Resonance in Organic Chemistry, New York, Pergamon, p. 85.
Lester, R. L., Hatefi, Y., Widmer, C., and Crane, F. L. (1959), *Biochim. Biophys. Acta 33*, 169.
Lev, M., and Reiter, B. (1962), *Nature 193*, 905.
Morton, R. A., and Elam, W. T. (1941), *J. Chem. Soc. 1941*, 159.
Packard, J. T., and Arnold, M. T. (1951), *J. Chem. Phys. 19*, 1608.
Rouser, G., O'Brien, J., and Heller, D. (1961), *J. Am. Oil Chemists' Soc. 38*, 14.
White, D. C. (1962), *J. Bacteriol. 83*, 851.
White, D. C., and Smith, L. (1962), *J. Biol. Chem. 237*, 1332.

The Incorporation of Amino Acids into Protein by Cell-free Extracts from Tobacco Leaves*

DONALD SPENCER† AND S. G. WILDMAN

From the Department of Botany and Plant Biochemistry, University of California, Los Angeles

Received February 4, 1964

The distribution of amino acid–incorporating activity in cell-free extracts of tobacco leaves has been studied. Extraction media and procedures were designed to give maximum preservation of organelle structures in the extracts. The major portion of the activity is associated with the 1000-*g* fraction, which contains principally nuclei and chloroplasts, together with some mitochondria, spherosomes, and fragments of the nuclei and chloroplasts. Evidence is presented that the nuclei are not contributing to amino acid incorporation by the 1000-*g* fraction. Incorporation of [^{14}C]valine into this fraction is strongly dependent on exogenous adenosine triphosphate, and in addition requires Mg^{2+}, a mixture of amino acids, a mixture of guanosine, uridine, and cytidine triphosphates, and an adenosine triphosphate–generating system for maximum activity. The system is extremely sensitive to ribonuclease (0.02 μg/ml gives 50% inhibition), puromycin, and chloramphenicol, but is relatively insensitive to deoxyribonuclease and actinomycin D. Incorporation rate is approximately linear for 30 minutes and falls to zero in about 60 minutes. Evidence is presented for the intermediate formation of the aminoacyl–soluble ribonucleic acid complex by the 1000-*g* fraction. Added tobacco mosaic virus–ribonucleic acid has no effect on incorporation, whereas polyuridylic acid stimulates the incorporation of [^{14}C]phenylalanine, but not of [^{14}C]valine.

Because most of the visible growth of a plant leaf is the result of cell expansion occurring after cell division has ceased, this organ affords the opportunity to study net protein synthesis in the absence of net nuclear synthesis. During the expansion phase tobacco leaves accumulate protein at an exponential rate (Dorner *et al.*, 1957). When expansion ceases net protein accumulation stops, and thereafter the amount of protein steadily declines as the leaf senesces. Thus, it appeared that young leaves in the process of rapid net protein synthesis might provide a readily accessible source of a system capable of *in vitro* synthesis of protein.

Recent studies in this laboratory (Honda *et al.*, 1962) have led to the development of extraction media which facilitate the preparation from higher plant leaves of cell-free extracts in which the microscopically visible organelles are preserved in a morphologically intact state, closely resembling their condition in the living cell. In these extracts a high proportion of nuclei are intact, many chloroplasts retain the outer mobile jacket seen in the living cell, and the pleomorphic character of mitochondria is also preserved. This finding suggested that such cell-free extracts from young leaves may provide an opportunity for the *in vitro* study of complex processes such as the synthesis of nucleic acids and proteins. This paper describes the distribution of amino acid–incorporating activity in such extracts, and some of the characteristics of an active system associated with the 1000-*g* fraction.

* This work was supported in part by research grants from the Division of Biology and Medicine, U. S. Atomic Energy Commission, Contract AT (11-1)-34, Project 8; the National Institutes of Health (AI-00536); and the National Science Foundation (B-14729).

† Permanent address: Division of Plant Industry, C.S.I.R.O., Canberra, Australia.

MATERIALS AND METHODS

Plant Material.—Both *Nicotiana glutinosa* or *N. tabacum* var. Turkish Samsun plants were grown under standard greenhouse conditions. The former were grown in 10-cm pots and were used when they had reached a height of about 15 cm. Leaves which were about 20–40 mm were used. The *N. tabacum* plants were grown in 7-cm peat pots containing vermiculite and were watered twice daily with a mineral nutrient solution. Leaves 50–70 mm in length were harvested when the plants had reached a height of about 7 cm.

