The Rate-Limiting Step of Protein Synthesis *in vivo* and *in vitro* and the Distribution of Growing Peptides between the Puromycin-Labile and Puromycin-Non-Labile Sites on Polyribosomes

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1. At 3 min after an intravenous injection of radioactive amino acids into the rat, the bulk of radioactivity associated with liver polyribosomes can be interpreted as growing peptides. 2. In an attempt to identify the rate-limiting step of protein synthesis *in vivo* and *in vitro*, use was made of the action of puromycin at 0°C, in releasing growing peptides only from the donor site, to study the distribution of growing peptides between the donor and acceptor sites. 3. Evidence is presented that all growing peptides in a population of liver polyribosomes labelled *in vivo* are similarly distributed between the donor and acceptor sites, and that the proportion released by puromycin is not an artifact of methodology. 4. The proportion released by puromycin is about 50% for both liver and muscle polyribosomes labelled *in vivo*, suggesting that neither the availability nor binding of aminoacyl-tRNA nor peptide bond synthesis nor translocation can limit the rate of protein synthesis *in vivo*. Attempts to alter this by starvation, hypophysectomy, growth hormone, alloxan, insulin and partial hepatectomy were unsuccessful. 5. Growing peptides on liver polyribosomes labelled in a cell-free system *in vitro* or by incubating hemidiaphragms *in vitro* were largely in the donor site, suggesting that either the availability or binding of aminoacyl-tRNA, or peptide bond synthesis, must be rate limiting *in vitro* and that the rate-limiting step differs from that *in vivo*. 6. Neither *in vivo* nor in the hemidiaphragm system *in vitro* was a correlation found between the proportion of growing peptides in the donor site and changes in the rate of incorporation of radioactivity into protein. This could indicate that the intracellular concentration of amino acids or aminoacyl-tRNA limits the rate of protein synthesis and that the increased incorporation results from a rise to a higher but still suboptimum concentration.

During protein synthesis in animal cells the active complex has been shown to be the polyribosome (Wettstein, Staehelin & Noll, 1963; Warner, Knopf & Rich, 1963; Manner, Kretsinger, Gould & Rich, 1967; Heywood & Rich, 1968; Earl & Morgan, 1968).

Warner & Rich (1964) have demonstrated that each ribosome in polyribosomes engaged in protein synthesis carries one growing peptide. Watson (1963) proposed that during the addition of each amino acid, the growing peptide migrates from one ribosomal site (the donor site) to another (the acceptor site) forming a new peptide bond with the aminoacyl-tRNA bound to this acceptor site. The peptide is then translocated back to the donor site, vacating the acceptor site for the next aminoacyl-tRNA. Traut & Munro (1964) and Cannon (1967) have provided evidence for this scheme and have shown that growing peptides in the donor site can

be released from the ribosome by puromycin at 0°C. When occupying the acceptor site, however, the growing peptides are not released under these conditions.

During protein synthesis each growing peptide will spend a proportion of its time in the puromycin-labile donor site and a proportion in the puromycin-non-labile acceptor site. At any particular time the distribution of growing peptides between the puromycin-labile and -non-labile sites, in a population of polyribosomes, will depend on the availability and binding of aminoacyl-tRNA, and the relative rates of reactions involved in the cyclic process of peptide bond formation.

Evidence is presented in this paper that the proportion of growing peptides released by puromycin is affected neither by the molecular size of peptides nor by the size of the polyribosomes to which they are attached. In the light of this work,

the proportion of growing peptides released by puromycin (or the puromycin lability) was studied in a variety of experimental conditions in an attempt to identify the reaction limiting the rate of protein synthesis *in vivo* and *in vitro*.

EXPERIMENTAL

Materials. Male Wistar rats of the Alderley Park strain were used. Transauricular hypophysectomy by the method of Falconi & Rossi (1964) was done by Dr S. E. Jagers and the animals were used 3 weeks later. The completeness of hypophysectomy was checked by lack of growth during this 3 week period and by anatomical examination after death.

Dr A. L. Walpole carried out partial hepatectomy by the removal of about 60% of the liver (Higgins & Anderson, 1931).

Diabetes was induced in rats, starved overnight, by an intravenous injection of alloxan (60 mg/kg body wt.). Animals were used 72 h later and had starvation blood-glucose concentrations of 300–700 mg/100 ml, by the method of Krebs, Dierks & Gascoyne (1964). Treatment of some alloxan-diabetic rats with a subcutaneous injection of 12 units of soluble insulin 24 h after alloxan and subsequently every 12 h resulted in starvation blood-glucose concentrations of 70–80 mg/100 ml.

Puromycin hydrochloride, bovine growth hormone and insulin were purchased from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

5-(4-Biphenyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) was obtained from CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K. Butyl-PBD (6 g) was dissolved in 1 litre of toluene for the toluene-based scintillation mixture, and 4 g of butyl-PBD and 100 g of scintillation-grade naphthalene (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) were dissolved in 1 litre of dioxan for the dioxan-based scintillation mixture. Degussa Aerosil silica (50 g) was added to 1 litre of the latter for the dioxan-silica scintillation mixture.

