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# Cell-free protein synthesis bioreactor*

**Abstract:** *In vivo* expression of foreign or synthetic genes can be subject to certain restrictions such as protein aggregation, degradation and toxicity. Conventional *in vitro* systems can overcome these problems, but in turn suffer from other limitations, in particular short life–time and low protein yield. In this review, two types of gene expression system are described. Both are based on the novel concept of enhanced expression from cell–free extracts where incubation is performed in the continuous flow of a feeding solution, rather than in a fixed volume of a test–tube. The first makes use of cell-free translation of mRNA templates in either prokaryotic or eukaryotic cell lysates. The second utilizes coupled transcription–translation of DNA templates, with genes transcribed by either endogenous or bacteriophage RNA polymerases. In both systems translation can be carried out over tens or hundreds of hours resulting in high protein yields.

## Introduction

Protein synthesis is a basic feature of all living beings. It is a highly complex process comprising interrelated events of nucleic acid transcription, its regulation, post–transcriptional alterations, nucleoprotein assembly, nucleic acid and nucleoprotein transport, mRNA translation, translational regulation, co– and post–translational polypeptide modifications, co– and post–translational protein folding, assembly, transport and secretion.

Translation is at the heart of this process and is often considered protein synthesis proper. It is a finely regulated multi–step sequence of events, performed by ribosomes moving along a mRNA chain. Many ribosomes moving along the same mRNA form a polyribosome. Ribosomes are specialized ribonucleoprotein particles with binding, catalytic and mechanical functions. Each translation cycle of the ribosome results in the synthesis of one polypeptide and involves three successive stages: initiation, elongation and termination.

It is remarkable that such a complex process can be entirely reproduced after disruption of a living cell, i.e. in a cell homogenate or extract. The first cell–free systems of protein synthesis were developed in the 1950's. Experiments in the 1960's demonstrated that exogenous template polynucleotides, added either directly as polyribonucleotide messengers (1) or as polydeoxyribonucleotide genes (2,3), could be expressed into polypeptide and protein products in cell-free translation or coupled transcription–translation systems, respectively. Since then cell-free systems have played the main role in unravelling molecular mechanisms of protein synthesis on ribosomes.

The 1970's and 80's witnessed the development of recombinant DNA technology and methods for *in vivo* expression of foreign and synthetic genes leading to the so–called Biotechnology revolution. Today, biologically active polypeptides and proteins of different origin, composition and properties can be produced from cell cultures harboring the appropriate cloned genes.

However, *in vivo* expression of foreign and synthetic genes inevitably encounters certain limitations. Heterologous genes may be unstable or poorly expressed because of host

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regulatory mechanisms. Many gene products are insoluble and aggregate as inclusion bodies. Other proteins are unstable and are degraded by intracellular proteases. Finally, some gene products are toxic to the host cell and therefore are not expressed.

In principle these and other problems of gene expression could be solved by the use of cell–free systems. Absence of cellular control mechanisms, ability to manipulate incubation mixture composition, i.e. introduction of protease and nuclease inhibitors, selective removal of undesired proteins and substances, as well as purification of protein-synthesizing components make cell-free systems look attractive. However, short life-time and low protein yield have been the major limitations preventing use of classical cell-free systems for preparative gene expression.

Recently it has been demonstrated that continuous translation over long periods resulting in high protein yields can be attained in cell-free systems when the incubation is performed not under static conditions in a fixed volume but in the flow of a feeding solution through the cell–free extract (4–6).

## Flow–through Bioreactors for Cell–Free Protein Synthesis

In our first experiments with translation of viral RNAs the standard Amicon 8 MC micro–ultrafiltration chamber was used as a bioreactor for establishing a continuous flow cell–free system (4). A 1 ml incubation mixture in the working chamber of the thermostated instrument was supplied with a constant flow (1 ml/hour) of feeding solution for translation (amino acids, ATP, GTP and buffer), while the reaction products were continuously removed at the same rate through an ultrafiltration membrane (Fig. 1).

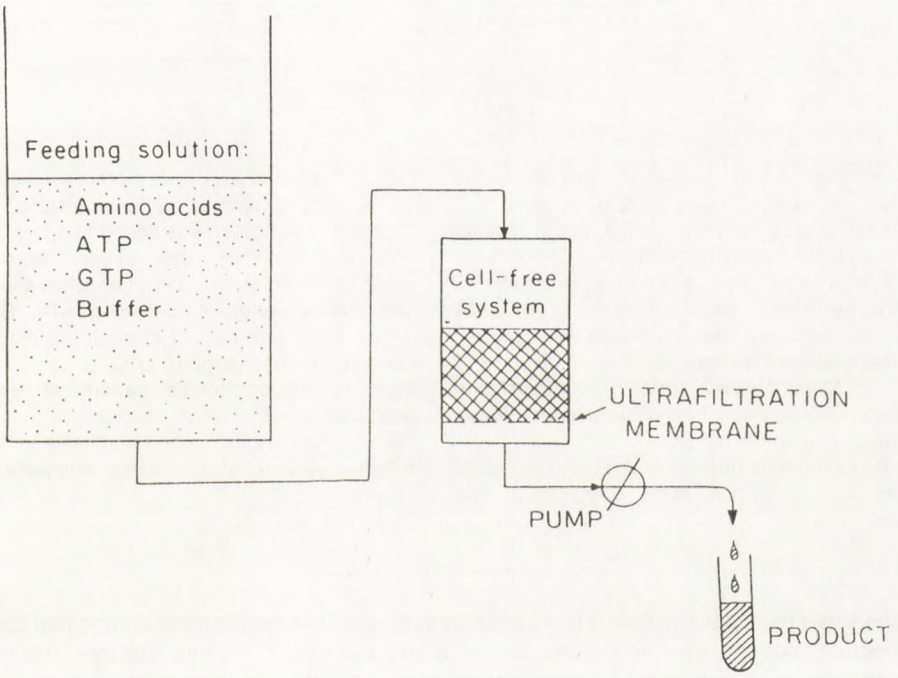


Fig. 1. Scheme of bioreactor for translation based on direct flow through ultrafiltration membrane.