Preparation of Extracts.—The midrib and large veins were cut out of the leaves and, after cooling in ice, 5 g fresh weight of the laminae together with 6 ml of extracting medium were placed in a flat-bottomed, shallow, polythene dish and chopped to a fine mince with a sharp razor blade. The object of the method was to cut the leaf cells so that their protoplasmic contents would be released into the extraction medium with as little crushing of the cells and organelles as possible. Mincing required about 10 minutes, during which time the chopping dish was standing on crushed ice. The extracting medium was essentially that developed by Dr. Shigeru Honda for maximum structural preservation of organelles. Honda medium consisted of ficoll (2.5%), dextran (5%) sucrose (0.25 M), Tris, *p*H 7.8 (0.025 M), magnesium acetate (1 mM), and mercaptoethanol (4 mM). The resulting *brei* was

filtered through three layers of fine cloth to remove unbroken cells. Microscopic examination of this filtrate showed it to be almost free of whole cells. An occasional remnant of a trichome was seen, but it was clear that the contribution from this source was extremely slight. The crude cell-free extract was then centrifuged at 1000 *g* for 5–10 minutes, the supernatant fraction was discarded, and the pellet was suspended in 3–4 ml of Honda medium. This will be referred to as the 1000-*g* fraction. Low temperature was maintained throughout.

Standard Assay of Amino Acid Incorporation.—The assay procedure was based on that of Sager *et al.* (1963). The reaction mixture consisted of 0.4 ml of leaf extract in Honda medium (1000-*g* fraction) together with Tris, *p*H 7.8 (2.3 μmoles), magnesium acetate (2.3 μmoles), KCl (13.8 μmoles), mercaptoethanol (1.3 μmoles), ATP[1] (0.2 μmole), phosphoenolpyruvate (1.25 μmoles), pyruvate kinase (10 μg), UTP (0.01 μmole), CTP (0.01 μmole), GTP (0.01 μmole), and a mixture of 0.0125 μmole each of nineteen amino acids (omitting valine). The final reaction mixture volume was 0.5 ml. [^{14}C]Valine (5 μl of a solution containing 0.1 mc and 0.5 μmole/ml) was added at zero time, and incubation was carried out for 45 minutes at 25°. Assays were conducted in duplicate and each experiment included control tubes which were treated in an identical manner except that the [^{14}C]valine was not added until the end of the incubation period. After incubation, tubes were rapidly cooled and 0.5 ml [^{12}C]valine (0.1 M) was added, followed by trichloroacetic acid to give a final concentration of 5%. After standing 15 minutes at 0° the trichloroacetic acid–insoluble fraction was sedimented at 12,000 *g* for 10 minutes, and the clear supernatant liquid was withdrawn and discarded. The pellets were placed in solid CO_2 for 15 minutes, thawed, resuspended in 5% trichloroacetic acid containing 0.05% [^{12}C]valine, transferred quantitatively to a 1.2-μ Millipore filter (Millipore Filter Corp., Bedford, Mass.), and washed on the filter with five aliquots of 5 ml of trichloroacetic acid–valine solution. The freezing step ensures rapid filtration of the precipitates. Without freezing, the precipitates tend to clog the filter and washing times are greatly protracted. The Millipore filter with the trichloroacetic acid–insoluble fraction was cemented to a planchet and dried, and the radioactivity was counted on a Nuclear Chicago gas flow counter fitted with a Micromil window. Approximately 5×10^5 cpm was added per assay, and radioactivity in precipitates from zero-time incubations was between zero and 10 cpm. It is critical to remove most of the unincorporated counts as a supernatant after the high-speed centrifuging of the trichloroacetic acid precipitates. If the bulk of the unincorporated [^{14}C]valine is passed through the filter with the trichloroacetic acid precipitate, large and variable background counts will be obtained, in some instances as high as 300 cpm. A hot trichloroacetic acid treatment was not routinely included since preliminary experiments indicated only a small fraction of the counts to be solubilized by this procedure. However, later experiments showed that the hot trichloroacetic acid–soluble fraction varied considerably between experiments (from 5 to 25% of the total cold trichloroacetic acid–insoluble counts), and thereafter hot trichloroacetic acid extraction (15 minutes at 90°) was routinely carried out. Amino acid incorporation activity is reported throughout as either hot trichloroacetic acid– or cold trichloroacetic acid–precipitable cpm incorporated per assay under the above standard conditions.

The 1000-*g* fraction contained 0.5–1.0 mg protein/ml as determined by the method of Lowry *et al.* (1951).

