Concentrations of major plasma proteins in serum and whole-tissue extracts of porcine fetuses during development

F. Lampreave and A. Piñeiro

Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, 50009 Zaragoza, Spain

Summary. In extracts from fetuses up to 32 days of gestation, the major serum proteins were fetuin, α-fetoprotein and α₁-antitrypsin, but albumin was not detected. The concentration of all proteins rose with age until 40–50 days of gestation; and then the serum concentration of α-fetoprotein (2.9 mg ml⁻¹), α₁-antitrypsin (4.4 mg ml⁻¹) and transferrin (2.6 mg ml⁻¹) fell progressively to about 1 mg ml⁻¹ at birth, whereas those of fetuin, albumin and α₁-acid glycoprotein increased. The patterns of serum proteins in fetuses at about the middle of gestation were similar in extracts and sera. At birth, the major proteins were α₁-acid glycoprotein and fetuin, which accounted for 45 and 18% of serum proteins, respectively. Albumin represented only 7% of serum proteins at this age. For most of the second gestational period, the six quantified proteins accounted for about 85% of total serum proteins. In early gestation, a significant proportion of serum proteins was intracellular.

Keywords: proteins; fetus; pig; fetal development

Introduction

The developmental changes in serum protein patterns have been studied extensively in man, because there are specific antisera against most human plasma proteins (Gitlin & Gitlin, 1975). In other mammals, data on serum proteins during fetal or adult life are limited.

The porcine fetus is a suitable model for studying the genetic expression and metabolic functions of serum proteins during development, because the epitheliochorial placenta of this species isolates the conceptus from maternal plasma proteins. The concentrations of serum proteins in fetal pigs are much lower (about 20 mg ml⁻¹) during most of the fetal period than in adults (Ingvarsson et al., 1978; Stone, 1981; Cavanagh et al., 1982; Lampreave & Piñeiro, 1982). In porcine fetuses, α-fetoprotein, α₁-protease inhibitor (α₁-antitrypsin), fetuin, transferrin and α₁-acid glycoprotein are predominant (Carlsson et al., 1976; Dziegielewska et al., 1980; Weström et al., 1982; Lampreave & Piñeiro, 1982, 1984). Albumin is a minor component in pigs during most of the gestational period (Karlsson, 1970; Ingvarsson et al., 1978; Dziegielewska et al., 1980; Stone, 1981; Lampreave & Piñeiro, 1982). However, quantitative data on the production of plasma proteins during pig development are limited (Cavanagh et al., 1982; Weström et al., 1982). In the present work, we quantified six major proteins immunologically in extracts and sera of porcine fetuses at different stages of gestation.

Materials and Methods

Serum samples and fetal extracts

Porcine fetuses of known gestational age, ranging from 23 days to birth, were removed from sows immediately after slaughter. Blood samples from fetuses aged about 50 days or more were collected from vessels in the axilla. Sera were prepared by centrifugation at 1500 g for 5 min after blood coagulation at room temperature and stored at −20°C. Sera from perinatal pigs were provided by the Surgery and Reproduction Institute of the Veterinary Faculty
of Zaragoza University. Fetuses at 23–57 days of gestation were weighed and frozen at −20°C. At each gestational age, pooled frozen fetuses from different litters were chopped and homogenized in a Waring blender after addition of cold phosphate-buffered saline (0.15 mol NaCl 1 -1 and 0.01 mol potassium phosphate 1 -1 at pH 7.2; 1 ml per 3 g of fetuses). After centrifugation at 10,000 g and 4°C for 15 min, the supernatant was carefully removed. The extraction was repeated twice with the pellets. The three supernatants were mixed, the total volume was recorded and aliquots were frozen until use. At some stages of gestation, extracts and serum samples were obtained from different fetuses of the same litters. Serum of adult pigs was obtained at the local slaughterhouse.

