Factors Controlling Aminoacyl-Transfer-Ribonucleic Acid Synthesis *in vitro* by a Plant System

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(Received 24 August 1970)

1. Factors affecting aminoacyl-tRNA synthesis *in vitro* by cell-free preparations from bean leaves were investigated. 2. Evidence was obtained that optimum concentrations as well as correct ratios of Mg^{2+} and ATP are required for aminoacyl-tRNA synthesis in the bean-leaf system. 3. The results indicated that pH is a controlling factor having differential effects on the formation of individual aminoacyl-tRNA species. The possible micro-regulatory function of pH in protein synthesis *in vivo* is discussed with special reference to alanyl-tRNA formation. 4. Very low rates of alanine-stimulated pyrophosphate exchange were observed in the absence of tRNA. This observation is discussed relative to proposals about the mechanism of aminoacyl-tRNA synthesis.

Since the formation of aminoacyl-tRNA is an essential step in the synthesis of protein, factors that affect this step also control the synthesis of protein to a greater or lesser extent. Henshall & Goodwin (1964) indicated that the activity of the aminoacyl-tRNA synthetase was a limiting factor, and the involvement of tRNA species in the control of protein synthesis has been studied in detail (Stent, 1964; Anderson, 1969; Sueoka & Kano-Sueoka, 1970). It is therefore apparent that an understanding of the conditions affecting aminoacyl-tRNA formation is essential to cell-free investigations on the mechanism of protein synthesis. Studies with microbial systems (Marshall & Zamecnik, 1969, 1970; Kosakowski, Neidhardt & Bock, 1970; Cole & Schimmel, 1970) have demonstrated good reaction rates *in vitro*. Similarly, the studies by Rubin, Kelmers & Goldstein (1967), Loftfield & Eigner (1968), Anderson (1969), Shearn & Horowitz (1969), Mehler (1970) and others, have clearly shown controlling effects of many factors on aminoacyl-tRNA synthesis.

Data from plant systems has previously been presented for the pyrophosphate exchange assay (Fowden & Frankton, 1968; Hall & Cocking, 1966) and direct assay of aminoacyl-tRNA synthesis (Attwood & Cocking, 1965; Moustafa & Lyttleton, 1963). However, during studies of the rates of aminoacyl-tRNA synthesis by bean-leaf systems (Hall & Tao, 1970) we observed that the effects of several factors such as potassium chloride concentration and ATP/ Mg^{2+} ratio differed from those reported for other organisms. The present paper

details these results and also describes the profound effect of pH noted in our system.

EXPERIMENTAL

Materials. Expanding leaves were used from *Phaseolus vulgaris* L. (var. Tendergreen) plants grown under normal greenhouse conditions or under artificial illumination (3500 ft-candles) with 16 h days (26°C day, 17°C night) in the University of Wisconsin Biotron.

Chemicals and techniques were as described previously (Hall & Tao, 1970) except as noted below. Cytoplasmic and chloroplast fractions were prepared as described by Arglebe & Hall (1969), and the cytoplasmic pH5 enzyme was isolated as described by Hall & Tao (1970). The chloroplast fraction was kept in a hypo-osmotic medium containing 10 mM-Hepes buffer [1-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid], pH 7.8, 15 mM-magnesium acetate and 4 mM-dithiothreitol for 30 min and then ground with a Virtis '45' homogenizer. Unbroken chloroplasts were removed by centrifugation at 26 000 g_{av} for 30 min, and the pH5 enzyme was obtained from the dark-green supernatant.

RNA was concentrated by precipitation with ethanolic potassium acetate rather than by Aquacide. The precipitate was suspended in 50 mM-Hepes buffer, pH 7.6, containing dithiothreitol (4 mM), and then dialysed as described by Hall & Tao (1970).

