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ON THE FUNCTIONS OF THE ENDOPLASMIC RETICULUM

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Abstract—Ribonucleoprotein (RNP) particles were purified in a highly active state from a number of tissues of the rat. Particle yield varied considerably for different tissues but the composition of particles was fairly similar. From the early blastula stage of *Psammechinus* eggs active particles were also obtained, and at a higher yield than from preceding stages of development. Mitochondria also yielded protein synthesizing particles in a study of other cell components. No significant amino acid incorporation was obtained with purified RNP particles when they were used alone. Mechanisms of amino acid incorporation are discussed. Experiments show the possibility of a functional interrelationship between endoplasmic particles and membranes.

A GOOD deal of evidence has accumulated in the last few years in favour of the idea that ribonucleoprotein particles (RNP-particles) are intimately involved whenever proteins become synthesized in the cytoplasm. These particles may occur in a free state, but they are usually associated with membranous structures of some kind. The majority of them are attached to the so-called *endoplasmic reticulum*, a system of submicroscopic ducts and vesicles, by which the cytoplasm is penetrated. Fragments of this reticulum can be isolated from tissue homogenates by differential centrifugation, becoming concentrated in the so-called *microsomal fraction*.

A little more than 10 years ago we observed that in liver the microsomal material was characterized by an extraordinarily rapid protein metabolism.[1] It has subsequently been shown, particularly by Zamecnik and his group in Boston, that under suitable conditions also isolated microsomes can give rise to an incorporation of labelled amino acids into protein.[2, 3]

It has been demonstrated in several different ways that the nucleoprotein constituents of the microsomal material are primarily involved in the incorporation reaction.[4–8] In spite of this fact, attempts to obtain amino acid incorporation *in vitro* by RNP-particles, free from adhering membranes, have not been very successful. Recently, however, a method has been worked out in our laboratory by Rendi and myself, by which it has been possible to prepare relatively pure RNP-particles in a highly active state (Fig. 1).

By use of this method RNP-particles have been prepared from a number of rat tissues. As may be anticipated, the yield of particles varied considerably from one kind of tissue to another. The composition of the particles, however, was fairly similar, with ribonucleic acid (RNA) contents in some cases approaching 40 per cent (Table 1). The capacity of these different kinds of particles to incorporate labelled amino acids

in vitro was also very much the same, when measured on the basis of equal RNA content (Table 2).

Particles have been prepared in this way not only from mammalian tissues but also from sea urchin eggs and embryos. These particles, also, proved quite active in incorporation systems. When particles were isolated from equal samples of embryos, taken

A: Mitochondria-free homogenate in:
0·15 M sucrose
0·025 M KCl
0·01 M $MgCl_2$
0·035 M tris pH 7·8 6·5 ml

mix with: (1) 2·5 M KCl in 0·01 M $MgCl_2$ 2·0 ml
(2) 10% Lubrol W in 0·01 M $MgCl_2$ 0·5 ml
(3) 10% deoxycholate 1·0 ml

B:
0·3 M sucrose
0·6 M KCl
0·01 M $MgCl_2$
0·035 M tris

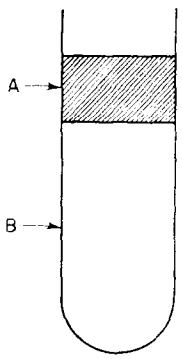

FIG. 1. Procedure for the preparation of active RNP-particles from liver microsomes.

at different periods from a developing culture, the total activity of the preparations for amino acid incorporation rapidly increased in the course of the early blastula stage (Table 3). The hypothesis has previously been advanced that populations of protein-synthesizing particles gradually develop in the cytoplasm of the early blastula cells in connexion with the primary determination, the process by which the extension of the main embryonic regions becomes irreversibly established.[9, 10] The present results seem to confirm this hypothesis.

TABLE 1. PROTEIN AND RNA CONTENT OF FRACTIONS OF RNP-PARTICLES, PREPARED FROM DIFFERENT TISSUES OF THE RAT BY THE METHOD ILLUSTRATED IN FIG. 1

Tissue	Fresh tissue (mg/g)		RNA %
	RNA	protein	
Liver	0·84	1·38	38
Kidney	0·46	1·46	24
Spleen	0·74	1·00	41
Brain	0·20	0·41	33
Testis	0·40	0·61	39

The method illustrated by Fig. 1 has been used for the preparation of RNP-particles not only from the endoplasmic reticulum but also from other cell components. In this way Rendi in our laboratory has obtained good evidence for the presence of such protein-synthesizing particles in mitochondria.[11] It may be justifiable, therefore, to propose that we are dealing here with a sort of biological elementary particles, universally responsible for the cytoplasmic production of cell proteins.

