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AN UNSTABLE INTERMEDIATE CARRYING INFORMATION FROM GENES TO RIBOSOMES FOR PROTEIN SYNTHESIS

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A LARGE amount of evidence suggests that genetic information for protein structure is encoded in deoxyribonucleic acid (DNA) while the actual assembling of amino-acids into proteins occurs in cytoplasmic ribonucleoprotein particles called ribosomes. The fact that proteins are not synthesized directly on genes demands the existence of an intermediate information carrier. This intermediate template is generally assumed to be a stable ribonucleic acid (RNA) and more specifically the RNA of the ribosomes. According to the present view, each gene controls the synthesis of one kind of specialized ribosome, which in turn directs the synthesis of the corresponding protein—a scheme which could be epitomized as the one gene–one ribosome–one protein hypothesis. In the past few years, however, this model has encountered some difficulties: (1) The remarkable homogeneity in size[1] and nucleotide composition[2] of the ribosomal RNA reflects neither the range of size of polypeptide chains nor the variation in the nucleotide composition observed in the DNA of different bacterial species[2,3]. (2) The capacity of bacteria to synthesize a given protein does not seem to survive beyond the integrity of the corresponding gene[4]. (3) Regulation of protein synthesis in bacteria seems to operate at the level of the synthesis of the information intermediate by the gene rather than at the level of the synthesis of the protein[5].

These results are scarcely compatible with the existence of stable RNA intermediates acting as templates for protein synthesis. The paradox, however, can be resolved by the hypothesis, put forward by Jacob and Monod[5], that the ribosomal RNA is not the intermediate carrier of information from gene to protein, but rather that ribosomes are non-specialized structures which receive genetic information from the gene in the form of an unstable intermediate or 'messenger'. We present here the results of experiments on phage-infected bacteria which give direct support to this hypothesis.

When growing bacteria are infected with a virulent bacteriophage such as *T*2, synthesis of DNA stops immediately, to resume 7 min. later[6], while protein synthesis continues at a constant rate[7]. After infection many bacterial enzymes are no longer produced[8]; in all likelihood, the new protein is genetically determined by the phage. A large number of new enzymatic activities appears in the infected cell during the first few minutes following infection[9], and from the tenth minute onwards some 60 per cent of the protein synthesized can be accounted for by the proteins of the phage coat[7]. Surprisingly enough, protein synthesis after infection is not accompanied, as in growing cells, by a net synthesis of RNA[10]. Using isotopic labelling, however, Volkin and Astrachan[11] were able to demonstrate high turnover in a minor RNA fraction after phage infection. Most remarkable is the fact that this RNA fraction has an apparent nucleotide composition which corresponds to that of the DNA of the phage and is markedly different from that of the host RNA[11]. Recently, it has been shown that the bulk of this RNA is associated with the ribosomes of the infected cell[12].

Phage-infected bacteria therefore provide a situation in which the synthesis of protein is suddenly

