The Extraction and Assay of Aminoacyl-Transfer-Ribonucleic Acid Synthetases of Tobacco Leaf

By J. W. ANDERSON* AND K. S. ROWAN

Botany School, University of Melbourne, Parkville, N. 2, Victoria, Australia

(Received 2 March 1966)

1. Aminoacyl-transfer-RNA synthetase activity in extracts prepared from tobacco leaf was increased 3–5-fold when sodium thioglycollate (30mM) and magnesium chloride (16mM) were included in the extraction medium. Omitting sucrose (0.45M) from the extraction medium did not alter the activity. 2. Activity was a linear function of enzyme concentration up to 1 disk (30mg. fresh wt.)/ml. and was not affected by dialysis at any concentration. 3. Activity increased about 13-fold above control values when a mixture of 21 amino acids and amides (1mM) was added to the reaction mixture. 4. Under the conditions used in the standard assay for aminoacyl-transfer-RNA synthetase activity K_m (ATP) was 0.65mM and K_m (L-amino acids) was 70 μ M. 5. Activity above the control value was found with all amino acids and amides tested except alanine, arginine, glutamic acid, glutamine and hydroxyproline. Activity was highest with leucine, isoleucine, valine, cysteine and histidine. Total activity with a mixture of 21 amino acids and amides was 20% lower than the total activity of the enzymes assayed separately.

Studies of amino acid-s-RNA[†] ligases (trivial name, aminoacyl-s-RNA synthetases) extracted from plants have been reviewed by Rowan (1966), and Henshall & Goodwin (1964) have studied synthetase activity in relation to germination. The enzymes specific for several amino acids in various species of plants have been purified and described (Moustafa, 1963; Peterson & Fowden, 1963, 1965; Attwood & Cocking, 1965) but the properties of the enzymes when assayed collectively have not been examined. The present paper describes the extraction and properties of aminoacyl-s-RNA synthetases extracted from tobacco leaf and precedes a study (Anderson & Rowan, 1966) of the synthetases in relation to senescence and to the mechanism of action of 6-furfurylaminopurine (kinetin) in delaying senescence of leaf tissue of tobacco. Preparations responded 10–15-fold to added amino acids and amides, and the enzyme activity could be assayed in less than 20mg. fresh wt. of tobacco leaf.

MATERIALS AND METHODS

Tobacco leaf. Tobacco plants (*Nicotiana tabacum* L., cultivar Virginian Gold) were grown in a glass house at

not less than 13°. Mature, dark-green, leaves were harvested and disks 1.3cm. in diameter cut from the leaves with a cork borer, the main veins being avoided. Replicate samples contained equal numbers of disks from each leaf; the approximate mass of each disk was 30mg.

Chemicals. Unless stated otherwise, alanine, aspartic acid, isoleucine, leucine, phenylalanine, tryptophan, tyrosine and valine were supplied by S. A. F. Hoffmann-La Roche, Basle, Switzerland. Histidine, lysine hydrochloride, methionine, proline, serine and threonine were supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Arginine hydrochloride, asparagine, cysteine hydrochloride, glutamic acid, glutamine and glycine were supplied by British Drug Houses Ltd., Poole, Dorset. Hydroxyproline was supplied by Mann Research Laboratories Inc., New York, N.Y., U.S.A.

ATP (disodium salt; 99% pure) was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A., and stock solutions (60mM) were adjusted to pH 7.2 with solid NaHCO₃.

[³²P]Orthophosphate was supplied carrier-free by The Radiochemical Centre, Amersham, Bucks., and converted into [³²P]pyrophosphate by pyrolysis at 400° (Lee Peng, 1956). [³²P]Pyrophosphate was diluted with unlabelled Na₄P₂O₇ (30mM) so as to contain 30–120 counts/min./m μ mole of pyrophosphate. When the [³²P]pyrophosphate was analysed by paper chromatography with propyl acetate-formic acid-water (11:5:3, by vol.) as solvent, 95% of the radioactivity was in pyrophosphate, 5% in orthophosphate and no [³²P]polyphosphate was formed.

Sodium thioglycollate was prepared by titrating thioglycollic acid (L.R. grade; British Drug Houses Ltd.) to pH 7.2 with 2N-NaOH. Activated charcoal was supplied by British Drug Houses Ltd.