Density-Gradient Centrifugation.—Based on procedures developed by S. I. Honda (private communication), nuclei were separated from other organelles in the cell-free extract by centrifugation at 25,000 *g* in a Spinco SW39 rotor for 15 minutes. The extract, or the whole reaction mixture, was placed on top of three graded layers of Honda medium in which only the ficoll concentration was changed. The three layers contained 40%, 30%, and 20% ficoll, respectively. This procedure yielded a gray nuclear pellet largely free of any chlorophyll-bearing particles.

Microscopy.—The composition of the various fractions of the leaf extracts were observed with a Zeiss GFL research microscope using both phase-contrast and fluorescence microscopy. Nuclei could be conveniently seen under ultraviolet light after staining with acridine orange (0.01 mg/ml). At magnifications used (up to 1200 ×) mitochondria and spherosomes were readily seen under phase contrast.

Special Reagents.—The sources of special reagents were as follows: Ficoll and dextran 40 from Pharmacia, Sweden; ATP and crystalline DNAase from Sigma Chemical Co; GTP, UTP, CTP, and poly-U from Calbiochem Corp.; puromycin from Nutritional Biochemicals Corp.; actinomycin D from Merck, Sharp and Dohme; PEP, tricyclohexylammonium salt, and crystalline pyruvate kinase from Boehringer und Soehne, Germany, L-[^{14}C]valine (200 μc/μmole) and L-[^{14}C]phenylalanine (350 μc/μmole) from New England Nuclear Corp.

Results

Distribution of Amino Acid–incorporating Activity in Cell-free Extracts.—A crude cell-free extract of tobacco leaves was fractionated by successive centrifugations at 1000 *g* for 10 minutes, 12,000 *g* for 30 minutes, and 144,000 *g* for 2 hours. The sedimented fractions were resuspended in the original volume of fresh extracting medium and their amino acid–incorporating activity was determined (Table I). By far the major part of the incorporating activity was associated with the 1000-*g* fraction. The 12,000-*g* and 144,000-*g* fractions showed about 3% and 18%, respectively, of the activity of the 1000-*g* fraction. The relatively low activity of the original cell-free extract may be owing to the presence of an endogenous inhibitor, or to

Table I

Distribution of Amino Acid–incorporating Activity in Cell-free Extracts of Tobacco Leaves[a]

Cell Fraction	[^{14}C]Valine Incorporation[b]
Cell-free extract	99
1,000-*g* fraction	1010
12,000-*g* fraction	31
144,000-*g* fraction	181
144,000-*g* supernatant	1

[a] Cell-free extract was centrifuged successively at 1000 *g* for 10 minutes, 12,000 *g* for 30 minutes, and 144,000 *g* for 2 hours, and each pellet was resuspended in its original volume. The assay procedure is described under Methods.
[b] Incorporation is expressed as hot trichloroacetic acid–insoluble cpm/assay.

[1] Abbreviations used in this paper: ATP, GTP, UTP, and CTP, the triphosphates of adenosine, guanosine, uridine, and cytidine, respectively; RNAase, ribonuclease; DNAase, deoxyribonuclease; RNA, ribonucleic acid; s-RNA, soluble ribonucleic acid; TMV, tobacco mosaic virus; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvic acid; poly-U, polyuridylic acid.

