

**Communication**

# Protein Synthesis in Isolated Mitochondria of Rice (*Oryza sativa* L.) Seedlings<sup>1</sup>

Hwa Dai, Yih-Shan Lo, Chiung-Yuan Wu, Chia-Lin Tsou, Gon-Shi Hsu, Chyr-Guan Chern, Manfred Ruddat, and Kwen-Sheng Chiang\*

*Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China (H.D., Y.-S.L., C.-Y.W., C.-L.T., G.S.H.); Taiwan Agriculture Research Institute, Wufeng, Taichung, Taiwan, Republic of China (C.-G.C.); and Departments of Ecology and Evolution (M.R.) and Molecular Genetics and Cell Biology (M.R., K.-S.C.), The University of Chicago, Chicago, Illinois 60637*

## ABSTRACT

For studies of *in organello* mitochondrial protein synthesis in rice, *Oryza sativa* L., conventional surface-sterilization procedures were demonstrated to be ineffective. Because of the overwhelmingly efficient [<sup>35</sup>S]methionine utilization by contaminating bacteria, even “essentially bacteria-free” rice mitochondria were shown to be unsuitable for the study of *in organello* protein synthesis. We developed a procedure to obtain a bacteria-free preparation of rice mitochondria. Such mitochondria favored a membrane-dependent ATP-generating system over an external ATP-generating system as the energy supplement for *in organello* protein synthesis. Two distinct classes of [<sup>35</sup>S]methionine-labeled, cycloheximide-insensitive products were detected: an electrophoretically unresolved population and a set of some 22 to 27 discrete polypeptide species, each with a characteristic electrophoretic mobility and relative abundance.

The problem of bacterial contamination in mitochondrial preparations had received little attention before plants were first employed for mitochondrial protein synthesis studies (2–4, 7). Low or no mitochondrial protein synthesis without a supplemented ATP-generating system was construed to indicate an “essentially bacteria-free” preparation. Whereas the concept of relying on energy dependency in determining bacterial contamination was well accepted, in practice such determination was neither convenient nor quantitative. A mitochondrial sample was considered to be essentially bacteria-free when protein synthesis in the presence of acetate (a substrate utilized by bacteria but not by mitochondria) was less than several percent of that under standard conditions with a supplemented ATP-generating system (7). Assessing the magnitude of the effect of bacterial contamination on mitochondrial protein synthesis has been difficult from one experiment to another, since autoradiograms were used routinely for documenting the result of mitochondrial protein synthesis. The lack of a straightforward, quantitative method

to determine the extent of bacterial contamination in plant mitochondrial samples has led to serious consequences. For instance, a 62 kD [<sup>35</sup>S]methionine-labeled polypeptide first reported to be synthesized by isolated maize mitochondria in response to heat shock (8, 10) was later shown to be the product of contaminating bacteria (9).

The present communication documents our characterization of protein synthesis by isolated rice mitochondria prepared from etiolated seedlings grown under different culture conditions. The presence of bacteria was monitored by plating the mitochondrial suspension and by determining [<sup>35</sup>S]methionine incorporation with different energy supplements and in the presence and absence of antibiotics. Conventional surface-sterilization procedures were shown to be ineffective for removing bacteria from rice seedlings. Bacterial contamination, although relatively low in numbers in rice seedlings grown under growth-chamber conditions, exerted an unexpectedly serious and detrimental effect on mitochondrial protein synthesis. To obtain rice mitochondria suitable for the study of protein synthesis, we found it necessary to germinate and grow dehulled, individually selected and rigorously surface-sterilized rice grains under aseptic conditions.

## MATERIALS AND METHODS

### Preparation of Rice Seedlings

Rice (*Oryza sativa* cv Tainun-67) grains were surface sterilized with 0.5% sodium hypochlorite (NaOCl) for 30 min at room temperature with constant agitation. Following two rinses with sterile distilled water, the grains were allowed to germinate in the dark in sterile distilled water for 3 d at 30°C and then planted on soil or vermiculite, supplemented with 0.1% HYPONeX #5 (N:P<sub>2</sub>O<sub>5</sub>:K<sub>2</sub>O, 30:10:10). Seedlings were grown in a growth chamber at 30°C in the dark.