* Present address: Department of Botany, University College London, Gower Street, London, W.C. 1.

[†] Abbreviations: s-RNA, transfer or 'soluble' RNA; ATPase, adenosine triphosphatase.

Extraction of aminoacyl-s-RNA synthetases. Unless stated otherwise, the extraction medium contained sodium thioglycollate (30 mM), MgCl_2 (16 mM) and sucrose (0.45 M) in 0.1 M-tris-maleic acid-NaOH buffer, pH 7.2. The tissue was ground for 3 min. with a glass pestle and mortar with 1 ml. of extraction medium/5 leaf disks. The enzyme solution was recovered by centrifugation at 15000g for 10 min. Particulate matter in the supernatant solution was removed by further centrifugation at 105000g for 1 hr. All procedures were performed at 0.5°. ATPase and pyrophosphatase activities and the concentration of soluble protein were determined in the undiluted extract, but unless stated otherwise aminoacyl-s-RNA synthetase activity was assayed at 0.8 disk/ml.

Assay of aminoacyl-s-RNA synthetase activity. Assays were performed at 40° with incubation mixtures containing 0.7 ml. of diluted enzyme extract (containing 11.2 μmoles of MgCl_2 , 21 μmoles of sodium thioglycollate and 70 μmoles of tris-maleic acid-NaOH buffer, pH 7.2), 6 μmoles of ATP, 3 μmoles of [^{32}P]pyrophosphate and 1 μmole of each of the 21 amino acids and amides. The final volume was 1.0 ml. Amino acids and amides were omitted from control incubation mixtures. After 15 min. 3.5 ml. of ice-cold 6% (w/v) trichloroacetic acid was added to precipitate protein. Opalescence, if present, was removed by centrifugation.

The incorporation of [^{32}P]pyrophosphate into ATP was measured by the method of Hele & Finch (1960) with micro-filter tubes supplied by A. Gallenkamp Ltd. (no. AG 12A $\times$ 3). [^{32}P]Orthophosphate eluted from the charcoal was counted with a liquid counting tube (20th Century Electronics Ltd., type M6 or M6H) coupled to a scaler (Phillips Electronics, type PW 4032 or 4035). A standard solution of [^{32}P]pyrophosphate (3 μM in 0.1 N-HCl) prepared from the sample used in the assay was used to convert counts/min. into μmoles of pyrophosphate incorporated into ATP/min. (aminoacyl-s-RNA synthetase units). Absolute and specific synthetase activities are expressed as the differences in units/ml. and units/mg. of protein respectively between the results obtained with incubation mixtures prepared with and without added amino acids. The charcoal adsorbed 95% of the ATP applied but did not adsorb pyrophosphate; 97% of the radioactivity eluted from the charcoal with 0.1 N-ammonia in 50% (v/v) ethanol was recovered in ATP and ADP when the eluate was examined by paper chromatography.

Assay of ATPase activity. ATPase activity was measured in undiluted extracts at 40°. Incubation mixtures contained 0.7 ml. of extract (containing 11.2 μmoles of MgCl_2 , 21 μmoles of sodium thioglycollate and 70 μmoles of tris-maleic acid-NaOH buffer, pH 7.2) and 1–12 μmoles of ATP in a final volume of 1.0 ml. After 15 min. 3.5 ml. of 6% (w/v) trichloroacetic acid was added. Protein was removed by centrifugation and a sample of the supernatant solution analysed for orthophosphate. Appropriate control measurements of orthophosphate were made in extract and ATP solutions.

Since thioglycollate interferes with the estimation of orthophosphate involving molybdate the following modification to the method of Allen (1940) was made. The sample containing orthophosphate and thioglycollate (1.0–4.0 ml.) was added to a 25 ml. volumetric flask with an equal volume of KIO_3 (14 mM) (i.e. 3 moles of KIO_3 /mole of thioglycollate). The mixture was diluted to approx. 15 ml. and shaken, releasing free iodine. This was reduced

to iodide when the reagents for Allen's (1940) method were added in the usual way. The solution was diluted to 25 ml. and E_{700} measured. Orthophosphate released from ATP ($\mu\text{moles/ml.}$ of reaction mixture) was calculated by using standard samples of orthophosphate containing sodium thioglycollate (14.1 μmoles).

