Differences in the Ribosomes Prepared from Lactating and Non-Lactating Bovine Mammary Gland

By M. D. HERRINGTON AND A. O. HAWTREY

Department of Biochemistry, University College of Rhodesia, Salisbury, Rhodesia

(Received 31 July 1970)

1. Ribosomes prepared from bovine lactating mammary gland are able to synthesize protein, whereas similar preparations from non-lactating glands are not. Washing the ribosome suspensions through a medium containing 0.5M-ammonium chloride enhanced their ability to incorporate phenylalanine into polyphenylalanine. 2. Ribosomes isolated from non-lactating bovine mammary gland, in contrast with those from rat liver and lactating mammary gland, contained significant amounts of extraneous nucleases. These enzymes could be removed by washing with a medium A buffer containing 0.5M-ammonium chloride. 3. Only those ribosomes from functionally active tissues were able to bind poly-uridylic acid and phenylalanyl-tRNA.

Ribosomes have a complex structure that is essential for protein synthesis (Hoagland, 1960) and consist of ribosomal RNA and a number of different structural proteins (Waller & Harris, 1961; Traut, 1966; Traut, Moore, Delius, Noller & Tissières, 1967; Fridlender & Wettstein, 1970). Only the complete ribosome consisting of both subunits can bring about the process of translation in the protein-synthesizing system. Neither of the ribosomal subparticles can replace the complete ribosome (Gilbert, 1963; Pestka & Nirenberg, 1966). These factors suggest a complexity in the functioning of the individual components present in the ribosomes.

It has been shown that unfertilized sea-urchin eggs are incapable of synthesizing protein (Maggio, Vittorelli, Rinaldi & Monroy, 1964). This is apparently prevented by some structural condition of the ribosomes that is changed upon fertilization, since trypsin treatment of the particles from the unfertilized egg enables amino acid incorporation to occur (Monroy, Maggio & Rinaldi, 1965). However, Stavy & Gross (1967) suggested that there is no general defect in egg ribosomes, nor is there an inhibitor or a deficiency in cofactors, but that the stored messenger RNA is not in a translatable state until after the egg is activated.

Since we have shown in a previous paper (Herrington & Hawtrey, 1969a) that microsomes prepared from non-lactating bovine mammary glands are unable to incorporate amino acids into protein, further studies were conducted to ascertain whether there were any physical or chemical differences between ribosomes of lactating and non-lactating mammary glands. Our approach to this

problem has been to investigate the mechanisms of protein synthesis with particular reference to the binding of either messenger RNA or the aminoacyl-tRNA complex to the ribosomes. Of particular interest was whether the protein-synthetic processes in the mammary gland were analogous to those found in sea-urchin eggs.

MATERIALS AND METHODS

Chemicals. ATP (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt) and crystalline GSH were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. GTP (sodium salt) and pyruvate kinase were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and puromycin dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. [³H]Phenylalanine (1.65 Ci/mmol) and poly(U) (potassium salt) were supplied by Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. and [³H]poly(U) (7.8 μ Ci/mmol) from Miles Laboratories Inc., Elkhart, Ind., U.S.A.

Solutions of ATP, GTP, GSH and phosphoenolpyruvate were prepared before use and adjusted to pH 7.6 with 0.3M-KOH.

Scintillation chemicals. 2,5-Diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) were obtained from Packard Instrument Co. Inc., La Grange, Ill., U.S.A.

Preparation of ribosomes. Rat liver homogenates and bovine mammary-gland homogenates were prepared in medium A [0.25M-sucrose+TKM (50mM-tris-HCl buffer, pH 7.6, 25mM-KCl and 5mM-MgCl₂)] as described by Hawtrey, Schirren & Dijkstra (1963) and Herrington & Hawtrey (1969a) respectively.

The tissue suspension was centrifuged at 1400g for 10min to remove cell debris and nuclei and the resultant

supernatant was centrifuged again at 10000g for 20 min (Spinco no. 30 rotor) to remove the mitochondria.

Ribosomes were prepared from the post-mitochondrial supernatant by adding 10% (w/v) sodium deoxycholate (General Biochemicals, Laboratory Park, Chagrin Falls, Ohio, U.S.A.) to a final concentration of 1% and allowing to stand at 0°C for 15 min. Approx. 15 ml of the supernatant was carefully layered over a preformed double-layered discontinuous gradient containing 11 ml of 0.8 M-sucrose in TKM buffer on top of 9 ml of 1.5 M-sucrose in TKM buffer. The gradients were centrifuged for 4 h at 92000g (Spinco no. 30 rotor) and yielded a pellet of crude or unwashed ribosomes. Each pellet of ribosomes was suspended in approx. 1 ml of medium A and left overnight.

