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Adenylate and Nicotinamide Nucleotides in Developing Soybean Seeds During Seed-Fill[1]

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ABSTRACT

Profiles of adenylate and nicotinamide nucleotides in soybean seeds were determined during seed-fill. The ATP content per seed increased during the early seed-filling stages to a level of 10 to 12 micrograms per seed. Seed ATP decreased after 40 days of development and reached its lowest level of less than 1 microgram at maturity. The ATP:ADP ratios were relatively constant at all seed development stages. Sharp increases in AMP levels during the late seed-fill stages were paralleled with a disappearance of ATP and ADP pools resulting in a reduced seed energy charge. Energy charge varied from the highest value of 0.78 at mid-seed-fill to less than 0.10 at maturity.

Of the oxidized (NAD, NADP) and reduced (NADH, NADPH) nicotinamide nucleotide forms, NAD was the most abundant. Levels as high as 17.5 micrograms per seed were observed during the mid-seed-filling stages. NADP was found almost exclusively in the reduced form with a NADP: NADPH ratio of less than 0.35, whereas the reverse was noted for NAD which was found mainly in the oxidized form with a NAD:NADH ratio in the range of 5 to 25. NADP was detected in low concentrations compared to the other adenylate and nicotinamide nucleotides. The nicotinamide redox charge defined as (NADH + NADPH)/(NAD + NADH) + (NADP + NADPH) was calculated to express the state of the energy balance between the oxidized and reduced nicotinamide nucleotide forms. The nicotinamide redox charge varied between 0.15 and 0.30 during seed development and was significantly lower than that found for the adenylate energy charge.

In spite of the studies in green plants emphasizing the importance of adenylates and nicotinamide nucleotides in photosynthesis and energy metabolism, little is known about their presence and function in developing seeds. Their concentrations may have an important directional influence on metabolism (9, 18), because they function as co-substrates for a variety of enzyme reactions. The central importance of nicotinamide nucleotides in biological oxidation-reduction reactions and changes in their endogenous levels and ratios in developing seeds are not well understood.

The amount of available energy stored in the adenylate nucleotide pools can be expressed according to Atkinson and Walton's (1) EC[3] units. Actively growing tissues have a high EC around 0.8 whereas the EC values of senescing tissues are less than 0.5. The relationship between nicotinamide nucleotide levels and tissue metabolism is not clear. This study reports the adenylate and nicotinamide nucleotide pools in developing soybean seeds during seed-fill. Adenylate energy charge, nicotinamide redox charge, and NADP content were low during certain seed-filling stages. A preliminary report of this study has been presented (16).

MATERIALS AND METHODS

Plant Culture. Soybeans (*Glycine max* [L.] Merr. cv. Wye) were grown to maturity in a controlled environment growth room under the following conditions: 12-h photoperiod, 4,300 ft-c, 24/18 C day-night temperature cycle and 75% RH. Seeds treated with *Rhizobium japonicum* inoculum were planted in 15-cm plastic pots containing a metro-mix potting medium. A modified Hoagland nutrient solution (6) was supplied daily. Samples of 20 to 30 pods were randomly selected from a population at regular intervals after full bloom (35 days postplanting) until seed maturation (60 days postanthesis). To avoid possible diurnal variation seed samples were harvested 1.5 h after the beginning of the photoperiod. The developing seeds were dissected from the pods in a 4 C cold room, frozen, and ground in liquid N_2 with a super-cooled mortar and pestle, and stored on Dry Ice for immediate extraction. Seed dry weights were determined by placing freshly weighed seeds in a 70 C drying oven and reweighing after 48 h.

Extraction of Adenylate Nucleotides from Soybean Seeds. The boiling glycine buffer procedure (10) was adapted for extraction of adenylate nucleotides from developing soybean seeds. Preliminary experiments using perchloric, sulfuric, and trichloroacetic acid extraction were less effective for soybean seeds and gave lower recovery of one or more of the adenylate standards. For extraction, 200-mg samples of freshly harvested, frozen, ground seed tissue were boiled for 1 min in 5 ml of 20 mM glycine buffer (pH 7.8), and immediately homogenized in a Pyrex tissue grinder and then rapidly chilled in an ice bath. The homogenizer was then rinsed with 1.5 ml of chilled buffer, and the homogenates were combined and centrifuged at 10,000*g* for 20 min at 4 C. The supernatant fraction was removed, diluted to a total volume of 9.3 ml with 0.2 M Tris buffer (pH 7.6), and stored at 0 C for assay on the same day. Samples boiled more than 2 min or stored frozen for greater than 6 h usually gave less reproducible results. The recovery of standard ATP, ADP, and AMP added to soybean seed samples during extraction and homogenization varied between 89 and 98% with a standard deviation of 5 to 8%.