### Surface Sterilization of Rice Seedlings Grown in a Growth Chamber

Fourteen- to 16-d-old seedlings were harvested by cutting the shoot just above the grain. The seedlings were washed extensively with sterile water, immersed in 0.9% w/v NaOCl for 5 min, and rinsed five times with cold, sterile distilled

<sup>1</sup> Supported by research grants from Academia Sinica and the National Science Council, Republic of China (to H. D.), and by a research fund from University of Chicago (to K. S. C.).

water. Plating cell homogenates and suspensions of purified mitochondria on Luria's Broth agar medium indicated that increasing the sodium hypochlorite concentration threefold (to 2.7%) did not reduce appreciably the amount of contaminating bacteria in the purified mitochondria preparation.

### Preparations of Rice Seedlings Grown under Aseptic Conditions

Twenty grams of rice grains were dehulled, selected individually, and surface sterilized by immersing in 70% ethanol for 30 s and then shaking vigorously with 250 mL of 0.75% sodium hypochlorite for 10 min. The NaOCl sterilization procedure was repeated twice. The grains were then rinsed five times with sterile distilled water and transferred aseptically to closed culture containers containing a sterile solid medium. This medium consisted of 1% (w/v) sucrose, 0.1% (w/v) HYPONeX #5, and 1.0% agar; the pH was adjusted to 5.6 with dilute sodium hydroxide before autoclaving. Grains germinated and seedlings grew well in this medium at 30°C in the dark.

### Composition of Solutions and Buffers

M-1 buffer contained 0.4 M mannitol, 25 mM MOPS-KOH (pH 7.8), 1 mM EGTA, 0.1% (w/v) BSA, 40 mM mercaptoethanol, and 8 mM cysteine (the last two added fresh just before use). M-2 buffer contained 0.4 M mannitol, 5 mM Mops-KOH (pH 7.5), 1 mM EGTA, and 0.1% BSA. M-3 buffer contained 10 mM Tricine-KOH (pH 7.2), 1 mM EGTA, and 0.1% BSA. M-4 buffer contained 0.2 M mannitol, 10 mM Tricine-KOH (pH 7.2), and 1 mM EGTA. M-5 buffer contained 0.4 M mannitol, 10 mM Tricine-KOH (pH 7.2), and 1 mM EGTA. Protein-synthesis chasing solution contained 10 mM methionine and 1.8 mM each of 19 other amino acids. Protein-synthesis stopping solution contained 0.4 M mannitol, 10 mM Tricine-KOH (pH 7.2), 10 mM methionine, 1 mM EGTA, 1 mM PMSF, and 0.1 mM each of five other protease inhibitors (pepstatin A, bestatin, tosyl lysine chloromethyl ketone, *N*-tosyl-L-phenylalanylchloromethyl ketone, and *t*-epoxysuccinyl-L-leucylamido(4-guanidino)butane. Sample loading buffer contained 63 mM Tris-HCl [pH 6.8], 2.5% (w/v) Ficoll 400, 2% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, 0.005% (w/v) bromophenol blue, 1 mM PMSF, 0.1 mM each of pepstatin A, bestatin, tosyl lysine chloromethyl ketone, *N*-tosyl-L-phenylalanylchloromethyl ketone, and *t*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

### Isolation and Purification of Rice Mitochondria

Mitochondria were isolated from 14- to 16-d-old rice seedlings (12–15 cm in length) by a preoptimized protocol based on a procedure by Leaver *et al.* (7). Buffers, solutions, glassware, plasticware, and all surfaces that touched seedlings and cell extract were sterilized and tested before use. All procedures were carried out at 4°C under aseptic conditions.

Seedlings were cut into approximately 0.5 cm<sup>2</sup> pieces and then homogenized in M-1 buffer (4 mL/g tissue) in a Waring Blendor with two to five 5 to 10 s high speed bursts. The homogenate was filtered through four layers of cheesecloth

and then through two layers of Miracloth. The filtrate was centrifuged to reach 4000g and then let coast to a halt without braking. The resulting supernatant was centrifuged again to reach 4000g and then let coast to a halt without braking. Crude mitochondria were precipitated by centrifuging the supernatant at 14,000g for 15 min, resuspended in M-2 buffer, centrifuged to reach 4000g, and coated to a halt without braking. After repeating the centrifugation one more time, the mitochondria were pelleted at 10,000g for 15 min, resuspended in a small volume of M-2 buffer, and fractionated on a continuous sucrose gradient (0.6–1.8 M in buffer M-3) at 52,000g for 60 to 90 min in a swinging bucket rotor.