Purified or washed ribosomes were prepared by suspending the crude ribosomes in 0.25 M-sucrose containing (final concns.) 0.5 M-NH₄Cl, 50 mM-tris-HCl buffer, pH 7.6, and 10 mM-MgCl₂ and centrifuging at 140000g for 45 min (Spinco no. 40 rotor). The washing process was repeated once again before suspending the ribosomal pellets by light homogenization in an appropriate volume of buffer.

Denatured material was removed from the crude and purified ribosomal suspensions by centrifugation at 1400g for 10 min. The final suspension of ribosomes was kept at 0°C and used within 2 days.

Preparation of pH5 enzymes. Rat liver cell sap was prepared by centrifugation of the homogenate at 100000g for 4 h and from this the pH5-enzyme fraction was made as described by Hawtrey *et al.* (1963).

Preparation of [³H]phenylalanyl-tRNA. The tRNA of rat liver pH5 enzyme was highly labelled with [³H]-phenylalanine by a modification of the method described by Hawtrey (1965). The reaction medium contained rat liver pH5 enzyme (approx. 250 mg of protein), 9.4 mM-ATP, pH 7.6, 0.16 M-sucrose, 31 mM-tris-HCl buffer, pH 7.6, 16 mM-KCl, 15 mM-MgCl₂, 3 mg of deacylated rat liver tRNA, and [³H]phenylalanine (50 μCi) in a total volume of 48 ml. Incubation was carried out for 30 min at 37°C with continuous shaking on a Warburg bath.

After cooling in ice for 15 min the mixture was adjusted to pH 5.2 with 1 M-acetic acid and allowed to stand for a further 15 min in ice before centrifuging. This procedure was repeated twice before extraction and isolation of the labelled RNA with an equal volume of 90% (w/v) phenol. After shaking at room temperature for 90 min the two phases were separated by centrifugation at 1400g for 30 min and the top aqueous layer collected. RNA was precipitated from this aqueous phase by the addition of 0.1 vol. of 20% (w/v) potassium acetate and 2.5 vol. of 96% (v/v) ethanol and left for 24 h at -15°C. The precipitated RNA was dissolved in a minimum volume of water and dialysed against water until free of phenol.

Assay of phenylalanine incorporation into ribosomes. The incubation medium for the incorporation of labelled phenylalanine into ribosomes is given in the legend to Fig. 1. Incubations were carried out under aerobic conditions at 37°C and after the predetermined times the reactions were terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The samples were kept overnight at 0°C before collection of the protein precipitates by centrifugation. Washing and counting of radioactivity was carried out as previously described (Herrington & Hawtrey, 1969b).

Reaction mixture for the binding of phenylalanyl-tRNA to ribosomes. The method devised by Nirenberg & Leder (1964) was used for the assay of binding of phenylalanyl-tRNA. The reaction mixture contained 83 mM-sucrose, 100 mM-tris-HCl buffer, pH 7.6, 50 mM-KCl, 20 mM-MgCl₂, 5 mM-phosphoenolpyruvate, 50 μg of pyruvate kinase, 0.875 mM-puromycin dihydrochloride, 500 μg of poly(U), [³H]phenylalanyl-tRNA (106130 c.p.m.) and an appropriate amount of the indicated ribosomes in a total volume of 3 ml. Binding was carried out aerobically at 25°C. At the indicated times, 0.5 ml samples were taken from the incubation medium and immediately placed into 3 ml of ice-cold Nirenberg buffer, containing 0.1 M-tris-HCl buffer, pH 7.6, 0.05 M-KCl and 0.02 M-MgCl₂. The diluted reaction mixture was poured on to Millipore HA 0.45 μm filters and washed with buffer under suction to remove unbound [³H]phenylalanyl-tRNA. The washing procedure was repeated at least three times. The filters were then dried in air for about 1 h, placed in counting vials with 10 ml of scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in chromatography-grade toluene] and their radioactivities counted in a liquid-scintillation spectrometer (Packard Tri-Carb, model 2002) at approx. 54% efficiency.