**Purified proteins**

Porcine albumin and α1-acid glycoprotein were isolated as previously reported (Lampreave et al., 1982; Lampreave & Piñeiro, 1984). The isolation of porcine α-fetoprotein has also been reported (Lampreave et al., 1982); this was based on chromatography of pooled fetal serum on a DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) column equilibrated with a buffer of 0.05 mol Tris-HCl 1 -1 and 0.05 mol NaCl 1 -1 at pH 8.8. Bound proteins were eluted with a saline gradient between 0.05 mol NaCl 1 -1 and 0.25 mol NaCl 1 -1 in the same Tris-HCl buffer at pH 8.8. Fractions eluted between 0.14 and 0.17 mol NaCl 1 -1 (pool A) contained fetuin, α1-acid glycoprotein and minor amounts of α1-antitrypsin and other components of high molecular mass. Those eluted between 0.19 and 0.23 mol NaCl 1 -1 (pool B) contained albumin, α1-fetoprotein and α1-antitrypsin. Pool B was the starting sample for the isolation of α1-antitrypsin. This was achieved as follows: albumin was adsorbed on insoluble Cibacron Blue (Naval et al., 1982); α1-antitrypsin and α-fetoprotein (30 mg total protein) were then separated by chromatography on a DEAE-Sephadex A-50 column (10 × 1.5 cm) equilibrated with 0.04 mol potassium phosphate 1 -1 buffer at pH 7.2. Elution was carried out with a saline gradient (200 ml from 0 to 0.3 mol NaCl 1 -1 ) in the same buffer. Fractions (3 ml each) containing only α1-antitrypsin were selected after analysis by immunodiffusion against specific antiserum to α-fetoprotein. Pool A (containing fetuin) was concentrated by ultrafiltration and then subjected to Sephadex G-100 (Pharmacia) chromatography. Fractions from the main elution peak, which contained fetuin, α1-acid glycoprotein and minor amounts of α1-antitrypsin, were selected. This sample, after quantification of total proteins, α1-acid glycoprotein and α1-antitrypsin, was used as fetuin standard. Porcine transferrin was provided by J. H. Brock (Glasgow University, UK).

The purity of isolated proteins was assessed by electrophoresis in gel slabs, prepared with 6% polyacrylamide and 0.8% agarose (Uriel, 1966), and by immunoelectrophoresis against rabbit antiserum to fetal pig serum. Total serum proteins and purified proteins were quantified by the method of Lowry et al. (1951), using bovine serum albumin as reference.

**Immunological methods**

Specific antisera against α-fetoprotein, albumin and α1-acid glycoprotein were raised in rabbits as described by Lampreave & Piñeiro (1982). Similar protocols were used to prepare antisera to total fetal serum proteins, transferrin, α1-antitrypsin and the fetuin preparation already indicated. The latter antiserum reacted not only with fetuin, but also against α1-antitrypsin and α1-acid glycoprotein. This antiserum was made specific for fetuin by incubation for 30 min at 37°C and then for 24 h at 4°C, with equivalent amounts of pure α1-acid glycoprotein and α1-antitrypsin solutions. After centrifugation at 5000 g the supernatant was used as specific anti-fetuin antiserum. Antiserum specificity was tested against fetal pig serum by immunoelectrophoresis. The determination of individual serum proteins was carried out by quantitative radial immunodiffusion in 1% agarose gels (Mancini et al., 1965), using specific antiserum and, as reference standards, the purified proteins containing 10–100 μg ml⁻¹. In these experimental conditions the coefficient of variation of the method was 7%.

**Statistical analysis**

Results for serum samples are expressed as means ± SD.

**Results**

**Proteins and antisera**

At 90 days of gestation, transferrin (lane 2 in Fig. 1) shows a characteristic multiband pattern; fetuin (lane 3) contained impurities, mainly α1-acid glycoprotein; lanes 4 to 7 correspond to pure preparations of α1-acid glycoprotein, albumin, α-fetoprotein and α1-antitrypsin, respectively; minor bands observed in albumin (lane 5) and α-fetoprotein (lane 6) patterns correspond to dimeric forms of the proteins. Antiser to individual proteins showed single precipitation lines when tested by immunoelectrophoresis against fetal serum (Fig. 2). These specific antisera were used for quantification of the proteins by radial immunodiffusion.
Fig. 1. Electrophoresis in polyacrylamide (6%) and agarose (0.8%) of (1) fetal pig serum at 90 days of gestation, (2) transferrin, (3) fetuin preparation, (4) $\alpha_1$-acid glycoprotein, (5) albumin, (6) $\alpha$-fetoprotein and (7) $\alpha_1$-antitrypsin.