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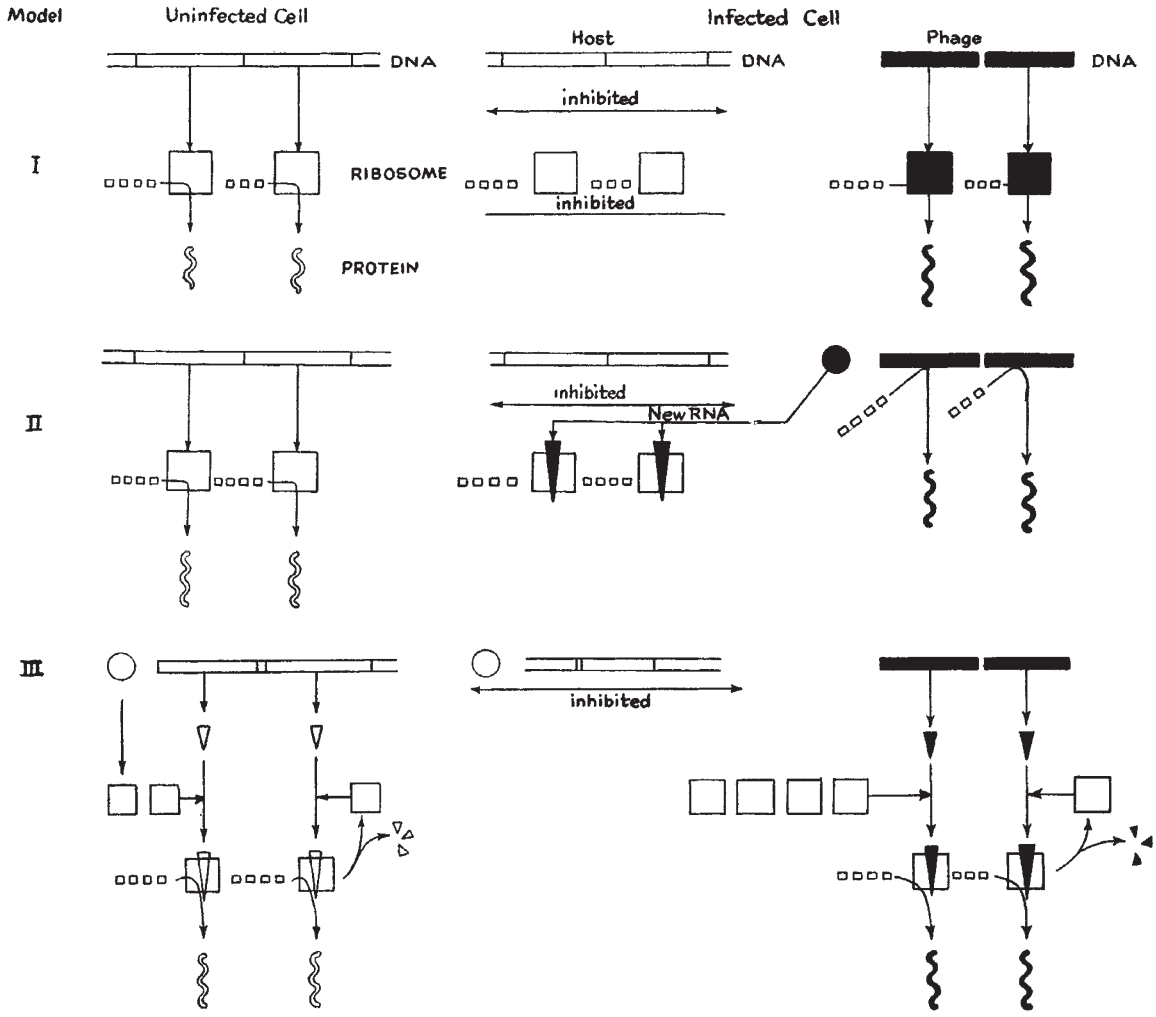

Fig. 1. Three models of information transfer in phage-infected cells

switched from bacterial to phage control and proceeds without the concomitant synthesis of stable RNA. *A priori*, three types of hypothesis may be considered to account for the known facts of phage protein synthesis (Fig. 1). Model I is the classical model. After infection the bacterial machinery is switched off, and new ribosomes are then synthesized by the phage genes. The *ad hoc* hypothesis has to be added that these ribosomes are unstable, to account for the turnover of RNA after phage infection. This is, in fact, the model favoured by Nomura *et al.*[12]. Model II assumes that in the particular case of phage the proteins are assembled directly on the DNA; the new RNA is a special molecule which enters old ribosomes and destroys their capacity for protein synthesis. At the same time, synthesis of ribosomes is switched off. Model III implies that a special type of RNA molecule, or 'messenger RNA', exists which brings genetic information from genes to non-specialized ribosomes and that the consequences of phage infection are two-fold: (*a*) to switch off the synthesis of new ribosomes; (*b*) to substitute phage messenger RNA for bacterial messenger RNA. This substitution can occur quickly only if messenger RNA is unstable; the RNA made after phage infection does turn over and appears, therefore, as a good candidate for the messenger.