Sedimentation analysis of [³H]poly(U) binding to ribosomes. The 16-34% (w/v) sucrose density gradients were prepared by the method of Henshaw, Bojarski & Hiatt (1963) as modified by Stead, Nourse & Hawtrey (1964). A 2 ml sample containing ribosomes plus [³H]-poly(U) (1 μCi), previously incubated for 5 min at 37°C, was layered on top of the linear gradient. The centrifugation was carried out at 60000g_{av} for 4 h (Spinco no. SW 25.1 rotor) with a rotor temperature of 4°C. After centrifugation, fractions (1 ml) were collected by using a hypodermic needle, diluted to 2 ml with water and their *E*₂₆₀ was measured in a spectrophotometer (Unicam model SP.500). The radioactivity of alternate samples (1 ml) were counted in Bray's (1960) solution.

Ribonuclease assays. The nuclease activity present on the ribosomes was measured as described by Herrington & Hawtrey (1970a).

Determinations. Protein was determined by the biuret method of Gornall, Bardawill & David (1949) with bovine serum albumin as standard. The concentration of ribosomes was measured spectrophotometrically at 260 nm (*E*_{1%¹cm} 135) (Nourse, 1969).

RESULTS

Incorporation of phenylalanine by crude and purified ribosomes. A number of workers have studied amino acid incorporation *in vitro* into ribonucleoprotein particles prepared from guinea-pig and rabbit lactating mammary glands (Frazer & Gutfreund, 1958; Brew & Campbell, 1967; Baird & Herriman, 1968). In all cases it has been proved to be an ideal system in which to study the mechanisms of protein biosynthesis.

The results presented in Fig. 1 show that [³H]-phenylalanine incorporation into rat liver and lactating bovine mammary-gland ribosomes, puri-

Fig. 1. Time-course of $[^3\text{H}]$ phenylalanine incorporation into ribosomes prepared from rat liver (▲), lactating bovine mammary gland (○) and non-lactating bovine mammary gland (●). ----, Crude unwashed ribosomes and —, purified washed ribosomes. The incubation mixture contained: $2\mu\text{mol}$ of ATP, $0.5\mu\text{mol}$ of GTP, $1.6\mu\text{mol}$ of GSH, $5\mu\text{mol}$ of phosphoenolpyruvate, $50\mu\text{g}$ of pyruvate kinase, 0.19M -sucrose, 37mM -tris-HCl buffer, pH 7.6, 18mM -KCl, 6mM - MgCl_2 , $100\mu\text{g}$ of poly(U), rat liver pH5 enzyme (0.5mg of protein), $[^3\text{H}]$ phenylalanine ($1\mu\text{Ci}$) and ribosomes (0.2 – 0.9mg) in a total volume of 1.6ml . All incubations were carried out in duplicate at 37°C for the indicated time. For each experiment, controls that contained the complete incubation mixture with the exception of the ribosomes were included.

fied by washing with a medium containing 0.5M -ammonium chloride, follows an almost linear response over a period of 20 min. However, the ribosomes prepared from non-lactating bovine mammary gland show a markedly low amount of amino acid incorporation. Although protein synthesis is obviously taking place, it is at a considerably decreased rate.

On the other hand if the ribosomes prepared from rat liver and lactating and non-lactating bovine mammary gland were not purified by washing, a similar trend in incorporation was observed although to a lesser extent. Washing of the lactating mammary-gland ribosomes increased phenylalanine incorporation considerably more than that shown

Fig. 2. Time-course of the binding of $[^3\text{H}]$ phenylalanyl-tRNA to purified ribosomes isolated from rat liver (▲), lactating bovine mammary gland (○) and non-lactating bovine mammary gland (●). See the Materials and Methods section for experimental details.

by rat liver, presumably due to the removal of milk proteins from the peptidyl binding sites of the ribosomes. The trends shown in Fig. 1 represent the average results obtained from two independent experiments, each experiment of which was carried out in duplicate.

Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958) showed that during protein synthesis, the amino acid is transferred from tRNA to uncharacterized protein chains being formed on the ribosomes. Whereas this is true for the rat liver system (Herrington & Hawtrey, 1970b), the ribosomes isolated from non-lactating bovine mammary gland are unable to transfer $[^3\text{H}]$ phenylalanine from $[^3\text{H}]$ phenylalanyl-tRNA (M. D. Herrington & A. O. Hawtrey, unpublished work). This result and those shown in Fig. 1 suggest that the ribosomal protein-synthetic mechanism in this tissue is unable to function efficiently.

