1
A History of Protein Biosynthesis and Ribosome Research

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1.1
Introduction

It is a challenge to write the history of protein synthesis, the structure and function of ribosomes and of the other components of translation (see Refs. [1, 11-18] for earlier accounts). Many researchers and research groups have been involved (see Refs. [2-10] for autobiographical accounts), and widely different experimental systems, methods, and traditions of skills have been involved. The efforts to elucidate the protein synthesis machinery were scattered all over the world. Nevertheless, a scientific community of surprising cohesion has developed over time, and a network of shared and standardized procedures has been established. Although the formal connection that kept it together was minimal, its meetings have been milestones of a vigorously ongoing process of investigation for several decades (the meetings took place at Cold Spring Harbor, New York 1969 and 1974, Madison, Wisconsin 1979, Port Aransas, Texas 1985, East Glacier Park, Montana 1989, Berlin, Germany 1992, Victoria, British Columbia, Canada 1995, and Helsingør, Denmark 1999; cf. Refs. [19–26]). Emerging to a considerable degree out of cancer research at its beginning, the field of protein synthesis research has only gradually become an integral part of molecular genetics. To trace the broader context of the emergence of the experimental culture of translation research is the aim of this introductory chapter. All those involved in the work of the period covered here but not mentioned will, if not excuse me, realize that I am aware of my limitations: selective reading, specific idiosyncrasies, and, above all, the structural constraints of writing the history of such a complex, empirically driven research field in such a compressed manner. My historical survey will mainly focus on the decades between 1940 and 1970. The more recent developments will only be summarized at the end, since they will be largely covered in the subsequent chapters of this book.

In May 1959, Paul Zamecnik, who can be regarded as the grand old man of protein synthesis research, had been invited to deliver one of the prestigious *Lectures* at the Harvey Society in New York. He chose to speak about "Historical and current aspects of protein synthesis", and he traced them back to "careful, patient studies" extending, as he said, "over half a century" [27, p. 256]. He then began with Franz Hofmeister [28] and Emil Fischer [29], who recognized the peptide bond structure of proteins;

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went on to Henry Borsook [30], who realized that peptide bond formation was of an endergonic nature; to Fritz Lipmann [31], who postulated the participation of a high-energy phosphate intermediate in protein synthesis; to Max Bergmann [32], who determined the specificity of proteolytic enzymes; to Rudolf Schoenheimer [33] and David Rittenberg [34], who pioneered the use of radioactive tracer techniques in following metabolic pathways; to Torbjörn Caspersson [35] and Jean Brachet [36], who became aware of the possible role of RNA in protein synthesis; to Frederick Sanger [37], who unraveled the first primary structure of a protein, showing the specificity and uniqueness of the amino acid composition of insulin; and finally to George Palade [38], who gave visual evidence for the particulate structures in the cytoplasm acting as the cellular sites of protein synthesis. This is an impressive list of pioneers, who all, according to Zamecnik, "blazed the trail to the present scene", which in his retrospect inadvertently had assumed the character of a royal path to present knowledge. It was not until the very end of the lecture that he relativized this linear perspective: "From a historical vantage point", he said, "too simple a mechanistic view [has] been taken in the past. [The] details of the mechanisms at present unfolding were *largely unanticipated*". We may ask, then, how the unanticipated was brought into being. Zamecnik's answer was: "By the direct experimental approach of the foot soldiers at work in the field" ([27, p. 278], emphasis added). I hope that the following lines offer at least a trace of the history of quirks and breaks that mark protein synthesis research as a collective and multidisciplinary endeavor whose outcome, as with science in general, cannot be told in advance. Scientists usually tell their stories from the point of view of those selected insights that have made their career. No historian can escape this retrospective valuation either, but we should, at least, try to remain aware of its shortcomings.

1.2 The Archaeology of Protein Synthesis – The 1940s: Forgotten Paradigms

The early 1940s were the heydays of what Lily Kay [39] has aptly described as the 'protein paradigm of life'. The transformation experiments of Oswald Avery and his colleagues at the Rockefeller Institute notwithstanding [40], proteins for quite some time continued to be seen as the key substances, not only of biochemical function, but also of hereditary transmission (from Delbrück [41] to Haurowitz [42]). It is surprising then to learn that, despite this early focus on proteins, the mechanism of protein synthesis largely remained a black box throughout the 1940s. Thoughts on mechanism during that decade mainly centered around the conception, favored by eminent biochemists of the time such as Max Bergmann and Joseph Fruton, that the mechanism of protein synthesis might be based on a reversal of proteolysis [43, 32, 44]. Max Bergmann, then at the Rockefeller Institute in New York, investigated the specificity of proteolytic enzymes, and it was in his laboratory that Paul Zamecnik, as a postdoctoral fellow in 1941–1942, became interested in protein synthesis. The proteolysis concept, however, remained a controversial issue, especially since it could hardly be reconciled with the endergonic

nature of peptide bond formation that appeared to be evident from Henry Borsook's investigations at the California Institute of Technology in Pasadena. His measurements favored the idea that the formation of peptide bonds might involve some sort of activation of amino acids prior to their condensation, a topic on which Fritz Lipmann [31] as well as Herman Kalckar [45] had speculated as early as at the beginning of the 1940s (for an assessment of the 'multi-enzyme program' of protein synthesis, its neglect in the history of biochemistry and its resurrection in biotechnology, see Ref. [46]). These considerations, however, remained without conclusive experimental evidence for the next 15 years. Classical biochemistry alone did not provide a definite handle on the question of the cellular mechanisms of protein biosynthesis, despite the growing sophistication of experimental enzymology and of the structural, physical, and chemical analysis of proteins, including powerful new devices such as chromatography, electrophoresis, and X-ray crystallography (see Refs. [47, 48] for historical accounts).

Some observations on the part of cytochemistry were intriguing but also remained erratic for the time being. Around 1940, Torbjörn Caspersson from Stockholm and Jack Schultz from the Kerckhoff Laboratories in Pasadena had developed techniques for measuring the UV absorption of nucleic acids within cells as well as UV microscopy of cells [35]. With that, they were able to correlate growth, i.e., the production of proteins, with the increased presence of ribonucleic acids at certain nuclear and cytoplasmic locations. Around the same time, Jean Brachet and his colleagues Raymond Jeener and Hubert Chantrenne in Brussels reached similar conclusions on the basis of differential staining and *in situ* RNase digestion of tissues [36].

The elucidation of the particulate structure of the cytoplasm by means of high-speed centrifugation dates back to the 1930s and derives from still other lines of research. Normand Hoerr and Robert Bensley in Chicago had used centrifugation to isolate and characterize mitochondria [49]. Albert Claude, in James Murphy's Laboratory at the Rockefeller Institute, was working on the isolation of Peyton Rous' chicken sarcoma agent when he, around 1938, incidentally realized that the particles he was sedimenting from *infected* cells had exactly the same chemical constitution than those sedimented from *normal* chick embryo tissue and thus were cellular constituents [50]. Figure 1-1 shows early dark-field microscopic images (segments 1 and 5) of preparations of Claude's "small particles". (All the figures in this introductory historical chapter are reproduced from the original publications, and in three cases from handwritten laboratory notes.) After tentatively identifying his high-speed sediment with mitochondria or fragments thereof for some years, he, in 1943, came to the conclusion that his pellet did contain another class of cytoplasmic particles. They were definitely smaller than mitochondria, and Claude termed them "microsomes" [51] accordingly. In contrast with the mitochondria, these particles were particularly rich in ribonucleic acid – Claude estimated them to consist of 50% lipids, about 35% proteins, and some 15% nucleic acids. Speculating that they might be self-replicating nucleoproteins, he was tempted to place them in the category of 'plasmagenes', a notion associated with the idea – widely discussed at the time – of some form of cytoplasmic inheritance [52]. But although these particles were reported to carry

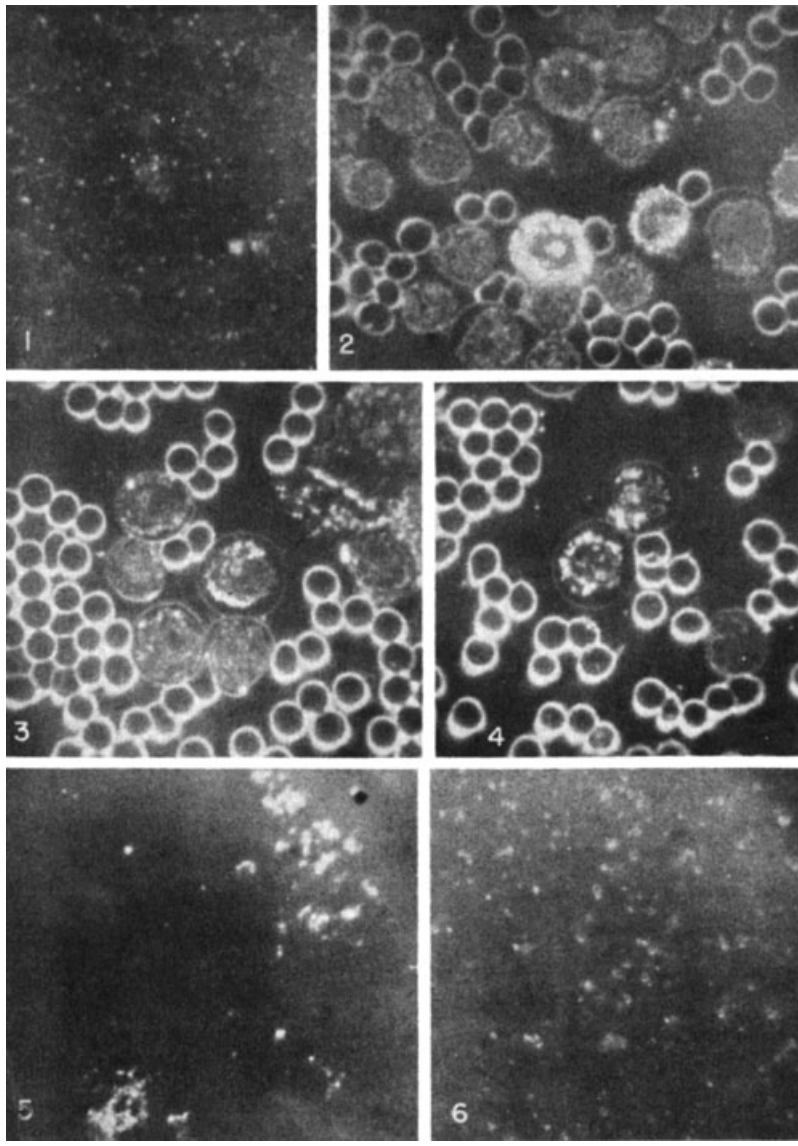

Figure 1-1 Cytoplasmic particles derived from differential high-speed centrifugation. 1–6, Dark-field photographs, magnifications 1000×. 1. Rat leukemia: 'small particles', purified, in neutral water; 2. rat leukemia: whole blood showing cytoplasmic granules in leukemic and normal cells; 3. rat leukemia: heparinated blood; cytoplasmic granules in lymphoid cells; 4. rat leukemia: heparinated blood; cytoplasmic granules in lymphoid cells; purified particles added to plasma; 5. Guinea pig liver: 'small particles' agglutinated at pH 6; phosphate buffer; 6. Guinea pig liver: purified 'Bensley' granules in neutral water. 'Small particles' are represented in segments 1 and 5 (Ref. [301], Figures 1–6).

varying amounts of oxidases and hydrolases [53], they tenaciously resisted all attempts by Claude and his collaborators, especially Walter Schneider and George Hogeboom, to correlate specific and unique enzymatic functions with them [54]. At that time, assigning functions essentially meant enzyme mapping. Unfortunately, this procedure did not work with microsomes. In contrast, however, the microsomes became preferential objects of ultracentrifugation. The centrifugation methods of Hubert Chantrenne [55] from Brachet's laboratory in Brussels, and of Cyrus Barnum and Robert Huseby [56] from the Division of Cancer Biology at the University of Minnesota in Minneapolis were more sophisticated than the Rockefeller method and pointed to a greatly varying size of the particles – if they had a definable size at all. Despite Brachet's recurrent claim of a close connection between microsomes and protein synthesis, no particular experimental efforts were made in all these studies to enforce this line of argument. Still, by the end of the 1940s, Albert Claude thought that microsomes were most probably involved in anaerobic glycolysis [57]. However, the various efforts of an *in vitro* characterization of the cytoplasm by means of ultracentrifugation resulted in a set of procedures for the gentle isolation of cytoplasmic fractions – especially centrifugation through sucrose solutions [58] – that soon proved very useful in a wide variety of other experimental contexts.

1.3 Basic Mechanisms – The 1950s

This situation was bound to change between 1945 and 1950 through still another approach to assess metabolic events. Right after World War II, low-energy radioactive tracers, especially ^{35}S, ^{32}P, ^{14}C, and ^{3}H, became available for research to a wider scientific public as a byproduct of expanding reactor technology. The ensuing new attack on the mechanism of protein synthesis by way of radioactive amino acids was embedded in a particular, historical conjuncture of interests that benefitted greatly from the vast resources made available for cancer research after the War [59], and from the efforts of the American Atomic Energy Commission to demonstrate the potentials of a peaceful use of radioactivity [60, 61]. In fact, cancer research programs provided the background for much of the protein synthesis research during those years. Cancer was related to abnormal growth, and growth was considered to be intimately linked with the metabolism of proteins. This constellation also explains why much of protein synthesis research during the decade between 1950 and 1960 was done on the basis of experimental systems derived from higher animals, especially rat liver, and not on bacteria, as might be expected from hindsight.

