Occurrence, Structure and Function of Intracellular Polyglucose in the Obligate Chemolithotroph Thiobacillus neapolitanus

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Abstract. Nitrogen-limited cells of the obligate chemolithotroph Thiobacillus neapolitanus formed an intracellular polymer during growth in the chemostat. This polymer was isolated and characterized as a branched polyglucose composed of units joined by \( \alpha-1 \rightarrow 4 \) and \( \alpha-1 \rightarrow 6 \) linkages. Polyglucose in T. neapolitanus can be considered a storage compound since formation of this compound took place during excess of energy and \( \text{CO}_2 \) whilst shortage of \( \text{CO}_2 \) resulted in rapid breakdown of polyglucose. Moreover the breakdown of polyglucose generated metabolically useful energy as could be demonstrated by polyglucose-dependent protein synthesis. Possession of polyglucose did not influence the viability of T. neapolitanus during prolonged periods of energy starvation. Activities of key enzymes of the oxidative pentose phosphate cycle, glucose-6-phosphate-dehydrogenase and 6-phospho-glucuronate-dehydrogenase, were demonstrated in cell free extracts of T. neapolitanus and appeared to increase 5- and 3-fold, respectively, during growth on \( \text{NO}_3^- \) instead of \( \text{NH}_4^+ \) as a nitrogen source.

Key words: Polyglucose — Chemolithotroph — Thiobacillus neapolitanus

Electron microscopical studies of the obligate chemolithotroph Thiobacillus neapolitanus revealed the presence of an intracellular polymer in this organism during nitrogen-limited growth conditions in the chemostat. This paper describes the isolation and characterization of this polymer which appeared to be polyglucose.

Storage compounds such as polyglucose and poly-\( \beta- \)hydroxybutyric acid accumulate in various bacteria when carbon and energy are in excess as for example during nitrogen-limited growth. The function of polyglucose as a storage compound has been extensively investigated in heterotrophic bacteria (for review see Dawes and Senior 1973). During periods of energy- or carbon limitation, or starvation, such storage compounds may generate carbon and energy to sustain the cell metabolism.

For bacteria with a comparable restricted metabolic capacity, such as the obligate photoautotrophic cyanobacterium Synechococcus sp. (Anacystis nidulans), it has been shown that intracellular polyglucose can serve as a carbon storage compound (Lehmann and Wöber 1976) whereas in the obligate methylotroph Methylococcus strain NCIB 11083 polyglucose can serve as an energy storage compound (Linton and Cripps 1978). In the light of the inability of obligate chemolithotrophic bacteria including T. neapolitanus to use exogenously supplied glucose as an energy source and the very limited capacity to use this compound as a carbon source (Matin and Rittenberg 1971; Smith and Hoare 1977; Matin 1978; Beudeker unpublished) it was of interest to investigate whether T. neapolitanus would be able to derive carbon and/or energy from its intracellular polyglucose. The results of these investigations are presented in this paper.

Materials and Methods

Medium and Growth Conditions

Thiobacillus neapolitanus strain X was grown in the chemostat as has been described by Kuenen and Veldkamp (1973). The basal growth medium for T. neapolitanus contained (g/l): \( \text{NH}_4\text{Cl}, 0.4; \text{MgSO}_4 \cdot 7 \text{H}_2\text{O}, 0.8; \text{KH}_2\text{PO}_4, 0.5; \text{K}_2\text{HPO}_4, 0.5; \text{Na}_2\text{S}_2\text{O}_3 \times 5 \text{H}_2\text{O}, 10.0. \) To a litre of this medium 2 ml of trace element mixture (Vishniac and Santer 1957) were added.

Ammonium limitation was achieved by lowering the \( \text{NH}_4\text{Cl} \) concentration 10-fold to 0.004 % (w/v) and by lowering the thiosulfate concentration to 0.65 % (w/v). At this concentration of thiosulfate T. neapolitanus was able to oxidize the excess of thiosulfate completely, whilst the nitrogen was still the only growth limiting compound. This made it possible to change over instantaneously from \( \text{NH}_4^+ \) limitation to thiosulfate limitation. NO\(_3^-\) and urea were also used by T. neapolitanus as a nitrogen source when supplied at nitrogen concentrations equivalent to those described for growth on ammonium as a nitrogen source. CO\(_2^-\) and thiosulfate limiting conditions were applied as has been described previously (Beudeker et al. 1980).

