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Intervarietal Differences in some Metabolic Functions Associated with Protein Accumulation in Rice Grains *

R. Mitra, P. Narahari, T. Gopalakrishna and C.R. Bhatia
Biology and Agriculture Division, Bhabha Atomic Research Centre, Bombay (India)

Summary. Total nitrogen, amino nitrogen, glutamic acid dehydrogenase (GDH) activity and incorporation of ^{3}H-uridine and ^{14}C-amino acids into RNA and proteins, respectively, were compared in the developing grains of three high-protein stocks (IR-480-5-9, GMPR-51 and Erythroceros) and a high-yielding, medium-protein cultivar IR-8. The above parameters were also independently studied in the developing grains of IR-8 grown at 0, 60 and 120 kg N/ha. In addition, mobilization of nitrogen from flag leaf during kernel development was compared in a separate experiment. Higher protein concentration, both in high-protein stocks and in IR-8 grown at 120 kg N/ha, was associated with increased levels of: soluble amino nitrogen, GDH activity, ^{3}H-uridine and ^{14}C-amino acid incorporation. Significant variation was found among the high protein stocks in mobilization of nitrogen from flag leaf.

Introduction

The major problem in rice breeding programmes aimed at improving the nutritional value of rice is to combine high grain yield with high protein concentration, without impairing the grain production of the plant or the biological value of the kernel proteins. Sizeable efforts are underway at several laboratories to increase the grain protein concentration of high-yielding rice varieties (Beachell *et al.* 1972a; Juliano 1972; Chu *et al.* 1973; Harn *et al.* 1973; Tanaka 1973). In rice most of the high protein types identified in the screening of the world collection at the International Rice Research Institute were not suitable for commercial cultivation (Beachell *et al.* 1972b). In general, there is a negative correlation between grain yield and protein concentration in cereals (Tanaka and Takagi 1970; McNeal *et al.* 1972; Whitehouse 1973; Riley 1974; Johnson and Lay 1974; Gomez and De Datta 1975). Nevertheless, there are certain rice stocks, such as IR-480-5-9, that combine high kernel protein concentration with reasonably good grain yield (Beachell *et al.* 1972a), though these grain yields are much lower than those of the high-yielding cultivars. Only such types can be readily used as the genetic source for high protein in breeding programmes. Our main interest is to identify the component characters of high grain protein genotypes in different stocks, to hybridize the genotypes where the increased protein has a different basis, and to study the genetics of component characters affecting grain protein concentration.

In this paper the results of investigations on some metabolic functions associated with the accumulation of proteins in developing grains are reported for three high-protein genotypes in comparison with IR-8, a high-yielding medium-protein cultivar. In another independent experiment the same metabolic functions were compared in IR-8 grown at 0, 60 and 120 kg N/ha. The following attributes have been compared in the developing grains: (a) total nitrogen, (b) amino nitrogen, (c) incorporation of ^{3}H-uridine into RNA, (d) incorporation of ^{14}C-amino acids into proteins, and (e) glutamic acid dehydrogenase (GDH) activity. In addition, the mobilization of nitrogen from flag leaf during grain development was compared in a separate experiment. Studies on protein metabolism in developing grains of rice have been made by Cruz *et al.* (1970) and Perez *et al.* (1972).

Materials and Methods

Foundation seed of IR-480 was obtained from the International Rice Research Institute, Los Baños, Philippines. GMPR-51 and Erythroceros seeds were received from the Central Rice Research Institute, Cuttack, India. Seeds of IR-8 were obtained from a crop grown in our experimental field and were originally received from the Co-ordinated Rice Improvement

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Project, Hyderabad. Labelled amino acid mixture (^{14}C-algal protein hydrolysate) and ^{3}H-uridine were obtained from the Isotopes Division of this Research Centre. All other chemicals used were of the analytical grade.

The investigations concerning IR-8 at low and high nitrogen levels were carried out during the wet season (June-November) of 1972. Experiments with the high-protein stocks were carried out during the dry (December-May) season of 1972-73. Seedlings were raised in nursery beds and 30-day-old seedlings were transplanted into the field, a single seedling per hill at a spacing of 30 × 20 cm. In the IR-8 experiment the plots ultimately received the equivalent of 0, 60 and 120 kg N/ha in the form of ammonium sulphate in three equal applications, at transplanting (30 days) and at 55 and 80 days after sowing. The high-protein stocks were transplanted to a field in which 80 kg N/ha was provided in three equal instalments.