Assay for aminoacyl-tRNA synthesis. Unless otherwise specified the incubation mixture contained bean-leaf pH5 synthetase fraction (50 µg of protein), ATP (64 nmol), $MgCl_2$ (500 nmol), KCl (4 µmol), RNA (30 µg) and labelled amino acid in a final volume of 0.1 ml. The reaction was carried out at 30°C for 30 min at pH 7.6 or as stated. All results are the mean of at least two replicates. The composition of the ^{14}C -labelled amino acid mixture was:

alanine (10%), arginine (6.5%), aspartate (9.0%), glutamate (12.5%), glycine (5%), leucine (12%), isoleucine (5%), lysine (5.5%), phenylalanine (7%), proline (6%), serine (5%), threonine (6%), tyrosine (3.5%), valine (7%). The reaction was terminated by pipetting a 25 μ l portion on to a filter-paper disc, previously wetted with 20% (w/v) trichloroacetic acid. For radioisotope counting, the filter-paper discs were air-dried and washed twice with 5% (w/v) trichloroacetic acid, and then once each in ethanol-ether (1:1, v/v) and ether for 10 min. The discs were again air-dried and then the radioactivity was assayed by scintillation counting (Davies & Hall, 1969) in a Beckman model LS 200 spectrometer.

RESULTS

Effect of ATP and Mg^{2+} concentrations. By using a fixed concentration of ATP but different concentrations of magnesium chloride relatively high aminoacylation rates were observed at concentration ratios of between 1 μ mol of ATP:1 μ mol of magnesium chloride and 1 μ mol of ATP:10 μ mol of magnesium chloride in a final volume of 0.1 ml (Fig. 1a). The optimum ATP/magnesium chloride concentration ratio (0.5 μ mol:4 μ mol in 0.1 ml; i.e. 5 mm:40 mm or 1:8) was defined by maintaining a fixed concentration of magnesium chloride and varying the concentration of ATP (Fig. 1b). When ATP and Mg^{2+} were present at this optimum ratio of 1:8, but at different concentrations, peaks were obtained at 640 μ M-ATP:5 mm-magnesium chloride and at 5 mm-ATP:40 mm-magnesium chloride (Fig. 2). However, at 20 mm-ATP:160 mm-magnesium chloride no activity could be detected even though the optimum ATP/magnesium chloride ratio was maintained.

Effect of potassium chloride concentration. The concentration of potassium chloride affected the rate of aminoacyl-tRNA synthesis in this plant system. The addition to the system of increasing amounts of potassium chloride up to 4 μ mol caused a steady increase in the rate of aminoacylation. The rate of amino [14 C]acyl-tRNA formation was nearly doubled by increasing the amount of potassium chloride from 1 μ mol to 4 μ mol. Further increases of potassium chloride concentration up to 10 μ mol gave varying, but lesser effects.

Effect of RNA concentration. The rate of amino- [14 C]acyl-tRNA formation increased steadily with the addition of increasing amounts of RNA up to 10 μ g/0.1 ml, when a plateau was reached (Fig. 3).

Effect of enzyme concentration. The effect of enzyme concentration on amino [14 C]acyl-tRNA formation is presented in Fig. 4. An increase from 25 μ g to 50 μ g of enzyme more than doubled the rate of formation of amino [14 C]acyl-tRNA. Further addition of pH 5 enzyme caused a decrease in aminoacyl-tRNA formation. This is in agreement with the cofactor requirement in aminoacyl-tRNA

Fig. 1. Effect of the ATP/ $MgCl_2$ ratio on aminoacyl-tRNA synthesis for an amino acid mixture. The incubation mixture (see the Experimental section) was modified to contain, in a final volume of 0.1 ml, bean-leaf pH 5-synthetase fraction (400 μ g of protein), KCl (1 μ mol), RNA (30 μ g) and 0.63 μ Ci of 14 C-labelled amino acid mixture. Either the $MgCl_2$ concentration was varied at a constant ATP concentration (a) or the ATP concentration varied at a constant $MgCl_2$ concentration (b).

Fig. 2. Effect of varying the amounts of ATP and $MgCl_2$ at a constant ratio of 1:8. The incubation mixture was as stated for Fig. 1.

formation, since the higher the concentration of the enzyme, the lower will be the relative concentration of free cofactors. The binding of cofactors by high concentrations of enzyme prevents them from reacting with the substrate and produces a considerable inhibition of the overall reaction. A similar curve was obtained by Anderson & Rowan (1966) using a tobacco-leaf system.