Both feeding and incubation solutions were sterile. When MS2 bacteriophage RNA was translated in DNA– and RNA–free extracts of *Escherichia coli* (0,06 nmoles of MS2 RNA per 0,6 nmoles of ribosomes) the phage coat protein was synthesized linearly over 20 hours at 37°C and passed through a PM–30 membrane as the only detectable protein of the eluate (Fig. 2). The yield was 100 copies of the protein per MS2 RNA molecule, i.e. ca 6 nmoles or 0,1 mg from 1 ml incubation mixture. In the case of brome mosaic virus (BMV) RNA 4 translation in wheat embryo extract at 27°C synthesis again continued for up to 20 hours; it was linear and yielded ca 10 nmoles or 0,2 mg of the BMV coat protein. Product was visualized as a predominant 20 kDa polypeptide in the filtrate which had passed through an XM–50 membrane; two additional bands, probably corresponding to abortive peptides, were also present (Fig. 3).

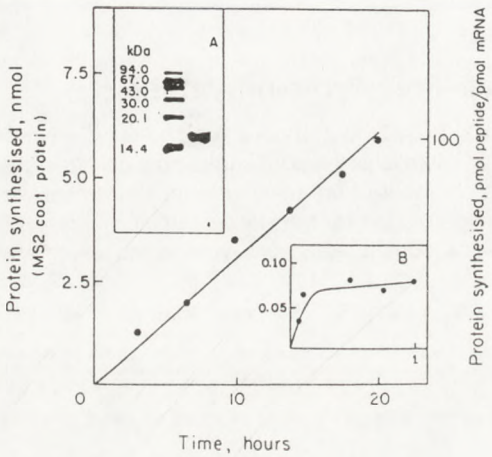


Fig. 2. Kinetics of MS2 coat protein synthesis in the *E. coli* continuous cell–free translation system.
0,06 nmol of MS2 RNA was added to a 1-ml reaction mixture containing 70S ribosomes, tRNA, S100 protein fraction and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP and amino acids was passed through the reactor at a constant rate of 1 ml/hour, 37°C.
Inset A: Electrophoretic pattern of translation products. Here and in Fig. 3–5 the left lane shows reference polypeptide bands.
Inset B: Kinetics of protein synthesis in standard cell-free system of the same composition and volume.

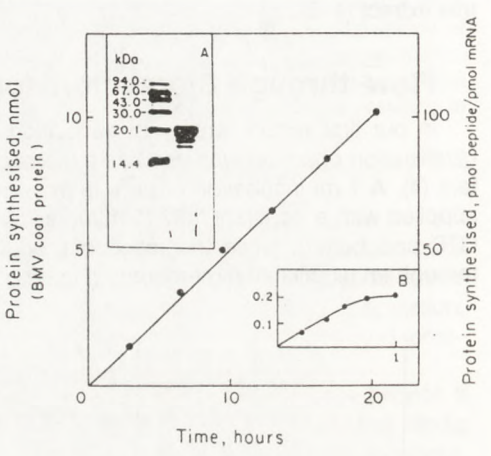


Fig. 3. Kinetics of BMV coat protein synthesis in the wheat germ continuous cell-free translation system.
0,1 nmol of BMV RNA was added to a 1–ml reaction mixture containing S30 extract from wheat embryos and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP and amino acids was passed through the reactor at a constant rate of 1 ml/hour, 27°C.
Inset A: Electrophoretic pattern of translation products.
Inset B: Kinetics of protein synthesis in standard cell-free system of the same composition and volume.

The same ultrafiltration–based bioreactor was used for the synthesis of unmodified calcitonin polypeptide, both in the prokaryotic (*E. coli*) and eukaryotic (wheat) cell-free systems (4). mRNAs were pre-synthesized by transcription from synthetic Val 8–calcitonin genes, with and without Shine–Dalgarno sequence, using bacteriophage SP6 RNA polymerase. Polypeptide synthesis was recorded for up to 40 hours of incubation at either 37°C (*E. coli* lysate) (Fig. 4) or

27°C (wheat embryo extract) (Fig. 5). Yields were about 300 copies of the polypeptide per mRNA molecule (18 nmoles or 60 mg of the product) and about 150 copies of the poplypeptide per mRNA molecules (9 nmoles or 30 $\mu$g of the product), respectively. The product was the only visible polypeptide passed through the PM–10 membrane used in the experiment, although some dimerisation was observed in the wheat germ system.

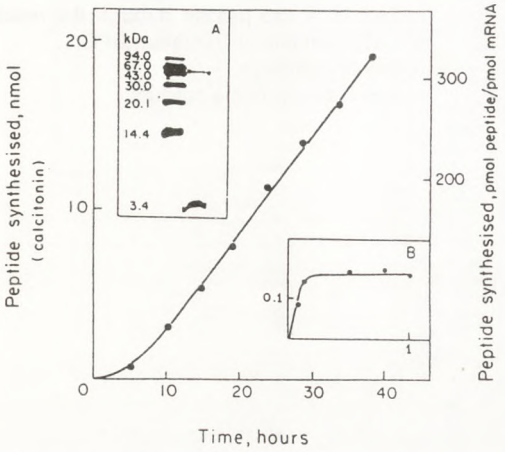


Fig. 4. Kinetics of calcitonin synthesis in the *E. coli* continuous cell–free translation system.
0,06 nmol of calcitonin mRNA was added to a 1–ml reaction mixture containing 70S ribosomes, tRNA, S100 protein fraction and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP and amino acids was passed through the reactor at a constant rate of 1 ml/hour, 37°C.
Inset A: Electrophoretic pattern of translation products.
Inset B: Kinetics of protein synthesis in standard cell–free systems of the same composition and volume.