The mitochondria population that banded at a density between 1.30 to 1.35 M sucrose was recovered and slowly diluted (over a 15–20 min period) by adding M-4 buffer to a give final osmotic concentration of 0.6 M. The purified mitochondria were recovered by centrifuging at 10,000g for 15 min and then suspended in M-5 buffer at a mitochondrial protein concentration of 1 to 2 mg/mL.

### Protein Synthesis by Isolated Mitochondria

Purified mitochondria (70–125  $\mu$ g of mitochondrial protein) were incubated in a final volume of 250  $\mu$ L at 30°C for 60 to 90 min with constant rotatory shaking. The filter-sterilized reaction mixture contained: 10 mM Tricine-KOH (pH 7.2), 5 mM potassium phosphate (pH 7.2), 250 mM mannitol, 90 mM KCl, 10 mM MgCl<sub>2</sub>; 1 mM EGTA, 2 mM DTT, 1 mM GTP, 25  $\mu$ M 19 amino acids except methionine, 225  $\mu$ Ci [<sup>35</sup>S]methionine (~1000 Ci/mM), and one of the following three neutralized energy-generating supplements: 6 mM ATP/8 mM phosphocreatine/4 units of creatine phosphokinase; 2 mM ADP/10 mM sodium succinate; or 20 mM sodium acetate.

### Assay of the [<sup>35</sup>S]Methionine Incorporation into Protein

A 2.5  $\mu$ L portion of the protein synthesis reaction mixture was added to 102.5  $\mu$ L 5.64% (w/v) TCA. After mixing, two independent 2.5  $\mu$ L aliquots of this mixture were spotted on two Whatman GF/C filter discs for scintillation counting the total [<sup>35</sup>S]methionine. To the remaining 100  $\mu$ L mitochondria suspension, 10  $\mu$ L of 0.22% (w/v) BSA was added, incubated for 30 min at 4°C then 15 min at 70°C, chilled, and then filtered through a Whatman GF/C filter disc for scintillation counting to determine the [<sup>35</sup>S]methionine incorporation into protein.

### Termination of the Protein Synthesis Reaction and Analysis of the Product

After a 60 to 90 min incubation period, 2.5  $\mu$ L of protein-synthesis chasing solution was added and the incubation continued at 30°C for 15 min. Protein synthesis was then terminated by adding 1 mL of protein-synthesis stopping solution. In some experiments, the addition of the chasing solution was intentionally omitted. Mitochondria were pelleted at 12,000g for 10 min, quick frozen in liquid nitrogen, and kept at -80°C. Immediately before analysis, the mitochondria pellet was solubilized in sample loading buffer by

heating at 90°C for 2 min (in some experiments the six protease inhibitors were omitted in this buffer). The mitochondrial proteins (40–125 µg protein) were analyzed by 15% (w/v) SDS-PAGE (acrylamide:bisacrylamide, 150:1) according to the method of Laemmli (6). Gels were stained with silver or Coomassie blue, immersed in 1 M sodium salicylate for 1 h, dried onto Whatman 3 MM filter paper, and then exposed to Kodak XAR-5 x-ray film at -70°C.

## RESULTS

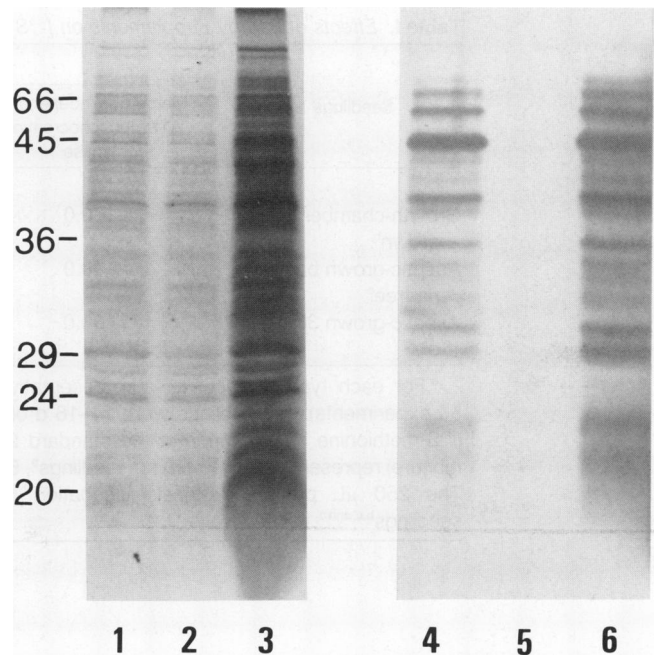
### Mitochondria Isolated from Growth-Chamber Grown Seedlings