When nearly 50 percent of the plants of each variety had flowered, tillers were labelled on the day of panicle emergence and random samples from these panicles were collected at 5 day intervals until the crop was mature. Six different stages of grain development were thus sampled. Grains of comparable size from the top half of the panicle were collected in order to avoid differences due to inter- and intra-panicle variability. Grain yield and protein characteristics of the plants from which the samples were collected are given in Table 1.

Protein estimation

Nitrogen estimations were made by the micro-Kjeldahl method and a factor of 5.95 was used to obtain the protein per cent (Vergara *et al.* 1970). Protein per grain was calculated from this value and 1000-grain weight.

Soluble amino nitrogen

Samples weighing 1-10 g of developing grains were dehulled by hand, and were then homogenized with ten volumes of 80 % ethanol at room temperature and centrifuged at 12000 × g for 15 min. The pellet was extracted thrice with 10 ml of 80 % ethanol. The pooled supernatant solutions were evaporated to dryness in a boiling water bath. The residue was dissolved in 2-3 ml of 0.1 M citrate buffer, pH 2.2, and an aliquot assayed for animo nitrogen after neutralization by the ninhydrin method of Moore and Stein (1954). DL leucine was used as a standard.

^{3}H-uridine and ^{14}C-amino acid incorporation

Incorporation studies of labelled amino acids into proteins and uridine into ribonucleoproteins were followed using the methods described by Chen and Osborne (1970). We were aware of the limitations of the technique as big pools and changing pool size can considerably influence the uptake and dilution of labelled material. In spite of these limitations the method is good for comparative studies.

Uridine incorporation

Freshly harvested kernels (6-30) were sliced into four pieces and incubated in small petri dishes at 25° C for 1h in light (275 ft. candles) in a reaction mixture consisting of : 3.8 ml of 0.005 M Tris-HCL buffer, pH 7.6, containing 0.02 M KCl, 0.002 M $MgCl_2$, 20 µg/ml sucrose, 20 units of penicillin and 0.2 ml ^{3}H-uridine (80 µc; 0.048 µ moles). Following incubation, the kernel slices were thoroughly washed, first with 100 ml of incubation mixture containing unlabelled uridine and then with 100 ml of distilled water. The slices were homogenized with 5 ml of 0.1 M Tris-HCl buffer, pH 7.8, containing 0.02 M KCl and 0.01 M $MgCl_2$ at 4 °C. Five ml of 10 % chilled trichloroacetic acid (TCA) were added to the homogenate and 2 ml of the resulting suspension was filtered through Whatman (GF/C) glass fibre filter and washed successively with 20 ml of 5 % TCA, 5 ml ethanol, 5 ml ether and 5 ml acetone. Filter papers were dried and the radioactivity on them was measured in a Beckman Liquid Scintillation System LS-100 using 0.5 % POPOP and 0.4 % PPO in toluene.

Amino acid incorporation

Freshly harvested kernels were sliced into four pieces like those for uridine incorporation and incubated for 1h at 25 °C in light (275 ft. candles). The composition of the incubation mixture was similar to that used for uridine incorporation except that, instead of labelled uridine, it contained ^{14}C-uniformly labelled amino acid mixture (algal protein hydrolysate 25 µc; 355 µ moles). One hundred µc (1420 µ moles) of ^{14}C amino acid mixture was used in control and nitrogen-treated kernels of IR-8. After the incubation period the kernel slices were washed with 30 ml Tris-HCl buffer, pH 7.6, containing 0.02 M KCl and 0.002 M $MgCl_2$ and 0.5 mg/ml cas-amino acids, and then with 30 ml of distilled water over a Buchner funnel lined with Saran screen. Washed kernel slices were homogenized in 8 ml chilled 5 % TCA, heated for 15 minutes at 90 °C and then cooled to 5 °C. An aliquot of 0.5 ml was filtered through Sartorius membrane filter (0.45 µ), washed with 30 ml of 5 % TCA taking 10 ml each time, dried and then counted for radioactivity as described under uridine incorporation.

Enzyme activity

The method given by Kanamori *et al.* (1972) was used to determine the GDH activity. The extraction buffer contained 0.1 M dithiothreitol. The activity of NADH-GDH (aminating) was measured.

