The Effect of Feeding with a Tryptophan-Free Amino Acid Mixture on Rat-Liver Polysomes and Ribosomal Ribonucleic Acid

By W. H. WUNNER, J. BELL AND H. N. MUNRO*

Department of Biochemistry, University of Glasgow, and Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.

(Received 29 March 1966)

1. Starving rats were given complete and tryptophan-deficient amino acid mixtures by stomach tube and were killed from 1 to 7 hr. later. The polysome profile in the livers of rats fed with the tryptophan-deficient mixture showed a shift in distribution such that the large aggregates were decreased and the small aggregates were increased, particularly dimers. This polysome shift was reversed when the complete amino acid mixture was given by stomach tube 2 hr. after administering the tryptophan-free amino acid mixture. 2. After removal of liver polysomes by centrifugation, some smaller ribosomal aggregates (oligosomes) remaining in suspension were harvested by prolonged centrifugation of the supernatant fluid. A large increase in the dimer population of this fraction was observed in the rats receiving the incomplete mixture. 3. When the polysome and oligosome fractions were incubated with cell sap, an energy-generating system and labelled amino acids DL-[1-¹⁴C]leucine and L-[Me-¹⁴C]tryptophan were incorporated into the cell fractions in the ratio 4.5:1. Preparations of polysomes and oligosomes from rats fed with the tryptophan-free amino acid mixture showed a decreased amino acid-incorporating activity compared with particulate preparations made from rats fed with the complete mixture. 4. The yield of free ribosomes prepared from the unfractionated liver microsomes by treatment with iso-octane was 40–50% greater in rats fed with the amino acid mixture deficient in tryptophan. 5. A post-microsomal fraction was prepared from cell sap and was shown to consist of ribosomal sub-units. When the animals were fed with the tryptophan-deficient mixture, there was an increase in content of this post-microsomal fraction and in the ratio 30s RNA/19s RNA. Rats were also given [5-³H]orotic acid at the time of feeding with the amino acids. Lack of tryptophan in the mixture caused a decrease in the specific activity of both RNA fractions which affected the 30s RNA more extensively than the 19s RNA. 6. These changes in the distribution and quantity of the cellular components engaged in protein synthesis are discussed in relation to RNA metabolism and amino acid-incorporating activity of the liver cell and their response to feeding with the tryptophan-free amino acid mixture.

Since the amount of protein in the liver is considerably influenced by the quantity and quality of the protein in the diet (Allison, Wannemacher, Banks & Wunner, 1964), the supply of amino acids may effect the mechanisms of protein biosynthesis. Nutritional studies of the effect of protein intake on body protein metabolism have now advanced to the point at which it is possible to examine the impact of amino acid supply on subcellular components engaged in protein synthesis. The polysomes of the hepatic cell are formed by ribosomes attached to strands of messenger RNA coding for

the amino acid sequences of proteins, and are the sites of polypeptide chain formation. As it is possible that amino acid availability may alter the rate of protein synthesis and thus the speed of attachment and detachment of ribosomes on the messenger strand (Noll, Staehelin & Wettstein, 1963), the lack of one amino acid required in the peptide sequence could alter polysome stability (Villa-Trevino, Farber, Staehelin, Wettstein & Noll, 1964).

From this Laboratory, studies have been reported in which rats were given either a nutritionally complete amino acid mixture or a similar mixture with one essential amino acid (tryptophan) omitted. The effects of these mixtures on rat-liver

* Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.

nucleic acid metabolism (Munro & Clark, 1959), amino acid incorporation into liver microsomal protein and the degree of polyribosomal aggregation (Fleck, Shepherd & Munro, 1965) were shown to be related to protein biosynthesis. In the present paper, these studies investigating the response at the subcellular level are extended to provide an integrated picture of the changes occurring in polysome patterns, free ribosomes and ribosomal sub-units and their contained RNA when a complete amino acid mixture or one lacking tryptophan is fed to rats. These changes have been related to the capacity of the liver cell to synthesize proteins, as demonstrated by alterations in the uptake of labelled amino acids into protein in a cell-protein system *in vitro* in which liver polysomes were employed from rats fed with the complete or tryptophan-free amino acid mixtures. The choice of tryptophan as the essential amino acid deleted from the mixture was dictated by our previous studies; we do not know whether removal of other essential amino acids would lead to similar changes.

MATERIALS AND METHODS

Materials. The following radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks.: [8-¹⁴C]adenine sulphate, DL-[1-¹⁴C]leucine, [5-³H]-orotic acid and L-[Me-¹⁴C]tryptophan. All amino acids in the feeding mixture were L-isomers purchased from British Drug Houses Ltd., Poole, Dorset. Sephadex G-25 (in the bead form) was obtained from Pharmacia, Uppsala, Sweden. All other chemicals were obtained through British Drug Houses Ltd.

Animals. Male albino rats (150 g.) were starved for 18 hr. Groups of three to five rats were fed by stomach tube with a mixture of amino acids made according to the relative composition of egg albumin (Table 1). The tryptophan in the complete mixture was four times the amount called for in the albumin pattern. Each rat received 1.2 g. of amino acids in 3 ml. of the mixture, a polyethylene stomach tube and syringe being used. One group of rats was given the nutritionally complete amino acid mixture, whereas the second group was given the mixture with tryptophan omitted. The rats were killed by decapitation 2 hr. after feeding, unless otherwise noted, and livers were removed for the preparation of polysomes and oligosomes, chilled in tris-HCl buffer, pH 7.6, containing 0.25 M-sucrose, 25 mM-KCl and 5 mM-MgCl₂, and homogenized as described by Fleck *et al.* (1965).

Polysome preparation. The rat-liver homogenate was centrifuged for 10 min. at 16000g. The post-mitochondrial supernatant, treated with sodium deoxycholate to final concentration 1.3%, was used to prepare 'C'-ribosomes (polysomes) (Wettstein, Staehelin & Noll, 1963) as described by Fleck *et al.* (1965). Portions of suspended polysomes (50 μ l.) representing the same amount of tissue were analysed on sucrose density gradients (15–34%, w/v) centrifuged at 118000g for 1 hr. in a 3 $\times$ 5 ml. swing-out rotor (MSE Superspeed 50), and the pattern of ultraviolet absorption at 260 m μ on the gradient was recorded auto-

Table 1. *Composition of amino acid mixture*

The complete amino acid mixture and the mixture with tryptophan omitted were administered in 3 ml. to rats by stomach tube. The first nine amino acids listed were dissolved in 2.09 g. of NaHCO₃/50 ml. of water; the remaining amino acids were slightly soluble or insoluble. All amino acids were in the L-isomer form.

