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Functional Ribosomal Unit of Gamma-Globulin Synthesis

Abstract. *Nascent protein synthesis is associated with polyribosomes in extracts of lymph node cells removed from hyperimmunized rabbits, if the ribonuclease activity in such extracts is inhibited. Prior treatment of the cells with actinomycin D markedly decreases protein synthesis by polyribosomes.*

A unique characteristic of antibody formation is that an individual animal, after appropriate stimulation with an antigen, can produce antibody molecules of a seemingly unlimited number of different specificities. The possibility has been considered, therefore, that the mechanism of γ-globulin synthesis differs from that of other mammalian proteins.

In the mammalian systems that have been examined under conditions which preserve ribosomal aggregates, protein synthesis occurs on clusters of ribosomes (polyribosomes) held together by a messenger RNA (mRNA) molecule (*1*, *2*). The average number of ribosomes forming a single polyribosome is approximately proportional to the size of the mRNA (*3*). Further, polyribosome-mediated protein synthesis slowly ceases when the synthesis of new RNA is prevented with actinomycin D (*2*).

The synthesis of antibody can be inhibited by actinomycin D (*4*); this suggests but does not prove (*5*) that γ-globulin synthesis is also dependent upon the continued synthesis of mRNA. Stenzel *et al.* (*6*) have studied the functional ribosomal unit of protein synthesis in spleen cells from immunized rabbits and have suggested that the polypeptides of γ-globulin are synthesized on single ribosomes and dimers, and thus the synthesis of γ-globulin differs from that of other mammalian proteins which have been investigated. In our experiments we show that protein synthesis by lymph node cells obtained from hyperimmune rabbits occurs on polyribosomes and that treatment of such cells in vitro with actinomycin D inhibits protein synthesis.

Rabbits were hyperimmunized with T2 phage and, 4 to 9 days after the last immunizing injection, cells were "teased" from the popliteal lymph nodes, washed, and resuspended (2 to 4×10^7 cells/ml) in either tris-buffered 1066 medium (*7*) supplemented with fetal-calf serum (20 percent) or Eagle's medium (*8*) containing 1/10 to 1/50 the standard concentrations of amino acids. Cells were stirred gently for 1 hour at 37°C and then exposed to 10 μc of C^{14}-labeled amino acids (*9*) for 1.5 to 3 minutes in order to label only nascent protein (*1*, *2*). The cells were washed three times in chilled Earle's saline solution, and 4×10^7 cells were resuspended in 1 ml of cold hypotonic buffer ($10^{-2}M$ KCl; $10^{-2}M$ tris-HCl, *p*H 7.4; $1.5 \times 10^{-3}M$ $MgCl_2$).

In the initial experiments, "pulse"-labeled lymphocytes were disrupted in a number of ways: (i) with a Dounce homogenizer after suspension of the cells in hypotonic buffer alone, or in buffer supplemented with either 10^{-4} mg of hydrocortisone per milliliter or 0.1 percent Tween 80 (*10*) (these additions increased the cell breakage); (ii) with a Potter-Elvehjem homogenizer after suspension of cells in either hypotonic buffer or the same buffer supplemented with $0.25M$ sucrose; (iii) with 0.5 percent deoxycholate.

The cultivation of S3 HeLa cells, the preparation of cytoplasmic extracts, the determination of ultraviolet absorbancy at 260 mμ, and the determination of the radioactivity insoluble in trichloroacetic acid (TCA) have already been described (*11*).

When extracts from labeled lymphocytes were prepared as described, almost all of the radioactivity was associated with either single ribosomes or small aggregates of two to three ribosomes. Since a minute amount of pancreatic ribonuclease causes similar breakdown of HeLa cell polyribosomes under the same conditions (*2*), experiments were undertaken to determine whether this association of nascent protein with single ribosomes was due to breakdown of polyribosomes. Lymphocyte extracts were mixed with HeLa cell cytoplasm which had been treated with C^{14}-labeled amino acids for 1.5 minutes, and the mixture was analyzed on sucrose gradients along with untreated HeLa cell cytoplasm (Fig. 1). In the cytoplasm of the control HeLa cell the ultraviolet-absorbing material (which is mostly due to ribosomal RNA) is in both the region of the single-ribosomes (74*S*) and in the lower part (100 to 350*S*) of the sucrose gradient. The heavier material contains polyribosomes composed of 5 to 30 ribosomes each (*12*). In agreement with the results of Penman *et al.* (*2*), nascent protein (as determined by TCA-precipitable radioactivity) is primarily associated with polyribosomes, or has been released from the ribosomes, and is found at the top of the gradient. Very little radioactivity is associated with single ribosomes. When the same HeLa cell cytoplasm is mixed with lymphocyte extracts (Fig. 1), both ultraviolet absorbancy and radioactivity are lost from the polyribosome fraction and are associated with single ribosomes and small aggregates.