Absolute activity was expressed as μmoles of orthophosphate released from ATP/ml. of incubating mixture/15 min. so that the amount of orthophosphate produced could be compared directly with the amount of ATP in the original mixture.

Assay of pyrophosphatase activity. Pyrophosphatase activity was measured in undiluted extracts with methods similar to those used above for ATPase except that the substrate was 3.0 μmoles of $\text{Na}_4\text{P}_2\text{O}_7$. Absolute activity was expressed as μmoles of orthophosphate released from pyrophosphate/ml. of incubating mixture/15 min.

Estimation of protein. The protein in 1.0 ml. of undiluted extract was precipitated with 0.5 ml. of ice-cold 30% (w/v) trichloroacetic acid. After centrifugation, the precipitate was washed with 10 ml. of 10% (w/v) trichloroacetic acid and twice with 5 ml. of acetone. Protein in the precipitate was determined by the method of Ellman (1962) as described by Anderson & Rowan (1965).

Estimation of orthophosphate. Orthophosphate in samples without thioglycollate was measured by the method of Allen (1940).

RESULTS

Effect of including sodium thioglycollate in the extraction medium on the aminoacyl-s-RNA synthetase activity. Since including sodium thioglycollate in the extraction medium increased the activity of peptidase extracted from tobacco leaf (Anderson & Rowan, 1965), the effect of concentration of thioglycollate (1–27 mM) in the extraction medium on synthetase activity was examined (Fig. 1). Activity increased and brown colour decreased in the extracts with increasing concentration of thioglycollate up to 5.5 mM. Activity did not increase or brown colour decrease at concentrations greater than 5.5 mM. Extracts prepared with the standard extraction medium containing thioglycollate (30 mM) are well within the range needed for optimum activity and contain 3–5 times the activity of extracts prepared without thioglycollate in the extraction medium.

Effect of omitting sucrose from the extraction medium on the aminoacyl-s-RNA synthetase activity. Francki, Boardman & Wildman (1965) showed that iso-osmotic extraction media were necessary for obtaining intact chloroplasts capable of incorporating amino acids into protein. However, although the extraction medium described in the present paper contains sucrose (0.45 M), omitting sucrose did not increase the yield of synthetases (Table 1). Further, extraction of synthetases with standard extraction medium followed by re-extraction from the residue with media without sucrose did not yield any more activity than

Fig. 1. Effect of sodium thioglycollate concentration in the extraction medium on the aminoacyl-s-RNA synthetase activity. Extracts (5.0 disks/ml.) were diluted to 0.83 disk/ml. before assay. Activity is expressed as units/ml. of diluted extract. The concentration of thioglycollate in the incubating mixture was 0.7 × concentration in the extraction medium.

Fig. 2. Effect of enzyme concentration (0.6–8.0 disks/ml.) on the aminoacyl-s-RNA synthetase activity (▲). The extract (10 disks/ml.) was diluted to the concentration shown. Activity is expressed as units/ml. of extract at the respective concentration. Activities with (●) and without (○) added amino acids are also included.

Table 1. Effect of omitting sucrose from the extraction medium on the aminoacyl-s-RNA synthetase activity

The synthetases were extracted from leaf disks (25) in 5.0ml. of extraction medium with or without sucrose, and the precipitates remaining were ground again in 5.0ml. of extraction medium with or without sucrose. All supernatant fractions were diluted to 0.83 disk/ml. and assayed for synthetase activity. Activities shown are means of duplicate extractions and are expressed/ml. of diluted extract.

First extraction		Second extraction	
Concn. of sucrose (M)	Activity (units/ml.)	Concn. of sucrose (M)	Activity (unit/ml.)
0.45	11.9	0.45	0.7
0.45	11.5	0	0.6
0	10.8	0	0.6

re-extracting with the standard medium containing sucrose (0.45 M).