U-¹⁴C-labelled amino acid mixture (52 mCi/mg-atom) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin solution was obtained from Armour Pharmaceutical Co. Ltd., Hampton Park, Eastbourne, Sussex, U.K. and used as standard for protein determinations.

Radioactivity was measured with a Packard Tri-Carb model 3320 scintillation spectrometer.

Preparation of polyribosomes labelled *in vivo* and *in vitro*. The U-¹⁴C-labelled amino acid mixture (6 μ Ci in 0.3 ml of 0.9% NaCl) was injected into the lateral tail vein of rats after overnight starvation. At 3 min after injection the rats were killed by cervical dislocation and polyribosomes isolated from the liver or gastrocnemius muscles by the method of Earl & Morgan (1968). Polyribosomes, similarly prepared but without prior injection of radioactive amino acids, were labelled by incubation *in vitro* in a cell-free system for 5 min at 37°C with radioactive amino acids and liver supernatant fraction such that the concentration of polyribosomes limited the rate of incorporation as described by Earl & Morgan (1968). The reaction was stopped by cooling in ice and the mixture was layered on 4 ml of ice-cold 1 M-sucrose containing buffer A [0.2 M-tris-HCl (pH 7.6) at 4°C–0.1 M-KCl–0.04 M-NaCl–0.006 M-

magnesium acetate–0.001 M-EDTA–0.006 M-2-mercaptoethanol] in polyallomer tubes for the 50Ti rotor of the Spinco model L2-65B ultracentrifuge. The tube was filled up with ice-cold buffer A and polyribosomes were re-isolated by centrifuging at 100 000g for 3 h.

Measurement of incorporation of radioactive amino acids into total liver protein *in vivo*. After overnight starvation, rats were given an intravenous injection (2 μ Ci/100 g body wt.) of the U-¹⁴C-labelled amino acid mixture. Then 30 min later the rats were killed by cervical dislocation and a lobe of the liver was removed and homogenized (Earl & Morgan, 1968) in 10 ml of ice-cold 0.9% NaCl. Protein was precipitated with HClO₄ and washed for assay of radioactivity by the method of Munro, Jackson & Korner (1964). The dried protein was finally dissolved in 5 ml of 0.1 M-NaOH and 1 ml was added to 10 ml of the dioxan-silica scintillation mixture. A counting efficiency of about 60% was obtained. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) and the results were expressed as c.p.m./mg of protein.

Determination of puromycin lability. Polyribosomes, labelled *in vivo* or *in vitro* with radioactive amino acids, were resuspended in ice-cold buffer A to give 5 mg of polyribosomes/ml, based on E_{260} 11.2 (Mathias, Williamson, Huxley & Page, 1964) for a solution of 1 mg of polyribosomes/ml. A sample (0.2 ml) was incubated in ice, with and without 0.2 mg of puromycin hydrochloride, in a total volume of 0.4 ml of buffer A for 30 min at 0°C (Cannon, 1967). After incubation, polyribosomes were re-isolated and separated from peptidyl-puromycin exactly as described in the preceding paragraph for the re-isolation of polyribosomes labelled by incubation *in vitro*.

The pellet was resuspended in 0.4 ml of ice-cold buffer A. Samples (0.1 ml) were diluted and polyribosomes estimated from the E_{260} as described before. Further 0.2 ml samples (containing about 0.4 mg of polyribosomes) were prepared for assay of hot-HClO₄-insoluble radioactivity by the filter-paper-disc method of Mans & Novelli (1961). With the toluene-based scintillation mixture the efficiency of counting was 80%. Results were calculated as c.p.m./mg of polyribosomes and the puromycin lability was expressed as the percentage of the radioactivity associated with the polyribosomes that was released by incubation with puromycin.

Polyribosomes labelled with radioactive amino acids *in vivo* had about 1000 c.p.m./mg of polyribosomes, whereas those labelled by incubation *in vitro* had about 5000 c.p.m./mg of polyribosomes.

Control incubations, as described above but without puromycin, resulted in no loss of radioactivity from the polyribosomes. Neither increasing the concentrations of puromycin or magnesium acetate in the incubation mixture, nor prolonging the time of incubation, affected the proportion of the original radioactivity released by puromycin.

Separation of most of the polyribosomal protein from radioactivity associated with polyribosomes. Polyribosomes labelled with radioactive amino acids were resuspended in buffer A with KCl replaced by NH₄Cl and re-isolated by centrifugation at 100 000g for 2 h. No radioactivity was lost. The pellets were resuspended in buffer B [0.02 M-tris-HCl (pH 7.4) at 4°C–0.5% sodium dodecyl sulphate–0.05 M-Li₂SO₄–0.015 M-EDTA] to give 20 mg of polyribosomes/ml. This procedure disrupts the polyribosome

structure releasing aminoacyl- and peptidyl-tRNA, ribosomal structural protein and ribosomal RNA (Gilbert, 1963). A portion (0.2 ml) of this solution was layered on linear sucrose gradients (10–20%, w/v) containing buffer B in polyallomer tubes for the SW65K rotor for the Spinco model L2-65B ultracentrifuge. After centrifugation at 60000 rev./min for 22 h at 7°C, the gradients were divided into 30 0.18 ml fractions by using an ISCO model 180 density-gradient fractionator. Three similar gradients were pooled. The protein content of each fraction was determined by the method of Lowry *et al.* (1951). Samples (0.1 ml) were taken for determination of radioactivity and pipetted directly into the dioxan-based scintillation mixture. A counting efficiency of 60% was obtained.