Measurement of Adenylate Nucleotides (ATP, ADP, AMP). A Du Pont Luminescence Biometer was used for measuring ATP concentration in soybean seed extracts. One vial of purified enzyme luciferase (Du Pont ATP kit) and the substrate luciferin was dissolved in 3 ml of 10 mM of Mops buffer and 10 mM $MgSO_4$ (pH 7.4). Ten μl of seed extract were added to reaction cuvettes containing 100 μl of the prepared luciferase-luciferin mixture, and

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[3] Abbreviations: EC, energy charge; Mops, morpholinopropane sulfonic acid; DCPIP, 2,6-dichlorophenolindophenol.

the resulting bioluminescence measured as previously described (8). The instrument was routinely calibrated with standard ATP solutions. ADP was measured by enzymic conversion of ADP to ATP by pyruvate kinase, and then assayed for ATP. AMP was measured similarly by its stepwise conversion to ADP and then to ATP by myokinase and pyruvate kinase, respectively. For ADP determinations, 0.8 ml of seed extract was diluted with 0.1 ml of a reaction buffer (0.5 M Hepes plus 0.5 M Mg acetate [pH 7.5]), and incubated with 0.1 ml of a solution containing 20 μg of pyruvate kinase plus 500 nmol of trisodium P-enolpyruvate at 30 C for 15 min. For AMP determinations, 0.8 ml of seed extract, 0.1 ml of reaction buffer, and 0.1 ml of a solution containing 20 μg of pyruvate kinase, 20 μg of myokinase and 500 nmol of P-enolpyruvate were incubated for 15 min at 30 C. Sample ADP content was calculated by subtracting the original sample ATP content from the ATP content of the pyruvate kinase-converted sample. Sample AMP content was calculated from the difference of the ATP content of the pyruvate kinase-converted sample and the myokinase-pyruvate kinase-converted sample. Enzyme activity efficiencies of pyruvate kinase and myokinase were routinely determined with standard ADP and AMP solutions. Efficiency varied between 85 to 95%.

Extraction of Nicotinamide Nucleotides from Soybean Seeds. Glock and McLean's (4) animal tissue extraction procedures were adapted to extract nicotinamide nucleotides from developing soybean seeds. The oxidized nucleotides (NAD, NADP) were separated from the reduced forms (NADH, NADPH) by differential acid and alkali extraction. This differential extraction procedure of the oxidized and reduced forms of the coenzymes is based on their stability at different pH levels. Oxidized nucleotides are stable in acid and labile in alkali, whereas the reduced forms are destroyed in acid and maintain their stability at pH values above 7.0. NAD and NADP were extracted with 5% trichloroacetic acid. Preliminary experiments using HCl or H_2SO_4 were less effective and gave lower standard recoveries. A 500-mg sample of freshly harvested frozen seed tissue was homogenized in a Pyrex tissue grinder containing 5 ml of 5% trichloroacetic acid for 2 min and then rapidly chilled in an ice bath. The homogenizer was then immediately rinsed with 1.5 ml of trichloroacetic acid, and the homogenates combined. One ml of 0.2 M Tris buffer (pH 7.6), was added to each homogenate and the solution brought to pH 7.5 with 0.1 N NaOH. The neutralized homogenates were then centrifuged at 10,000*g* at 4 C for 20 min, and the supernatant fraction was stored at 0 C for assay on the same day. NADH and NADPH were extracted with 0.1 N NaOH at 70 C for 7 min, and these samples were treated similarly and neutralized with 0.1 N HCl. The recovery of NAD, NADH, NADP, and NADPH standards added to the seed sample during extraction and homogenization varied between 85 to 90% with a standard deviation of 6 to 11%. Frozen extracts could be stored (−20 C) overnight without appreciable changes in nicotinamide nucleotide content. Storage for more than 24 h usually gave lower values.