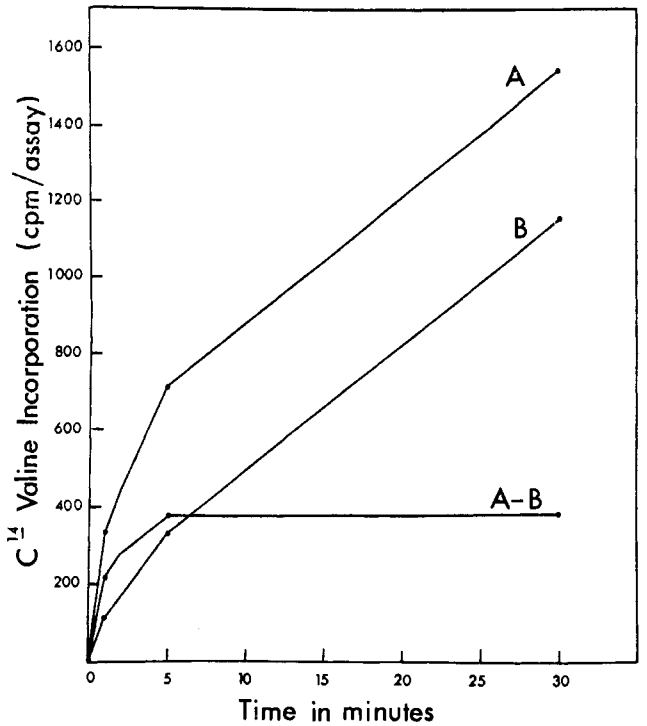

FIG. 1.—Time course of incorporation of [^{14}C]valine into cold and hot trichloroacetic acid–insoluble products by the 1000-*g* fraction from tobacco leaves. Curve A = cold trichloroacetic acid–insoluble. Curve B = hot trichloroacetic acid –insoluble. Curve A-B = hot trichloroacetic acid–soluble fraction of the total cold trichloroacetic acid–insoluble products.

large pools of [^{12}C]valine in the unfractionated extracts. Since these results implicated some component of the 1000-*g* fraction as a major site of protein synthesis in the leaf cell, subsequent experiments were confined to this fraction.

Microscopically, the 1000-*g* fraction was seen to contain well-preserved chloroplasts and nuclei, a number of small fragments originating from chloroplasts, together with many small particles of the size of mitochondria and spherosomes. This fraction contained all the nuclei and 70–80% of the intact chloroplasts present in the crude homogenate.

Characteristics of the Amino Acid–incorporating System.—The effect of various components of the reaction mixture on the activity of the 1000-*g* fraction from tobacco leaves is shown in Tables II and III. All additions tested were required for maximum activity, the most striking effect being that of ATP, the absence of which caused an 85% decrease in activity.

TABLE II
EFFECT OF EXOGENOUS COMPONENTS ON AMINO ACID INCORPORATION BY THE 1000-*g* FRACTION OF TOBACCO LEAVES[a]

Assay System	[^{14}C]Valine Incorporation[b]
Complete reaction mixture	454
Omit GTP, UTP, and CTP	417
Omit [^{12}C]amino acids	343
Omit PEP and pyruvate kinase	290
Omit ATP, PEP, and pyruvate kinase	66
Omit all additions	24

[a] Composition of complete reaction mixture is given under Methods. [b] Incorporation is expressed as cold trichloroacetic acid–insoluble cpm/assay.

TABLE III
EFFECT OF MAGNESIUM CONCENTRATION ON AMINO ACID INCORPORATION BY THE 1000-*g* FRACTION OF TOBACCO LEAVES[a]

Additions	[^{14}C]Valine Incorporation[b]
Nil	465
10^{-3} M Mg acetate	462
5×10^{-3} M Mg acetate	976
10^{-2} M Mg acetate	978

[a] Extract was prepared in absence of added Mg^{2+}. [b] Incorporation expressed as cold trichloroacetic acid–insoluble cpm/assay.

A lack of added Mg^{2+} resulted in a 52% reduction, while the omission of a mixture of nineteen amino acids and of an ATP-generating system also caused a significant decrease in activity. The stimulation by a mixture of GTP, UTP, and CTP, although small with fractions from tobacco, was more apparent with the 1000-*g* fraction from *N. glutinosa*. This presumably is a reflection of different pool sizes in the two sources.

In the standard assay the rate of incorporation by *N. glutinosa* extracts approached linearity for 30–45 minutes. Tobacco extracts did not show strict linearity (Fig. 1). Incorporating activity is heat labile as shown by a 61% reduction in incorporation following 5 minutes' preincubation at 50°.

The effect of a range of inhibitors is shown in Table IV. The system is extremely sensitive to RNAase with measurable inhibition at 2×10^{-4} μg/ml and maximum inhibition at about 0.1 μg/ml. In contrast, the system was comparatively insensitive to DNAase action. The slight inhibition noted at high concentration could be due to contamination of the DNAase with RNAase. Chloramphenicol and puromycin were potent inhibitors whereas actinomycin D caused only a slight reduction in activity.