Amino acid	Dose/rat (mg.)
Glutamic acid	200
Lysine hydrochloride	120
Histidine hydrochloride	43
Arginine	80
Aspartic acid	20
Proline	20
Glycine	10
Alanine	20
Serine	10
Cystine	40
Methionine	56
Threonine	66
Phenylalanine	75
Tyrosine	45
Valine	100
Leucine	118
Isoleucine	86
Tryptophan	53

matically by a flow-cell device in conjunction with the Spectronic 505 spectrophotometer (Bausch and Lomb).

Post-polysome pellet (oligosomes). Since the method of preparing polysomes results in the loss of a large proportion of monomers and small ribosomal aggregates by failure to sediment through the discontinuous gradient of 0.25 M-, 0.5 M- and 2 M-sucrose (Fleck *et al.* 1965), the post-polysome supernatant, which contains the three layers of sucrose, was mixed and then centrifuged for 3 hr. at 105000g to sediment a post-polysome pellet (oligosomes). The oligosomes were washed on the surface and suspended in 0.15 ml. of 5 mM-tris-HCl buffer, pH 7.6, containing 25 mM-KCl and 5 mM-MgCl₂ (medium A) (Littlefield & Keller, 1957). Portions of suspended oligosomes (50 μ l.) representing the same amount of tissue were layered on 4.6 ml. of a sucrose density gradient (5–25%, w/v) and centrifuged in the 3 $\times$ 5 ml. swing-out rotor for 2 hr. at 90000g and 1° (MSE Superspeed 50). The ultraviolet absorption of the gradient at 260 m μ was recorded as described for the polysomes. The absorption of material at 320 m μ on an identical gradient was measured to correct for the presence of ferritin (Jackson, Munro & Korner, 1964).

Evaluation of polysome and oligosome patterns. The distribution of ribosomal aggregates in a single pattern was estimated from the area under the curve by using a compensating polar planimeter. The resolution of ribosomal aggregates from monomer to hexamer was clearly discernible by the reasonably symmetrical individual peaks. A standard of comparison was adapted for these tracings from the procedure described by Webb, Blobel & Potter (1964). Perpendicular lines were drawn by inspection from the trough between successive peaks up to and including the hexamer. The heavier polyribosomes were estimated

under the area, $n > 6$, which extended to the end of the tracing. The area under the curve bounded by the perpendicular and base lines was traced with the compensating polar planimeter and recorded as a percentage of the total area of the polysome or oligosome tracing. A comparison of the percentage contribution of any one peak to the entire population of aggregate sizes on the sucrose gradient was used as a measure of polysome response to amino acid mixtures.

Polysome and oligosome amino acid-incorporating system. Samples of polysomes and oligosomes containing 0.45 mg. of protein were incubated at 37° with cell sap (13 mg. of protein), which had been passed through Sephadex G-25 to remove inhibitors of protein synthesis (Munro, Jackson & Korner, 1964). The incubation medium consisted of 0.25 M-sucrose, 25 mM-KCl and 5 mM-MgCl₂ in 0.05 M-tris buffer, pH 7.6. The incubation mixture (1 ml.) contained 2 μ moles of guanosine triphosphate, 14.8 μ moles of creatine phosphate, 20 μ g. of creatine phosphokinase and either L-[Me-¹⁴C]tryptophan or DL-[¹⁴C]leucine. To supply the system with equal quantities of each ¹⁴C-labelled L-amino acid (25 μ moles), 0.57 μ Ci of L-[Me-¹⁴C]tryptophan and 1.83 μ Ci of DL-[¹⁴C]leucine were added. The amino acid-incorporating reactions were stopped after 30 min. in a solid CO₂-ethanol bath.

The reaction mixtures were then treated with an equal volume of cold 0.6 N-HClO₄ containing 1 mg. of either unlabelled DL-leucine or unlabelled L-tryptophan. After two washings with 0.3 N-HClO₄, the RNA was hydrolysed in 0.3 N-KOH for 1 hr. at 37°. The protein was recovered quantitatively by acidification of the alkaline digest, separated by centrifugation and redissolved in alkali (Fleck *et al.* 1965). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), and radioactivity was measured in a Nuclear-Chicago gas-flow counter. All the data were corrected for the activity of the zero-time controls and background activity, which were low. Results are expressed in terms of μ moles of radioactive amino acid incorporated/mg. of protein.

Preparation of free ribosomes from rat liver. Livers from rats given the complete and incomplete amino acid mixtures were homogenized in 0.88 M-sucrose. Free ribosomes, i.e. those not attached to the membrane of the endoplasmic reticulum, were obtained from the liver homogenate with iso-octane treatment according to the method of Hallinan & Munro (1965a). The free ribosomes were suspended in a tris-buffered medium, pH 7.6, containing 0.25 M-sucrose, 25 mM-KCl and 2.5 mM-MgCl₂ in 35 mM-tris-HCl, centrifuged at 105 000 g for 1 hr. and resuspended. The RNA/protein ratio 0.365 for the washed free liver ribosomes from untreated rats compared favourably with previous preparations (Hallinan & Munro, 1965a). Successive washes resulted in some loss of protein, whereas the RNA was recovered without loss. This loss of protein can account for higher RNA/protein ratios. The identity of the free ribosomes as monomers was confirmed on sucrose-density gradients (5–25%, w/v) in the suspension medium by comparison with the position of monomers of the oligosome pellet after a 2 hr. centrifugation at 90 000 g in the SW 39 swing-out rotor (Spinco model L ultracentrifuge).

RNA and ribosomal sub-unit particles from the post-microsomal fraction of rat liver. The post-microsomal pellet was sedimented by prolonged centrifugation of cell sap prepared from rat livers. These were first homogenized in

2.5 vol. of 0.35 M-sucrose in 0.02 M-potassium phosphate buffer, pH 7.8, containing 0.03 M-KHCO₃, 25 mM-KCl and 1.5 mM-MgCl₂, and cell sap was prepared by removing microsomes in a 1 hr. centrifugation at 105 000 g. The PMP* was separated from the 105 000 g supernatant as described by Hird, McLean & Munro (1964). The pellet was either suspended in the homogenizing medium without magnesium and containing 2% (w/v) sucrose for gradient analysis of sub-unit particles, or was suspended in 0.01 M-tris-HCl, pH 7.5, containing 0.1% of bentonite for the extraction of RNA with phenol.

