Stimulatory Activity of High Molecular Weight Ribonucleic Acid in the Rabbit Reticulocyte Cell-Free System*

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ABSTRACT: The stimulation of protein synthesis in a rabbit reticulocyte cell-free system by ribonucleic acid (RNA) extracted from reticulocyte ribosomes by different methods and the product of the stimulation observed using RNA extracted from rabbit reticulocytes, bone marrow, and liver were examined. Chromatography of the ribosome-free supernatant fraction of the cell-free system stimulated by RNA extracted from these sources, on DEAE-cellulose, showed no difference between the stimulation observed by bone marrow erythropoietic cell RNA or liver RNA and reticulocyte RNA. In all cases hemoglobin synthesis was stimulated. It is concluded that the addition of high molecular weight RNA to the reticulocyte cell-free system stimulates hemoglobin synthesis by stimulation of the endogenous messenger ribonucleic acid (mRNA) activity on the ribosomes and not by acting as messenger ribonucleic acid itself. This conclusion is borne out by comparison of these results with previous results reported by other workers.

Many attempts have been made to isolate mRNA for a specific protein. Most of this work has concentrated on the mRNA for hemoglobin (Arnstein *et al.*, 1964; Kruh *et al.*, 1964a; Shaeffer *et al.*, 1964; Drach and Lingrel, 1966a,b; Brawerman *et al.*, 1965; Marbaix *et al.*, 1966), since it can be demonstrated that hemoglobin constitutes about 90% of the protein synthesized by reticulocytes from phenylhydrazine-treated rabbits (Kruh and Borsook, 1956) and about 70% of the released protein in the cell-free system from reticulocytes (Arnstein *et al.*, 1964).

Cell-free systems from *Escherichia coli* (Schaeffer *et al.*, 1964; Drach and Lingrel, 1966a,b; Brawerman *et al.*, 1965) and from rabbit reticulocytes (Arnstein *et al.*, 1964; Kruh *et al.*, 1964a) have been used to test RNA extracted from reticulocytes and reticulocyte ribosomes for its ability to stimulate protein synthesis and to locate the mRNA activity after fractionation by sucrose gradient centrifugation (Cox and Arnstein, 1964; Drach and Lingrel, 1966a; Brawerman *et al.*, 1965). Using the *E. coli* cell-free system, neither Schaeffer *et al.* (1964) nor Drach and Lingrel (1966b) were able to demonstrate the synthesis of globin induced by reticulocyte RNA while both Arnstein *et al.* (1964) and Kruh *et al.* (1964a) have claimed that rRNA from reticulocytes induces the synthesis of hemoglobin in the reticulocyte cell-free system.

The present paper describes experiments in which the product of the stimulation by unfractionated RNA extracted from rabbit reticulocytes, liver, and bone marrow cells in the reticulocyte cell-free system was characterized. The possibility that the effect of high molecular weight RNA on the reticulocyte cell-free system is a nonspecific protection or stimulation of endogenous mRNA on monoribosomes is discussed in relation to these results and the results of other workers.

Materials

Chemicals. ATP,[1] GTP, glutathione, trisodium phosphoenolpyruvate, and pyruvate kinase were obtained from Sigma Chemical Co. Pyruvate kinase was also obtained from C. F. Boehringer through Calbiochem. ^{14}C-labeled amino acids were obtained through Nuclear-Chicago Corp. [^{14}C]Valine had a specific activity of 160 or 107 mc/mmole.

Buffers. The following buffers were made from reagent grade materials: medium A_1, 0.25 M sucrose–0.025 M KCl–0.05 M Tris-HCl (pH 7.6 at 25°)–0.001 M $MgCl_2$; medium A_5 was the same as medium A_1 except that the concentration of $MgCl_2$ was 0.005 M.

Buffers for Chromatography. PHOSPHATE (0.005 M)–KCN (pH 8.6). Na_2HPO_4 (947 ml of 0.005 M) 53 ml of 0.005 M NaH_2PO_4, and 100 mg of KCN adjusted to pH 8.6 with 0.2 M NaH_2PO_4.

PHOSPHATE (0.01 M)–0.6 M NaCl-KCN (pH 6.4). Na_2HPO_4 (375 ml of 0.01 M), 625 ml of 0.01 M NaH_2PO_4, 100 mg of KCN, and 35.1 g of NaCl had a final pH of 6.4.