Localization of Amino Acid–incorporating Activity in 1000-g Fraction. The heterogeneity of the 1000-*g*

TABLE IV
EFFECT OF INHIBITORS ON AMINO ACID INCORPORATION BY 1000-*g* FRACTION OF TOBACCO LEAVES[a]

Experiment	Additions (μg/ml)	[^{14}C]Valine Incorporation[b] (cpm)	[^{14}C]Valine Incorporation[b] (% inhibition)
1	Nil	879	
	RNAase: 0.0002	821	7
	0.0008	720	18
	0.002	680	23
	0.08	314	64
	0.2	213	74
	8	176	80
	20	184	78
2	Nil	856	
	DNAase: 10	733	14
	100	644	25
3	Nil	2028	
	Chloramphenicol: 200	615	70
	Actinomycin D: 20	1670	18
4	Nil	1045	
	Puromycin: 1	624	40
	10	319	69
	100	296	72

[a] Complete reaction mixture is given under Methods. [b] Incorporation is expressed as cold trichloroacetic acid–insoluble cpm/assay

TABLE V

THE NONPARTICIPATION OF NUCLEI IN AMINO ACID INCORPORATION BY THE 1000-*g* FRACTION OF TOBACCO LEAVES[a]

Experiment	Fraction	[^{14}C]Valine Incorporation[b] (cpm/assay)	[^{14}C]Valine Incorporation[b] (cpm/mg chlorophyll)
1	200-*g* pellet (nuclei, chloroplasts, and small organelles)	1407	29,500
	1000-*g* pellet (chloroplasts and small organelles)	1220	22,500
2	1000-*g* fraction	1469	
	Nuclei separated from 1000-*g* fraction by density-gradient centrifugation	18	
3	1000-*g* fraction	1631	
	Nuclei separated from 1000-*g* fraction by Triton X-100 treatment	126	

[a] In expt 1 the two fractions were separated before incubation. In expts 2 and 3 the nuclei were separated from the remainder of the 1000-*g* fraction after incubation. [b] Incorporation is expressed as cold trichloroacetic acid–insoluble cpm.

fraction, as revealed microscopically, posed the question as to which of its several classes of organelle possessed the incorporating activity. This has proved extremely difficult to resolve. Although chloroplasts are by far the major component of this fraction there is present, in addition to nuclei, a significant proportion of smaller organelles, and no satisfactory method has yet been found to obtain a chloroplast preparation free of these smaller organelles.

The evidence to date all suggests that the nuclei are not involved in amino acid incorporation by this fraction. Table V, expt 1, shows the results of an experiment in which the standard 1000-*g* fraction was subdivided into two fractions, one of which contained all the whole nuclei, while both fractions contained approximately equal amounts of chloroplasts and other components. This was achieved by first centrifuging the cell-free extract at 200 *g* for 4 minutes to sediment all the whole nuclei together with some chloroplasts, and then recentrifuging the resultant supernatant at 1000 *g* for 5 minutes to sediment a nuclei-free chloroplast pellet. These fractions showed equal capacity for amino acid incorporation both on a volume basis and on the basis of chlorophyll concentration. This result argues against the participation of the nuclei in the process, and suggests that this activity is a property of the chloroplasts or the smaller organelles.

This view is supported by the distribution of incorporated [^{14}C]valine in the 1000-*g* fraction after incubation. Intact nuclei, well-preserved and in a highly purified state, can be obtained when the 1000-*g* fraction is centrifuged through a density gradient as described under Materials and Methods. When an incubated reaction mixture was treated in this way and the incorporation into the nuclei was compared with the total incorporation it was found that fewer than 1% of the incorporated counts were associated with the nuclei (Table V, expt 2).

Triton-X 100 (alkylphenoxypolyethoxyethanol, Rohm and Haas) at 5% final concentration completely solubilizes tobacco chloroplasts, but has no microscopically detectable effect on nuclei. The latter can now be readily separated by low-speed centrifugation. Consistent with the above results, when an incubated 1000-*g* fraction was treated with Triton X-100, the isolated unwashed nuclei were found to contain only 7% of all the incorporated counts (Table V, expt 3).

TABLE VI

THE EFFECT OF TMV-RNA AND POLYURIDYLIC ACID ON AMINO ACID INCORPORATION BY THE 1000-*g* FRACTION OF TOBACCO LEAVES[a]

Condition	[^{14}C]Amino Acid Incorporation Expt 1[b]	[^{14}C]Amino Acid Incorporation Expt 2[c]
[^{14}C]valine	1824	
[^{14}C]valine + poly-U (200 μg/ml)	1674	
[^{14}C]valine + TMV-RNA (250 μg/ml)	1692	
[^{14}C]phenylalanine	2418	1289
[^{14}C]phenylalanine + poly-U (200 μg/ml)	3703	3079

[a] Approximately 10^6 cpm of [^{14}C]phenylalanine was added in expt 1 and 5×10^5 cpm in expt 2. Otherwise assay conditions were as given under Methods. [b] Incorporation expressed as cold trichloroacetic acid–insoluble cpm/assay. [c] Incorporation expressed as hot trichloroacetic acid–insoluble cpm/assay.

