Elution of Monomers from Provisional Composite Materials

Simon Daniel Schulz, Tobias Laquai, Klaus Kümmerer, Richard Bolek, Volker Mersch-Sundermann, and Olga Polydorou

1Department of Oral Biotechnology, University Medical Center Freiburg, Center of Dental Medicine, Hugstetter Straße 55, 79106 Freiburg, Germany
2Department of Operative Dentistry and Periodontology, University Medical Center Freiburg, Center of Dental Medicine, Hugstetter Straße 55, 79106 Freiburg, Germany
3Department of Environmental Health Sciences, University Medical Center Freiburg, Breisacher Straße 115 b, 79106 Freiburg, Germany
4Institute of Sustainable and Environmental Chemistry, Leuphana University Lüneburg, Scharnhorststraße 1, C13.311b, 21335 Lüneburg, Germany

Correspondence should be addressed to Olga Polydorou; olga.polydorou@uniklinik-freiburg.de

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The aim of this study was to evaluate the elution of substances from different materials used for the manufacturing of temporary indirect restorations, after storage in saliva and ethanol 75%. 10 samples of three chemically cured materials (Protemp 3 Garant, Systemp.c&b, and Trim) and one light-cured material (Clip F) were stored in saliva and ethanol 75% for 24 h, 7, and days 28 days. From the storage media at each time period, samples were prepared and analysed by LC-MS/MS, in order to access the elution of monomers. The results differed among the materials ($P \leq 0.05$). No monomers were detected in the samples of Protemp 3 Garant and Clip F. Substances were detected only in ethanol samples of Systemp.c&b and Trim. The amount of BisGMA, TEGDMA, and UDMA2 released from Systemp.c&b was higher compared to Trim. Storage time affected the release of substances ($P \leq 0.05$). The highest release was observed within the first 24 h. It can be concluded that provisional resin composite materials do not show high release of monomers and this release is material dependent. However, the detection of additional peaks during the analysis, suggesting the formation of by-products of the eluted substances, may not be in favour of these materials with respect to their toxicity.

1. Introduction

The disadvantages of using amalgam for dental restorations and the rapid development of dental materials and techniques in the past decades combined with the concept of minimal invasive therapy have resulted in excessive use of resin composites in daily clinical practice. Resin composites are indicated for several applications in dentistry, for example, not only materials for definitive restorations but also for manufacturing of temporary restorations such as inlays, crowns, and bridges after the tooth preparation until the cementation of the definitive indirect restoration. These provisional restorations might remain in the oral cavity for some days up to some weeks until the final restorations have been prepared.

The importance of the provisional restorations for the success of the final indirect restorations has been stated in the past [1]. The composite materials used for manufacturing of temporary dental restorations should meet biological, esthetical, and mechanical requirements, resembling the form and function of the planned definitive treatment [1]. The proper material selection for the manufacturing of the provisional restorations is influenced by the mechanical and physical properties of the material, its handling, and its biocompatibility [2]. Basically, dental resin composites consist of a resin matrix, inorganic filler, and a coupling
agent. Common monomers used in the resin matrix are Bis-GMA (bisphenol A glycol dimethacrylate), UDMA (urethane dimethacrylate), TEGDMA (triethylene glycol dimethacrylate), and BisEMA (bisphenol A ethoxylated dimethacrylate). In the market, two different kinds of temporary crown and bridge materials are available: powder/liquid hand mixed methacrylate resins and paste/paste mainly automixed (not hand mixed) resin composite based materials [1]. Generally, the kinds of the monomers used in the composition of the composite materials influence the reactivity, viscosity, polymerization shrinkage, and the mechanical properties of the composite materials [3]. It has been shown that the mechanical stability of the composite materials used for manufacturing of temporary crown and bridge restorations is comparably low, especially in the first hours after fabrication [4]. The degree of polymerization of the composite materials is not affecting completely the physical properties and the clinical performance of resin composite materials [5, 6]. Beside the physical and chemical properties of the composite materials, their biocompatibility is an important parameter regarding the choice of the material used for a restoration. The release of substances, such as BisGMA, TEGDMA, HEMA (2-hydroxyethyl methacrylate), and UDMA from dental composite materials, has been studied extensively in the literature [7–14]. Eluted substances have been shown to induce cytotoxicity [15] and apoptosis to human dental pulp cells [16–19]. Besides that, the release of bisphenol A from composite materials [9, 10, 12], knowing to act as estrogen-receptor antagonist causing endocrine disruption, rises concerns about its possible implications on human health. Additionally, the material softening caused by the exposure to plaque acids, foods, and salivary enzymes in the oral environment [20, 21] might lead to further release of substances and degradation products.