RNA Preparations. PAS RNA was extracted from ribosomes and rabbit liver by method 2 of Kirby (1965) (see also Wilkinson and Kirby, 1966), using 4-aminosalicylate and phenol–cresol, either at room temperature (25°) or 0°. PAS RNA was extracted from fractionated

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[1] ATP and GTP, adenosine and guanosine triphosphates; TCA, trichloroacetic acid; PAS, *p*-aminosalicylate.

cells from bone marrow by method 2 of Kirby (1965) except that the total nucleic acids were dissolved in 0.1 M NaCl and NaCl was added to 3 M instead of extracting the precipitate with 3 M sodium acetate.

GUANIDINIUM CHLORIDE RNA was extracted from ribosomes by precipitation with 4 M guanidinium chloride as described by Cox (1966) (see also Hunt, 1965).

Estimation of Protein and RNA. Protein content of pH 5 enzymes and cell sap was estimated from a nomogram using absorbance values at 260 and 280 mμ (Warburg and Christian, 1942). The $A_{260}^{1\,cm}$ for ribosomes as taken as 11.2/mg and for RNA as 20.0/mg.

Determination of Radioactivity. Aliquots of the solutions to be measured were pipetted onto 2 × 6 cm pieces of Whatman 3MM paper (each paper held up to 0.25 ml) dried, and washed according to the method of Mans and Novelli (1961) using 0.1 M valine in the first 10% trichloracetic acid wash. The washed papers were placed in glass vials covered with 10 ml of scintillator solution 0.5% (w/v) 2,5-diphenyloxazole, 0.05% 1,4-bis(4-methyl-5-phenyloxazol-2-yl) in reagent grade toluene, and counted in a Packard series 3314 liquid scintillation spectrometer. The efficiency of counting varied between 50 and 60%. Quenching due to color on the papers was monitored by use of the automatic external standard but the efficiency was never less than 90% of the efficiency of standard [^{14}C]valine on unwashed paper. A blank paper washed with the others usually had a count of 100–200 cpm for the cell-free system but 10–20 cpm for chromatographic fractions.

Preparation of Subcellular Fractions. Reticulocytes were obtained from New Zealand white rabbits by five daily injections of 2.5% phenylhydrazine. H_{60} and L_{60} ribosome fractions and pH 5 enzymes were prepared according to Arnstein *et al.* (1964) care being taken to readjust the pH 5 enzyme fraction to pH 8.2 at 0° after dissolving it in medium A_5; because of the temperature dependence of Tris buffer this corresponds to pH 7.6 at 25°. Ribosome fractions were resuspended in medium A_1.

Cell-Free System. The cell-free system was as described by Nair and Arnstein (1965). Usually each tube contained 0.25 mg of L_{60} ribosomes and 1 or 2 mg of pH 5 enzymes in 0.25 ml. Each milliliter of incubation mixture contained 50 μmoles of KCl, 5 μmoles of $MgCl_2$, 25 μmoles of Tris-HCl (pH 7.6), 10 μmoles of glutathione, 0.2 μmole each of 19 L-amino acids excluding the labeled one, 1 μc of labeled amino acid, 5 μmoles of sodium phosphoenolpyruvate, 0.25 μmole of ATP, 0.0625 μmole of GTP, and 100 μg of pyruvate kinase. Incubation was for 1 hr at 37° in open tubes.

Chromatography. After incubation of a cell-free system containing 5 mg of ribosomes and 20 mg of pH 5 enzymes in 5 ml, 5 ml of bone marrow lysate (approximately 9.6 mg of protein/ml) was added and the material was centrifuged in the Spinco Ti50 head at 50,000 rpm (165,000*g*) for 1 hr to sediment the ribosomes. The supernatant was then dialyzed four times against 100 volumes of 0.005 M phosphate–KCN (pH 8.6) buffer over 2 days and frozen. The total volume after dialysis was increased to 12–13 ml. DEAE-cellulose Serva (0.68 mequiv/g, obtained through Gallard-Schlesinger, Garden City, N. Y.), which was held on a 200 mesh sieve was suspended in starting buffer and poured at room temperature under 7 psi of pressure to make a column of 54 × 1.1 cm. Starting buffer (200 ml) was passed through the column at room temperature at 10 psi and the column was transferred to a 4° room when 500 ml of starting buffer was passed through the column at 30 ml/hr. The dialyzed sample (10 ml) was put on the column and a 400-ml linear gradient from 0.005 M phosphate–KCN (pH 8.6) to 0.01 M phosphate–0.6 M NaCl-KCN (pH 6.4) was run through the column at approximately 30 ml/hr. Fractions of 3 or 4 ml were collected. At the end of the gradient 0.5 N NaOH was passed through the column until no more material was eluted. The fractions were assayed for absorbancy at 280 and 415 mμ and 0.5-ml aliquots were taken for radioactive analysis on two 2 × 6 cm Whatman 3MM papers.