Fractionation of radioactive peptides from polyribosomes, labelled *in vivo*, by chromatography on Sephadex G-100. A 2.5 ml portion of polyribosome suspension (10 mg/ml) in 0.05 M-Na₂HPO₄ (pH 10.5)–0.005 M-EDTA–6 M-urea: was incubated at 37°C for 2 h to hydrolyse RNA and release the growing peptides from tRNA (Gilbert, 1963). The mixture was pumped (by using upward flow) on to a column (75 cm × 2.5 cm) of Sephadex G-100 previously equilibrated with the same buffer. Development of the column was continued at 60 ml/h to fractionate the growing peptides by molecular size, and 6 ml fractions were collected by using a BTL Chromapump and Chromafac fraction cutter. Growing peptides were detected by their radioactivity, which was determined in each fraction by pipetting 2.5 ml samples directly into 17 ml of the dioxan-based scintillation mixture.

Fractionation of polyribosomes by sucrose-density-gradient centrifugation. Samples (0.2 ml) of polyribosomes, resuspended in buffer A, were layered on linear sucrose gradients (15–30%, w/v) in buffer A without 2-mercaptoethanol, in polyallomer tubes for the SW65K rotor of the Spinco model L2-65B ultracentrifuge. After centrifugation at 40000 rev./min for 65 min at 4°C, the gradients were divided into 30 fractions by using an ISCO model 180 density-gradient fractionator.

Labelling of polyribosomes by incubating hemidiaphragms *in vitro*. Hemidiaphragms were prepared essentially as described by Randle & Smith (1958). Four hemidiaphragms were incubated at 37°C in each of a series of 50 ml flasks with 8 ml of Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932), containing 1 mg of glucose/ml and 0.4 μ Ci of ¹⁴C-labelled amino acid mixture. To half the flasks insulin was added to give 1 mU/ml. After 30 min, polyribosomes were prepared from pools of 20 hemidiaphragms incubated with or without insulin and samples of the homogenate were taken for measurement of radioactivity in total diaphragm protein by methods described above.

RESULTS

Nature of radioactivity associated with liver polyribosomes labelled *in vivo*. The interpretation of the puromycin lability of radioactivity associated with polyribosomes, in terms of the proportion of growing peptides occupying the donor site, depends on two major assumptions on the nature of this radioactivity: (a) that polyribosomal radioactivity represents growing peptides and not structural or adsorbed protein; (b) that all growing peptides, in a

Fig. 1. Separation by sucrose-density-gradient centrifugation of the bulk of polyribosomal protein from radioactivity associated with liver polyribosomes labelled *in vivo*. Liver polyribosomes isolated 3 min after an intravenous injection of radioactive amino acids were resuspended in buffer B containing 0.5% sodium dodecyl sulphate. Samples were layered on linear 10–20% (w/v) sucrose density gradients and centrifuged at 60000 rev./min for 22 h at 7°C. Δ , Distribution of radioactivity; $\circ$, distribution of protein.

population of polyribosomes, are similarly distributed between the acceptor and donor sites and that the proportion released by puromycin is similar for all.

In this section evidence is presented that 3 min after an intravenous injection of radioactive amino acids most of the radioactivity associated with polyribosomes has properties expected of growing peptide chains. Very little is incorporated into the bulk of the ribosomal structural protein.

Fig. 1 shows the distribution of protein and radioactivity after sucrose-density-gradient centrifugation of liver polyribosomes labelled *in vivo* and solubilized in a buffer containing sodium dodecyl sulphate to disrupt polyribosome structure. This procedure has separated most of the 1.4S ribosomal protein (Möller & Chrambach, 1967) from peptidyl-tRNA of greater size than 4S (Phillips, 1966). The ribosomal RNA forms a precipitate at the bottom of the tube (Gilbert, 1963). Recovery of polyribosomal protein and radioactivity was virtually complete (Table 1).

Fractions 1–13, containing 80% of the polyribosomal protein on the gradient, were pooled. The radioactivity in these fractions could be associated with this protein or with low-molecular-weight peptides attached to tRNA. To distinguish between these possibilities, the pooled fractions were adjusted to pH 10 with 0.1 M-sodium hydroxide and incubated at 37°C for 2 h to hydrolyse

Table 1. *Summary of experiments to demonstrate that radioactivity associated with polyribosomes labelled in vivo is not in the bulk of the structural protein*

Liver polyribosomes were prepared 3 min after an intravenous injection of radioactive amino acids and solubilized in buffer B containing 0.5% sodium dodecyl sulphate. A 0.2 ml sample of this solution was layered on linear sucrose gradients containing the same buffer and centrifuged at 60000 rev./min for 22 h at 7°C (see Fig. 1). Fractions containing the bulk of protein from the gradient were pooled and divided into two. One part was adjusted to pH 10 and incubated at 37°C for 2 h to hydrolyse tRNA. Both parts were then subjected to chromatography on Sephadex G-25. The recovery of radioactivity and protein is summarized below.

