Biochem. J. (1965) **97**, 595

Further Observations on the Polynucleotide-Induced Stimulation of Protein Synthesis by Cell-Free Preparations from Rabbit Reticulocytes

By K. G. NAIR* and H. R. V. ARNSTEIN
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 18 *March* 1965)

1. The effect of high-molecular-weight RNA from reticulocyte polyribosomes (messenger RNA) on protein synthesis by subcellular fractions derived from reticulocytes, reported by Arnstein, Cox & Hunt (1964), has now been studied in detail. Optimum response of the cell-free system requires 30–50mM-K^+ and approx. 5mM-Mg^{2+} in the pH range 7·4–7·6. 2. RNA stimulates the incorporation into protein of both free amino acids and of aminoacyl residues from s-RNA. Stimulation by either RNA or polyuridylic acid is dependent on a labile factor or enzyme, which is present in the 'pH 5 fraction' and may be concerned with the formation of new polysomes. Quantitatively the response of the cell-free system to RNA is similar to that of polyuridylic acid, and there appears to be competition between messenger RNA and polyuridylic acid or polyadenylic acid.

The stimulation of haemoglobin formation by preparations containing m-RNA† and the induction of polyphenylalanine synthesis by polyuridylic acid in a cell-free system derived from rabbit reticulocytes have been described by Arnstein, Cox & Hunt (1962, 1964). In the present study, the conditions required for stimulation of the cell-free system by m-RNA have been investigated in greater detail. Evidence is presented for the formation of new polyribosomes in the presence of m-RNA, which appears to be dependent on a factor present in the 'pH 5 enzyme' fraction. Aging of this fraction affects the interaction of RNA with ribosomes with a consequent decrease in the stimulation of amino acid incorporation into protein, as reported briefly in a preliminary communication (Arnstein & Nair, 1964).

EXPERIMENTAL

Materials

Buffers. The following buffered media were used: medium A, 0·25M-sucrose–50mM-tris–HCl buffer (pH 7·5)–25mM-KCl–1mM-$MgCl_2$; medium A_5, as medium A, but with the $MgCl_2$ concentration 5mM; medium D, as medium A, but containing in addition glutathione (10mM) (neutralized to pH 7·5 with KOH) and MgEDTA (1mM).

Chemicals. The following materials were obtained commercially: glutathione and silver barium phosphoenolpyruvate from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); ATP (dipotassium salt) and GTP (sodium salt) from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and Sigma Chemical Co. (St Louis, Mo., U.S.A.) respectively; polyuridylic acid from Miles Chemical Corp. (Elkhart, Ind., U.S.A.); ^{14}C-labelled amino acids from The Radiochemical Centre (Amersham, Bucks.).

Polyadenylic acid was given by Dr R. A. Cox, National Institute for Medical Research.

The solution of potassium phosphoenolpyruvate (100μ-moles/ml.), ATP (5μmoles/ml.) and GTP (1·25μmoles/ml.) used in the cell-free system was prepared as described by Arnstein *et al.* (1964).

Estimation of protein and RNA. The protein content of the pH 5 enzyme fraction and of cell sap, and the amount of ribosomes in medium A, were estimated by u.v. spectrophotometry at 260mμ and 280mμ, as described by Arnstein *et al.* (1964).

RNA preparations. High-molecular-weight RNA was isolated from polysomes and ribosomes by extraction with guanidinium chloride (Cox & Arnstein, 1963). Except for preparations used in the experiments reported in Tables 5–8, the RNA was further purified by dissolving it in 6M-guanidinium chloride–10mM-tris–HCl buffer, pH 7·3, at 20° and reprecipitation with ethanol (2 vol.) at −18°. All RNA preparations were converted into the potassium salt by precipitation from solutions in 2% (w/v) potassium acetate with ethanol (2 vol.) at −18°.

s-RNA was obtained from ribosome-free cell sap of rabbit reticulocytes by extraction with phenol or from rabbit liver by the method of Cantoni, Gelboin, Luborsky, Richards & Singer (1962). [^{14}C]Phenylalanyl-s-RNA was prepared by charging the s-RNA (after stripping off amino acids) with L-[U-^{14}C]phenylalanine (specific radioactivity 96μc/μmole) and the remaining 19 unlabelled L-amino acids (Ehrenstein & Lipmann, 1961). The 'pH 5 enzymes' from rabbit reticulocytes were used for loading the s-RNA. A sample of the

* Present address: Department of Medicine, University of Chicago, Chicago 37, Ill., U.S.A.

† Abbreviations: m-RNA, messenger RNA; s-RNA, transfer RNA.

labelled s-RNA was dried on lens paper in a 2·5 cm.^{2} polythene planchet and counted in a Dekatron scaler (counting efficiency 7%). A 1 mg. portion of s-RNA gave 3780 counts/min., corresponding to approx. 23·5 mμc.

Isolation of subcellular fractions

Reticulocytes were obtained from anaemic rabbits (sandy-lop or Dutch strain), washed and lysed, and the lysate was fractionated into pH 5 enzymes, and 'heavy' (polysome-rich) and 'light' (ribosome-rich) ribonucleoprotein fractions (H- and L-ribosomes respectively), by differential centrifugation at 105000*g* for 60 min., as described by Arnstein *et al.* (1964). In most experiments the pH 5 enzymes were dissolved in medium D, but in some (Tables 6–8) medium A_5 was used. Unless otherwise stated ribosomes were washed by resuspension in medium A and centrifuging at 105000*g* for 60 min.

Aging of pH 5 *enzymes.* The pH 5 enzymes were dissolved in medium A_5, giving a protein concentration of 9·8 mg./ml. One half of the solution was stored at 4°, usually for about 20 hr. (aged enzymes); the other half was frozen immediately after isolation by using a mixture of solid CO_2 and acetone (frozen enzyme).

Amino acid incorporation into protein by subcellular fractions

Standard cell-free system. Unless otherwise stated, the incubation mixture (0·4–0·6 ml.) for testing the incorporation of labelled amino acids into protein had the following composition: KCl (50 mM), $MgCl_2$ (5 mM), glutathione (10 mM), tris–HCl buffer, pH 7·6 (25 mM), 0·05 ml. of an amino acid mixture containing one ^{14}C-labelled amino acid (in amounts given for each experiment) and 19 unlabelled protein amino acids (0·1 μmole of each), ribosomes (0·25–1 mg.), pH 5 enzymes (1–4 mg.) and an ATP-generating system consisting of sodium phosphoenolpyruvate (5 or 2·5 μmoles), ATP and GTP in the molar proportions 80:4:1) and pyruvate kinase (25–50 μg.). The incubation was carried out in air at 37° for 1 hr.

Messenger-competition experiment. The effect of polyuridylic acid or polyadenylic acid on m-RNA (Table 4) was investigated by mixing once-washed L-ribosomes (0·5 mg. in 0·1 ml. of medium A made 7 mM with respect to $MgCl_2$) with either polyuridylic acid (0·05 mg. in 0·025 ml. of water) or reticulocyte RNA (0·165 mg. in 0·025 ml. of water). As a control, polyuridylic acid (0·05 mg. in 0·025 ml. of water) was mixed with reticulocyte RNA (0·165 mg. in 0·025 ml. of water) in the absence of ribosomes. After 20 min. at 0°, the other components of the cell-free system were added in the following order: (1) polyuridylic acid or polyadenylic acid (0·05 mg. in 0·025 ml. of water) or reticulocyte RNA (0·165 mg. in 0·025 ml. of water) or water (0·025 ml.) or L-ribosomes (0·5 mg. in 0·1 ml. of medium A made 7 mM with respect to $MgCl_2$); (2) pH 5 enzymes in medium D (0·15 ml.) containing pyruvate kinase (0·2 mg./ml.); (3) a mixture (0·2 ml.) containing the usual salts, tris–HCl buffer, glutathione (3·5 μmoles), amino acids (0·05 ml.), phosphoenolpyruvate (2·5 μmoles), ATP (0·125 μmole) and GTP (0·03 μmole).