The PMP suspension (3 E units; E units = vol. applied $\times E_{1\text{ cm. at } 260\text{ m}\mu}$) was applied to a sucrose density gradient (5–25%, w/v) which contained 0.03 M-KHCO₃, 25 mM-KCl and 0.02 M-phosphate buffer, pH 7.8. Magnesium was omitted from the gradient medium since, when included during long centrifugation periods, the precipitation of magnesium phosphate was observed. PMP sub-unit patterns obtained from Mg²⁺-free gradients were identical with those obtained on gradients containing 1.5 mM-Mg²⁺ in 0.02 M-tris-HCl buffer, pH 7.4; concentrations of Mg²⁺ of 5 mM or above caused aggregation of sub-unit particles. The gradients were centrifuged in the SW 39 rotor (Spinco model L ultracentrifuge) at 90 000 g for 4 hr. and either drop-counted as 30 fractions to measure ultraviolet absorption at 260 m μ and 280 m μ , or passed through a flow-cell device in conjunction with the Spectronic 505 spectrophotometer to record automatically the ultraviolet-absorption pattern at 260 m μ . Gradients were occasionally checked for ferritin content by either absorption at 320 m μ or by assay for ferritin iron (Drysdales & Munro, 1966).

RNA was extracted from the PMP by the phenol method of Kirby (1956). To the PMP suspended in 0.01 M-tris-HCl, pH 7.5, containing 0.1% of bentonite, a freshly prepared solution of sodium dodecyl sulphate was added to give final concentration 0.5%. An equal volume of phenol (90%, w/v) containing 0.1% of 8-hydroxyquinoline was added (Kirby, 1962). The extracted RNA was desiccated over P₂O₅ and then dissolved in 0.01 M-sodium acetate buffer, pH 5.2, containing 0.05 M-NaCl. A sample of 3 E units was layered on a sucrose gradient (3–20%, w/v) in acetate-NaCl. The gradients were centrifuged either for 4 hr. at 103 000 g or 16 hr. at 32 000 g in the SW 39 rotor in the Spinco model L ultracentrifuge.

To measure the quantitative yield of RNA and protein in the post-microsomal pellet and free ribosomes, RNA was separated from protein according to the modified Schmidt-Thannhauser procedure of Fleck & Munro (1962) and estimated by the ultraviolet-absorption method of Fleck & Begg (1965). Protein was determined from the alkaline digest by the Lowry method (Lowry *et al.* 1951).

Incorporation of RNA precursor in vivo. Rats were injected intraperitoneally with [5-³H]jorotic acid (75 μ Ci/100 g. body wt.) at the time of feeding with the complete and tryptophan-free amino acid mixtures. The uptake of radioactivity into the RNA fractions of the post-microsomal pellet was measured by using the methods of RNA separation on sucrose gradients described above. The gradients were drop-counted into 15 fractions, and 0.5 ml. of water was added to each fraction for ultraviolet-absorption measurements at 260 m μ . To a sample (0.5 ml.) of the diluted fraction, 8 ml. of Scintstant NE572 in

* Abbreviation: PMP, post-microsomal pellet.

dioxan, scintillation grade (Nuclear Enterprises Ltd., Edinburgh), was added in glass liquid-scintillation vials. The samples were counted in a Nuclear-Chicago liquid-scintillation counter and corrections were made for background and a 13% counting efficiency.

RESULTS

Effect of administered amino acid mixtures on polysome patterns. Difficulties were experienced in isolating polysomes from the livers of fed animals with large amounts of liver glycogen. Consequently, only animals starved overnight were used. An example of a typical sucrose-gradient (15–34%, w/v) pattern of polysomes derived from livers 2 hr. after giving the complete amino acid mixture to one group of rats and the tryptophan-free mixture to the other group is shown in Fig. 1. The polysome profile of the group fed with the amino acid mixture lacking tryptophan is characterized by the high proportion of small ribosomal aggregates (oligosomes) compared with starved animals. The group fed with the complete mixture, however, has a smaller proportion of oligosomes and a greater proportion of the larger ribosomal aggregates than the starving or tryptophan-deficient rats. A qualitative difference in these patterns compared with those shown by Fleck *et al.* (1965) is the presence of an additional component marked 1+ in Fig. 1. The total area under the curve of the polysome tracing was used as a measure of the quantitative yield of ultraviolet-absorbing material in the polysome suspension per unit weight of liver. This area was 2.19 ± 0.16 (mean $\pm$ s.e.m.) units of

the polar planimeter for polysomes isolated from rats fed with the complete mixture and 2.94 ± 0.19 units when rats were fed with the tryptophan-deficient mixture. The recoveries of polysomes from liver were incomplete under the preparative conditions. However, by strictly controlling the conditions under which polysomes were prepared, we felt justified in assuming that the recoveries from both groups of rats were comparable.

The distribution of ribosomal aggregates 2 hr. after giving the complete amino acid mixture to one group of rats and the tryptophan-free mixture to the other group is shown in Table 2. The percentage of the area under the curve represented by each size of ribosomal aggregate in the polysome pattern relative to the total area of the polysome population was averaged from eight groups of rats which received the amino acid mixtures. The amount of ultraviolet-absorbing material in the region of oligosomes (< seven ribosomes aggregated) relative to the total portion applied to the sucrose gradient increased by 14% in the preparation of polysomes from the rats lacking tryptophan compared with those fed with the complete mixture. The dimers ($n=2$) accounted for nearly half of the 14% increase in oligosomes in the tryptophan-deficient rats. Based on the greater quantitative yield of polysome material per unit weight of liver in the tryptophan-deficient rats, the relative increase in oligosomes is an absolute increase.

The duration of the effect of amino acid feeding on polysome patterns was examined. In Fig. 2, the accumulation of dimers in the population of polysomes prepared from rat livers at 1, 2, 3, 4, 5 and 7 hr. after feeding with the tryptophan-free mixture has been compared with the response to the complete mixture. Rats fed with the tryptophan-free amino acid mixture over a 7 hr. period showed a maximum accumulation of dimers at 2 and 3 hr., and a decreasing percentage of dimers thereafter in the remaining 4 hr. after feeding. The continuous curve (tryptophan-deficient group) would thus suggest an adaptation within the animal, whereby a more satisfactory pattern of free amino acids required for protein biosynthesis was provided after some time through the endogenous supply of tryptophan.