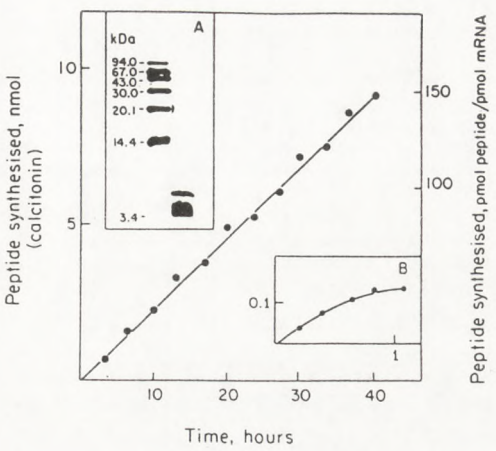


Fig. 5. Kinetics of calcitonin synthesis in the *wheat germ* continuous cell–free translation system.
0,06 nmol of calcitonin mRNA was added to a 1–ml reaction mixture containing S30 extract from wheat embryos and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP and amino acids was passed through the reactor at a constant rate of 1 ml/hour, 27°C.
Inset A: Electrophoretic pattern of the translation products.
Inset B: Kinetics of protein synthesis in standard cell–free systems of the same composition and volume.

An important consideration concerns the functional activity of proteins synthesized in such systems. To test functional activity, a 0,5 ml S30 wheat germ extract containing 0,1 nmoles of dihydrofolate reductase (DHFR) mRNA was incubated at 24°C for 18h in the flow (1ml/hour) of feeding solution. In parallel with monitoring protein synthesis, its activity was continuously measured (Fig. 6). Throughout the whole period of synthesis, the protein was found to possess functional activity.

More recently, an automated large–scale bioreactor (100 ml of incubation mixture) for cell–free translation based on the principle of ultrafiltration through a membrane has been constructed and tested with the synthesis of interleukin 4 in the Institute of Protein Research, Pushchino (Yu. B. Alakhov, S. Yu. Ovodov and L. M. Vinokurov). However, instead of direct flow, a reverse pulse principle was utilized; feeding solution was first pumped under pressure into the

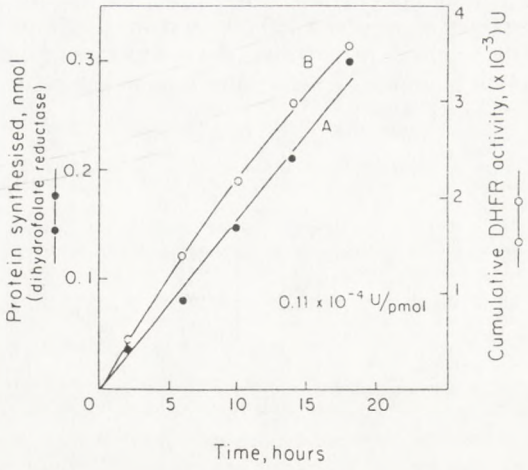


Fig. 6. Kinetics of dihydrofolate reductase synthesis in the *wheat germ* continuous cell–free translation system.
0,1 nmol of *E. coli* dihydrofolate reductase mRNA was added to a 0,5–ml reaction mixture containing S30 extract from wheat embryos and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP and amino acids was passed through the reactor at a constant rate of 1 ml/hour, 24°C.
A: Protein synthesis.
B: DHFR activity of the product.

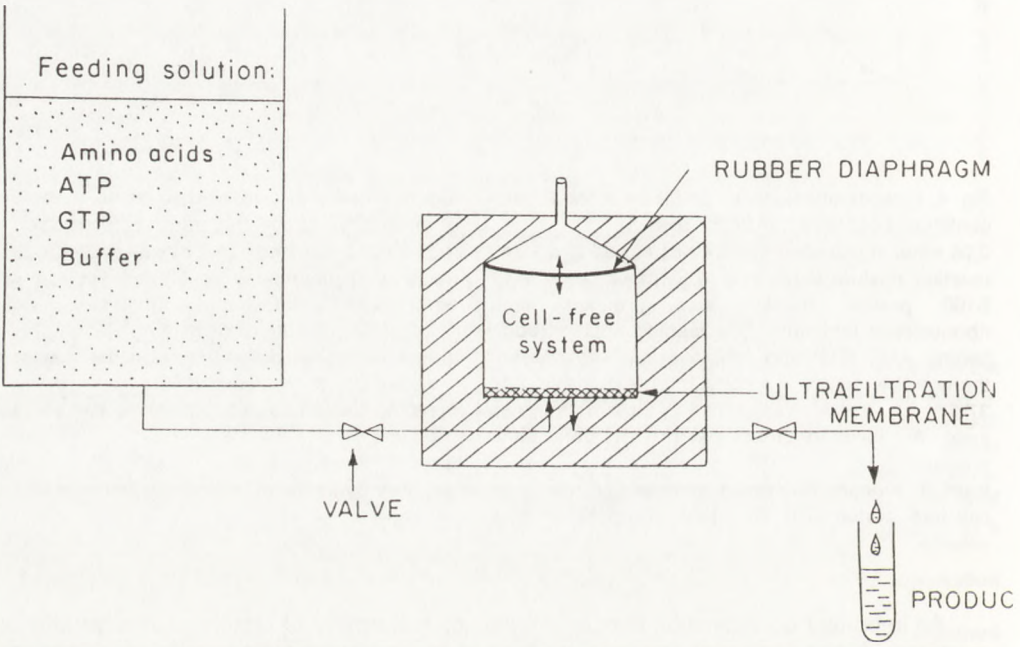


Fig. 7. Scheme of bioreactor for translation based on reverse pulse flow through ultrafiltration membrane.