The final volume was 0·5 ml. and the concentrations of salts and buffer were those given for the standard cell-free system. Incubation was at 37° for 1 hr.

Isolation of labelled protein from incubation mixtures. After incubation, 0·2 ml. of N-NaOH containing 25 μmoles of unlabelled amino acid/ml. was added to the reaction mixture (0·6 ml.), which was then kept for 1 hr. at room temperature and divided into three portions for a comparison of the following procedures, method 1 having been used in previous work.

Method 1. Ribosome-free lysate from reticulocytes (0·4 ml. containing 26 mg. of protein) was added as carrier to 0·6 ml. of the mixture, which was then acidified with 30% (w/v) trichloroacetic acid (0·5 ml.). The precipitated protein was washed as described by Arnstein *et al.* (1964), except that the step involving redissolution in alkali was omitted since treatment with alkali had been carried out initially.

Method 2. To a second portion (0·1 ml.) 10% (w/v) trichloroacetic acid (2 ml.) was added and after standing at 4° overnight the protein was filtered on to a 3 cm.-diam. Oxoid membrane, and washed with cold 5% trichloroacetic acid (2 × 5 ml.), hot 5% trichloroacetic acid (2 × 5 ml.) and cold water (2 × 5 ml.).

Method 3. Another portion (0·1 ml.) was similarly treated with 5% (w/v) trichloroacetic acid containing sodium tungstate (0·25%) (Gardner *et al.* 1962). The protein was collected and washed as described above (method 2).

Radioactivity measurements. An automatic ultra-thin end-window low-background counter (model C–110B; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) was used for assay of radioactive proteins. The counting efficiency for ^{14}C was 15% and samples were counted for at least 1000 counts. The background was approx. 2 counts/min.

Amino acid pool. The amino acid pool of the cell-free system (x) was calculated from the radioactivities of protein (R_a and R_b) when different amounts (a and b) of a labelled amino acid containing the same radioactivity (R) were used in otherwise identical incubation mixtures, by using the equation:

$$x = \frac{bR_b - aR_a}{R_a - R_b}$$

The equation is derived as follows, m being the amount of amino acid converted into protein:

$$R_a = \frac{mR}{a+x} \quad \text{and} \quad R_b = \frac{mR}{b+x}$$

$$\therefore \frac{R_a}{R_b} = \frac{b+x}{a+x} \quad \text{and} \quad x = \frac{bR_b - aR_a}{R_a - R_b}$$

In this calculation the assumption is made that the concentration of precursor amino acid has no effect on protein synthesis. Allen & Schweet (1962) have shown that, when dialysed enzymes were used, leucine at 35, 70 or 140 mμmoles/ml. was incorporated into protein independently of the concentration. This range was, however, somewhat higher than the amounts used in the present experiments.

Analysis of incubation mixtures by density-gradient centrifugation

Cell-free incubation system. The incubation was carried out under standard conditions, but on a larger scale.

In the experiment reported in Fig. 6 each tube contained (in 1·8 ml.): 90 μmoles of KCl, 9·0 μmoles of $MgCl_2$, 45 μmoles of tris–HCl buffer, pH 7·6, 18 μmoles of glutathione, 50 μg. of pyruvate kinase, 32 μmoles of phosphoenolpyruvate, 1·6 μmoles of ATP, 0·4 μmole of GTP, 12·8 mg. of pH 5

enzymes, 3·6mg. of L-ribosomes, and 1μc of L-[^{14}C]valine (specific radioactivity 161μc/μmole) in a mixture containing 0·2μmole of each of the other 19 protein amino acids. High-molecular-weight RNA (obtained from ribosomes or polysomes) was added at a concentration of 210μg. of RNA/mg. of ribosomes.

In the experiment with labelled s-RNA (Fig. 7) each tube contained (in 1·5ml.): 75μmoles of KCl, 7·5μmoles of $MgCl_2$, 37·5μmoles of tris–HCl buffer, pH7·6, 35μg. of pyruvate kinase, 40μmoles of phosphoenolpyruvate, 2μmoles of ATP, 0·5μmole of GTP, 0·1ml. of a mixture containing 0·2μmole of each of 20 unlabelled protein amino acids, 15·0μmoles of glutathione, 3·0mg. of ribosomes, 3·0mg. of pH5 enzymes that had been stored either frozen at −18° (Fig. 7*a*) or at 4° (Fig. 7*b*) for 20hr., 400μg. of rabbit-liver s-RNA loaded with unlabelled amino acids and L-[^{14}C]phenylalanine (23·5mμc/mg. of s-RNA), and 660μg. of high-molecular-weight reticulocyte RNA.

In both experiments the tubes were incubated at 37° for 10min. with shaking. The reaction was terminated by chilling the tubes rapidly in ice and the mixtures were then layered on the gradients as described below.

Sucrose density gradients. Linear sucrose density gradients were obtained by carefully layering 8ml. each of 30%, 25%, 20% and 15% (w/v) sucrose solutions in 10mM-tris–HCl buffer (pH7·6)–10mM-KCl–1·5mM-$MgCl_2$ (Charlwood, 1963).

A simple syringe assembly was used for layering the solutions in Spinco centrifuge tubes. The gradients were left at 4° for 18hr. and then cooled to 0° before use. The incubated material (cooled to 0° by chilling in ice) was layered on the top of each of the gradients and centrifuged in the no. 30 angle-head rotor of a Spinco model L preparative ultracentrifuge at 25000rev./min. (54450$g_{av.}$) for 110–120min. at 0°. Immediately after centrifugation 1·5ml. fractions (Fig. 6) or 1·6ml. fractions (Figs. 7–9) were collected by using a semi-automatic syringe device for emptying the tube from the bottom upwards. Samples (0·2ml.) were diluted with 2·5ml. of distilled water for extinction readings at 260mμ in a Hilger Uvispek spectrophotometer.

Counting of samples from density gradients. A sample (0·75ml. in Fig. 6 and 1·0ml. in Figs. 7–9) was taken from each fraction and dried on previously numbered Whatman 3MM paper strips (3·5in. × 1in.). The papers were mounted in serial order on straight pins to prevent them from adhering to each other. When dry the strips of paper were immersed in ice-cold 10% (w/v) trichloroacetic acid containing 0·1% of the appropriate unlabelled amino acid as diluent. After standing for several hours the trichloroacetic acid solution (approx. 10ml./strip of paper) was decanted and the strips were washed with the same volume of 5% (w/v) trichloroacetic acid for 15min. After decanting the trichloroacetic acid the strips were immersed in 5% trichloroacetic acid (containing 0·1% of unlabelled amino acid) at 90° for 30min. to hydrolyse aminoacyl-s-RNA. The paper strips were washed once again in 5% trichloroacetic acid for 15min. and then treated with ether–ethanol (1:1, v/v) at room temperature for 1hr. to remove the trichloroacetic acid. After a second treatment with ether–ethanol (2:1, v/v) for 15min. the paper strips were dried with a hair-dryer in a stream of warm air. The adequacy of this method has been shown by Mans & Novelli (1960).