The distribution of liver polysomes in the rats lacking tryptophan in their diet was shown to be reversible. One group of three rats was fed with the complete amino acid mixture and four other groups received the mixture lacking tryptophan. Groups receiving the incomplete mixture were killed after 2 hr. and 4 hr., and the group on the complete mixture was also killed after 4 hr. The remaining two groups, both of which had received the incomplete mixture, were aspirated by stomach tube after 2 hr., and one group was then re-fed with

Fig. 1. Polysome patterns on sucrose density gradients. Polysomes were isolated from rat livers 2 hr. after feeding with the complete amino acid mixture (—) to one group of starved rats and the mixture lacking tryptophan (---) to another group. The polysome patterns are compared with the pattern of polysomes from starved rats (....) on sucrose gradients (15–34%, w/v) in 0.05M-tris-HCl, pH 7.6, 25mM-KCl and 5mM-MgCl₂. $E_{280\text{ m}\mu}$ of the sample on the gradient was recorded automatically by using a flow-cell device in conjunction with a recording spectrophotometer. The peaks of ultraviolet-absorbing material correspond to the aggregate size of polysomes, where n is the number of ribosomes aggregated; $n=1+$, intermediate between dimer ($n=2$) and monomer ($n=1$); $n=f$, ferritin.

Table 2. *Distribution of ribosome aggregates in rat-liver polysome and oligosome fractions after giving amino acid mixtures by tube*

An amino acid mixture lacking tryptophan and a complete amino acid mixture were fed by stomach tube to groups of three to five rats previously starved for 18 hr. The rats were decapitated 2 hr. later, and the polysome and oligosome fractions were analysed as described in the Materials and Methods section. Monomers and small (< 6) ribosomal aggregates not sedimenting with the polysomes were recovered in a post-polysome pellet by prolonged centrifugation of the polysome supernatant and analysed as outlined in the Materials and Methods section. *n*, Number of ribosomes per aggregate. Results are expressed as a percentage (mean $\pm$ s.e.m.) of the total polysome or oligosome population.

Ribosome fraction	Polysome fraction (%)		Oligosome fraction (%)	
	Complete	Tryptophan-free	Complete	Tryptophan-free
Ferritin	1.0 $\pm$ 0.1	0.7 $\pm$ 0.1	28.1 $\pm$ 4.0	10.4 $\pm$ 1.4
<i>n</i> = 1	4.0 $\pm$ 0.3	4.7 $\pm$ 0.5	22.4 $\pm$ 0.9	14.3 $\pm$ 1.2
<i>n</i> = 1 +	1.4 $\pm$ 0.1	3.3 $\pm$ 0.3	9.2 $\pm$ 0.5	10.2 $\pm$ 1.0
<i>n</i> = 2	3.8 $\pm$ 0.3	10.5 $\pm$ 0.6	23.4 $\pm$ 1.7	45.6 $\pm$ 3.8
<i>n</i> = 3	3.7 $\pm$ 0.2	6.8 $\pm$ 0.4	7.9 $\pm$ 0.8	11.1 $\pm$ 1.3
<i>n</i> = 4	4.3 $\pm$ 0.2	5.8 $\pm$ 0.2	6.2 $\pm$ 1.1	6.2 $\pm$ 1.1
<i>n</i> = 5	5.3 $\pm$ 0.2	6.1 $\pm$ 0.2	5.1 $\pm$ 0.9	4.1 $\pm$ 1.4
<i>n</i> = 6	5.9 $\pm$ 0.2	6.0 $\pm$ 0.3	—	—
<i>n</i> > 6	70.4 $\pm$ 1.4	56.2 $\pm$ 1.3	—	—

Fig. 2. Percentage of dimers in the preparation of rat-liver polysomes in response to feeding with the complete and tryptophan-free amino acid mixtures. Patterns of the polysomes prepared from the two dietary groups of rats were compared 1–7 hr. after giving the amino acid mixtures by tube. The proportion of dimers in the polysome population of the tryptophan-deficient groups (▲—▲) and groups receiving the complete amino acid mixture (○—○) was measured with a compensating polar planimeter (see the Materials and Methods section). Two hours after giving the tryptophan-deficient amino acid mixture to a group of starving rats, their stomachs were aspirated and half of the group was re-fed with the tryptophan-deficient mixture (▲---▲) and the other half was fed with the complete amino acid mixture (▲---○).

the incomplete mixture, whereas the other was given the complete amino acid mixture; the rats from these two groups were killed 2 hr. later. The

liver polysome profiles from all five groups were assessed by the percentage distribution of aggregated sizes. The percentage of dimers in the polysome population for the various groups is shown in Fig. 2. Re-feeding with the tryptophan-free amino acid mixture maintained the proportion of dimers that was observed at 2 hr. and 4 hr. in the groups allowed to absorb the tryptophan-free mixture of amino acids undisturbed, whereas administering the mixture with tryptophan restored the state of polyribosome aggregation found in the group receiving the complete amino acid mixture alone (Fig. 2).

Oligosome patterns. After sedimentation of the polysomes by centrifugation, the supernatant fluid was centrifuged at 105 000*g* for a further period of 3 hr. to precipitate the remaining oligosomes as a post-polysome pellet. In contrast with our preliminary study, which was confined to polysomes (Fleck *et al.* 1965), in the present studies the oligosomes were also harvested, and changes in distribution of ribosomal aggregates were related to dietary amino acid supply. A typical pattern of oligosomes from livers of rats fed with the complete and tryptophan-free amino acid mixtures is presented in Fig. 3. The amount of ultraviolet-absorbing material in the oligosome suspension per unit weight of liver was measured by the total area under the curve of the oligosome tracing. This area was 0.67 $\pm$ 0.08 unit of the polar planimeter for oligosomes harvested from rats fed with the complete mixture and 1.81 $\pm$ 0.30 units when rats were fed with the tryptophan-deficient mixture. The percentage distribution of ribosomal aggregates in the post-polysome patterns after feeding with the