Effect of enzyme concentration on the aminoacyl-s-RNA synthetase activity. An extract (10 disks/ml.) was diluted with extraction medium (0.6–8.0 disks/ml.) and assayed for synthetase activity (Fig. 2). Without amino acids added, the amount of pyro-

phosphate incorporated was a linear function of enzyme concentration up to 4 disks/ml. Increasing the concentration of the extract further did not increase incorporation. With amino acids and amides added (1 mM) incorporation increased up to 3 disks/ml. but decreased again at higher concentrations. At 8 disks/ml. incorporation was essentially the same as without amino acids added. Precipitates formed in incubation mixtures containing higher concentrations of enzyme and were denser in incubations containing added amino acids. Dialysis did not alter activity in dilute or concentrated extracts. Varying the enzyme concentration by diluting a concentrated extract or by extracting tissue at the required concentration gave preparations with equal activity. Enzyme activity was a linear function of concentration up to 1 disk/ml. (Fig. 3). Accordingly, assays were made with extracts diluted to approx. 0.8 disk/ml.

Effect of duration of incubation on the incorporation of pyrophosphate. The rate of incorporation of pyrophosphate at 0.8 disk/ml. was constant for at least 25 min. and increased approx. 13-fold when amino acids and amides were added. At 5 disks/ml. the rate of incorporation was constant for only 4 min. and decreased rapidly after that time.

ATPase and pyrophosphatase activities in extracts

containing aminoacyl-s-RNA synthetases. The undiluted extract (5 disks/ml.) contained an active ATPase and activity was decreased in proportion

Fig. 3. Effect of enzyme concentration (0.25–2.0 disks/ml.) on the aminoacyl-s-RNA synthetase activity (▲). The extract (2.0 disks/ml.) was diluted to the concentration shown. Activity is expressed as units/ml. of extract at the respective concentration. Activities with (●) and without (○) added amino acids are also included.

Table 2. ATPase, pyrophosphatase and aminoacyl-s-RNA synthetase activities in concentrated (5.0 disks/ml.) and dilute (0.8 disk/ml.) enzyme extracts

ATPase and pyrophosphatase activities are expressed as the substrate-dependent increase in orthophosphate in μ moles/ml. of reaction mixture/15 min. Aminoacyl-s-RNA synthetase activity is expressed as μ moles of pyrophosphate incorporated into ATP/ml. of reaction mixture/15 min.

Enzyme	Concn. of enzyme (disks/ml.)	Substrate	Activity
ATPase	0.8	ATP (6 mM)	1.11
	5.0		6.83
Pyrophosphatase	0.8	Pyrophosphate (3 mM)	0.52
	5.0		0.20
Aminoacyl-s-RNA synthetases	0.8	Standard assay	0.018
	5.0	without amino acids	0.159
	0.8	Standard assay	0.142
	5.0	with amino acids	0.265

when the extract was diluted to 0.8 disk/ml. (Table 2). The rate of production of orthophosphate from ATP was constant for approx. 20 min. Pyrophosphatase activity was slight.

Determination of the Michaelis constant for ATP and for L-amino acids and amides in the aminoacyl-s-RNA synthetase reaction. With the concentration of reagents used in the standard assay for synthetase, K_m (ATP) was 0.65 mM and K_m (L-amino

Fig. 4. Determination of K_m (ATP) for aminoacyl-s-RNA synthetases by the method of Lineweaver & Burk (1934). The extract (5 disks/ml.) was diluted to 0.8 disk/ml. K_m (ATP) was 0.65 mM. s , Concn. of ATP (mM); v , units/ml. of diluted enzyme.

Fig. 5. Determination of K_m (L-amino acids) for aminoacyl-s-RNA synthetases by the method of Lineweaver & Burk (1934). The extract (5 disks/ml.) was diluted to 0.8 disk/ml. K_m (L-amino acids) was 70 μ M. s , Concn. of L-amino acids; v , units/ml. of diluted enzyme, corrected for activity without added amino acids.

acids) was 70 μ M (Lineweaver & Burk, 1934) (Figs. 4 and 5). ATP at high concentration inhibited activity slightly and values of s above 7 mm were neglected. ATPase action released orthophosphate equivalent to only 3–5% of the ATP in the incubation mixture without amino acids added, so that activity of this enzyme did not affect the value of K_m (ATP) significantly. The apparent K_m (ATP) for synthetases with concentrated enzyme (5 disks/ml.) was 1.2 mm but the value was affected by hydrolysis of ATP through the activity of ATPase (Table 2).

Effect of Mg^{2+} concentration on the aminoacyl-s-RNA synthetase activity. Extracts assayed without Mg^{2+} added were virtually inactive and activity increased as the concentration of magnesium chloride in the incubation medium was raised to 16 mm (Fig. 6). Including magnesium chloride

(16 mm) in the extraction medium and assaying at a final concentration of 9.6 mm increased the activity still further and magnesium chloride (16 mm) was included in the extraction medium in subsequent experiments.