CO\(_2\) starvation was achieved by stripping CO\(_2\) from the air with sodiumarosinate and using 1 N NaOH for titration, instead of Na\(_2\)CO\(_3\).

Thiosulfate-starving conditions were created by switching off the medium supply; cysteine (final concentration 0.2 mM) was added to the culture as a sulphur source.

Simultaneous with change-over conditions from \( \text{NH}_4^+ \) limitation to other limitations or starvation, \( \text{NH}_4\text{Cl} \) (0.04 %, w/v) was added to the culture.

Isolation of Polysaccharide

Intracellular polysaccharide was isolated from T. neapolitanus by the procedure of Zevenhoven (1956). The effluent from an ammonium-limited chemostat was collected at \( 4^\circ\text{C} \). It appeared necessary to start with about 1,200 mg dry weight to obtain a significant amount of polysaccharide (80 mg). The cells were ruptured by sonication in the presence of 1:1 (w/v) ballonite beads (0.11 mm) and the suspension was centrifuged for 20 min at 40,000 \( \times g \) (4 \( ^\circ\text{C} \)). The extract was deproteinized by adding iccoid trichloroacetic acid (TCA) and the precipitated protein

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was removed by centrifugation for 20 min at 40,000 × g (4°C). To the clear supernatant 1:1 (v/v) ethanol was added after which the polysaccharide precipitated; it was collected by centrifugation for 20 min at 40,000 × g (4°C). The pellet was washed twice with ethanol (100%) and once with diethyl ether and dried at 100°C.

Analysis of the Isolated Polysaccharide

Isolated polysaccharide (10 mg) was hydrolyzed initially for 18 h in 2 ml 24 N H₂SO₄ at 4°C. The partly hydrolyzed polysaccharide was hydrolyzed further by diluting the mixture to 1 N H₂SO₄ with water and boiling this suspension for 10 h (Sietsema et al. 1969). The acidic solution was neutralized with solid BaCO₃ and the BaSO₄ precipitate was removed by filtration. The neutral solution was subjected to chromatography using cellulose thin layer plates (Merck). References containing 10 μg of glucose, fructose, sucrose or maltose were used. The chromatograms were developed at room temperature in a mixture of equal amounts of solutions A (375 ml n-butanol + 233 ml water) and B (176 ml propionic acid + 224 ml water). Sugars were located by spraying with p-anisidine phosphate. The solution was assayed for glucose using the glucose oxidase system (Boehringer, Mannheim, FRG).

The iodine-poly saccharide complex was prepared by adding 400 μg of polysaccharide to 3 ml of iodine reagent according to Zevenhuizen (1966).

Storage Polysaccharide Content

Total sugar was assayed with the anthrone reagent (Fairbairn 1953) with glucose serving as a standard. The amount of storage polysaccharide in T. neapolitanus cells was calculated by subtracting the sugar content of cells without storage polysaccharide.

Infrared Analysis

The infrared spectrum of isolated polysaccharide of T. neapolitanus was recorded with a Unicam 1100 infrared spectrophotometer and compared with that of glycogen (Merck, Darmstadt, FRG) using the KBr-disc technique (Barker et al. 1956).

Conditions of Amyolysis

The actions of β-amylase (β-1,4-glucan maltodextrinase EC 3.2.1.2) and α-amylase (α-1,4-glucan 4 glucanohydrolase EC 3.2.1.1) on the isolated polysaccharide of T. neapolitanus were tested as has been described by Linton and Cripps (1978). The action of amyloglucosidase (α-1,4-α-glucan glucohydrolase EC 3.2.1.3) on the isolated polysaccharide from T. neapolitanus was tested in an identical way as has been described for α-amylase. Reducing sugars were determined by the method of Somogyi (1952) using maltose as a standard.