Fig. 3. Patterns of oligosomes from the post-polysome pellet on sucrose density gradients. Oligosomes from livers of rats fed with the complete amino acid mixture (----) and tryptophan-free mixture (—) were harvested from the post-polysome supernatant into a post-polysome pellet by centrifugation at 105000g for 3 hr. Pellets from the two groups were suspended in 5 mM-tris-HCl, pH 7.6, 25 mM-KCl and 5 mM-MgCl₂ and analysed on sucrose gradients (5–25%, w/v) in the suspension medium. $E_{260\text{ m}\mu}$ was recorded automatically by using a flow-cell device in conjunction with a recording spectrophotometer. The peaks of ultraviolet-absorbing material correspond to the aggregate size of oligosomes, where n is the number of ribosomes aggregated; $n=1+$, intermediate between dimer ($n=2$) and monomer ($n=1$); $n=f$, ferritin.

respective amino acid mixtures for 2 hr. in six experiments is shown in Table 2. The dimers in this fraction increased from 23% of the total ultraviolet-absorbing material in rats given the complete mixture to 46% in the tryptophan-deficient rats. Based on the greater amount of ultraviolet-absorbing material in the post-polysome fraction from tryptophan-deficient rats, there is an absolute increase in dimers. Thus the polysome and oligosome fractions both accumulate dimers under conditions of tryptophan deficiency. A peak of 260 m μ -absorbing material intermediate between $n=1$ (monomer, estimated 73s) and $n=2$ (dimer, $S_{20,w}$ 113s, as determined by analysis on the Spinco model E ultracentrifuge), which occurred consistently in the polysome pattern, was also evident in the post-polysome pattern and was more marked after the tryptophan-deficient amino acid feeding. This intermediate peak did not appear to be an artifact due to the aggregation of sub-units in 5 mM-Mg²⁺, since the entire procedure for oligosome preparation was also performed in the presence of 1.5 mM-Mg²⁺ for comparison, and even under these conditions the intermediate peak remained clearly discernible and of the same magnitude.

It is generally recognized that polysomes are ribosomes held together by single-stranded messenger RNA. This concept is supported by the evidence that polysomes are broken down to single ribosomes after the addition of trace amounts of pancreatic ribonuclease (EC 2.7.7.16) (Wettstein *et al.* 1963; Gierer, 1963). The effects of ribonuclease

Fig. 4. Demonstration of the lability of the intermediate ribosomal aggregate when oligosomes are incubated with ribonuclease. Oligosomes (0.35 mg.) of the post-polysome pellet (—) from tryptophan-deficient rats were incubated at 37° with 0.0075 μ g. of ribonuclease in 0.1 ml. for 5 min. (----) and 10 min. (-.-.-). The incubation mixtures were chilled to stop the reaction and 0.05 ml. was layered on 4.6 ml. of 5–25% (w/v) sucrose gradient in 5 mM-tris-HCl, pH 7.6, 25 mM-KCl and 5 mM-MgCl₂. Analysis of the sucrose-gradient patterns is described in Fig. 3.

on our oligosome fraction were also examined. Oligosomes (0.34–0.4 mg. of RNA) were incubated with ribonuclease (0.0075 μ g.) in 0.1 ml. for 5 and 10 min. at 37°. A portion (0.05 ml.) of the digest was layered on to a sucrose gradient (5–25%, w/v) and centrifuged for 2 hr. at 90000g. Under these mild conditions of enzymic hydrolysis of the single-stranded RNA, partial breakdown of the aggregates to single ribosomes (73s) was observed (Fig. 4). The rate of disappearance of the intermediate peak (1+, Figs. 1 and 3), however, was slightly greater than for the dimer.

The position of ferritin on the gradient of fractionated oligosomes was assessed by the following evidence. The position of the fraction marked $n=f$ (Fig. 3), which is lighter than the monomer (73s), coincides with the sedimentation coefficient 65s for ferritin (Petermann & Hamilton, 1957). This peak is the only fraction on the gradient which absorbs ultraviolet light at 320 m μ , characteristic of ferritin (Jackson *et al.* 1964), and it resists digestion with ribonuclease (Fig. 4). Consequently we consider this peak to consist mainly of ferritin.

Amino acid-incorporating activity of polysomes and oligosomes. From the change in polysome and oligosome patterns with diet, it might be expected that the capacity of these cell particles to incorporate labelled amino acids in a cell-free system would be different. A change from the complete to tryptophan-free amino acid mixture affected the amino acid-incorporating capacity of isolated liver polysomes and oligosomes in the same manner as shown previously for liver microsomes (Fleck *et al.* 1965). This correlation in incorporating activity between the liver microsome fraction and the poly-

somes was noted for hypophysectomized and normal rats by Staehelin (1965), but was not observed by Korner (1961, 1964) and Moolenburgh, Otten, Kassenaar & Querido (1965).

A comparison was made between the effect of amino acid supply on cell sap and on polysomes in relation to their capacity to support amino acid incorporation into polysome protein in a cell-free system. Both polysomes and oligosomes from rats previously fed with either amino acid mixture incorporated DL-[1- ^{14}C]leucine and L-[Me- ^{14}C]tryptophan (μmoles per mg. of protein) in the molar ratio 4.5:1. The polysomes from the tryptophan-deficient rats incorporated 20% less leucine and 14% less tryptophan than did polysomes from the rats fed with the complete mixture (Table 3). These relative polysome activities were maintained whether the cell sap used came from either group of rats fed with the amino acid mixtures. Maximum incorporation, however, was established with the cell-sap preparation from the rats fed with the complete mixture. By pooling the cell saps, and thus eliminating the difference in pool size of free amino acids, amino acid incorporation with polysomes was expected to be at an intermediate level. In fact, it was less than with cell sap prepared from tryptophan-deficient rats. The evidence clearly suggests that the capacity of polysomes for amino acid incorporation varied in response to the amino acid mixtures fed to the rats. It was also recognized from these data that factors in the cell sap contributed to the effective capacity for amino acid incorporation in the complete system (Hoagland & Askonas, 1963; Bont, Razelman & Bloemendal, 1965).

When the post-polysome pellet fraction (oligo-

somes) replaced the polysomes (0.45 mg. of protein) in the cell-free incorporating system, the amino acid-incorporating activity expressed as μmoles of [^{14}C]leucine or of [^{14}C]tryptophan/mg. of protein was 43% of that observed for polysomes (Table 3). Oligosomes harvested from rats fed with the tryptophan-free amino acid mixture were less active by 9% for the incorporation of radioactive leucine and 6% for tryptophan compared with those from rats fed with the complete mixture. Thus the effect of feeding with the incomplete amino acid mixture was reflected in the amino acid-incorporating capacity of small ribosomal aggregates as well.