1.3.1 Steps toward an *in vitro* Protein Synthesis System

The first attempts at approaching protein synthesis via tracing consisted in administering radioactive amino acids to test animals and in following the incorporation of the label in to the proteins of different tissues. However, radioactively labeled amino acids were not yet commerically sold and were therefore available only in limited

amounts at that time. In addition, the tracing technique posed problems of controlling the experimental conditions. One of the biggest concerns of these early radioactive *in vivo* studies was to maintain control over the specific activity of the injected material. Consequently, researchers in the field attempted to establish test tube protein synthesizing systems from animal tissues. Among the first to use tissue slices – a kind of hybrid system between *in vivo* and *in vitro* – were Jacklyn Melchior and Harold Tarver [62], as well as Theodore Winnick, Felix Friedberg and David Greenberg [63], all from the University of California Medical School at Berkeley. Going one step further, attempts to incorporate amino acids into proteins of tissue homogenates were also made at that time by Melchior and Tarver [62], by Friedberg et al. [64], and by Henry Borsook's team at Caltech [65]. Initially, they all used different amino acids: sulfur-labeled cysteine and methionine (Tarver), carbon-labeled glycine (Greenberg and Winnick), and carbon-labeled lysine (Borsook). All these labels were incorporated, but some of the amino acid 'incorporations' in these early *in vitro* studies turned out to be due to amino acid turnover reactions that were not related to peptide bond formation. Granting that the experimental observation of amino acid 'uptake' indeed meant peptide bond formation became one of the biggest concerns of all those trying protein synthesis in the test tube between 1950 and 1955.

I cannot follow all these activities in detail here. Instead, I will organize my narrative around the efforts of one particular group, thereby illustrating the conjuncture of centrifugation and radioactive tracing through which microsomes became linked to protein biosynthesis. The group is Paul Zamecnik's at the Massachusetts General Hospital (MGH) in Boston, whose work can rightly be considered to have been at the cutting edge of the field for the decade between 1950 and 1960. Zamecnik started his work on protein synthesis in 1945. As a medical doctor, he had an interest in the action of carcinogenic agents. Protein metabolism seemed to him to be a suitable target for studying the differences between normal and neoplastic tissue. The choice of rat liver followed from this comparative interest; a standardized procedure of inducing hepatomas in rat belonged to the laboratory routines at MGH.

In 1948, Robert Loftfield, an organic chemist from the Radioactivity Center at MIT, joined the staff of the Massachusetts General Hospital as part of a collaboration of the Center with the Huntington Laboratories. In the preceding 2 years at MIT, he had worked out a suitable method for the synthesis of ^{14}C-alanine and glycine [66]. Together with Loftfield, Warren Miller, and Ivan Frantz, Zamecnik started to introduce radioactive amino acids into the livers of rats. Miller, from the Physics Department of MIT, had been involved in the development of a new method of radioactive carbon gas counting. Ivan Frantz, who belonged to the Huntington Laboratories, was an expert in the technique of incubating sliced livers.

Right in the first series of these liver slice experiments, cancer tissues proved to be considerably more active than normal liver in taking up radioactive amino acids. But the signal that redirected the research process came from a control. In the laboratory of Fritz Lipmann, who was a neighbor of Zamecnik's at MGH, William Loomis had just shown that dinitrophenol (DNP) specifically interfered with the process of phosphorylation [67]. When the Zamecnik group included DNP into one of their slice

experiments, it stopped all amino acid incorporation activity. The result suggested that, as Lipmann had assumed for a long time, protein synthesis was indeed coupled with the utilization of phosphate bond energy [68]. At that point, the research perspective of Zamecnik's group began to shift from the cancer-related problem of malignant growth to the bioenergetic aspects in the making of proteins.

There was no chance, however, to approach the problem by further manipulating liver slices. But to proceed along the lines of cell homogenization meant, as Zamecnik remarked, to enter a "biochemical bog" [69]. It was a largely unexplored experimental field, and the MGH group worked for 3 years, from 1948 to 1951, to arrive at something that could be taken as the 'incorporation' via peptide bond formation of radioactive amino acids into protein in the test tube. In 1951, Philip Siekevitz, who had joined Zamecnik's group in 1949, had achieved a preliminary fractionation of the liver homogenate by means of a regular Sorvall laboratory centrifuge [70]. His main fractions were a mitochondrial fraction, a fraction enriched in what was taken to be 'microsomes', and a supernatant fluid. None of the fractions was fully active when incubated alone. But when all of them were put together again, as can be seen in Fig. 1-2, the activity of the homogenate was restored, although the signal was extremely faint.

In these efforts, the combination of two methodologies had been instrumental: radioactive tracing and differential centrifugation. From a superposition of them, the system acquired dynamic capacities. In 1953, a tiny but decisive detail was incorporated into the system at MGH. It consisted of a slightly altered, gentle homogenization procedure [71]. 'Loose homogenization' enhanced the activity of the cell-free protein synthesis system by a factor of 10. During the same year, the laboratory centrifuge was replaced by a high-speed ultracentrifuge. The new instrument made a quantitative sedimentation of the microsomes possible, leaving behind a non-particulate, soluble enzyme supernatant. As shown in Fig. 1-3, incorporation activity was restored from these two fractions under the condition that the test tube was supplemented with ATP and an ATP-regenerating system [72, 73]. (The investigation of mitochondrial and chloroplast protein synthesis will not be pursued here. It was investigated in parallel. It should also not be forgotten that the nucleus, too, continued to be considered a site of protein synthesis throughout the 1950s; cf. e.g., Ref. [74]).

1.3.2
Amino Acid Activation and the Emergence of Soluble RNA

Towards the end of 1953, Mahlon Hoagland took up his work in Zamecnik's lab, after having spent a year with Lipmann. Figure 1-4 shows Hoagland, Zamecnik, and Mary Stephenson in their laboratory in the mid-1950s. In what later appeared to Hoagland as one of those "vagaries of fortune in science" [10, p. 71], he realized that he could use the technique of 'phosphate-ATP-exchange' developed in Lipmann's lab as a tool in Zamecnik's rat liver system. He proceeded to graft this technique onto the fractionated protein synthesis setup. Within a year, a first partial, molecular

Fraction	Protein per 100 mg. wet weight tissue	Per cent weight	O_2 per hr.	Qo_2	PO_4 esterified per 5 min.	α-Ketoglutarate disappearing per 60 min.	C.p.m. per mg. protein	
							Minus α-ketoglutarate	Plus α-ketoglutarate
	mg.		*microatoms*		*μM*	*μM*		
Homogenate	17.6	100	37.3	24	3.6	19.1	1.4	10.8*
Nuclei + cells	3.3	18	1.6	5	0.0		1.2	2.9
Mitochondria	2.5	15	7.3	36	3.4	4.0	0.9	1.3
Mixed fraction	2.2	14	1.1	6	0.0		1.7	1.1
Microsomes	1.5	12	0.1	1	0.0	0.0	1.6	1.0
Supernatant	7.8	40	1.2	1	0.0	0.0	0.1	0.4
Mitochondria + microsomes	4.0	24	13.8	39	3.0	6.6	1.1	4.2
" + supernatant	10.3	55	17.1	19	4.6	7.9	1.0	6.6
" + microsomes + supernatant	11.8	64	20.8	20	3.5	10.7	0.8	9.8
All fractions	17.3	98	38.9	25	3.4	18.8	0.8	10.5

* 0.012 μM of L-alanine per gm. of protein per 30 minutes.

Figure 1-2 Ability of various rat liver fractions to incorporate radioactive alanine into their proteins. Each sample contained 3.2 μM of adenosine-5-phosphate, 10 μM of MgCl2, 40 μM of α-ketoglutarate, 0.5 ml of phosphate-sucrose solution containing 15 μM of phosphate, and isotonic sucrose to a final volume of 2.0 ml. Each radioactivity vessel also contained 0.08 mg of [1-^{14}C]DL-alanine (760 000 c.p.m.) in addition. Incubation at 37°C. As seen from a comparison of the last two columns, the activity was dependent on the addition of α-ketoglutarate, an oxidative substrate for mitochondria (Ref. [70], Table 1).

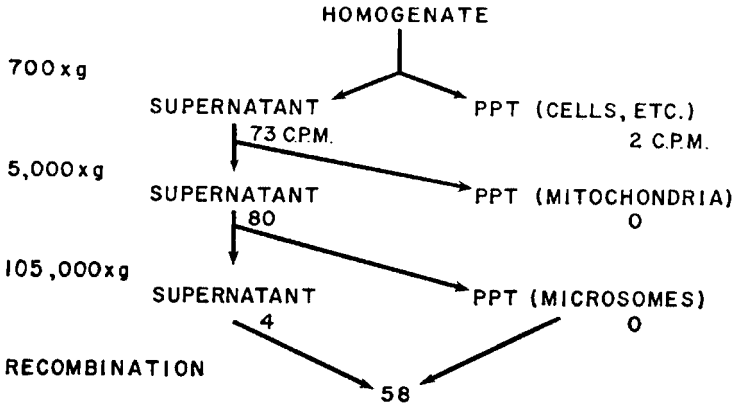

Figure 1-3 Fractionation and recombination of a rat liver extract. The homogenization medium was as follows: 0.004 M $MgCl_2$, 0.04 M potasium phosophate buffer (pH 7.4), 0.01 M HDP, and 0.25 M sucrose. All fractions were prepared from the same homogenization and were incubated simultaneously for 30 min at 37°C in 95% N_2–5% CO_2 (Ref. [103], Figure 2).

model of protein synthesis emerged [75]. The combination of the phosphate-exchange reaction with another model reaction, that of amino acids with hydroxylamine, suggested an activation by ATP of the amino acids as represented in Fig. 1-5. These experiments induced a major turn in the representation of the fractionated system. Its energy requirement became linked to a particular fraction. What until then had been the 'soluble fraction', or the '105 000 × *g* supernatant', or the 'pH 5 precipitate', became now viewed as a set of activating enzymes. With that, amino acid activation began to attract the attention of a larger scientific community.

Several other groups quickly added similar observations obtained in other systems. David Novelli, who had moved from Lipmann's lab to the Department of Microbiology at Case Western Reserve University in Cleveland, established an amino acid-dependent PP/ATP-exchange reaction with microbial extracts [76]. Paul Berg,

Figure 1-4 Left to right: Mahlon Hoagland, Paul Zamecnik and Mary Stephenson, about 1956 [302].

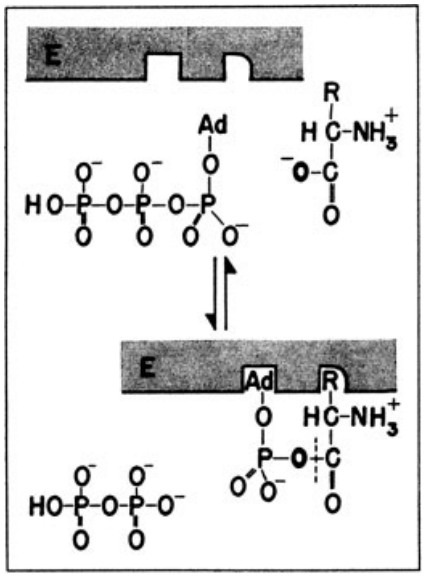

Figure 1-5 Schematic representation of amino acid carboxyl activation by ATP and the pH 5 fraction. Ad, adenosine. The heavily drawn O indicates the attacking carboxyl oxygen which would remain with the nucleotide moiety upon subsequent splitting of the activated compound (dashed line) (Ref. [79], Figure 5).

from Washington University School of Medicine in St. Louis, reported on the activation of methionine in yeast extracts [77, 78]. Lipmann's lab took up the task of isolating and purifying one of the amino acid-activating enzymes. Within half a year, the general character of the carboxyl-activation mechanism appeared to be established [79].

At this point, the participation of ribonucleic acids in protein synthesis still appeared as a black box conveniently termed 'ribonucleoprotein' (cf. e.g., the representation in Fig. 1-6). This black box now attracted the attention of both biochemists and geneticists. Microsomal RNA, by 1955, was generally assumed to play the role of an ordering device, jig, or 'template' for the assembly of the amino acids. The actual point of discussion at that time, however, to which Sol Spiegelman from the University of Illinois at Urbana and Ernest Gale from Cambridge repeatedly referred, was accumulating indirect evidence for a coupling of the synthesis of proteins with the actual synthesis of RNA [80–82]. Also in 1955, Marianne Grunberg-Manago, in Severo Ochoa's laboratory in New York, identified an enzyme which was able to synthesize RNA from nucleoside diphosphates [83]. For the first time, an RNA-synthesizing enzyme had been isolated.