reaction chamber through the membrane; the ultrafiltrate then was forced from the chamber through the same membrane by a reverse flow (Fig. 7). Such pulses prevented obstruction of membrane pores by protein aggregates. After 20 hours of translation the interleukin 4 of 85% purity was obtained giving a yield of 50 mg per liter.

A further development involves a hollow fiber bioreactor invented jointly by H. Bauer, Tübingen University, and Yu. B. Alakhov, S. Yu. Ovodov and V. I. Baranov, Institute of Protein Research, Pushchino. Here the feeding solution flows along an ultrafiltration hollow fiber coil

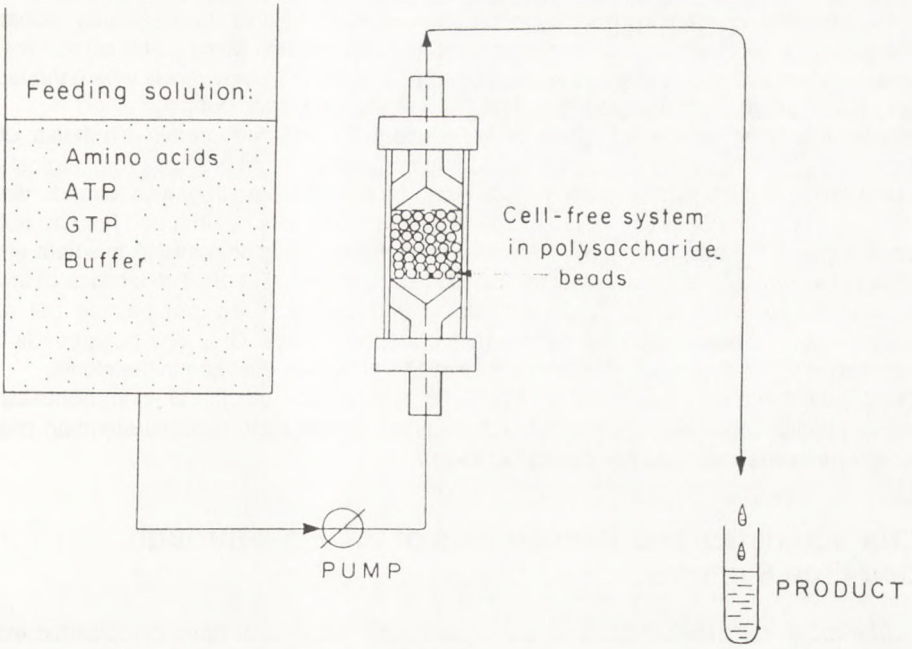


Fig. 8. Scheme of column bioreactor for translation

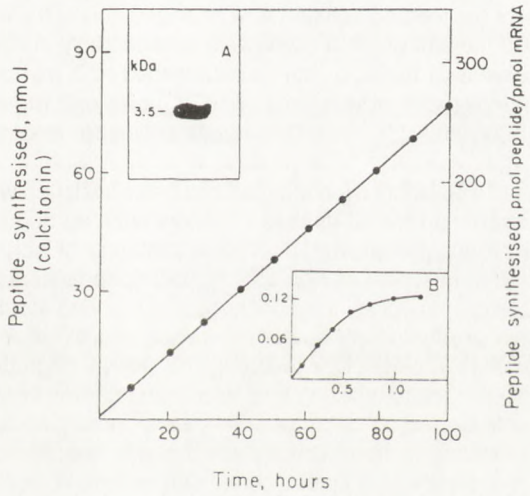


Fig 9. Kinetics of calcitonin synthesis in the wheat germ continuous cell-free translation system with the use of polysaccharide encapsulated incubation mixture.
0,3 nmol of calcitonin mRNA was added to a 5–ml reaction mixture containing S30 extract from wheat embryos and protease and ribonuclease inhibitors. The mixture was encapsulated in zosterin beads of 0,1 – 0,2 mm in diameter. The beads were packed in a column. The feeding solution containing ATP, GTP and amino acids was passed through the column at a constant rate of 2,5 ml/hour, 25°C.
Inset A: Electrophoretic pattern of the translation products.
Inset B: Kinetics of protein synthesis in standard cell-free system of the same composition and volume.

within the chamber containing the incubation mixture. Exchange of the translation substrates and the products between incubation mixture and feeding solution takes place across the fiber membrane. A bioreactor of this type can also operate in a reverse pulse mode where the feeding solution in-flow under pressure alternates with the product ultrafiltrate out-flow.

One of the most promising types of bioreactors for cell-free protein synthesis utilizes a column technique. Here the incubation mixture is embedded in gel granules (e.g. alginate). Alternatively drops of the liquid incubation mixture can be encapsulated in polysaccharide vesicles (e.g. zosterin). In both cases the resulting beads are packed into a column and feeding solution is passed through the beads (Fig. 8). Translation of synthetic calcitonin mRNA in wheat embryo extract was performed in a column reactor for 100 hours at 25°C (Fig. 9). The kinetics of the synthesis was shown to be strictly linear producing 260 copies of the polypeptide per mRNA molecule, i.e. 80 nmoles or 250 mg from 5 ml incubation mixture. Only one polypeptide band, corresponding to the molecular mass of calcitonin (3,5 kDa) was detected in the eluate.