Material	Polyribosomal protein (mg)	Radioactivity (c.p.m./mg of protein)	Total radioactivity (c.p.m.)
Polyribosomal protein loaded on to sucrose density gradient	6	2260	13500
Recovered from gradient	5.9	2090	12300
Pool of fractions 1-13 (Fig. 1) containing bulk of protein	4.8 (82%)	625	2990 (24%)
Material from pool, voided by Sephadex G-25 after hydrolysis at pH 10	3.8	38*	144
Material from pool, voided by Sephadex G-25 without hydrolysis	4.2	540	2270

* 1.7% of radioactivity associated with original polyribosomal protein.

tRNA and release growing peptides (Gilbert, 1963). The pH was then brought back to 7 with 0.1M-hydrochloric acid and each pool was loaded on to a column (47 cm × 2.5 cm) of Sephadex G-25 previously equilibrated with 0.02M-tris-HCl (pH 7 at 4°C)-0.15M-lithium chloride-0.05% sodium dodecyl sulphate. Development of the column was continued with the same solution at 4°C. Portions of the voided fractions were adjusted to 5% (v/v) with ice-cold perchloric acid and the precipitate was collected by centrifugation and dissolved in 1 ml of 0.1M-sodium hydroxide. Samples were taken for measurement of radioactivity in dioxan scintillation mixture and for protein determination (Lowry *et al.* 1951). The recovery of protein was about 75% of that loaded on to the column (Table 1). About 94% of the radioactivity associated with this protein in the sucrose density gradient was hydrolysed to low-molecular-weight material, included in the Sephadex G-25. This strongly suggests its attachment to tRNA rather than to the structural protein. Thus 3 min after an intravenous injection of radioactive amino acids less than 1.7% of the radioactivity associated with polyribosomes can be in the bulk of the structural protein.

Various attempts to verify this result by other means were tried. Polyribosomes labelled *in vivo* and *in vitro*, where label in structural protein would not be expected (Phillips, 1966), were extracted by the method of Waller & Harris (1961), to isolate structural protein. Radioactivity was found in the extracts from both preparations, suggesting that this technique also extracts growing peptides. However, the contention was supported by the observation that polyribosomes labelled *in vivo* were found

to have lost 90% of their original radioactivity on re-isolation after incubation for 2 min *in vitro* in the presence of puromycin under conditions that allow protein synthesis (Earl & Morgan, 1968). Incubation under these conditions for longer than 2 min resulted in a progressively declining loss of radioactivity from the polyribosomes. This can be explained by the readsorption of peptidyl-puromycin to polyribosomes noted by Traut & Monro (1964) and Schneider, Raeburn & Maxwell (1968).

Growing peptides of different molecular sizes and from different classes of polyribosomes show similar puromycin labilities. In this section evidence is reported that a constant proportion of all the growing peptides in a population of polyribosomes is released by puromycin, irrespective of molecular size. This suggests that all growing peptides are similarly distributed between the acceptor and donor sites.

A suspension of liver polyribosomes, labelled *in vivo* with radioactive amino acids, was divided into two parts. To one part, puromycin dissolved in buffer A was added to give a final concentration of 0.2 mg of puromycin/mg of polyribosomes. An equivalent volume of buffer A was added to the other part. After incubation in ice for 30 min, the polyribosomes in both mixtures were re-isolated by centrifugation through 1M-sucrose in buffer A, and resuspended in buffer A to give the same concentration for both. Equal volumes of each were incubated at pH 10.5, as described in the Experimental section, to hydrolyse RNA and release growing peptides (Gilbert, 1963), and the peptides fractionated by chromatography on Sephadex G-100. The growing peptides were detected by their radioactivity.

Fig. 2. Fractionation of radioactivity from liver polyribosomes labelled *in vivo*, by column chromatography on Sephadex G-100. Liver polyribosomes isolated 3 min after an intravenous injection of radioactive amino acids were resuspended in 0.05 M- Na_2HPO_4 (pH 10.5)–0.005 M-EDTA–6 M-urea and incubated at 37°C for 2 h to hydrolyse RNA. The growing peptides were fractionated by column chromatography on Sephadex G-100 and detected by radioactivity. Fraction 30 is the void volume.

A typical elution profile is shown in Fig. 2. Peptides from both puromycin-treated and -untreated polyribosomes gave similar profiles, except that puromycin treatment removed some 49% of the radioactivity. The recovery of radioactivity loaded on to the columns was about 85% for both columns. Thus direct comparison of the columns was justified. The puromycin lability was calculated for pools of five consecutive samples as the percentage loss of radioactivity caused by incubation with puromycin. Representative results averaged for three repeat runs are shown in Table 2. The puromycin lability was found to be constant, irrespective of position of elution of the peptide from Sephadex G-100, and essentially the same as that of the original polyribosomes.