The paper strips were then placed in a 20ml. glass vial (low-potassium glass) and covered with 15ml. of a scintillation mixture consisting of 0·5% (w/v) 2,5-diphenyloxazole and 0·05% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene in toluene and counted with 50% efficiency in a Tri-Carb scintillation counter (model 3003; Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.). The gate settings of the counter were 50–1000 and the gain was 12·5%. The background was 30counts/min.

The radioactivity of protein in each gradient fraction is usually given as counts/min. The results of the experiment described in Fig. 8, however, are expressed as specific radioactivity (counts/min./mg. of ribosomes in each fraction) because of the unequal loading of the gradients.

Table 1. *Comparison of different methods for measuring the incorporation of radioactive amino acid into protein*

Standard incubation mixtures (volume 0·6ml.) were used with 1mg. of ribosomes, 3·25mg. of pH5 enzymes and L-[^{14}C]leucine (3·1μmoles, 0·5μc). After incubation for 1hr. at 37°, protein was isolated by the three procedures described in the Experimental section.

	Incorporation of L-[^{14}C]leucine into protein (mμc)		
Ribosome fraction	Method 1	Method 2	Method 3
Polysome-rich (H-ribosomes)	73·8	97·6	93·5
Polysome-rich (H-ribosomes)	74·9	101	99·9
Ribosome-rich (L-ribosomes)	6·0	8·4	7·6
Ribosome-rich (L-ribosomes)	5·7	7·7	7·2

RESULTS

A comparison of different methods for isolating and counting the protein from the incubation mixture (Table 1) shows that precipitation with 5% trichloroacetic acid is as effective as that with the trichloroacetic acid–tungstate reagent, which has been reported to give a more complete recovery of labelled polylysine in the cell-free incorporation system from *E. coli* (Gardner *et al.* 1962). The method involving addition of cell sap as carrier, which was used by Arnstein *et al.* (1964), appears to give a lower recovery of radioactivity in the protein, but this result may be due to low values being obtained in the estimation of protein in the cell sap by u.v. spectrophotometry. It is concluded that filtration of protein on to membranes after precipitation with trichloroacetic acid is satisfactory and convenient. This procedure was therefore adopted for the work reported in the present paper. Usually N-sodium hydroxide (0·2ml.) was added to each tube after incubation, and the total protein was precipitated with trichloroacetic acid and used for radioactivity assays.

Estimation of the free amino acid pool present in the cell-free system (Table 2) gave values of approx. 3mμmoles for phenylalanine and 7mμ-

Table 2. *Estimation of the free amino acid pool of the cell-free incubation mixture*

Standard experimental conditions were used for the incubation with the following modifications in addition to those shown in the Table. Expt. 1: the volume was 0·9 ml.; phosphoenolpyruvate, ATP, GTP and pyruvate kinase were present in double the usual amounts; 3·6 mg. of pH 5 enzymes was used. Expt. 2: the volume was 1·0 ml.; 10 μmoles of $MgCl_2$, 13 μmoles of tris, 5 μmoles of glutathione, 7 μmoles of phosphoenolpyruvate, 0·35 μmole of ATP, 0·08 μmole of GTP, 100 μg. of pyruvate kinase and 2 mg. of pH 5 enzymes were used. Expt. 3: the conditions were as described in Table 1.

Expt. no.	Labelled amino acid added	Specific radioactivity (μc/μmole)	(μc)	(mμmoles)	Amount of ribosomes (mg./tube)	Incorporation of amino acid into protein (mμc/mg. of ribosomes)	Amino acid pool (mμmoles)
1	L-[^{14}C]Phenylalanine	10	1	100	1·4	1·95	2·7
		80	1	12·5	1·4	12·5	
2	L-[^{14}C]Phenylalanine	7·3	0·17	23·4	1·7	2·1	2·8
		48·8	0·17	3·5	1·7	8·6	
3	L-[^{14}C]Leucine	10·2	0·5	49	1·0	13·5	7·0
		161	0·5	3·1	1·0	75·3	

Table 3. *Inhibition of protein synthesis in the cell-free system by excess of phosphoenolpyruvate*

Each tube contained 0·24 mg. of a polysome-rich fraction, 1·0 mg. of pH 5 enzymes and L-[^{14}C]leucine (specific radioactivity 30 μc/μmole, 0·1 μc/tube) in 0·4 ml. The other components of the cell-free system were standard except that the amounts of phosphoenolpyruvate, ATP and GTP were varied, the proportions of the three components (80:4:1) being kept constant.

Addition to standard cell-free system	Amount of phosphoenolpyruvate (μmoles)	Incorporation of L-[^{14}C]leucine into protein ($\mu\mu$moles/mg. of ribosomes)
None	1·0	675
None	2·5	670
None	5·0	595
None	10·0	57
$MgCl_2$ (10 μmoles)	10·0	260

moles for leucine. These amounts are small in relation to the labelled precursor when amino acids of low specific radioactivity are used and the quantities added are correspondingly large, but there is considerable dilution when small amounts of high-specific-activity amino acids are used as precursor. To calculate the amount of amino acids incorporated into protein it would be necessary to correct for this pool of amino acids, but since its size may be expected to vary somewhat from experiment to experiment no correction has been applied in the present work. The values reported therefore represent the uncorrected minimum incorporation of the relevant amino acid into protein.

The optimum amount of phosphoenolpyruvate is less than 6 μmoles/ml. (Table 3). At 12·5 μmoles/

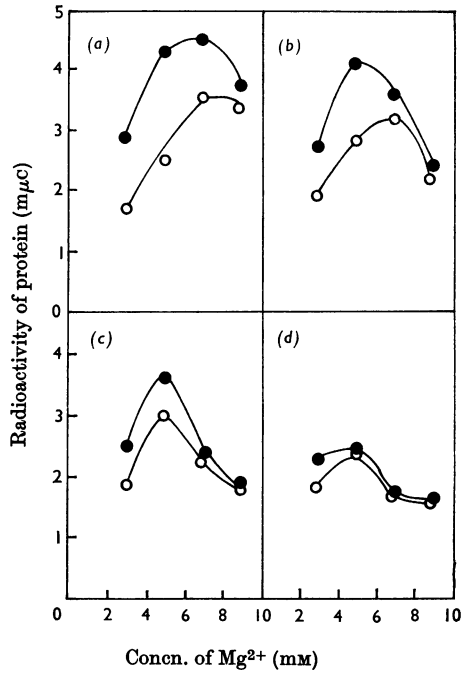

Fig. 1. Stimulation of protein biosynthesis by rabbit reticulocyte RNA at different concentrations of K^+ and Mg^{2+}. The cell-free system consisted of the standard incubation mixture (0·4 ml.) containing 0·73 mg. of L-ribosomes, 1·4 mg. of pH 5 enzymes, L-[^{14}C]leucine (0·125 μc, specific radioactivity 30 μc/μmole), phosphoenolpyruvate (2·5 μmoles), ATP (0·125 μmole), GTP (0·03 μmole), pyruvate kinase (50 μg.) and various concentrations of KCl [(*a*) 30 mM; (*b*) 50 mM; (*c*) 70 mM; (*d*) 90 mM] and $MgCl_2$. ○, L-ribosomes; ●, L-ribosomes with rabbit reticulocyte RNA (preparation no. 176, 67 μg.).

ml. there is some decrease in the amino acid incorporation and at 25 μmoles/ml. the system is almost inactive. The addition of extra Mg^{2+} partially restores the original activity.