Electron Microscopy

Pellets were embedded in Epon as has been described previously (Beudeker et al. 1980). To demonstrate carbohydrate compounds the thin sections were stained with aqueous solutions of phosphotungstic acid using golden grids according to Schade (1973).

Demonstration of the Function of Polyglucose in T. neapolitanus as an Energy Source

In a typical experiment nitrogen-limited cells (containing 2.4 mg of protein) were incubated at 28°C for 24 h in basal growth medium without thiosulfate. For the detection of polyglucose dependent protein synthesis 35S-cystine (100 μCi) was added at a concentration of 0.2 mM to the cell suspensions. Incorporation of 35S-cystine into several cell fractions concurrent with polyglucose metabolism was followed. Cell fractionation was carried out according to Cripps (1973). 35S-cysteine was taken as a tracer molecule because this compound can be used as a sulphur source by T. neapolitanus. The organism is unable to carry out assimilatory sulfate reduction and S from 35S-cysteine can only be incorporated into protein and into lipid-protein complexes and not into other cell fractions. Controls were taken with thiosulfate-limited cells which do not contain polyglucose. Incorporation of 35S-cysteine into T. neapolitanus was also followed in cells without polyglucose but in the presence of the energy source (thiosulfate). The thiosulfate was exhausted after 1 h of incubation. For comparison a similar experiment has been carried out with addition of 1-14C-acetate as a tracer molecule instead of cysteine.

Preparation of Cell-Free Extracts

Steady state cultures were harvested by centrifugation for 40 min at 20,000 × g (4°C) and washed in the assay buffer. Cell-free extracts were made by sonication for 5 × 30 s at 0°C, in the presence of 1:1 (v/v) bohlini beads (0.11 mm diameter). Beads and debris were removed by centrifugation for 20 min at 40,000 × g (4°C).

Enzyme Assays

D-Glucose-6-phosphate: NADP oxidoreductase (EC 1.1.1.49) reaction mixture contained 36 mM tri(hydroxymethyl)aminomethane (TRIS)-HCl pH 7.4; 12.5 mM MgCl₂; 6 mM glucose-6-phosphate; 1 mM NADP and cell-free extract (0.2 mg protein/ml of reaction mixture). Glucose-6-phosphate dependent NADP reduction was measured in a Perkin Elmer 124 double beam spectrophotometer at 30°C.

D-Phospho-D-gluconate: NADP oxidoreductase (EC 1.1.1.43) test mixture contained 36 mM TRIS-HCl pH 7.4; 12.5 mM MgCl₂; 1.5 mM D-phosphogluconate; 1 mM NADP and cell-free extract (0.2 mg protein/ml of test mixture). The reaction was followed as described above.

Protein Determination

Protein of cell-free extracts was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Protein of whole cells was determined with a modification of the microburet method of Goo (1953) as has been described by Kuenen and Veldkamp (1973).

Contamination by Other Bacteria

The cultures were frequently checked for contaminants by methods described by Kuenen and Veldkamp (1973).

Viability

Viability was measured on appropriate agar media by the method of Postgate (1969). Agar plates were incubated at 28°C and contained the basal growth medium for T. neapolitanus.

Chemicals

All radioisotopes were purchased from the Radiochemical Centre Amersham (England). Amyloglucosidase and α-amylase were obtained from Merck (Darmstadt, FRG). β-Amylase came from Sigma Chemicals Co. (St. Louis, Missouri, USA).

Results

Structure of the Intracellular Polyglucose

Polyglucose was isolated from cells of Thiobacillus neapolitanus, which had been grown under ammonium limitation at
a dilution rate of 0.07 h⁻¹ using standard procedures as described in Materials and Methods.

Thin layer chromatograms of the hydrolysed polysaccharide from *T. neapolitanus* showed only one spot with a *Rf* value of 0.28. This *Rf* value was identical to that of a standard glucose solution. Quantitative determination of the glucose from the hydrolysed polysaccharide using glucose oxidase confirmed the observation that the intracellular polysaccharide from *T. neapolitanus* consisted for more than 99% of glucose. The infrared spectrum of the polysaccharide taken in the region 960 – 720 cm⁻¹ showed absorbance peaks at 765, 850 – 860 and at 930 cm⁻¹. Commercial glycogen showed an identical spectrum (Fig. 1). The absorption at 850 – 860 cm⁻¹ is characteristic for derivatives with α-glucosidic linkages. Polysaccharides containing β-linkages do not show an absorbance peak at this wavelength. These data provide convincing evidence that the *Thiobacillus neapolitanus* polysaccharide consists of α-linked glucose units.