Development and construction of an ATP–GTP–regeneration block and an immuno–adsorption concentration block are in progress. For all types of bioreactor special attention must be paid to the maintenance of sterility during incubation.

## Characteristics and Peculiarities of the Flow-through Cell-Free Systems

Flow-through cell-free translation systems based on bacterial or plant cytoplasmic extracts have been described above. Fig 10 demonstrates the application of the same flow principle for the translation of isolated globin mRNA in RNA–free rabbit reticulocyte extract (5). A 1 ml incubation mixture in Amicon 8 MC micro-ultrafiltration chamber with XM–100 membrane was used at 30°C. By chance the rate of the feeding solution flow changed during the above experiment from 1 ml/hour to 2 ml/hour and the rate of globin synthesis unexpectedly increased more than twice. Therefore, in the next experiment the flow rate was increased to 3 ml/hour from the beginning (Fig. 11). This resulted in maximum protein yield of 100 nmoles or 2 mg of product from 0,5 ml incubation mixture after 100 hours (5). Thus flow rate is critical for maximizing yield of cell-free protein synthesis.

Prolonged incubation of mRNA during enhanced cell-free translation was expected to lead to problems with mRNA degradation since RNAase inhibitors such as human placental ribonuclease inhibitor cannot completely protect mRNA. This is because of non-complete inhibition of RNAases, presence of different types of non-specific phosphodiesterase activities in the extracts, and the removal of the inhibitor by the continuous flow during incubation. This apprehension seems to be the main psychological barrier preventing use of cell-free translation systems in reactions with long incubation times. Unexpectedly, no mRNA degradation was observed in any type of cell–free translation system during tens and even hundreds of hours at elevated temperatures. In all experiments limiting amounts of mRNA were present compared to other components of the protein–synthesizing system. This means that any degradation of mRNA would be immediately reflected in the decline of a kinetic curve. One plausible explanation is that the actively working protein–synthesizing machinery, or ribosomes themselves, protect the mRNA against degradation.

More surprising was the absence of significant leakage of translation factors and tRNAs through the ultrafiltration membrane during incubation. Initially, ultrafiltration membranes with pore sizes as small as possible were chosen to retard the leakage of the components of the protein–synthesizing machinery: PM–10 in the case of calcitonin polypeptide synthesis, or PM–30 in the case of MS2 coat protein synthesis. Eventually it was realized, however, that there was no need for this precaution. For example, when globin synthesis was performed using a high

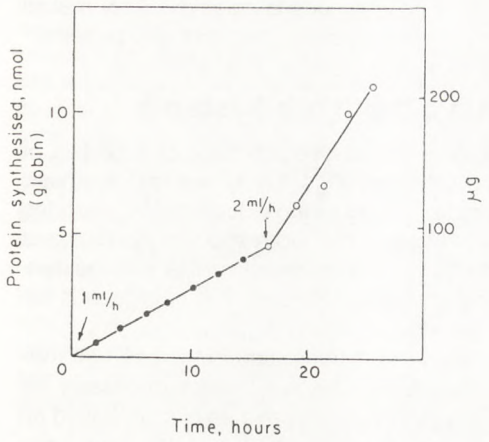


Fig.10. Kinetics of globin synthesis in the rabbit reticulocyte continuous cell-free translation system.
15 $\mu$g of globin mRNA was added to a 1–ml reaction mixture containing micrococcal nuclease-treated rabbit reticulocyte lysate. The feeding solution containing ATP, GTP and amino acids was passed through the membrane reactor at a rate of 1 ml/hour for the first 16 hours and then at a rate of 2 ml/hour, 30°C.

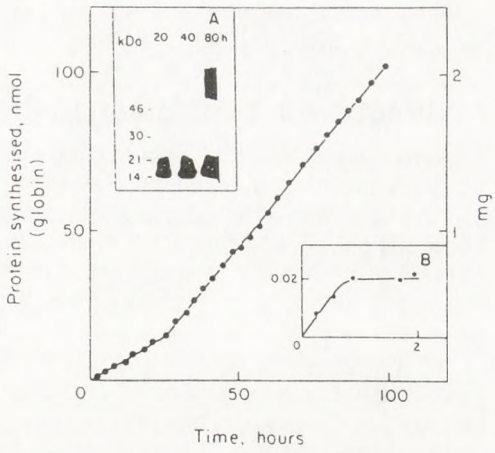


Fig.11. Kinetics of globin synthesis in the rabbit reticulocyte continuous cell–free translation system.
3 $\mu$g of globin mRNA was added to a 0,5–ml reaction mixture containing micrococcal nuclease–treated rabbit reticulocyte lysate and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP and amino acids was passed through the membrane reactor at a constant rate of 3 ml/hour, 30°C.
Inset A: Electrophoretic pattern of the translation products at 20, 40 and 80 hours of incubation.
Inset B: Kinetics of protein synthesis in standard cell-free system of the same composition and volume.

flow rate for 100 hours with an XM–100 membrane (5) no serious leakage of proteins involved in translation was recorded. Now YM–100 and XM–300 membranes are routinely used by our group in all types of systems (bacterial, plant and animal origin) for the synthesis of various proteins with different molecular masses. Polysaccharide capsules may have even larger pores, but tRNAs and proteins required for translation are still retained. This leads us to speculate that the actively working translational machinery is organized in dynamic multi-component complexes, so that individual elements are not free for most of the time.