Mobilization of nitrogen from flag leaf

Flag leaves were collected at 5-day intervals up to 35 days after panicle emergence and their nitrogen concentration was determined. Mobilization of nitrogen was determined by the difference in the nitrogen concentration of flag leaf at 5 days after panicle emergence, and at the time of harvest. Three high protein stocks, CRHP 1A, 1B and 1C, received from the Central Rice Research Institute, Cuttack, were included in this experiment.

Replications

^{14}C-amino acid and ^{3}H-uridine incorporation was studied in two replicates. Other observations are the

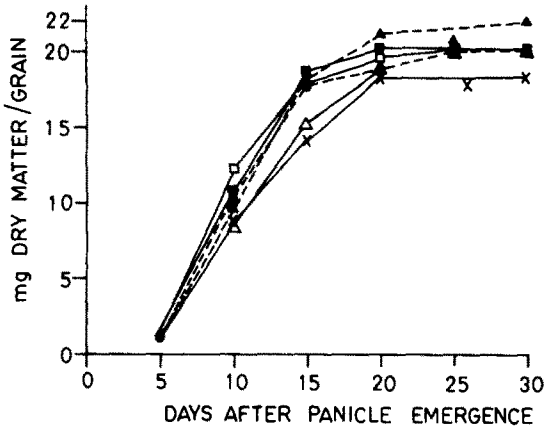

Fig. 1. Increase in dry weight during grain development.
X——X Erythroceros
Δ——Δ GMPR-51
□——□ IR-480-5-9 and
■——■ IR-8 grown along with the above stocks at 80 kgN/ha during the dry season of 1972-73.
▲·····▲ IR-8 grown at 120 kgN/ha and
●·····● IR-8 grown at 0 kgN/ha in an independent experiment during the wet season of 1972

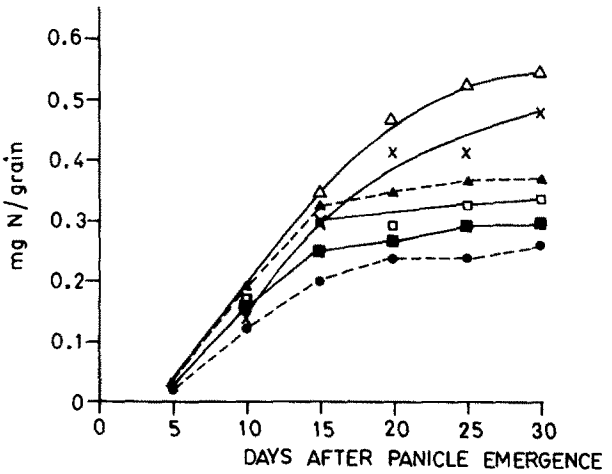

Fig. 2. Nitrogen accumulation during grain development. Details as in Fig. 1

Table 1. Grain yield and grain protein of the stocks used in this study

Stock	Duration to flowering (days)	Grain yield [a] kg/ha	relative to IR-8	Thousand grain weight (grams)	Protein Percent
1972 wet season					
IR-8 - 0 kgN/ha	96	not estimated		26.4 ± 0.2	7.3 ± 0.1
IR-8 - 120 kgN/ha	98	not estimated		26.6 ± 0.2	10.1 ± 0.1
1972-73 dry season					
IR-8	125	8155	100	26.5 ± 0.3	9.9 ± 0.2
IR-480	123	6000	73.6	28.8 ± 0.2	11.8 ± 0.4
GMPR-51	100	4711	57.8	27.2 ± 0.2	12.2 ± 0.2
Erythroceros	92	4511	55.3	25.6 ± 0.1	11.5 ± 0.1
1973 wet season					
IR-8	98	5978	100	27.1 ± 0.1	9.2 ± 0.2
IR-480	93	4633	77.5	29.5 ± 0.3	11.8 ± 0.1
CRHP 1A	78	3000	50.2	17.0 ± 0.2	11.8 ± 0.03
CRHP 1B	77	2778	46.5	16.9 ± 0.3	10.7 ± 0.2
CRHP 1C	77	2778	46.5	17.3 ± 0.2	11.9 ± 0.1

[a] Estimated yield from 10 plants harvested in the middle of central rows in each plot

means of 3-4 replications. As the results of 60 kg N/ha were intermediate to the values of 0 and 120 kg N/ha, only the results of the latter treatments are given here. Grains harvested from plants grown at 0 and 120 kg N/ha are hereafter designated as coming from low and high nitrogen treatments, respectively.