No significant amino acid incorporation is obtained, however, with the purified RNP-particles alone, since these have no (or very small) ability to activate the amino acids. I feel that the already classical amino acid activating enzymes of Hoagland[12], which mainly occur in the so-called pH 5 fraction of the cell sap, are too well known at present to be more than briefly discussed in this connexion. As is well known, these

TABLE 2. INCORPORATION OF LABELLED AMINO ACIDS INTO PROTEIN BY RNP-PARTICLES PREPARED FROM DIFFERENT TISSUES OF THE RAT

(The incubation mixture contained: 0·08 μmoles of ^{14}C-amino acids, 1 μmole of ATP, 10 μmoles of phosphoenolpyruvate, rat liver supernate, containing 2 mg of protein, and RNP-particles prepared from different tissues and containing approximately 2mg of RNA.)

Tissue	Total counts incorporated	
	^{14}C-L-leucine	^{14}C-L-valine
Liver	3700	6200
Kidney	2700	—
Brain	2400	4800
Spleen	2100	3700
Testis	3000	4000

TABLE 3. INCORPORATION OF ^{14}C-L-LEUCINE INTO PROTEIN BY FRACTIONS OF RNP-PARTICLES PREPARED FROM *Psammechinus* EGGS AT DIFFERENT STAGES OF DEVELOPMENT

(The fractions of particles were incubated in a system of the same kind as in Table 2, with the difference, however, that the samples of added liver supernate contained about 10 mg of protein.)

Particles prepared from	Incorporation (counts/min per cm^2)
Unfertilized eggs	45
Fertilized eggs, 30 min	58
Blastulae, before hatching	275

enzymes (E) are able to activate amino acids (aa) by splitting off pyrophosphate (PP) from adenosine triphosphate (ATP):

$$\text{aa} + \text{ATP} + \text{E} \rightleftharpoons \text{E} - \text{AMP} \sim \text{aa} + \text{PP} \qquad (1)$$

The reaction is reversible, and in the presence of amino acids an isotope exchange between added ^{32}P-pyrophosphate and ATP is catalysed by the enzymes. This pyrophosphate exchange reaction can easily be measured, and it has frequently been used both for qualitative and quantitative studies of the activation reaction.[12–16] It has been demonstrated by experiments of this kind that separate enzymes are responsible for the activation of different amino acids, although some overlapping may be observed under certain conditions.[12–14, 17–21]

By the activation reaction adenylates of the amino acids are formed.[17, 19, 20] Amino acid adenylates have been prepared also by chemical synthesis.[17, 22, 23] Since these

compounds have the chemical character of anhydrides, they are highly reactive and spontaneously transfer the amino acid residue to acceptor molecules of different kinds, even proteins.[20, 22] However, no such spontaneous transfer of labelled amino acids to protein takes place in the enzymic activation systems, and no traces of free adenylates occur.[23] The adenylates, therefore, must be present only in catalytic amounts, firmly bound to the enzymes.

We observed some years ago that the incorporation of labelled amino acids into protein by cytoplasmic systems is accomplished by two successive steps:[24]

(1) In the presence of the soluble fraction and a suitable energy supply a derivative of the amino acid accumulates in the system, without equilibrating very rapidly with the free amino acid pool.

(2) When microsomes are then added, the amino acid is directly transferred from this intermediate derivative and incorporated into protein (Fig. 2). It has been shown