In the second part of this experiment extracts prepared from labeled lymphocytes were mixed with unlabeled HeLa cell cytoplasm (Fig. 2). The HeLa cell polyribosomes were broken down as before, and lymphocyte nascent proteins were again associated with single ribosomes and small aggregates (Fig. 2). This was true of lymphocyte extracts

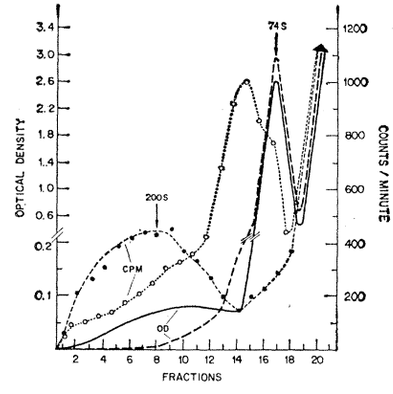

Fig. 1. Breakdown of HeLa cell polyribosomes by lymph node cell extracts. HeLa cells (8×10^7) were treated (pulse) with C^{14}-labeled amino acids, and cytoplasm was prepared and divided into two portions. One portion was mixed with the lysate from 4×10^7 lymph node cells, and both the treated sample and the untreated control were layered on a sucrose gradient (15 to 30 percent) and were centrifuged for 120 minutes at 24,000 rev/min in a SW 25.1 swinging-bucket rotor. The bottom of the gradient is on the left and the top is on the right. ——, $O.D._{260}$ of untreated HeLa cell cytoplasm; ●----●, TCA-precipitable radioactive fraction from untreated HeLa cell cytoplasm; – – – –, $O.D._{260}$ of lymph node cell plus treated HeLa cell cytoplasm; ○···○, TCA-precipitable radioactive fraction of treated HeLa cell cytoplasm.

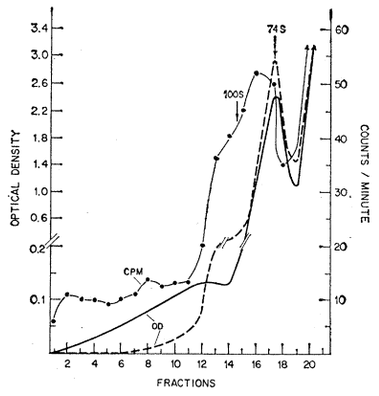

Fig. 2. Breakdown of HeLa cell polyribosomes by pulse-labeled lymph node cells. This is the same as experiment described in Fig. 1 except that the HeLa cells were unlabeled and the lymph node cells were labeled. ——, O.D.$_{260}$ of untreated HeLa cell cytoplasm; – – – –, O.D.$_{260}$ of treated HeLa cell cytoplasm; ●——●, TCA-precipitable radioactive fraction of pulse-labeled lymph node cells.

prepared by all of the methods described.

To demonstrate the presence of ribonuclease activity, extracts of lymphocytes were incubated with C^{14}-labeled RNA (*13*). All of the radioactive material that was sensitive to pancreatic ribonuclease was degraded to acid-soluble material.

Subsequently, the breakdown of lymphocyte polyribosomes was prevented by disrupting small numbers of lymphocytes with deoxycholate in the presence of large excesses of HeLa cell cytoplasm and immediately centrifuging. Under these conditions, HeLa cell polyribosomes were preserved, and lymphocyte nascent protein synthesis was almost entirely associated with polyribosomes (Fig. 3). The peak of radioactivity was in the 200*S* fraction (about eight ribosomes per polyribosome) but nascent protein was also associated with polyribosomes with sedimentation constants of 100 to 350*S*. Considering that some radioactive material from the adjoining portions of the gradient extends into the single ribosome fraction, it can be estimated that about 6 percent of the ribosome-associated radioactivity is present in the single ribosome part of the sucrose gradient.

When exogenous ribonuclease was added to a mixture of labeled lymphocyte extract and unlabeled HeLa cell cytoplasm (as in Fig. 3), all the radioactivity and the ultraviolet-absorbing material were in the part of the sucrose gradient occupied by single ribosomes and small aggregates.

The possibility was considered that the association of the nascent protein of the lymphocyte with the polyribosome area of the sucrose gradient may have been caused by binding in vitro to HeLa cell polyribosomes. When labeled lymphocyte material consisting of either single ribosomes with attached polypeptides or released polypeptide chains was recovered from sucrose gradients and rerun, either alone or mixed with HeLa cell cytoplasm (Table 1), there was no significant binding in vitro.