Response to Exogenous Polynucleotides.—One aim of this work has been to induce the synthesis of tobacco mosaic virus protein in extracts of healthy plants using exogenous TMV-RNA as the messenger. To date, no consistent response in incorporation has been obtained with TMV-RNA added to any fraction of healthy leaf extracts. However, as seen in Table VI, the 1000-*g* fraction is capable of being programed by the synthetic messenger, polyuridylic acid. As in the case of other incorporating systems (Nirenberg and Matthaei, 1961), polyuridylic directs the incorporation of phenylalanine but not of valine.

Role of s-RNA.—The participation of s-RNA in protein synthesis by the 1000-*g* fraction is indicated by two observations. First, the presence of a rapidly labeled component of the 1000-*g* fraction, insoluble in cold trichloroacetic acid but soluble in hot trichloroacetic acid, is demonstrated in Figure 1. Incorporation into this component, which presumably represents the aminoacyl-s-RNA complex, reached a maximum after 5 minutes' incubation, and thereafter remained at this maximum level (curve A-B, Fig. 1), while incorporation into the hot trichloroacetic acid–insoluble fraction was still increasing after 30 minutes (curve B). During the first 7 minutes more counts were incorporated in the hot trichloroacetic acid–soluble fraction than into the hot trichloroacetic acid–insoluble fraction. The aminoacyl-s-RNA fraction thus accounted for 67% of the total cold trichloroacetic acid–insoluble counts after 1 minute and only 25% after 30 minutes.

Second, extraction of the standard 1000-*g* pellet with either water or a solution containing 20 mM Tris, *p*H 7.8, 4 mM mercaptoethanol, and 1 mM magnesium acetate, followed by centrifugation at 144,000 *g* for 2 hours, yielded a supernatant solution which, under standard assay conditions, incorporated 200–400 cpm into a cold trichloroacetic acid–insoluble product. This product was completely soluble in hot trichloroacetic acid. This soluble extract of the 1000-*g* fraction could be stored at −70° for 2 weeks without loss of activity.

These results indicate that the soluble components of the protein-synthesising system are sedimented

along with the 1000-*g* fraction, presumably in association with the large organelles. If the supernatant suspension remaining after sedimentation of the 1000-*g* fraction was further centrifuged at 144,000 *g*, and the resultant supernatant was dialyzed overnight, this fraction also had an appreciable capacity for incorporation into a cold trichloroacetic acid–insoluble, hot trichloroacetic acid–soluble product. Without prior dialysis no incorporation was seen, suggesting the presence of an endogenous inhibitor or a large pool of [^{12}C]valine, as also indicated by the results in Table I.

Discussion

The amino acid–incorporating activity of the 1000-*g* fraction of tobacco leaf extracts has many characteristics of cell-free systems from other sources with respect to its dependence on ATP, Mg^{2+}, and amino acids, and its sensitivity to RNAase, chloramphenicol, and puromycin. Although a number of cell-free amino acid–incorporating systems have been isolated from higher plant sources (Webster, 1959; Raacke, 1959; Rabson and Novelli, 1960), relatively little was known of the distribution and characteristics of this process in higher leaves. Stephenson *et al.* (1956) demonstrated incorporation of labeled amino acids into a number of centrifugal fractions prepared from cell-free extracts of tobacco leaves. On a protein basis they found the 1000-*g* fraction to be the most active. The relationship between their results and those reported here is not clear since many of the characteristics of the incorporating systems appear to differ. In contrast to the present data, Stephenson *et al.* obtained no enhancement with exogenous ATP or a mixture of nonlabeled amino acids, and ribonuclease at 50 μg/ml was without effect. Although variations in pool sizes could explain lack of response to exogenous components, the marked contrast in RNAase sensitivity leaves a major difference. One explanation may lie in the plant material used. For reasons given in the Introduction, we have made use of young expanding leaves from plants approximately 4 weeks old, whereas Stephenson *et al.* employed 3- to 5-month-old plants. In our experience the activity of extracts from older plants is appreciably lower. Again in contrast to their results, we have observed no stimulation by light of incorporation in the 1000-*g* fraction under our standard assay conditions.