The disadvantages of the chemically cured composite materials, such as poor storage stability, poor physical properties, and increased air porosities caused by mixing and decreased degree of conversion compared to light-cured materials [22, 23], increase the concerns about their biocompatibility. Previous data [24] concerning the evaluation of core built-up materials have shown that light-cured materials are less critical with respect to the elution of monomers compared to the chemically cured or dual cured (the polymerization can be activated by both light initiation and chemical initiation) materials. Although the elution of substances from dental composite materials has been widely studied during the past years, no data exist concerning the release of substances from materials used for manufacturing of temporary inlays, crown, and bridges.

The aim of the present study was to evaluate the elution of monomers from four different resin composite materials used for manufacturing of temporary indirect restorations. The elution of substances was studied using LC-MS/MS. The null hypotheses tested were as follows: (a) the elution of substances is material dependent and (b) the analysis based on standards of the substances used for manufacturing of composite materials is adequate in order to evaluate effectively the release of monomers from these materials.

2. Materials and Methods

In the present study, four different materials for manufacturing of provisional restorations were used: the three chemically cured materials Protemp 3 Garant (3M ESPE Dental Products, Seefeld, Germany), Systemp c&b (Ivoclar Vivadent, FL-Schaan, Liechtenstein), and Trim (Bosworth Company, Skokie, IL, USA) and a light-cured material, Clip F (VOCO GmbH, Cuxhaven, Germany). Information about the composition of the composite materials as provided by the manufacturers is given in Table 1.

Two different storage media were used in the present study in order to evaluate the release of substances: (i) human pooled saliva gathered from people without composite restorations and (ii) ethanol 75%. From each tested material, two groups (n = 10 each) were manufactured for each tested storage medium. For the manufacturing of the composite specimens, moulds with a diameter 4.5 mm and 2 mm thickness were used, allowing for the production of standardized cylindrical specimens. These moulds were positioned on a transparent plastic matrix strip on top of a glass plate. Then, they were filled with the respective composite materials. The samples were built up in one increment. After inserting the materials into the moulds, a transparent plastic matrix strip (Kerr Hawe, Switzerland) was placed on top of them in order to avoid an oxygen-inhibited superficial layer. Additionally, a glass slide was used in order to flatten the surface. The materials were used according to the manufacturers’ instructions. For the polymerization of the samples manufactured by Clip F, a halogen unit (Elipar Highlight, 3M ESPE, Seefeld, Germany) was used. Its light intensity was 780–800 mW/cm². The spectral irradiance was determined with a visible curing light meter (Cure Rite; Dentsply, USA). The polymerization of the samples took place for 20 sec, according to the manufacturer’s instructions. The other three tested composite materials were chemically cured materials. Protemp 3 Garant and Systemp c&b are non-hand mixed materials as they are mixed due to an automixed double cartridge, and their samples were left undisturbed for 5 minutes to allow curing, as recommended by the manufacturers’ instructions. Trim is a hand mixed material (powder/liquid), and therefore the paste was prepared per hand according to the manufacturer instructions: 7 mL of Trim liquid was placed into a mixing cup and 13 mL (by volume) powder was added and the paste was mixed for almost 1 min. After inserting the paste into the molds like it was done for the other materials, the samples remained undisturbed for 5 min. After further 10 min after curing (chemical or light), each sample was immediately immersed in 1 mL of the respective storage medium according to the group they belong to. The samples were stored in a dark box at room temperature and the storage medium was renewed after 1 day, 7 days, and 28 days after the polymerization. From the storage medium removed, liquid samples were prepared and stored until analysis at 4°C in the dark.

