Molecular Biology Through Discovery
Companion to Brenner et al (1961) *An unstable intermediate…*
Nature 190:576-581

In March 1960, many of the luminaries of molecular biology met in Sydney Brenner's apartment to escape from a meeting in London that they were attending. By the end of the evening, our understanding of the connection between DNA and protein had been shattered and replaced with what we now call mRNA. You can read more about it if you like in The Discovery of mRNA, which describes the world through the eyes of Francis Crick, focusing on his view of an article by Belozersky and Spirin.

A. Summary of the state of affairs at the March 1960 meeting

Going into the March 1960 meeting, Crick and Brenner (and many others) held the following views:[1]

- DNA is the genetic material
- DNA resides exclusively in the nucleus of eukaryotes
- Ribosomes are the sites of protein synthesis
- Ribosomes reside exclusively in the cytoplasm
- Ribosomes contain RNA, the cytoplasmic sister of DNA
- Ribosomes are similar to certain viruses – RNA encased by a protein shell
- Ribosomes may act similarly as viruses – directing the synthesis of specific protein and perhaps even replicating themselves
- One ribosome is responsible for the synthesis of one protein

SQ1. Compare each of these views to your own. If any differ, replace it with a current belief concerning protein synthesis.

Certain recently published results were difficult to accommodate into this world view:

- Volkin and Astrachan (1956)[2] found that phage infection of E. coli led to the production of RNA whose base composition is different from that of the host DNA and similar to that of the phage DNA. However, this RNA was a very small fraction of total RNA, and so it was difficult to explain the fact that almost all protein produced was phage-specific protein.
- Belozersky and Spirin (1957)[3] found that the base composition of RNA is similar amongst a broad cross section of bacteria, but the base composition of their DNA is highly variable. If RNA is the mirror of DNA, then one would expect their compositions to be the same.

Jacob (**Fig. 1**) brought to the meeting the idea[4] developed by him and Monod that information for protein synthesis was conveyed from DNA via an unstable intermediate, which they eventually christened messenger RNA but at the time called X. The evidence for the notion was indirect. Pardee, Jacob, and Monod had found[5] that when the *lacZ* gene, encoding the enzyme β-galactosidase, was introduced by conjugation (bacterial mating) from one E. coli to another lacking the gene, synthesis of the enzyme in the new host began almost immediately.

Figure 1: Francois Jacob, 1953. (courtesy of Cold Spring Harbor LaboratoryArchives)

SQ2. Diagram the experiment, using different panels to represent different snapshots in time. If the one-ribosome-one-protein idea were correct, then what would Jacob's result imply?

Furthermore, the rate of enzyme synthesis was constant from the beginning if the number of recipients gaining *lacZ* was not allowed to increase (**Fig. 2**).[6] If the number does increase (because new matings are continuously initiated over time), then you'd expect an increasing rate of enzyme synthesis, just as building new car factories would increase the rate of car production. However, if introduction of the gene led to the production of new ribosomes (protein factories), you'd still expect an increasing rate of enzyme synthesis as new ribosomes were progressively brought into production, One might imagine that new ribosomal factories might be constructed and destroyed at the same rate, leading to a constant rate of enzyme production, but this contradicted the known properties of ribosomes. They and their RNA components were known to be highly stable.[7] It seemed that the one-ribosome-one-protein hypothesis was incompatible with the kinetics of enzyme production and with the messenger RNA postulated by Jacob.

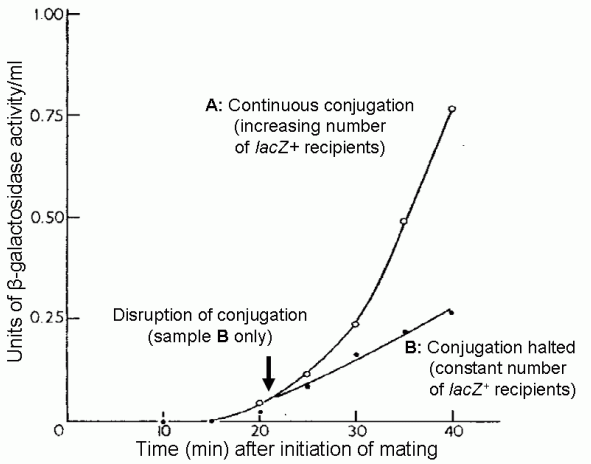

Figure 2: Time course of enzyme production. Production of β-galactosidase due to introduction by conjugation of *lacZ* from donor *E. coli* into recipient lacking the gene. The *lacZ* begins entering recipients approximately 18 min after the initiation of mating. The arrow indicates the point in time when mating was disrupted in Sample **B** (taken from reference 6).

