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The involvement of RNA in the synthesis of proteins

Nobel Lecture, December 11, 1962

Prologue

I arrived in Cambridge in the fall of 1951. Though my previous interests were largely genetic, Luria had arranged for me to work with John Kendrew. I was becoming frustrated with phage experiments and wanted to learn more about the actual structures of the molecules which the geneticists talked about so passionately. At the same time John needed a student and hoped that I should help him with his X-ray studies on myoglobin. I thus became a research student of Clare College with John as my supervisor.

But almost as soon as I set foot in the Cavendish, I inwardly knew I would never be of much help to John. For I had already started talking with Francis. Perhaps even without Francis, I would have quickly bored of myoglobin. But with Francis to talk to, my fate was sealed. For we quickly discovered that we thought the same way about biology. The center of biology was the gene and its control of cellular metabolism. The main challenge in biology was to understand gene replication and the way in which genes control protein synthesis. It was obvious that these problems could be logically attacked only when the structure of the gene became known. This meant solving the structure of DNA. Then this objective seemed out of reach to the interested geneticists. But in our cold, dark Cavendish lab, we thought the job could be done, quite possibly within a few months. Our optimism was partly based on Linus Pauling's feat[1] in deducing the α-helix, largely by following the rules of theoretical chemistry so persuasively explained in his classical *The Nature of the Chemical Bond.* We also knew that Maurice 'Wilkins had crystalline X-ray diffraction photographs from DNA and so it must have a well-defined structure. There was thus an answer for somebody to get.

During the next eighteen months, until the double-helical structure became elucidated, we frequently discussed the necessity that the correct structure have the capacity for self-replication. And in pessimistic moods, we often worried that the correct structure might be dull. That is, it would

suggest absolutely nothing and excite us no more than something inert like collagen.

The finding of the double helix[2] thus brought us not only joy but great relief. It was unbelievably interesting and immediately allowed us to make a serious proposal[3] for the mechanism of gene duplication. Furthermore, this replication scheme involved thoroughly understood conventional chemical forces. Previously, some theoretical physicists, among them Pascual Jordan[4], had proposed that many biological phenomena, particularly gene replication, might be based on still undiscovered long-range forces arising from quantum mechanical resonance interactions. Pauling[5] thoroughly disliked this conjecture and firmly insisted that known short-range forces between complementary surfaces would be the basis of biological replication.

The establishment of the DNA structure reinforced our belief that Pauling's arguments were sound and that long-range forces, or for that matter any form of mysticism, would not be involved in protein synthesis. But for the protein replication problem mere inspection of the DNA structure then gave no immediate bonus, This, however, did not worry us since there was much speculation that RNA, not DNA, was involved in protein synthesis.

Introduction

The notion that RNA is involved in protein synthesis goes back over twenty years to the pioneering experiments of Brachet and Caspersson[6] who showed that cells actively synthesizing protein are rich in RNA. Later when radioactive amino acids became available, this conjecture was strengthened by the observation[7] that the cellular site of protein synthesis is the microsomal component, composed in large part of spherical particles rich in RNA. Still later experiments* revealed that these ribonucleoprotein particles (now conveniently called ribosomes), not the lipoprotein membranes to which they are often attached, are the sites where polypeptide bonds are made. Most ribosomes are found in the cytoplasm and correspondingly most cellular protein synthesis occurs without the direct intervention of the nuclear-located DNA. The possibility was thus raised that the genetic specificity present in DNA is first transferred to RNA intermediates which then function as templates controlling assembly of specific amino acids into proteins.

We became able to state this hypothesis in more precise form when the structure of DNA became known in 1953. We then realized that DNA's

genetic specificity resides in the complementary base sequences along its two intertwined chains. One or both of these complementary chains must serve as templates for specific RNA molecules whose genetic information again must reside in specific base sequences. These RNA molecules would then assume 3-dimensional configurations containing surfaces complementary to the side groups of the 20 specific amino acids.

X-ray Studies on RNA and RNA-containing Viruses

The direct way to test this hypothesis was to solve the RNA structure. Already in 1952, I had taken some preliminary X-ray diffraction pictures of RNA. These, however, were very diffuse, and it was not until I returned to the United States in the fall of 1953 that serious X-ray studies on RNA began. Alexander Rich and I, then both at the California Institute of Technology, obtained RNA samples from various cellular sources. We[9] were first very encouraged that all the RNA samples, no matter their cellular origin, give similar X-ray diffraction pattern. A general RNA structure thus existed; This gave us hope that the structure, when solved, would be interesting. Our first pictures already showed large systematic absence of reflections on the meridian, suggesting a helical structure. But despite much effort to obtain native undegraded high molecular weight samples, no satisfactory X-ray diffraction pattern was obtained. The reflections were always diffuse, no evidence of crystallinity was seen. Though there were marked similarities to the DNA pattern, we had no solid grounds for believing that these arose from a similar helical molecule. The problem whether RNA was a one- or several-chained structure remained unanswered.

We then considered the possibility that RNA might have a regular structure only when combined with protein. At that time (1955) there was no good evidence for RNA existing free from protein; All RNA was thought to exist either as a viral component or to be combined with protein in ribonucleoprotein particles. It thus seemed logical to turn attention to a study of ribonucleoprotein particles (ribosomes) since upon their surfaces protein was synthesized. Our hope again was that the establishment of their structure would reveal the long-sought-after cavities specific for the amino acids.