It is possible to distinguish experimentally between these three models in the following way: Bacteria are grown in heavy isotopes so that all cell constituents are uniformly labelled 'heavy'. They are infected with phage and transferred immediately to a medium containing light isotopes so that all constituents synthesized after infection are 'light'. The distribution of new RNA and new protein, labelled with radioactive isotopes, is then followed by density gradient centrifugation[13] of purified ribosomes.

Density gradient centrifugation was carried out in a preparative centrifuge, and the ribosomes were stabilized by including magnesium acetate (0·01–0·06 *M*) in the cæsium chloride solution. Ribosomes show two bands, a heavier *A* band and a lighter *B* band, the relative proportions of which, for a given preparation, depend on the magnesium concentration used. The lower the magnesium concentration, the smaller the proportion of *B* band ribosomes and the larger the proportion of *A* band ribosomes.

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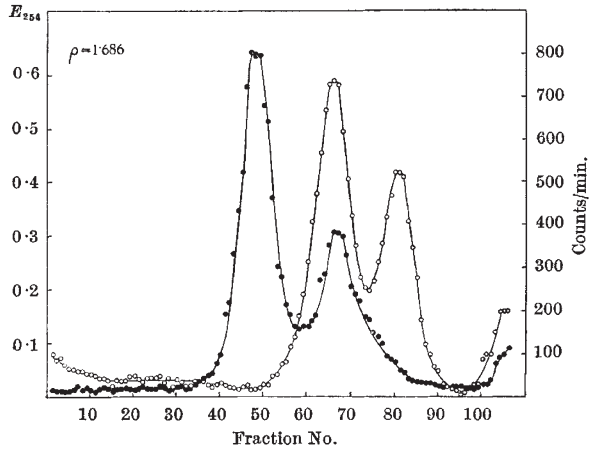

Fig. 2. Distribution of heavy and light ribosomes in a density gradient. *E. coli* B, grown in 5 ml. of a medium containing ^{15}N (99 per cent) and ^{13}C (60 per cent) algal hydrolysate and $^{32}PO_4$, were mixed with a fifty-fold excess of cells grown in nutrient broth, the ribosomes extracted by alumina grinding in the presence of 0·01 *M* Mg^{++} and purified by centrifugation. 1 mgm. of ribosomes was centrifuged in 3 ml. of cæsium chloride buffered to *p*H 7·2 with 0·1 *M tris* and containing 0·03 *M* magnesium acetate for 35 hr. at 37,000 r.p.m. in the *SW*39 rotor of the Spinco model *L* ultracentrifuge. After the run, a hole was pierced in the bottom of the tube and drops sequentially collected. Ultra-violet absorption at 254 mμ detects the excess of light ribosomes (○), ^{32}P counts detect the heavy ribosomes (●)

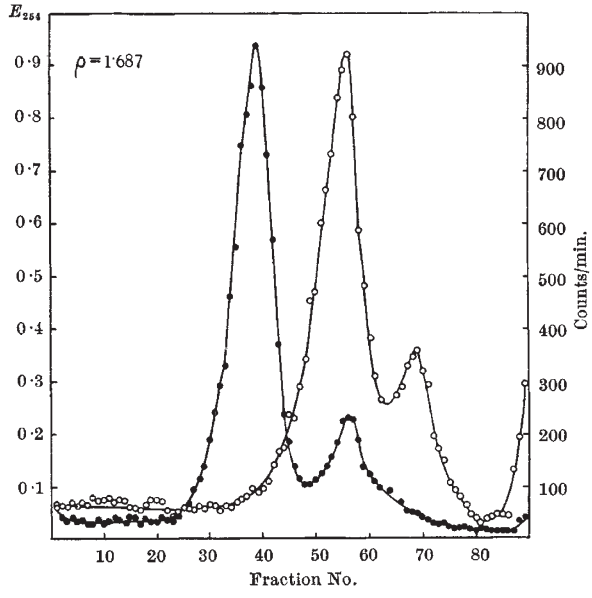

Fig. 3. Distribution of randomized heavy and light ribosomes in a density gradient. The mixture of $^{15}N^{13}C^{32}P$ and $^{14}N^{12}C^{31}P$ ribosomes was dialysed first for 18 hr. against 0·0005 *M* magnesium acetate in 0·01 *M* phosphate buffer *p*H 7·0, and then for 24 hr. against two changes of 0·01 *M* magnesium acetate in 0·001 *M tris* buffer *p*H 7·4. 1 mgm. of ribosomes was centrifuged for 38 hr. at 37,000 r.p.m. in cæsium chloride containing 0·03 *M* magnesium acetate. The drops were assayed for ultra-violet absorption (○) and ^{32}P content (●)