For analysis, a previous published method [25] using a high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) was used. A triple quadrupole mass spectrometer (Model 1200L) from Varian Inc., combined
Table 1: Materials tested.

<table>
<thead>
<tr>
<th>Material</th>
<th>Category</th>
<th>Main monomer(s)†</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protemp 3 Garant</td>
<td>Paste/paste system self-curing</td>
<td>Dimethacrylate</td>
<td>3M ESPEDental Products, Seefeld, Germany</td>
</tr>
<tr>
<td>Systemp.c&amp;b</td>
<td>Paste/paste system self-curing</td>
<td>BisGMA, methacrylate, and polyfunctional acrylates</td>
<td>Ivoclar Vivadent, FL-Schaan, Liechtenstein</td>
</tr>
<tr>
<td>Trim</td>
<td>Powder/liquid system self-curing</td>
<td>Poly(ethyl methacrylate), methyl methacrylate</td>
<td>Bosworth Company, Skokie, IL, USA</td>
</tr>
<tr>
<td>Clip F</td>
<td>One component system light curing</td>
<td>2-Hydroxyethyl methacrylate, and acrylate ester</td>
<td>VOCO GmbH, Cuxhaven, Germany</td>
</tr>
</tbody>
</table>

† According to manufacturers’ information.

Table 2: Monomers used as reference standards.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Name</th>
<th>Elemental formula</th>
<th>Mol. weight*</th>
<th>CAS-number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BisGMA</td>
<td>Bisphenol A glycol dimethacrylate</td>
<td>C&lt;sub&gt;29&lt;/sub&gt;H&lt;sub&gt;36&lt;/sub&gt;O&lt;sub&gt;8&lt;/sub&gt;</td>
<td>513.0 g/mol</td>
<td>1565-94-2</td>
</tr>
<tr>
<td>TEGDMA</td>
<td>Triethylene glycol dimethacrylate</td>
<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>286.32 g/mol</td>
<td>109-16-0</td>
</tr>
<tr>
<td>UDMA 1</td>
<td>Urethane dimethacrylate product</td>
<td>C&lt;sub&gt;26&lt;/sub&gt;H&lt;sub&gt;42&lt;/sub&gt;O&lt;sub&gt;8&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>498.0 g/mol</td>
<td>—</td>
</tr>
<tr>
<td>UDMA 2</td>
<td>Urethane dimethacrylate</td>
<td>C&lt;sub&gt;23&lt;/sub&gt;H&lt;sub&gt;34&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>470.56 g/mol</td>
<td>41137-60-4 or 72869-86-4</td>
</tr>
<tr>
<td>MMA</td>
<td>Methyl methacrylate</td>
<td>C&lt;sub&gt;5&lt;/sub&gt;H&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>100.12 g/mol</td>
<td>80-62-6</td>
</tr>
</tbody>
</table>

* Information according to the manufacturers.

With an HPLC, was used. The separation of the monomers took place with a CC 70/3 Nucleodur 100-3 Cl<sub>8</sub>ec HLC-Column (Macherey-Nagel, Düren, Germany) using a gradient program with 0.1% (v/v) formic acid and acetonitrile as solvents. External calibrations with standards were obtained with the help of the peak areas. As reference standards, bisphenol A, bisphenol-A-glycidyldimethacrylate (BisGMA), two different forms of urethane dimethacrylate (UDMA 1 and UDMA 2), triethylene glycol dimethacrylate (TEGDMA), and methyl methacrylate (MMA) were used. The information on the substances used as reference standards is given in Table 2. Identification of monomers was performed by retention time and MRM experiments in MS/MS mode (Figure 1). The limits of quantification of all tested substances were found to be 0.005 µg/mL. Positive signals beyond this level could not be qualified. Additionally, the basic monomers TEGDMA, BisGMA, UDMA 1, and UDMA 2 were mixed in order to evaluate the extra peaks that might exist in the case that the eluted monomers react with each other.