Measurement of Nicotinamide Nucleotides. The NAD, NADH, NADP, and NADPH concentrations in developing soybean seeds were determined enzymically coupling to specific coenzyme diaphorases with DCPIP reduction using modifications of Yamamoto's procedure (17). The NAD and NADH concentrations were determined with the enzyme alcohol dehydrogenase coupled to NADH-specific diaphorase with measurement of DCPIP reduction. The assay mixture contained 0.2 ml of the neutralized acid or alkaline extract incubated with 0.8 ml of 0.2 M Tris-HCl buffer (pH 8.7), 0.2 ml of 2.4 mM DCPIP, 0.1 ml alcohol dehydrogenase (160 U/ml), 0.05 ml NADH diaphorase (28 U/ml), and 0.1 ml ethanol. These were added to a cuvette in consecutive order for a total volume of 3.0 ml in H_2O. Extracts usually contained reducing substances which immediately oxidized some of the added DCPIP. This was overcome by titrating small amounts of DCPIP until a faint blue color persisted. The titrated DCPIP tissue extract mixture was allowed to stand briefly and then the other enzyme components added in the indicated order. NADP and NADPH were determined by a similar procedure in which glucose-6-P was coupled to a NADPH specific diaphorase, and DCPIP reduction was measured. The assay reaction mixture consisted of 0.5 ml of the neutralized acid or alkaline extract incubated with 0.5 ml of 0.2 M Tris-HCl (pH 7.6), 0.02 ml of 2.4 mM DCPIP, 0.3 ml glucose-6-P (3.3 mg/ml), 0.05 ml glucose -6-P dehydrogenase (200 U/ml), 0.1 ml NADPH diaphorase (10 U/ml). These were added to a cuvette in consecutive order for a total volume of 3.0 ml in water. Assay procedures were standardized daily with various concentration levels of each coenzyme. The DCPIP reduction rates were measured at 610 nm in a Cary model 14, spectrophotometer equipped with a high intensity light source. The NAD, NADH, NADP, NADPH tissue concentrations were calculated from corresponding standard curves of authentic substances.

RESULTS AND DISCUSSION

Fresh and dry weight changes of developing soybean seeds during seed-fill are shown in Figure 1. Seed fresh and dry weight changes and the dry matter accumulation rate during seed-fill were similar to those reported recently (5, 14, 15). The rate of seed filling varied from less than 1 mg/seed dry weight·day at 15 days postanthesis to a maximum of 8.0 mg/seed dry weight·day at 25 and 38 days postanthesis. At 58 days postanthesis (leaf senescence) the seed-fill rate decreased to less than 0.5 mg/seed dry weight·day. Changes in adenylate and nicotinamide nucleotide levels throughout seed growth are shown in Figures 2 and 3. The levels show a number of significant features in relation to seed growth and seed-filling.

The total adenylate pool as well as the concentrations of all three adenosine nucleotides (ATP, ADP, and AMP) show rela-

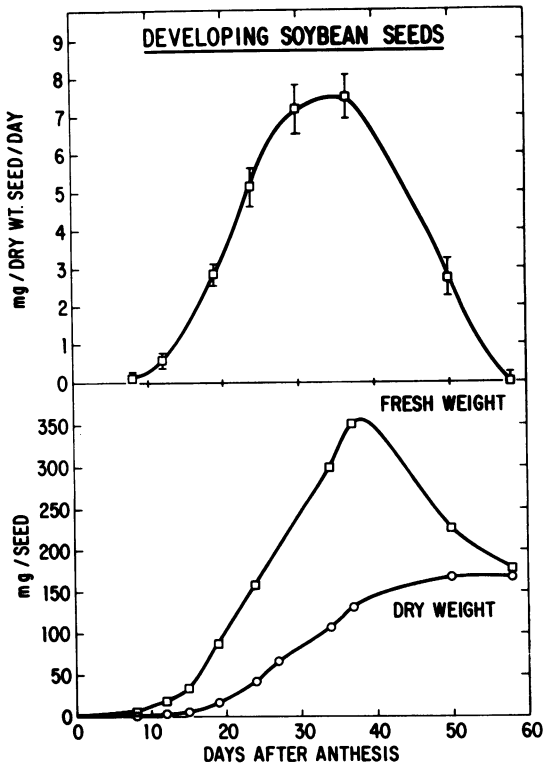

FIG. 1. Seed fresh and dry weight changes and the dry matter accumulation rate of developing soybean seeds during seed-fill samples at regular intervals from postanthesis to seed maturation. Each point is a mean of two replications with 20 to 30 pods/sample.