Late in 1955, Zamecnik began to look for RNA synthesis activity in his fractionated protein synthesis system. He added radioactive ATP to a mixture of the

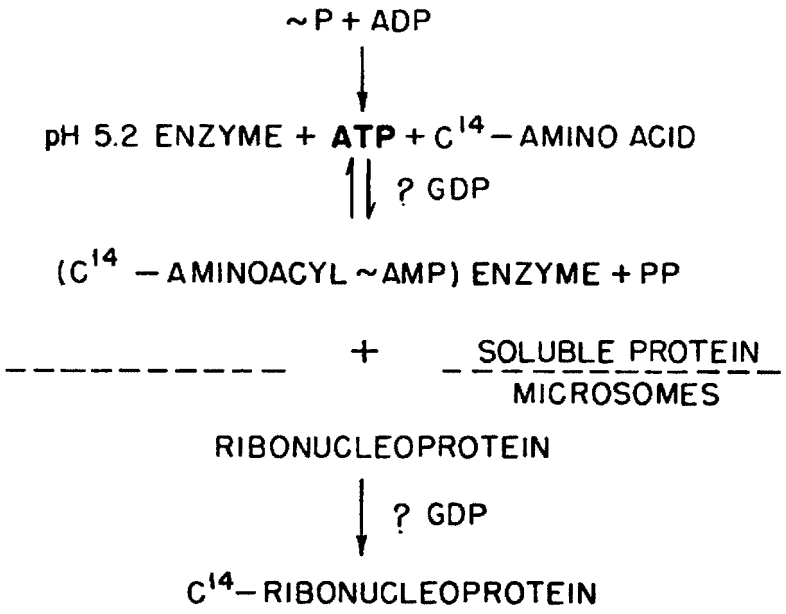

Figure 1-6 Intermediate steps in protein synthesis as seen in 1956 (Ref. [303], Figure 5).

enzyme supernatant and the microsomal fraction. To his astonishment, the nucleotide indeed labeled an RNA component of the system. But there was another, even more puzzling observation. In a parallel experiment, Zamecnik had incubated non-radioactive ATP and ^{14}C-labeled leucine instead of non-radioactive leucine and ^{14}C-labeled ATP together with the fractions. As Zamecnik recorded in his notebook (see Fig. 1-7), the assay suggested – quite contrary to his expectation – that radioactive leucine also became attached to the RNA. In fact, it took another year before Zamecnik, in collaboration with Mary Stephenson and Hoagland, became convinced of the significance of the finding and was ready to publish it [84]. Zamecnik had searched for hints of a *synthesis of RNA* on the microsomes. What he had found was an RNA in the soluble fraction to which *amino acids* were attached. For the time being, the new entity was termed 'soluble RNA'.

Soluble RNA immediately helped to focus research under way in a variety of other laboratories and in a variety of similar systems. The further differentiation of the cell-free protein synthesis system now became the working field for a growing protein synthesis 'industry'. In 1956, evidence for the presence of an RNA intermediate in protein synthesis was being gathered by Robert Holley, from Cornell University. He had found a ribonuclease-sensitive step in the alanine-dependent conversion of AMP into ATP [85]. Paul Berg, soon joined by James Ofengand, went ahead with studies on the amino acid incorporation into soluble RNA of *Escherichia coli* [86]. In 1956, Tore Hultin from the Wenner-Gren Institute in Stockholm had obtained independent evidence for an intermediate step in protein synthesis from kinetic isotope

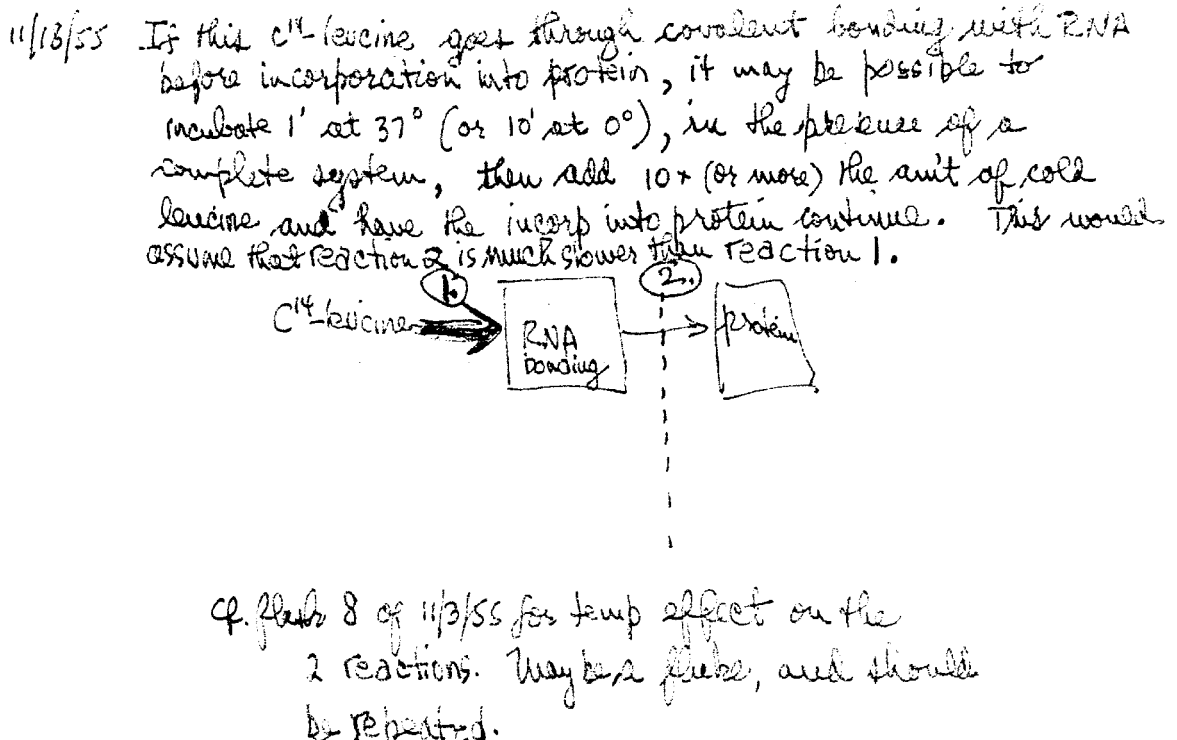

Figure 1-7 First representation of S-RNA as an 'intermediate' in protein synthesis (Zamecnik, laboratory notebook, 10 November 1955, with kind permission from the author).

dilution studies [87]. Kikuo Ogata and Hiroyoshi Nohara at the Niigata University School of Medicine in Japan also had collected hints for an RNA-connected intermediate in protein synthesis [88]. By the end of 1957, amino acid–oligonucleotide compounds were being investigated by at least three other research groups: Victor Koningsberger, Olav Van der Grinten, and Johannes Overbeek [89] at the Van't Hoff Laboratory in Utrecht; Richard Schweet, Freeman Bovard, Esther Allen, and Edward Glassman [90] at the Biological Division of Caltech; and Samuel Weiss, George Acs, and Fritz Lipmann [91], who had moved from the Massachusetts General Hospital to the Rockefeller Institute in New York. All of them joined the race for adding items to the list of what these molecules and their activating enzymes did and what they failed to do. In the process, what had emerged as a biochemical intermediate in protein synthesis soon turned into one of those big missing pieces within the flow scheme of the expression of molecular information. At Richard Schweet's suggestion, the molecule was later referred to as transfer RNA [92], and it became identified with what, based on considerations rooted in the double-helical structure of DNA, Francis Crick had postulated as an adaptor of some sort of the genetic code [93–95]. Figure 1-8 represents the interaction of soluble RNA and microsomal RNA as seen by Zamecnik at the end of the 1950s.

1.3.3
From Microsomes to Ribosomes

As we have seen, it was not until the beginning of the 1950s, and in a context quite different from their original characterization, that the 'small particles' or 'microsomes', operationally defined in terms of fractional sedimentation, optical inspection,

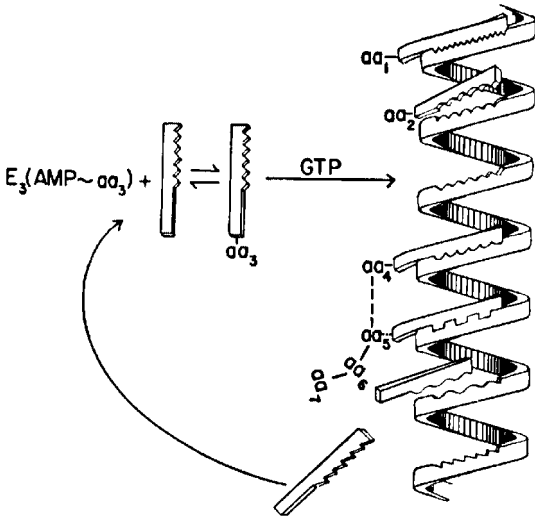

Figure 1-8 A scheme for the interaction of microsomal RNA and soluble RNA-amino acid (Ref. [27], Figure 5).

and chemical composition, became linked on experimental grounds to protein synthesis *in vivo* [97–102] and *in vitro* [70, 96, 102, 103]. Around this time, hints were also accumulating that eukaryotic microsomal material was quite heterogenous in size as well as in composition. It took another decade before the the isolation of active cytoplasmic particles through sucrose-gradient centrifugtion became a laboratory standard. To obtain 'purified' microsomes became one of *the* major issues in the development of cell-free protein synthesis around 1955 [104].

For purification, Zamecnik's colleague John Littlefield took advantage of the detergent sodium deoxycholate which solubilized the protein–lipid aggregates of the microsomal fraction. The RNA-to-protein content (1 : 1) of his particles corresponded to the value given by Howard Schachman from Wendell Stanley's Virus Laboratory in Berkeley for *Pseudomonas fluorescens* particles [105] and by Mary Petermann from the Sloan Kettering Institute in New York for rat liver and spleen particles [106].

Around the same time, George Palade [38], by using an ensemble of advanced specimen preparation techniques, was able to visualize small, electron dense particles on the surface of the endoplasmatic reticulum *in situ* by means of electron microscopy (see Fig. 1-9). Philip Siekevitz had joined Palade in 1954. He added his biochemical expertise to the work at the Rockefeller Institute which aimed at a correlation of the "cytochemical concepts" of microsomal particles and "morphological concepts" derived from electron microscopy [107 pp. 171–172].

Besides electron microscopy, the calibration of these 'macromolecules' involved velocity sedimentation and electrophoretic mobility [106, 108–110]. These structures became a synonym for cytoplasmic RNA, although the postmicrosomal supernatant invariably also contained RNA – approximately 10% of the cell's total RNA [107]. From analytical ultracentrifugation, a sedimentation pattern emerged, and a sedimentation coefficient of the particles could be calculated. Littlefield's rat liver particles appeared as a major 47S peak in the optical record, similar to the main macromolecular component already described by Petermann and their co-workers between 1952 and 1954 (see Fig. 1-10 for Petermann's pattern). A broader peak running ahead of the 47S particle disappeared upon treatment of the material with deoxycholate. However, there was also an additional smaller peak running behind the 47S particle which was not deoxycholate-sensitive. Thus, the suspicion was reinforced that the particulate portion of the microsomal fraction might be in itself heterogenous.

The ribonucleoprotein particles gradually took shape by a comparison of the representations delivered by different biophysical and biochemical techniques applied in different laboratories. The main problem was that the material was no longer active in the test tube after the different isolation procedures. This meant that for the time being there was no functional reference available for comparison. The 'deoxycholate particle', for instance, entered the field of *in vitro* protein synthesis around 1953, and around 1956 it disappeared again from the scene because nobody had succeeded in rendering it functionally active. Preparation procedures played a dominant role, and the terminology faithfully reflected their operational character. Successively, the cellular component at issue had changed from a sedimentable entity no longer visible

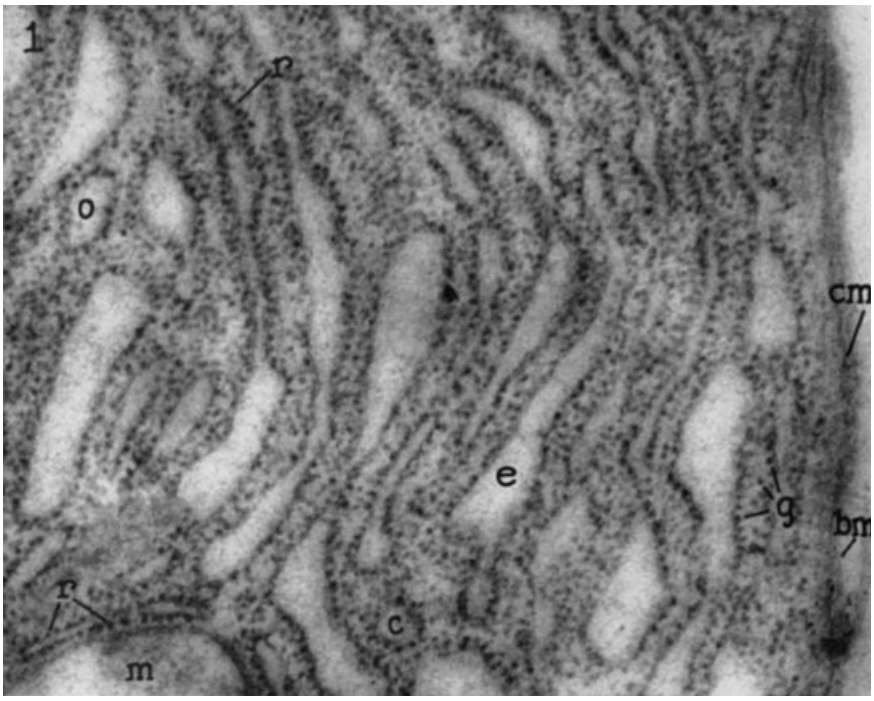

Figure 1-9 Electron micrograph of a limited field in the basal region of an acinar cell of the pancreas (rat). The cell membrane (cm) is coated towards the exterior by a poorly defined layer of dense material (bm), which may be the equivalent of a basement membrane. Part of a mitochondrial profile appears at m. The rest of the field is taken up by elongated (e), oval (o), and circular (c) profiles of the endoplasmic reticulum. Note that in the matrix there are numerous small and dense granules (g) which appear to have particular affinity for the membrane limiting the cavities of the endoplasmic reticulum. The outside surface of this membrane is actually covered by many such particles which in a few places (r) appear to be more or less regularly disposed in rows. Magnification 73,000 × (Ref. [38], Figure 1).

under the light microscope, the 'microsome', to a granular cytoplasmic constituent which was 'deoxycholate-insoluble', and finally to a 'ribonucleoprotein particle' presumably involved in amino acid incorporation into protein, consisting of half protein and half RNA, and visible under the electron microscope. Following a lingering trajectory, the different means and modes of representation eventually produced particles that became firmly linked with subcellular morphology, in particular the endoplasmic reticulum, and to the biochemistry of protein synthesis. The match was, however, hardly perfect.