2.1. Statistical Analysis. A mixed model [26] was fitted with a random intercept. The continuous response variable is modelled as a linear function of storage medium and time and the corresponding interactions as explanatory variables, separately for each material. Variance components were used as covariance structure. Least-square means are calculated. The P values for the pairwise comparison of the storage media were adjusted by the Tukey method. All calculations have been done using the PROC MIXED procedure from the statistical software SAS 9.1.2. The statistical analysis was carried out at a significance level of 0.05.

3. Results

The results differed among the four tested materials. With the analytical method used in the present study, none of the examined monomers were found to be eluted from Protemp 3 Garant and Clip F, in any of the two tested storage media. As far as the other two tested materials are concerned, no substances were detected in saliva, but in ethanol 75% elution of substances was observed.

After the elution periods of 24 h and 7 days, the storage media were exchanged to separately measure eluted...
Table 3: Substances released from System p.c&b and Trim in ethanol 75% [mean values (μg/mL) ± SD].

<table>
<thead>
<tr>
<th>Material</th>
<th>Period 1 Day</th>
<th>Period 2 Days</th>
<th>Period 3 Days</th>
<th>Period 1 Day</th>
<th>Period 2 Days</th>
<th>Period 3 Days</th>
<th>Period 1 Day</th>
<th>Period 2 Days</th>
<th>Period 3 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>System p.c&amp;b</td>
<td>0.614 ± 0.23</td>
<td>0.128 ± 0.27</td>
<td>nd</td>
<td>1.839 ± 0.202</td>
<td>1.544 ± 0.203</td>
<td>16.931 ± 0.203</td>
<td>11.020 ± 0.203</td>
<td>9.968 ± 0.203</td>
<td>0.341 ± 0.321</td>
</tr>
<tr>
<td>Trim</td>
<td>0.026 ± 0.028</td>
<td>0.024 ± 0.051</td>
<td>nd</td>
<td>0.345 ± 0.123</td>
<td>nd</td>
<td>0.126 ± 0.083</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

nd = not detected.

Table 4: Tukey’s test (adjusted P values).

<table>
<thead>
<tr>
<th>Material</th>
<th>Pairs compared</th>
<th>BisGMA</th>
<th>TEGDMA</th>
<th>UDMA 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>System p.c&amp;b</td>
<td>Period 1 versus 2</td>
<td>&lt;0.0001</td>
<td>0.155</td>
<td>0.2655</td>
</tr>
<tr>
<td></td>
<td>Period 1 versus 3</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Period 2 versus 3</td>
<td>0.356</td>
<td>&lt;0.0001</td>
<td>0.0204</td>
</tr>
<tr>
<td>Trim</td>
<td>Period 1 versus 2</td>
<td>0.994</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Period 1 versus 3</td>
<td>0.219</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Period 2 versus 3</td>
<td>0.257</td>
<td>0.991</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

3.1. System p.c&b. The amount of BisGMA, TEGDMA, and UDMA 2 released from System p.c&b was higher than the ones detected in the ethanol samples of Trim. The amount of UDMA 2 released from System p.c&b was significantly higher (P ≤ 0.05) than the amount of BisGMA and TEGDMA. The highest release of substances was observed in the first 24 h. BisGMA was found to be released up to 7 days after polymerization, while as far as TEGDMA and UDMA 2 are concerned, small amounts were released after 28 days storage in ethanol 75%.