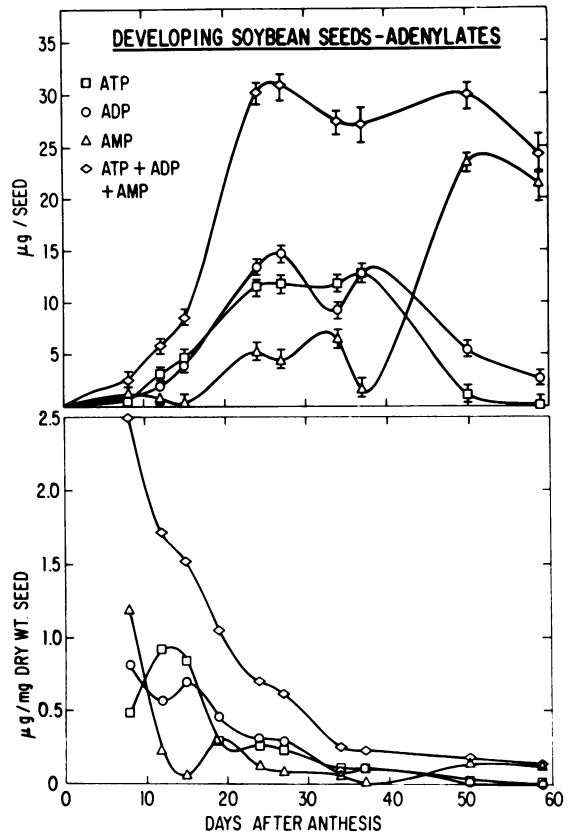

FIG. 2. Changes in content of total adenylate pools (ATP, ADP, AMP) in developing soybean seeds determined during seed-fill. Each point is the mean of two samples with each sample replicated twice during analysis.

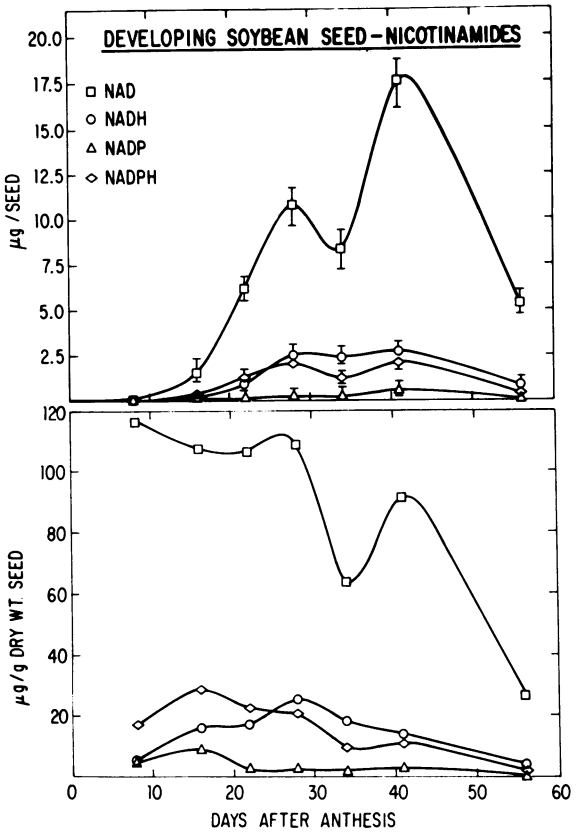

FIG. 3. Changes in content of oxidized (NAD, NADP) and reduced (NADH, NADPH) nicotinamide nucleotide forms in developing soybean seeds determined during seed-fill. Each point is the mean of two samples with each sample replicated twice during analysis.