For determining the optimum ionic conditions for stimulation of the cell-free system by RNA (Fig. 1) 100 μg. of RNA/mg. of ribosomes was used since this amount does not saturate the cell-free system (Fig. 2). When due allowance is made for the higher specific radioactivity of the precursor leucine used for the experiment in Fig. 2, the response of the cell-free system to RNA is found to be similar in both experiments, although the endogenous incorporation is somewhat lower in Fig. 2. The response of the amino acid incorporation to RNA appeared to be greatest when the Mg^{2+} concentration was 5mM and the K^+ concentration was between 30 and 50mM. At high Mg^{2+} or K^+ concentrations the endogenous incorporation was diminished and the response to RNA almost completely abolished. Most experiments were therefore carried out at 5mM-Mg^{2+} and 50mM-K^+.

The stimulation of amino acid incorporation was found to have a pH optimum of 7·5–7·6 (Fig. 3), which is a little lower than that required for optimum endogenous incorporation by either H- or L-ribosomes (pH 7·8). For this reason, the cell-free system was usually incubated at pH 7·6.

Fig. 4 gives a comparison of the effect of different amounts of high-molecular-weight RNA on the incorporation of [^{14}C]leucine into total trichloroacetic acid-insoluble and into alkali-stable radioactive material. On the assumption that the former represents incorporation into protein plus s-RNA and the latter into protein, it is evident that addition of high-molecular-weight RNA stimulated only protein synthesis but had no effect on the loading of s-RNA, since the two curves are superimposable.

A study of the competition between RNA and synthetic polynucleotides (Table 4) shows that polyuridylic acid decreased the stimulation of the incorporation of leucine into protein due to m-RNA to 24% of the control value. Polyadenylic acid also decreased the effect of m-RNA, but to a smaller degree (50% of the control value with leucine, 74% with phenylalanine).

The possibility that preincubation of ribosomes in the complete cell-free system under relatively mild conditions might remove m-RNA and yield a preparation having a low-background incorporation but capable of responding to the addition of m-RNA was examined by testing the response of polysomes to polyuridylic acid after preincubation. It was found, however, that preincubation decreased

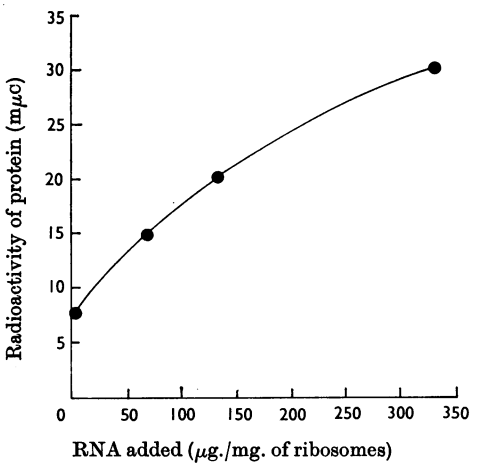

Fig. 2. Response of cell-free system to reticulocyte RNA. The same RNA preparation as in Fig. 1 was used in the standard incubation mixture containing 1mg. of L-ribosomes, 3·25mg. of pH5 enzymes, L-[^{14}C]leucine (0·5 μc, specific radioactivity 161 μc/μmole), phosphoenolpyruvate (5 μmoles), ATP (0·25 μmole), GTP (0·06 μmole) and pyruvate kinase (50 μg.) in 0·6ml.

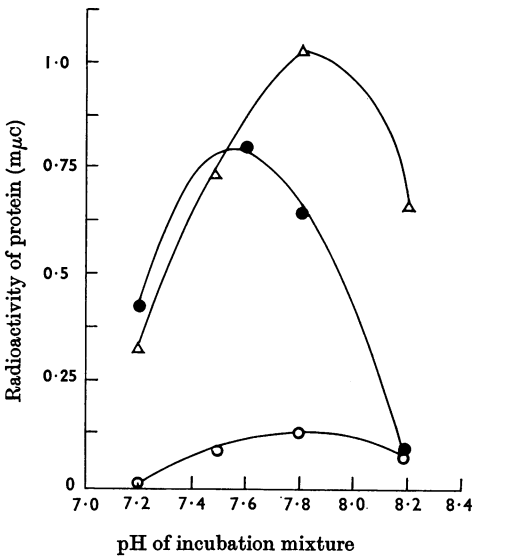

Fig. 3. Effect of pH on protein synthesis and its stimulation by RNA. The standard incubation mixture contained 0·25mg. of H- or L-ribosomes, 1·15mg. of pH5 enzymes, L-[^{14}C]phenylalanine (0·25 μc, specific radioactivity 2·5 μc/μmole), phosphoenolpyruvate (2·5 μmoles), ATP (0·125 μmole), GTP (0·03 μmole) and pyruvate kinase (33 μg.) in 0·4ml. The pH was adjusted by adding N-acetic acid or N-KOH in amounts that were predetermined with portions of the incubation mixture to give the stated pH at room temperature. ○, L-ribosomes; ●, L-ribosomes+reticulocyte RNA (100 μg.); △, H-ribosomes.

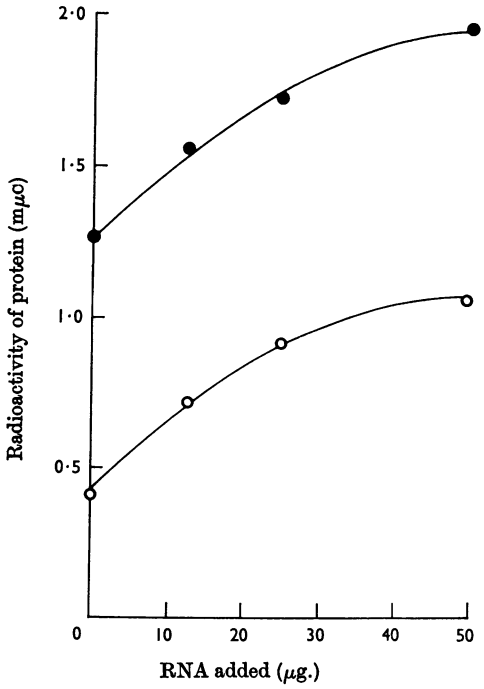

Fig. 4. Effect of RNA on the incorporation into alkali-labile and alkali-stable products. Each tube contained L-ribosomes (0·25 mg.), pH 5 enzymes (1·3 mg.) and L-[^{14}C]-leucine (0·125 μc, specific activity 30 μc/mole) in the standard incubation mixture (0·5 ml.). Protein was precipitated with trichloroacetic acid by the usual method, but treatment with N-NaOH after incubation was omitted in the series of tubes used for measuring incorporation into total (alkali-stable plus alkali-labile) trichloroacetic acid-insoluble products. ●, Radioactivity of trichloroacetic acid-insoluble products before hydrolysis with NaOH; ○, radioactivity of protein after treatment with NaOH.