Bone Marrow Cell Fractionation. Bone marrow cells from anemic rabbits were fractionated by the method of H. Borsook (private communication). Dispersed cells were centrifuged on 40-ml linear density gradients of bovine serum albumin in plasma and isotonic salt solutions (see also Leif and Vinograd, 1964) using densities from 1.04 to 1.08 at 3500 rpm in the SW 25.2 rotor of the Spinco Model L2 at 20°, for 30 min. The two top fractions were recentrifuged under the same conditions for 60 min. Smears of the fractionated cells were stained with Wright's stain (Aloe Scientific) and differential counts were made of about 500 nucleated cells.

Results

Products of RNA Stimulation. Because it was felt that the stimulation of the reticulocyte cell-free system by

TABLE I: Stimulation of Incorporation by RNA from Bone Marrow Fraction $2C_{II}$ and RNA from Liver and Reticulocytes.[a]

RNA Sample	Amt Added/ μg mg of Ribosomes	Incorp of mμmoles of Valine/ mg of Ribosomes	Increase with RNA (μμmoles of valine/ 100 μg of RNA)
None	—	0.191	—
$2C_{II}$	190	0.312	64
Cold PAS reticulocyte	194	0.421	118
PAS liver	204	0.340	72
GHCl reticulocyte	200	0.390	99

[a] Each tube contained 5 mg of ribosomes and 20 mg of pH 5 enzyme in 5 ml.

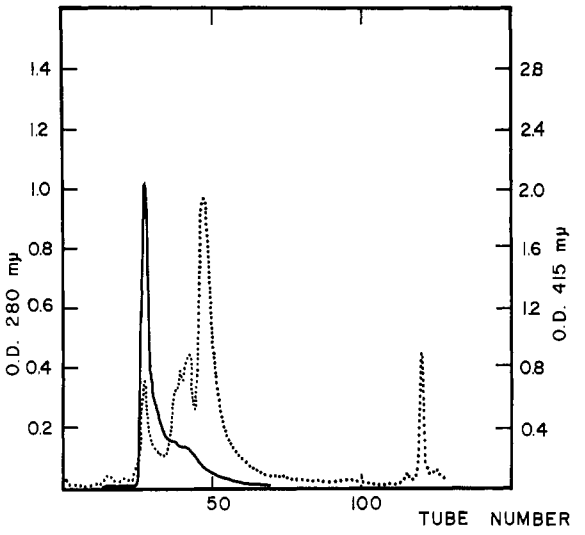

FIGURE 1: Separation of rabbit hemoglobin from rabbit plasma proteins on a column of DEAE-cellulose (for details see text).

TABLE II: Distribution of Incorporated Valine between Ribosomes and Supernatant after Centrifuging for 1 hr at 50,000 rpm.

RNA Sample	Incorporation of Valine (mμmoles of valine/mg of ribosome) In Ribosomes	In Recovd Supernatant	% in Supernatant
None	0.037	0.116	76
$2C_{II}$	0.051	0.192	79
Cold PAS reticulocyte	0.066	0.242	79
PAS liver	0.055	0.204	79
GHCl reticulocyte	0.069	0.233	77

RNA may be nonspecific the products of the stimulation by RNA extracted from different sources were characterized by column chromatography on DEAE-cellulose. The RNA samples tested were PAS–phenol-extracted rabbit liver RNA, reticulocyte RNA, RNA from fraction $2C_{II}$ of bone marrow cells and guanidinium chloride RNA from reticulocytes. The cell composition of fraction $2C_{II}$ was as follows: pronormoblasts (6%), basophilic erythroblasts (50%), polychromatophilic normoblasts (27%), white cells (7%), and nonnucleated cells (10%).

Table I shows the degree of stimulation by the RNA from various sources. Although the background incorporation was variable the stimulation of incorporation/100 μg of RNA was constant for each batch of RNA. Table II shows the distribution of radioactivity between the ribosomes and the supernatant and indicates that the stimulation equally affects ribosomes and supernatant.

The chromatographic system used was chosen for its ability to separate hemoglobin from bone marrow cell proteins and from rabbit serum proteins. As can be seen from Figures 1 and 2 this aim was accomplished using a pH and salt gradient on DEAE-cellulose. For the separation using rabbit plasma a ratio of plasma protein to hemoglobin of 8:1 was used, with bone marrow lysate the ratio of hemoglobin to other proteins was approximately 1:1.