During the first few minutes of incubation many ballast proteins and other substances were eluted from the protein-synthesizing mixture. Subsequently, however, the outflow contained only or predominantly the product polypeptide. This results in a unique advantage of the flow-through cell–free system: the protein or polypeptide synthesized is found in an almost pure state, rather than in a complex cell homogenate. Hence there is no need for special purification procedures. This product homogeneity in the eluate is especially important when the properties of the expressed protein or polypeptide are unknown or modified, e.g., in the case of the expression of an unidentified open reading frame or at some steps of protein engineering.

Finally, it is worth emphasizing the long life-time of flow-through cell-free protein-synthesizing systems. As a consequence, a high yield of proteins or polypeptides is achieved. This makes such cell-free systems highly promising for preparative syntheses of polypeptides and proteins.

## Direct Gene Expression in Preparative Cell-Free Systems

Availability of individual mRNA is the limiting factor in preparative synthesis of a protein or a polypeptide in cell-free translation systems. Extrapolation from Figs. 2–5 shows that synthesis of 1 mg of protein over 100 hours requires approximately 20 $\mu$g of individual mRNA, provided about 500 polypeptide copies are synthesized per mRNA molecule. Correspondingly, 20 mg of mRNA will be required to synthesize 1 g of a protein. Such amounts of mRNA can be synthesized *in vitro* by bacteriophage SP6 or T7 RNA polymerases. However, this approach is expensive.

An alternative approach makes use of coupled transcription–translation in the cell–free mixture. Bacterial extracts are known to contain DNA–dependent RNA polymerase necessary for transcription. Several versions of the coupled transcription–translation cell–free system based on crude bacterial extracts (2,3) or on more or less purified components of it (7–10) have been described. The system proved useful but was short–lived, and protein yield was low. However, application of the flow–through principle to coupled transcription–translation using endogenous RNA polymerase and exogenous DNA (genes) has increased the feasibility of using such systems (6). Fig. 12 demonstrates the DNA–directed synthesis of both $\beta$–lactamase (Bla) and dihydrofolate reductase (DHFR) in nucleic acid–free *E. coli* extract supplemented with plasmid pDF34 (pUD18) carrying the two corresponding genes (N. V. Murzina, A. T. Gudkov). The feeding solution containing all four nucleoside triphosphates and twenty amino acids was passed through the incubation chamber (1 ml volume, XM–100 membrane, in the Amicon 8 MC microultrafiltrator). Protein synthesis at 37°C was monitored for 50 hours. Protein production was continuous and synthesis rate was directly dependent on the flow rate: the switch from 3 ml/hour to 2 ml/hour decreased the synthesis rate about two fold, and the subsequent switch from 2 ml/hour to 3 ml/hour restored the previous synthesis rate. Total protein yield was more than 0,2 mg. Only two bands, corresponding to $\beta$–lactamase and dihydrofolate reductase were detected in approximately equimolar amounts by electrophoretic analysis.

Hence, the coupled transcription-translation system described above retains the advantages of the flow-through cell-free translation systems, resulting in the production of preparative

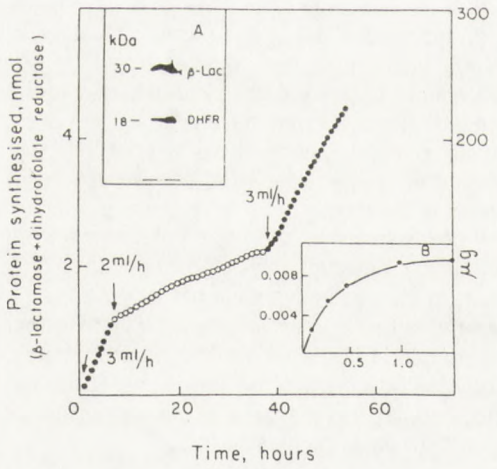


Fig.12. Kinetics of expression of $\beta$–lactamase and dihydrofolate reductase–encoding genes in the *E. coli* continuous cell–free system with endogenous RNA polymerase.
150 $\mu$g of plasmid (pDF34) was added to a 1–ml reaction mixture containing *E. coli* S30 extract and additional tRNA. The feeding solution containing ATP, GTP CTP, UTP and amino acids was passed through the reactor at rates of 3 ml/hour and 2 ml/hour, 37°C.
Inset A: Electrophoretic pattern of translation products.
Inset B: Kinetics of protein synthesis in standard cell–free system of the same composition and volume.

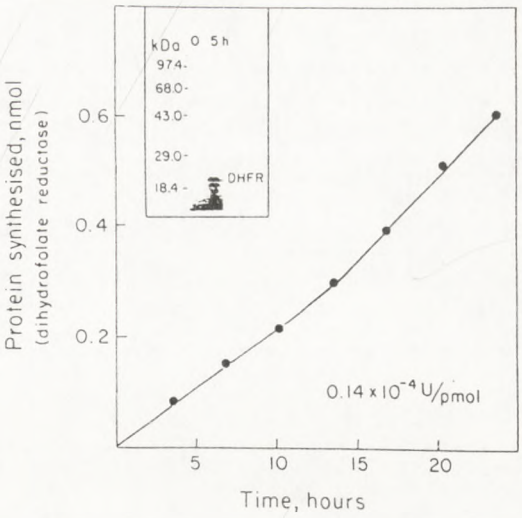


Fig.13. Kinetics of expression of dihydrofolate reductase–encoding genes in the *E. coli* continuous cell–free system with bacteriophage SP6 RNA polymerase.
50 $\mu$g of plasmid (pSP65) with the *E. coli* DHFR gene under SP6 promotor and Bla gene under *E. coli* RNA polymerase promotor was added to a 0,5–ml reaction mixture containing *E. coli* S30 extract SP6 polymerase, additional tRNA and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP CTP, UTP and amino acids was passed through the membrane reactor at a constant rate of 1,5 ml/hour, 37°C.
Inset: Electrophoretic pattern of translation products at 0 and 5 hours of incubation.