Fig. 2. Immunoelectrophoretic analysis of fetal pig serum (at 90 days of gestation) against (1) anti-fetal-pig serum, (2) anti-transferrin, (3) anti-albumin, (4) anti-\textalpha-fetoprotein, (5) anti-\textalpha$_1$-antitrypsin, (6) anti-\textalpha$_1$-acid glycoprotein and (7) anti-fetuin.
Serum proteins in fetal extracts

In the extracts from fetuses at 23 days of gestation, α-fetoprotein, α₁-antitrypsin and fetuin occurred at the highest concentrations of the six proteins analysed, at about 0.4–0.5 mg ml⁻¹ (Table 1). The concentration of these proteins rapidly rose with age reaching maximal values in fetuses at 47 days. At this age, fetuin and α₁-antitrypsin were predominant (1.9 and 1.7 mg ml⁻¹, respectively). Transferrin followed a similar pattern, although its concentration was lower than that of the other three proteins during this period. The concentration of α₁-acid glycoprotein was low at 23 days (0.14 mg ml⁻¹), but increased with age, reaching a value of 0.75 mg ml⁻¹ in extracts from 57-day-old fetuses. With the quantitative method used, albumin could not be measured in extracts until 37 days; then the concentrations of this protein increased progressively.

Table 1. Concentration (mg ml⁻¹) of individual serum proteins in extracts of porcine fetuses at different stages of gestation

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>23</th>
<th>26</th>
<th>32</th>
<th>37</th>
<th>47</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fetuses in each homogenate*</td>
<td>5</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total weight (g)</td>
<td>1.45</td>
<td>7.6</td>
<td>13.8</td>
<td>62.2</td>
<td>107</td>
<td>361</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>0.49</td>
<td>0.96</td>
<td>1.15</td>
<td>1.34</td>
<td>1.29</td>
<td>0.80</td>
</tr>
<tr>
<td>Albumin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.09</td>
<td>0.15</td>
<td>0.28</td>
</tr>
<tr>
<td>α₁-Antitrypsin</td>
<td>0.38</td>
<td>0.85</td>
<td>1.30</td>
<td>1.74</td>
<td>1.68</td>
<td>1.37</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.27</td>
<td>0.44</td>
<td>0.61</td>
<td>1.10</td>
<td>1.20</td>
<td>1.22</td>
</tr>
<tr>
<td>Fetuin</td>
<td>0.55</td>
<td>0.76</td>
<td>1.45</td>
<td>1.60</td>
<td>1.90</td>
<td>1.80</td>
</tr>
<tr>
<td>α₁-Acid glycoprotein</td>
<td>0.14</td>
<td>0.24</td>
<td>0.26</td>
<td>0.31</td>
<td>0.42</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*From at least two litters.
†Refers to all the fetuses used.
ND, not detected.

Proteins in sera

The concentration of total serum proteins was about 19–20 mg ml⁻¹ during most of the period studied, although a small decrease was observed at 75 days of gestation (Table 2). In the last 2 weeks before birth, the total serum proteins increased considerably (to 27.5 mg ml⁻¹ at birth). In serum from fetuses at 50 days, the major proteins were α₁-antitrypsin and fetuin, both at about 4 mg ml⁻¹, which together represented 40% of total serum proteins. The concentration of fetuin remains high and almost constant during the period studied, but that of α₁-antitrypsin fell steadily until birth. A similar pattern was observed for α-fetoprotein, the concentration declining from 2.9 mg ml⁻¹ at 50 days to 1 mg ml⁻¹ at birth. The main content of transferrin (about 3 mg ml⁻¹) was in serum from fetuses at 50–57 days of gestation. As for α₁-antitrypsin and α-fetoprotein, serum concentration of transferrin decreased in the second part of fetal life, with a minimum (1.1 mg ml⁻¹) near term. The concentration of albumin was low and almost constant (0.4–0.5 mg ml⁻¹) from 50 to 75 days of gestation and then progressively rose to 1.8 mg ml⁻¹ at birth. The concentration of α₁-acid glycoprotein was similar to those of α-fetoprotein and transferrin and lower than those of α₁-antitrypsin and fetuin in fetuses at 50–57 days of gestation but from 90 days of gestation to birth the concentration of α₁-acid glycoprotein was higher than that of any other protein. At birth, α₁-acid glycoprotein represented 45% of serum proteins.