Results

Increase in dry weight

The pattern of dry matter accumulation during grain development was similar in all four genotypes (Fig. 1). Kernel growth was very rapid between 5 and 15 days and the maximum dry matter accumulated by 20 days after panicle emergence. Grain weights at harvest are given in Table 1.

Nitrogen accumulation

Nitrogen accumulation was parallel to the increase in dry weight during grain development. In compa-

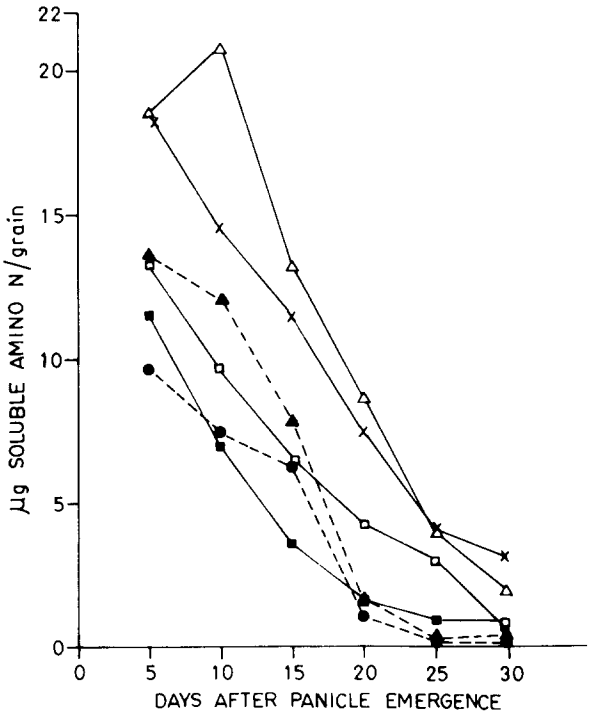

Fig.3. Soluble amino nitrogen in developing grains. Details as in Fig.1

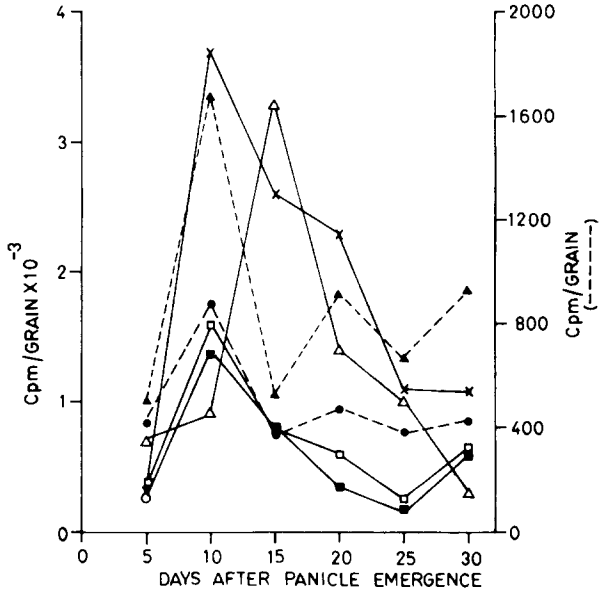

Fig.4. Incorporation of ^{3}H-uridine into nucleoproteins during grain development. Details as in Fig.1

rison with IR-8 and IR-480, where after 20 days of panicle emergence nitrogen content did not increase, Erythroceros and GMPR-51 showed a continued increase in nitrogen content up to 30 days (Fig.2). In the IR-8 experiment the rate of nitrogen accumulation was greater in grains from high nitrogen treatments than in grains from low nitrogen treatment. The protein concentration in the grains at harvest is given in Table 1.

Soluble amino nitrogen

The high protein stocks showed increased levels of soluble amino nitrogen compared with IR-8 at all stages of grain development (Fig.3). Amino nitrogen content was higher during the early phases of grain development in all four genotypes and has greatly diminished after 20 days of panicle emergence. Increased levels of amino nitrogen were also observed in IR-8 grains from high nitrogen treatment.

Incorporation of uridine

The peak of uridine incorporation was observed at 10 days after panicle emergence in IR-8, IR-480 and Erythroceros (Fig.4). GMPR-51 had the peak uridine incorporation at 15 days. Considerably greater incorporation was observed in GMPR-51 and Erythroceros compared with IR-8 and IR-480. Uridine incorporation was higher in grains from high nitrogen than in those from low nitrogen treatment in the IR-8 experiment.