Purified mitochondria of etiolated 14 to 16-d-old rice seedling appeared as a homogeneous population under a phase contrast microscope without apparent contaminants. The incorporation of [<sup>35</sup>S]methionine into polypeptides increased with the number of bacteria as determined by plating (Fig. 1). While laborious surface sterilization of rice seedlings resulted in a considerable reduction of [<sup>35</sup>S]methionine incorporation, it failed to eliminate the effect of bacterial contamination on mitochondrial protein synthesis. The size and relative amount of [<sup>35</sup>S]methionine-labeled polypeptides were virtually the same with or without surface sterilization. Further increasing the surface-sterilization stringency produced only more quantitative reduction of [<sup>35</sup>S]methionine incorporation without any qualitative change in the composition of the newly synthesized polypeptides. A typical result is presented in Figure 1. Chloramphenicol at a concentration of 100 µM effectively eliminated the [<sup>35</sup>S]methionine incorporation into proteins, whereas erythromycin and cycloheximide exhibited little effect (Fig. 1, lanes 2, 4, and 5).

Cell homogenates and mitochondrial suspensions plated onto Lucia's broth medium at different stages of mitochondria purification revealed qualitative as well as quantitative variations of contaminating bacteria in independently isolated mitochondrial samples. According to colony morphology, there were some 10 different types of bacteria with marked variation in relative abundance. This was consistent with the observation that the size and relative abundance of [<sup>35</sup>S]methionine-labeled polypeptides were not always the same among different mitochondrial samples isolated under identical experimental conditions but from different batches of seedlings (Fig. 1).

The efficiency of [<sup>35</sup>S]methionine incorporation into acid precipitable counts was approximately the same irrespective of the energy source used in growth-chamber grown seedlings (Table I). This is consistent with the results from gel electrophoresis that in the presence of [<sup>35</sup>S]methionine, the size and relative amount of polypeptides synthesized appeared to be the same irrespective of the supplemental energy sources (data not shown).

Taken together, these results indicate that: (a) most of the [<sup>35</sup>S]methionine-labeled polypeptides shown in Figure 1 were not synthesized by rice mitochondria; (b) methionine was preferentially utilized by contaminating bacteria for their own protein synthesis; and (c) the bias of methionine utilization was overwhelming as the number of bacteria was insignificant compared with that of mitochondria.



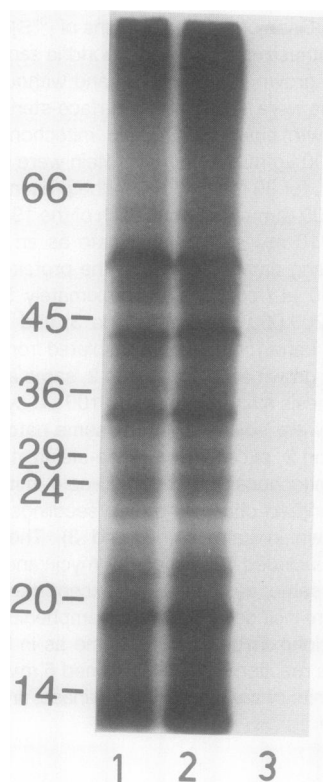
**Figure 1.** Comparison of autoradiographs of [<sup>35</sup>S]methionine-labeled polypeptides synthesized by rice mitochondria samples isolated from growth-chamber grown seedlings with and without surface sterilization. Mitochondria were isolated from surface-sterilized rice seedlings grown in a growth chamber. Purified mitochondria equivalent to approximately 100 µg mitochondrial protein were incubated in a final volume of 250 µL for 90 min at 30°C in the presence of 225 µCi [<sup>35</sup>S]methionine (~1000 Ci/mM), 25 µmol each of the 19 other amino acids, and 2 mM ADP/10 mM sodium succinate as an energy-generating supplement. Plating data indicate that the protein-synthesis incubation mixture (250 µL) contained approximately 50,000 bacteria in lanes 1 and 2; 200,000 bacteria in lane 3; and 10,000 bacteria in lanes 4, 5, and 6. Lane 1, mitochondria isolated from surface-sterilized growth-chamber grown seedlings. Lane 2, same as in lane 1, except the protein-synthesis mixture contained 100 µM cycloheximide. Lane 3, mitochondria were isolated from the same batch of rice seedlings used in lane 1 and 2, except the surface-sterilization procedure was omitted before mitochondria isolation. Lane 4, mitochondria isolated from surface-sterilized chamber grown seedlings (a different batch from those shown in Lanes 1, 2, and 3). The protein-synthesis reaction mixture included 100 µM erythromycin and 100 µM cycloheximide. Lane 5, same as in lane 4, except the protein-synthesis incubation mixture included 100 µM chloramphenicol instead of erythromycin and cycloheximide. Lane 6, same as in lane 4, except the protein-synthesis reaction mixture contained 6 mM ATP, 8 mM phosphocreatine, 4 units of creatine phosphokinase, and no erythromycin or cycloheximide.

