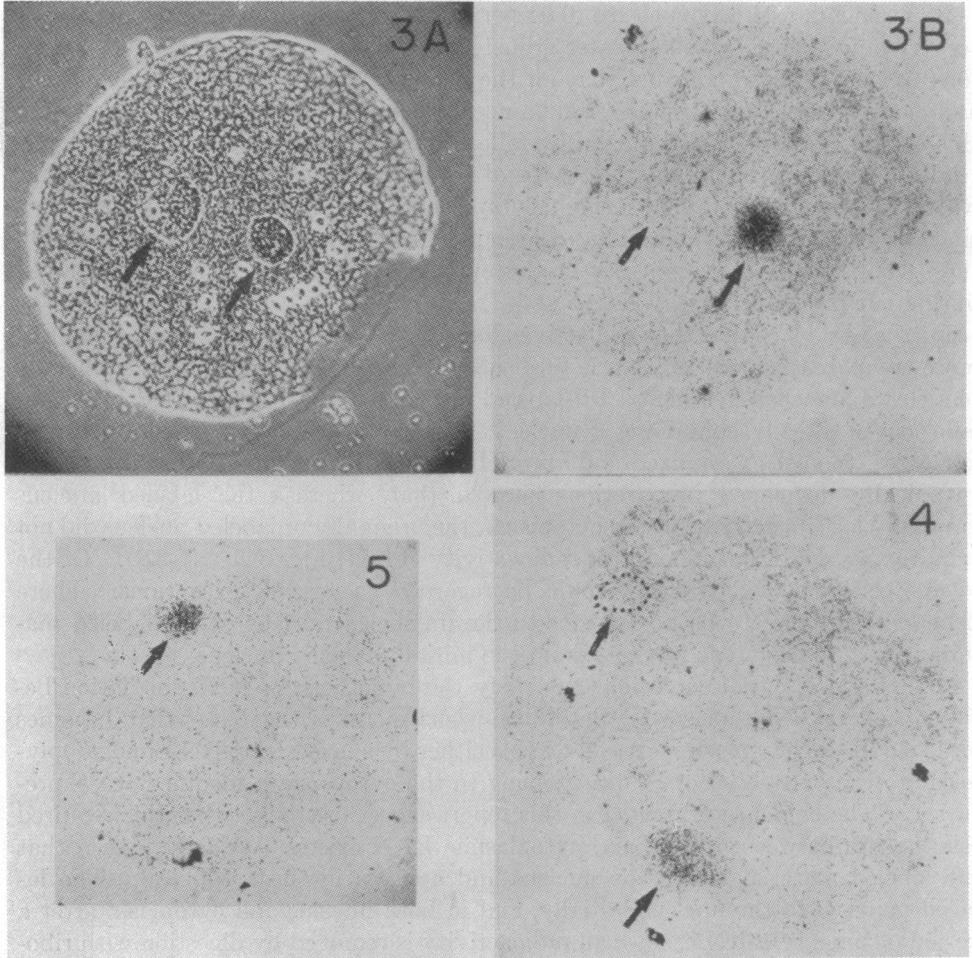


FIG. 3A.—Photomicrograph of phase-contrast view of a binucleate with one  $P^{32}$ -labeled nucleus grafted  $42\frac{1}{2}$  hours before fixation ( $\times 117$ ). FIG. 3B.—Photomicrograph of autoradiograph of same amoeba as in Fig. 3A ( $\times 117$ ). The grains visible over the left nucleus are attributable to the radioactivity in the overlying and underlying cytoplasm. FIG. 4.—Photomicrograph of autoradiograph of a binucleate amoeba with one  $P^{32}$ -labeled nucleus grafted  $43\frac{1}{2}$  hours before fixation ( $\times 117$ ). The dotted outline indicates the region of the originally unlabeled nucleus. (Phase-contrast view not presented because of insufficient contrast for photographic reproduction.) FIG. 5.—Photomicrograph of autoradiograph of an unoperated amoeba which was incubated in a  $C^{14}$ -adenine solution for 12 hours prior to fixation ( $\times 120$ ). (Arrows indicate the location of the nuclei in each case.)

and the consequent availability of some labeled precursor for resynthesis by the nucleus. It follows, then, that the nucleus synthesizes its RNA and that, while the nuclear RNA label appears in cytoplasmic RNA, the transfer proceeds in that direction only.