To obtain an impression about the branching character of the polysaccharide from *T. neapolitanus* the spectrum of the iodine-polysaccharide complex was measured. The more branching is present in a polysaccharide, the shorter the wavelength at which the absorbance maximum is reached (Zevenhuizen 1966). Addition of the iodine reagent to the polysaccharide isolated from *T. neapolitanus* yielded a red-brown solution with an absorbance maximum at 470 nm, characteristic for a highly branched polysaccharide (Zevenhuizen 1966; Linton and Crips 1978).

The actions of α- and β-amylase as well as amyloglucosidase on the isolated polysaccharide from *T. neapolitanus* were followed to detect whether α-1 → 4 and α-1 → 6 linkages existed. The percentage of the polysaccharide released as maltose or glucose is shown in Table 1. When α-amylase acts on polysaccharide α-1 → 4 linkages will be hydrolysed at random, yielding maltose, whilst β-amylase only hydrolyses the outer glucose chains to maltose. Neither amylase attacks α-1 → 6 linkages. In contrast, amyloglucosidase will hydrolyse not only the α-1 → 4 linkages but also the α-1 → 6 bonds.

As shown in Table 1 the amylases did not hydrolyse polysaccharide from *T. neapolitanus* completely. However, α-amylase plus amyloglucosidase did, indicating that the polysaccharide from *T. neapolitanus* is composed of α-1 → 4 and α-1 → 6 linked glucose molecules. The amylolysis limit (that is the amount of polysaccharide hydrolysed by the β-amylase) for the polysaccharide from *T. neapolitanus* was found to be 50% (Table 1). To visualize the deposition of polysaccharide within the cells ultrathin sections were prepared for electron microscopy (Fig. 2). Carbohydrate was stained specifically (Fig. 3) showing rosette-like granules of more or less uniform size (25 nm). Each individual cell contained the granules. Poly-β-hydroxybutyrate was never detected in *T. neapolitanus* grown under ammonium limitation in the chemostat (D = 0.07; pO₂ = 50% air saturation).

### Table 1. The percentage of polyglucose released as maltose by the action of α- and β-amylase (amylolysis limit) and the percentage of polyglucose released as glucose by the combined action of amyloglucosidase and α-amylase on polyglucose isolated from *Thiobacillus neapolitanus*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Maltose</th>
<th>% Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amylase</td>
<td>71</td>
<td>—</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>50</td>
<td>—</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>—</td>
<td>99</td>
</tr>
</tbody>
</table>
content of the culture grown at a fixed dilution rate of 0.07 h\(^{-1}\) was independent of the nitrogen source applied and amounted to approximately 8\% of the dry weight. The polyglucose content of an ammonium limited culture expressed per mg of protein varied inversely with the applied growth rate (Fig. 4). This phenomenon which has also been observed in other organisms (Herbert 1961; Linton and Cripps 1978) is most likely explained by the relatively high overcapacity of \(T. neapolitanus\) to oxidize thiosulfate at low dilution rate (Beudeker and Kuenen, unpublished results). During thiosulfate- or CO\(_2\)-limitation in the presence of excess ammonia (7.7 mM) polyglucose was not detected in \(T. neapolitanus\). However, when CO\(_2\)-limited chemostat cultures of \(T. neapolitanus\) were shifted to CO\(_2\)-excess and thiosulfate limitation a transient accumulation of polyglucose was observed which disappeared after prolonged cultivation under thiosulfate limitation.