Amount of free ribosomes in livers of rats given the complete and incomplete amino acid mixtures. A quantitative analysis was made of RNA and protein in the free-ribosome fraction prepared from rat liver by the iso-octane procedure of Hallinan & Munro (1965a). Table 4 shows the effect of feeding with the two amino acid mixtures on the amount of ribosomes not attached to membrane of the endoplasmic reticulum. Although the RNA/protein ratio remained the same in the free-ribosome preparations irrespective of amino acid supply, there was a 40–50% increase in the yield of RNA and protein of this liver fraction in rats given the mixture lacking tryptophan when compared with the complete mixture. Fig. 5 shows that the RNA of the fraction, when extracted with phenol and distributed on a sucrose density gradient, has the customary three components in the same proportions as those found in whole microsome pellet (Hallinan & Munro, 1965b).

Ribosomal sub-units and RNA in the post-microsomal fraction. Munro, McLean & Hird (1964) described a post-microsomal fraction which can be

Table 3. *Incorporation of [^{14}C]leucine and [^{14}C]tryptophan into polysomes and post-polysome pellets in vitro*

Amino acid mixture		Incorporation of ^{14}C -labelled amino acids			
		Leucine		Tryptophan	
		($\mu\text{moles}/\text{mg.}$ of protein)	% Difference	($\mu\text{moles}/\text{mg.}$ of protein)	% Difference
Polysomes	Cell sap				
Complete	Complete	1029		202	
Tryptophan-free	Complete	816	–21	176	–13
Complete	Tryptophan-free	823		170	
Tryptophan-free	Tryptophan-free	670	–19	145	–15
Oligosomes	Cell sap				
Complete	Pooled	318		74	
Tryptophan-free	Pooled	290	–9	70	–6

Liver polysomes and oligosomes of the post-polysome pellet from rats given either an amino acid mixture lacking tryptophan or the complete mixture were tested for amino acid-incorporating activity. Cell sap was obtained from groups of rats fed with similar mixtures. The incubation mixture and conditions for the cell-free system are described in the Materials and Methods section. Results represent the mean data from two concordant experiments.

Table 4. *Effect of complete and incomplete amino acid mixtures on the free ribosome content of rat liver*

Groups of three or four rats were fed as described in Table 2 and killed 2 hr. later. The liver free-ribosome fraction was prepared by the iso-octane procedure, and the RNA and protein contents were measured. Results of two experiments (A and B) are recorded separately.

Amino acid mixture	RNA content (mg./g. of liver)		Protein content (mg./g. of liver)		Ratio $\frac{\text{RNA}}{\text{protein}}$	
	A	B	A	B	A	B
Complete	0.352	0.390	0.62	0.71	0.56	0.55
Tryptophan-free	0.463	0.640	0.82	1.11	0.56	0.58
Increase (%) after tryptophan-free mixture	32	64	32	56

Fig. 5. RNA fractions from liver ribosomes and the post-microsomal pellet. RNA was extracted from the post-microsomal pellet with 90% (w/v) phenol containing 0.1% hydroxyquinoline (see the Materials and Methods section) and compared with ribosomal RNA. Sucrose-gradient (3–20%, w/v) analysis of the ribosomal RNA (----) and post-microsomal RNA (—) reveals three peaks of RNA in different proportions recorded automatically at 260 mμ with a flow-cell device in conjunction with a recording spectrophotometer. The RNA fractions were isolated from the gradients, and sedimentation coefficients were determined by the Spinco model E ultracentrifuge.

sedimented by prolonged centrifugation from cell sap that was prepared by centrifuging for 1 hr. at 105 000g in the conventional manner. The amount of RNA in this fraction changed with diet; during starvation, the RNA content increased, and diminished again when the animals were absorbing amino acids after a meal of protein. No nutritional effects were observed in the RNA of the cell sap remaining after removal of the post-microsomal fraction. H. N. Munro *et al.* (1964) suggested that the post-microsomal fraction accumulates RNA from breakdown of endoplasmic reticulum. Accordingly, we examined the effect of feeding with complete and tryptophan-deficient amino acid mixtures on the RNA of this fraction. Table 5 shows that the RNA content per g. of liver in the post-microsomal fraction was 34% greater in the

animals fed with the tryptophan-deficient mixture; there was no change in the RNA content of the residual cell sap left after removal of the fraction. Absence of tryptophan thus has the same effect as starvation in increasing the amount of RNA in this fraction.

The sedimentation characteristics of the post-microsomal fraction were examined by Hird *et al.* (1964). Particles with sedimentation coefficients 57s, 32s and 21s were observed. These particles have been analysed on sucrose density gradients. The post-microsomal fraction, suspended in a Mg^{2+} -free phosphate buffer, pH 7.8, containing 0.03 M-potassium hydrogen carbonate, 25 mM-potassium chloride and 2% (w/v) sucrose, was applied to a gradient of 5–25% (w/v) sucrose. In addition, some experiments were performed with homogenizing and suspending media and sucrose gradients containing 1.5 or 5 mM- Mg^{2+} , to determine whether the particles observed in the absence of Mg^{2+} were artifacts caused by breakdown of ribosomes. The density-gradient analyses gave essentially similar results to those obtained in the absence of Mg^{2+} , and the possibility of whole ribosomes being present in the post-microsomal fraction can be excluded. Accordingly, the 57s and 32s components have been identified as ribosomal sub-units. This has been confirmed by extraction of the RNA from the post-microsomal fraction. The RNA components were fractionated on a sucrose density gradient (3–20%, w/v), and sedimentation coefficients for the isolated components were determined by the Spinco model E ultracentrifuge. This RNA yielded 30s, 19s and 4s components, similar to those found in the RNA from intact ribosomes (Fig. 5). However, in contrast with the intact ribosomes, the post-microsomal fraction has a larger component of 19s RNA than of 30s RNA, and the 4s RNA component was largely a contaminant from the cell sap. An attempt was made to identify the 30s and 19s species with the 57s and 32s sub-unit particles

Table 5. *Effect of feeding with complete and tryptophan-free amino acid mixtures on the RNA content of the post-microsomal fraction*

Groups of three or four rats were fed as described in Table 2 and killed 1 hr. later. The liver post-microsomal fraction was prepared from '1 hr.' cell sap, and the RNA content was measured in the '3 hr.' cell sap and PMP as described in the Materials and Methods section. Extracted RNA was measured by ultraviolet absorption at 260 m μ . Results are recorded as the means $\pm$ s.e.m. of four experiments.