SQ3. Why should the constant increase in new recipients that have gained *lacZ* lead to an exponential increase in β-galactosidase activity over time?

SQ4. Why should the constant increase in new ribosomes capable of producing β-galactosidase lead to an exponential increase in β-galactosidase activity over time?

Crick and Brenner had heard about this experiment before. They found Jacob's conclusion difficult to accept and struggled to find a way out. Perhaps a few stable ribosomes were made quickly after the introduction of *lacZ* and then production of new ribosomes stopped? That would lead to a constant rate of enzyme synthesis.

SQ5. Incorporate Crick and Brenner's idea into your series of snapshots from SQ2. What new behavior of the *lacZ* gene is required by their idea?

No, said Jacob, that wouldn't work, and he proceeded to describe the results of an experiment new to Crick and Brenner, one that Arthur Pardee (**Fig. 3**) and his graduate student Monica Riley had just completed.

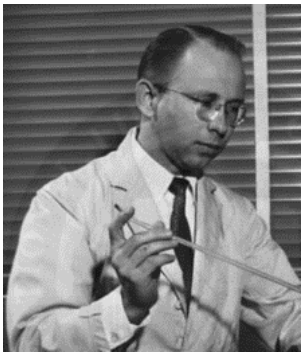

Figure 3: Arthur Pardee, (courtesy of National Library of Medicine)

After a sabbatical at Institut Pasteur, Pardee returned home to Berkeley, taking with him an experiment that was the complement of the one he did in Paris with Jacob and Monod. In the original experiment, they learned how fast β-galactosidase was expressed when suddenly introduced into E. coli. In the new experiment, he hoped to learn what happened to β-galactosidase expression when the gene was suddenly removed. If expression persisted, then the idea of a few stable ribosomes could still be entertained.

But how to remove a gene from a cell? The trick was to heavily label donor E. coli DNA with radioactive phosphate, ^{32}P. Since only DNA is transferred during conjugation, the recipient E. coli would receive the labeled DNA, but the rest of the donor cell would not be radioactive. Once β-galactosidase expression began, the cells were frozen, putting the E. coli in suspended animation. However, the process of radioactive decay is not affected by temperature. Over the course of weeks, the radioactive ^{32}P would decay to ^{32}S, causing breaks in the DNA,[8] but only the DNA introduced during conjugation, not the host DNA. Indeed, Riley and Pardee found[6] that when E.coli were brought back to life, β-galactosidase expression was depressed to a degree proportional to the time ^{32}P had been allowed to decay. It appeared that the presence of an active gene was required for β-galactosidase activity, excluding the new stable ribosome hypothesis.

SQ6. Incorporate the ^{32}P-decay procedure into your series of snapshots.

SQ7. What alternative explanation for these Riley and Pardee's results can you propose that can rescue the new stable ribosome hypothesis? (Riley and Pardee thought of it too[9])

At this Brenner suddenly became a convert to the messenger idea and initiated one of the most celebrated blowouts in the history of science.[10,11,12] He jumped up, agitated, saying it's Volkin and Astrachan! And Crick jumped up with the same realization that Jacob's unstable messenger was the same thing as Volkin and Astrachan's minor phage-induced RNA that matched the phage DNA. Ribosomes were just inert tape recorders,[*] while a small fraction of the RNA served as the tape. And if that were the case, then Belozersky and Spirin's results made perfect sense!

SQ8. How can Jacob's idea about an unstable messenger explain Volkin and Astrachan's results?

SQ9. How can it explain the results of Belozersky and Spirin? (See Problem Set 6 #1)

That evening, Brenner, Jacob and Crick planned the experiment they would do to test the idea. These experiments were begun later that spring at Cal Tech, in the lab of Matt Meselson.

[*] Crick missed this in his 1958 review article. See Crick (1958) companion, Fig. 2.

B. Brenner, Jacob, and Meselson (1961)[13]

B.1. Introduction

We've already gone through an extensive introduction, but skim the first page of this article.

SQ10. Any surprises?

On the second page, Brenner et al put forth three competing hypotheses to explain how phage DNA directs the synthesis of phage-specific proteins. I find their figure somewhat confusing, so I redrew it (**Fig. 4**).

SQ11. Identify each icon shown in Fig. 4 and speculate why they are drawn in the way they are.

SQ12. What are the critical differences distinguishing the three models?

SQ13. Model I differs in one important way from the classical one-ribosome-one-protein hypothesis. What is that difference (explained in the text but not reflected in the graphical representation of the model)? Does that disturb you?