Then we were struck by the morphological similarity between ribosomes and small RNA-containing viruses like Turnip Yellow Mosaic Virus or Poliomyelitis Virus. By then (1955-1956) I was back in Cambridge with

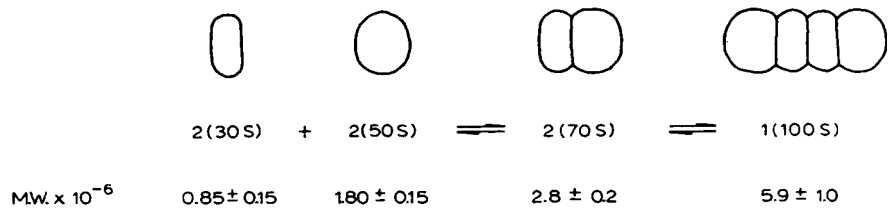
Crick to finish formulating some general principles on viral structure[10]. Our main idea was that the finite nucleic acid content of viruses severely restricted the number of amino acids they could code for. As a consequence, the protein coat could not be constructed from a very large number of different protein molecules. Instead it must be constructed from a number of identical small sub-units arranged in a regular manner. These ideas already held for Tobacco Mosaic Virus, a rod-shaped virus, and we were very pleased when D. L. D. Caspar[11], then working with us at the Cavendish, took some elegant diffraction pictures of Bushy Stunt Virus crystals and extended experimental support to the spherical viruses.

Structural Studies on Ribosomes

At that time almost no structural studies had been done with ribosomes. They were chiefly characterized by their sedimentation constants; those from higher organisms[12] in the 70s-80s range, while those from bacteria[13] appeared smaller and to be of two sizes (30s and 50s). Because the bacterial particles seemed smaller, they seemed preferable for structural studies. Thus when Alfred Tissières and I came to Harvard's Biological Laboratories in 1956, we initiated research on the ribosomes of the commonly studied bacteria *Escherichia coli.* We hoped that their structure would show similarities with the small spherical RNA viruses. Then we might have a good chance to crystallize them and to eventually use X-ray diffraction techniques to establish their 3-dimensional structure.

Ribosome sub-units

But from the beginning of our Harvard experiments, it was obvious that ribosome structure would be more complicated than RNA virus structure. Depending upon the concentration of divalent cations (in all our experiments Mg^{++}), 4 classes of *E. coli* ribosomes were found, characterized by sedimentation constants of 30s, 50s, 70s and 100s. Our first experiments in 10^{-4} *M* Mg^{++} revealed 30s and 50s ribosomes. At the same time Bolton[14], at the Carnegie Institute of Washington employing higher Mg++ levels, saw faster sedimenting ribosomes and suggested that they were observing aggregates of the smaller particles. Soon after, our experiments's revealed that, as the Mg^{++} concentration is raised, one 30s particle and one 50s particle



All particles are composed of 64%. RNA and 36% protein

Fig. 1. Diagrammatic representation of *E. coli* ribosome sub-units and their aggregation products. (The molecular weight data are from Tissières *et al.*[15])

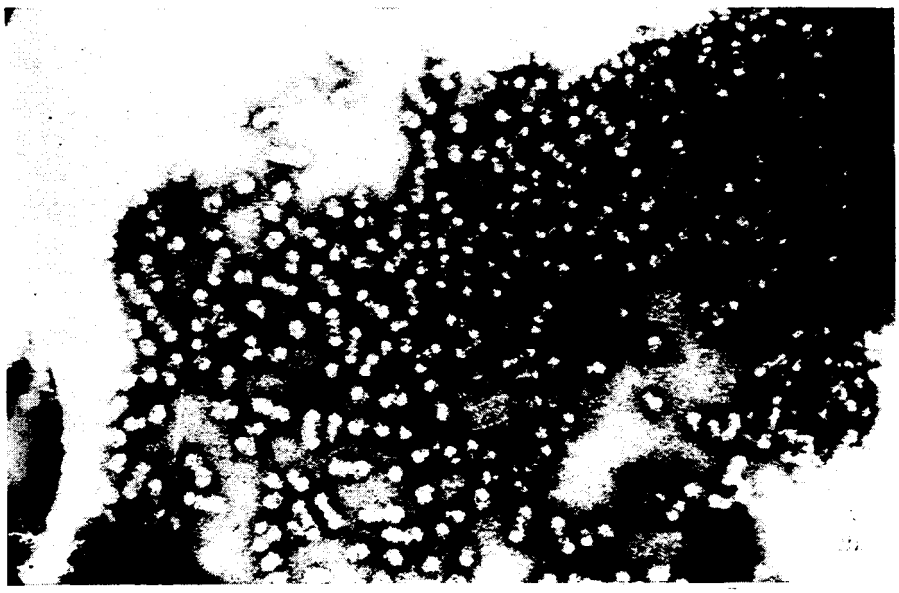


Fig. 2. Electron micrograph of negatively stained *E. coli* ribosomes (Huxley and Zubay's). Two particle types are predominant: (1) 70s containing two sub-units of unequal size, and (2) 100s consisting of two 70s ribosomes joined together at their smaller (30s) sub-units.

combine to form a 70s ribosome. At still higher Mg^{++} concentrations, two 70s ribosomes dimerize to form a 100s ribosome. (Figs. 1 and 2).

Ribosomes from every cellular source have a similar sub-unit construction. As with *E. coli* ribosomes, the level of divalent cations determines which ribosomes exist, Bacterial ribosomes seem to require higher Mg^{++} levels in order to aggregate into the larger sizes. Conversely they break down much

faster to the 30s and 50s forms when the Mg^{++} level is lowered. It is often convenient[16] when using mammalian ribosomes to add a chelating agent to rapidly break down the 80s ribosomes (homologous to the 70s ribosomes of bacteria) to their 40s and 60s sub-units. Bacterial ribosomes are thus not significantly smaller than mammalian ribosomes. It is merely easier to observe the smaller sub-units in bacterial systems.