Activity of aminoacyl-s-RNA synthetases specific for individual amino acids and amides. With L-amino acids and amides supplied by Mann Research Laboratories Inc., the activities of the synthetases specific for each amino acid and amide were assayed separately (Table 3). The same extract was assayed also in the usual way for total activity of the synthetases. Only alanine, arginine, glutamic acid, glutamine and hydroxyproline failed to show activity significantly greater than that in the control incubation. The enzymes specific for leucine, isoleucine, valine, cysteine and histidine accounted for most of the activity in the preparation. Total activity of the synthetases assayed with the complete mixture of amino acids and amides was 20% less than the total activity of the enzymes assayed separately.

DISCUSSION

Assaying aminoacyl-s-RNA synthetases by measuring either aminoacyl hydroxamate formed from hydroxylamine or pyrophosphate exchanged into ATP (Novelli, 1958, 1960) depends on the difference in activity observed with and without amino acids added to the incubation mixtures.

Fig. 6. Effect of $MgCl_2$ concentration in the incubation medium on the aminoacyl-s-RNA synthetase activity. Extracts were prepared with and without $MgCl_2$ (16 mm) in the extraction medium and diluted to 0.8 disk/ml. Extracts prepared without $MgCl_2$ were assayed in incubation mixtures (1.0 ml.) containing 6 μ moles of ATP, 3 μ moles of pyrophosphate, 0.6 ml. of extract and 0–16 μ moles of $MgCl_2$, with and without 1 μ mole of each of the 21 amino acids and amides. The extract containing $MgCl_2$ was assayed as above, but without adding further $MgCl_2$ (final concn. of $MgCl_2$ 9.6 mm). The activity of this extract was 12.5 units/ml. of diluted extract.

Table 3. Activities of the aminoacyl-s-RNA synthetases specific for individual L-amino acids and amides extracted from tobacco leaf

Activity is expressed as μ moles of pyrophosphate incorporated into ATP/mg. of protein/min. Each value has been corrected for activity without amino acids or amides added (7.1 μ moles/mg. of protein/min.). Enzymes were assayed at 0.8 disk/ml. with amino acid and amide concn. 1 mm. Activities are means of duplicate determinations.

L-Amino acid or amide	Activity	L-Amino acid or amide	Activity
Alanine	0	Lysine	1.0
Arginine	0.1	Methionine	1.0
Asparagine	3.1	Phenylalanine	2.0
Aspartic acid	0.8	Proline	3.6
Cysteine	7.2	Serine	2.6
Glutamic acid	0	Threonine	0.9
Glutamine	0	Tryptophan	2.2
Glycine	2.9	Tyrosine	4.9
Histidine	6.7	Valine	10.1
Hydroxyproline	0	Total	81.9
Isoleucine	14.6	Complete mixture	65.3
Leucine	18.2		

High concentrations of endogenous amino acids in crude concentrated extracts make estimates of activity by both methods misleading (Clark, 1958; Novelli, 1958), and both methods will underestimate activity of enzymes with abnormal characteristics when activity is determined with a complete mixture of amino acids. For example, Attwood & Cocking (1965) found that alanyl-s-RNA synthetase from tomato root was not saturated at a concentration of alanine below 0.1M and that the optimum pH was 8.1; on the other hand, Moustafa & Lyttleton (1963) found pH optima between 6.2 and 7.1 for most enzymes extracted from wheat germ. Elliott & Coleman (1962) assayed synthetases in crude extracts by separating the basic aminoacyl hydroxamates formed by both endogenous and added amino acids from hydroxamates of other classes of compounds not forming basic hydroxamates. This method does not assay aspartyl- and glutamyl-s-RNA synthetases since they do not form basic hydroxamates. The present method is sensitive, since adding amino acids increases activity 10–15-fold, and is convenient for assaying large numbers of extracts prepared in a single experiment from small samples of tissue.