SQ14. What unsettling assumption is central to Model II?

SQ15. Why are the tight squiggles located in the middle of the two blobs in Models I and II but only overlapping the blobs in Model III?

SQ16. What unsettling assumptions are evident in Models I and II?

B.2. Experimental system

If you skim through the paper (always a good idea!) you'll see that all the figures bear a close resemblance: fraction number on the bottom and E_{254} on the left and counts/min to the right. Evidently there's only one kind of experiment in this article to figure out – good news! That experiment is described in broad outline in the right column of p.577.

SQ17. What is the goal of the authors and how does the experiment address it?

SQ18. What parts of the description don't you understand?

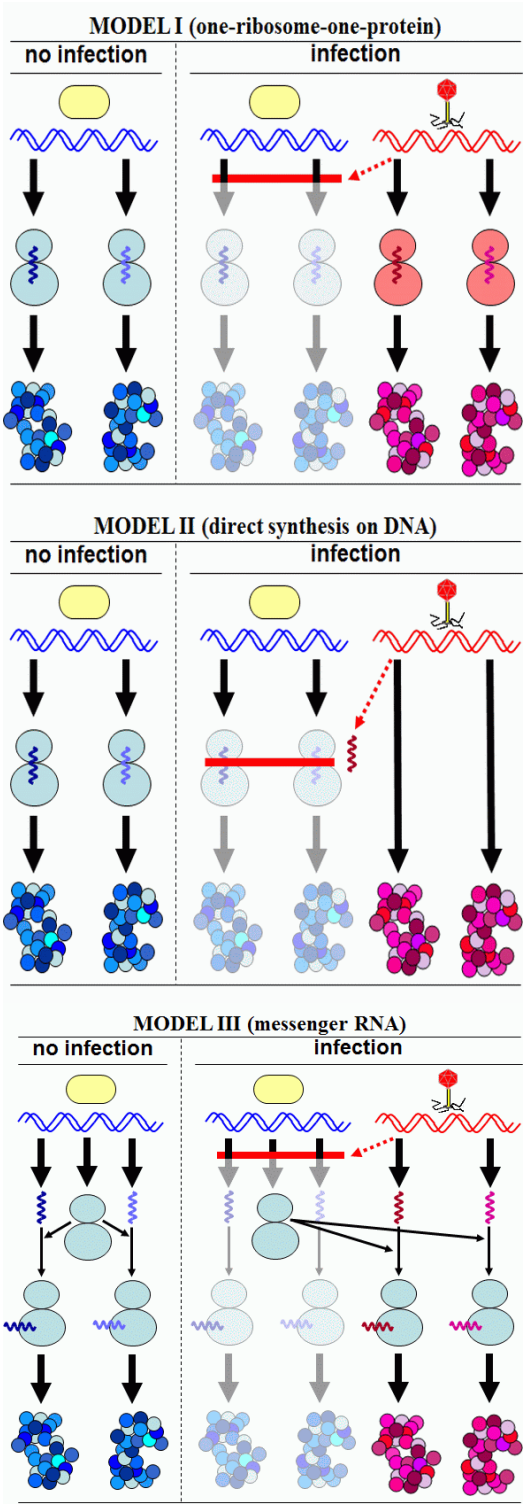

Figure 4: Three models of protein synthesis per Brenner et al (1961), Figure 1.

I count just two basic techniques: density gradient centrifugation and labeling with heavy and radioactive isotopes. Both should be familiar to you from Meselson and Stahl (1958),[14] which we considered a month ago. Most find density gradient centrifugation less intuitive, so I'll spend some time now on that.

Fig. 5A, shows the scheme of a typical experiment presented by Brenner et al. E. coli is grown in a medium containing heavy isotopes of nitrogen and carbon. The culture is infected with bacteriophage T4, and at the same time it is diluted into a large quantity of medium with the usual light isotopes of nitrogen. All macromolecules in the cell contain nitrogen and/or carbon, so every macromolecule synthesized after the switch from heavy medium to light medium will be less dense than the equivalent macromolecule synthesized before the switch. A major goal of the experiment is to determine whether the T4 phage directs the synthesis of new ribosomes after infection. The labeling with heavy isotopes allowed density of the ribosomes in the cell to be a diagnostic of ages of the ribosomes.

SQ19. Would ribosomes be labeled by ^{14}N and ^{13}C? By ^{32}P? How about DNA? RNA?

SQ20. Suppose you found that after the shift from heavy to light medium there was a ribosome that contained both heavy and light N and C. What explanations for this could you put forth?