Ribosomal RNA

Already in 1958 there were several reports[17] that ribosomal RNA from higher organisms sedimented as two distinct components (18s and 28s). We thought that the smaller molecules most likely arose from the smaller sub-unit while the faster sedimenting RNA came from the larger of the ribosomal sub-units. Experiments of Mr. Kurland[18] quickly confirmed this hunch. The *E. coli* 30s ribosome was found to contain one RNA chain (16s) with a molecular weight of 5.5×10^5. Correspondingly a larger RNA molecule (23s) of mol. wt. 1.1×10^6 was found in most 50s ribosomes (Fig. 3).

Ribosome proteins

Analysis of the protein component revealed a much more complicated picture. In contrast to the small RNA viruses, where the protein coat is constructed from the regular arrangement of a large number of identical protein molecules, each ribosome most likely contains a large number of different polypeptide chains. At first, our results suggested a simple answer when Drs. Waller and J. I. Harris analysed *E. coli* ribosomes for their amino terminal groups. Only alanine, methionine, with smaller amounts of serine, were present in significant amounts. This hinted that only several classes of protein molecules were used for ribosomal construction. Further experiments of Dr. Waller[19], however, suggested the contrary. When ribosomal protein fractions were analysed in starch-gel electrophoresis, more than 20 distinct bands were seen. Almost all these proteins migrated towards the anode at pH 7 confirming the net basic charge of ribosomal protein[20]. A variety of control experiments suggested that these bands represent distinct polypeptide chains, not merely aggregated states of several fundamental sub-units. Moreover, the band pattern from 30s ribosomes was radically different from that of 50s proteins.

As yet we have no solid proof that each 70s ribosome contains all the

various protein components found in the total population. But so far, all attempts by Dr. Waller to separate chromatographically intact ribosomes into fractions with different starch-gel patterns have failed. The total protein component of a 70s ribosome amounts to about 9 x 10^5 daltons. Since the end group analysis suggests an average mol. wt. of about 30,000, approximately 20 polypeptide chains are used in 50s construction and 10 for the 30s ribosome. It is possible that all the polypeptide chains in a 30s particle are different. Waller already has evidence for 10 distinct components in 30s ribosomes and the present failure to observe more in the 50s protein fraction may merely mean that the same electrophoretic mobility is shared by several polypeptide chains.

We believe that all these proteins have primarily a structural role. That is, they are not enzymes but largely function to hold the ribosomal RNA and necessary intermediates in the correct position for peptide bond formation. In addition a number of enzymes are bound tightly to ribosomes. As yet their function is unclear. One such is a bacterial ribonuclease, found by Elsonar to be specifically attached to 30s ribosomes in a latent form. No ribonuclease activity is present until ribosome breakdown. Dr. Spahr[22] in our laboratories has purified this enzyme, shown its specificity and from specific activity measurements, concludes that it is present on less than one in twenty

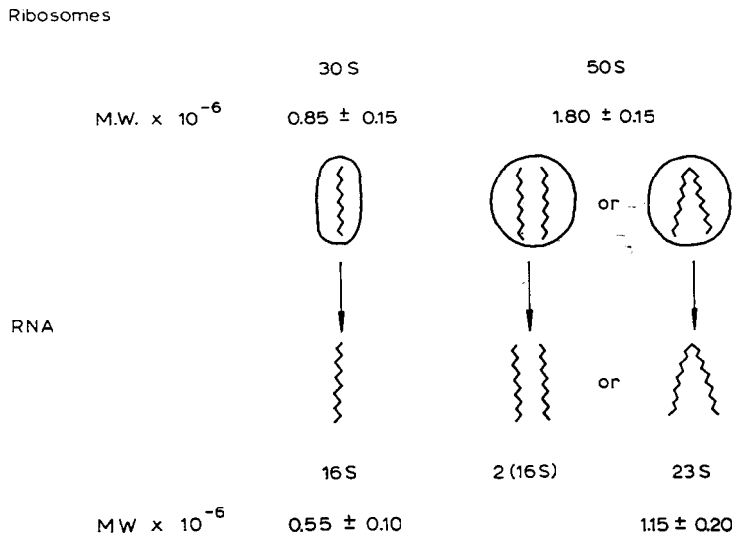


Fig. 3. Molecular weights of RNA isolated from *E. coli* ribosomes. (This picture is diagrammatic and does not represent the true conformation of ribosomal RNA.)

30s particles. It is clear that this enzyme ifpresent in a free active form, would be rapidly lethal to its host cell. Thus its presence in latent form is expected. But why it is stuck to ribosomes is still a complete mystery.

Chemical Intermediates in Protein Synthesis

Our early experiments with ribosomes were almost unrelated to the efforts of biochemists. At that time our research objects seemed very different. The enzymologically oriented biochemists hoped to find the intermediates and enzymes necessary for peptide bond formation. On the contrary, those of us with a genetic orientation wanted to see the template and discover how it picked out the correct amino acid. Very soon, however, these separate. paths came together, partly because of a breakthrough in the nature of the amino acid intermediates, and partly from an incisive thought by Crick.

The biochemical advances arose from work in Paul Zamecnik's laboratory at the Massachusetts General Hospital. There was developed a reproducible *in vitro* system[23] containing ribosomes, supernatant factors, and ATP which incorporated amino acids into protein. Using these systems Hoagland made two important discoveries. Firstly, he[24] showed that amino acids are initially activated by ATP to form high-energy AA-AMP complexes. Secondly, he demonstrated[25] that the activated amino acids are then transferred to low molecular weight RNA molecules (now known as soluble or transfer RNA), again in an activated form. These amino-acyl-sRNA compounds then function as the direct intermediate for peptide bond formation (Fig. 4).