In order to show that there is no aggregation of ribosomes during preparation and density gradient centrifugation an experiment was carried out on ribosomes extracted from a mixture of $^{15}N^{13}C$ and $^{14}N^{12}C$ bacteria. The results are shown in Fig. 2, from which it can be seen that ribosomes of different isotopic compositions band independently and that there are no intermediate classes. The same preparation was then dialysed against low magnesium to dissociate the ribosomes into their 50 *S* and 30 *S* components and then against high magnesium to re-associate the sub-units[14]. This should have resulted in distributing heavy 30 *S* and 50 *S* sub-units into mixed 70 *S* and 100 *S* ribosomes. Surprisingly enough, density gradient centrifugation of this preparation (Fig. 3) yields the same bands as found in the original ribosomes except for a decrease in the proportion of the *B* bands. This means that both bands contain units which do not undergo reversible association and dissociation and that the mixed 70 *S* ribosomes prepared by dialysis separate

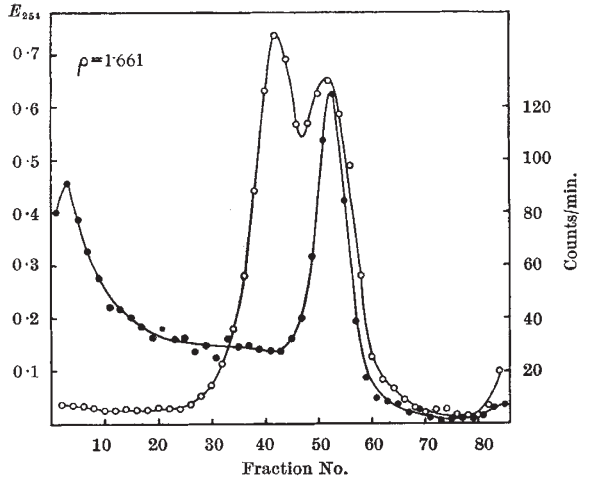

Fig. 4

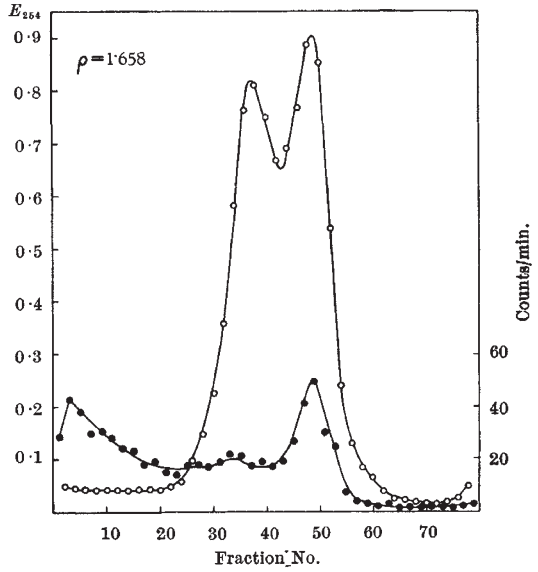

Fig. 5

Figs. 4 and 5. Distribution and turnover of RNA formed after phage infection. A 600 ml. culture of *E. coli B*6 (mutant requiring arginine and uracil) was infected with *T4D* (multiplicity 30) and fed ^{14}C-uracil (10 mc./m*M*) from third to fifth min. after infection. One half of the culture was removed and ribosomes prepared (Fig. 4). The other half received a two hundred-fold excess of ^{12}C-uridine for a further 16 min. and ribosomes prepared (Fig. 5). In both experiments approximately 3 mgm. of purified ribosomes were centrifuged for 42 hr. at 37,000 r.p.m. in cæsium chloride containing 0·05 *M* magnesium acetate. Alternate drops were collected in *tris*-magnesium buffer for ultra-violet absorption (○) and on to 0·5 ml. of frozen 5 per cent trichloroacetic acid. These tubes were thawed, 1 mgm. of serum albumin added, and the precipitates separated and washed by filtration on membrane filters for assay of radioactivity (●)

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into their components in the density gradient. Other experiments to be reported elsewhere suggest that the *A* band is composed of free 50 *S* and 30 *S* ribosomes and that the *B* band contains undissociated 70 *S* particles.