Binding of $[^3\text{H}]$ phenylalanyl-tRNA to ribosomes. The binding of either aminoacyl-tRNA or peptidyl-tRNA to ribosomes has been studied in many laboratories (Kaji & Kaji, 1963; Bretscher & Marcker, 1966; Ghosh & Khorana, 1967; Jonak & Rychlik, 1970). The assays of the results shown in

Fig. 2 are based upon that described by Nirenberg & Leder (1964) in which the ribosomes and [^3H]-phenylalanyl-tRNA bound to ribosomes is retained by Millipore filters.

As can be seen from the results (Fig. 2) both rat liver and lactating bovine mammary-gland ribosomes were capable of binding [^3H]phenylalanyl-tRNA in the presence of poly(U). Since the rate of interaction increases with temperature, studies were carried out at 25°C to prevent the maximum being attained much before 15 min. However, ribosomes from non-lactating bovine mammary gland, that had been purified of all extraneous protein by washing with ammonium chloride were almost incapable of binding the activated aminoacyl-tRNA complexes.

Interaction of ribosomes and poly(U). It has been reported that synthetic messenger RNA such as poly(U) is able to associate with both 70S ribosomes and with 30S ribosomal subunits (Barondes & Nirenberg, 1962; Okamoto & Takanami, 1963;

Kaji, Suzuka & Kaji, 1966a). Other synthetic polyribonucleotides have also been shown to bind to ribosomes (Takanami & Okamoto, 1963; Moore, 1966).

The results of Fig. 3 show that although crude, unwashed lactating and non-lactating bovine mammary-gland ribosomes both exhibit a sedimentation coefficient of approx. 73S, only the former were able to associate with the synthetic poly(U) message (Fig. 3b). Those ribosomes prepared from non-lactating bovine mammary gland were unable to interact with poly(U). This inability to bind poly(U) may be associated with the high nuclease activity present on non-lactating bovine mammary-gland ribosomes (Table 1) since corresponding preparations from lactating mammary glands have a low nuclease activity and are able to interact with the synthetic message.

Nevertheless, when ribosomes prepared from rat liver, lactating and non-lactating bovine mammary gland were washed twice in a medium containing

Fig. 3. Sucrose-density-gradient centrifugation of crude unwashed ribosomes prepared from non-lactating (a) and lactating (b) bovine mammary gland and the binding of [^3H]poly(U) to such ribosomes. Gradients contained 16–34% (w/v) sucrose, 50 mM-tris-HCl buffer, pH 7.6, and 5 mM-MgCl₂. Centrifugation was carried out at 60 000 g_{av} for 4 h (Spinco no. SW 25.1 rotor). ●, E_{260} ; ----, radioactivity.

Table 1. Nuclease activity exhibited by different ribosome preparations

The reaction medium contained: 0.27 M-sucrose, 54 mM-tris-HCl buffer, pH 7.6, 16 mM-KCl, 11 mM-MgCl₂, 500 μg of deacylated rat liver tRNA and an appropriate amount of the indicated ribosomes in a total volume of 1.13 ml. All incubations were carried out in duplicate at 37°C for 20 min.

Source of ribosomes	Treatment	Nuclease activity	
		% degradation	Increase in E_{260} /mg of ribosome
Non-lactating mammary gland	Crude, unwashed	8.82	4.171
Lactating mammary gland	Crude, unwashed	0.51	0.06
Rat liver	Purified, washed	0.12	0.01
Non-lactating mammary gland	Purified, washed	0.12	0.15
Lactating mammary gland	Purified, washed	0.44	0.04

Fig. 4. Sucrose-density-gradient centrifugation of purified washed ribosomes prepared from rat liver (a), non-lactating (b) and lactating (c) bovine mammary gland and the binding of [^3H]poly(U) to such ribosomes. Details of gradient centrifugation are identical with those given in Fig. 3. ●, E_{260} ; ----, radioactivity.

0.5M-ammonium chloride, only ribosomes from rat liver and lactating glands were able to form a message-ribosome complex (Figs. 4a and 4c). Although the extraneous ribonuclease on the non-lactating bovine mammary-gland ribosomes had been removed (Table 1) they were nevertheless still incapable of binding poly(U) (Fig. 4b).

DISCUSSION

The lactating mammary gland, because of its rapid anabolic metabolism, is an ideal system in which to study the mechanisms of protein synthesis. However, little is known of the biochemical changes that occur in the transition period between conception and lactation.