It is important to realize that ^{15}N and ^{13}C are not radioactive isotopes – they're just heavier than the isotope found most frequently in nature. It is also important to realize that heavy isotopes are not something you can pick up at Walmart on the way into the lab. Meselson got the limited supply for these experiments from Linus Pauling, who obtained them while on a trip to the Soviet Union.[10] They had to be used in the smallest quantities possible. Therefore, in almost all cases in which E. coli was grown in heavy isotopes, the volume was small, so small that it was not possible to isolate from it ribosomes that could be detected by conventional means. They were detected instead by labeling them with radioactivity.

To separate old (dense) ribosomes from new (less dense) ribosomes, they were added to a cesium chloride solution and centrifuged (**Fig. 5B**). Cesium lies in the same column of the periodic table as sodium but is much heavier and denser. It's so dense that high speed centrifugation can push cesium atoms towards the bottom of the centrifuge

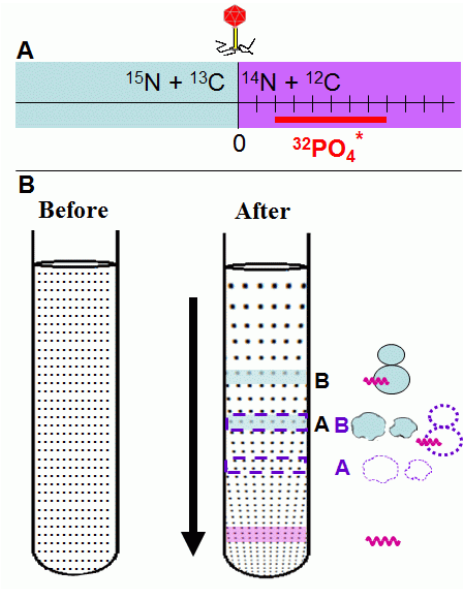

Figure 5: Principle behind experiment. (**A**) Density shift time course: In some experiments, E. coli was grown in heavy medium and shifted at the time of phage infection to light medium. A radioactively labeled compound, in this example radioactive phosphate, was sometimes added. (**B**) Density gradient centrifugation: A preparation of ribosomes taken from E. coli was put into a centrifuge tube containing a uniform concentration of CsCl (before) and centrifuged. The centrifugal force (in the direction of the arrow) caused a CsCl gradient to form, with greater density at the bottom of the tube. Ribosomes band at a level at which the density of CsCl equals their density. The **B** bands contained intact ribosomes. The **A** bands contained subunits that have lost proteins.[16] Large quantities of light ribosomes were added to visualize their positions (blue bands). The heavy ribosomes were present in very small quantities, detectable only by radioactivity (dotted bands). Nucleic acids (pink band) were present at position of great CsCl density.

tube faster than they can return by diffusion. The dots in the tube of the right hand centrifuge tube of **Fig. 5B** are intended to represent the gradient of cesium that results from high speed centrifugation – concentrated CsCl on the bottom and more dilute CsCl on the top. A ribosome placed at the top of the gradient would sink until it reached a level where the density of the CsCl equaled the density of the ribosome. Naturally, ribosomes composed of ^{15}N and ^{13}C would be denser than ribosomes composed of ^{14}N and ^{12}C.

SQ21. What about RNA? The cartoon shows RNA being denser than ribosomes. Does that make sense? Remember, we're talking about *denser* not *heavier*. Heavier doesn't make any difference (battleships float).

SQ22. What about broken ribosomes? Why should they be denser than intact ribosomes? Again, denser and heavier are two different things. Think about what ribosomes are composed of and notice that the broken ribosomes are missing some parts.

How to detect these ribosomes? After centrifugation, a small hole was punched in the bottom of the centrifuge tube so that the contents of the tube could drip out. The first drops (or fractions) came from the bottom and so were the heaviest. The drops were examined in two ways. First, ribosomes were detected ribosomes by absorbance of ultraviolet light (wavelength of 254 nm). This was possible because ribosomes contain proteins, and proteins containing aromatic amino acids (i.e. almost all proteins) absorb UV. Second, the amount of radioactivity was measured in each fraction, for purposes that will become apparent.

With that in mind, look at Brenner et al's Figure 2.

SQ23. Read the legend to Fig. 2 and diagram the procedure of the experiment whose results are displayed in the figure. Your summary should relate the conditions of two separate cultures.

SQ24. The legend says that the two cultures were mixed in the ratio of 1:50. Why not 1:1?

SQ25. Label the four peaks shown in Fig. 2.

SQ26. From the actual ratios of the two cultures, calculate how much absorbance (in E_{254} units) you'd expect for heavy band A. Do you see that amount?