Effect of supplied amino acid concentration. An increase in the amount of supplied 14 C-labelled amino acid mixture from 0.385 μ g-atom of carbon to 24.64 μ g-atom of carbon (0.02 mCi–0.64 mCi) resulted in an increase in apparent aminoacyl-tRNA formation (Fig. 5). However, if the amount of supplied amino acid was rate-limiting, higher rates

Fig. 3. Effect of RNA concentration on aminoacyl-tRNA formation. The standard incubation mixture was modified to contain, in a final volume of 0.1ml, bean-leaf pH 5-synthetase fraction (300 μg), ATP (63 nmol), MgCl₂ (0.5 μmol), KCl (1 μmol), ¹⁴C-labelled amino acid mixture (0.63 μCi) and the amounts of RNA as stated.

Fig. 4. Effect of enzyme concentration on aminoacyl-tRNA formation. The conditions were as described in the Experimental section, but with different amounts of pH 5-synthetase fraction as stated.

of incorporation into aminoacyl-tRNA would be obtained up to a maximum at which the system would be saturated with amino acid. Since no

Fig. 5. Effect of different amounts of amino acid mixture on aminoacyl-tRNA formation. The conditions were as described in the Experimental section, but with different amounts of ¹⁴C-labelled amino acid mixture as stated.

Fig. 6. Effect of temperature on aminoacyl-tRNA formation. The conditions were as described in the Experimental section, but with an amino acid mixture containing 1.6×10^6 d.p.m. and under different incubation temperatures. □, 1°C; ●, 10°C; △, 26°C; ○, 30°C; ▲, 35°C.

maximum was observed, and also since the percentage of supplied amino acid incorporated into aminoacyl-tRNA decreased at the higher amounts (from 5.35% when 0.385 μg-atom of ¹⁴C-labelled amino acid mixture was supplied to 1.15% when 24.64 μg-atom was supplied) it is clear that the contamination of the system with endogenous unlabelled amino acids (Hall & Tao, 1970) remains a problem with our system.

Effect of temperature. Linear reaction rates were obtained for up to 40 min incubation at temperatures up to an optimum value of 30°C (Fig. 6). Incubation at 35°C gave a slightly higher initial

Fig. 7. Effect of pH on aminoacyl-tRNA formation for an amino acid mixture. The conditions were as described in the Experimental section.

Fig. 8. Effect of pH on the formation of alanyl-tRNA and tyrosyl-tRNA. The conditions were as described in the Experimental section, but with 550 ng of [14 C]alanine or 3 ng of [14 C]tyrosine. $\circ$, Alanyl-tRNA; $\bullet$, tyrosyl-tRNA.

velocity, but the reaction only continued for some 20 min under these conditions. At 1°C , a low reaction rate was observed; hence during the preparation of the incubation systems, and just before the reaction was stopped, the error caused by the difference of reaction time was minimized by keeping the tubes at 1°C .

Effect of pH on the rate of aminoacyl-tRNA synthesis. When the formation of aminoacyl-tRNA was assayed over a range of pH values, activity was detected between pH 5.2 and 8.2 (Fig. 7). Two peaks (pH 5.8 and 7.6) were obtained for a mixture of amino acids; however, when individual amino acids were used as substrates at various pH values, different curves were obtained (Fig. 8). Usually, at pH 8.2 or pH values below 5.2, there was little or no enzyme activity. Low activity at pH 8.2 may result from rapid hydrolysis of the aminoacyl-tRNA at this pH. Short half-lives for many aminoacyl-

Fig. 9. Derivation of the Michaelis constant for alanyl-tRNA synthetase at pH 5.8 and pH 7.6. The conditions were as stated for Fig. 8. $\circ$, Observed value at pH 5.8; $\bullet$, value predicted by computer at pH 5.8; $\triangle$, observed value at pH 7.6; $\blacktriangle$, value predicted by computer at pH 7.6.

tRNA species at pH values higher than 8.2 have been reported by Gatica, Allende, Mora, Allende & Medina (1966). Since some tyrosyl-tRNA synthesis was observed at pH 8.2 (Fig. 8), it is possible that tyrosyl-tRNA is comparatively stable at this pH. As the aminoacyl-tRNA synthetase preparation was obtained by precipitation at pH 5, the lack of activity at pH 5.2 was not unexpected. Both phenylalanyl-tRNA and alanyl-tRNA formation showed peaks at pH 5.8 and 7.6, but there was only one peak (pH 7.6) for tyrosyl-tRNA formation.