In previous work it has been shown that a major portion of the tRNA from non-lactating glands lacks the terminal trinucleotide sequence -pCpCpA (Herrington & Hawtrey, 1970a,b) and as such, is unable to form activated aminoacyl-tRNA complexes. At the onset of lactation, however, the -pCpCpA group is replaced, thereby allowing protein synthesis to proceed as normal. An analogous observation has also been reported by Turkington (1969) who showed that RNA extracted from explants of mammary glands in tissue culture increased in their ability to accept amino acids as the tissue proceeded from non-lactation through to lactation.

In addition to this mechanism for the control of milk-protein biosynthesis, it appears from the results published previously (Herrington & Hawtrey, 1969a) and in the present paper that the ribosomes from non-lactating bovine mammary gland are unable to incorporate amino acids into protein. This is in agreement with Baird & Herriman (1968) who reported that ribosomal protein synthesis

markedly increased 30-fold from 2 days before birth to 12 days after birth. In all cases, however, no explanation has been offered as to why pre-partum ribosomes are unable to synthesize protein.

The results presented show that neither the synthetic poly(U) message nor the activated aminoacyl-tRNA complex were able to bind to ribosomes prepared from non-lactating bovine mammary gland. On the other hand both components are able to associate with 70S ribosomes from rat liver and lactating mammary gland, suggesting some structural difference between ribosomes from functional tissue and essentially non-functional tissue.

The question of whether nucleases present on non-lactating bovine mammary-gland ribosomes played any significant role in altering the structure of the ribosomes, and hence their ability to bind message, required special consideration. Such activity could lead to degradation of the ribosomal RNA and breakdown of the particles. When, however, these ribosomes were prepared in the presence of rat liver cell sap, which contains an inhibitor of ribonuclease, they were still incapable of synthesizing protein and binding message. This suggests that the extraneous ribonucleases do not affect the ribosomal structure; and even when the nucleases are removed by washing the ribonucleo-protein particles remain almost completely non-functional.

It is noteworthy that although ribosomes from non-lactating mammary glands contain extraneous nucleases, those from lactating tissue and rat liver do not. It seems possible that in mammary-gland tissue these enzymes decrease in activity after conception although no explanation can be offered as to the role that they play in the tissue before the hormonal initiation of lactation.

In order for the ribosomes to serve as an apparatus for translation they should satisfy three basic requirements: (a) be able to associate into the form of 73S particles which consist of two associated subparticles, (b) have an intact chain of ribosomal RNA and (c) contain the complete set of ribosomal structural proteins.

Both lactating mammary gland and rat liver ribosomes must satisfy these requirements since they are able to incorporate amino acids into protein. Ribosomes prepared from non-lactating glands, however, definitely satisfy the first two requirements since they exhibit a sedimentation coefficient of approx. 73S and their ribosomal RNA is intact having sedimentation coefficients of approx. 18S and 28S (Herrington & Hawtreay, 1969a). It appears, therefore, that the malfunction lies with the protein moiety of these ribosomes.

Monroy *et al.* (1965) reported that unfertilized sea-urchin eggs when treated with trypsin gained the ability to incorporate amino acids into protein, presumably due to the removal of a protein coat around the ribosomes that prevented their functioning. This condition, however, was not found to exist in non-lactating mammary-gland ribosomes, since trypsin treatment had no effect on their ability to synthesize protein (M. D. Herrington & A. O. Hawtreay, unpublished work).

Another alternative was that the ribosomes lacked a few proteins, since Nomura & Traub (1966) have shown that the separation of a definite portion of the ribosomal protein from the 30S subunit deprived it of its ability to bind the template polynucleotide. Similarly, very mild treatment of the ribosomes destroys its ability to bind poly(U) (Kaji, Suzuka & Kaji, 1966b; Zak, Nair & Rabinowitz, 1966). The absence of certain proteins from non-lactating mammary-gland ribosomes would hardly be expected to affect drastically their sedimentation coefficient but would critically influence their ability to synthesize protein.

Polyacrylamide-gel electrophoresis of proteins isolated from rat liver and lactating mammary-gland ribosomes gave approx. 20 bands. Proteins isolated from non-lactating mammary-gland ribosomes would not migrate. Different protein solvents were tried as well as 7.5 and 15% (w/v) polyacrylamide gels, but to no avail. For this reason it is difficult to state categorically that vital proteins are missing but the circumstantial evidence obtained suggests so.