Further experiments were done to check that growing peptide chains associated with different classes of polyribosomes had similar puromycin liabilities. A suspension of liver polyribosomes, labelled *in vivo* with radioactive amino acids, in ice-cold buffer A was divided into two parts and one part incubated with puromycin as just described. On reisolation, the polyribosomes were resuspended in ice-cold buffer A to give the same concentrations for both and equal volumes from each were subjected to sucrose-density-gradient centrifugation.

Incubations of polyribosomes with puromycin in

Table 2. Puromycin lability of growing peptides isolated from liver polyribosomes labelled *in vivo* and fractionated by Sephadex G-100

Labelled polyribosomes were isolated from liver 3 min after an intravenous injection of radioactive amino acids, resuspended, incubated with or without puromycin at 0°C for 30 min and re-isolated. Equal amounts were resuspended in 0.05 M- Na_2HPO_4 (pH 10.5)–0.005 M-EDTA–6 M-urea and incubated at 37°C for 2 h to hydrolyse RNA and release remaining growing peptides, which were fractionated by column chromatography on Sephadex G-100 and detected by radioactivity (see Fig. 2). The puromycin lability was determined by comparison of the radioactivity in pools of five consecutive fractions from chromatography of material previously treated with puromycin with that not treated. The elution profile and recovery of radioactivity loaded on to the column was similar for both. The results shown are averaged from three separate experiments. Fraction 30 is the void volume.

Fraction no.	Puromycin lability (%)
30–34	43
35–39	49
40–44	55
45–49	53
50–54	51
55–59	55
60–64	60
65–69	57
70–74	58
75–79	56
Original polyribosomes	49

ice had no detectable effect on the distribution of polyribosomes in the density gradient (Fig. 3). Fractions were pooled in threes and samples taken for assay of radioactivity in the dioxan-based scintillation mixture. Recovery of radioactivity from both gradients was 90%. The radioactivity in pooled samples from the gradients of polyribosomes incubated in the presence or absence of puromycin were compared and the puromycin liabilities determined along the gradient. The results (Table 3) show that the proportion of radioactivity released by puromycin is constant over the major part of the gradient.

*Puromycin lability of growing peptides associated with polyribosomes labelled *in vivo* under a variety of conditions.* The results are shown in Table 4. The proportion of growing peptide chains associated with polyribosomes from liver and muscle from normal animals that can be released by puromycin is about 50%. A variety of conditions expected to alter the rate of protein synthesis was examined (Table 4). It proved impossible to change the puromycin lability of growing peptides to any great extent. The radioactivity, in c.p.m./mg of

Fig. 3. Fractionation by sucrose-density-gradient centrifugation of liver polyribosomes, labelled *in vivo* after incubation in ice with and without puromycin. Liver polyribosomes isolated 3 min after an intravenous injection of radioactive amino acids were resuspended and incubated with or without puromycin at 0°C for 30 min and re-isolated. Samples were layered on linear 5–30% (w/v) sucrose density gradients and centrifuged at 40 000 rev./min for 65 min at 4°C. ○, Polyribosomes incubated with puromycin; Δ, polyribosomes incubated without puromycin.

polyribosomes (3 min after the intravenous injection of radioactive amino acids), is presented merely for illustration. No meaningful comment can be made on the differences between the groups. The body weights of the animals varied, and no allowance for this was made in the dose of radioactive amino acids given. For this reason no statistics are presented. In some experiments, normal and hypophysectomized rats with and without growth hormone treatment were left for 30 min after an intravenous injection of radioactive amino acids (2 μ Ci of U-¹⁴C-labelled amino acid mixture/100 g body wt.) to assay the incorporation of radioactivity into total liver protein.

Hypophysectomy caused a small but significant rise in the puromycin liability, but treatment with growth hormone (0.3 mg/day subcutaneously for 5 days; Korner, 1960) failed to return the puromycin liability toward normal even though an enhanced incorporation into total liver protein was detected (Korner, 1960).

Table 3. Puromycin liability of growing peptides associated with liver polyribosomes fractionated by sucrose-density-gradient centrifugation

Polyribosomes were isolated from liver 3 min after an intravenous injection of radioactive amino acids, incubated with or without puromycin at 0°C for 30 min and re-isolated. Equal amounts were subjected to sucrose-density-gradient centrifugation. The E_{260} profiles shown in Fig. 3 were similar for both. The puromycin liabilities were determined by comparison of the radioactivity in the two gradients.

Fraction no.	Puromycin liability (%)
Original polyribosomes	48
1–3	—
4–6	56
7–9	52
10–12	56
13–15	55
16–18	50
19–21	51
22–24	48
25–27	63
28–30	—

Krahl (1953) showed that starvation diminishes the ability of the liver to incorporate amino acids into protein. Starvation for 48 h did not change the puromycin liability. Alloxan-diabetes depresses the rate of protein synthesis in liver and insulin treatment restores it toward normal (Krahl, 1956). The puromycin liability was not affected by diabetes with or without insulin treatment.