The bulk of the RNA synthesized after infection is found in the ribosome fraction, provided that the extraction is carried out in 0·01 *M* magnesium ions[12]. We have confirmed this finding and have studied the distribution of the new RNA among the ribosomal units found in the density gradient. Fig. 4 shows that this RNA, labelled with ^{14}C-uracil, bands in the same position as *B* band ribosomes. There is no peak corresponding to the *A* band. In addition, there is radioactivity at the bottom of the cell. This is free RNA as its density is greater than 1·8, and, moreover, it must have a reasonably high molecular weight to have sedimented in the gradient. Lowering of the magnesium concentration in the gradient, or dialysing the particles against low magnesium, produces a decrease of the *B* band and an increase of the *A* band. At the same time, the radioactive RNA leaves the *B* band to appear at the bottom of the gradient. This shows that the uracil has labelled a species of RNA distinct from that of the bulk of *B* band ribosomes, since the specific activity of the RNA at the bottom of the cell is much higher than that of the *B* band. Fig. 5 shows that this RNA turns over during phage growth. There is a decrease by a factor of four in the specific activity of the *B* band after 16 min. of growth in ^{12}C-uridine. Similar results have been obtained using $^{32}PO_4$ as a label.

These results do not distinguish between a messenger fraction and a small proportion of new

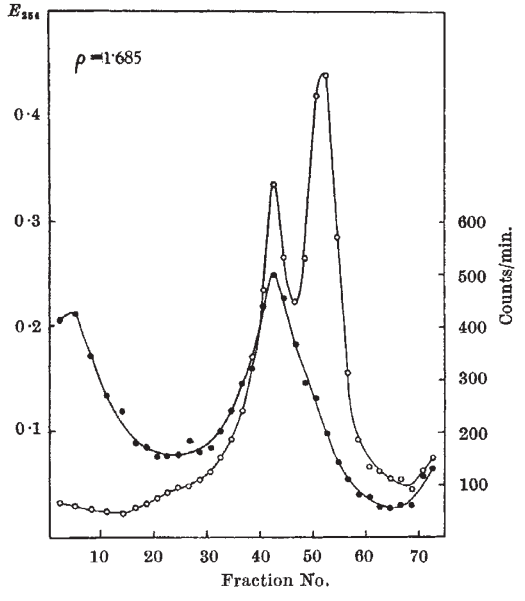

Fig. 6. RNA after isotope transfer in phage-infected cells. *E. coli* B grown in 10 ml. of ^{15}N (99 per cent) and ^{13}C (60 per cent) algal hydrolysate medium were starved in buffer, infected with *T*4 and growth initiated by addition of glucose and dephosphorylated broth (^{14}N^{12}C). $^{32}PO_4$ was fed from the second to seventh min. after infection. The culture was mixed with a fifty-fold excess of *E. coli* B grown in nutrient broth, infected in buffer and then grown for 7 min. in dephosphorylated broth medium. 1 mgm. of purified ribosomes was centrifuged for 36 hr. at 37,000 r.p.m. in cæsium chloride containing 0·03 *M* magnesium acetate. Ultra-violet absorption (○) detects the ^{14}N^{12}C^{31}P carrier, radioactivity (●) detects the new RNA in the heavy cells transferred to light medium

