Biochimica et Biophysica Acta, 1000 (1989) 103–105
Elsevier

Commentary by

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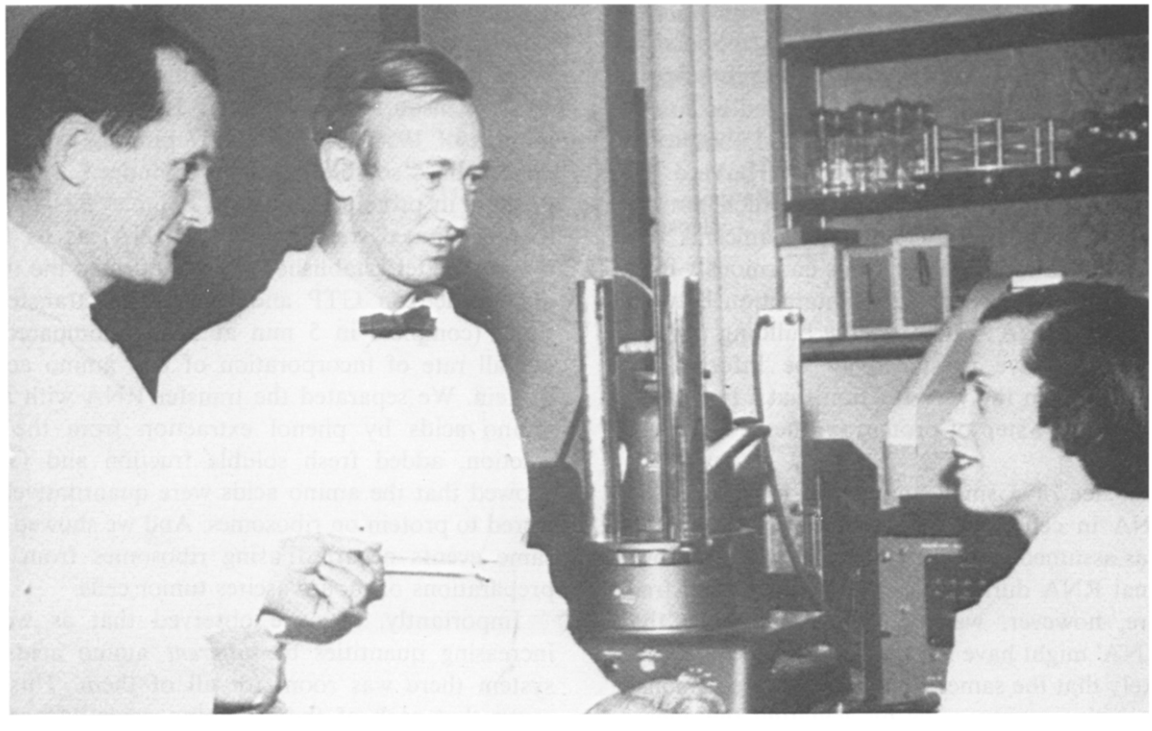
on 'Intermediate reactions in protein biosynthesis'
by M.B. Hoagland, P.C. Zamecnik and M.L. Stephenson
Biochim. Biophys. Acta 24 (1957) 215–216

I first became aware of the challenge of the problem of protein biosynthesis in 1949 at the Huntington Laboratories at the Massachusetts General Hospital, Boston. Paul Zamecnik and a small group of associates were then pioneering the use of ^{14}C-labeled amino acids to explore protein synthesis in mammalian cells. They were progressing from in vivo systems to tissue slices and thence to the earliest cell-free preparations. They were at pains to show that incorporation of isotopes into protein represented true peptide bond formation, and they had established that the process was coupled to oxidative energy production.

I had just finished medical school after a 2-year bout with tuberculosis and had got a postdoctoral position at the Huntington with the thought of returning to medicine in a year or two. I worked on the carcinogenic and other biological effects of the metal beryllium – a project that convinced me I wanted to continue to do science – and over the next 2 years became increasingly interested in Zamecnik's work. He encouraged me to get some in-depth exposure to protein chemistry and to biochemical energetics. I spent a year with Kaj Linderstrøm-Lang at the Carlsberg Laboratory in Copenhagen and another year with Fritz Lipmann at the Biochem-



(Left to right) Mahlon Hoagland, Paul Zamecnik and Mary Stephenson (about 1956).

ical Research Laboratories back at the MGH. My exposure to the latter's interest in the ATP-dependent activation of acetate was an important factor in leading me to my discovery of amino-acid activation in 1954, soon after my return to the Huntington. By then the Zamecnik group had delineated the protein synthesizing system of rat liver as consisting of microsomes (ribosomes), a soluble fraction (in which I had found the activating enzymes), ATP, an ATP generating system, and GTP.