A small increase in the puromycin liability was observed after partial hepatectomy, but only after 3 days. At 20 h after partial hepatectomy, when the rate of protein synthesis in the liver remnant is maximal (Tsukada, Moriyama, Umeda & Lieberman, 1968; Majumdar, Tsukada & Lieberman, 1967), the puromycin liability is not significantly different from normal.

Puromycin liability of growing peptides labelled in vitro. The puromycin liability of liver and muscle polyribosomes labelled by incubation at 37°C with radioactive amino acids in a cell-free system *in vitro*, such that the concentration of polyribosomes limited the rate of incorporation, was about 90% (Table 5).

The puromycin liability of growing peptides associated with polyribosomes isolated from hemidiaphragms incubated with radioactive amino acids *in vitro* was 76% (Table 6). The puromycin liability was unaffected by the addition of insulin, even though the incorporation of radioactivity into protein was doubled (Manchester & Young, 1958). Hemidiaphragms from hypophysectomized rats similarly labelled were not significantly different in behaviour from normal although the incorpora-

Table 4. Puromycin lability of growing peptides associated with polyribosomes labelled in vivo under a variety of conditions

Rats were starved overnight and given an intravenous injection of $3 \mu\text{Ci}$ of $\text{U-}^{14}\text{C}$ -labelled amino acid mixture. At 3 min after injection polyribosomes were isolated from the liver or gastrocnemius muscle. These polyribosomes were incubated at 0°C for 30 min in the presence of puromycin and re-isolated. The puromycin lability is expressed as the percentage of the radioactivity released from the polyribosomes by puromycin. Results are shown $\pm$ s.e.m., with numbers of experiments in parentheses. Student's *t* test was used as a test of significance; * $P < 0.01$ compared with normal; † $P < 0.01$ compared with hypophysectomized. Growth hormone was given by subcutaneous injection 0.3 mg/day for 5 days. The incorporation into total liver protein was measured 30 min after an intravenous injection of radioactive amino acids ($2 \mu\text{Ci}$ of $\text{U-}^{14}\text{C}$ -labelled amino acid mixture/100 g body wt.).

Source of polyribosomes	Condition of animals	Radioactivity (c.p.m./mg of polyribosomes)	Puromycin lability (%)	Radioactivity (c.p.m./mg of total liver protein) 30 min after injection
Liver	Normal	909	48.5 ± 1.5 (11)	329 ± 15
	Starved 48 h	1194	48 ± 3 (4)	—
	Hypophysectomized	1503	59.3 ± 5.4 (6)*	$240 \pm 12^*$
	Hypophysectomized + growth hormone	1950	57.3 ± 5.5 (6)	$383 \pm 17^\dagger$
	Alloxan-diabetic	939	51 ± 3.5 (4)	—
	Alloxan-diabetic + insulin	1292	51.5 ± 6 (4)	—
	20 h after partial hepatectomy	1680	42 ± 3.1 (6)	—
	2 days after partial hepatectomy	1738	50.6 ± 4 (6)	—
	3 days after partial hepatectomy	1567	61.3 ± 2.7 (6)*	—
	Normal	1250	57 ± 5 (3)	—
Gastrocnemius muscle				

tion into total diaphragm protein was depressed by 50% (Kostyo & Knobil, 1959). Previous treatment of the rat with growth hormone enhanced the incorporation of radioactivity into total diaphragm protein, but did not alter the puromycin lability.

DISCUSSION

The use of the puromycin reaction to determine the distribution of growing peptides between the donor and acceptor sites can be questioned. The mechanism of peptide bond synthesis and the action of puromycin are not fully understood. Cannon (1967) has provided evidence that growing peptides occupying only the donor site are released by puromycin at 0°C . On incubation of polyribosomes with puromycin at 37°C further growing peptides become available for reaction by translocation from the acceptor sites (Baglia, Cohen & Munro, 1970).

If puromycin is bound to the acceptor site in place of aminoacyl-tRNA before reacting with the growing peptide in the donor site, then the puromycin lability could be a measure of vacant acceptor sites rather than of growing peptides occupying donor sites. This is unlikely for Cannon, Krug & Gilbert (1963) and Wettstein & Noll (1965) have presented evidence suggesting that the acceptor site is not involved in the puromycin reaction. A further assumption that there is no random equilibration of peptidyl-tRNA between donor and acceptor sites is also unlikely because of the high puromycin lability observed after incubating hemidiaphragms *in vitro*.

If the mechanism of peptide bond formation and the action of puromycin described above are correct, then studies of the proportion of growing peptides occupying the donor site, and therefore puromycin-labile, can be used to investigate the rate-limiting step of protein synthesis. The distribution of growing peptides between the puromycin-labile donor site and the puromycin-non-labile acceptor site will depend on the availability and binding of aminoacyl-tRNA and the relative rates of reactions involved in peptide bond synthesis and translocation. If in the steady state the growing peptides were largely puromycin-labile, then the availability or binding of aminoacyl-tRNA or the rate of peptide bond synthesis might limit the overall rate of protein synthesis. If the major portion were puromycin-stable then translocation might be limiting.