tively large increases during early seed development reaching maximum values about 25 days after anthesis. However, when expressed on a μg adenylate/g dry weight basis, they decreased with development (Fig. 2). ATP and ADP levels of 10 to 12 μg/seed were maintained for about 15 days and then began decreasing after 40 days of development and reached their lowest levels of less than 1 μg for ATP and 2 μg for ADP at maturity. ATP concentration on a μg/g dry weight of seed increased from 0.5 at 8 days postanthesis to 0.9 at 12 days and then decreased steadily with development to its lowest value at maturity. A similar rise and fall in ATP concentrations was observed in developing rape and wheat seeds (3, 7). Despite the large increases in seed ATP content, especially during the most active growth stage, the ATP:ADP ratio remained remarkably constant. AMP levels were relatively low during early seed growth and increased to 5 μg/seed at 32 days. The levels then decreased to 2 μg/seed at 38 days followed by a 10- to 12-fold increase during late seed-filling. Sharp increases in AMP during seed maturation were paralleled with the disappearance of ATP and ADP pools without an increase in total adenylate content.

From the results of the three individual adenosine nucleotide concentrations, the amount of energy in the total adenylate pool available for seed growth and maintenance of cell energy balance can be calculated according to Atkinson and Walton's (1) EC units, where EC is defined as (ATP + 0.5 ADP)/(ATP + ADP + AMP). These calculated values are thought to reflect the degree of saturation of energy-rich bonds in the adenylate pool. Growing and dividing tissues maintain a high EC around 0.8, whereas senescing or dormant tissues maintain an EC of less than 0.5 (2). The EC variations for developing soybean seeds during seed-fill are shown in Figure 4. Relatively low EC values were found during several key periods of seed-fill. The EC values of less than

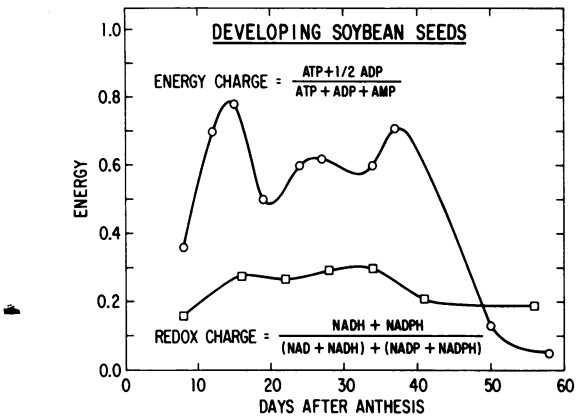

FIG. 4. Variations in the adenylate energy charge and the nicotinamide redox charge of developing soybean seeds during seed-fill.

0.5 were noted during early seed growth. Values increased from 0.38 to 0.78 by 15 days of development and then decreased to 0.50 at 20 days. This decline was followed by a gradual increase to 0.75 at 38 days, then a rapid decrease to its lowest value of 0.1 during leaf senescence and seed maturation. The average EC for the entire seed growth cycle was 0.52 which is lower than the 0.8 average values found in bacteria, yeast, animal tissue, and leaves (2). A variation in the EC from 0.78 to less than 0.10 at maturity for developing soybean seeds during the seed-fill process seems to be caused mainly by the large AMP concentration changes and not by the other adenylate pools, since ADP:ATP ratios remained almost constant. The high AMP content at maturity may represent a latent ADP and ATP pool available for the rapid and extensive

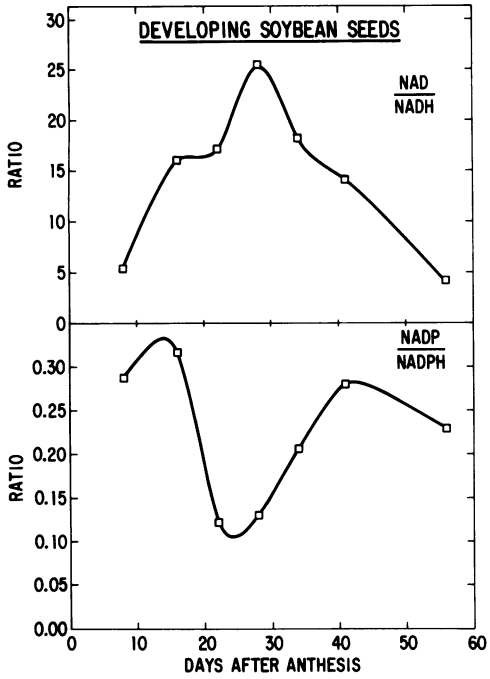

FIG. 5. The nicotinamide nucleotide ratios of oxidized to reduced for NAD and NADP of developing soybean seeds during seed-fill.