amounts of almost pure proteins. It uses DNA molecules directly as templates for mRNA transcription by endogenous RNA polymerase, thereby reducing the number of steps involved in *in vitro* gene expression. In addition, this system creates new possibilities for the study of transcriptional and translational regulation mechanisms, as well as of the coupling of transcription and translation.

However, there are several limitations in the use of the endogenous RNA polymerase of bacterial extracts. Most of them are caused by problems of transcriptional regulation, proper promoters, termination of transcription, etc. As a rule, a complete circular plasmid with the gene of interest (e.g., DHFR), a selection gene (e.g., Bla) and all necessary regulatory elements is used in coupled transcription–translation experiments. This means the selection gene is also transcribed and translated.

A simpler version of the coupled transcription–translation system can be produced utilizing bacteriophage SP6 or T7 RNA polymerases instead of endogenous cellular RNA polymerase. Fig. 13 shows expression in an *E. coli* extract with added SP6 polymerase of plasmid DNA specifying the DHFR gene under the control of the SP6 polymerase promoter and of the Bla gene under the control of the *E. coli* RNA polymerase promoter. Rifampicin was added to suppress the endogenous RNA polymerase activity. The feeding solution containing all four nucleoside triphosphates and twenty amino acids was passed through the working chamber (0,5 ml volume, YM–100 membrane). Protein synthesis at 37°C was monitored for 24 hours. Proteins were synthesized continuously. One main band corresponding to DHFR was detected by electrophoretic analysis indicating that only dihydrofolate reductase was synthesized (L. A. Ryabova, O. B. Yarchuck, V. I. Baranov, A. S. Spirin). Enzyme activity was measured (11) in parallel with protein yield from the eluate. Total protein yield was about 0,6 nmol and the specific activity of DHFR was about $0{,}14 \times 10^{-4}$ U/pmol.

A further major limitation of coupled transcription–translation systems seemed to be their sole application to bacterial (prokaryotic) extracts. In eukaryotes, transcription and translation are known to be uncoupled and even spatially separated. Following the experiments described above, coupling of bacteriophage enzymatic activity with eukaryotic translational machinery was proposed (V. I. Baranov, L. A. Ryabova, O. B. Yarchuck, A. S. Spirin). Fig. 14 illustrates expression of the DHFR gene in wheat embryo extract supplemented with SP6 RNA polymerase. The feeding solution containing all four nucleoside triphosphates and twenty amino acids was pas-

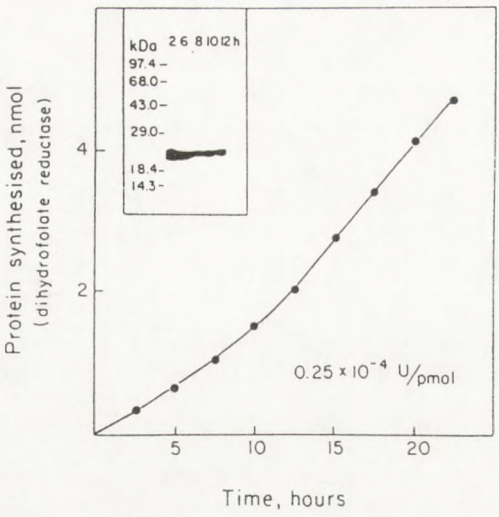


Fig.14. Kinetics of expression of dihydrofolate reductase–encoding genes in the *wheat germ* continuous cell–free system with bacteriophage SP6 RNA polymerase.
50 $\mu$g of plasmid (the same as in Fig. 13) was added to a 0,5–ml reaction mixture containing S30 extract from wheat embryos, SP6 polymerase and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP CTP, UTP and amino acids was passed through the membrane reactor at a constant rate of 1,5 ml/hour, 24°C.
Inset: Electrophoretic pattern of translation products at 2, 6, 8, 10 and 12 hours of incubation.

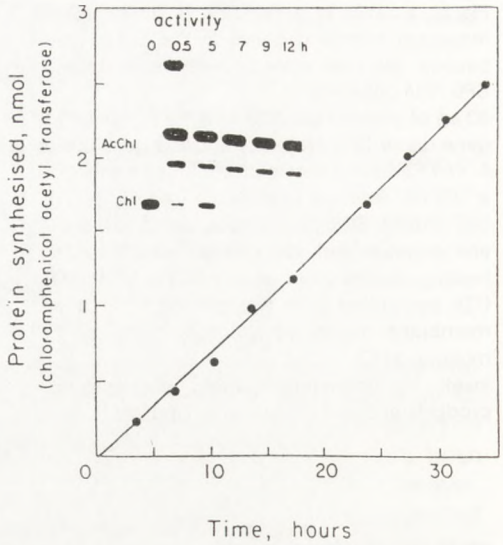


Fig.15. Kinetics of expression of chloramphenicol acetyl transferase–encoding gene in the rabbit reticulocyte continuous cell–free system with bacteriophage SP6 RNA polymerase.
50 $\mu$g of plasmid (pSP65) with the TN5 CAT gene under SP6 promotor and Bla gene under *E. coli* RNA polymerase promotor added to a 0,5–ml reaction mixture containing micrococcal nuclease–treated rabbit reticulocyte lysate and protease and ribonuclease inhibitors. The feeding solution containing ATP, GTP CTP, UTP and amino acids was passed through the membrane reactor at a constant rate 1,5 ml/hour, 34°C.
Inset: Thin–layer chromatography of chloramphenicol acetyl transferase assay products. Functional activity of protein synthesized in the continuous system was measured after 0, 0,5, 5, 7, 9, and 12 hours of incubation.