In all experiments polyribosomes were used, being the active complex in animal cells. Evidence is presented that 3 min after an intravenous injection of labelled amino acids radioactivity associated with polyribosomes can be interpreted as growing peptides. It can be distinguished from ribosomal structural protein and most can be removed

Table 5. *Puromycin lability of growing peptides associated with polyribosomes labelled by incubation in a cell-free system in vitro*

Liver and muscle polyribosomes were incubated in a cell-free system for 5 min at 37°C with radioactive amino acids and liver supernatant fraction such that the concentration of polyribosomes limited the rate of incorporation (see Earl & Morgan, 1968). The reaction was stopped by cooling in ice. The polyribosomes were separated, incubated with puromycin at 0°C for 30 min and re-isolated. The puromycin lability is expressed as the percentage of the radioactivity released from polyribosomes by puromycin and is presented $\pm$ s.e.m., with numbers of experiments in parentheses.

Source of polyribosomes	Radioactivity (c.p.m./mg of polyribosomes)	Puromycin lability (%)
Liver	5420 $\pm$ 160	90 $\pm$ 4 (10)
Gastrocnemius muscle	4575 $\pm$ 181	91 $\pm$ 5 (6)

Table 6. *Puromycin lability of growing peptides associated with polyribosomes labelled by incubation of hemidiaphragms in vitro*

Four hemidiaphragms were incubated at 37°C for 30 min in each of a series of 50 ml flasks with 8 ml of Krebs-Henseleit bicarbonate buffer containing 0.4 μ Ci of 14 C-labelled amino acid mixture. Insulin was added to half the flasks at 1 mU/ml. After incubation polyribosomes were isolated from pooled hemidiaphragms, treated with puromycin at 0°C for 30 min and re-isolated. Puromycin lability is expressed as the percentage of the radioactivity released by puromycin. Results are expressed $\pm$ s.e.m. * $P < 0.01$ compared with normal; † $P < 0.01$ compared with hypophysectomized.

Source of hemidiaphragms	Incubation conditions	Radioactivity (c.p.m./mg of polyribosomes)	Puromycin lability (%)	Radioactivity (c.p.m./mg of diaphragm protein)
Normal rats	No additions	1730	76 $\pm$ 7	654 $\pm$ 71
Normal rats	+insulin	2680	70 $\pm$ 9	1286 $\pm$ 103*
Hypophysectomized rats	No additions	1041	82 $\pm$ 11	420 $\pm$ 62*
Hypophysectomized rats treated with growth hormone (0.3 mg/day for 5 days)	No additions	1430	82 $\pm$ 9	606 $\pm$ 83†

from polyribosomes by incubation *in vitro* with puromycin under conditions allowing peptide bond formation.

It has been shown that the puromycin lability of growing peptides identified by radioactivity is constant, despite differences in molecular size and association with different classes of polyribosomes. It is concluded that the puromycin lability of radioactivity associated with polyribosomes could be interpreted in terms of the distribution of all growing peptides in a population of polyribosomes between the donor and acceptor sites.

There is still the problem of whether the observed lability is an artifact of methodology. This cannot be discounted. However, it has been shown that the presence or absence of deoxycholate in the isolation procedure did not affect the puromycin lability (49% and 54% respectively). Further, the re-isolation of polyribosomes labelled in a cell-free system *in vitro*, and their isolation from hemidiaphragms labelled *in vitro*, gave puromycin labilities very different from those observed with polyribosomes labelled *in vivo*. Thus available evidence

suggests that the puromycin lability is not an artifact of methodology.

The proportion of growing peptides associated with normal liver and muscle polyribosomes *in vivo* that can be released by puromycin was about 50%. This result suggests that aminoacyl-tRNA is freely available in these tissues *in vivo* and that neither its supply nor its rate of binding to the codon-specific acceptor site can limit the rate of protein synthesis. Further, the rates of the translocation reaction and peptide bond formation must be similar and neither can limit the rate of protein synthesis *in vivo*. If any of these factors were limiting, then an unbalanced distribution of growing peptides might be expected, tending to 100% labile or 100% stable.

The possibility that one of these factors might become rate limiting *in vivo* under experimental conditions known to alter the rate of protein synthesis was investigated in liver. Hypophysectomy depresses the rate of protein synthesis in liver (Korner, 1960) and resulted in a small increase in the puromycin lability. Treatment of

the hypophysectomized rat with growth hormone (Korner, 1960) failed to cause a return of the puromycin lability to normal even though an increased incorporation of radioactive amino acids into protein was observed.

The rate of protein synthesis in liver is diminished by starvation (Krahl, 1953) and alloxan-diabetes (Krahl, 1956) and is restored toward normal by treatment of the diabetic animal with insulin. None of these procedures altered the puromycin lability. A small increase in the puromycin lability was observed 3 days after partial hepatectomy. At 20 h after partial hepatectomy, when the rate of growth and protein synthesis in the liver remnant is reported to be maximal (Majumdar *et al.* 1967; Tsukada *et al.* 1968), the puromycin lability was not significantly different from normal.

It is concluded that *in vivo* neither the availability nor the rate of binding of aminoacyl-tRNA nor peptide bond synthesis nor translocation can limit the rate of protein synthesis in any of the conditions studied. This conclusion is further supported by the apparent lack of correlation between changes in puromycin lability and changes in the overall rate of protein synthesis.