This experiment was a test of the system. Was it possible to separate old (heavy) ribosomes from new (light) ribosomes by cesium density centrifugation? It would not be possible if the components of the two types of ribosomes dissociated and reassembled during the course of the experiment.

SQ27. Draw the two curves (absorbance and radioactivity) that you would expect to see if light and heavy ribosomes could disassemble and reassemble.

B.3. Results

With this experimental system in hand, Brenner et al were finally able to address the three models set forth in **Fig. 4** and in particular the objection to the idea of messenger RNA raised at the 1960 meeting (p.2 of these notes): Could it be that new gene expression was accounted for by a small subset of newly made ribosomes? To do this, they performed three experiments whose results are presented in the following figures from Brenner et al:

- Figs. 4 and 5: Is new RNA synthesized after phage infection incorporated into stable ribosomes?
- Fig. 6: Is new RNA synthesized after phage infection associated with new or old ribosomes?
- Fig. 7 and 8: Is new protein synthesized after phage infection associated with ribosomes, and if so, are they new or old ribosomes?

SQ28. Which of the three models (Fig. 4) are distinguished by the first question? Second question? Third?

To address the first question, Brenner et al made use of what is called a pulse-chase experiment. In such an experiment, a pulse of radioactive compound is added to a culture, then, sometime thereafter, it is chased by an excess amount of the same compound without radioactivity.[†] This procedure allows you to follow the fate of the compound over time. Now look at the experiment described in the legend to Figs. 4 and 5.

SQ29. Diagram the procedure of the experiment whose results are shown in Fig. 4 at the end of the pulse period and in Fig. 5 sixteen minutes into the chase period.

SQ30. How does this experiment differ from the one whose results are shown in Fig. 2?

SQ31. What macromolecule(s) would radioactive ^{14}C-uracil be expected to label?

SQ32. Label the four peaks in Fig. 4. Provide a biological interpretation for the finding that radioactivity was found primarily in two positions in the cesium gradient.

SQ33. If ^{14}C-uracil had been incorporated into stable ribosomes, what would be your expectation of its position and quantity during the chase period? How do you interpret Fig. 5?

SQ34. What if the ribosomes made during the pulse period were not stable? Are all the results obtained to this point consistent with that idea?

SQ35. What conclusions can you draw regarding any of the three models? Why?

Brenner et al were more concerned than we would be about the possibility that the pulse-chase experiment labeled the RNA within special ribosomes that were much less stable than known ribosomes. Regardless of their reasons, they performed the experiment whose results are shown in Fig. 6 of their article. Take a look at the figure legend.

SQ36. Diagram the procedure of the experiment whose results are shown in Fig. 6.

SQ37. How does this experiment differ from the one whose results are shown in Fig. 4?

SQ38. What macromolecule(s) would radioactive $^{32}PO_4$ be expected to label?

SQ39. Label the four peaks in Fig. 6. Provide a biological interpretation for the finding that radioactivity was found primarily in the position of the left absorbance peak. (A refresher on Brenner et al's Fig. 2 might be helpful at this point). If mRNA were associated with old ribosomes, where would you expect to see the radioactive peak? If it were associated with new ribosomes?

[†] I think the origin of the term is the use of "chaser" to mean a glass of water taken after liquor.

SQ40. What conclusions can you draw regarding any of the three models? Why?

Brenner et al performed a pulse-chase experiment in order to determine where new proteins are synthesized. The results of these experiments are shown in their Figs 7 and 8. Look at the legend to these figures.

SQ41. Diagram the procedure of the experiment whose results are shown in Fig. 7 and 8. Brenner et al must have thought this experiment was hugely important, because they devoted a huge amount of scarce resources to it. What do I mean by that?

SQ42. How does this experiment differ from previous experiments?

SQ43. What macromolecule(s) would radioactive $^{32}SO_4$ be expected to label? (What macromolecules contain sulfur?)

SQ44. Label the four peaks in Fig. 7. Provide a biological interpretation for the positions of the two radioactive peaks.

SQ45. How does Fig. 8 differ from Fig. 7? Provide a biological interpretation for this difference.

SQ46. What conclusions can you draw regarding any of the three models? Why?

B.4. Rest of the article

The remainder of the article (starting on p.580, paragraph 3) consists of a summary of major findings, a brief discussion of results for which they did not present data, a discussion of a sister article (Gros et al, 1961[15]), and some predictions.

SQ47. Consider each of the three numbered findings and point to experimental results that support them.

SQ48. Note the last sentence of the paper. How did their speculations pan out?

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[14] Meselson Stahl
[15] Gros