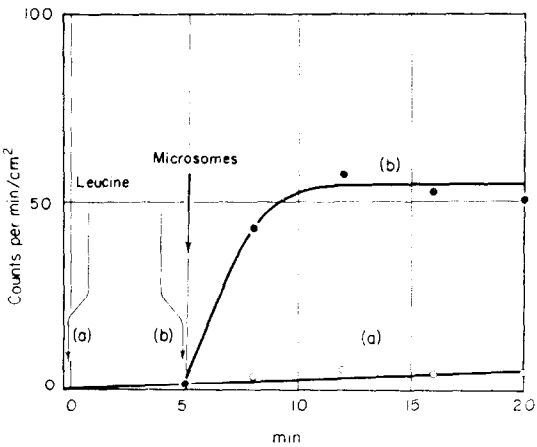

FIG. 2. The incorporation of ^{14}C-L-leucine into protein by a rat liver system, visualized as a two-step reaction. The incubation started with a system containing 0·6 ml of cell sap, 0·02 μmoles of ^{14}C-L-leucine, 1 μmole of ATP and 10 μmoles of phosphoenolpyruvate. A 1000-fold isotope dilution was effected by the addition of 20 μmoles of L-leucine (a) at zero time, (b) after 5 min of incubation. In both cases microsomes were added after 5 min of incubation. No significant incorporation was obtained without added microsomes. Trichloroacetic acid was added to the tubes at the periods indicated.

by the Boston workers[25] that the intermediate amino acid carrier is a ribopolynucleotide, to which the amino acid is specifically transferred from the enzyme-bound adenylate:

$$E - AMP \sim aa + polynuc \rightleftharpoons (E-)\ polynuc - aa + AMP \quad (2)$$

[26]

The polynucleotide is normally associated with the enzyme.[25, 27] It can be isolated, however, from the pH 5 fraction, and purified without losing its activity as amino acid carrier in the incorporation system.[25, 28] There is good reason to believe that the interaction between a polynucleotide of this kind and the corresponding enzyme is specific, and that different kinds of polynucleotides act as carriers for different kinds of amino acids.[28, 29]

When a purified polynucleotide preparation carrying a labelled amino acid is incubated with microsomes or with RNP-particles, the amino acid becomes incorporated into protein. For this to happen, however, the system must contain: (1) a proper energy-generating system; (2) guanosine triphosphate; (3) a soluble enzyme preparation:

$$(E-)\ \text{polynuc} - \text{aa} + \text{RNP} - \text{protein} \underset{?}{\overset{\text{GTP, enzyme}}{\rightleftharpoons}} \text{RNP} - \text{protein} - \text{aa} + ? \quad (3)$$

A little more than 2 years ago we became interested in the question: what happens with the carrier polynucleotide itself in the course of this final incorporation step? Some isotope experiments, carried out by von der Decken and myself[30, 31] pertain to this question. We observed that under the same exacting conditions (cf. above) required so that the nucleotide-bound amino acid could become incorporated into protein by a RNP-particle, the *polynucleotide itself became associated with the particle* (Tables 4 and 5).

TABLE 4. REQUIREMENTS FOR ISOTOPE TRANSFER FROM SOLUBLE POLYNUCLEOTIDES TO MICROSOMAL NUCLEOPROTEIN BY A RAT LIVER SYSTEM

(The prelabelled soluble fraction had been dialysed for 20 hr. The microsomes had been prepared from non-labelled, regenerating liver. The tubes (final volumes 5·0 ml) were incubated for 8 min at 35 °C, except the control tubes, which were left in the ice-bath. PEP = phosphoenolpyruvate (30 μ moles); ATP, GTP, UTP, CTP = adenosine, guanosine, uridine and cytidine triphosphate (3 μ moles))

	Supernatant prelabelled with			
	^{32}P-phosphate		^{14}C-adenine	
	Counts/min per 100 μg phosphate in		Counts/min per mg RNA	
	Cytidylic acid	Uridylic acid		
Microsomes, labelled supernatant			21,	11
plus ATP	230	260		
PEP	130	220		
PEP, ATP	870	800	51,	35
PEP, ATP, amino acids	750	830	52	
PEP, ATP, GTP, UTP	960	915		
PEP, ATP, GTP, UTP, amino acids	970	930		
PEP, ATP, GTP, UTP, CTP				43
PEP, ATP, GTP, UTP, CTP, amino acids			58,	46
kept in ice-bath during incubation	200	360	17,	14

It is commonly believed that the function of RNA in protein synthesis is to arrange the different amino acids in proper order before they become incorporated into a developing peptide chain. Chemically, however, it is difficult to understand, how a RNA-molecule could act directly upon individual amino acids in this highly discriminating way. The experiments just mentioned suggest that specific carrier-polynucleotides may pilot the amino acids into their proper sites on the RNA-"template" by *becoming associated themselves* with identical or complementary nucleotide

sequences along the RNA-molecule. We have often tried to illustrate this idea by the scheme shown in Fig. 3. On the basis of such mutual interactions between nucleotide sequences alone it seems possible to give the template function in protein synthesis a chemically intelligible interpretation. Evidence in favour of a "coding" mechanism of this kind has been presented also by Hoagland[32].