The preferential utilization of [<sup>35</sup>S]methionine by bacteria made even minor bacterial contamination a serious problem for the study of rice mitochondria protein synthesis *in organello*. Conventional procedures, *e.g.* surface-sterilization of seeds, autoclaving the preplanting and planting media, administering antibiotics at a non-growth limiting concentration, etc., failed to solve this problem. Therefore, rice mitochondria “essentially free of bacteria” isolated from surface-sterilized, growth-chamber grown seedlings are not suitable for studies on *in organello* protein synthesis.

**Table I.** Effects of Energy Supplements on [<sup>35</sup>S]Methionine Incorporation into Mitochondrial Protein<sup>a</sup>

Seedlings	Energy Supplement		
	Membrane-independent ATP/phosphocreatine/ kinase	Membrane-dependent ADP/succinate	Nonmitochondria sodium acetate
		% of incorporation	
Growth-chamber grown <sup>b</sup>	100.0	110.3	96.6
Aseptic-grown bacte- ria-free <sup>c</sup>	100.0	150.4	37.7
Aseptic-grown 30-d old <sup>d</sup>	100.0	43.6	53.6

<sup>a</sup> For each type of seedling, a representative set of data was presented (from a total of some 50 experiments). All seedlings were 14–16 d old unless otherwise stated. <sup>b,c,d</sup> The value of 100% [<sup>35</sup>S]methionine incorporation in the standard 2.5  $\mu$ L assay (from 250  $\mu$ L protein-synthesis reaction mixture) represents 49,641 cpm for seedlings<sup>b</sup>, 6,760 cpm for seedlings<sup>c</sup>, and 3,854 cpm for seedlings<sup>d</sup>. The 250  $\mu$ L protein synthesis incubation mixture contained ~1,000, 0, and 0 bacteria for seedlings<sup>b,c, and d</sup>, respectively.



**Figure 2.** An autoradiograph of [<sup>35</sup>S]methionine-labeled polypeptides synthesized by mitochondria prepared from rice seedlings grown under aseptic conditions. Experimental conditions for protein synthesis were the same as in Figure 1, except mitochondria equivalent to 80  $\mu$ g mitochondria protein were used. Plating 5  $\mu$ L out of 250  $\mu$ L protein-synthesis incubation mixture produced no bacterial colony. Lane 1, 6 mM ATP, 8 mM phosphocreatine, 4 units of creatine phosphokinase. Lane 2, 2 mM ADP, 10 mM sodium succinate. Lane 3, 20 mM sodium acetate.

### Mitochondria Isolated from Aseptically Grown Rice Seedlings

Following extensive preliminary testing, we developed a method to isolate bacteria-free mitochondria from rice seedlings grown under aseptic conditions. Two crucial aspects of this method were: (a) sterilizing dehulled, individually selected grains by lengthy and vigorous agitation with sodium hypochlorite to exterminate bacteria; and (b) developing a procedure to germinate and cultivate such grains under aseptic conditions. Mitochondria isolated from seedlings grown in this way usually contained no detectable bacteria as measured by direct plating.

The efficiency of [<sup>35</sup>S]methionine incorporation into acid-precipitable protein in the bacteria-free mitochondrial protein synthesis reaction varied according to the energy supplement employed. Acetate, when supplemented, gave approximately 60% less [<sup>35</sup>S]methionine incorporation than an external ATP-generating system, which in turn was 1.5 to 2.8-fold lower than that of an internal ATP-generating system with ADP and succinate. Such differences were not observed for mitochondria samples isolated from growth-chamber grown rice seedlings (Table I). The ratio of [<sup>35</sup>S]methionine incorporation with an internal ATP-generating system was almost always higher than with an external one and somewhat variable with different mitochondria samples. The only exception came from a mitochondrial sample isolated from 30-d old, unhealthy seedlings grown under aseptic conditions (Table I).