Table 4. *Competition between homopolynucleotides and m-RNA*

Each tube contained L-ribosomes (0·5 mg.), pH 5 enzymes (5 mg.) and either L-[^{14}C]leucine (0·18 μC, specific radioactivity 7 μc/μmole) or L-[^{14}C]phenylalanine (0·125 μc, specific radioactivity 5 μc/mole) in the standard cell-free system. The final volume was 0·5 ml. Reticulocyte RNA (165 μg.) and/or homopolynucleotides (50 μg.) were interacted at 0° with ribosomes (in medium A made 7 mM with respect to Mg^{2+}) before adding the other components of the incubation mixture, as described in the Experimental section. Incubation was at 37° for 1 hr.

	Incorporation of amino acid into protein (μμmoles)	
Polynucleotides added	Leucine	Phenyl-alanine
None	62	99
Polyuridylic acid	87	2680
RNA	178	186
RNA and polyuridylic acid*	94	2570
Polyuridylic acid and RNA*	91	2350
RNA and polyuridylic acid†	93	2560
RNA and polyadenylic acid	121	163

* Added 20 min. after mixing ribosomes and first polynucleotide at 0°.

† Both polynucleotides were kept at 0° for 20 min. after mixing before the addition of ribosomes and other constituents of the cell-free incubation mixture.

both the endogenous incorporation and the response to polynucleotide messenger to a similar extent (Fig. 5).

Storage of ribosomes either frozen or at 4° for 20 hr. had no effect on their capacity to incorporate amino acids into protein (Table 5), but the activity of the pH 5 enzyme fraction in the cell-free system decreased somewhat when the enzymes were stored at 4° for 20 hr. The most striking result of storage of the pH 5 enzymes at 4°, however, is the almost complete inactivation of a factor required for the stimulation of the cell-free system by exogenous m-RNA (Table 5, column 6). A similar result was obtained when polyuridylic acid was substituted for m-RNA (Table 6). However, the stimulation of H-ribosomes by polyuridylic acid was affected much less by storage of the enzyme at 4° than that of the L-ribosome fraction, suggesting that this factor may be present not only in the pH 5 enzymes but also in the H-ribosome fraction or, alternatively, that H-ribosomes do not require the factor. The experiment reported in Table 7 shows that cell sap is particularly rich in this or a similar factor which is required for the response of the cell-free system to m-RNA. In this case, however, the factor appeared to be comparatively stable at 4°.

To locate the step for which the factor is required, the effect of storing the pH 5 enzymes at 4° was investigated with aminoacyl-s-RNA as the protein precursor instead of the free amino acid (Table 8). In two different experiments, aging of the enzyme preparation resulted in a decrease in the stimulation of protein synthesis from phenylalanyl-s-RNA by high-molecular-weight RNA. In both cases a greater effect was obtained with the cell-free system containing L-ribosomes than with those containing H-ribosomes.

The formation of polysomes *in vitro* was studied by using a cell-free system containing 80 s ribosomes and a preparation of high-molecular-weight RNA at a concentration of 200–400 μg./mg. of ribosomes. Higher concentrations of RNA produced non-specific aggregates of the 80 s particles.

Kinetic studies based on sucrose-density-gradient analysis of incubation mixtures similar to those shown in Figs. 6 and 7 revealed that 10 min. of

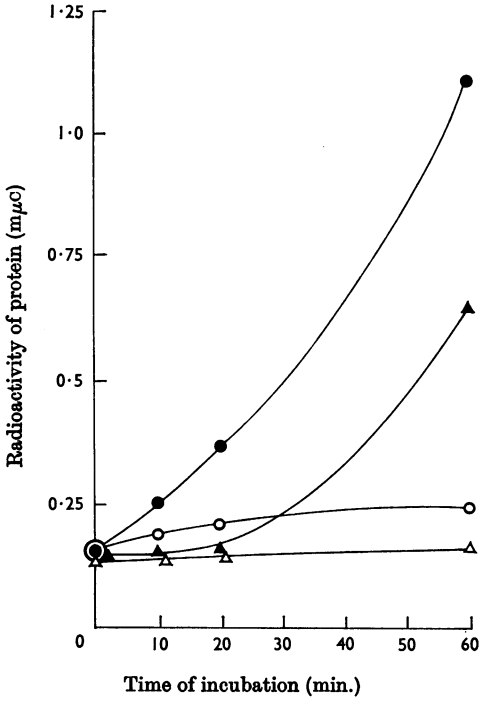

Fig. 5. Response to preincubated cell-free system to polyuridylic acid. Each tube contained H-ribosomes (0·25 mg.) and pH 5 enzymes (1·9 mg.) in the complete standard incubation mixture (0·4 ml.) with unlabelled amino acids except phenylalanine (0·02 μmole of each). After incubation at 37° for 0 or 30 min., the tubes were cooled in ice. Polyuridylic acid (25 μg.), amino acids with L-[^{14}C]phenylalanine (7·5 mμc, specific radioactivity 5 μc/μmole) and additional $MgCl_2$ to make the final concentration 8 mM were added, polyuridylic acid being omitted from control tubes. Incubation was then continued at 24°. ○, No preincubation, no polyuridylic acid added; ●, no preincubation, polyuridylic acid added; △, 30 min. preincubation, no polyuridylic acid added; ▲, 30 min. preincubation, polyuridylic acid added.

incubation at 37° was the most suitable time period for demonstrating polysome formation *in vitro*. Incubation for longer periods produced a sucrose-density-gradient profile in which the label appeared chiefly under the monomer peak. In previous kinetic studies on the RNA effect (Arnstein *et al.* 1964) a similar time was found for optimum incorporation of labelled leucine into the ribosome-bound nascent proteins. The formation of new polysomes by the addition of RNA from either 80 s ribosomes or polysomes to L-ribosomes is shown in Fig. 6. There was a 2–3-fold increase in the radioactivity over the polysome region (fractions 4–10) and a similar increase in the activity of the released protein. In this experiment, RNA from polysomes (Fig. 6*b*) appeared to be no more active

Table 5. *Effect of storage of enzymes and ribosomes on the stimulation of amino acid incorporation by RNA*

Each tube contained 0·69 mg. of L-ribosomes or 0·74 mg. of H-ribosomes, 2·25 mg. of pH 5 enzymes, 2·5 μmoles of glutathione and L-[^{14}C]phenylalanine (0·5 μc, 7·6 mμmoles) in 0·05 ml. of the usual amino acid mixture. The final volume was 0·4 ml. Phosphoenolpyruvate (2·5 μmoles) and pyruvate kinase (50 μg.) were used in the ATP-generating system. Ribosomes in medium A and pH 5 enzymes in medium A_5 were stored for 18 hr. either at −18° after rapid freezing, or at 4°. The cell-free system was also tested with the same subcellular fractions before storage (Expt. 1) but with a different batch of reticulocyte RNA.

Expt. no.	Conditions of storage of subcellular fractions: Ribosomes	Enzymes	Incorporation of L-[^{14}C]-phenylalanine into protein (μμmoles/mg. of ribosomes): H-ribosomes	L-ribosomes	L-ribosomes + RNA*
1	Fresh	Fresh	392	26	394
2	Frozen	Frozen	415	56	223
	Frozen	4°	262	22	28
	4°	Frozen	445	60	271
	4°	4°	229	21	25

* Reticulocyte RNA preparation no. 80 (320 μg./mg. of ribosomes) was used in Expt. 1 and preparation no. 115 (200 μg./g. of ribosomes) in Expt. 2.

than RNA isolated from 80 s ribosomes (Fig. 6*c*). The addition of RNA to the cell-free system did not, however, give any appreciable increase in the extinction in the polysomal region.