In conclusion it appears that non-lactating bovine mammary-gland ribosomes are non-functional owing to a deficiency of certain ribosomal proteins that would cause the particles to remain inactive. Once conception occurs, however, hormonal interaction may result in the missing proteins being replaced thereby forming functional ribosomes.

The authors are indebted to Dr R. C. Elliott and to Henderson Research Station for supplying valuable dairy cows.

REFERENCES

- Baird, G. D. & Herriman, I. D. (1968). *Biochim. biophys. Acta*, **166**, 162.
- Barondes, S. H. & Nirenberg, M. W. (1962). *Science, N.Y.*, **138**, 813.
- Bray, A. G. (1960). *Analyt. Biochem.* **1**, 279.
- Bretscher, M. S. & Marcker, K. A. (1966). *Nature, Lond.*, **211**, 380.
- Brew, K. & Campbell, P. N. (1967). *Biochem. J.* **102**, 265.
- Frazer, M. J. & Gutfreund, H. (1958). *Proc. R. Soc. B.* **149**, 392.
- Fridlander, B. R. & Wettstein, F. O. (1970). *Biochem. biophys. Res. Commun.* **39**, 247.
- Ghosh, H. P. & Khorana, H. G. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 2455.
- Gilbert, W. (1963). *J. molec. Biol.* **6**, 374.
- Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Hawtreay, A. O. (1965). *S. Afr. J. med. Sci.* **30**, 100.
- Hawtreay, A. O., Schirren, V. & Dijkstra, J. (1963). *Biochem. J.* **88**, 106.
- Henshaw, E. C., Bojarski, T. B. & Hiatt, H. H. (1963). *J. molec. Biol.* **7**, 122.
- Herrington, M. D. & Hawtreay, A. O. (1969a). *S. Afr. J. med. Sci.* **34**, 49.
- Herrington, M. D. & Hawtreay, A. O. (1969b). *Biochem. J.* **115**, 671.
- Herrington, M. D. & Hawtreay, A. O. (1970a). *Biochem. J.* **119**, 323.
- Herrington, M. D. & Hawtreay, A. O. (1970b). *Biochem. J.* **116**, 405.
- Hoagland, M. (1960). In *The Nucleic Acids*, vol. 3, p. 349. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
- Jonak, J. & Rychlik, I. (1970). *Biochim. biophys. Acta*, **199**, 421.
- Kaji, A. & Kaji, H. (1963). *Biochem. biophys. Res. Commun.* **13**, 186.
- Kaji, H., Suzuka, I. & Kaji, A. (1966a). *J. biol. Chem.* **241**, 1251.
- Kaji, H., Suzuka, I. & Kaji, A. (1966b). *J. molec. Biol.* **18**, 219.
- Maggio, R., Vittorelli, M. L., Rinaldi, A. M. & Monroy, A. (1964). *Biochem. biophys. Res. Commun.* **15**, 436.
- Monroy, A., Maggio, R. & Rinaldi, A. M. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 107.
- Moore, P. B. (1966). *J. molec. Biol.* **18**, 8.
- Nirenberg, M. W. & Leder, P. (1964). *Science, N.Y.*, **145**, 1399.
- Nomura, M. & Traub, P. (1966). *Organizational Bio-synthesis*. New York: Academic Press Inc.
- Nourse, L. D. (1969). Ph.D. Thesis: University of Natal.
- Okamoto, T. & Takanami, M. (1963). *Biochim. biophys. Acta*, **68**, 325.
- Pestka, S. & Nirenberg, M. (1966). *Cold Spring Harb. Symp. quant. Biol.* **31**, 641.

- Stavy, L. & Gross, P. R. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 735.
- Stead, R., Nourse, L. D. & Hawtrey, A. O. (1964). *S. Afr. J. med. Sci.* **29**, 79.
- Takanami, M. & Okamoto, T. (1963). *Biochem. biophys. Res. Commun.* **13**, 297.
- Traut, R. R. (1966). *J. molec. Biol.* **21**, 571.
- Traut, R. R., Moore, P. B., Delius, H., Noller, H. & Tissières, A. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 1294.
- Turkington, R. W. (1969). *J. biol. Chem.* **244**, 5140.
- Waller, J. P. & Harris, J. J. (1961). *Proc. natn. Acad. Sci. U.S.A.* **47**, 18.
- Zak, R., Nair, K. & Rabinowitz, M. (1966). *Nature, Lond.*, **210**, 169.