Extracts prepared from tobacco leaf contain an active ATPase (Shaw, 1965), but with concentrated extracts (5 disks/ml.) the ATP concentration decreased during assay at the most from 6mM to 3.6mM through the action of ATPase. Since the apparent K_m (ATP) of the synthetases with concentrated extracts was only 1.2mM the activity of ATPase appears unlikely to contribute substantially to the lower specific activity of aminoacyl-s-RNA synthetases in the concentrated extracts (Fig. 2). Moustafa & Proctor (1962) also reported that the specific activity of synthetases extracted from root nodules of *Galega officinalis* L. decreased with increasing concentration of enzyme.

Clark (1958) concluded that aminoacyl-s-RNA synthetases extracted from spinach leaves were not thiol enzymes since thiol inhibitors were inactive and thiols added to the incubation medium did not increase activity.

Spencer & Wildman (1964), Boardman, Francki & Wildman (1965), Francki *et al.* (1965) and Spencer (1965) have shown that chloroplasts are the major site of protein synthesis in leaves, and Bové & Raacke (1959) and Marcus (1959) have recovered a considerable proportion of the aminoacyl-s-RNA synthetases of spinach leaves in the soluble fraction isolated from chloroplasts. Francki *et al.* (1965) found that tobacco-leaf chloroplasts retain the enzymes for incorporating amino acids into protein only when extracted in iso-osmotic media. Since the extraction medium used in the present study

contained sucrose (0.45M), appreciable synthetase activity might have been retained in chloroplasts remaining intact after extraction. However, activity in the supernatant fraction did not increase when sucrose was omitted from the extraction medium (Table 1) or when ultrasonic treatment followed grinding of tissue in the extraction medium (Anderson & Rowan, 1966). We attribute recovery of synthetases in the supernatant fraction to differences between the method of extraction used in the present work and that of Francki *et al.* (1965) since the concentrations of Mg^{2+} and thiol in the extraction medium were higher and grinding was used rather than chopping.

This work was supported by grants from the Rural Credits Development Fund of Australia, the Anti-Cancer Council of Victoria and the Cattle and Beef Research Committee.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
- Anderson, J. W. & Rowan, K. S. (1965). *Biochem. J.* **97**, 741.
- Anderson, J. W. & Rowan, K. S. (1966). *Biochem. J.* **101**, 15.
- Attwood, M. M. & Cocking, E. C. (1965). *Biochem. J.* **96**, 616.
- Boardman, N. K., Francki, R. I. B. & Wildman, S. G. (1965). *Biochemistry*, **4**, 872.
- Bové, J. & Raacke, I. D. (1959). *Arch. Biochem. Biophys.* **85**, 521.
- Clark, J. M. (1958). *J. biol. Chem.* **233**, 421.
- Elliott, W. H. & Coleman, G. (1962). *Biochim. biophys. Acta*, **57**, 236.
- Ellman, G. L. (1962). *Analyt. Biochem.* **3**, 40.
- Francki, R. I. B., Boardman, N. K. & Wildman, S. G. (1965). *Biochemistry*, **4**, 865.
- Hele, P. & Finch, L. R. (1960). *Biochem. J.* **75**, 352.
- Henshall, J. D. & Goodwin, T. W. (1964). *Phytochemistry*, **3**, 677.
- Lee Peng, G. H. (1956). *Biochim. biophys. Acta*, **22**, 42.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Marcus, A. (1959). *J. biol. Chem.* **234**, 1238.
- Moustafa, E. (1963). *Biochim. biophys. Acta*, **76**, 280.
- Moustafa, E. & Lyttleton, J. W. (1963). *Biochim. biophys. Acta*, **68**, 45.
- Moustafa, E. & Proctor, M. H. (1962). *Biochim. biophys. Acta*, **63**, 93.
- Novelli, G. D. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 86.
- Novelli, G. D. (1960). *Annu. Rev. Microbiol.* **14**, 65.
- Peterson, P. J. & Fowden, L. (1963). *Nature, Lond.*, **200**, 148.
- Peterson, P. J. & Fowden, L. (1965). *Biochem. J.* **97**, 112.
- Rowan, K. S. (1966). *Int. Rev. Cytol.* **19**, 313.
- Shaw, J. G. (1965). *Arch. Biochem. Biophys.* **109**, 627.
- Spencer, D. (1965). *Arch. Biochem. Biophys.* **111**, 381.
- Spencer, D. & Wildman, S. G. (1964). *Biochemistry*, **3**, 954.