Discussion

We quantified six major proteins (α-fetoprotein, albumin, α₁-antitrypsin, transferrin, fetuin and α₁-acid glycoprotein) in fetal pig serum from 50 days of gestation to birth. These proteins
<table>
<thead>
<tr>
<th>Sample</th>
<th>Fetus</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (days)</td>
<td>50</td>
<td>57</td>
</tr>
<tr>
<td>n*</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Total proteins</td>
<td>19.40 ± 1.3</td>
<td>19.10 ± 1.1</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>2.91 ± 0.30</td>
<td>2.41 ± 0.25</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.39 ± 0.10</td>
<td>0.49 ± 0.10</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
<td>4.36 ± 0.80</td>
<td>3.41 ± 0.42</td>
</tr>
<tr>
<td>Transferrin</td>
<td>2.59 ± 0.60</td>
<td>3.04 ± 0.45</td>
</tr>
<tr>
<td>Fetuin</td>
<td>3.48 ± 0.50</td>
<td>4.30 ± 0.35</td>
</tr>
<tr>
<td>α1-Acid glycoprotein</td>
<td>2.70 ± 0.34</td>
<td>2.62 ± 0.50</td>
</tr>
</tbody>
</table>

*Number of samples analysed at each age from at least three litters.
The % of each individual protein with respect to the total proteins is shown in parentheses.
ND, not detected.
accounted for about 85% of total serum proteins during the period studied. This figure is reasonable considering that, although other proteins, such as α_{2}-macroglobulin (Weström, 1979), ceruloplasmin (Dziegielewksa et al., 1980), haptoglobin (Stone, 1989), immunoglobulins (Bourne, 1974) and lipoproteins (Johansson & Karlsson, 1982), have also been characterized, in fetal pig serum they are all minor components. The same six proteins were also determined in total extracts from fetuses of 23 days or more, which is the major part of the gestational period of pigs after implantation. All the proteins except albumin were detected in high amounts in these early fetuses, (see Table 2) suggesting that these proteins may play an important role during development.

**Albumin and α-fetoprotein**

Albumin and α-fetoprotein have been extensively studied in porcine fetuses (Karlsson, 1970; Stone, 1981; Cavanagh et al., 1982; Lampreave & Piñeiro, 1982). Albumin was not detected in extracts from fetuses until 31 days of gestation as previously reported by others (Stone, 1981; Cavanagh et al., 1982). The contribution of albumin to total plasma proteins was small during fetal life. At birth, albumin represented <7% of total serum proteins. The developmental pattern of α-fetoprotein was similar to that found by others, but, although it is a major protein in extracts from early fetuses, its concentration was lower than those of fetuin and α_{1}-antitrypsin. Maximal concentrations of α-fetoprotein were found in extracts from 37-day-old fetuses.

Alpha-fetoprotein has been considered to be the first plasma protein in fetal life (Gitlin & Boesman, 1967), but, at least in porcine fetuses, α-fetoprotein shares this priority with fetuin, α_{1}-antitrypsin and transferrin. Alpha-fetoprotein, like albumin, binds long-chain fatty acids (Parmelee et al., 1978; Piñeiro et al., 1979; Lampreave et al., 1982), but it shows higher affinity than albumin for arachidonic acid and mainly for 4,7,10,13,16,19-docosahexanoic acid (Anel et al., 1989). Both fatty acids, which are derivatives of the essential linoleic and linolenic acids, respectively, accumulate during fetal development in lipids of neural and other tissues. The function of α-fetoprotein is probably to facilitate the transfer of these fatty acids from maternal circulation to the fetus (Calvo et al., 1988).