The amino acids are bound to the polynucleotide carriers by ester linkages with relatively low energy content.[28, 33] The function of guanosine triphosphate in the final

TABLE 5. REQUIREMENTS FOR ISOTOPE TRANSFER FROM SOLUBLE POLYNUCLEOTIDES TO MICROSOMAL RNP-PARTICLES BY A RAT LIVER SYSTEM

(The incubation system contained in final volumes of 3 ml : 200 μg of labelled, purified polynucleotides, microsomes corresponding to 2·5 g of fresh liver, 120 μg of pyruvate kinase, 200 μg of myokinase. Mg-concentration 0·005 M. After the incubation RNP-particles were isolated.)

	Counts/min per mg ribonucleotide
Microsomes, ^{32}P-labelled polynucleotide plus PEP, ATP, GTP	255
supernatant, GTP	110
ATP, GTP	120
PEP, GTP	205
PEP, ATP, GTP	330
PEP, ATP, GTP	130*
pH 5 fraction, PEP, ATP	195
PEP, ATP, GTP	310

* Kept in ice bath during incubation.

incorporation step (3) may be to reactivate the amino acid, possibly by forming an amino acid guanylate capable of immediate reaction *in situ* with the neighbouring amino end group of the developing peptide chain. The specificity may thus be gained in the incorporation process at the expense of a repeated energy investment.

It is only possible to speculate, at present, about the ultimate fate of the carrier polynucleotide. One possible inference of the polynucleotide transfer (Tables 4, 5) may be of some interest, however, for the debate on the postulated self-reduplication of the RNP-particles. When the carrier polynucleotides are stripped of their amino acids at the moment of incorporation, the polynucleotide residues themselves may become linked together under formation of a new RNA-molecule. This possibility is consistent with a number of isotope experiments *in vivo*, indicating that polynucleotides in the cell sap become labelled earlier than the RNA of the particles.[34–36]

It has been shown by Sachs[37] that labelled amino acids may become incorporated into protein by liver microsomes under the influence of a polynucleotide-free preparation of soluble enzymes. This preparation ("s-protein") has been further studied in Campbell's laboratory,[38] and it has recently been somewhat purified by Rendi and myself[39]. The purified s-protein enhanced the amino acid incorporation both by microsomes and by isolated RNP-particles. In spite of this, only a small amino acid dependent pyrophosphate exchange was catalysed by this enzyme preparation, and it could be demonstrated by ^{32}P-labelled preparations that no nucleotides were present.

A characteristic property of the s-protein is its marked sensitivity to the redox level of the medium. Its action becomes greatly stimulated by sulphydryl compounds like mercaptoethylamine and inhibited by oxidative agents (Table 6). As a rule, the amino acid in corporation in the presence of s-protein became stimulated by added polynucleotide. Whether polynucleotide was added to the system or not, the amino acid incorporation by s-protein was strongly inhibited by ribonuclease, and guanosine triphosphate could be shown to stimulate the incorporation process. This seems to indicate

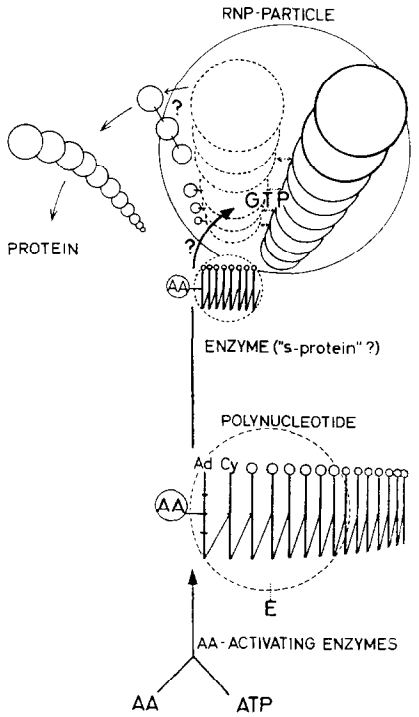

FIG. 3. Diagram representing the conception of the incorporation process, underlying the polynucleotide transfer experiments mentioned in the text. The carrier-polynucleotides (symbolized by dotted circles) are supposed to become specifically attracted by identical or complementary nucleotide sequences within the RNA-template (system of continuous circles). The incorporation by the s-protein system may occurr independently of the polynucleotide mechanism. Since the incorporation is strongly inhibited by ribonuclease, the template-RNA may participate in the reaction. It is difficult, however, at present to visualize how this may occur.

that the two kinds of incorporation systems discussed here, the system with particles and pH 5 fraction and the system with particles and purified s-protein, have at least the final enzymic path in common (Fig. 3).