Both the internal and the external ATP-generating systems supported the production of polypeptides by bacteria-free rice mitochondria. Two distinct classes of [<sup>35</sup>S]methionine-labeled products were detected by autoradiography: an electrophoretically unresolved population and a set of 22 to 27 discrete polypeptide species, each showing a characteristic electrophoretic mobility and relative abundance (Fig. 2). No products were detected by autoradiography when acetate was used as the energy source even though [<sup>35</sup>S]methionine was incorporated into acid precipitable non-RNA polymers (Table I). The appearance of the two distinct classes of [<sup>35</sup>S]methionine-

labeled products as well as the electrophoretic mobility and relative abundance of the 22 to 27 polypeptides were not appreciably affected by erythromycin and cycloheximide, whereas chloramphenicol practically abolished such [<sup>35</sup>S]methionine labeled products (data not shown).

### DISCUSSION

For plant mitochondrial protein synthesis, it has been customary to employ surface sterilization to minimize bacterial contamination. Because bacteria may not only be on the outer surface of a plant, the effectiveness of surface sterilization is not always predictable. Surface-sterilization procedures developed for a particular plant or certain tissues of that plant may not necessarily be effective for another plant or tissue, since differences in species as well as in the locale of the contaminating bacteria species and the surface characteristics of the plant may be considerable. Presumably because of the porous surface, high silicon content, and other structural features of the rice leaf (1, 5), surface sterilization was not as effective in removing bacteria from rice seedlings as it was in many other flowering plants (7).

Repeated differential and sucrose-gradient centrifugation proved also inadequate to eliminate the effect of bacteria contamination in mitochondrial protein synthesis. This unexpected seriousness results presumably in part from the relatively small size of rice mitochondria and, far more importantly, from the preferential utilization of labeled methionine by contaminating bacteria. At 225  $\mu$ M, the concentration of [<sup>35</sup>S]methionine used in our mitochondrial protein-synthesis reactions, although substantially higher (4.4–22.9  $\mu$ M) than what was used in most previous studies (2–4, 7), is still several orders of magnitude lower than that of the 19 other amino acids (25  $\mu$ M) and most likely the rate-limiting factor of the protein synthesis reaction. It is unlikely that an intra-organellar amino acid concentration bias would compensate this inequity. The ability to import methionine efficiently becomes prerequisite for protein synthesis to proceed. The fact that nearly all [<sup>35</sup>S]methionine-labeled polypeptide products of “essentially bacteria-free” mitochondria samples were of bacterial origin indicates that surface-contaminating bacteria in rice seedlings possessed a much higher efficiency to import methionine than rice mitochondria. Thus, a widely used mitochondria-isolation procedure based on surface sterilization useful for *in organello* protein synthesis in several other plants (7) could not be employed with rice seedlings. Cultivating rice seedlings under aseptic conditions, although labor intensive, became necessary.

The addition of 10 to 1000-fold excess cold methionine to the protein-synthesis reaction of “essentially bacteria-free” or bacteria-free mitochondria did not alter significantly the [<sup>35</sup>S]methionine incorporation and autoradiographic results, re-

spectively (data not shown). Thus, methionine, at a concentration of 225 nM, appeared still to be the rate-limiting factor for the contaminating bacterial as well as for the mitochondrial protein synthesis reaction. This preferential utilization of amino acid by contaminating bacteria underscores the seriousness of the problem of bacterial contamination in mitochondrial preparations not only in rice but perhaps also in other experimental systems.

Rice mitochondria utilized sodium succinate and ADP to generate ATP considerably more efficiently than a membrane-independent, external ATP-regenerating system for the synthesis of [<sup>35</sup>S]methionine-labeled polypeptides, whereas acetate failed to support such a synthesis (Fig. 2, Table I). The general consistency between the autoradiographic and the quantitative [<sup>35</sup>S]methionine-incorporation data reinforces the original concept of relying on energy-dependency in determining bacterial contamination in mitochondrial protein synthesis *in organello* (7), although plating of mitochondrial suspensions could be used to achieve the same purpose. The preference for an internal over an external ATP-generating system as energy source indicates that despite the rigorous organelle purification procedure, isolated rice mitochondria retained a high degree of structural integrity and, by extension, a high fidelity of protein synthesizing capability. The present work has thus provided the necessary groundwork for the study on possible correlation of mitochondrial protein-synthesizing activity/pattern with cytoplasmic male sterility in rice.

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