Function of the Intracellular Polyglucose in the Obligate Chemolithothroph \(T. neapolitanus\)

The possible function of polyglucose in \(T. neapolitanus\) as a carbon storage compound was examined by change-over experiments from NH\(_4\)-limitation in the chemostat to CO\(_2\) limitation and CO\(_2\) starvation (\(D = 0.07\); P\(_{O_2}\) = 50\% air saturation). Figure 5 shows that polyglucose disappeared from the culture at a rate considerably faster than the dilution (wash-out) rate. During change-over from NH\(_4\)-limitation to CO\(_2\) starvation 40\% of the polyglucose was degraded within 20 min. This was followed by a reproducible short period of approximately 60 min, during which no further degradation was detected. After this period the breakdown resumed at a much slower rate. Protein content, viability and carbon content of the cells did not change significantly during these experiments (not shown).

The degradation of polyglucose during the change-over from NH\(_4\)-limitation to thiosulfate limitation (or starvation) appeared to be slower as compared to the change-over to CO\(_2\) limitation (or starvation) but was still significantly faster than would be expected from mere washout of the culture.

The degradation of the intracellular polyglucose in \(T. neapolitanus\) after transfer of NH\(_4\)-limited cells (\(D = 0.07\); P\(_{O_2}\) = 50\% air saturation) to conditions of energy starvation in batch culture appeared to yield metabolically useful energy. The magnitude of \(\text{35}^\text{S}\)-cysteine incorporation was very similar in cells containing polyglucose and cells supplied with thiosulfate (Table 2), in cells containing polyglucose or cells provided with thiosulfate also the percentage of label incorporated into protein was comparable. These results clearly showed that the breakdown of polyglucose allowed protein synthesis. Cells without polyglucose or thiosulfate hardly incorporated any \(\text{35}^\text{S}\)-cysteine (Table 2) whilst they were able to do so after addition of thiosulfate as an energy source (not shown). The uptake and incorporation of 1-\(^{14}\text{C}\)-acetate by \(T. neapolitanus\) in an identical experiment showed similar results (not shown).

### Table 2. Effect of intracellular polyglucose on the incorporation of radioactive cysteine in the obligate chemolithotroph \(T. neapolitanus\).

<table>
<thead>
<tr>
<th></th>
<th>Cells with polyglucose</th>
<th>Cells without polyglucose</th>
<th>Cells without polyglucose but with thiosulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total uptake of (\text{35}^\text{S})-cysteine</td>
<td>1,000</td>
<td>55</td>
<td>1,125</td>
</tr>
<tr>
<td>Low molecular weight compounds fraction (^*)</td>
<td>244</td>
<td>9</td>
<td>292</td>
</tr>
<tr>
<td>Lipid fraction (^*)</td>
<td>221</td>
<td>30</td>
<td>224</td>
</tr>
<tr>
<td>Nucleic acids fraction (^*)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Protein fraction (^*)</td>
<td>535</td>
<td>16</td>
<td>609</td>
</tr>
</tbody>
</table>

Effect of the Presence of Intracellular Polyglucose on the Viability of \(T. neapolitanus\)

The effect of intracellularly stored polyglucose on the viability of \(T. neapolitanus\) incubated in 0.05\% (w/v) phosphate buffer at 28°C is shown in Fig. 6. Though polyglucose disappeared from the cells no significant difference in viability could be detected between cells containing polyglucose and cells without polyglucose. Even after 2 months of starvation the viability of both types of cells was still approximately 40\%.
Fig. 6. The effect of prolonged starvation in 0.05 % phosphate buffer on the viability of cells of *T. neapolitanus* with (x) or without (o) intracellular polyglucose.

Table 3. Activities of glucose-6-phosphate dehydrogenase and 6-phospho-glucuronate dehydrogenase measured in cell-free extracts of *Thiobacillus neapolitanus* grown in the chemostat (D = 0.07 h⁻¹; pO₂ = 90 % air saturation) during NH₄⁺ and NO₃⁻ limitation

<table>
<thead>
<tr>
<th>Growth limiting substrate</th>
<th>Glucose-6-phosphate dehydrogenase</th>
<th>6-Phosphogluconate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>77</td>
<td>86</td>
</tr>
</tbody>
</table>