In ruminating upon the activation reaction, Zamecnik wondered if the enzymes involved might also participate in the synthesis of RNA. Could adenyl-amino acids be, in a sense, double-barreled – donating their amino acids to the protein polymerizing machinery or, alternatively, their adenyl portion to a nucleotide polymerizing system? To test the idea, Zamecnik and Mary L. Stephenson, his long-term associate at the Huntington, incubated ^{14}C-labeled ATP with ribosomes and soluble fraction and looked to see if RNA became labeled. It did. (This phenomenon was later shown by Zamecnik and Lisalotte Hecht to be due to a special reaction: the terminal adenylation of transfer RNA.) They were pleased with the result, but suspicious: could the AMP moiety of ATP, or indeed the whole ATP molecule, be attached to RNA in some trivial, non-specific, non-convalent linkage? They did a control experiment incubating the same system with ^{14}C-labeled amino acids and found to their surprise that they, too, became bound to RNA. Furthermore, when the two cell fractions were *separately* incubated with labeled amino acids the soluble fraction labeled its indigenous RNA startlingly more actively than the ribosomal fraction labeled its RNA.

At this point – January 1956 – Zamecnik stopped doing experiments. He'd just been appointed as Joseph Aub's successor as head of the Huntington Laboratories and Professor of Oncologic Medicine at Harvard. He also had clinical duties in the hospital which put additional demands on his time. In June, Zamecnik told me of the 5-month-old findings. I was enormously intrigued by what was clearly a direct interaction between amino acids and RNA – between the building units of protein and what we presumed to be information-bearing molecules in the very fraction that I had found carried out the first step of protein synthesis. I pursued the lead.

The existence of a small amount of low-molecular-weight RNA in cells had already been noted by that time. It was assumed to be a product of the breakdown of ribosomal RNA during the preparation of cell fractions. Here, however, was unexpected evidence that 'soluble RNA' might have some very special function. It seemed likely that the same enzymes that were responsible for activating amino acids by acylating the adenyl portion of ATP were transferring the amino acyl groups to soluble RNA. This was borne out by my early studies reported in the cited BBA paper: the aminoacylation of soluble RNA was specifically dependent on ATP (GTP, CTP and UTP wouldn't do); the addition of pyrophosphate caused a rapid deacylation of the RNA (presumably by pushing the reaction to the left to form ATP and free amino acid); and the product was acid-stable and alkali-labile, did not contain free amino acid, and produced a hyroxamic acid when incubated with hydroxylamine.

The exciting broader interpretation of the phenomenon was that we had found in this small amino-acid-binding RNA a key intermediate in protein synthesis – a molecule or molecules that accepted activated amino acids and carried them to the ribosomes. (Other possibilities – that the binding was an in vitro artifact or that the RNA was some kind of a waste disposal unit picking up unwanted amino acids – were sufficiently unattractive to be set aside.) That such a hypothetical intermediate might have a role in the mysterious process of ordering amino acids, we had no doubt – and no evidence. The crucial first test was obvious: see if amino acids could be transferred from soluble RNA to peptide linkage in protein in the absence of ATP.

In the most suspenseful and exciting few hours of my professional life, I did an experiment that came out essentially as reported in the cited paper: having labeled the indigenous RNA of the soluble fraction with amino acids in the presence of ATP, reisolated the fraction's protein and RNA free of amino acids and ATP by reprecipitation at pH 5, I then incubated it with microsomes. To my delight, most of the counts on RNA were rapidly transferred to protein, in a reaction that specifically required GTP and proceeded in the presence of a large excess of free unlabelled amino acid.

Stephenson, Zamecnik and I spent the remaining months of 1956 and early 1957 pinning down the evidence that 'soluble RNA' was indeed a key intermediate in protein synthesis. ('Soluble' RNA was soon to be renamed transfer RNA (tRNA) as its function became better established.) We confirmed the transfer's dependence on GTP and showed the transfer to be rapid (complete in 5 min at 37 °C) compared to the overall rate of incorporation of free amino acids into protein. We separated the transfer RNA with attached amino acids by phenol extraction from the soluble fraction, added fresh soluble fraction and GTP and showed that the amino acids were quantitatively transferred to protein on ribosomes. And we showed that the same events occurred using ribosomes from cell-free preparations of mouse ascites tumor cells.

Importantly, too, we observed that as we added increasing quantities of *different* amino acids to the system there was room for all of them. This had to mean that each of the 20 amino acids was separately bound to some site on the RNA molecules. The RNA

must, then, either be a single species with binding sites for all 20 of the amino acids *or* 20 (or more) different RNAs, each with its own specific binding site for each amino acid. The latter, of course, proved to be the case but we couldn't make the distinction then. We were convinced we had uncovered the second major step in the cell's machinery for making protein.