In the course of the 1950s, RNA-containing particles had attracted more and more attention. Around 1955, their RNA was generally assumed to provide the template upon which the amino acids were assembled into protein threads. In 1958, Howard Dintzis coined the term 'ribosome' for purified microsomes devoid of membrane fragments (Wim Möller, pers. comm.; see also Refs. [111, 112]). During the following

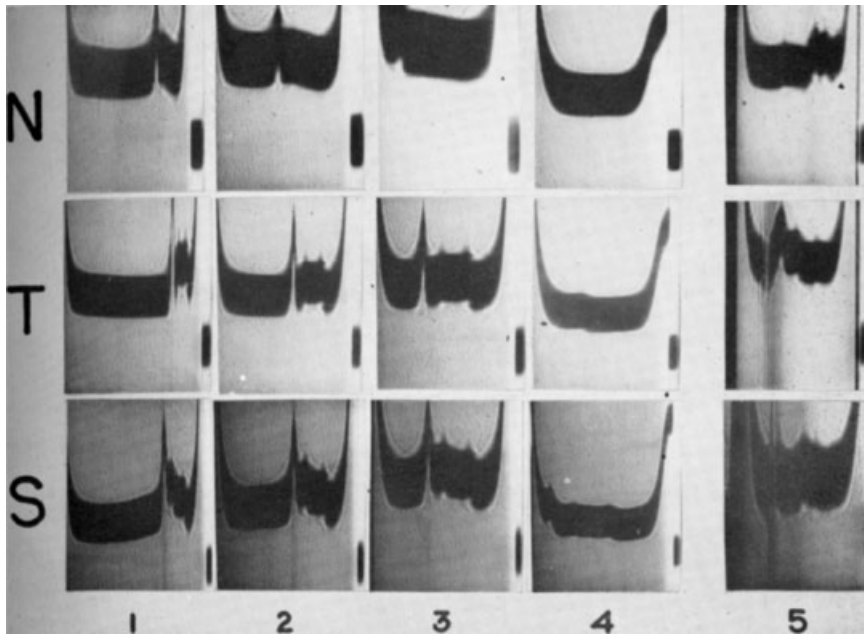

Figure 1-10 Sedimentation patterns of normal and leukemic spleen particles. The direction of sedimentation is to the left: N, normal spleen; T, transplanted leukemia; S, spontaneous leukemia (Ref. [108], Figure 1).

years, this neologism made its way into the laboratories and into the literature. The reason for changing the name was the presumed role of the particle's RNA. The new designation no longer reflected a mere technical representation, but a biological function. Like 'transfer RNA', the 'ribosome' began to relocate protein synthesis from biochemistry to molecular genetics, transforming it into an integral part of what Crick, apparently without minding about the theological connotations of the term, had called the "central dogma" of molecular biology [113, p. 153]. It codified the notion that the genetic information makes its way from DNA to RNA to protein and that, once in the protein, it cannot get back into DNA. The central dogma subsumed the process of protein synthesis as the final, translational, step in the overarching process of gene expression.

With respect to their physical parameters, the protein synthesizing particles considerably changed their appearance between 1955 and 1960. Around 1956 and after many trials, Schachman had found yeast microsomes sedimenting with a velocity constant (*S*) of 80 and to dissociate reproducibly into two unequal portions of 60S and 40S [114]. In a similar manner, Petermann and co-workers were able to separate 78S liver ribosomes into 62S and 46S particles [115]. Alfred Tissières and James Watson, at Harvard, had started to work with *E. coli* ribosomes and had their bacterial particles sediment with 70S. Most interestingly, they could dissociate them

reversibly into a 50S and a 30S component [116, 117]. Gradually, in a decade of painstaking isolation attempts, in which sucrose-gradient centrifugation came to occupy a central place, the confusion about the size of the RNP particles cleared up, and it was realized that the secret of stabilization lay chiefly in the concentration of divalent Mg^{2+} ions. Work on a variety of particles from other sources began to converge on two distinguishing features: bacterial particles (roughly 70S) were consistently smaller than their eukaryotic counterparts (roughly 80S), but both could be separated into something that began to be recognized as a small and a large ribosomal subunit.

1.3.4 Models

The state-of-the-art of protein synthesis, as a process of translation of genetic information, was conceptually re-framed by Francis Crick and his colleagues, especially Sidney Brenner, between 1955 and 1957, and summarized by Crick in his seminal paper of 1958. After years of theorizing from template models, starting with, among others, Hans Friedrich-Freksa [118] and Max Delbrück [41], and continuing with Hubert Chantrenne [119], Felix Haurowitz [42], Alexander Dounce [120], Victor Koningsberger and Johannes Overbeek [121], Fritz Lipmann [122], George Gamow [123], Henry Borsook [124] and Robert Loftfield [125], Crick had come up with a new proposal. During the 1940s, models of autocatalytic protein replication were at the forefront (cf., e.g., Delbrück's scheme [41] and Haurowitz' [42]), as seen in Fig. 1-11. At that time, nucleic acids were still considered, if at all, as structural scaffolds facilitating protein replication. Gene duplication thus meant protein duplication. Friedrich-Freksa [118] had envisaged a protein copying process whereby nucleic acid bases

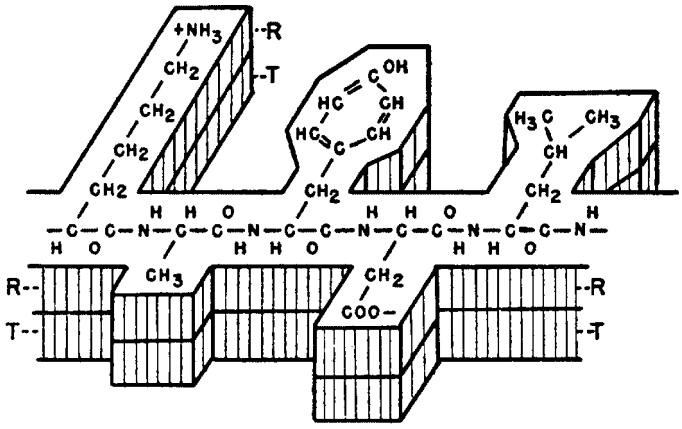

Figure 1-11 Model of protein as template. Replication of a peptide chain formed by lysine, alanine, tyrosine, aspartic acid, and leucine: T, template; R, replica (Ref. [42], Figure 2).

served as a kind of intermediate 'mirror-image'. Later models (such as that of Koningsberger and Overbeek [121] seen in Fig. 1-12) conceived the process of molecular information transfer in terms of a physicochemical interaction between ribonucleic acids and amino acids involving covalent bonding. In the aftermath of the Watson and Crick [126] seminal model of the DNA double helix, Gamow [123] proposed an interaction between DNA and amino acids based on the geometrical shape of holes in the double helix (cf. Fig. 1-13). Crick, thinking of the complementarity features of the DNA double helix, now envisaged what he called "adaptation", i.e., a specific base-pairing interaction between an amino acid-carrying nucleic acid adaptor exposing a signature complementary to the code of a template nucleic acid (cf. Fig. 1-14). It is interesting to note that at the time Crick launched his adaptor hypothesis, he

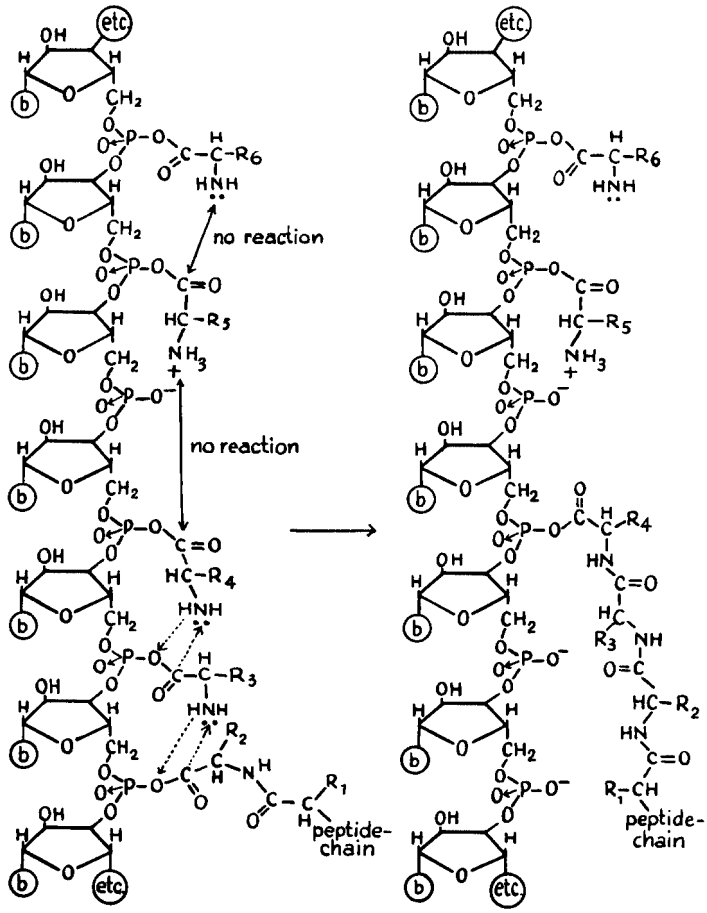

Figure 1-12 RNA as template. Schematic outline of the synthesis of a polypeptide chain starting from an amino acid–nucleic acid compound: b, purine and pyrimidine derivatives (Ref. [121], Figure 2).

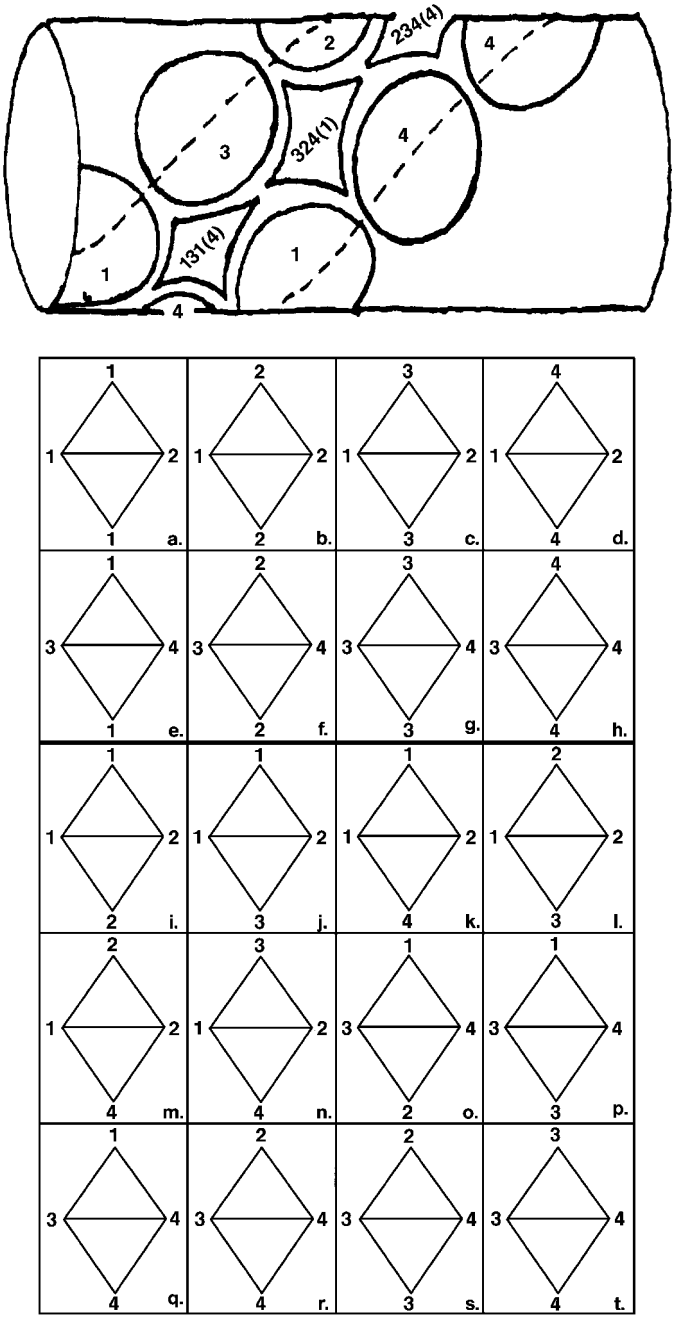

Figure 1-13 DNA as template. Diamond-shaped holes in the double helix of DNA, and the coding scheme for protein synthesis. The rhombs represent the 20 possibilities of arranging the four bases (numbered 1–4) to form niches for the 20 amino acids [123].