Amino acid mixture	RNA (μ g./g. of liver)		RNA of post-microsomal pellet RNA of '3 hr.' cell sap
	In post-microsomal pellet	In '3 hr.' cell sap	
Complete	166 $\pm$ 13	681 $\pm$ 28	0.25 $\pm$ 0.03
Tryptophan-free	223 $\pm$ 13	709 $\pm$ 27	0.31 $\pm$ 0.02
Increase (%) due to tryptophan-free mixture	34	4	24

Table 6. *Uptake of [3 H]orotic acid by RNA of the post-microsomal pellet in response to feeding with incomplete and complete amino acid mixtures*

Groups of three or four rats were injected intraperitoneally with 75 μ C of [3 H]orotic acid/100 g. body wt. immediately after giving either the incomplete or complete amino acid mixtures. The rats were killed 1 hr. later, RNA fractions were extracted from the post-microsomal pellet, and 3 *E* units (*E* unit = vol. applied $\times$ E_{1cm} at 260 m μ) were analysed on sucrose gradients (3–20%, w/v) in acetate–NaCl buffer, pH 5.2 (see the Materials and Methods section). The area under the respective peaks of 30s and 19s RNA was expressed in *E* units, and the specific activity in the RNA fractions was expressed as counts/min./*E* unit. Results are recorded as the means $\pm$ s.e.m. of three experiments.

Amino acid mixture	RNA fractions		
	30s (counts/min./ <i>E</i> unit)	19s	Ratio 30s/19s
Complete	701 $\pm$ 15	1689 $\pm$ 176	0.43 $\pm$ 0.04
Tryptophan-free	385 $\pm$ 47	1067 $\pm$ 141	0.36 $\pm$ 0.00
Decrease (%) after tryptophan-free mixture	45	37	16

isolated individually from a gradient and extracted with phenol to yield free RNA. The 57s particle contained mainly 30s RNA with a trace of 19s RNA. The 32s sub-unit contained some 19s RNA, which was readily degraded to about 8s RNA.

The effect of feeding with complete and tryptophan-deficient amino acid mixtures on the RNA of the 57s and 32s particles was explored. In three separate experiments, the ratio 30s RNA/19s RNA in the post-microsomal pellet was 0.88 (range 0.49–1.36) in animals fed with the complete amino acid mixture and 1.11 (range 0.62–1.70) in those receiving the tryptophan-free mixture. Despite the large variation in these values, in each experiment, the ratio 30s RNA/19s RNA for the tryptophan-deficient groups was always 26% greater than that of the groups fed with the complete mixture. This indicates a larger increment in 57s sub-units in the post-microsomal fraction of tryptophan-deficient animals. In these experiments, the rats were also injected with [5- 3 H]orotic acid imme-

diately after feeding with the amino acid mixtures. No attempt was made to measure specific radioactivity in the acid-soluble nucleotides of the liver. The specific activity of the RNA in the post-microsomal fraction was lower in animals fed with the tryptophan-deficient diet, the decrease being 45% for the 30s RNA and 37% for the 19s RNA (Table 6). This may be related to the larger amount of 30s RNA added to the post-microsomal fraction on feeding with the tryptophan-deficient mixture, and suggests that changes not explicable by variations in pool radioactivity must occur with deletion of tryptophan from the mixture. An effect of the tryptophan-free amino acid mixture on the entry of [3 H]orotic acid into the liver that results in a lower specific activity of the RNA is possible in the short interval of 2 hr. but unlikely. It is also possible, though difficult to assess, that an effect due to an alteration of the RNA precursor pools is a possible explanation of the results. This would have to be considered in future work.

DISCUSSION

In earlier experiments in which amino acids were fed to rats (Munro & Clark, 1959), incorporation of [^{14}C]glycine into the purine bases and [^{14}C]orotic acid into the pyrimidine bases of liver RNA was found to be considerably greater when a complete amino acid mixture was given than when a mixture lacking tryptophan was fed. On the basis of evidence available from other studies of the relationship of amino acid supply to liver RNA metabolism (Clark, Naismith & Munro, 1957), it was concluded that deletion of tryptophan from the mixture had resulted in breakdown of part of the liver-cell RNA with consequent dilution of label in the precursor pool. Thus the completeness of the supply of amino acids may affect the stability of RNA in a part of the liver cell.

The present experiments support these earlier studies by demonstrating that the polysomes in the liver cell are sensitive to amino acid supply. When the various particulate fractions are compared 1 hr. or more after giving either a complete amino acid mixture or a mixture lacking trypto-

phan, the tryptophan-deficient groups show a loss of large polysomes (Fig. 1), an absolute increase in the amount of oligosomes per unit weight of liver (Figs. 1 and 3), an increase in free ribosomes (Table 4) and an accumulation of RNA from ribosomal sub-units in the post-microsomal pellet (Table 5). Fig. 6 displays these various sub-cellular particles without necessarily implying the precise metabolic relationships between them. The response to tryptophan deficiency is readily reversible by feeding with a complete amino acid mixture (Fig. 2). This is of interest, since Revel, Hiatt & Revel (1964) have claimed that the release of monomers and dimers from polysomes is an artifact of homogenization. The fact that the pattern can be reversed on giving a complete amino acid mixture 2 hr. after administering the incomplete mixture is consistent with re-aggregation to polysomes from monomers and dimers as a function of the living cell. A similar reversal of polysome disaggregation has been noted in rats previously treated with ethionine: this compound causes breakdown of polysomes, which can be restored by giving adenine or methionine (Villa-

Fig. 6. Summary of changes comparing responses in fractions to giving the tryptophan-free amino acid mixture and the complete amino acid mixture to starved rats. The flow diagram describes the procedure for preparing particulate fractions, whereas the insert illustrates: (a) the increased amount of small ribosomal aggregates in polysomes of tryptophan-deficient animals, (b) the corresponding polysome patterns which support the illustrations in (a) by showing the increase in oligosomes relative to the total polysome population under the tryptophan-deficient conditions, (c) the increased amount of free ribosomes after feeding with the amino acid mixture lacking tryptophan and (d) the difference in ribosomal sub-unit RNA patterns on sucrose gradients showing an increased proportion of 30 s RNA in the post-microsomal pellet of tryptophan-deficient rats.