Limited conclusions can be drawn from our data concerning the localization of protein-synthesizing activity within the leaf cell. Under our conditions the 1000-*g* fraction was more than five times as active as the ribosomal (144,000-*g*) pellet on a volume (and hence per cell) basis (Table I). Further work is needed to compare the activity of the two fractions fully supplemented with soluble components. However, it appears that with our procedures for making cell-free extracts the necessary soluble components (amino acid–activating enzymes and s-RNA) are associated with the 1000-*g* pellet. This raises the possibility that in the intact leaf cell a similar distribution occurs, and that the contribution of the cytoplasmic ribosome fraction to protein synthesis is relatively slight.

Our results eliminate the nuclei as significant contributors to protein synthesis by the 1000-*g* fraction. Since the remaining major component of this fraction is the chloroplasts, it seems likely that these are the major site of protein synthesis in the extracts, and possibly in the intact leaf cell. The finding by Lyttleton (1962) of a unique chloroplast ribosome fraction, together with recent evidence of DNA in chloroplasts (Chun *et al.*, 1963), provides added support for this suggestion. The reservation should be added that, in addition to chloroplasts and nuclei, the 1000-*g* fraction also contains a significant proportion of microscopically visible components which include mitochondria, spherosomes, and small fragments of the larger organelles, and may also contain smaller organelles, such as ribosomes, which are not resolved by the light microscope. The possibility cannot be excluded that these smaller components are in fact the site of protein synthesis in the 1000-*g* fraction.

It is rather surprising that the 1000-*g* fraction, prepared in such a way as to preserve the organelle structure and provide minimum opportunity for loss of soluble components, should readily display a dependence on exogenous low-molecular-weight compounds such as ATP and amino acids. This is in marked contrast to ribosomal particles from pea seedlings, which require repeated washing before addition of these compounds will enhance incorporation (Raacke, 1961).

Another interesting feature of the 1000-*g* fraction of tobacco leaves is its extreme sensitivity to exogenous RNAase, which in turn implies a very low endogenous RNAase level. It seems possible that where organelle structures are preserved the release of latent RNAase is avoided. Mans and Novelli (1961) reported that 200 μg/ml of RNAase caused 80% inhibition of incorporation by a ribosomal fraction from maize seedlings, whereas incorporation by tobacco leaf extracts was maximally inhibited at 0.1 μg/ml. In this respect the tobacco leaf fraction resembles *E. coli* ribosomes (Matthaei and Nirenberg, 1961).

The possibility of bacterial contamination in experiments on protein synthesis in cell-free extracts is particularly pertinent when the starting material is a relatively nonsterile tissue such as a leaf. Bacterial contamination does not appear to be a significant factor in our experiments for several reasons. First, microscopic examination of the tobacco extracts did not reveal bacteria; second, the time course of the reaction does not resemble a bacterial growth curve; and third, the strong dependence upon ATP and an ATP-generating system and the extreme sensitivity to RNAase are indicative of a genuine cell-free system.

It is obvious from the data that in absolute terms only a minute amount—of the order of 5–10 $\mu\mu$mole/assay or 10–20 $\mu\mu$mole/mg protein—of amino acid incorporation occurs in our standard assay. This estimate of the capacity of the system is low for two reasons. No account is taken of the size of the endogenous pool of free [^{12}C]amino acid in the relatively crude extracts, and further, the molar concentration of the labeled amino acid was not fully saturating, since a 10-fold increase in [^{14}C]valine concentration resulted in a 75% increase in incorporation by the 1000-*g* fraction. However, the tobacco leaf system is clearly in contrast to a pea-seedling system–ribosome fraction which has been reported to carry out net protein synthesis during short incubation periods (Webster, 1959).

Current work involves a study of the mechanism of synthesis of a specific protein, namely, tobacco mosaic virus protein, in leaf extracts.

References

Chun, E. H. L., Vaughan, M. H., and Rich, A. (1963), *J. Mol. Biol. 7*, 130.

Dorner, R. W., Kahn, A., and Wildman, S. G. (1957), *J. Biol. Chem. 229*, 945.

Honda, S. I., Hongladarom, T., and Wildman, S. G. (1962), *Plant Physiol. 37*, xli.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lyttleton, J. W. (1962), *Exptl. Cell Res. 26*, 312.
Mans, R. J., and Novelli, G. D. (1961), *Biochim. Biophys. Acta 50*, 287.
Matthaei, J. H., and Nirenberg, M. W. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1580.
Nirenberg, M. W., and Matthaei, J. H. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1588.
Raacke, I. D. (1959), *Biochim. Biophys. Acta 34*, 1.
Raacke, I. D. (1961), *Biochim. Biophys. Acta 51*, 73.
Rabson, R., and Novelli, G. D. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 434.
Sager, R., Weinstein, I. B., and Ashkenazi, Y. (1963), *Science 140*, 304.
Stephenson, M. L., Thimann, K. V., and Zamecnik, P. C. (1956), *Arch. Biochem. Biophys. 65*, 194.
Webster, G. C. (1959), *Arch. Biochem. Biophys. 85*, 159.