It had previously been obvious that amino acid activation would have to occur. However, Hoagland's second discovery (in 1956) of the involvement of a hitherto undiscovered RNA form (sRNA) was unanticipated by almost everybody. Several years previously (in 1954), Leslie Orgel and I spent a quite frustrating fall attempting to construct hypothetical RNA structures which contained cavities complementary in shape to the amino acid side groups. Not only did plausible configurations for the RNA backbone fail to result in good cavities, but even when we disregarded the backbone, we also failed to find convincing holes which might effectively distinguish between such amino acids as valine and isoleucine. Crick, at the same time (early 1955) sensed the same dilemma, and suggested a radical solution to the paradox. He proposed[26] that the amino acids do not combine with the template. Instead each should first combine with a specific adaptor molecule,

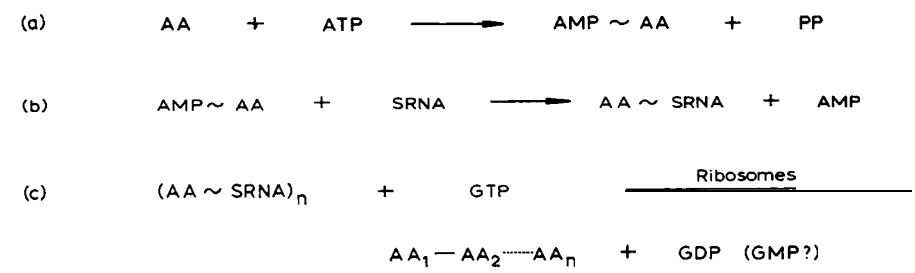


Fig. 4. Enzymatic steps in protein peptide bond formation. Steps (a) and (b) are catalyzed by single enzymes. The number of enzymes required in (c) is unknown.

capable of selectively interacting with the hydrogen bonding surfaces provided by RNA's purine and pyrimidine bases. This scheme requires at least twenty different adaptors, each specific for a given amino acid. These are very neatly provided by the specific sRNA molecules. Soon after Hoagland's discovery of sRNA, many experiments, particularly by Hoagland and Paul Berg[27], established that the sRNA molecules are in fact specific for a given amino acid. It thus became possible to imagine, following Crick's reasoning, that the ribosomal template for protein synthesis combined not with the amino acid side groups, but instead with a specific group of bases on the soluble RNA portion of the amino-acyl-sRNA precursors.

Participation of Active Ribosomes in Protein Synthesis

Very little protein synthesis occurred in the cell-free system developed by the Massachusetts General Hospital Group. Only by using radioactive amino acids could they convincingly demonstrate amino acid incorporation into proteins. This fact, initially seemed trivial and there was much hope that when better experimental conditions were found, significant net synthesis would occur. But despite optimistic claims from several laboratories, no real improvement in the efficiency of cell-free synthesis resulted. Some experiments (1959) of Dr. Tissières and Mr. Schlessinger[28] with *E. coli* extracts illustrate well this point. At 30°C, cell-free synthesis occurs linearly for 5-10 minutes and then gradually stops. During this interval the newly synthesized protein amounts to 1-3 γ of protein per mg of ribosomes. Of this about one third was released from the ribosomes, the remainder being ribosomal bound.

Cell-free synthesis in *E. coli* extracts requires the high ($\sim 10^{-2} M$) Mg^{++}

levels which favor the formation of 70s ribosomes from their 30s and 50s sub-units. Following incorporation, those ribosomes possessing nascent polypeptide chains become less susceptible to breakdown to 30s and 50s ribosomes. When cell-free extracts (following synthesis) are briefly dialyzed against 10^{-4} *M* Mg^{++}, about 80-90% of the 30s and 50s ribosomes become free. There remain, however, 10-20% of the original 70s ribosomes and it is upon these "stuck" ribosomes that most ribosomal bound nascent protein is located. This firstly suggests that protein synthesis occurs on 70s ribosomes, not upon free 30s or 50s ribosomes. Secondly, in the commonly studied *E. coli* extract, only a small ribosome fraction is functional. Tissières and Schlessinger named these particles "active ribosomes" and suggested, they contained a functional component lacking in other ribosomes.

Each active ribosome synthesizes on the average between 15,000 and 50,000 daltons of protein. This is in the size range ofnaturally occurring polypeptide chains. Thus while we remained unsatisfied by the small net synthesis, sufficient synthesis occurs to open the possibility that some complete protein molecules are made. This encouraged us to look for synthesis of β-galactosidase. None, however, was then found[29] despite much effort.

Another important point emerged from these early (1959) incorporation studies with *E. coli* extracts. Addition of small amounts of purified deoxyribonuclease decreased protein synthesis to values 20-40% that found in untreated extract[28]. This was completely unanticipated, for it suggested that high molecular weight DNA functions in the commonly studied bacterial extracts. But since a basal synthetic level occurs after DNA is destroyed by deoxyribonuclease, the DNA itself must not be directly involved in peptide bond formation. Instead, this suggested synthesis of new template RNA upon DNA in untreated extracts. If true, this would raise the possibility, previously not seriously considered by biochemists that the RNA templates themselves might be unstable, and hence a limiting factor in cell-free protein synthesis.

Metabolic Stability of Ribosomal RNA

All our early ribosome experiments had assumed that the ribosomal RNA was the template. Abundant evidence existed that proteins were synthesized on ribosomes and since the template must be RNA, it was natural to assume that it was ribdsomal RNA. Under this hypothesis ribosomal RNA was a

collection of molecules of different base sequences, synthesized on the functioning regions of chromosomal DNA. Following their synthesis, they combined with the basic ribosomal proteins to form ribosomes. We thus visualized that the seemingly morphological identical ribosomes were, in fact, a collection of a very large number of genetically distinct particles masked by the similarity of their protein component.