Amino acid incorporation

Amino acid incorporation was rapid between 5 and 20 days after panicle emergence (Fig.5). IR-480 and Erythroceros incorporated higher amounts of radioactivity into the TCA insoluble proteins at the period of peak activity, around 10 days after panicle emergence, than did IR-8. In GMPR-51 peak incorporation was at 15 days after panicle emergence. Amino acid incorporation was also higher in the grains from high nitrogen plants of IR-8. The duration of protein synthesis remained the same and peak incorporation was observed both in grains from high and low nitrogen plants at 10 days after panicle emergence.

GDH activity

The high protein stocks showed increased GDH activi-

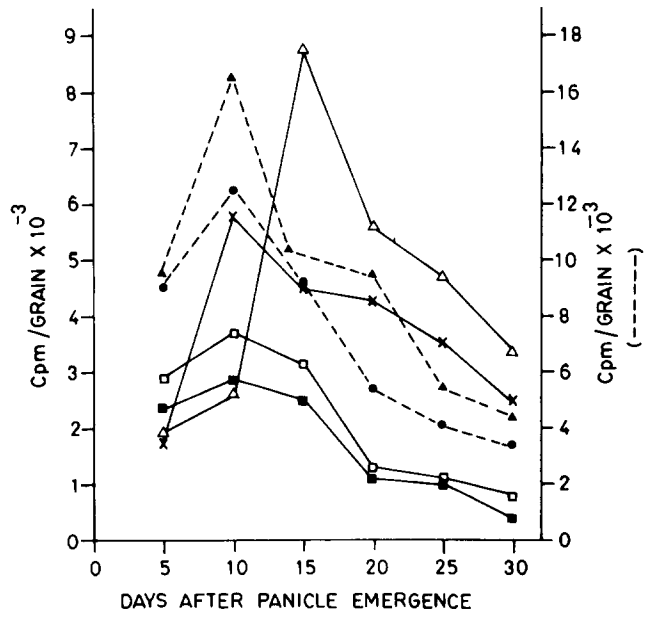

Fig.5. Incorporation of ^{14}C-amino acids into TCA insoluble proteins. Details as in Fig.1

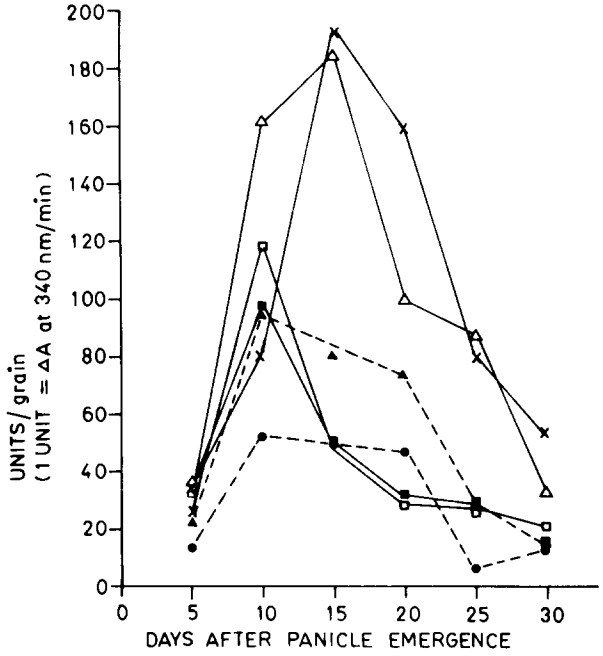

Fig.6. Glutamic acid dehydrogenase activity in the developing grains. Details as in Fig.1

Table 2. Nitrogen as percent dry matter in flag leaf and its mobilization during grain development

Variety	Days after panicle emergence					Percent difference between 5 and 35 days
	5	10	15	25	35	
IR-8	3.08±0.06	2.73±0.08	2.15±0.07	0.95±0.04	0.86±0.09	71.4±3.1
IR-480	2.70±0.05	2.54±0.14	1.87±0.15	0.94±0.07	0.71±0.04	73.7±1.8
GMPR-51	4.07±0.11	3.15±0.18	2.38±0.11	1.59±0.06	1.38±0.07	66.1±1.9
CRHP-1A	3.38±0.16	2.70±0.13	1.78±0.13	1.51±0.07	1.21±0.04	64.2±1.9
CRHP-1B	3.54±0.14	3.14±0.21	2.13±0.95	1.31±0.08	1.22±0.08	65.5±4.4
CRHP-1C	3.63±0.12	2.97±0.09	2.26±0.17	1.34±0.12	1.32±0.04	63.6±2.4

ty compared with IR-8 (Fig.6). At the time of maximum activity GMPR-51 and Erythroceros had almost twice the activity observed in IR-8. GDH activity was also greater in the grains of IR-8 from high than from the low nitrogen treatment.