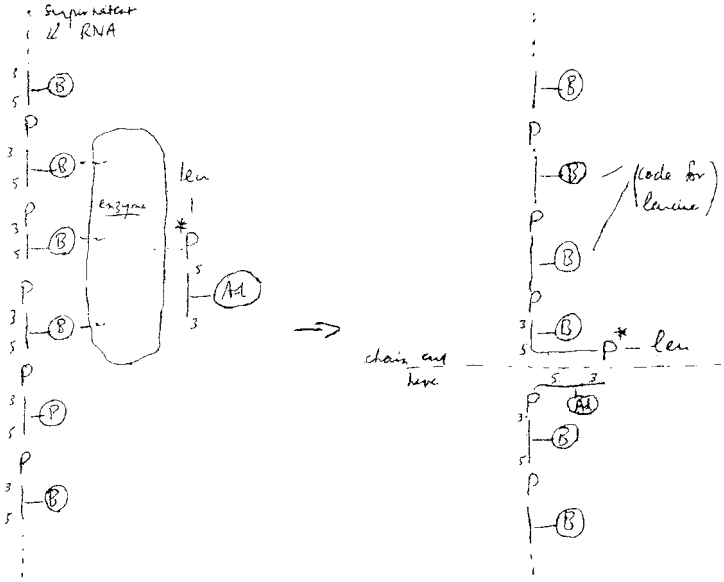

Figure 1-14 Crick's scheme of chopping soluble RNA into trinucleotide adaptors (reprinted from a letter of Crick to Hoagland, January 1957, with kind permission from the author).

obviously did not judge it important enough to be published. It was only its linkage to soluble RNA that made it a prominent concept and a prophecy as seen in hindsight. According to Crick, information-carrying nucleic acids, or templates, and the corresponding proteins were, first, co-linear and second, characterized by strict sequence specificity. But the code itself, the correlation between the building blocks of nucleic acids and those of proteins, remained elusive.

With the surprising emergence of soluble, amino acid-carrying RNAs, the attractive possibility of cracking the code seemed to appear on the horizon. Immediately after Crick had heard the news on soluble RNA from the Massachusetts General Hospital, he invited Hoagland to spend a year with him at Cambridge to isolate an individual S-RNA molecule and to determine its 'signature'. These efforts remained without success. While protein synthesis research during the previous years had certainly not been guided by the theoretical coding discussion, the first attempt to solve the code on the basis of a molecule involved in protein synthesis also failed.

Meanwhile, Zamecnik and Liza Hecht had established as a common feature of all S-RNAs a common 3′-end to which the amino acids became attached: an invariable -CCA trinucleotide [127]. This was anything but a distinct code! Hoagland had hoped to have, with transfer RNA, the "Rosetta Stone" for deciphering the code in his hands [128, p. 61]. But trying to obtain the code through transfer RNA with a direct experimental approach led only to a dead end. Ernest Gale and Joan Folkes at Cambridge, who were analyzing the relation between protein synthesis and the synthesis of nucleic acids in a staphylococcal *in vitro* system, also got stuck with the

characterization of their 'incorporation factors', i.e., nucleic acid fractions that stimulated the incorporation of amino acids into protein [129–131]. And Robert Holley, who since 1957 had put all his efforts into isolating, purifying and sequencing the S-RNA specific for alanine from yeast, took many years and a massive crew of co-workers to arrive at the primary sequence of the first transfer RNA [132]. When he presented the sequence, the code had already been solved by following a completely different experimental track.

1.4 The Golden Age of Translation – The 1960s

The genetic code was solved between 1961 and 1965 with a breathtaking velocity that nobody would have dared to predict even a year before the decisive events. The 1960s also saw the emergence of messenger RNA, the dissection of the ribosome into its components, and the resolution of the translational process into partial functions. Through transfer RNA, messenger RNA, and the code, the biochemistry of protein synthesis merged and for a while even tended to become synonymous with molecular biology, a situation that had been unimaginable a decade earlier when a gap still loomed large between those who considered themselves to be the avantgarde of molecular biology and those who did the messy work of experimentally draining the 'bog' of nucleic acid or protein biochemistry and metabolism [10].

In vitro systems remained central to the field, but the procedures changed. The main strategies of the 1950s had been grounded in what might be called a pursuit of 'integral requirements'. As long as virtually all fractions of the translational system remained black boxes, it would have been deleterious to reduce the system, since this way one could lose essential information. During the 1960s, however, an opposite strategy of 'minimal requirements' became feasible. It was based on the preliminary partitioning of the translational machinery that had been achieved around 1960, the historical stages of which are depicted in Fig. 1-15. It was greatly facilitated through the transition from mammalian systems to bacterial, especially *E. coli* systems of protein synthesis (for the further development of this reductive type of system, see the historical review of Spirin [9]). *E. coli*, so central as a *genetic model* throughout the 1950s, was not yet a *model of translation* during this decade. It was only around 1960 that molecular genetics and protein synthesis research joined forces on the basis of one single model organism. With that, the stage was set for the characterization of some fundamental features of the translational apparatus that still constitute present-day textbook knowledge (see Ref. [133] for an overview of the field around 1960).

1.4.1 From Enzymatic Adaptation to Gene Regulation: Messenger RNA

Towards the end of the 1950s, the work of Jacques Monod and François Jacob at the Pasteur Institute in Paris acquired a new and unforeseen direction and resulted in a

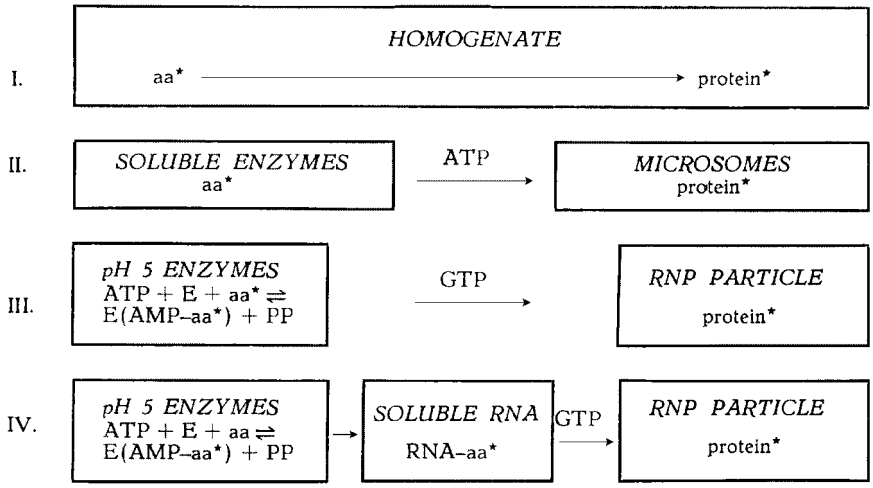

Figure 1-15 Historical stages in the dissection of the rat-liver cell-free system for the incorporation of [^{14}C]-amino acids (aa*) into protein: I, end of the 1940s; II, ca. 1952; III, ca. 1955; IV, end of the 1950s (Ref. [304], Figure 1).

major contribution to understanding protein synthesis and its regulation. Since the beginning of the 1940s, Monod had studied 'enzyme adaptation' in *E. coli*, i.e., the enzymatic response of these bacteria to changes in nutritional conditions. Using a range of mutants, he concentrated on the lactose complex as a model system. At the beginning, Monod's ideas on the subject were shaped by the contemporary theories of immunological instruction. (According to the 'instructional' view, it was the antigen which imprinted the appropriate three-dimensional conformation onto the antibody.) Monod gradually swtiched, at the beginning of the 1950s, to the idea of a genetic control of enzyme synthesis. Conceptually, this resulted in the transition from 'enzyme adaptation' to 'enzyme induction' [134]. Around 1955, Monod and his co-worker Georges Cohen distinguished three genes: the y-gene specifying a permease responsible for the import of lactose into the bacterial cell, the z-gene responsible for the sugar-decomposing ß-galactosidase, and an i-factor responsible for the induction of the system.

François Jacob had started his work on the viral phenomenon of lysogeny in the laboratory of André Lwoff at the Pasteur Institute in 1950. Soon he developed a tight cooperation with Elie Wollman, who had returned from Caltech where he had worked on phage infection in the laboratory of Max Delbrück. Around that time, decisive developments in bacterial genetics were about to take shape. William Hayes in London and Luca Cavalli-Sforza in Milan found hints for a sexual differentiation in *E. coli* bacteria and learned to distinguish between donor and recipient cells during conjugation. In 1951, Joshua Lederberg and Norton Zinder described the phenomenon of viral 'transduction', and Esther Lederberg observed lysogeny in *E. coli* K12. The phage involved in the process was termed 'lambda'. In 1953, Hayes

characterized a high-frequency recombinant donor variant of K12 (Hfr). Soon thereafter, Wollman and Jacob started to work with this lysogenic system. In the process of doing recombination kinetics with multiple mutants of K12, they invented a trick that proved to be highly consequential: If the process of conjugation was interrupted at certain time intervals by mechanical agitation in a mixer, the transfer of different characters could be resolved in a linear fashion. 'Mapping by mating' became a clue to the genetic mapping of bacterial chromosomes [135]. The gene for ß-galactosidase turned out to be in the vicinity of the insertion site of the phage lambda. It was precisely this proximity that allowed an efficient coupling of the systems of Monod and Jacob, respectively.

The collaboration began in 1957 and included Arthur Pardee from the virus laboratory of the University of California at Berkeley. It resulted in the famous series of 'Pa-Ja-Mo' experiments which led to the operon model of gene expression. The experiments suggested that Cohen and Monod's 'i-factor' was responsible for the production of a cytoplasmic substance influencing the structural gene or its product. It was for this special, regulatory substance that Pardee, Jacob and Monod used, for the first time, the term 'cytoplasmic messenger'. 'Messenger' therefore was a concept that originated in the framework of regulatory phenomena and not in a framework of genetic information transfer to begin with. Additional observations pointed to a functionally "*unstable* intermediate" responsible for the expression of the structural genes as well [136, p. 224].

It took some time before Jacob and Sidney Brenner arrived at drawing a parallel between these experiments and the observation of Lazarus Astrachan and Elliot Volkin [137] from the Oak Ridge National Laboratory of a quickly metabolizing RNA that appeared after infection of their bacteria with T2 phages. The question became whether this unstable intermediate was some sort of an "information carrying RNA" [136, p. 225], which transiently combined with existing microsomes, thus inducing the immediate synthesis of a specific protein [6].

There had been hints in the literature pointing towards quickly metabolizing RNAs for quite some time, but obviously they had not been taken into serious account, either by the Pasteur group or by Crick and his molecular biologist colleagues in Cambridge and elsewhere. As early as 1955, microbiologist Ernest Gale, who was a neighbor of Crick in Cambridge, had claimed that in inducible systems, protein synthesis is accompanied by or even dependent upon RNA synthesis [138]. In addition, Sol Spiegelman, who also worked on enzyme induction, had assumed that the RNA templates of induced enzymes are unstable [139].

The concept of microsomes had emerged from eukaryotic *in vitro* systems with reduced metabolic activity, and as it had gained currency towards the end of the 1950s, it was clearly at odds with these observations on bacterial metabolism. Microsomal RNA appeared to be inert, and for all those working on cells from higher organisms, the ribosome represented "a stable factory", already containing an RNA transcript of DNA [10, p. 107], or to use Crick's words at that time: "'Template RNA' is located inside the microsomal particles" [113, p. 157]. Implicit in this vision was a kind of 'one-microsome-one-enzyme-hypothesis', meaning that a particular

ribosome was engaged in the fabrication of one specific protein or set of proteins. Moreover, bacterial *in vitro* systems had a bad reputation in the leading circles of protein synthesis workers in the late 1950s. They were considered 'dirty' systems which were difficult to control [125].

The decisive experiment establishing the role of messenger RNA came from a joint effort of Jacob, Brenner and Matthew Meselson at Caltech: They grew bacteria on heavy isotopes to tag the ribosomes and infected the *E. coli* cells with a virulent phage in the presence of radioactive isotopes. What they found was that newly synthesized radioactive phage RNA indeed became associated with pre-existing heavy ribosomes [140]. 'Messenger RNA' [141] now assumed the general meaning of a molecular information transmitter whose transcription was controlled by feedback loops according to the operon model (see Fig. 1-16). Around the same time, Masayasu Nomura and Benjamin Hall, in Spiegelman's laboratory at Urbana, had characterized a 'soluble' form of RNA synthesized in *E. coli* after bacteriophage T2 infection. It became associated with ribosomes in the presence of high magnesium concentrations [142]. They, however, drew no conclusions with respect to its function. As Nomura recalls, he was "unaware of the new developments, both experimental and conceptual, that were taking place in Cambridge, England, as well as in Paris" [8, p. 5]. And François Gros, Walter Gilbert, and Chuck Kurland, in the laboratory of Watson at Harvard, showed that unstable

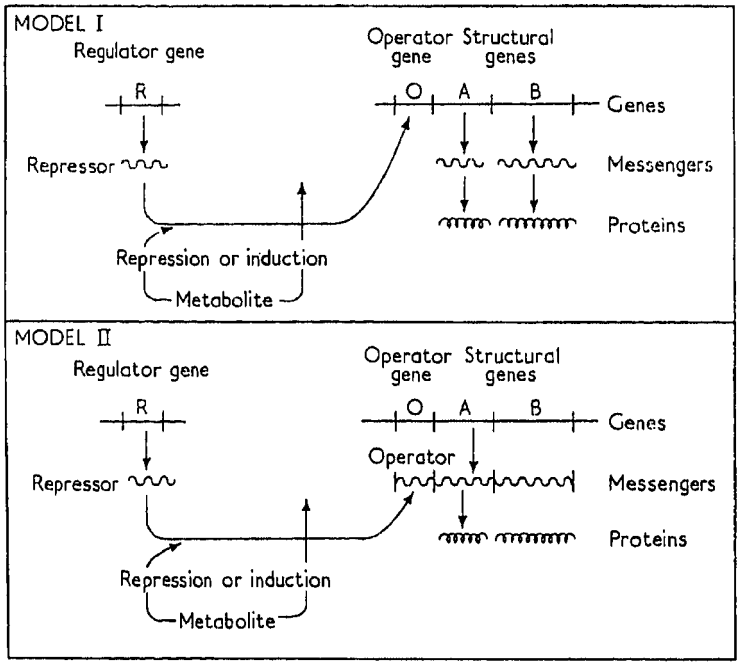

Figure 1-16 Operon models of the regulation of protein synthesis (Ref. [141], Figure 6).