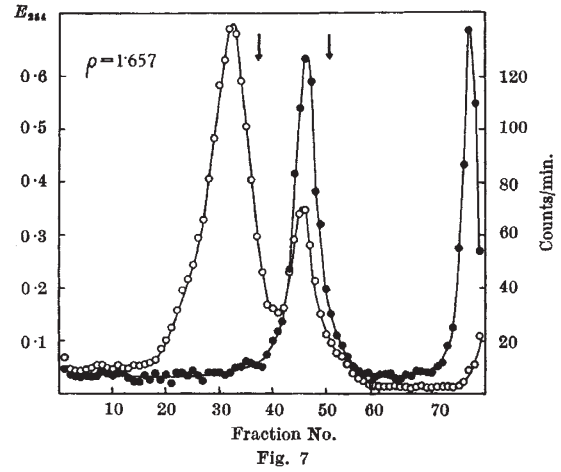

Fig. 7

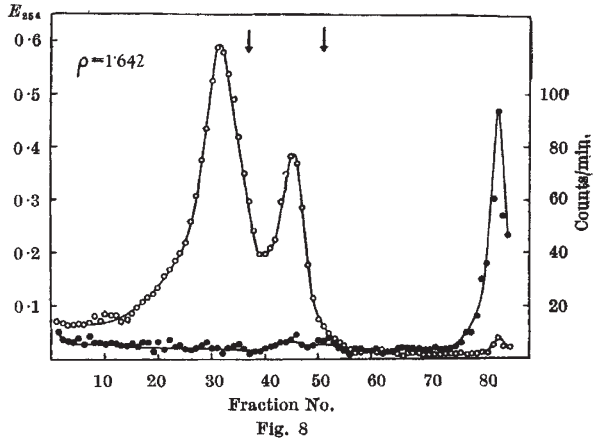

Fig. 8

Figs. 7 and 8. Distribution and turnover of newly synthesized protein in ribosomes of phage-infected cells after transfer. *E. coli* B grown in 600 ml. of a salt glucose medium containing $^{15}NH_4Cl$ (99 per cent) were starved in buffer, infected with *T*4 and transferred to $^{14}NH_4Cl$ medium. $^{35}SO_4$ was fed for the first 2 min. of infection and one half of the culture removed (Fig. 7). The other half received an excess of $^{32}SO_4$ and ^{32}S-methionine and growth was continued for a further 8 min. (Fig. 8). 1 mgm. of purified ribosomes was centrifuged for 39 hr. at 37,000 r.p.m. in cæsium chloride containing 0·05 *M* magnesium acetate. Drops were assayed for ultra-violet absorption (○) and for radioactivity (●). The arrows mark the expected positions for the peaks of ^{14}N *A* and *B* bands. The radioactivity at the top of the gradient is contaminating protein

ribosomes which are fragile in cæsium chloride and which are also metabolically unstable. In order to make the distinction, the experiment was carried out with an isotope transfer, in the following manner: Cells grown in a small volume of ^{15}N^{13}C medium were infected with *T* 4, transferred to ^{14}N^{12}C medium and fed $^{32}PO_4$ from the second to the seventh minute. They were mixed with a fifty-fold excess of cells grown and infected in ^{14}N^{12}C^{31}P medium. Fig. 6 shows that the RNA formed after infection in the heavy cells has a density greater than that of the *B* band of the carrier. Its peak corresponds exactly with the density of the *B* band of ^{15}N^{13}C ribosomes (Fig. 2) although it is skewed to lighter density, and its response to changing the magnesium concentration was that of a *B* band. There is no radioactive peak corresponding to the *B* band of the carrier: this means that no wholly new ribosomes are synthesized after phage infection. As already shown, the new RNA does not represent random labelling of *B* band ribosomes; therefore it con-

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stitutes a fraction which is added to pre-existing ribosomes the bulk of the material of which has been assimilated before infection. This result conclusively eliminates model I.