Then there existed much suggestive evidence that ribosomal RNA molecules were stable in growing bacteria. As early as 1949, experiments showed that RNA precursors, once incorporated into RNA, remained in RNA. Then the distinction between ribosomal and soluble RNA was not known, but later experiments by the ribosome group of the Carnegie Institute of Washington and at Harvard indicated similar stabilities of both fractions. These experiments, however, did not follow the fate of single molecules, and the possibility remained that a special trick allowed ribosomal RNA chains to be broken down to fragments that were preferentially re-used to make new ribosomal RNA molecules. Davern and Meselson[30], however, ruled out this possibility by growing ribosomal RNA in heavy (^{13}C,^{15}N) medium, followed by several generations of growth in light (^{12}C, ^{14}N) medium. They then separated light from heavy ribosomal RNA in cesium formate density gradients and showed that the heavy molecules remained completely intact for at least two generations. This result predicts, assuming ribosomes to be genetically specific, that the protein templates should persist indefinitely in growing bacteria.

Experiments Suggesting Unstable Protein Templates

But already by the time of the Davern & Meselson experiment (1959), evidence began to accumulate, chiefly at the Institut Pasteur, that some, if not all, bacterial templates were unstable with lives only several per cent of a generation time. None of these experiments, by themselves, were convincing. Each could be interpreted in other ways which retained the concept of stable templates. But taken together, they argued a strong case.

These experiments were of several types. One studied the effect of suddenly adding or destroying specific DNA molecules. Sudden introduction was achieved by having a male donor introduce a specific chromosomal region absent in the recipient female. Simultaneously the ability of the male gene to function (produce an enzymatically active protein) in the female cell

was measured. Riley, Pardee, Jacob, and Monod[31] obtained the striking finding that β-galactosidase, genetically determined by a specific male gene, began to be synthesized at its maximum rate within several minutes after gene transfer. Thus the steady state number of β-galactosidase templates was achieved almost immediately. Conversely when the *E. coli* chromosome was inactivated by decay of ^{32}P atoms incorporated into DNA, they observed that active enzyme formation stops within several minutes. It thus appeared that the ribosomal templates could not function without concomitant DNA function.

At the same time, François Gros discovered[32] that bacteria grown in 5-fluorouracil produced abnormal proteins, most likely altered in amino acid sequences. 5-Fluorouracil is readily incorporated into bacterial RNA and its presence in RNA templates may drastically raise the mistake level. More unexpected was the observation that following 5-fluorouacil addition the production of all normal proteins ceases within several minutes. Again this argues against the persistance of *any* stable templates.

Unstable RNA Molecules in Phage Infected Cells

At first it was thought that no RNA synthesis occurred in T2 infected cells. But in 1952 Hershey[33] observed that new RNA molecules are synthesized at a rapid rate. But no net accumulation occurs since there is a correspondingly fast breakdown. Surprisingly almost everybody ignored this discovery. This oversight was partly due to the tendency, still then prevalent, to suspect that the metabolism of virus infected cells might be qualitatively different from that of uninfected cells.

Volkin and Astrachan[34] were the first (1956) to treat Hershey's unstable fraction seriously. They measured its base composition and found it different from that of uninfected *E. coli* cells. It bore a great resemblance to the infecting viral DNA which suggested that it was synthesized on T2 DNA templates. Moreover, and *most importantly,* this RNA fraction must be the template for phage specific proteins. Unless we assume that RNA is not involved in phage protein synthesis, it necessarily follows that the Volkin-Astrachan DNA-like RNA provides the information for determining amino acid sequences in phage specific proteins.

Not till the late summer of 1959 was its physical form investigated. Then Nomura, Hall, and Spiegelmanss examined its relationship to the already

characterized soluble and ribosomal RNA's. Immediately they observed that none of the T2 RNA was incorporated into stable ribosomes. Instead, in low Mg^{++} (10^{-4} *M*) it existed free while in 10^{-2} *M* Mg^{++} they thought it became part of 30s ribosomal like particles. At the same time, Mr. Risebrough in our laboratories began studying T2 RNA, also using sucrose gradient centrifugation. He also found that T2 RNA was not typical ribosomal RNA. In addition, he was the first to notice (in early spring 1960) that in 10^{-2} *M* Mg^{++}, most T2 RNA sedimented not with 30s particles but with the larger 70s and 100s ribosomes.

His result leads naturally to the hypothesis that phage protein synthesis takes place on genetically non-specific ribosomes to which are attached metabolically unstable template RNA molecules. Independently of our work, Brenner and Jacob motivated by the above-mentioned metabolic and genetic experiments from the Institut Pasteur, were equally convinced that conditions were ripe for the direct demonstration of metabolically unstable RNA templates to which Jacob and Monod[36] gave the name *messenger RNA*. In June of 1960, they travelled to Pasadena for a crucial experiment in Meselson's laboratory. They argued that all the T2 messenger RNA should be attached to old ribosomes synthesized before infection. This they elegantly demonstrated[37] by T2 infecting heavy (^{13}C and ^{15}N) labeled bacteria in light (^{12}C and ^{14}N) medium. Subsequent CsCl equilibrium centrifugation revealed that most of the T2 messenger RNA was indeed attached to "old" ribosomes, as was all the ribosomal bound nascent protein, labeled by pulse exposure to radioactive amino acids.

Demonstration of Messenger RNA Molecules in Uninfected Bacteria

We were equally convinced that similar messenger RNA would be found in uninfected bacteria. Its demonstration then presented greater problems, because of the simultaneous synthesis of ribosomal and soluble RNA. François Gros had then (May 1960) just arrived for a visit to our laboratory. Together with Mr. Kurland and Dr. Gilbert, we decided to look for labeled messenger molecules in cells briefly exposed to a radioactive RNA precursor. Experiments with T2 infected cells suggested that the T2 messenger comprised about 2-4% of the total RNA and that most of its molecules had lives less than several minutes. If a similar situation, held for uninfected cells, then during any short interval, most RNA synthesis would be messenger. There

would be no significant accumulation since it would be broken down almost as fast as it was made.