The puromycin lability of radioactivity associated with ribosomes labelled *in vivo* was found to be 33% compared to 70% reported by Andrews & Tata (1968). The reason for this discrepancy is not clear. Skogerson & Moldave (1968) studied the incorporation of [³H]puromycin into peptidyl-puromycin by liver ribosomes at 37°C and concluded that *in vivo* only 20% of the growing peptides are in the donor site. This result compares with 33% for ribosomes measured by the techniques described here, but makes no allowance for the presence of single ribosomes free of growing peptides.

When muscle or liver polyribosomes were labelled by using a cell-free system *in vitro*, under conditions in which the concentration of polyribosomes limited the rate of incorporation, the puromycin lability was about 90%. This result suggests that either the availability or binding of aminoacyl-tRNA or the rate of peptide bond synthesis limits the rate of incorporation in this cell-free system. As the addition of more polyribosomes will increase the rate of incorporation under the conditions used, the rate of peptide bond synthesis, catalysed by a polyribosome-bound enzyme (Sutter & Moldave, 1966; Skogerson & Moldave, 1967) might be implicated. However, the system used was extremely crude. It is notable that even under nominally optimum conditions for incorporation in a cell-free system *in vitro*, the rate-limiting reaction appears to be different from that pertaining *in vivo*.

Allen & Zamecnik (1962), Morris, Favelukes, Arlinghaus & Schweet (1962) and Andrews & Tata (1968) have incubated ribosomes labelled *in vitro*

in the presence of puromycin, cell sap and excess of non-radioactive amino acids at 37°C, and observed the release of only 30–40% of the radioactivity associated with the ribosomes. This result is considerably lower than that reported here for polyribosomes labelled *in vitro* and incubated with puromycin alone at 0°C. In this system ribosomes labelled *in vitro* have always released less of the radioactivity (60%) than polyribosomes. The reason for this is not known. Wettstein & Noll (1965) observed the release of 80% of the radioactivity associated with polyribosomes labelled *in vitro*, when incubated at 37°C for 6 min in the presence of puromycin, cell sap and non-radioactive amino acids. This confirms the difference between ribosomes and polyribosomes discussed here. It has also been shown that incubation at 37°C for longer than 2 min under these conditions results in a progressive readsorption of peptidyl-puromycin to the polyribosomes (Traut & Monro, 1964; Schneider *et al.* 1968), leading to an erroneously low value for the release of radioactivity by puromycin.

Hultin (1966) observed a release of 35% of the radioactivity associated with ribosomes labelled *in vitro* and subsequently incubated for 4 min at 0°C in the presence of cell sap and spermine to inhibit further incorporation. Again differences in methodology make comparison difficult.

The high puromycin lability observed after labelling *in vitro* is not due to the release of growing peptides from the acceptor site during the labelling procedure. Liver polyribosomes labelled *in vivo* and subsequently incubated *in vitro* at 37°C for 5 min under conditions allowing peptide synthesis (Earl & Morgan, 1968) with non-radioactive amino acids did not release significant amounts of radioactivity. Further, the puromycin lability is increased by this procedure from 50% to 85% without loss of radioactivity. Similar results were obtained by incubating *in vitro* liver polyribosomes labelled *in vivo* with liver cell sap (Earl & Morgan, 1968) and GTP. Here no peptide bonds are formed, the increase in the puromycin lability presumably being the result of translocation of the radioactive peptidyl-tRNA from the acceptor to the donor site (Skogerson & Moldave, 1967).

When polyribosomes were isolated from hemidiaphragms after incubation *in vitro* with radioactive amino acids the puromycin lability was substantially higher than that observed with muscle polyribosomes labelled *in vivo*, again indicating a different rate-limiting step. In hemidiaphragms incubated *in vitro* either the availability or binding of aminoacyl-tRNA or peptide bond synthesis becomes rate-limiting. The addition of insulin, although stimulating the overall rate of incorporation of radioactive amino acids into protein, did not

alter the puromycin lability. Previous hypophysectomy of the rat depressed the incorporation of radioactive amino acids into protein of the hemidiaphragm, whereas prior treatment of the hypophysectomized animal with growth hormone stimulated incorporation. However, in neither case was the puromycin lability significantly different from normal.

Thus in this hemidiaphragm system it has also proved impossible to correlate changes in the rate of incorporation of radioactive amino acids into protein with changes in the puromycin lability. This is a problematical situation. In the hemidiaphragm incubated *in vitro* a puromycin lability of about 80% was observed, indicating that a reaction thought to affect the puromycin lability was rate limiting, and an increase in the rate of protein synthesis might have been expected to result in a change in the puromycin lability. However, this lack of correlation could be understood if the intracellular concentration of amino acids or aminoacyl-tRNA or the binding of the latter to the acceptor site limited the rate of protein synthesis. For an increase in the concentration of amino acids or aminoacyl-tRNA or in the rate of the binding reaction to a higher but still suboptimum state would increase the rate of incorporation of radioactivity into protein without necessarily altering the rate-limiting reaction or the puromycin lability.

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