Computer analysis of the results presented in Fig. 9 gave Michaelis constants of $4.8 \mu\text{M}$ for alanyl-tRNA formation at pH 7.6 and $34.5 \mu\text{M}$ at pH 5.8. (The program used was an iteration procedure to arrive at a least squares solution for best fit of the Michaelis-Menten equation. It was written in FORTRAN 11-D language for use on an IBM 1620 computer. A limited number of copies of this program are available on request by letter. A similar program is on file at the University of Wisconsin Computing Center, with the title of UWHAUS.) The possibility that these differences might result from the use of a synthetase fraction prepared from a mixture of cytoplasmic and chloroplast fractions was checked by careful separation of chloroplasts from the cytoplasm before preparation of the pH 5 enzymes. However, both cytoplasmic pH 5-enzyme fraction and chloroplast pH 5-enzyme fraction yielded double peaks for alanyl-tRNA synthesis similar to those shown in Fig. 8 obtained by using an extract that was not resolved into chloroplast and cytoplasmic fractions.

Fig. 10. Effect of changing pH value during incubation on tyrosyl-tRNA and alanyl-tRNA formation. The conditions were as stated in the Experimental section, but with a mixture of [^3H]alanine (1×10^6 d.p.m., 5.4 Ci/mmol) and [^{14}C]tyrosine (0.75×10^6 d.p.m., 43 Ci/mmol). The pH was adjusted from 6.4 to 7.6 after 15 min by addition of 1M-KOH solution. ●, Formation of [^3H]alanyl-tRNA at pH 6.4; ○, formation of [^{14}C]alanyl-tRNA at pH 7.6; ▲, formation of [^{14}C]tyrosyl-tRNA at pH 6.4; △, formation of [^{14}C]tyrosyl-tRNA at pH 7.6.

Fig. 12. Effect of pH on the alanine-stimulated pyrophosphate exchange. Conditions were as stated in Fig. 11.

Fig. 11. Effect of RNA concentration on alanine-stimulated pyrophosphate exchange. The mixture contained, in a final volume of 1 ml, bean-leaf pH5-synthetase fraction (350 μg of protein), ATP (10 μmol), MgCl_2 (10 μmol), KCl (8 μmol), alanine (100 μmol), [^{32}P]PP $_i$ (10 μmol ; 1.6×10^6 c.p.m.) and RNA (80 μg). Incubation was for 30 min at pH 8.0 and 30°C.

Since pH evidently had a major effect on the reaction it was decided to determine whether relatively small changes in acidity would have differential effects on the charging reaction for the individual amino acids. Lowering the pH from 7.6 to 6.4 greatly decreased the rate of alanyl-tRNA formation (Fig. 8). When a mixture of [^{14}C]tyrosine and [^3H]alanine was incubated, inactivation of alanyl-tRNA formation occurred at pH 6.4, but tyrosyl-tRNA was readily synthesized after changing the pH to 7.6 (Fig. 10). Differential effects

were also apparent when a mixture of [^{14}C]tyrosine and [^3H]alanine were incubated at pH 6.4 or pH 7.6.

Effect of pH on alanine-stimulated pyrophosphate exchange. Because of the marked effects of pH on alanyl-tRNA synthesis it was decided to determine if the adenylation reaction (Marshall & Zamecnik, 1970) was similarly affected. This was tested by observing the rate of pyrophosphate exchange stimulated by alanine. The reaction was dependent on the amount of tRNA present (Fig. 11) and also on the pH (Fig. 12).

DISCUSSION

The enzyme-catalysed attachment of amino acids to tRNA is well recognized as an essential step in the incorporation of amino acids into proteins. The reaction is conveniently studied by measuring the incorporation of ^{14}C -labelled amino acids into tRNA (Eisenstadt & Brawerman, 1964; Anderson & Fowden, 1969). Factors affecting the reaction include pH and Mg^{2+} , ATP, potassium chloride, enzyme and RNA concentrations. Novelli (1967) has emphasized the importance of ascertaining the optimum ATP/ Mg^{2+} concentration ratio for aminoacyl-tRNA formation, since the requirements may differ widely for the various aminoacyl-tRNA synthetases. The optimum ATP/ Mg^{2+} concentration ratio for a mixture of amino acids for the bean-leaf cell-free system was found to be 1:8 (Fig. 1). An important observation appears to be that maximal activity for aminoacyl-tRNA synthetase depended not only upon the optimum ATP/ Mg^{2+} ratio but also upon the absolute concentrations of Mg^{2+} and ATP present in the system. If Mg^{2+} and ATP were present in inappropriate amounts, no activity at all was observed, even though ATP and