In order to identify γ-globulin polypeptides on ribosomes in each fraction, the sucrose was removed by dialysis, and the fraction was made 0.14*M* with NaCl and reacted with guinea pig antiserum to reduced and alkylated γ-globulin (*14*). The resulting soluble complexes were precipitated with rabbit antiserum to guinea pig γ-globulin, and the precipitates were analyzed for radioactivity (*11*). The amount of radioactivity precipitable (by the same rabbit antiserum) with normal guinea pig serum (control) was determined for each sample and subtracted from that precipitated with the guinea pig antiserums. The ratio of the radioactivity in the antiserum precipitate to that in normal serum precipitate was at least 2 to 1. The concentration of the immunological reagents had been adjusted so that the aforementioned precipitates were formed in antibody excess, and each bound the same amount of radioactive material in the presence of labeled HeLa and KB cells (*11*).

In sucrose gradients (as in Fig. 3) the amounts of radioactivity were too small to obtain reliable results. In order both to increase the amount of radioactivity and to concentrate it in a few fractions, larger numbers of immune lymph node cells were "pulse"-labeled, disrupted, allowed to break down to single ribosomes, and then analyzed for nascent γ-globulin. Nascent protein immunologically identifiable as γ-globulin was associated with small aggregates and single ribosomes, and it was at the top of the gradient (Fig. 4). Since there is little radioactivity in the single ribosome portion in undegraded preparations, this immunologically precipitable material must originally have been polyribosome-associated.

The fact that only a small percentage of the total ribosome-associated radioactivity is specifically precipitable is not unexpected. In a relatively homogeneous population of HeLa cells infected with either poliovirus or vaccinia virus, only a slightly larger percentage of the polyribosome-associated polypeptides were immunologically precipitable (*11*). In lymph node cells, only 30 percent of the protein synthesized is γ-globulin and not all of the polypeptide chains are complete enough to react with antiserum.

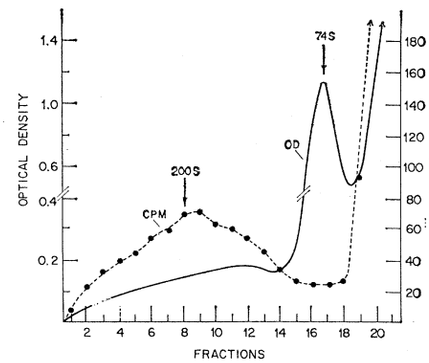

Fig. 3. Location of nascent protein synthesis in lymph node cells. Cytoplasm was prepared from 1 × 10^8 HeLa cells and treated with 0.5 percent deoxycholate. Lymph node cells (2 × 10^7) were then exposed to C^{14}-labeled amino acids for 3 minutes, washed, added to the DOC treated HeLa cell cytoplasm, and the mixture was immediately centrifuged. ——, O.D.$_{260}$ of HeLa cell cytoplasm; ●– – – –●, TCA-precipitable radioactivity of labeled lymph node cells.

In order to study the effect of actinomycin D on polyribosome-mediated protein synthesis, lymph node cells were incubated in vitro with actinomycin D (0.5 μg/ml). At 5 and 7 hours after addition of the drug, treated and control cells were treated for 3 minutes with C^{14}-labeled amino acids, lysed in

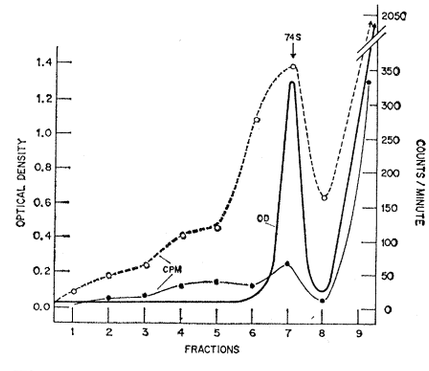

Fig. 4. Immunological precipitation of γ-globulin attached to ribosomes. Lymph node cells (8 × 10^7) were labeled with C^{14}-amino acids for 3 minutes, washed, and disrupted with a Dounce homogenizer in hypotonic buffer containing 0.1 percent Tween, and then centrifuged in a 15 to 30 percent sucrose gradient. ——, O.D.$_{260}$; ○– – – –○, TCA-precipitable radioactivity; ●——●, specifically precipitable radioactivity.

Table 1. Effect of extracts of unlabeled HeLa cells on distribution of labeled lymphocyte material in the sucrose gradient. Released polypeptides, 0–45*S*; single ribosomes, 45–130*S*; polyribosomes, 130–300*S*.