A Cell-free Amino Acid-incorporating System from *Saccharomyces cerevisiae*. Variation in Ribosomal Activity and in RNA Synthesis during Logarithmic Growth*

JEAN M. LUCAS,† A. H. W. M. SCHUURS,‡ AND MELVIN V. SIMPSON§

From the Departments of Biochemistry, Yale University, New Haven, Connecticut, and Dartmouth Medical School, Hanover, New Hampshire

Received February 14, 1964

A cell-free ribosomal system has been obtained from *S. cerevisiae* by lysis of protoplasts and by grinding the cells with alumina. The ribosomes can incorporate labeled amino acids added either as free amino acids or as aminoacyl-s-RNA and the properties of both types of system have been summarized. The amino acid–incorporating activity of the system varies during the logarithmic phase of growth. The ribosomes are responsible for this variation. Paralleling this change in activity is a change in the cellular content of ribosomes and in the rate of synthesis of ^{32}P-pulse-labeled RNA, whose base composition points to an identity with messenger RNA, at least in part. This greatly increased capacity for protein synthesis does not appear to be reflected in an actual increase in the protein content of the intact cell. A beginning has been made in seeking the biochemical factors associated with the ribosome which cause the variation in its activity. The base composition of the RNA does not appear to play such a role as it does not vary throughout the log phase. In view of such variation in messenger RNA synthesis, the ribosomal content of this RNA is being investigated.

Yeast offers a number of potential advantages in studies on protein synthesis. Its amino acid–activating enzymes, s-RNA, and ribonucleoprotein particles have been isolated and extensively studied. In addition, a large number of highly purified and well-characterized proteins, some of which are inducible, have been isolated from this microorganism, thus presenting a wide range of choice for the study of the biosynthesis of a specific protein in a cell-free system. Because an active cell-free preparation from yeast was not available when this work was begun, our efforts were directed toward the development of such a system. This communication describes the isolation of active ribosomes from *S. cerevisiae*, briefly summarizes the properties of the system, and reports on the following unexpected finding.

Early in these studies, it was noted that the activity of the system varied considerably with the batch of yeast used, and a tentative correlation began to appear between the extent of this activity and the age of the yeast culture from which the cell-free system had been prepared. Inasmuch as the cells were always harvested at some point in the log phase, this meant that the activity of the cell-free system and therefore the potential capacity of the cell for protein synthesis was changing during the log phase. A variation in so fundamental a property during a period of growth in which the cells are reproducing at a constant rate makes this phenomenon one of particular interest in terms of the regulatory mechanisms of the cell. The further finding, that the variation in activity of the cell-free system could be attributed to the ribosomes themselves, raises the interesting question of the factor or factors associated with the ribosome which regulates this activity. The changes observed in ribosomal activity are accompanied by parallel changes in the RNA content of the cell and in the rate of synthesis of what is probably messenger RNA. Work is in progress to elucidate the mechanisms involved.

* This investigation was supported by a grant (AM 07124-02) from the United States Public Health Service. A preliminary report of these studies has been published (Lucas *et al.*, 1963).

† Special Trainee of the United States Public Health Service.

‡ Present address: H. V. Organon, Oss, The Netherlands.

§ Present address: Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire.

Experimental Procedure

Materials.—Phosphoenolpyruvate, pyruvate kinase, GTP, ATP, CTP, spermine, GSH, puromycin, and unlabeled amino acids were obtained from the California Corp. for Biochemical Research, [1-^{14}C]leucine from the New England Nuclear Corp., vitamins from Nutritional Biochemicals Corp., agar, bactopeptone, and yeast extract from Difco Laboratories, and RNase and DNase from Worthington Biochemical Corp. The amino acid mixture used in these experiments consisted of the stated amounts of glycine, L-alanine, L-serine, L-threonine, L-isoleucine, L-valine, L-glutamic acid, L-aspartic acid, L-lysine, L-arginine, L-histidine, L-methionine, L-cysteine, L-proline, L-tryptophan, L-phenylalanine, L-tyrosine, L-glutamine, and L-asparagine. [P^{32}]orthophosphate was obtained carrier-free from the Union Carbon and Carbide Co., Oak Ridge,