'messenger RNA templates' (cf. the sedimentation pattern in Fig. 1-17) also belonged to the metabolic makeup of uninfected *E. coli* cells [143].

1.4.2
A Bacterial *in vitro* System of Protein Synthesis and the Cracking of the Genetic Code

The differentiation of reliable bacterial *in vitro* systems occurred in parallel, but independent of the experimental context of enzyme induction. The first to report on a system based on *E. coli* were Dietrich Schachtschabel and Wolfram Zillig at the Max Planck Institute for Biochemistry in Munich [144]. Published in German, this paper was ignored by most of the protein synthesis community. In 1958, Marvin Lamborg, a postdoctoral Fellow of the National Cancer Institute from NIH, had come to work with Zamecnik. Zamecnik had tried to obtain a reliable protein synthesizing system from broken *E. coli* cells as early as 1951, but had failed to clean it sufficiently from intact bacteria. Lamborg finally managed to establish a cell-free protein synthesis

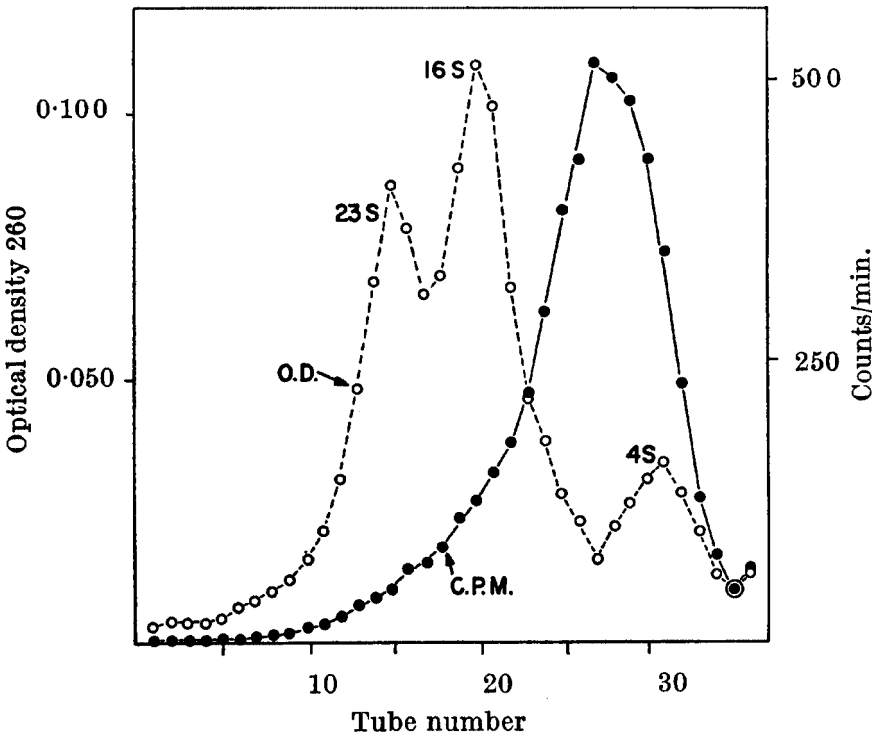

Figure 1-17 Sedimentation of [^{14}C]uracil pulse-labeled (unstable) RNA of *E. coli*. The RNA was run on a sucrose gradient for 10 h at 25 000 rpm: O.D., optical density; 23S, RNA of the large ribosomal subunit; 16S, RNA of the small ribosomal subunit; 4S, soluble RNA (Ref. [143], Figure 8).

system based on *E. coli* extracts [145]. In a rapid dissemination, the Lamborg–Zamecnik type of system made its way into other laboratories and soon became a leading model system for protein synthesis research. Besides Tissières in Watson's lab [146], among the first to use such a system were David Novelli at the Oak Ridge National Laboratory [147], Daniel Nathans and Fritz Lipmann [148] at the Rockefeller Institute in New York, Kenichi Matsubara and Itaru Watanabe [149] at the University of Tokyo and at Kyoto University, and James Ofengand, then on a fellowship at the Medical Research Council Unit for Molecular Biology in Cambridge [150]. In 1962, there were no less than six reports from five laboratories using the *E. coli* system in a rapid publication journal such as *Biochem. Biophys. Res. Commun.*, and seven reports from five laboratories in the biochemical *Fed. Proc.* In Watson's group, with Tissières, Schlessinger, Kurland, Gros, and Gilbert, the structure and function of bacterial ribosomes and messenger RNA had moved to the center of attention. But the *E. coli* system was also being introduced at the National Institutes of Health in Bethesda. The days of the rat-liver system as a pace-maker for unprecedented events were over. Its role was displaced from representation to demonstration: it became marginal. In contrast, the *E. coli* system shifted in the opposite direction. It allowed investigators to bring the genetic code into the realm of experimental manipulation, in a surprising move which left behind all those who had tried to tackle the code through procedures based on virus and phage mutation.

Marshall Nirenberg, at NIH, had just started to establish a cell-free *E. coli* system when Heinrich Matthaei joined him in the fall of 1960. Nirenberg had set himself the task of investigating the steps that connect DNA, RNA and proteins, and synthesizing, in a cell-free system, a specific protein [151]. Despite many efforts (cf., e.g., Ref. [152]), the synthesis of a defined and complete protein *in vitro* had remained a challenge – and a dream – for all those concerned with protein synthesis ever since the end of the 1940s.

If the system was to express a specific protein, conditions had to be found under which it responded to specific templates. This appears to have been the crucial clue in the Nirenberg and Matthaei advances. With respect to the initial phase of the work, there is every reason to assume that Nirenberg and Matthaei proceeded well within the context of the prevailing picture of the ribosome, its RNA still being assumed to play the role of a template. A minor, but finally decisive procedure set the stage for their accomplishment: the preincubation of the bacterial cell extract. Matthaei and Nirenberg put the system to work until its endogenous activity came to a halt. Only then did they add the exogenous RNA. First they found a small, but specific effect with superadded ribosomal RNA. Then, according to a principle of variation, they introduced additional RNAs into the system, such as viral RNA, and finally artificial homopolymers and heteropolymers. It was a lucky coincidence that the synthesis of RNA fell into the special expertise of Leon Heppel, who was the director of the laboratory in which Nirenberg and Matthaei were working. With these polymers at their disposal, they needed only a few months until they, by systematically varying their radioactive amino acids, had deciphered the first code word: The homopolymer polyuridylic acid coded for the artificial protein poly-phenylalanine [18, 153]. Figure 1-18 shows the decisive experiment from Matthaei's laboratory notebook.

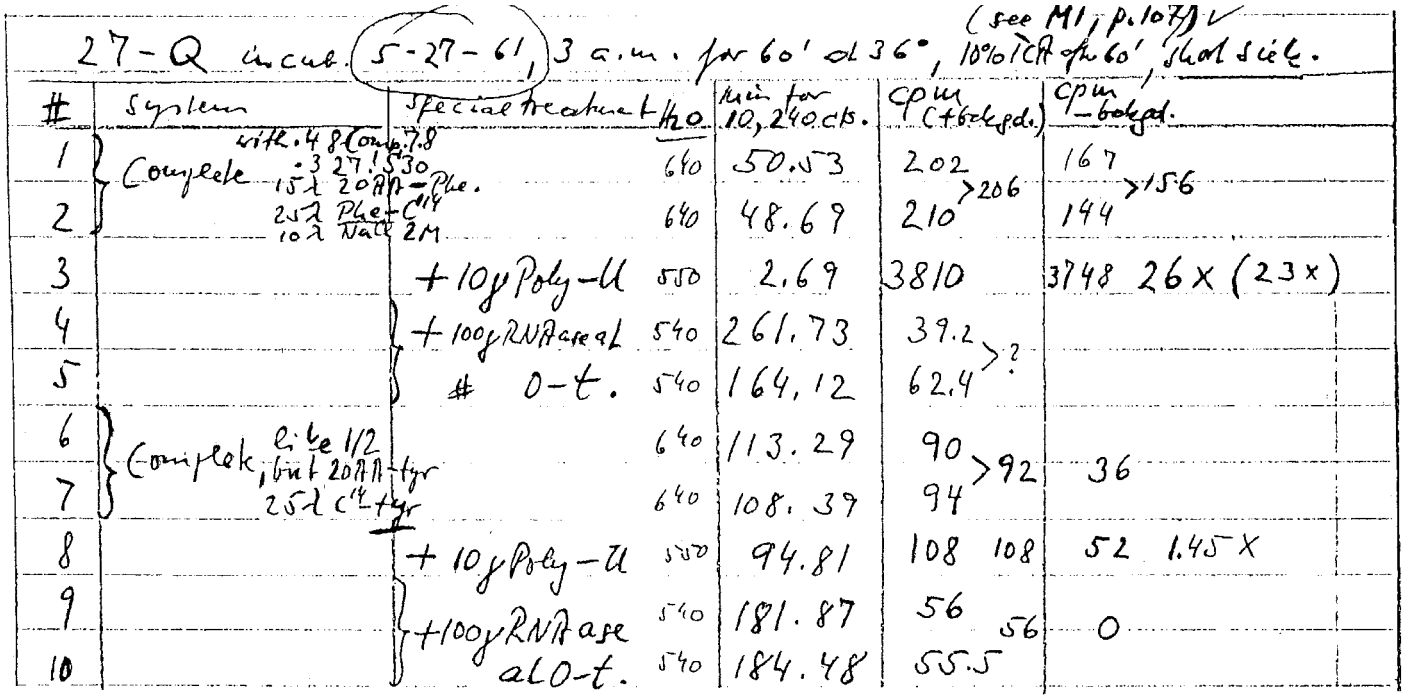

Figure 1-18 First poly(U) assay identifying phenylalanine as the corresponding amino acid (Matthaei, Notebook M2, p. 54, Experiment 27Q from 27 May 1961, with kind permission from the author).

Both Matthaei and Nirenberg, like Nomura, claim to have been unaware of the news from Paris concerning a cytoplasmic messenger at that time [12]. Thus, we have to assume that the concept of messenger arose at least twice in the history of molecular biology. It emerged from two experimental contexts that could not have been more different: from a delicate, genetically triggered *in vivo* system of enzyme induction, and from a comparatively modest, fractionated *in vitro* system of protein synthesis. Despite these radical breakthroughs, microsomal RNA continued to be considered for quite a while as a possible template, at least for ribosomal proteins. We still find this idea, e.g., in the first edition of Watson's *Molecular Biology of the Gene* in 1965 [154].

After the Fifth International Congress of Biochemistry in August 1961 in Moscow, where Nirenberg reported the findings from his laboratory, the other attempts at deciphering the code by genetic and chemical microanalysis of phage mutants in Cambridge and of tobacco mosaic virus mutants in Berkeley and Tübingen could be dropped (see, e.g., Refs. [155–158]). The subsequent hunt for the different code words became a matter of refining the experimental conditions of the *E. coli* system. The triplet binding assay of Philip Leder was one of the key accomplishments in the years to come [159]. Besides Nirenberg, it was mainly Severo Ochoa and his co-workers in New York and Gobind Khorana in Wisconsin who, on the basis of their experience with polymer synthesis, were able to join the race ([160], see Ref. [161] for a review). An initiation codon and the corresponding, formylated initiator tRNA [162, 163] as well as special codons functioning as stop signals were soon identified genetically [164, 165] and biochemically [166–168]. By 1967, the complete code was in place. For the next 10 years, the new findings on translation resulted, along the lines of ever new twists, quirks, and refinements, from the dissection of bacterial systems. After the initial technical difficulties in handling bacterial extracts had been mastered, these systems proved less complex, easier to take apart and simpler to entertain. The relation between eukaryotic and procaryotic systems was reversed. At the end of the decade, it was self-evident that a volume on *The Mechanism of Protein Synthesis* would deal primarily with bacteria, devoting just one special section of 116 pages out of a total of 855 to "Mammalian Systems" [19].