sed through the working chamber (0,5 ml volume, YM–100 membrane). Protein synthesis at 24°C was monitored over 24 hours and again the system continuously synthesized the protein giving a final yield of about 5 nmol. Enzyme activity of DHFR was measured in parallel with protein yield from the eluate; the specific activity was of about $0{,}25 \times 10^{-4}$ U/pmol.

In the second experiment a chloramphenicol acetyl transferase (CAT) gene was expressed in a rabbit reticulocyte lysate supplemented with SP6 RNA polymerase (Fig. 15).

Feeding solution containing all four nucleoside triphosphates and twenty amino acids was passed through the working chamber (0,5 ml volume, XM–300 membrane) and synthesis rate in the continuous action system was constant for at least 35 hours at 34°C. Total protein yield was about 2,5 nmol. CAT activity was measured (12) in parallel with protein yield from the eluate and again the enzyme was functionally active.

The advantages of combining bacteriophage RNA polymerase with cellular extracts (prokaryotic or eukaryotic) are obvious. The simplest genetic constructions, e.g. those containing an

SP6 or T7 promoter upstream from any gene on a linear DNA fragment, can be used for expression. Products of chemical DNA synthesis or DNA polymerase chain reaction can be directly added to the system for expression. Expression products appear as single pure proteins or polypeptides in the eluate throughout prolongated incubation. The system is stable and effective enough to provide preparative amounts of the expression product. The cell-free character of the expression system allows manipulation of all conditions of incubation, regardless of cell control.

## Conclusion

Preparative cell-free protein synthesis opens up new possibilities and areas in science and biotechnology. Firstly, this technology will be indispensable for biosynthesis of polypeptides and proteins which cannot be produced in cells because of instability, e.g., *in vivo* protease degradation or induced aggregation. The same applies to cytotoxic proteins and to a number of key proteins where overproduction is lethal. Proteins poorly expressed in cells because of genetic control also can be produced in cell-free systems. Difficulties with isolation or *in vivo* synthesis of some antigenic proteins and polypeptides can also be easily overcome by the use of the preparative cell–free systems.

A second potential use concerns easy identification and investigation of expression products from open reading frames, since the outflow of the reactor contains the product as the sole polypeptide. Similarly, polypeptides or proteins with invented amino acid sequences and therefore unknown properties can be collected in a pure state and studied thus permitting wide screening of newly designed proteins.

Thirdly, a specifically scientific application of continuous flow cell–free systems involves the *in vitro* study of transcriptional and translational regulation mechanisms. The long life–time of such systems and their dynamic (flux) character provide special advantages for such studies. For example, different effectors can be given transiently, imitating the *in vivo* condition, and after-effects can be traced for a long time.

One more important scientific application could be preparative syntheses of protein folding intermediates. Protein folding is a physical problem whose solution requires large amounts of the material for physical studies. It is likely that preparative cell–free translation systems can serve as a source of polypeptides terminated at definite stages of their natural folding pathways.

Preparative biosynthesis of proteins with massive or specified substitutions of unnatural or modified amino acids is a very promising application for the use of flow-through cell-free systems. This can be achieved by introducing amino acid derivatives recognizable by aminoacyl–tRNA synthetases, aminoacyl–residue–modified aminoacyl–tRNA, or artificially formed aminoacyl–tRNA with unnatural amino acid and special anticodon, etc. In any case, such substitutions are important for physical studies of proteins, for functional investigations of molecular mechanisms of protein activities, and for practical protein engineering.

Finally, preparative cell–free protein synthesis systems may initiate a new era in protein engineering: (1) a gene specifying an engineered protein can be directly expressed *in vitro*, without the need for *in vivo* expression; multiple, engineered versions of a chemically synthesized gene can be expressed directly; (2) all variants of a gene under investigation can be transcribed and translated without limitations imposed by cellular control mechanisms, proteolytic degradation, etc.; (3) all engineered versions of a protein can be easily visualized and tested directly in the outflow of the reactor without requiring isolation and purification. From this, rapid screening of altered proteins can be achieved; in addition protein variants with altered physico–chemical properties will not be excluded and protein versions toxic to cells can be obtained.

The invention of flow-through systems allows total automation of cell–free protein synthesis. I hope that automated protein–synthesizing bioreactors will be soon constructed and introduced into practice. A future development would link a DNA synthesizer, a gene multiplication instru-

ment based on the DNA polymerase chain reaction, and an automated cell–free protein synthesis bioreactor.

Of course, the most difficult and critical problem in cell–free protein biosynthesis concerns the correct co-translational and post-translational modification of a synthesized polypeptide. A solution to this problem is still under investigation. One approach might involve cell-free protein synthesis in vesicles made of natural endoplasmic reticulum membranes, with the system of polypeptide transport and modification intact. In principle, a flow-through protein synthesis bioreactor could be developed based on such vesicles placed in a column.

In any case, I believe that future biotechnology will be mainly cell-free biotechnology, including cell-free gene cloning, cell-free gene multiplication, cell-free gene expression, and cell-free protein modification.

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