Pathway of Glucose Metabolism

The key enzymes of the Embden-Meyerhof glycolytic pathway and Entner-Doudoroff pathway have been shown to be absent in *T. neapolitanus*, whereas the enzymes for the oxidative pentose phosphate cycle could be detected (Math and Rittenberg 1971). Therefore, only the key enzymes of the oxidative pentose phosphate cycle were tested for activity. Table 3 shows the activities of both glucose-6-phosphate dehydrogenase and 6-phospho-glucuronate dehydrogenase in cell-free extracts of *T. neapolitanus* grown in the chemostat under nitrogen-limitation. During NO₃⁻-limited growth these activities were 5 and 3 times higher, respectively, as compared with NH₄⁺-limited growth. The activities measured are high enough to account for the observed breakdown of (poly)glucose through the oxidative pentose-phosphate cycle. Attempts to elucidate the pathway of glucose metabolism in *T. neapolitanus* by radiospectrometry according to Wood et al. (1977) were unsuccessful due to the very limited uptake of exogenous 14C-glucose by *T. neapolitanus*.

Discussion

According to the criteria of Wilkinson (1959) the intracellular polyglucose of *T. neapolitanus* can be considered to be a storage compound:

1. Polyglucose was formed in *T. neapolitanus* during N-limitation growth with excess of carbon and energy.

2. Polyglucose functioned as a storage carbon compound by *T. neapolitanus* as can be concluded from the rapid degradation of polyglucose during change-over from NH₄⁺-limitation to CO₂ starvation or to CO₂-limitation (Fig. 5). The observed lag period during the rapid degradation of polyglucose in *T. neapolitanus* during CO₂ starving conditions might be needed to induce enzymes for the further degradation of polyglucose.

3. Degradation of polyglucose resulted in production of metabolically useful energy as could be judged from the polyglucose dependent protein synthesis (Table 2).

The importance of polyglucose for the survival of *T. neapolitanus* obviously lies in its role as reserve material during short periods of energy- or carbon starvation. The importance of polyglucose for the maintenance of viability is less obvious since *T. neapolitanus* turned out to be extremely resistant to starvation (Fig. 6). For heterotrophic bacteria such as *Streptococcus mitis* (van Houte and Jansen 1970) and *Escherichia coli* (Dawes and Ribbons 1963) it has been shown that the possession of glycogen resulted in a prolonged viability during energy-starvation periods. It has been shown that the facultatively anaerobic cyanobacterium *Oscillatoria limnetica* is able to ferment glucose derived from intracellular polyglucose under anaerobic conditions (Oren and Shilo 1979). The possibility that the aerobic *T. neapolitanus* would be capable of anaerobic metabolism of polyglucose for survival under anaerobic conditions is presently under investigation.

Polyglucose in cyanobacteria is metabolized aerobically through the oxidative pentose phosphate cycle as was shown by radiospectrometric experiments combined with enzyme studies (Pelroy et al. 1972). Radiospectrometric studies in *T. neapolitanus* have been unsuccessful hitherto due to the extremely low uptake of 14C-glucose by these cells. All enzyme data, however, indicated that glucose metabolism in *T. neapolitanus* proceeds through the oxidative pentose phosphate cycle (Table 3). The increase in activities of the key enzymes of this cycle during growth on NO₃⁻ as a nitrogen source was not due to the presence of activators in the cell-free extract as was concluded from experiments using mixed extracts. Whether glucose-6-phosphate dehydrogenase and 6-phosphogluconate-dehydrogenase were increased in activity by modification or by enzyme induction in this organism is still unknown. The increase in activities of both enzymes has also been found in plants during growth on NO₃⁻ as a nitrogen source and is most likely explained by the demand for NADPH needed for the reduction of NO₃⁻ to NH₄⁺ (Sarkissian and Fowler 1974).

The clear demonstration of the ability of *T. neapolitanus* to derive both energy and carbon from intracellular (poly)glucose is the first report on the importance of polyglucose as reserve material in an obligately chemolithotroph. This is another example of the interesting parallels between obligately photosynthetic and the obligately chemolithotrophs (see reviews Smith and Iloare 1977; Doolittle 1979). Both groups of organisms are not able to grow on glucose which is due to the absence of specific carriers for glucose in obligately photosynthetic cyanobacteria (Beaupler and Smith 1978).

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