The results of experiments on the effect of aging of the pH 5 enzymes are shown in Figs. 7–9. When unwashed L-ribosomes were used, there was only a small difference between the radioactivity incorporated from labelled phenylalanyl-s-RNA into ribosome-bound nascent protein in the presence of frozen or aged enzyme (Figs. 7*a* and 7*b* respectively), but the release of soluble radioactive protein from ribosomes was diminished by aging. Since unwashed ribosomes contain sufficient enzymes for almost optimum incorporation of amino acids into protein (Arnstein *et al.* 1964), it is not surprising that in this case aging of the pH 5 enzyme fraction made little difference to the response of the cell-free system to RNA. Aging of the enzymes also had no effect on the incorporation of amino acids into protein when H-ribosomes were used (Fig. 8). In contrast, when L-ribosomes that had been washed by resuspension in medium A were used with labelled phenylalanyl-s-RNA as precursor, replacement of fresh by aged enzymes resulted in a substantial decrease in the radioactivity both in the ribosomal region and in the released protein (Figs. 9*a* and 9*b* respectively).

Table 6. *Response of cell-free system to polyuridylic acid and RNA after aging of* pH 5 *enzymes*

Each tube contained 1·36 mg. of ribosomes, 3·6 mg. of pH 5 enzymes, L-[^{14}C]phenylalanine (0·5 μc, specific radioactivity 66 μc/μmole, except in tubes with polyuridylic acid when phenylalanine of specific activity 9·8 μc/μmole was used) in 0·1 ml. of the usual amino acid mixture, 25 μmoles of tris–HCl buffer, pH 7·6, 5 μmoles of glutathione, 10 μmoles of phosphoenolpyruvate and 100 μg. of pyruvate kinase. The other constituents were as given for the standard incubation mixture. The final volume was 1 ml.

Ribosome fraction	Conditions of storage of pH 5 enzymes	Addition to cell-free system	Incorporation of phenylalanine ($\mu\mu$moles/mg. of ribosomes)	With enzyme stored at 4° (as % with frozen enzyme)
H	Frozen	None	195	87%
H	4°	None	170	
H	Frozen	Polyuridylic acid (50 μg.)	810	79%
H	4°	Polyuridylic acid (50 μg.)	640	
L	Frozen	None	27	74%
L	4°	None	20	
L	Frozen	Polyuridylic acid (50 μg.)	1310	40%
L	4°	Polyuridylic acid (50 μg.)	519	
L	Frozen	Reticulocyte RNA (200 μg.)	121	30%
L	4°	Reticulocyte RNA (200 μg.)	36	

Table 7. *Effect of cell sap on the response of the cell-free system to RNA*

Experimental conditions were the same as described in Table 6. L-ribosomes were used throughout and the radioactive amino acid was L-[^{14}C]phenylalanine (specific radioactivity 66 μc/μmole). Cell sap was obtained by neutralizing the supernatant, after removal of ribosomes and pH 5 enzymes, with N-KOH to pH 7·5, and 0·2 ml. (13·2 mg. of protein) was added to each tube where indicated. The reticulocyte RNA preparation was the same as in Table 6, 200 μg. being added.

Conditions of storage		Incorporation of phenylalanine ($\mu\mu$moles/mg. of ribosomes)	
pH 5 enzymes	Cell sap	Without RNA	With RNA
Frozen	None added	27	120
4°	None added	20	36
4°*	None added	17	46
4°	Frozen	53	343
4°	4°	49	372

* In the presence of 3 μmoles of glutathione/ml.

DISCUSSION

The present experiments extend previous observations (Arnstein *et al.* 1964) on the effect of high-molecular-weight RNA on the incorporation of amino acids into protein by subcellular fractions from rabbit reticulocytes, and provide additional evidence for the view that the observed stimulation is due to m-RNA.

It has now been found that the optimum stimulation of the cell-free system by RNA takes place over a relatively narrow pH range (approx. 7·4–7.6) and is dependent also on the concentrations of K^+ and Mg^{2+}, the greatest effect being obtained at 30–50 mM-K^+ and approx. 5 mM-Mg^{2+}. This optimum Mg^{2+} concentration is somewhat lower than that required for maximal response to polyuridylic acid, which is approx. 8 mM (Arnstein *et al.* 1962), and considerably lower than the concentration used in the work with cell-free extracts from bacteria (see, e.g., Nirenberg & Matthaei, 1961; Ofengand & Haselkorn, 1961; Martin & Ames, 1962). Excess of phosphoenolpyruvate appears to be deleterious, probably because the effective Mg^{2+} concentration is decreased. Another difference between the bacterial and reticulocyte incorporation systems is that preincubation of the reticulocyte cell-free system decreases both the endogenous incorporation and the response to messenger.

Stimulation of amino acid incorporation by reticulocyte RNA takes place into an alkali-stable product, presumably protein, whereas there is no increase in the alkali-labile fraction (aminoacyl-s-RNA). These results show that the reticulocyte RNA has no amino acid-acceptor activity, and are in agreement with fractionation studies (Cox & Arnstein, 1964) indicating that the stimulatory RNA is of high molecular weight.

The results of the messenger-competition experiment (Table 4) suggest that ribosomes have a much greater affinity for polyuridylic acid than for m-RNA, since the latter decreases the polyuridylic acid-induced incorporation of phenylalanine only very little if at all, whereas polyuridylic acid inhibits the effect of m-RNA almost completely. Polyadenylic acid also decreases the stimulation of the cell-free system by m-RNA; in this connexion, polyadenylic acid has been reported to prevent the reattachment of ribosomes that are released from reticulocyte polysomes when the synthesis of a polypeptide chain is finished (Hardesty, Hutton, Arlinghaus & Schweet, 1963). Homopolynucleo-

Table 8. *Incorporation of phenylalanine from aminoacyl-s-RNA into protein with stored* pH 5 *enzymes, and response to high-molecular-weight RNA*

In Expt. 1, each tube (final volume 0·3 ml.) contained L-[^{14}C]phenylalanyl-s-RNA (1·38 mμc) in 45 μg. of total s-RNA from reticulocytes, ribosomes (0·25 mg.), pH 5 enzymes (2 mg. stored either frozen or at 4° for 18 hr.), glutathione (3 μmoles), KCl (15 μmoles), $MgCl_2$ (1·5 μmoles), tris–HCl buffer, pH 7·8 (7·5 μmoles), phosphoenolpyruvate (2·5 μmoles), ATP (0·125 μmole), GTP (0·03 μmole) and pyruvate kinase (25 μg.). High-molecular-weight RNA from reticulocyte ribosomes (70 μg.; preparation no. 140/1) was added where shown and the cell-free system was incubated for 1 hr. at 37°. The experimental conditions for Expt. 2 were the same, except that 1·8 mμc of L-[^{14}C]phenylalanyl-s-RNA in 72·5 μg. of total s-RNA and 1·4 mg. of pH 5 enzymes were used. N.D., Not determined.