**Fetuin**

Fetuin, first characterized in fetal bovine serum (Pedersen, 1944), has also been identified in other mammals, including pigs and man (Dziegielewksa et al., 1980; Christie et al., 1987). Bovine fetuin inhibits trypsin, but not chymotrypsin (Galemebeck & Cann, 1974; Rohrlich & Rifkin, 1981) and this could be related to its biological function which is still ill defined. A major trypsin inhibitor (α_{2}-antitrypsin) was previously characterized in serum of fetal and adult pigs (Weström, 1979). This trypsin inhibitor and pig fetuin (Dziegielewksa et al., 1980) are the same protein (Lampreave & Piñeiro, 1982). Cavanagh et al. (1982) reported quantitative data on fetuin in fetal pigs, concluding that this is the major serum protein during the last part of the gestation. Although in the present work fetuin was an important protein in fetal pig serum, its concentration was never >25% of total proteins and at birth represented 18% of total proteins. At this age, by contrast, α_{1}-acid glycoprotein represented 45% of total serum proteins. Weström et al. (1982) reported that α_{2}-antitrypsin (fetuin) was 4–5 times more concentrated in serum of fetuses than in adults. A similar trend was found in our quantitative data.

The high concentration of fetuin in porcine fetuses and in fetal ruminants (Cavanagh et al., 1982) is interesting from the physiological point of view. Fetuin and other protease inhibitors (particularly α_{1}-antitrypsin) could protect fetuses from proteolytic activity released from growing cells. As suggested by Weström et al. (1982), newborn pigs as well as newborn ruminants absorb undigested proteins, including probably intestinal proteases, during the few first days after birth. The deleterious effects of these proteases could be avoided by fetuin and other protease inhibitors.
\( \alpha_1 \)-Antitrypsin

\( \alpha_1 \)-Antitrypsin is a major serine protease inhibitor which probably participates in the inhibition of cellular proteases delivered to blood circulation, particularly elastase (Laurell & Jeppsson, 1975); it is a major protein in the serum of porcine fetuses at any age. High concentrations of this protease inhibitor have also been observed in ovine fetuses (Dziegielewska & Saunders, 1981). The developmental changes in \( \alpha_1 \)-antitrypsin concentration in fetal serum, reported in the present work, closely resemble those observed by Weström et al. (1982), though those were in relative terms with respect to the adult. In serum of 50-day-old fetuses, the \( \alpha_1 \)-antitrypsin was about 4 mg ml\(^{-1}\). This concentration was, as reported by Weström et al. (1982), about 15 times higher than in the adult. This relationship could be even higher for fetuses at about 37 days of gestation, since in fetal extracts the highest concentration of \( \alpha_1 \)-antitrypsin was observed at this age. The decrease of \( \alpha_1 \)-antitrypsin in the second part of the gestational period was previously interpreted as a loss of synthetic activity in the yolk sac (Weström et al., 1982). However, the synthesis of this protein also decreases in the fetal liver during this period, as observed by Stone (1989) using hybridization techniques with cloned cDNA for \( \alpha_1 \)-antitrypsin.

\( \alpha_1 \)-Acid glycoprotein

Previous results from our laboratory indicate that \( \alpha_1 \)-acid glycoprotein was predominant in serum from porcine fetuses in the last third of gestation (Lamprea & Piñeiro, 1982). It represented about 50% of total serum proteins at birth. The present data confirm previous work and extend the analysis to the first half of the gestational period in pigs. We characterized this protein by comparing its physicochemical properties with those of human \( \alpha_1 \)-acid glycoprotein (Lamprea & Piñeiro, 1984). Stone & Maurer (1987) cloned the cDNA for this protein and confirmed that its sequence is similar to that of human \( \alpha_1 \)-acid glycoprotein; the developmental changes for liver mRNA to this protein paralleled the changes in serum concentration of the protein shown here. As indicated by Stone (1989), it may be important for porcine physiology that \( \alpha_1 \)-acid glycoprotein was expressed at high levels during the perinatal period. In other species, \( \alpha_1 \)-acid glycoprotein is a positive acute-phase protein (Koj, 1974). Serum concentration of acute-phase proteins dramatically rises after inflammatory and other traumatic stimuli. Thus, the high content of \( \alpha_1 \)-acid glycoprotein just before birth may be an adaptative response to prepare the newborn pig for extrauterine life.