The supernatants from the experiment in Table I were chromatographed in the presence of bone marrow lysate as shown in Figure 2. Each chromatogram was divided into four main regions (I–IV). Table III shows the distribution of radioactivity in these regions for the stimulation by RNA samples $2C_{II}$, PAS reticulocyte, and liver as well as the control without RNA. Because the recovery of radioactivity from the chromatography is somewhat variable (67–80%) it is not easy to correlate the radioactivity between the areas from each fractionation. However, the main hemoglobin peak accounts for about 40% of the recovered radioactivity and the specific activity of the hemoglobin peaks (cpm/OD 415 mμ) increases by the same amount as for the total protein. It is also likely that some of area III contains hemoglobin with a higher specific activity than the main hemoglobin peak. However, the most striking feature of these chromatograms is that there is no significant difference be-

TABLE III: Distribution of Radioactivity of Soluble Proteins after Chromatography on DEAE-cellulose.

RNA Sample	Cpm on Column	% Recovd from Column	% in Fractions I	II	III	IV	Sp Act. of Peak II (cpm/OD 415 mμ)
None	63,800	80	5	33	43	19	129
$2C_{II}$	105,100	67	5	43	36	16	206
Cold PAS reticulocyte	132,600	73	5	43	35	17	290
PAS liver	111,900	78	10	40	33	17	214