1.4.3 The Functional Dissection of Translation

With the isolation of ribosomes, the purification of specific transfer RNAs and their corresponding synthetases, and the beginning of a deliberate manipulation of viral and synthetic messengers, the stage was set for the dissection of ribosomal function [169, 170]. From the first observations onwards [171], one of the big riddles concerning the energy turnover of peptide elongation had been the involvement of GTP in the process. Around 1960, it had become clear that GTP was not involved in the amino acid-activation reaction *per se*. In a manner still not understood, GTP did interfere with the amino acid transfer mechanism (see discussion in Ref. [172]). The transfer depended on a partial fraction of the pH 5 enzyme supernatant [173]. But attempts to clarify the role of GTP as a co-factor for a presumed 'transfer factor'

had failed so far [148]. It took another 3 years until Jorge Allende and Robin Monro in Lipmann's lab identified an enzyme fraction in *E. coli* whose transfer activity overlapped with a GTPase activity [174] and was termed 'G factor' ([175, 176], see Ref. [3] for a review). At the same time, a complementary 'T factor' was resolved into a temperature-stable component Ts and an unstable component Tu [177]. In bacteria, they became known as elongation factors EF-G and EF-Tu/EF-Ts (EF2 and EF1A/EF1B, respectively, in eukaryotes). In a reticulocyte system, Boyd Hardesty and Richard Schweet, a few years earlier, had already identified two fractions, TF-1 and TF-2, that were involved in the GTP-dependent interaction of Phe-tRNA with poly(U)-programmed ribosomes [178]. The identification of three factors required for the initiation [179, 180], and of factors required for the termination of the translation process soon followed [181]. The characterization, in terms of function and primary as well as tertiary structure, of these transient ribosomal-binding factors continued well into the next two decades. They became model proteins for the study of RNA–protein interactions.

Transfer RNA binding to ribosomes and to their subunits became a major subfield for studying ribosomal function. At the beginning, these studies were still done with eukaryotic microsomes. Among the pioneers were Tore Hultin in Stockholm and Leendert Bosch in Leiden [182–185]. Around the same time, Hoagland, in the process of performing one of the first experiments in which a doubly labeled tRNA was used, observed a "background" phenomenon which he found "difficult to reduce" [186]. The binding of transfer RNA to the rat liver microsomes occurred at zero time, and it took place prior to the amino acid incorporation reaction. A control experiment revealed that uncharged S-RNA bound to the microsomes as well as did S-RNA charged with amino acids. This finding opened the door for detailed studies of the interaction between tRNA and ribosomes.

The majority of the ensuing tRNA binding studies was done in bacterial systems, where the poly(U)-dependent Phe-tRNA binding assay became by far the most prominent. Soon, Walter Gilbert showed that the tRNA carrying the growing polypeptide is associated with the 50S subunit [187], whereas the binding of poly(U) apparently involved the small subunit [188], and the binding of transfer RNA in general depended on the presence of a messenger [189]. Jonathan Warner and Alex Rich found active reticulocyte ribosomes carrying two transfer RNAs [190].

Quantification in these early binding studies was difficult: too many parameters were not yet standardized, and stoichiometric relations could only be estimated. In this situation, a functional and clearcut distinction between two different binding sites of charged tRNAs on the ribosome would be of considerable advantage. Robert Traut and Robin Monro [191] provided it with the puromycin-peptidyltransferase assay which allowed investigators to distinguish a puromycin-sensitive (B-site, later named P-site) and a puromycin-insensitive binding state (A-site) of aminoacylated tRNA (see Fig. 1-19). Based on this observation, the two-site model of ribosomal elongation as shown in Fig. 1-20 became codified by Watson [192] and continued to serve as a reference system for research on ribosomal function well into the 1980s. Many features of translational initiation [193], elongation [194–196]

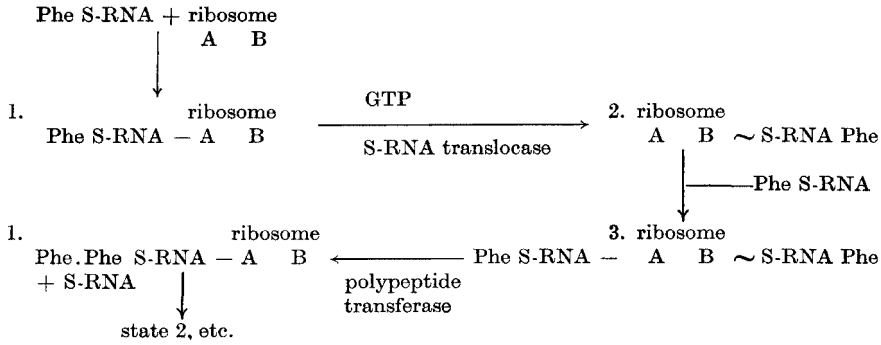

Figure 1-19 Model of the ribosomal elongation cycle as derived from the reaction with puromycin identifying two ribosomal tRNA binding sites, B and A, respectively [191].

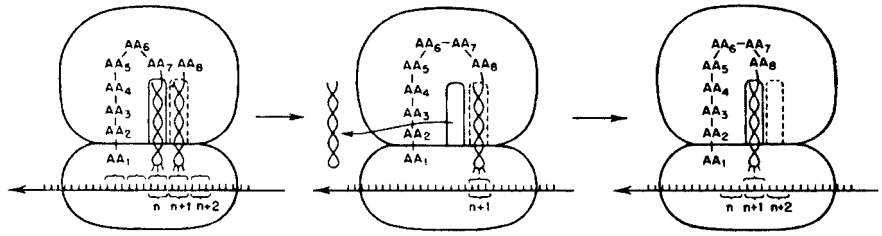

Figure 1-20 Model of the ribosomal elongation cycle comprising two tRNA binding sites (Ref. [192], Figure 20).

and termination [197] were outlined in more and more sophisticated and reduced partial *in vitro* systems (see Ref. [9] for a more recent survey), with acetylated Phe-tRNA (AcPhe-tRNA) becoming a generally accepted analogue for an initiator tRNA and a peptidyl-tRNA analog in the poly(U) system [198].

Antibiotics revealed themselves to be invaluable tools for the dissection of partial ribosomal functions as well as for the ongoing *in vivo* studies concerning regulation, speed, and accuracy of protein synthesis. Among the prominent drugs were puromycin as an elongation terminating agent (see Refs. [199–201] for early studies); chloramphenicol as a specific inhibitor of bacterial peptidyltransferase [202–204]; fusidic acid as interfering with the translocation factor EF-G [205, 206]; and streptomycin as inducing misreading [207, 208]. One of the earliest realistic measurements concerning the accuracy of the process of polypeptide formation came from Robert Loftfield [209]. (For more details about antibiotic effects on ribosomes see Chap. 12).

In the context of pursuing ribosomal function, and after the mRNA concept had been established, gentle isolation of messenger–ribosome complexes became a matter of priority in the early 1960s. Particles larger than 70S or 80S appeared on sucrose-gradient patterns and electron microscopic images (see Fig. 1-21 as an

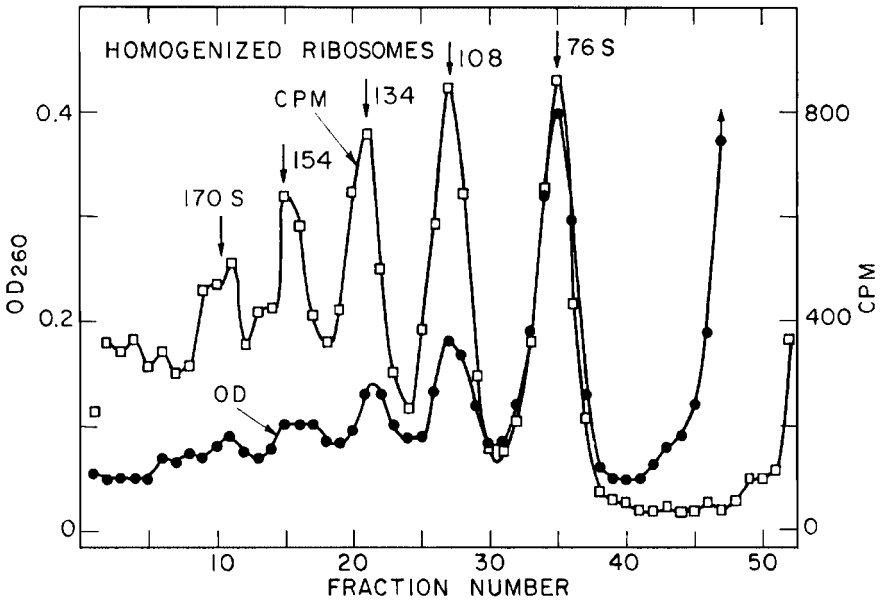

Figure 1-21 Polysomes. Sucrose gradient of lysed reticulocytes after incubation with [^{3}H]leucine. After lysis and low-speed centrifugation, the ribosomes were pelleted three times at 28 000 rpm and resuspended with a homogenizer. A 5–20% sucrose gradient was used. The numbers next to the arrows represent the sedimentation constants associated wih each peak (Ref. [214], Figure 3).

example). They were variously termed 'ribosomal clusters' [210], 'active complexes' [211], 'ergosomes' [212], or 'aggregated ribosomes' [213], before Warner and Rich coined the term 'polysomes' [214] which quickly came into general use. Polysomes appeared to consist of strings of ribosomes occupying a particular messenger RNA. Special isolation procedures were required to prevent them from breaking down to monosomes during fractionation. On the other hand, *in vivo* and *in vitro* evidence grew that ribosomes dissociated and reassociated during their functional cycle [215, 216], and that initiation started on the 30S subunit [217].

Around the same time, Peter Traub, together with Nomura, found the right temperature and ionic conditions for reconstituting the small ribosomal subunit of *E. coli* in the test tube [218] from its RNA and protein moieties, respectively. After the much simpler, symmetric TMV in the early 1950s, the highly asymmetric ribosome became the emblem of molecular self-assembly in the late 1960s and early 1970s (see Fig. 1-22 for Nomura's assembly map of the 30S subunit).

In the following years, multiple attempts to repeat the procedure for total reconstitution of the large 50S subunit from *E. coli* remained without success. A reason for these failures was suspected to be the high activation energy necessary for the 50S

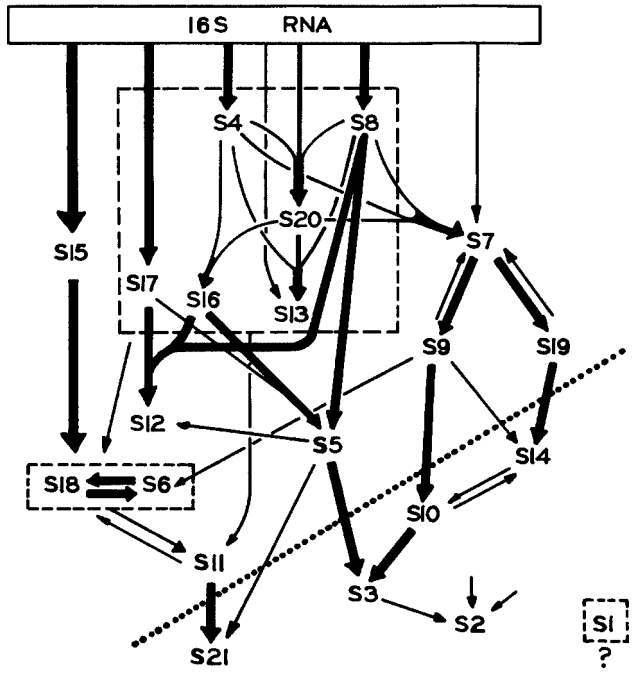

Figure 1-22 Assembly map of *E. coli* 30S ribosomal proteins. Arrows between proteins indicate the facilitating effect of one protein on the binding of another – a thick arrow indicates a major facilitating effect (Ref. [305], Figure 1).

assembly *in vitro*. Accordingly, Nomura and his coworkers shifted their interest to the thermophilic bacterium *Bacillus stearothermophilus*. In 1970, they achieved the first total reconstitution of a large ribosomal subunit [219]. In view of the widespread use of the *E. coli* ribosome as a model organelle, however, the search for a way to reconstitute the *E. coli* 50S ribosomal subunit continued. Four years later, Knud Nierhaus and Ferdinand Dohme succeeded in this task. They developed an alternative, two-step reconstitution method thus obviating the need for incubation at high temperatures above 50°C that would have been required in the previous one-step procedure [220].

The possibility of *in vitro* ribosome assembly opened the field for a multiplicity of structure–function correlation studies at a previously unknown level, including the construction of assembly pathways and maps, detailed interactions between ribosomal proteins and ribosomal RNA, and functional reconstitution experiments where one or more components were omitted (see Chap. 3.1 for details). The hope, however, that a particular ribosomal protein might be singled out as responsible for the peptidyl transferase reaction did not materialize (see Chap. 8.4).

1.4.4
The Structural Dissection of the Ribosome

Throughout the 1950s, the macromolecular composition of microsomes and ribonucleoprotein particles had remained obscure. The original assumption of Watson at Harvard, Schachman in Berkeley, and others who started to analyze bacterial particles, had been that their structure might be analogous to that of RNA viruses: an RNA moiety wrapped with multiple copies of a coat protein. The virus analogy dates back all the way to the beginning of microsome research. Although it had lost its early connotation of a cytoplasmic replicator, the analogy continued to play the role of an obstacle rather than that of a research promoting conceptual tool. It had certainly not been favorable either to the emergence of the concept of messenger RNA, or to the emergence of the view of an asymmetric particle consisting of many different proteins. Resisting the viral analogy, neither ribosomes nor their protein subunits seemed regular enough to form crystals, as had been the case with, e.g., tobacco mosaic virus.