Mg²⁺ were at an optimum ratio (Fig. 2). The two peaks of activity observed on variation of the concentrations of ATP and Mg²⁺ (with an optimum ATP/Mg²⁺ ratio) may reflect different cofactor requirements of aminoacyl-tRNA synthetases for the individual amino acids, or for the separate stages in the overall aminoacylation of tRNA. Attention to both Mg²⁺/ATP ratio and concentration may, therefore, be essential to demonstrate aminoacyl-tRNA synthesis in a cell-free system.

The bean-leaf system differs from the *Escherichia coli* system in that K⁺ had a marked effect on the rate of reaction, whereas no effect was observed with the microbial system (Rubin *et al.* 1967). Significant effects of tRNA and enzyme concentrations on aminoacyl-tRNA formation were also observed (Figs. 3 and 4). Optimum conditions including temperature and pH are presented in the Experimental section. Optimization of reaction conditions gave a 24-fold increase in the observed rate, from 0.6 pmol of amino acids incorporated/min per mg of RNA (Hall & Tao, 1970, Table 6) to 15.3 pmol of amino acids incorporated/min per mg of RNA (Fig. 7, pH 7.6).

The critical dependence of aminoacyl-tRNA formation on pH in the bean-leaf system tested is particularly notable. Variation in activity with pH is evident for a mixture of amino acids (Fig. 7), but it is more clearly demonstrated for individual amino acids (Fig. 8). For alanine virtually no activity was observed when aminoacylation was at pH 6.4, but high rates were obtained at pH 5.8 and 7.6. The lack of arginyl-tRNA synthetase observed in a mung-bean system (Fowden & Frankton, 1968) may have resulted from either this pH effect or the cofactor dependence discussed above. The sharpness of the peaks is unusual, but since the formation of aminoacyl-tRNA comprises at least two stages (kinase-like activation and phosphorylase-like attachment of adenylated amino acid to tRNA), or a triple-transfer mechanism (Dixon & Webb, 1964), the sharpness may not conflict with theoretical considerations. A lesser, but evident, effect of pH on the adenylation of an amino acid mixture was confirmed by using the pyrophosphate-exchange reaction (Fig. 12). The rate of pyrophosphate exchange stimulated by alanine was enhanced in the presence of added tRNA (Fig. 11). This indicates that alternative mechanisms of aminoacyl-tRNA formation, such as those discussed by Cole & Schimmel (1970) and Marshall & Zamecnik (1970), exist not only in the micro-organism but also in the plant system *in vitro*.

Some support for the hypothesis that the pH effects reflect activities of different enzymes is provided by the different Michaelis constants calculated for alanyl-tRNA synthesis (Fig. 9); that for the reaction at pH 7.6 being much lower (4.8 mM)

than that at pH 5.8 (34.5 mM). Our value for the reaction at pH 7.6 is much lower than that observed by Attwood & Cocking (1965) using the hydroxamate and pyrophosphate-exchange assays; this is partially a result of the use of a purified enzyme fraction in their studies, and indicates the importance of further purification for our system.

It seems possible that our results may reflect differential charging of codon-specific tRNA species (e.g. GCU, GCC and GCA and GCG for alanine), or the activities of different aminoacyl-tRNA synthetases. Separation of chloroplasts from cytoplasm and subsequent preparation of synthetase-enzyme fractions yielded results similar to the activity observed from the total synthetase preparation by using a mixture of amino acids (Fig. 7). It does not seem likely, therefore, that the effects are due to a mixture of synthetases obtained from different cellular locations. However, as with the studies of Marshall & Zamecnik (1970) further purification of both the synthetase and tRNA fractions is required before definite conclusions can be drawn.