I wrote up and submitted the cited BBA account in late 1956. By that time, T. Hultin in Sweden was obtaining indirect evidence that amino acids passed into a state experimentally distinguishable from free amino acid before they entered protein. Kikuo Ogata in Japan, and Paul Berg and Robert Holley in the U.S.A. were also finding signs of steps in which amino acids were bound to RNA after activation. A more definitive account of our work appeared in 1958 [1].

What justified our inference that transfer RNA had anything to do with determining order? The evidence was then only circumstantial: if DNA carried genetic information specifying amino-acid sequence, and if RNA carried out DNA's role in the cytoplasm – which everyone by then assumed it must – it followed that the observed direct chemical association of amino acids with RNA *must* have implications for sequencing.

In late 1956, I had my first visit from a card-carrying 'molecular biologist'. (We at the Huntington considered ourselves biochemists.) Jim Watson had just become Professor of Biology at Harvard and was probing the structure of the ribosome. He had heard rumors of our discovery of transfer RNA and I jubilantly told him of our findings. He was restlessly attentive and when I'd finished he told me that Francis Crick had forecast the existence of transfer RNA-like molecules! Hadn't I heard of the *adaptor hypothesis*? I was astonished and admitted that I hadn't. Watson explained that Crick had been wrestling with the question: if the machinery of protein synthesis contained information specifying the order of amino acids in protein – presumably in the form of DNA-derived RNA acting as the ordering template – how would the template 'know' – i.e., chemically recognize – an amino acid if it bumped into it? This concern was a natural extension into chemistry – into structure – of Crick's fascination with the genetic code. There wasn't a trace of chemical homology or complementarity between amino acids on the one hand, and RNA on the other. Suppose, thought Crick, an enzyme first attached each of the amino acids to a special, unique short length of RNA. The amino acid would then have had conferred upon it a chemical identity that could be recognized by an ordering RNA template by hydrogen bonding between complimentarily shaped bases. Crick envisioned that the postulated small molecules, which he called *adaptors* – in that they adapted the amino acids so as to be recognizable by an ordering template – would be really quite small, perhaps only 3 to 10 bases in length. According to coding theory, three would be the minimum number of bases needed to code for a single amino acid. (The theory glossed over the problem of how the system would 'know' how to attach the amino acid to the correct RNA adaptor – vaguely relegating the job to the versatility of an enzyme.)

I was bowled over by the ingenuity and beauty of the idea and sensed it had to be the explanation of our experimental findings. An image arose in my mind: we biochemical explorers slashing our way through a dense jungle to discover a beautiful temple while Francis Crick, floating gracefully overhead on gossamer wings of theory, waited patiently for us to see the goal that he was already gazing down upon!

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Preliminary Notes

Intermediate reactions in protein biosynthesis*, **

Previous studies in this laboratory furnished evidence that L-amino acids are activated as amino acyl-adenylate compounds bound to specific enzymes derived from the soluble protein of rat liver[1]. Further substance has been given this hypothesis by the finding that synthetic amino acyl-adenylate compounds, when incubated with activating enzymes and pyrophosphate (PP), are able to form ATP***[2]. This paper presents evidence for another step in the reaction sequence between amino acid activation and peptide bond condensation.

The rat liver activating enzyme preparation[1] contains ribonucleic acid (RNA): about 5 mg

TABLE I

TRANSFER OF LEUCINE-^{14}C FROM PRELABELED ACTIVATING ENZYME FRACTION TO MICROSOMAL PROTEIN

Microsomes and pH 5 enzymes (activating enzymes) were prepared from rat liver as previously described[5]. Labeled pH 5 enzymes were prepared by incubating pH 5 enzymes (approximately 100 mg protein) for 10 min at 37° C with 0.01 *M* $MgNa_2$ ATP (Sigma), 0.1 m*M* ^{14}C-leucine ($1.8 \cdot 10^6$ c.p.m./μmole) and the medium[5] at pH 7.5 in a total volume of 20 ml. The reaction mixture was then diluted to 60 ml with cold water and the pH brought to 5.2 with *M* acetic acid to precipitate the enzymes. This dilution and precipitation was repeated after redissolving at pH 7.5. The final precipitate was dissolved in 4 ml of medium. The microsomes were suspended in 4 ml of the same medium. RNA was determined on an aliquot of each. 0.6 ml of microsome suspension (14 mg of protein; 2.3 mg RNA) and 0.4 ml of pH 5 enzyme fraction containing the labeled S-RNA (6 mg protein, 0.3 mg RNA) were then incubated in a volume of 2 ml for 15 min at 37° C with 0.5 m*M* nucleoside triphosphates as indicated, plus the triphosphate generating system (0.01 *M* phosphoenol pyruvate and 0.04 mg pyruvate kinase[5]). ATP, not shown here, is also inert. The reaction was stopped by the addition of $HClO_4$ (final concentration, 0.4 *M*), the precipitate washed 4 times with cold 0.2 *M* $HClO_4$, once each with 5:1 ethanol: 0.2 *M* $HClO_4$, ethanol (at 25° C) and 3:1 ethanol-ether at 50° C[6]. RNA was extracted with 10% NaCl at 100° for 30 min and then precipitated twice with 60% ethanol at —10° C. The final alcohol suspension was filtered onto paper discs. The dried RNA was counted using a Nuclear "Micromil" window gas flow counter. The RNA was then eluted from the paper with dilute alkali, and the 260/280 mμ absorption ratio of the extract determined in a Beckman spectrophotometer. Protein was washed, weighed and counted as previously described[5]. The total counts in RNA were multiplied by the ratio of the amount of RNA initially added to the amount recovered.