In view of the complex protein make-up of ribosomes, it is not surprising that RNA was the first ribosomal component to be characterized physically and chemically in terms of sedimentation behavior, molecular weight, and overall base composition. As for the ribosomes, so for rRNA, too, sucrose-gradient centrifugation was crucial. Around 1960, there was still considerable uncertainty about the identity of ribosomal RNA. As long as it was considered to represent the template(s) for protein synthesis, there had been, understandably, no reason to assume that rRNA might be homogenous and well defined. In contrast, credit was given to the idea that ribosomal RNA might be composed of smaller templates that became linked within the particle later on, either non-covalently or covalently. Before RNase-free strains of bacteria became available [221, 222], the problem of RNA breakdown during preparation could hardly be mastered. Yet the introduction of the separation of RNA from cellular protein by phenol extraction had already greatly facilitated laboratory manipulation of RNA. This method came into quick and general use soon after its publication [223, 224]. In 1959, Paul Ts'o [225] separated rRNA from pea seedlings and rabbit reticulocytes into two major 28S and 18S peaks. A series of careful studies on *E. coli* ribosomes in Watson's laboratory led Kurland to propose that ribosomal RNA came in two large species, 16S and 23S, respectively [226]. Alexander Spirin in Moscow had reached basically the same conclusion [227]. The question however whether this represented the 'native' state of ribosomal RNA, whether originally they were made up from smaller fragments or derived from a large precursor, continued to be a matter of debate for several years [228]. The controversy eventually came to a satisfactory end when it became evident that mature ribosomal RNA originated from a large transcript that was processed in the event of ribosome formation, and that, indeed, a small defined RNA, 5S RNA, was part of the 50S subunit [229]. Subsequently, 5S rRNA became the first ribosomal RNA molecule to be completely sequenced in 1968 [230]. This breakthrough had been made possible through Sanger's 2D fractionation procedure for radioactive nucleotides [231]. It took 3 years

to determine its 120 nucleotides. In comparison, sequencing the first transfer RNA (yeast $tRNA^{Ala}$) with slightly more than half the number of nucleotides had taken Holley and his co-workers (see Fig. 1-23 for its sequence and putative secondary

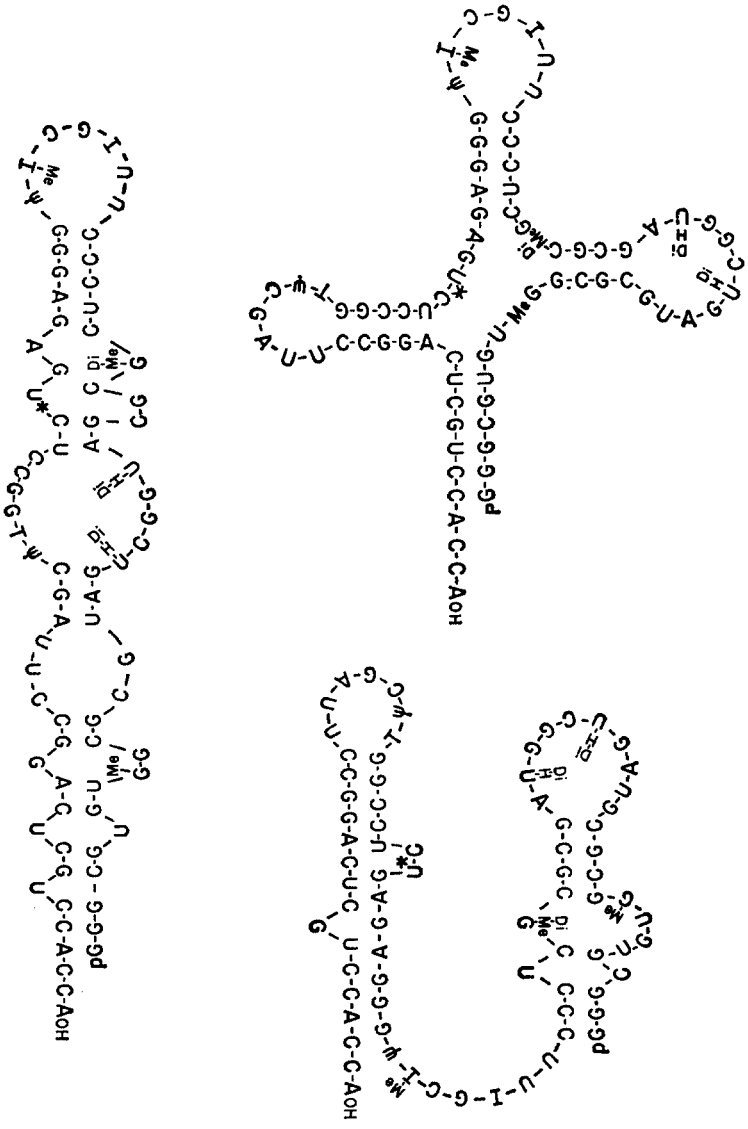

Figure 1-23 Proposed secondary structures for the first tRNA sequenced – $tRNA^{Ala}$ from yeast (Ref. [132], Figure 2). The cloverleaf structure was proposed by Elizabeth Keller who had moved from Zamecnik's laboratory to Holley's (cf. Ref. [11, pp. 282–285]).

structures) some eight years [132]. Other groups soon followed with other tRNA species [232, 233]. The detailed functional elucidation of these molecules, however, had to await further studies. Their crystallization proved to be a major prerequisite for moving forward in this direction (see Refs. [234, 235] among others).

Whether the protein moiety of ribosomes was made up of multiple copies of a single species or of many different proteins, and whether all ribosomes had the same protein composition, was still an open question at the beginning of the 1960s. Serious analysis, on the basis of starch-gel electrophoresis, of the protein composition of ribosomes goes back to the work of Jean-Pierre Waller [236] and to the fractionation studies of David Elson [237] and Pnina Spitnik-Elson [238]. One of the first ribosomal proteins to be characterized individually was the acidic A-protein of the large subunit studied by Wim Moeller and later known as L7 (L12) [239]. Major efforts to develop methods for separating and purifying individual proteins came, among others, from Heinz Günter Wittmann and Brigitte Wittmann-Liebold's laboratory in Berlin [240], Tissière's in Geneva [241], and Kurland's in Wisconsin [242]. A prominent achievement in this endeavor was the separation of all ribosomal proteins by 2D polyacrylamidegel electrophoresis [243] as shown in Fig. 1-24. It served as an efficient and economizing standardization vehicle in the field of ribosomal protein identification.

1.5
1970–1990s: A Brief Synopsis

The survey of the following three decades from the 1970s to the 1990s will be very brief. There is no need to go into the details of an ongoing research in this historical introduction, since the major events during these decades will be extensively dealt with in the following chapters. The 1970s can be regarded as the period of the elucidation of the primary structure of the components of the translational apparatus. Indeed, around the turn of the decade, the ribosome of *E. coli* became the first cellular organelle whose RNA [244–246] and protein components [247] were completely sequenced. Sequencing the complete ribosomal RNA became a feasible task only after the new sequencing methods of Maxam and Gilbert [248], and of Sanger [249] had been introduced.

The emergent recombinant DNA technologies helped to construct a detailed genetic map of the components involved in protein biosynthesis. The ribosomal RNA genes, however, were mapped before the era of recombinant DNA technology. A dozen years had elapsed between their first identification in 1962 [250] and their precise mapping [251]. Knowledge about ribosomal protein genes and operons rapidly accumulated after the subsequent isolation of protein gene-transducing lambda phages [252]. Another source of information was provided by the systematic work with ribosomal protein mutants [253, 254].

Molecular details of ribosomal function also became available, such as the interaction of mRNA with 16S RNA during initiation [255, 256], and the mechanisms by which ribosomes achieve their accuracy [257, 258]. The regulation of ribosome biosynthesis, starting with the early findings on the genetics of RNA synthesis [259],

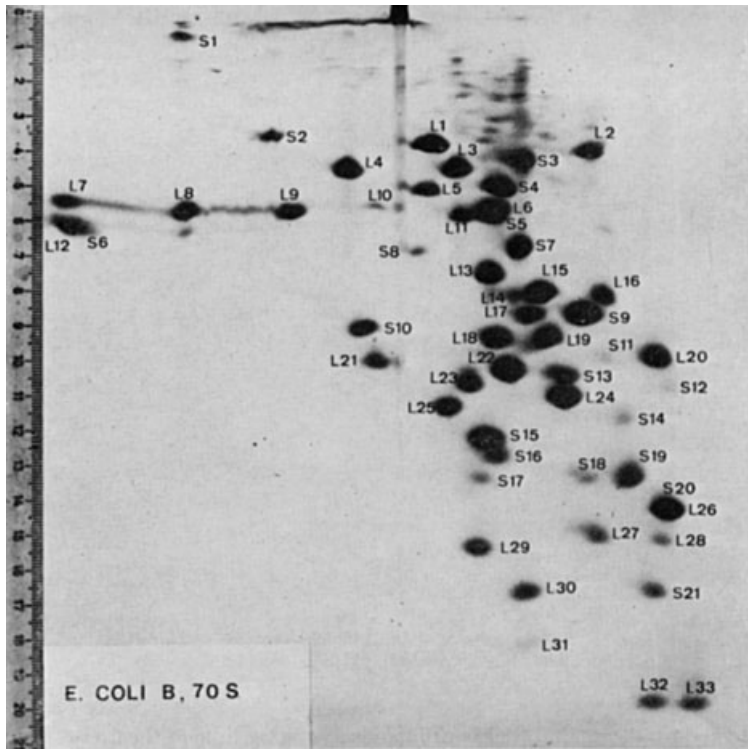

Figure 1-24 2-D electrophoretogram of 70S proteins of *E. coli* B: First dimension, 4% acrylamide, pH 8.6; second dimension, 18% acrylamide, pH 4.6 (Ref. [243], Figure 4).

also became a major field of investigation during the 1970s [260–262]. Over the years, a detailed view, first of transcriptional, then of translational feedback regulation mechanisms emerged. Since Monod and Jacob's work on the lac operon, transcriptional control had been the leading paradigm. The shift of interest from transcriptional to translational regulation was indeed an unprecedented turn. The major events in this area have both been initiated and reviewed some time ago by Nomura [8].

During the 1970s, ribosome research became a focus for the development and application of numerous advanced biochemical, biophysical and biological techniques. *In vitro* reconstitution of ribosomes [263] and *in situ* localization of ribosomal components via immunoelectron microscopy [264, 265], scattering studies [266], cross-linking [267] and affinity labeling [268] led to early insights into the quaternary structure of the protein synthesizing organelle and its functional characteristics such as factor binding and the constitution of the peptidyltransferase center.

The 1980s, on the one hand, were characterized by an increasing backshift of emphasis towards eukaryotic systems (see Ref. [269] for a contemporary overview).

Ira Wool in Chicago had pioneered mammalian ribosomal proteins during the era of *E. coli* (see Ref. [270] for a review), Rudi Planta in Amsterdan had done much of the genetic and structural work on yeast ribosomal RNA (see Ref. [271] for a review). On the other hand, after a lag period, the tedious and time-consuming task of secondary, tertiary, and quaternary structure modelling came to fruition and became linked to ribosomal function. Protein–protein crosslinking [272], protein–RNA crosslinking [273, 274], protection and modification studies [275], neutron scattering [276, 277], electron microscopy [278], and ribosome crystallization [279] figure prominently among the methods involved in this continuing endeavor. On the functional side, exhaustive tRNA-binding studies led to new model conceptions of the elongation cycle involving a third tRNA binding site [280–283, 275]. Peter Moore has judged on this topic: "The two-site model for the ribosome, which the world has accepted for a generation is dead. The existence of a third site for tRNA binding, the exit site, is now established beyond reasonable doubt. This is unquestionably the most significant advance in our understanding of the ribosomal events of protein synthesis in many years" [284].

Finally, the 1990s were dominated by major efforts to carry the structural analysis of ribosomes to atomic resolution. The availability of suitable crystals of ribosomes and ribosomal subunits, particularly from thermophilic and halophilic sources, and the solution of the phasing problem led to a proliferation of X-ray crystallographic studies to which Wittmann and Ada Yonath [285] in Berlin and the group at Pushchino [286] had laid the ground with their ribosome crystallization initiatives in the 1980s. After almost 20 years of continued efforts, atomic resolution has now been achieved for the large ribosomal subunit from *Haloarcula marismortui* [287] and *Deinococcus radiodurans* [288], the small subunit from *Thermus thermophilus* [289, 290], and near-atomic resolution for the 70S-tRNA–mRNA complex [291]. In parallel, the development of cryoelectron microscopic image reconstruction has helped to refine the overall 3D shape of ribosomal particles, in particular as related to specific functional states [292, 293]. Thus a dynamic picture of elongation is emerging.

Awareness of the involvement of rRNA in ribosomal functions has grown during the last decades. Seminal in this context was certainly Carl Woese with his speculations on the origin of the protein synthetic machinery [294]. But it was the characterization of catalytic activities of precursor ribosomal RNA initiated by Thomas Cech [295] that turned the 'protein paradigm' of the ribosome, prevalent in the 1960s and 1970s, back into an 'RNA paradigm'. (Indeed, in the early days of ribosomology, rRNA had been closely associated with ribosomal function. That function — of a template — however, did not survive history.) Indications accumulated that 23S RNA is involved in the peptidyltransferase reaction [296, 297], which until then was thought to be a domain of the ribosomal proteins. Efforts to achieve peptidyltransfer activity with ribosomal RNA alone have so far not been successful [298, 299]. The atomic model of the 50S subunit now appears to suggest that ribosomal RNA may indeed be able to do the job without direct involvement of proteins [300]. On this view, the ribosome would finally turn out to be a veritable ribozyme. However, to adduce direct biochemical evidence concerning the catalytic mechanism of the transfer reaction remains a task for the future.

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