Expt. no.	Cell-free system	Ribosomes	Conditions of storage of pH 5 enzymes	Incorporation of L-[^{14}C]/phenylalanyl-s-RNA into protein ($\mu\mu$c) Without RNA	With RNA	Increase with RNA*
1	Complete	L	Frozen	71·2	456	385
	Complete	L	4°	96·0	294	198 (51%)
	Complete	H	Frozen	390	568	178
	Complete	H	4°	350	472	122 (69%)
	Ribosomes omitted	—	Frozen	4·4	N.D.	
	Ribosomes omitted	—	4°	2·2	N.D.	
	Complete, not incubated	L	Frozen	0	N.D.	
	Complete, not incubated	L	4°	1·9	N.D.	
	Complete, not incubated	H	Frozen	27·4	N.D.	
	Complete, not incubated	H	4°	22·8	N.D.	
2	Complete	L	Frozen	130	218	88
	Complete	L	4°	129	135	6 (7%)
	Complete	H	Frozen	546	628	82
	Complete	H	4°	499	567	68 (83%)
	Enzyme omitted	H	—	163	N.D.	
	Enzyme heated for 10 min. at 55°	H	Frozen	198	N.D.	
	Ribosomes omitted	—	Frozen	130	N.D.	
	ATP and ATP-generating system omitted	H	Frozen	53	N.D.	

* The values in parentheses give incorporation with enzyme stored at 4° as a percentage of that with frozen enzyme.

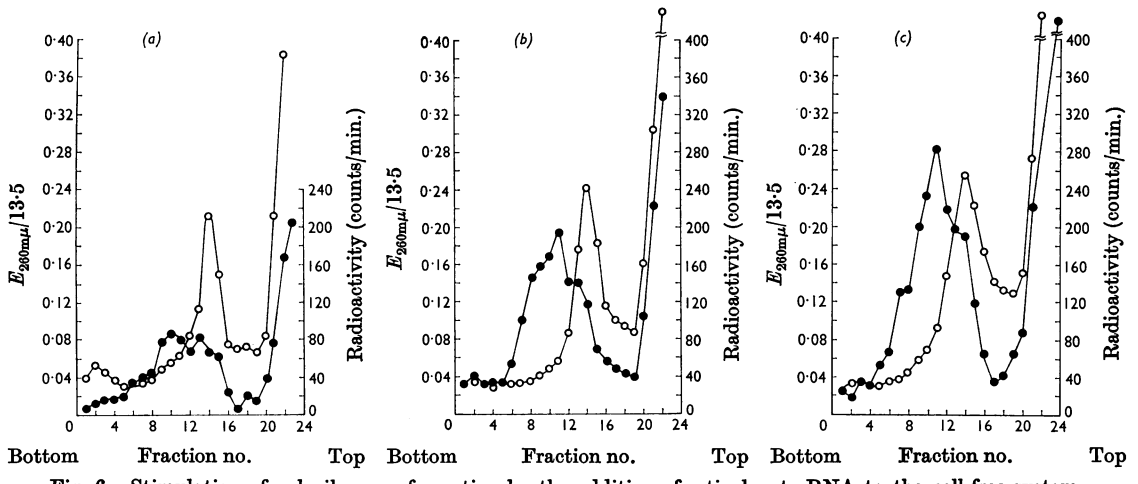

Fig. 6. Stimulation of polyribosome formation by the addition of reticulocyte RNA to the cell-free system. The incubation mixture contained L-ribosomes (*a*) without RNA, (*b*) with RNA from polysomes or (*c*) with RNA from ribosomes in the presence of L-[^{14}C]valine and the usual components of the standard cell-free system (for details see the Experimental section). After incubation at 37° for 10 min. the reaction mixture was cooled in ice and analysed by density-gradient centrifugation (see the text). ○, $E_{260m\mu}$ after 13·5-fold dilution of each fraction; ●, radioactivity (counts/min./fraction).

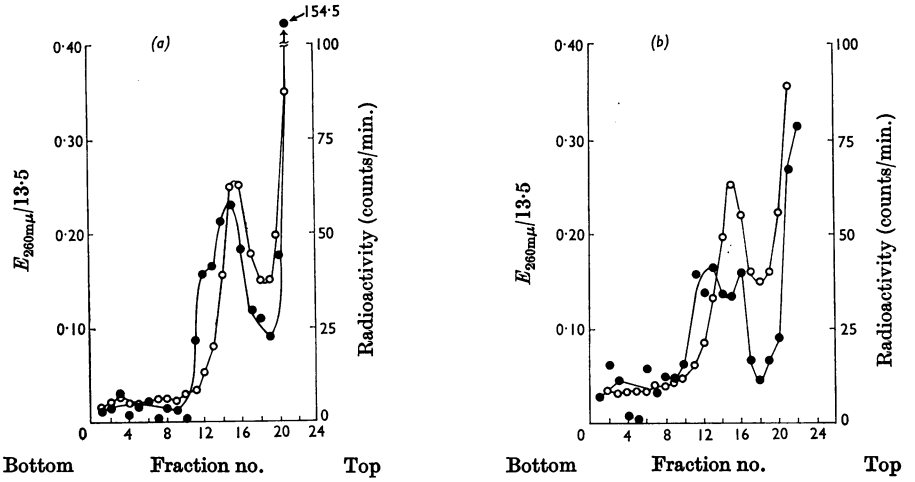

Fig. 7. Effect of aging of enzymes on the incorporation of L-[^{14}C]phenylalanyl-s-RNA into protein. The cell-free system contained pH5 enzymes that had been stored for 20hr. either at 4° (*b*) or frozen at −18° (*a*) and unwashed L-ribosomes, together with high-molecular-weight RNA and the remaining standard components (see the Experimental section). After incubation at 37° for 10min. the mixture was cooled in ice and analysed by density-gradient centrifugation (see the text). ○, $E_{260m\mu}$ after 13·5-fold dilution of each fraction; ●, radioactivity (counts/min./fraction).

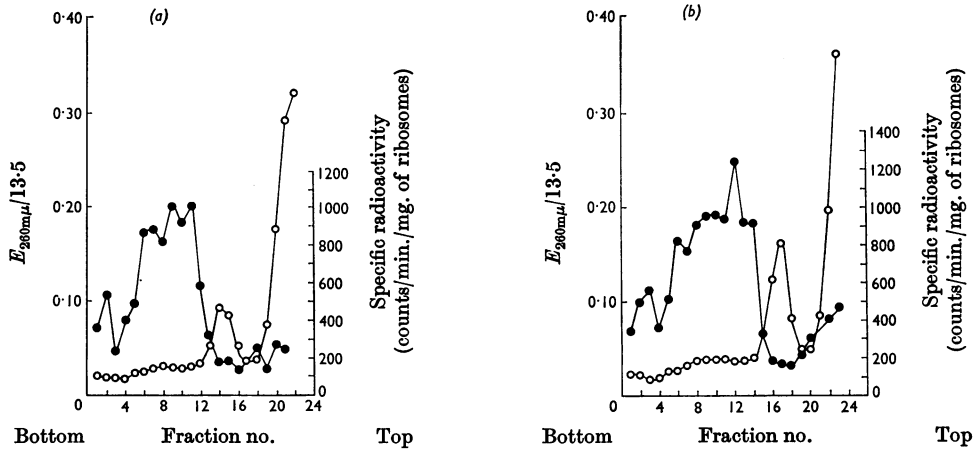

Fig. 8. Sucrose-density-gradient and radioactivity profiles of H-ribosomes incubated with either frozen (*a*) or aged (*b*) pH5 enzymes. The precursor was L-[^{14}C]phenylalanyl-s-RNA and the incubation conditions were identical with those used in Fig. 7, except that the mixture contained H-ribosomes (3mg./tube) and no RNA was added. ○, $E_{260m\mu}$ after 13·5-fold dilution of each fraction; ●, specific radioactivity (counts/min./mg. of ribosomes).

tides have also been reported to inhibit the effect of bacteriophage RNA on amino acid incorporation by a cell-free system from *E. coli* (Möller & Ehrenstein, 1963).