	Total counts in	
	RNA	*Protein*
Complete system (before incubation)	489	30
Complete system (after incubation)	180	374
Complete system, *minus* GTP	111	40
Complete system, *minus* generating system	72	155
Complete system, *minus* both GTP and generating system	23	30
Complete system, *minus* generating system but with 5 × GTP	145	129
Complete system, CTP replacing GTP	96	44
Complete system, UTP replacing GTP	101	53
Complete system, *plus* 0.005 *M* ^{12}C-leucine	183	314

* This is publication No. 889 of the Cancer Commission of Harvard University.
** This work was supported by grants from the U.S. Public Health Service and the Atomic Energy Commission.
*** ATP, GTP, CTP, UTP are the triphosphates of adenosine, guanosine, cytidine, and uridine respectively.

per 100 mg protein. This is apparently a low molecular weight RNA (S-RNA) with different metabolic properties from the high molecular weight RNA of the ribonucleoprotein of the microsomes. When the amino acid activating enzyme preparation is incubated with ATP and ^{14}C-carboxyl labeled leucine, at pH 7.5, the S-RNA subsequently isolated from this fraction is found to be labeled (0.02 to 0.05 μmoles leucine per mg RNA). The time curve of labeling is linear for 2 min, maximal at 10 min, and thereafter the ^{14}C-leucine label is rapidly lost. Microsomal RNA is labeled at approximately a tenth this rate. 0.005 M leucine and 0.01 M ATP saturate the system, and ribonuclease is completely inhibitory. Labeling is additive when 0.005 M ^{14}C-valine and 0.005 M ^{14}C-glycine are also incubated in the same preparation. Yeast RNA, microsomal RNA, and degraded microsomal RNA (prepared by mild alkaline hydrolysis of microsomes) do not give increased labeling when added to the system.

Leucine-labeled S-RNA so obtained from the activating enzymes preparation is non-dialysable and is charcoal and Dowex-1 adsorbable. The ^{14}C-leucine-RNA bond is acid stable and alkali labile, and does not exchange with free ^{12}C-leucine. The ninhydrin-CO_2 method indicates no free ^{14}C-leucine. When labeled S-RNA is incubated with anhydrous hydroxylamine and the products are chromatographed on paper a spot corresponding to leucine hydroxamic acid contains all the radioactivity.

The activating enzyme preparation labeled with ^{14}C-leucine (and reprecipitated twice at pH 5.2 from dilute solution to remove free ^{14}C-leucine and ATP) will transfer the bound ^{14}C-leucine to microsome protein upon subsequent incubation with microsomes and GTP (Table I). The GTP effect is apparently specific and is potentiated by addition of a nucleotide triphosphate generating system. With this complete system a high percentage of the S-RNA bound ^{14}C-leucine is transferred to protein, the rest remaining in S-RNA.

^{14}C-Leucine-S-RNA prepared by a phenol method[3] has been found to transfer ^{14}C-leucine to microsome protein in the absence of added activating enzymes, provided GTP is present.

Preliminary results, using an ascites tumor *in vivo* incorporation system[4], reveal that S-RNA becomes labeled with ^{14}C-leucine more rapidly than does the protein of the ribonucleoprotein particles of the microsomes, the most rapidly labeled protein fraction in this system.

These experiments suggest that incorporation of labeled amino acids into protein is indeed dependent upon the amino acid activation system. The initial formation of an enzyme-bound amino acyl-AMP compound, as originally suggested, accounts for hydroxamic acid formation and PP–ATP exchange[1]. It is now further postulated that this initial activation of amino acids is followed by a transfer of activated amino acid to S-RNA. This latter reaction is ribonuclease sensitive, while the former is not. GTP mediates the transfer of this activated amino acid to peptide linkage via the microsome by a mechanism as yet unknown.

The authors wish to thank Miss MARION HORTON for her able technical assistance.

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Received January 16th, 1957

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