The immediate effects of varying the pH value during the reaction (Fig. 10) and the differential effects of pH on individual amino acids suggest the probability that pH may function as a control factor during protein synthesis through its effect on aminoacylation of tRNA. This could be particularly effective if the aminoacylation of tRNA species corresponding to 'regulation codons' (Anderson, 1969), present in rate-limiting amounts, were highly pH-sensitive. Such systems could be particularly valuable in highly organized tissues, especially in higher-plant cells where the localization of intermediary metabolites and hence of pH within the cell is well established (MacLennan, Beevers & Harley, 1963; Oaks & Bidwell, 1970). Such control systems would preclude the aminoacylation of each and every tRNA at a single pH value, and could well account, in addition to cofactor dependence discussed above, for the general failure to obtain synthesis of defined peptides and proteins by cell-free preparations from higher plants. Such a microregulation system (Sueoka & Kano-Sueoka, 1970) could operate *in vivo* by localized pH changes produced by newly synthesized protein. The rate of aminoacylation of tRNA species specifically required in the synthesis of the protein would be decreased by unfavourable pH values, thereby decreasing the rate of synthesis of that protein.

This paper is published with the approval of the Director, Research Program Division, College of Agricultural and Life Sciences, and supported in part with funds administered by the University of Wisconsin Research Committee. We thank Dr P. J. Kaesberg for his continued interest, Miss A. R. Rastogi for help in the isolation of

tRNA, Mr R. M. Rice and Mr R. R. Daniels for the computer program for Michaelis constants, and Mr R. C. McLeester for excellent technical assistance.

REFERENCES

- Anderson, W. F. (1969). *Proc. natn. Acad. Sci. U.S.A.* **62**, 566.
- Anderson, J. W. & Fowden, L. (1969). *Pl. Physiol., Lancaster*, **44**, 60.
- Anderson, J. W. & Rowan, K. S. (1966). *Biochem. J.* **101**, 9.
- Arglebe, C. & Hall, T. C. (1969). *Pl. Cell Physiol., Tokyo*, **10**, 171.
- Attwood, M. M. & Cocking, E. C. (1965). *Biochem. J.* **96**, 616.
- Cole, F. X. & Schimmel, P. R. (1970). *Biochemistry, Easton*, **9**, 480.
- Davies, J. W. & Hall, T. C. (1969). *Analyt. Biochem.* **27**, 77.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 297. New York: Academic Press Inc.
- Eisenstadt, J. & Brawerman, G. (1964). *Biochim. biophys. Acta*, **80**, 463.
- Fowden, L. & Frankton, J. B. (1968). *Phytochemistry*, **7**, 1077.
- Gatica, M., Allende, C. C., Mora, G., Allende, J. E. & Medina, J. (1966). *Biochim. biophys. Acta*, **129**, 201.
- Hall, T. C. & Cocking, E. C. (1966). *Pl. Cell Physiol., Tokyo*, **7**, 343.
- Hall, T. C. & Tao, K. L. (1970). *Biochem. J.* **117**, 853.
- Henshall, J. D. & Goodwin, T. W. (1964). *Phytochemistry*, **3**, 677.
- Kosakowski, M. H. J. E., Neidhardt, F. C. & Bock, A. (1970). *Eur. J. Biochem.* **12**, 74.
- Loftfield, R. B. & Eigner, E. (1968). *Biochemistry, Easton*, **7**, 110.
- MacLennan, D. H., Beevers, H. & Harley, J. L. (1963). *Biochem. J.* **89**, 316.
- Marshall, R. D. & Zamecnik, P. C. (1969). *Biochim. biophys. Acta*, **181**, 454.
- Marshall, R. D. & Zamecnik, P. C. (1970). *Biochim. biophys. Acta*, **198**, 376.
- Mehler, A. H. (1970). *Prog. nucleic Acid molec. Biol.* **10**, 1.
- Moustafa, E. & Lyttleton, J. W. (1963). *Biochim. biophys. Acta*, **68**, 45.
- Novelli, G. D. (1967). *A. Rev. Biochem.* **36**, 449.
- Oaks, A. & Bidwell, R. G. S. (1970). *A. Rev. Pl. Physiol.* **21**, 43.
- Rubin, I. B., Kelmers, A. D. & Goldstein, G. (1967). *Analyt. Biochem.* **20**, 533.
- Shearn, A. & Horowitz, N. H. (1969). *Biochemistry, Easton*, **8**, 295.
- Stent, G. S. (1964). *Science, N.Y.*, **44**, 816.
- Sueoka, N. & Kano-Sueoka, T. (1970). *Prog. nucleic Acid molec. Biol.* **10**, 23.