The highest polyuridylic acid-induced incorporation of phenylalanine (Table 4) was approx. 5mμmoles/mg. of ribosomes, which is similar to that obtained by other workers with reticulocyte ribosomes (Arlinghaus & Schweet, 1962). In this experiment, reticulocyte RNA increased the incorporation of phenylalanine by 0·174mμmole/mg. of ribosomes, which corresponds to a total amino acid

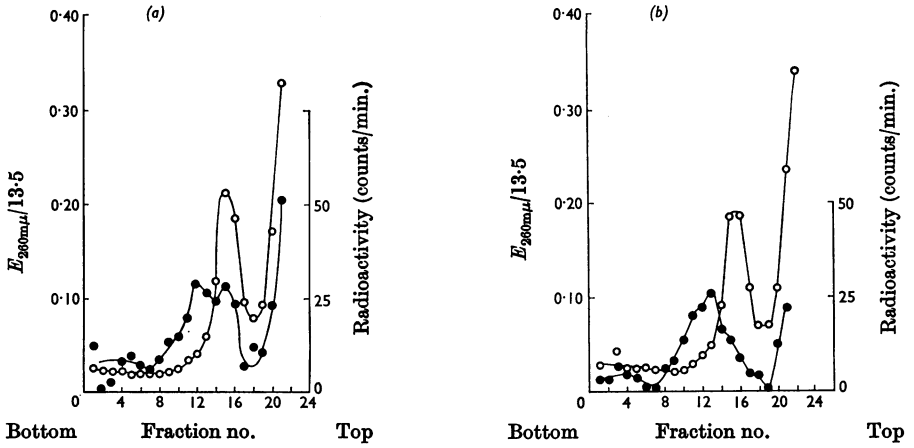

Fig. 9. Effect of aging of enzymes on the incorporation of phenylalanyl-s-RNA into protein by L-ribosomes: sucrose-density-gradient profiles of incubation mixture containing either fresh (*a*) or aged (*b*) enzymes under conditions identical with those given for Fig. 7, except that no high-molecular-weight RNA was added and the ribosomes were washed once by resuspension in medium A. ○, $E_{260m\mu}$ after 13·5-fold dilution of each fraction; ●, radioactivity (counts/min./fraction).

incorporation of 3·5 mμmoles/mg. of ribosomes since haemoglobin contains 5% of phenylalanine. The messenger activity of reticulocyte RNA thus appears to be quantitatively similar to that of polyuridylic acid. A further point of similarity between synthetic and natural messengers in the reticulocyte system is the observation (Table 6) that both polyuridylic acid and reticulocyte RNA require a factor present in the pH 5 enzyme fraction for stimulation of the cell-free system.

The stimulation of leucine incorporation by polyuridylic acid is very low compared with that of phenylalanine (approx. 1%) and very much less than that found in the *E. coli* system (Bretscher & Grunberg-Manago, 1962). It has been shown that the effect of polyuridylic acid on the incorporation of leucine is enhanced by high concentrations of Mg^{2+} (Friedman & Weinstein, 1964), and it is likely therefore that our results are explained by the relatively low Mg^{2+} concentrations used.

In the reticulocyte cell-free system stimulation of protein synthesis by RNA involves both the formation of new polysomes (probably only dimers or trimers) and an increase in the synthesis of soluble protein (Fig. 6; see also Arnstein *et al.* 1964). It is known that the attachment of ribosomes to polysomes is both temperature- and energy-dependent (Goodman & Rich, 1963; Hardesty *et al.* 1963), and our experiments on the effect of aging the pH 5 enzyme fraction at 4° indicate that a labile factor or enzyme may also be involved in the attachment of ribosomes to m-RNA. This factor is present also in the cell sap, although in this case it appears to be more stable than in the pH 5 fraction. Since aging of the pH 5 fraction decreases the RNA-induced protein synthesis from aminoacyl-s-RNA as well as from free amino acids, it is clear that the factor is not involved in the early stages of protein synthesis leading to the formation of aminoacyl-s-RNA. It may be required therefore either for the interaction of ribosomes with m-RNA, as mentioned above, or for the initiation of new polypeptide chains. The observation that amino acid incorporation by polysomes is not greatly decreased by aging of the enzyme excludes its direct participation in the synthesis of peptide bonds. According to current ideas, polysomes are complexes of m-RNA and ribosomes to which nascent peptide chains are attached by s-RNA (Warner, Knopf & Rich, 1963; Goodman & Rich, 1963). Since most of the amino acid incorporation by polysomes in the cell-free system consists of the completion of peptide chains that have already been started in the intact cell, either of these postulated functions of the factor would be compatible with our results. A factor with an apparently similar function has been reported to be present in *E. coli* (Zillig, Traub & Palm, 1964).

We thank Mrs B. Higginson for skilled technical assistance.

REFERENCES

Allen, E. H. & Schweet, R. S. (1962). *J. biol. Chem.* **237**, 760.
Arlinghaus, R. & Schweet, R. S. (1962). *Biochem. biophys. Res. Commun.* **9**, 482.
Arnstein, H. R. V., Cox, R. A. & Hunt, J. A. (1962). *Nature, Lond.*, **194**, 1042.

Arnstein, H. R. V., Cox, R. A. & Hunt, J. A. (1964). *Biochem. J.* **92**, 648.
Arnstein, H. R. V. & Nair, K. G. (1964). *Biochem. J.* **91**, 29c.
Bretscher, M. S. & Grunberg-Manago, M. (1962). *Nature, Lond.*, **195**, 283.
Cantoni, G. L., Gelboin, H. V., Luborsky, S. W., Richards, H. H. & Singer, M. F. (1962). *Biochim. biophys. Acta*, **61**, 354.
Charlwood, P. A. (1963). *Analyt. Biochem.* **5**, 226.
Cox, R. A. & Arnstein, H. R. V. (1963). *Biochem. J.* **89**, 574.
Cox, R. A. & Arnstein, H. R. V. (1964). *Biochem. J.* **93**, 33c.
Ehrenstein, G. von & Lipmann, F. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 941.
Friedman, S. M. & Weinstein, I. B. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 988.
Gardner, R. S., Wahba, A. J., Basilio, C., Miller, R. S., Lengyel, P. & Speyer, J. F. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2087.
Goodman, H. M. & Rich, A. (1963). *Nature, Lond.*, **199**, 318.
Hardesty, B., Hutton, J. J., Arlinghaus, R. & Schweet, R. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 1078.
Mans, J. R. & Novelli, G. D. (1960). *Biochem. biophys. Res. Commun.* **5**, 540.
Martin, R. G. & Ames, B. N. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2172.
Möller, W. J. & Ehrenstein, G. von (1963). *Biochem. biophys. Res. Commun.* **11**, 325.
Nirenberg, M. W. & Matthaei, J. H. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1588.
Ofengand, J. & Haselkorn, R. (1961). *Biochem. biophys. Res. Commun.* **6**, 469.
Warner, J. R., Knopf, P. M. & Rich, A. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 122.
Zillig, W., Traub, P. & Palm, P. (1964). *Abstr. 6th int. Congr. Biochem., New York*, vol. 1, p. 34.