The problem arises, whether purified RNP-particles are capable of synthesizing specific, fully differentiated proteins. This question seems particularly justified in the case of the incorporation systems with particles and purified s-protein, freed from carrier polynucleotides. We are trying at present to answer this question by studying the ability of different systems to incorporate labelled amino acids, not only into acid-precipitable protein matter but into specific, characterizable species of protein molecules.[40–43]

Two different incorporation systems have been tried so far:

(1) The incorporation of labelled amino acids into haemoglobin by reticulocyte particles.[41]

(2) The incorporation of labelled amino acids into serologically characterizable protein antigens by means of microsomes or RNP-particles from liver.[42, 43]

The experiments, which are carried out by Morgan, von der Decken, Perlmann and myself, are still in a preliminary state. So far, no incorporation of ^{14}C-L-leucine into

TABLE 6. EFFECTS OF POLYNUCLEOTIDE AND REDUCING AGENTS ON THE INCORPORATION OF ^{14}C-L-LYSINE INTO PROTEIN BY RNP-PARTICLES AND S-PROTEIN

(The incubation system contained in 1 ml, 0·08 μmoles ^{14}C-L-lysine, 1 μmole ATP, 10 μmoles phosphoenolpyruvate. MEA = mercaptoethylamine; DPN, DPNH = diphosphopyridine nucleotide, oxidized and reduced; TPN, TPNH = triphosphopyridine nucleotide, oxidized and reduced.)

	Counts/min per cm^2
Controls	67, 69
with MEA (12 μmoles)	218
polynucleotide (50 μg)	136
MEA + polynucleotide	273
DPNH (2 μmoles)	97
DPN (2 μmoles)	56
TPNH (2 μmoles)	93
TPN (2 μmoles)	63

specific proteins has been observed with certainty in the absence of carrier nucleotides, but the experiments are being continued with other amino acids.

As was mentioned before, the RNP-particles are generally attached to the membranes of the endoplasmic reticulum. The fact that such RNP-particles, which have been freed from the membranes, are also able to incorporate labelled amino acids into protein, does not mean that the membrane material is without any influence on the incorporation process under physiological conditions. This fact became particularly obvious in the incorporation experiments with subcellular preparations from sea urchin eggs. It was observed that total homogenates or mitochondria-free extracts from fertilized eggs had a much higher ability to incorporate ^{14}C-L-leucine into protein than the corresponding preparations from the unfertilized eggs. In parallel experiments with isolated RNP-particles this difference was less significant. It is tempting to assume that the endoplasmic membranes in the preparations were responsible for the greatly reduced incorporation capacity before fertilization.

We have observed that in liver the ability of the microsomes to incorporate labelled amino acids into protein may become influenced by enzymatic processes taking place in the membranes. It has been shown that a number of oxidative and reductive reactions, related to metabolic detoxication, are catalysed by enzymes in the endoplasmic membranes.[44–48] Under certain conditions such reactions may diminish the activity of the particles in incorporation systems. This has been observed in experiments with the water-soluble liver carcinogen dimethylnitrosamine,[49–50] and further examples of inhibitions of this kind by liver carcinogens and by other compounds

metabolized by the endoplasmic membranes of the liver have been presented by Arrhenius and myself[51] previously at this meeting.

The experiments discussed here indicate the possibility of a functional interrelationship between the endoplasmic particles and membranes. It is generally assumed that the rate of protein metabolism in a tissue cell is determined under the influence of manifold, integrated regulation factors. A factor of supreme importance for the total capacity of protein synthesis may obviously be the number of RNP-particles. As was mentioned before an increased rate of protein metabolism due to a higher content of active RNP-particles can be demonstrated in sea urchin embryos (Table 3 [10]), and a similar situation has previously been observed in regenerating rat liver.[15] It is our feeling that the particle–membrane interrelationship in the endoplasmic reticulum may affect the level of protein metabolism in a more direct and flexible way.

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