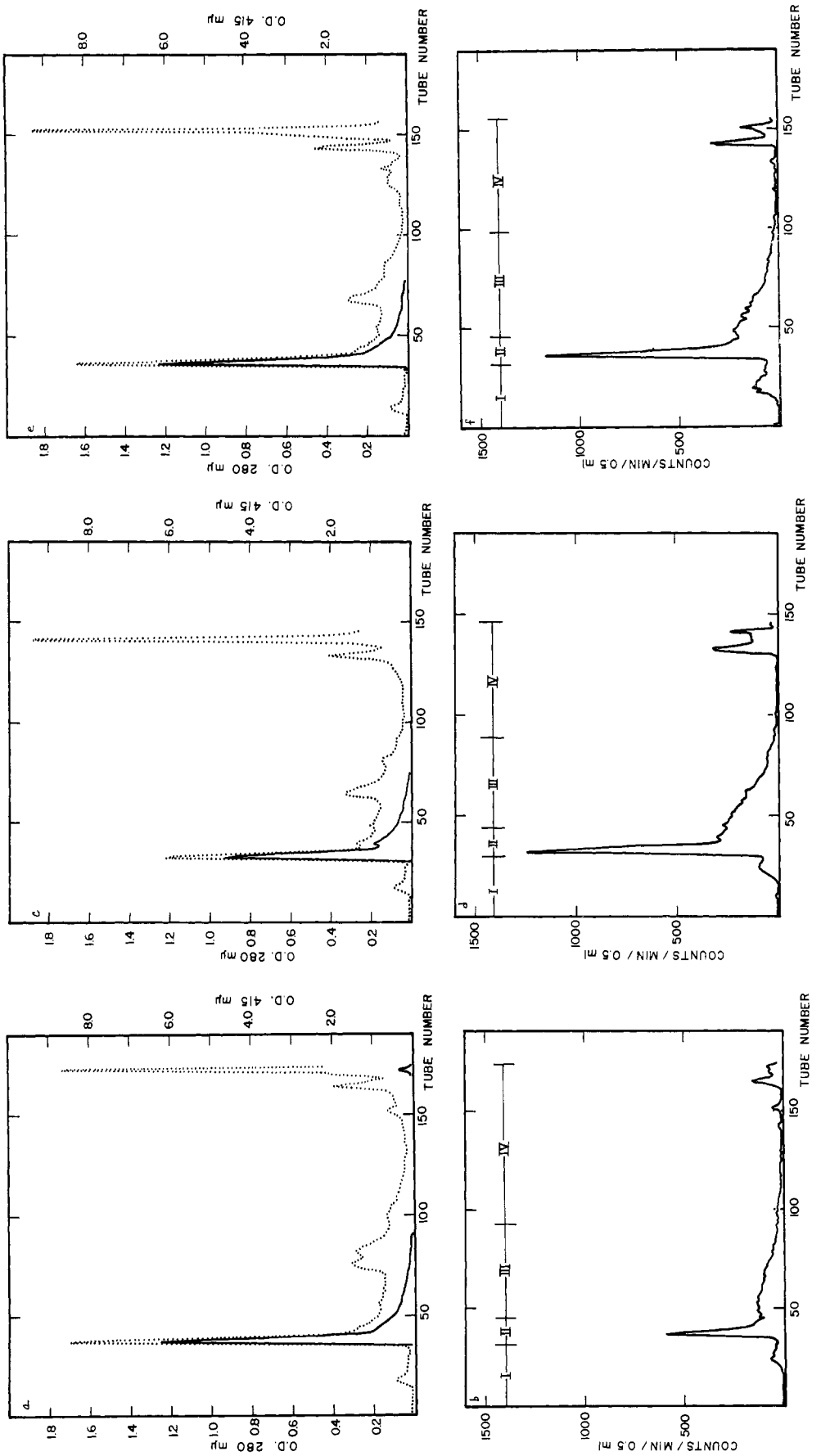

FIGURE 2: Separation of supernatant proteins from cell-free systems stimulated by various RNAs (for details see text). (a) (control) No RNA added. (——) OD 415 mμ. (·····) OD 280 mμ. (b) (control) No RNA added. (——) Radioactivity in TCA-insoluble material. (c) Addition of PAS reticulocyte RNA. (– – –) OD 415 mμ. (·····) OD 280 mμ. (d) Addition of PAS reticulocyte RNA. (——) Radioactivity in TCA-insoluble material. (e) Addition of rabbit liver RNA. (——) OD 415 mμ. (·····) OD 280 mμ. (f) Addition of rabbit liver RNA. (——) Radioactivity in TCA-insoluble material.

tween the stimulation given by reticulocyte RNA, bone marrow RNA, and liver RNA and that at least 50% of this stimulation is in hemoglobin. As a final test that the main peaks II contained only hemoglobin the peaks were concentrated and subjected to paper electrophoresis at pH 8.6 in 0.05 M Veronal buffer. The radioactive profile corresponded with the hemoglobin bands.

Discussion

The experiments described were initiated because it was felt that there was no conclusive proof available that the stimulation of protein synthesis observed by addition of rRNA to a reticulocyte cell-free system was the mRNA-induced synthesis of new protein. The ability of liver RNA to stimulate synthesis of hemoglobin but not to stimulate liver protein synthesis would indicate that the effect is nonspecific, and may be due to a protection or stimulation of endogenous mRNA.

Kruh *et al.* (1964b) found stimulation of hemoglobin synthesis by RNA extracted from rabbit reticulocytes, kidney, liver, or intestines but not by guinea pig reticulocyte or guinea pig, rat, or chicken liver RNA. They claim from these results that rabbit kidney, liver, and intestinal RNA contain mRNA for hemoglobin. Unfortunately their results are only concerned with the isolated hemoglobin and it is not at all clear whether they have an over-all stimulation of protein synthesis or a transfer of incorporated amino acids from ribosomal protein to hemoglobin. Thus their results tend to confirm our conclusions although they have not characterized the total products of their stimulation. Arnstein *et al.* (1964) using rabbit reticulocyte RNA in the rabbit reticulocyte cell-free system, found stimulation of hemoglobin synthesis with formation of new hemoglobin chains. But the L_{60} ribosomes which were stimulated also made completely new chains of hemoglobin (see Table 12 lines 1 and 2 of Arnstein *et al.*, 1964). Again there is no proof of the mRNA stimulation of hemoglobin synthesis. The best proof would be obtained by adding RNA from the reticulocytes of another species whose hemoglobins were separable. Kruh *et al.* (1964a), Schapira *et al.* (1966), and H. R. V. Arnstein and J. A. Hunt (unpublished data) used guinea pig reticulocyte RNA in a rabbit reticulocyte cell-free system but were unable to demonstrate synthesis of guinea pig hemoglobin although Arnstein and Hunt were able to show the stimulation of rabbit hemoglobin synthesis.