Transferrin

It is well known that transferrin carries iron ions to the cells (Brock, 1985). This protein seems to be essential to cells in growing states, such as occurs in the fetal period. Transferrin has been determined in adult and neonatal pigs (Thoren-Tolling & Martinsson, 1974) and in fetal pig serum (Cavanagh et al., 1982). Our results confirm that transferrin is a major plasma protein in the first half of the gestational period in pigs (Cavanagh et al., 1982). During the second half of gestation the serum concentration of transferrin continuously decreases to reach 1.1 mg ml\(^{-1}\) at birth. This figure was lower than that reported by Cavanagh et al. (1982), but similar to that described by Thoren-Tolling & Martinsson (1974). The lower concentration of transferrin in the perinatal period than in the adult is consistent with the transitory iron deficiency that is known to occur in neonatal pigs (Thoren-Tolling & Martinsson, 1974).

Serum proteins in fetal extracts

To compare the values for the concentration of serum proteins in fetal extracts in Table 1 with those of Table 2 it is necessary to introduce some corrections for the dilution due to homogenization processes. In total, 1 ml of homogenization buffer was added per gram of fetal.
tissue. It was assumed that water content was 89% of body weight for fetuses until 47 days of gestation or 85% for 57-day-old fetuses (Dickerson & Widdowson, 1960). Dilution of the soluble proteins in homogenized fetuses would then be 2.17 times as a maximum ((1 + 0.85)/0.85). In practice, we obtained, on average, only 1.5 ml extract g⁻¹ fetal tissue because part of the water was retained in the centrifugation pellet. That figure corresponds to a minimum dilution factor of 1.68 times (1.5/0.89). It was also assumed that the six proteins measured accounted for, on average, 85% of total serum proteins. The concentration of total proteins in serum of homogenized fetuses can be a maximum of 19 mg ml⁻¹, as inferred from our data and those of Cavanagh et al. (1982). The volume of the body water that contains serum proteins at the same concentration as blood serum can be calculated from these data. The equation used to calculate this volume was: (Σ concentration of individual proteins/0.85 × 19) × 1.68 × 100. For fetuses at 37–57 days of gestation, at least 65% of body water seems to be part of a compartment that contains serum proteins at the same concentration as serum. Although in these fetuses the plasma and other extracellular fluids represented a higher percentage of body weight than in the adult, the minimal figure of 65% of the body water equivalent to blood serum is outstanding. However, the concentration of plasma proteins in cerebrospinal and amniotic fluids is lower than in plasma (Cavanagh et al., 1982). It is known that during fetal development cells from several organs contain plasma proteins of extracellular origin (Mollgard et al., 1979; Trojan & Uriel, 1979, 1982; Toran-Allerand, 1980; Piñeiro et al., 1982). The high concentration of serum proteins in fetal extracts reported here may be explained if the content of plasma proteins in cells other than those specialized in their synthesis are higher than previously suspected and general for most plasma proteins. Earlier studies on the ion composition of fetal pig and human fetuses (Dickerson & Widdowson, 1960) also concluded that during fetal development there is more Cl⁻ in the intracellular space and more K⁺ in the extracellular space than in the adult. Our results, as well as those of Dickerson & Widdowson (1960), suggest that in immature cells the mechanisms for transport of substances through cell membranes differ from those in differentiated cells.

Fetal pig serum contains, in addition to α-fetoprotein, which is absent in the adult serum, two protease inhibitors, α₁-antitrypsin and fetuin, and α₁-acid glycoprotein at concentrations much higher than in adult serum. More experimental work is needed to define the role of these proteins during porcine fetal development and to determine whether α₁-acid glycoprotein is an acute-phase protein in pigs as in other animals.

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References


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