Again the messenger hypothesis was confirmed[38]. The RNA labeled during pulse exposures was largely attached to 70s and 100s ribosomes in 10^{-2} *M* Mg^{++}. In low Mg^{++} (10^{-4} *M*), it came off the ribosomes and sedimented free with an average sedimentation constant of 14s. Base ratio analysis revealed DNA like RNA molecules in agreement with the expectation that it was produced on very many DNA templates along the bacterial chromosome. Soon afterwards, Hall and Spiegelman[39] formed artificial T_2 DNA; T_2 messenger RNA hybrid molecules and in several laboratories[40], hybrid molecules were subsequently formed between *E. coli* DNA and *E. coli* pulse RNA. The DNA template origin for messenger RNA was thus established beyond doubt.

The Role of Messenger RNA in Cell-Free Protein Synthesis

It was then possible to suggest why deoxyribonuclease partially inhibits amino acid incorporation in *E. coli* extracts. The messenger hypothesis prompts the idea that DNA in the extract is a template for messenger RNA. This newly made messenger then attaches to ribosomes where it serves as additional protein templates. Since deoxyribonuclease only destroys the capacity to make messenger, it has no effect upon the messenger present at the time of extract formation. Hence, no matter how high the deoxyribonuclease concentration employed, a residual fraction of synthesis will always occur. Experiments by Tissières and Hopkins[41] in our laboratories and by Berg, Chamberlain, and Wood[42] at Stanford confirmed these ideas. First it was shown that addition of DNA to extracts previously denuded of DNA significantly increased amino acid incorporation. Secondly, RNA synthesis occurs simultaneously with *in vitro* protein synthesis. This RNA has a DNA like composition, attaches to ribosomes in 10^{-2} *M* Mg^{++}, and physically resembles *in vivo* synthesized messenger RNA.

Furthermore, Tissières showed that addition of fractions rich in messenger RNA stimulated *in vitro* protein synthesis 2-5 fold. More striking results came from Nirenberg and Matthaei[43]. They reasoned that *in vitro* messenger destruction might be the principal cause why cell-free systems stopped synthesizing protein. If so, *preincubated extracts* deficient in natural messenger should respond more to new messenger addition. This way they became

able to demonstrate a 20-fold increase in protein synthesis following addition of phenol-purified *E. coli* RNA. Like Tissières' active fraction, their stimulating fraction sedimented heterogeneously arguing against an effect due to either ribosomal or soluble RNA. More convincing support came when they next added TMV RNA to preincubated *E. coli* extracts. Again a 10-20 fold stimulation occurred. Here there could be no confusion with possible ribosomal RNA templates. Even more dramatic[44] was the effect of polyuridylic acid (like TMV RNA single stranded) addition. This specifically directed the incorporation of phenylalanine into polyphenylalanine. With this experiment (June 1961) the messenger concept became a fact. Direct proof then existed that single stranded messenger was the protein template.

Presence of Messenger RNA in Active Ribosomes

In *in vitro* systems ordinarily only 10-20% of *E. coli* ribosomes contain attached messenger RNA. This first was shown in experiments of Risebrough's who centrifuged extracts of T2 infected cells through a sucrose gradient. Ribosomes containing labeled messenger were found to centrifuge faster than ordinary ribosomes. Similarly, Gilbert[46] showed that these faster sedimenting ribosomes are "active", that is, able to incorporate amino acids into proteins. A fresh cell-free extract was centrifuged through a sucrose gradient. Samples along the gradient were collected and then tested for their ability to make protein. A complete parallel was found between "activity" and the presence of messenger.

Furthermore, if an extract is centrifuged *after* it has incorporated amino acids, the nascent protein chains also sediment attached to a small fraction of fast sedimenting ribosomes[45]. These ribosomes still contain messenger RNA. For when the messenger molecules are destroyed by ribonucleae (ribosomes remain intact in the presence of γ amounts of ribonuclease), the ribosomal bound nascent protein sediments as 70s ribosomes. The nascent proteinis thus not attached to messenger RNA but must be directly bound to ribosomes.

Binding of sRNA to Ribosomes

Experiments by Schweet[47] and Dintzes[48] show that proteins grow by stepwise addition of individual amino acids beginning at the ammo terminal end.

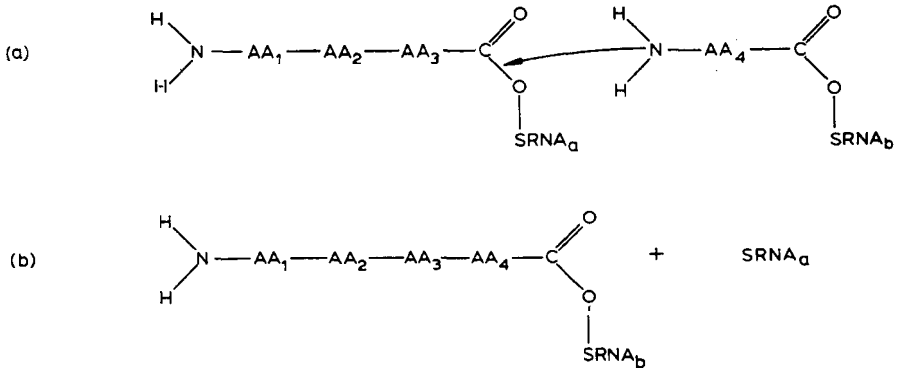


Fig. 5. Stepwise growth of a polypeptide chain. Initiation begins at the free NH, end with the growing point terminated by a sRNA molecule.

Since the immediate precursors are amino-acyl-sRNA molecules, their result predicts that the polypeptide chain is terminated at its carboxyl growing end by an sRNA molecule (Fig. 5). To test this scheme, we began some studies to see whether sRNA bound specifically to ribosomes. Cannon and Krug[49] first examined binding in the absence of protein synthesis. They showed that in 10^{-2} *M* Mg^{++} each 50s sub-unit of the 70s ribosome reversibly bound one sRNA molecule. The same amount of reversible binding occurs with amino-acyl-sRNA or with free sRNA and in the presence or absence of protein synthesis.