Trevino, Shull & Farber, 1963; Villa-Trevino *et al.* 1964; Baglio & Farber, 1965). In the amino acid-feeding experiments, it was clearly demonstrated that the supply of tryptophan in the complete amino acid mixture stimulated the restoration of polysomes.

Our studies on polysome response to amino acid mixtures amplify the preliminary studies of Fleck, *et al.* (1965). These authors also noted that microsomes prepared from the livers of rats given a complete amino acid mixture incorporated [^{14}C]leucine *in vitro* more actively than microsomes obtained from animals fed with a tryptophan-deficient mixture. In the present series of studies, polysomes prepared from these two nutritional groups of rats showed a corresponding difference in their protein-biosynthetic capacity (Table 3), indicating that the microsomes owe this difference in activity to their contained polysomes. The oligosome fraction, which had only 43% of the activity of the polysomes, also differed in activity according to the prior feeding of the rats, although the difference was less marked (Table 3). Both polysome and oligosome fractions incorporated leucine and tryptophan in the molar ratio 4.5:1, which approximates to the relative requirements of these two amino acids by the rat (Hegsted, 1964). This suggests that both the polysome and oligosome fractions are synthesizing protein *in vitro* containing average proportions of these two amino acids.

The nature of the particles accumulating during polysome breakdown merits some comment. In the polysome and oligosome fractions, the major change occurred among the dimers (Figs. 1 and 3). The reason for this in preference to monomer accumulation is not clear, but Howell, Loeb & Tomkins (1964) also observed dimer accumulation in a fraction of liver prepared from adrenalectomized rats. The dimer form may be an artifact of aggregation of monomers, but treatment with ribonuclease suggests that it can be broken down to monomers by using a concentration of this enzyme which attacks only the messenger strand (unpublished observations), and may therefore be a functional entity within the cell. Deficiency of tryptophan also results in an accumulation of ribosomal sub-units in the post-microsomal pellet fraction of the liver homogenate, with a preferential increase in the larger sub-unit. The smaller sub-unit may be unstable because of the dissociation of the ribosome (Tashiro & Siekvitz, 1965) and therefore may not accumulate. The relationship of these sub-units to the genesis of polysomes, as revealed by recent studies on HeLa cells (Joklik & Becker, 1965; Girard, Latham, Penman & Darnell, 1965), will require consideration in the exploration of the mechanism by which amino acid supply

affects the proportions of different subcellular particles in the liver.

The work was supported by U.S. Public Health Service Fellowship Grant 1-F2-CA-28000-01 from the National Cancer Institute and U.S. Public Health Service Grant CA-07087-03.

REFERENCES

- Allison, J. B., Wannemacher, R. W., jun., Banks, W. L., jun. & Wunner, W. H. (1964). *J. Nutr.* **84**, 383.
- Baglio, C. M. & Farber, E. (1965). *J. molec. Biol.* **12**, 466.
- Bont, W. S., Razelman, G. & Bloemendal, H. (1965). *Biochem. J.* **95**, 15c.
- Clark, C. M., Naismith, D. J. & Munro, H. N. (1957). *Biochim. biophys. Acta*, **23**, 587.
- Drysdale, J. W. & Munro, H. N. (1966). *J. biol. Chem.* (in the Press).
- Fleck, A. & Begg, D. (1965). *Biochim. biophys. Acta*, **108**, 333.
- Fleck, A. & Munro, H. N. (1962). *Biochim. biophys. Acta*, **55**, 571.
- Fleck, A., Shepherd, J. & Munro, H. N. (1965). *Science*, **150**, 628.
- Gierer, A. (1963). *J. molec. Biol.* **6**, 148.
- Girard, M., Latham, H., Penman, S. & Darnell, J. E. (1965). *J. molec. Biol.* **11**, 187.
- Hallinan, T. & Munro, H. N. (1965a). *Quart. J. exp. Physiol.* **50**, 93.
- Hallinan, T. & Munro, H. N. (1965b). *Biochim. biophys. Acta*, **108**, 285.
- Hegsted, D. M. (1964). In *Mammalian Protein Metabolism*, vol. 2, p. 163. Ed. by Allison, J. B. & Munro, H. N. New York: Academic Press Inc.
- Hird, H. J., McLean, E. J. T. & Munro, H. N. (1964). *Biochim. biophys. Acta*, **87**, 219.
- Hoagland, M. B. & Askonas, B. A. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 130.
- Howell, R. R., Loeb, J. N. & Tomkins, G. M. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 241.
- Jackson, R. J., Munro, A. J. & Korner, A. (1964). *Biochim. biophys. Acta*, **91**, 666.
- Joklik, W. K. & Becker, Y. (1965). *J. molec. Biol.* **13**, 511.
- Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
- Kirby, K. S. (1962). *Biochim. biophys. Acta*, **55**, 545.
- Korner, A. (1961). *Biochem. J.* **81**, 292.
- Korner, A. (1964). *Biochem. J.* **92**, 449.
- Littlefield, J. W. & Keller, E. B. (1957). *J. biol. Chem.* **224**, 13.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Moolenburgh, M. C. W., Otten, J., Kassenaar, A. A. H. & Querido, A. (1965). *Proc. kon. ned. Akad. Wetenschappen*, **68**, 251.
- Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.
- Munro, H. N. & Clark, C. M. (1959). *Biochim. biophys. Acta*, **33**, 551.
- Munro, H. N., McLean, E. J. T. & Hird, H. J. (1964). *J. Nutr.* **83**, 186.

- Noll, H., Staehelin, T. & Wettstein, F. O. (1963). *Nature, Lond.*, **198**, 632.
- Petermann, M. L. & Hamilton, M. G. (1957). *J. biol. Chem.* **224**, 725.
- Revel, M., Hiatt, H. H. & Revel, J. (1964). *Science*, **146**, 1311.
- Staehelin, M. (1965). *Biochem. Z.* **342**, 459.
- Tashiro, Y. & Siekevitz, P. (1965). *J. molec. Biol.* **11**, 149.
- Villa-Trevino, S., Farber, E., Staehelin, T., Wettstein, F. O. & Noll, H. (1964). *J. biol. Chem.* **239**, 3826.
- Villa-Trevino, S., Shull, K. H. & Farber, E. (1963). *J. biol. Chem.* **238**, 1757.
- Webb, T. E., Blobel, G. & Potter, V. R. (1964). *Cancer Res.* **24**, 1229.
- Wettstein, F. O., Staehelin, T. & Noll, H. (1963). *Nature, Lond.*, **197**, 430.