increase in respiratory activity that accompanies germination. These high AMP concentrations are consistent with the rapid rise in EC and ATP levels noted for germinating seeds following inbibition (11–13). In spite of the relatively low EC values found during certain key growth stages, developing soybean seeds continue to grow and develop at an unarrested rate. This suggests that developing seeds may have a lower critical EC threshold for growth than that noted for other tissues, or EC may not directly relate to seed growth.

Another important energy source regulating the state of energy balance and electron transport in the cell is the nicotinamide nucleotide content. The changes in oxidized (NAD, NADP) and reduced (NADH, NADPH) nicotinamide nucleotide forms in soybean seeds during seed-fill are shown in Figure 3. The concentration changes and calculated ratios (Fig. 5) show a number of significant features related to seed growth and development. Of the four nicotinamide nucleotides, NAD was the most abundant at all growth stages (Fig. 3). It increased from 0.02 μg/seed at 8 days after anthesis to an initial peak of 11.0 μg at 28 days of development, and then decreased to 7.5 μg at 36 days. This was followed by a rapid rise to its highest level of 17.5 μg at 42 days after which it decreased to a lower level of 5.0 μg at maturity. The concentrations of NADH, NADP, and NADPH all increased steadily with growth during the first 28 days of development, remained at their highest peak for about 15 days, and then decreased to lower levels during the advanced stages of seed-fill and maturity. Concentration changes for each nicotinamide nucleotide expressed on a μg/g dry weight were less dramatic. They remained at a constant level or increased slightly during the early seed development stages and then decreased at maturity. The most noticeable feature of the changing nicotinamide nucleotide patterns was the low endogenous NADP levels detected at all seed development stages compared to the other nicotinamide nucleotides. NADP was present predominantly and sometimes almost exclusively in the reduced form with a NADP:NADPH ratio considerably less than 1, whereas the reverse was noted for NAD which appears mainly in the oxidized form with a NAD:NADH ratio in the range of 5 to 25 (Fig. 4).

The state of the energy balance between the oxidized and reduced nicotinamide nucleotide forms can be an expression of the reducing power or redox charge indicating the degree of electron saturation. It can be calculated in a similar manner as the adenylate energy charge and can be defined as the ratio of (NADH + NADPH)/(NAD + NADH) + (NADP + NADPH) which can vary between a value of 0 and 1. The nicotinamide redox charge in comparison to the adenylate energy charge for soybean seeds during seed-filling is shown in Figure 4. The redox charge varied between 0.15 and 0.30 and was significantly lower than that found for the adenylate energy charge. The lowest redox charge was detected during early seed growth. The calculated values increased from an initial low of 0.15 to its highest level of 0.30 at 16 days of development and remained at this level until 35 days, and then decreased during the late seed-fill stages and maturity. The most noticeable feature of the changing nicotinamide nucleotide pattern causing a reduction in the redox charge was a relatively high level of NAD compared to the other nicotinamide nucleotide pools. NAD accumulation suggests that total oxidiation and reduction of the nicotinamide nucleotides are not in equilibrium for developing soybean seeds, and the ratio obtained is more typical of an energy-consuming system rather than an energy-yielding or storage system.

The metabolically available energy and the role of the adenylate and nicotinamide nucleotides in accepting, storing, and delivering energy are poorly understood, and little information is available for developing seeds. This is the first report comparting the variation and content of the adenylate and nicotinamide nucleotide forms and their ratios for developing seeds during seed-fill. These results show that developing soybean seed adenylate energy charge, redox charge, and NADP content are relatively low during the seed-filling compared to other plant parts. Since seed-fill includes cell growth and division which are energy-requiring processes, the seed-filling process requires certain energy forms. It is not known how this energy is made available or in what form it exists in the developing seed. Further studies on the *in vivo* concentrations and turnover rates of these coenzymes and their compartmentation within the seed are necessary to establish their physiological significance in photosynthate partitioning during the seed-filling processes.

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