HeLa cells added	Radioactivity in sucrose gradients (%)		
	0–45*S*	45–130*S*	130–300*S*
*Released polypeptides**			
0	99	1	0
+	98	2	0
Polypeptides attached to single ribosomes†			
0	18	80	2
+	10	82	8

* The labeled lymphocyte polypeptide chains were obtained from the top of a gradient in which labeled lymphocyte material had been run in the absence of HeLa cytoplasm. † Single ribosomes were obtained from the 60–80*S* portion of the aforesaid sucrose gradient.

the presence of unlabeled HeLa cell cytoplasm and analyzed (legend Fig. 3). Five hours of actinomycin treatment caused a 63-percent decrease in polyribosome-associated protein synthesis, whereas 7 hours of treatment resulted in an 83-percent reduction.

These experiments show that extracts of hyperimmune lymph node cells can cause the breakdown of HeLa cell polyribosomes, presumably by ribonuclease activity which has been detected in lymph node cell lysates. The presence of ribonuclease activity may explain the results of other investigators who have reported that protein synthesis in rabbit spleen cells is associated with single ribosomes and small aggregates (*6*).

When the breakdown was prevented by the addition of a large amount of HeLa cell cytoplasm, nascent C^{14}-labeled polypeptides in hyperimmune lymph node cells were associated with the polyribosome area of sucrose gradients. Two additional facts suggest that the observed radioactivity reflects polyribosome-mediated protein synthesis: (i) when exogenous ribonuclease is added to lymphocyte HeLa cell cytoplasm mixtures there is a shift of TCA-precipitable radioactivity from the polyribosome area of the sucrose gradient to the fractions occupied by the single ribosomes; this suggests that the lymphocyte single ribosomes are held together by a ribonuclease-sensitive structure. (ii) Addition of actinomycin D to cell suspensions causes a marked decrease in incorporation of amino acids in the material in the polyribosome region of the sucrose gradient. This corresponds to the decrease in total protein and γ-globulin synthesis in these cells after actinomycin treatment (*15*).

Since approximately 30 percent of the total protein made by the cells in these experiments is identifiable immunologically as γ-globulin (*15*) and since virtually all nascent protein is formed on polyribosomes, it can be concluded that γ-globulin synthesis occurs on polyribosomes. This was confirmed by immunological identification of γ-globulin polypeptides associated with ribosomes. Thus, the translation step of γ-globulin synthesis occurs on large aggregates of ribosomes held together by a ribonuclease-sensitive structure. This ribonuclease-sensitive structure is presumably mRNA, but this can only be proved by detailed studies of its synthesis and physical-chemical characteristics.

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Respiratory and Electrical Responses to Light Stimulation in the Retina of the Frog

Abstract. *Isolated retinas from frogs' eyes were preserved in a circulating medium; transretinal electrical potential and ultraviolet absorbancy were monitored. In response to visible stimulation, changes in absorbancy were observed which correlate with the c-wave of the electroretinogram. They are tentatively identified as cyclic oxidations of pyridine nucleotides reflecting the energy expenditure associated with evoked neuronal activity.*

The isolated retina in a perfusion system can be kept responsive to adequate stimulation and be subjected to various experimental procedures under stationary conditions for several hours (*1*). Specific requirements for optimum survival, tolerances toward controlled deviations, and changes in the perfusate effected by the retina pointed to the importance of energy yielding processes in the generation of the electroretinogram (ERG) (*2*). Therefore, an analysis for respiratory pigments seemed indicated on account of their role in intermediary metabolic reactions and of the speed of their spectroscopic detectability (*3*).

Diphosphopyridine nucleotide (DPN) was the first pigment sought. Its reduced form, DPNH, exhibits strong absorption at 340 nm with a resulting emission of bluish fluorescence. It was hoped that the analyzing procedure would not interfere with visual excitability (*4*). Preliminary experiments, however, showed that the isolated retina does respond electrically to flashes in the near ultraviolet region of the spectrum. Thus analyzing with beams of low intensities was mandatory. Because many substances in the retina are strongly fluorescent, giving a large background of steady fluorescence, it was not possible to detect changes in DPNH by changes in fluorescence. However, changes in ultraviolet absorption as a result of stimulation in the visible range could be measured easily.

In dim red illumination frog retinas were isolated from opened, submerged (*5*) eye cups and clamped at their peripheries to a tantalum grid which occupied the central opening of a black plastic support (Fig. 1, inset). The support also carried fluid and electrical connections into a modified tissue cul-