Protein synthesis, however, effects the binding observed in 10^{-4} *M* Mg++. In the absence of protein synthesis no sRNA remains ribosomal bound when the Mg^{++} level is lowered from 10^{-2} *M* to 10^{-4} *M*. On the contrary, following amino acid incorporation, sRNA molecules become tightly fixed to the "stuck" 70s ribosomes, whose nascent polypeptide chains prevent easy dissociation to 30s and 50s ribosomes. One sRNA molecule appears to be attached to each stuck ribosome. Prolonged dialysis against 10^{-4} *M* Mg^{++} eventually breaks apart the stuck ribosomes. Then all the bound sRNA as well as almost all the nascent protein is seen attached to the 50s component supporting the hypothesis that these bound sRNA molecules are directly attached to nascent chains (Fig. 6). Direct proof comes from recent experiments in which Gilbert[50] used the detergent duponol to further dissociate the 50s ribosomes to their protein and RNA components. Then the nascent protein and bound sRNA remained together during both sucrose gradient centrifugation and separation on G200 Sephadex columns. Following ex-

posure, however, to either weak alkali or to hydroxylamine, treatments known to break amino-acyl-bonds, the sRNA and nascent proteins move separately.

The significance of the reversible binding by non-active (no messenger) ribosomes is not known. Conceivably inside growing cells, all ribosomes have attached messenger and synthesize protein. Under these conditions, only those sRNA molecules corresponding to the specific messenger sequence can slip into the ribosomal cavities. But when most ribosomes lack messenger templates, as in our in *vitro* extracts, then any sRNA molecule, charged or uncharged, may fill the empty site.

All evidence suggests that covalent bonds are not involved in holding nascent chains to ribosome. Instead it seems probable that the point of firm attachment involves the terminal sRNA residue, bound by Mg^{++} dependent

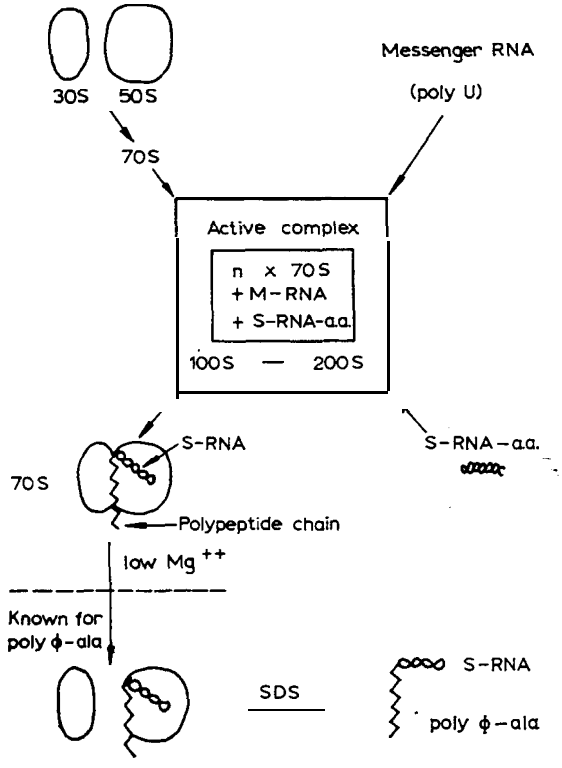


Fig. 6. Diagrammatic summary of ribosome participation in protein synthesis. (The active complex is pictured in Fig. 7.)

secondary forces to a cavity in the 50s ribosome. Extensive dialysis against $5 \times 10^{-5} M$ Mg^{++} (which leaves intact 30s and 50s ribosomes) strips the nascent chains off the 50s ribosomes[50,51]. The released polypeptides sediment about 4s and if the latent ribonuclease is not activated, most likely still have terminally bound sRNA. When the Mg^{++} level is again brought to $10^{-2} M$ many released chains again stick to ribosomes.

Movement of the Messenger Template over the Ribosomal Surface

At any given time, each functioning ribosome thus contains only one nascent chain. As elongation proceeds, the NH_3-terminal end moves away from the point of peptide bond formation and conceivably may assume much of its final three-dimensional configuration before the terminal amino acids are added to the carboxyl end. The messenger RNA must be so attached that only the correct amino-acyl-sRNA molecules are inserted into position for possible peptide bond formation. This demands formation of specific hydrogen bonds (base-pairs?) between the messenger template and several (most likely three) nucleotides along the sRNA molecule. Then, in the presence of the necessary enzymes, the amino-acyl linkage to the then terminal sRNA breaks and a peptide bond forms with the correctly placed incoming amino-acyl-RNA (Fig.5). This must create an energetically unfavorable environment for the now free sRNA molecule, causing it to be ejected from the sRNA binding site. The new terminal sRNA then moves into this site completing a cycle of synthesis. It is not known whether the messenger template remains attached to the newly inserted amino-acyl-sRNA. But if so, the messenger necessarily moves the correct distance over the ribosomal surface to place its next group of specific nucleotides in position to correctly select the next amino acid. No matter, however, what the mechanism is, the messenger tape necessarily moves over the ribosome. They cannot remain in static orientation if there is only one specific ribosomal site for peptide bond formation.