More information can be obtained from the data of Cox and Arnstein (1964) who tested various fractions of reticulocyte RNA separated by sucrose gradients in the reticulocyte cell-free system. The stimulation of protein synthesis closely followed the main 28S and 17S peaks of RNA with a small region of high specific activity in the 8–16S region. These results have been confirmed in these laboratories for RNA extracted at 0 and 20° by the PAS–phenol method as well as by guanidinium chloride extraction. But when Cox and Arnstein (1964) extracted RNA from polysomes which had been subjected to mild ribonuclease treatment, reducing their endogenous protein synthetic activity to 10%, the pattern of stimulation on the sucrose gradient was identical with nonribonuclease-treated material except for the loss of less than 10% of the total activity in the 8–16S region. If one assumes that the mRNA was 90% degraded by the ribonuclease treatment it would be expected that the stimulation of protein synthesis by the isolated RNA would be reduced by a similar amount which is clearly not found. Again it must be concluded that the stimulation by added RNA is probably not an mRNA-induced effect. Although Drach and Lingrel (1966a) and Brawerman *et al.* (1965) found a single fraction of reticulocyte RNA which stimulated protein synthesis in the *E. coli* cell-free system, neither Drach and Lingrel (1966b) nor Schaeffer *et al.* (1964) have been able to demonstrate the synthesis of globin or globin peptides in the stimulated system. It is notable that the only groups who have been able to demonstrate a synthesis of different species hemoglobin by adding cell-free extracts to a reticulocyte cell-free system (Weisberger and Armentrout, 1966; Schapira *et al.*, 1966) have used postribosomal supernatant fractions with some evidence (Weisberger and Armentrout 1966) that the factor involved is a small ribonucleoprotein particle.

It must be concluded that the stimulation of protein synthesis observed in the reticulocyte cell-free system by addition of high molecular weight RNA is not an mRNA-induced synthesis of protein but is due to the protection or stimulation of endogenous mRNA. This conclusion does not prove that the reticulocyte cell-free system cannot respond to mRNA since it is known that polyuridylic acid stimulates polyphenylalanine synthesis (Arnstein *et al.*, 1964; Hardesty *et al.*, 1963) and tobacco yellow mosaic virus ribonucleic acid stimulates the incorporation of valine and isoleucine in different ratios from those found by reticulocyte RNA stimulation (Betheil and Arnstein, 1965). However, it does show the need to characterize the products of any RNA stimulation of a cell-free system before this effect is ascribed to mRNA.

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2,4-Dinitrophenol and Azide as Inhibitors of Protein and Ribonucleic Acid Synthesis in Anaerobic Yeast*

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ABSTRACT: When *Saccharomyces cerevisiae* was grown under anaerobic conditions with energy and carbon supplied by glucose, it was established that two classic uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and azide, are potent inhibitors of protein and ribonucleic acid (RNA) synthesis. During the inhibition it was found that the cells maintained high levels of adenosine triphosphate (ATP), amino acids, and nucleotides. All of the reactions of *in vitro* amino acid incorporation and stimulation of incorporation by addition of polyuridylic acid (poly U) were insensitive to the uncouplers. The incorporation of added uracil into uridine triphosphate (UTP) and the activity of an isolated deoxyribonucleic acid dependent RNA polymerase were unaffected by the poisons. A uniform depression of [^{14}C]uracil and [^{14}C]leucine incorporation throughout the polysomes and monosomes was revealed by sucrose density gradient studies. Several possible explanations are considered including the idea that high-energy intermediates of oxidative phosphorylation common to ATP production and macromolecular synthesis may exist.

In facultative anaerobes, energy for anabolic processes can be produced either aerobically through the coupled processes of electron transport and oxidative phosphorylation or anaerobically by a fermentation process involving substrate phosphorylation. Both processes culminate in the formation of ATP.[1] Endergonic processes which directly employ ATP should then proceed irrespective of its route of formation.

In this paper we will show that despite high levels of ATP present in yeast under conditions of anaerobic fermentation of glucose, two classic uncouplers of oxidative phosphorylation (in concentrations where they normally would uncouple oxidative phosphorylation in aerobic cells), are capable of preventing protein and RNA synthesis. Previous workers have reported that 2,4-dinitrophenol (2,4-DNP) and azide will inhibit growth and induced enzyme synthesis in yeast under anaerobic conditions (Kovac and Istenesova, 1964; Spiegelman, 1947; Reiner and Spiegelman, 1947). These observations have been confirmed and extended. We have tested the effects of these agents on the maintenance of amino acid and nucleotide pools formed from glucose, the concentration of ATP, all *in vitro* reactions of protein synthesis, with and without stimulation by mRNA, the activity of yeast DNA-dependent RNA polymerase, and the distribution of radioactivity from uracil or leucine in the cytoplasmic supernatant fluid of cells after incubation, by analysis in a sucrose density gradient.

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; TCA, trichloroacetic acid; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; AMP, adenosine monophosphate; PPO, 2,5-diphenyloxazole.