We do not know the identity of the  $P^{32}$ -containing entity which is supplied to the nucleus in the initial process of labeling. We know, however, that it is incorporated into the nuclear RNA. Similarly, we do not know the nature of the labeled material leaving the nucleus, but we know that it appears in cytoplasmic RNA. It cannot, however, be identical with the labeled entity originally incorporated into nuclear RNA. Were it identical, we would expect that it would be incorporated into the RNA of the second nucleus of a binucleate, as it was into that of the first nucleus, resulting in two labeled nuclei. This is not the case. It follows, therefore, that the labeled entity which appears first in nuclear RNA and subsequently in cytoplasmic RNA is modified by the nucleus. Thus we have shown that the relationship between nuclear and cytoplasmic RNA necessary to satisfy the hypothesis of RNA intermediacy between gene and cytoplasm exists: the evidence demonstrates that the product furnished to the cytoplasm by the nucleus, while not completely characterized, must be at least a nucleus-modified RNA precursor, if not RNA as such.

We have not proved that the labeled material migrating from nucleus to cytoplasm is the RNA as it actually existed in the nucleus, although this conclusion is consistent with our findings. Since we have shown that the nucleus is capable of synthesizing the finished RNA molecule, it appears to us most likely that it is RNA and not a precursor which is transferred. (These observations do not answer the question whether RNA as such or as ribonucleoprotein is transmitted.<sup>12</sup>) Moreover, the possibility of the complete synthesis of *some* RNA in the cytoplasm is not ruled out by our data. In point of fact, such synthesis has been suggested for *Acetabularia* by Brachet.<sup>5</sup> It would not be surprising if the amount of cytoplasmic RNA synthesis varied widely among cell types. The presence of some cytoplasmic synthesis of RNA could account for the data indicating differences in purine and pyrimidine composition of the RNA derived, respectively, from cytoplasm and nucleus. These heterogeneity data, now thought by some to rule out the possibility of nuclear synthesis of cytoplasmic RNA, could then be explained on the basis of contributory synthesis by the cytoplasm, although alternative explanations are not ruled out.

*Summary.*—A more direct experimental design than has heretofore been employed has been developed to test the hypothesis of nuclear synthesis of RNA and its transfer to the cytoplasm. The RNA of the nucleus was labeled with radioactive tracer, and the nucleus was grafted into a cell whose RNA was unlabeled. Transmission of labeled material from nucleus to cytoplasm was then traced directly by autoradiography. The evidence presented shows that RNA is synthesized in the nucleus and that RNA, or at least a nucleus-modified precursor of RNA, is transmitted to the cytoplasm.

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## THE ENZYMATIC ACTIVATION OF SULFATE\*

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### INTRODUCTION

The problem of sulfate activation is somewhat unusual in so far as it represents an ATP-linked activation of an inorganic compound other than phosphate. DeMeio *et al.*<sup>1</sup> and Bernstein and McGilvery<sup>2</sup> showed that the over-all reaction is ATP-linked. Furthermore, a preliminary separation into two fractions has been reported.<sup>3</sup> In view of certain similarities to the carboxyl activation, we have recently turned our attention to this unusual reaction and will report here experiments which are still somewhat preliminary but have already led to a tentative identification of the active sulfate intermediary as an adenylyl sulfate.

Lamb liver was found to be a rather good source of the over-all transfer reaction to nitrophenol. The sulfate activation could, furthermore, be studied with *Neurospora* extracts, which were found to contain a strong sulfate-activation system but no transfer enzyme to nitrophenol.

### ENZYME PREPARATIONS

*Test System for Nitrophenyl Sulfate Synthesis.*—Assay mixture: 50  $\mu$ M imidazole-HCl buffer, pH 7.0; 6.25  $\mu$ M MgCl<sub>2</sub>; 0.50  $\mu$ M nitrophenol; 5  $\mu$ M cysteine; 5  $\mu$ M K<sub>4</sub>ATP; 5-10  $\mu$ M K<sub>2</sub>SO<sub>4</sub>; and enzyme solution in a final volume of 0.50 ml. or the doubled amounts in 1.0 ml.

After incubation at 38° for 60 minutes, the reaction is stopped by the addition of 2 ml. of alcohol, and the mixture is freed of the precipitated protein by centrifugation. To 2.5 ml. of 0.1 N KOH is added 0.50 ml. of the supernatant, and, after mixing, the extinction is measured at 420  $\mu$ M in the Klett-Summerson photometer