To distinguish between models II and III an experiment was carried out to see whether pre-existing ribosomes participate in protein synthesis after phage infection. Cells were grown in ^{15}N medium, infected with phage, transferred to ^{14}N medium and fed $^{35}SO_4$ for the first 2 min. of phage growth. Fig. 7 shows that only the *B* band of pre-existing ribosomes becomes labelled with ^{35}S and there is no peak corresponding to a ^{14}N *B* band. All this label can be removed by growth in non-radioactive sulphate and methionine (Fig. 8). In this experiment, the incorporation of ^{35}S into the total extract was measured and the amount in the *B* band found to correspond to 10 sec. of protein synthesis. This is probably an over-estimate since it is unlikely that pool equilibration was attained instantaneously. This value corresponds quite closely with the ribosome passage time of 5–7 sec. for the nascent protein in uninfected cells[15]. In addition, electrophoresis of chymotrypsin digests of *B* band ribosomes shows that the radioactivity is already contained in a variety of peptides. It would therefore appear that most, if not all, protein synthesis in the infected cell occurs in ribosomes. The experiment also shows that pre-existing ribosomes are used for synthesis and that no new ribosomes containing stable sulphur-35 are synthesized. This result effectively eliminates model II.

We may summarize our findings as follows: (1) After phage infection no new ribosomes can be detected. (2) A new RNA with a relatively rapid turnover is synthesized after phage infection. This RNA, which has a base composition corresponding to that of the phage DNA, is added to pre-existing ribosomes, from which it can be detached in a cæsium chloride gradient by lowering the magnesium concentration. (3) Most, and perhaps all, protein synthesis in the infected cell occurs in pre-existing ribosomes.

These conclusions are compatible only with model III (Fig. 1), which implies that protein synthesis occurs by a similar mechanism in uninfected cells. This, indeed, appears to be the case: exposure of uninfected cells to a 10-sec. pulse of $^{32}PO_4$ results in labelling of the RNA in the *B* band and not in the *A* band of ribosomes, and this RNA can be detached from the ribosomes by lowering the concentration of magnesium ions. Similarly the nascent protein can be labelled by a short pulse of $^{35}SO_4$; it is located in the *B* band and most of the label is removed by growth in non-radioactive sulphate. In contrast to what was observed in infected cells, residual stable radioactivity is found in both bands, reflecting the synthesis of new ribosomes.

In order to act as an intermediate carrier of information from genes to ribosomes, the messenger has to fulfil certain prerequisites of size, turnover and nucleotide composition. In the accompanying article, Gros *et al.*[16] have analysed the distribution of pulse-labelled RNA in sucrose gradients. They have shown that in uninfected cells there is an RNA fraction which has a rapid turnover and which can become attached reversibly to ribosomes depending on the magnesium concentration. The *T2* phage-specific RNA shows the same behaviour and both are physically similar, with sedimentation constants of 14–16 *S*. We have carried out similar experiments[17] independently and our results confirm their findings. These suggest that, although the messenger RNA is a minor fraction of the total RNA (not more than 4 per cent), it is not uniformly distributed over all ribosomes, and may be large enough to code for long polypeptide chains. When ribosomes, from phage-infected cells labelled with $^{32}PO_4$ for five min., are separated by centrifugation in a sucrose density gradient[18] containing 0·01 *M* Mg^{++}, most of the messenger is found in 70 and 100 *S* ribosomes, contrary to previous reports[12]. When the magnesium concentration is lowered, the radioactivity is found in three peaks of roughly equal amount: (1) corresponding to a small residual number of 70 *S* ribosomes; (2) corresponding to 30 *S* ribosomes; (3) a peak of very high specific activity at 12 *S*. Separation of the RNA extracted from such ribosomes with detergent shows all the counts to be located in a peak at 12 *S*, skewed towards the heavier side. These results suggest that the messenger is heterogeneous in size and may have a minimum molecular weight of about $\frac{1}{4}$ to $\frac{1}{2}$ million. Similar results have been obtained in uninfected cells[17].

The undissociable 70 *S* ribosomes are enriched for messenger RNA over the total ribosomes of this type and it has been shown that they are also enriched for the nascent protein[17]. These ribosomes have been called "active 70 *S*" ribosomes, by Tissières *et al.*[19], and they appear to be the only ribosomes which preserve the ability to synthesize protein *in vitro*. This leads one to suspect that there is a series of successive events involved in protein synthesis, and that at any time we investigate a temporal cross-section of the process.