Attachment of Single Messenger RNA Molecules to Several Ribosomes

Addition of the synthetic messenger poly U to extracts containing predominantly 70s ribosomes creates new active ribosomes which sediment in

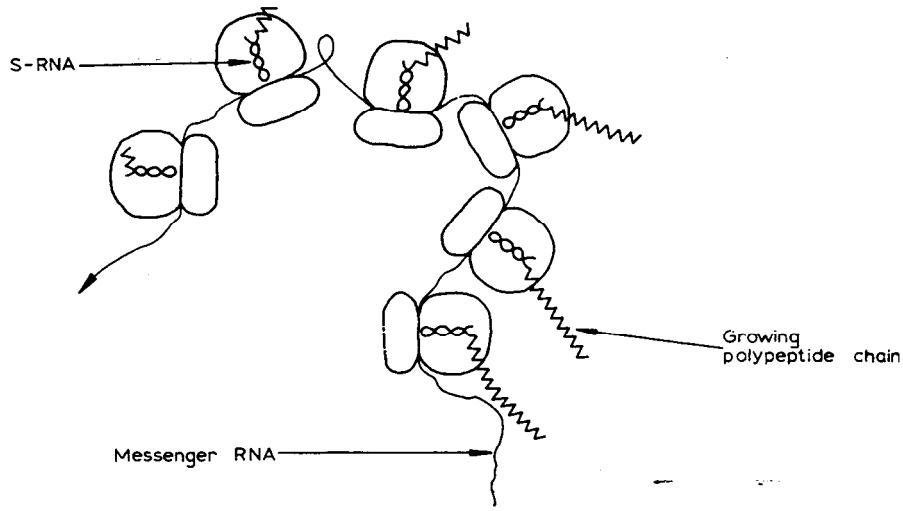


Fig. 7. Messenger RNA attachment to several ribosomes. (Thisillustration is schematic since the site of messenger attachment to ribosomes is not known.)

the 150-200s region[52]. Fixation of a single poly U molecule (mol. wt. = 100,000) to a 70s ribosome (mol. wt. = 3 x 10^6) should not significantly increase ribosomal sedimentation. Nor is it likely that a very large number of poly U molecules have combined with individual ribosomes. In these experiments, the molar ratio of fixed poly U to 70s ribosomes was less than $\frac{1}{5}$. Instead, the only plausible explanation involves formation of ribosomal aggregates attached to single poly U molecules. The 300 nucleotides in a poly U molecule of mol. wt. - 105 will have a contour length of about 1000 Å if the average intemucleotide distance is 3.4 Å. Simultaneous attachment is thus possible to groups of 4-8 ribosomes (diameter - 200 Å) depending upon the way the messenger passes over (through) the ribosomal surface. This estimate agrees well with the average aggregate size suggested by the sedimentation rate of the "active" complexes. Sedimentation of extracts *after* incorporation reveals most polyphenylalanine attached to the rapidly sedimenting "active" ribosomes.

Single messenger molecules thus most likely move simultaneously over the surfaces of several ribosomes, functioning on each as protein templates (Fig.7). A progression of increasingly long polypeptide chains should be attached to successive ribosomes depending upon the fraction of the messenger tape to which they were exposed. When all the messenger has moved across the site of synthesis, some mechanism, perhaps itself triggered by a

specific template nucleotide sequence must release the finished protein. The now vacant ribosome then becomes competent to receive the free end of another (or perhaps even the same) messenger molecule and start a new cycle of protein synthesis.

The realization that a single messenger molecule attaches to many ribosomes resolves a bothersome paradox which accompanied the messenger hypothesis. About 2-4% of E. *coli* RNA is messenger[40,53]. Its average sedimentation constant of 14s[54] suggests an average molecular weight about 500,000. This value may be too low since it is very difficult to completely prevent all enzymatic degradation. There thus must be *at least* 6-8 70s ribosomes for every messenger molecule. It was very difficult to believe that only 10-20% of the ribosomes functioned at a given moment. For, under a variety of conditions, the rate of protein synthesis is proportional to ribosome concentration[55]. Instead, it seems much more likely that, *in vivo,* almost all ribosomes are active. During the preparation of cell extracts, however, many ribosomes may lose their messenger and become inactive. If true, we may expect that use of more gentle techniques to break open *E. coli* cells will reveal larger fractions of fast-sedimenting active material. Already there are reports[56] that over 50% of mammalian reticulocyte ribosomes exist as aggregates of 5-6 80s particles. Furthermore, it is these aggregated ribosomes which make protein, both *in vivo* and *in vitro.*

Template Lifetime

Under the above scheme a messenger molecule might function indefinitely. On the contrary, however, the unstable bacterial templates function on the average only 10-20 times. This fact comes from experiments done in Levinthal's laboratory[57] where new messenger synthesis Gas blocked by addition of the antibiotic antinomycin D. Preexisting messenger *(Bacillus subtilus* growing with a 60 minute generation time) then broke down with a half-life of 2 minutes. Correspondingly, protein synthesis ceased at the expected rate. A mechanism(s) must thus exist to specifically degrade messenger molecules. Several enzymes (polynucleotide phosphorylase and a K+ dependent diesterase) which rapidly degrade free messenger are active in bacterial cell extract[58]. They function, however, much less efficiently when the messenger is attached to ribosomes[59]. Conceivably, a random choice exists whether the free forward-moving end of a messenger tape attaches to a vacant ribosome,

or is enzymatically degraded. If so, this important decision is settled by a chance event unrelated to the biological need for specific messengers.

Conclusion

We can now have considerable confidence that the broad features of protein synthesis are understood. RNA's involvement is very much more complicated than imagined in 1953. There is not one functional RNA. Instead, protein synthesis demands the ordered interaction of three classes of RNA - ribosomal, soluble, and messenger. Many important aspects, however, remain unanswered. For instance, there is no theoretical framework for the ribosoma1 sub-units nor, for that matter, do we understand the functional significance of ribosomal RNA. Most satisfying is the realization that all the steps in protein replication will be shown to involve well-understood chemical forces. As yet we do not know all the details. For example, are the DNA base-pairs involved in messenger RNA selection of the corresponding amino-acyl-sRNA? With luck, this will soon be known. We should thus have every expectation that future progress in understanding selective protein synthesis (and its consequences for embryology) will have a similar well-defined and, when understood, easy-to-comprehend chemical basis.

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