Mobilization of N from flag leaf

At the time of panicle emergence nitrogen concentration of the flag leaves on dry weight basis was higher in GMPR-51 and lower in IR-480, compared with IR-8 (Table 2). With the growth of kernels there was a gradual loss of nitrogen from the flag leaf, and at harvest the nitrogen concentrations were considerably reduced. IR-480 showed greater mobilization of nitrogen from the flag leaf compared with IR-8 and GMPR-51. Erythroceros could be included for this study. CRHP 1A, 1B and 1C also transported less nitrogenous materials than did IR-8.

Discussion

In our earlier studies with wheat grown at 0 and 120 kg N/ha level, we had observed that an increase in grain protein was associated with (a) greater uptake of nitrogen, (b) increased levels of amino nitrogen and free amino acids, and (c) higher rate of RNA and protein synthesis during kernel development (Mitra and Bhatia 1973). Of course, these parameters are inter-related, and also dependent on other metabolic functions. The IR-8 experiment confirmed the above observations. The specific aim of investigating diffe-

rent high protein stocks was to find if variation in some of the above parameters could be detected among them. The results obtained show that there could be significant variation in mobilization of nitrogen from the flag leaf.

Nitrogen mobilization from flag leaf

Flag leaf nitrogen concentration at the time of heading differed with variety but this was not correlated with grain protein per cent. The mobilization of nitrogen from the flag leaf during grain development also varied significantly. GMPR-51 had higher nitrogen per cent in the flag leaf but mobilized less. Initially IR-480 had less nitrogen in the flag leaf but mobilized a greater proportion of it. Perez *et al.* (1972) reported a higher nitrogen per cent in first leaf blades of IR-480 and IR-8 and the former also had more efficient translocation of nitrogen from the leaf blades. Mobilization of nitrogen, not only from the flag leaf but also from other leaves and the culm, appears to be an important factor contributing to grain protein in rice. This is also true in wheat (see Evans *et al.* 1975). Experiments to investigate the mobilization of nitrogenous materials (amino acids) from leaves and culm in the high protein stocks used in this study are in progress and will be reported later.

Nitrogen utilization during grain development

Higher GDH activity was observed in developing grains of GMPR-51, Erythroceros and IR-480 than in those of IR-8. Increased GDH activity was also observed in grains from high-nitrogen plants in the IR-8 experiment. GDH is the key enzyme which catalyzes the fixation of ammoniacal nitrogen to organic nitrogen. The induced formation of GDH in rice plant roots by addition of ammonia to the media has been shown (Kanamori *et al.* 1972). It was also reported that the treatment caused not only an increase in soluble GDH activity, but also the development of new GDH bands in zymograms. GDH is believed to be a more important enzyme than nitrate reductase in nitrogen uptake and fixation in rice grown under flooded conditions (Perez *et al.* 1972). Considering the inducible nature of the enzyme the higher GDH activity observed in the developing grains of certain stocks implies greater availability of ammoniacal nitrogen, probably transported directly from the roots.

It is reported that the rice crop before heading takes up over 90 % of the total nitrogen required for average yield, while nitrogen application at the heading stage increases the nitrogen per cent in the grain (Murata and Matsushima 1975). Higher protein content has also been reported in wheat under certain conditions where nitrogen uptake continued throughout the grain filling period (Evans *et al.* 1975).

Other characters associated with increased protein accumulation, both in the grains from high-nitrogen plants of IR-8 and in high-protein stocks, were increased RNA and protein synthesis. However, these seem to result from increased availability of amino acids and, as such, these parameters do not seem to be the limiting factor. A considerable increase in RNA and protein synthesis was induced in IR-8 grains by nitrogen application. Another parameter which influences the protein content is the duration of grain development after panicle emergence. In the present study, Erythroceros and GMPR-51 showed a longer duration, 38 and 35 days respectively, for grain maturity compared with 30 days in IR-8 and IR-480. At least under our conditions, the period of grain development is limited to about 30-35 days in the commercially grown cultivars.

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Dr. R. Mitra
Biology and Agriculture Division
Bhabha Atomic Research Centre
Bombay 400 085 (India)