The exact determination of the rate of turnover of the messenger RNA should give information about the process of protein synthesis. This might be stoichiometric, in the sense that each messenger molecule functions only once in information transfer before it is destroyed. Its rate of turnover should then be the same as that of the nascent protein; but experiments to test this idea have been limited by difficulties in pool equilibration with nucleotide precursors.

It is a prediction of the hypothesis that the messenger RNA should be a simple copy of the gene, and its nucleotide composition should therefore correspond to that of the DNA. This appears to be the case in phage-infected cells[11,20], and recently Ycas and Vincent[21] have found a rapidly labelled RNA fraction with this property in yeast cells. If this turns out to be universally true, interesting implications for coding mechanisms will be raised.

One last point deserves emphasis. Although the details of the mechanism of information transfer by messenger are not clear, the experiments with phage-infected cells show unequivocally that information for protein synthesis cannot be encoded in the chemical sequence of the ribosomal RNA. Ribosomes are non-specialized structures which synthesize, at a given time, the protein dictated by the messenger they happen to contain. The function of the ribosomal RNA in this process is unknown and there are also no restrictions on its origin in the cell: it may be synthesized by nuclear genes or by enzymes or it may be endowed with self-replicating ability.

This work was initiated while two of us (S. B. and F. J.) were guest investigators in the Division of

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UNSTABLE RIBONUCLEIC ACID REVEALED BY PULSE LABELLING OF *ESCHERICHIA COLI*

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WHEN *Escherichia coli* cells are infected with *T* even bacteriophage particles, synthesis of host proteins stops[1], and much if not all new protein synthesis is phage specific[2]. This system thus provides an ideal model for observing the synthesis of new proteins following the introduction of specific DNA. In particular, we should expect the appearance of phage-specific RNA, since it is generally assumed that DNA is not a direct template for protein synthesis but that its genetic information is transmitted to a specific sequence of bases in RNA. It was thus considered paradoxical when it was first noticed[3] that, following infection by the *T* even phages, net RNA synthesis stops even though protein synthesis continues at the rate of the uninfected bacterium. This could mean that DNA sometimes serves as a direct template for protein synthesis. Alternatively, net RNA synthesis may not be necessary so long as there exists the synthesis of a genetically specific RNA that turns over rapidly. This possibility was first suggested by experiments of Hershey[4], who, in 1953, reported that *T*2 infected cells contain a metabolically active RNA fraction comprising about 1 per cent of the total RNA. Several years later, Volkin and Astrachan[5] reported that this metabolic RNA possessed base ratios similar, if not identical (considering uracil formally equivalent to thymine), to those of the infecting *T*2 DNA. By 1958 they[6] extended their observation to *T*7 infected cells, where again the RNA synthesized after phage infection had base ratios similar to those of the phage DNA.

During these years, evidence[7] accumulated that the sites of much, if not all, protein synthesis are the ribosomal particles, and it was thought most likely that ribosomal RNA was genetically specific, with each ribosome possessing a base sequence which coded for a specific amino-acid sequence (one ribosome–one protein hypothesis). Direct verification of this hypothesis was lacking, and its proponents[8] were troubled by the fact that, except for phage-specific RNA, it was impossible to find any correlation within a given organism between the base ratios of DNA and RNA. Moreover, there was no evidence that phage-specific RNA was ribosomal RNA.

Nomura, Hall and Spiegelman[9] have recently discovered that following *T*2 infection there is no synthesis of typical (see below) ribosomal RNA and that the phage-specific RNA sediments at a slower rate (8*s*) than ribosomal RNA (16*s* and 23*s*). The genetic information for the synthesis of phage-specific proteins does not reside in the usual ribosomal RNA. Instead, if we assume that the synthesis of phage-specific proteins also occurs on ribosomes, then the phage-specific RNA might be viewed as a 'messenger' (to use the terminology of Monod and Jacob[10]) which carries the genetic information to the ribosomes. Furthermore, unless we postulate that there exist two different mechanisms for protein synthesis, there should also exist within uninfected normal cells RNA molecules physically similar to the phage-specific RNA and having base ratios similar to its specific DNA.

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