3.2. Trim. Low concentrations of substances were detected. The highest release was observed for TEGDMA, followed by UDMA 2 and then by BisGMA. BisGMA was released up to 7 d storage while TEGDMA and UDMA 2 were detected only in the ethanol samples after 24 h. According to Turkey’s test, the storage time had no significant effect on the elution of substances.

3.3. Mixture of Basic Monomers. In Figure 2, the peaks of BisGMA (1), TEGDMA (2), and the two kinds of UDMA (3) in one chromatogram are presented and identified according to the separate peaks and retention times (BisGMA: 10.6 min; TEGDMA: 5.3 min; UDMA: 10 min; 4 = new peak “reaction product”: ~13 min). The intensity of substances for the 3 different elution periods. In Table 3, the average concentration (±standard deviation) of each monomer detected in the ethanol solutions from Trim and System p.c&b at each tested instantaneous elution period is presented. The amounts of substances released differed significantly among the materials (P ≤ 0.05). In Table 4, the results for the pairwise analysis for Trim and System p.c&b for each detected substance are presented.

4. Discussion

In the present study, four different materials used in the daily dental praxis for manufacturing of provisional indirect...
restorations were tested concerning the elution of substances up to 28 days after curing. Although the elution of monomers from composite materials is widely tested, in the literature there is no information concerning the release of monomers from such materials. The elution of substances from materials used in the oral cavity, like those tested in the present study, is of great importance as it is combined with effects on their mechanical properties and implications on human health.

The present findings showed a significant difference among the tested materials as only two of them showed a release of substances. The materials tested differed according to the mixing process and the kind of curing. One of the tested materials (Trim) belongs to the category of the powder/liquid, hand mixed methacrylate materials, and two of them (Protemp 3 Garant and Systemp.c&b) belong to the category of paste/paste nonhand mixed (automixed) materials, and one of them (Clip F) belong to the category of one paste light-cured composite materials. Both materials that showed release of substances were chemically cured composite materials, being in accordance with the reported disadvantages of the chemically cured composite materials, as they show poor storage stability, poor physical properties, increased air porosities caused by mixing, and decreased degree of conversion compared to light-cured materials [24]. No substances were detected in the storage media from the light-cured material suggesting that the light-cured composite material results in a more cross-linked network, not allowing the unpolymerized monomers to elute. The release of monomers in the present study was material dependent and therefore the first hypothesis made in the beginning of the study has been proved to be true.

Like it is reported in the past by Ferracane [28], the elution of monomers relates to the extent of the polymerization reaction, the chemistry of the solvent used, and the size and the chemical nature of the released components. The composition of the materials has an important influence on the elution of monomers [29, 30]. Protemp 3 Garant has a BisGMA resin matrix while Systemp.c&b has a different mixture of methacrylates [31]. BisGMA used in the bisacryl composite materials has a rigid central structure which reduces its ability to rotate and participate in the polymerization reaction [31, 32], and therefore materials that contain BisGMA cannot be easily influenced during the polymerization process, compared to the other kinds of materials [32]. According to these, the resin matrix of Systemp.c&b, based on polyurethane dimethacrylate, might be more susceptible to changes [33].

In the study of Akova et al. [31], all tested provisional restorative materials (among them: Protemp 3 Garant and Systemp.c&b) were influenced by storage in ethanol 75%, concerning their hardness and their flexural strength, being in contrast to the findings of the present study, as no substances were found to be eluted in the ethanol solutions of Protemp 3 Garant. The degree of polymerization affects the physical properties and the clinical performance of resin composite materials [5, 6], playing an important role in determining the ultimate success of the restoration [34]. According to the study of Balkenhol et al. [4], the degree of conversion does not really reflect the stability of their mechanical properties, although the mechanical properties of provisional restorative materials were shown to depend on the storage time after mixing. In the past, we could show that the degree of conversion does not definitely influence the release of substances from dental restorative materials [24]. A similar level of conversion might result in different elution rates while as mentioned above the kind of existed network may affect the release of monomers [24]. In the case of a heavily cross-linked network more monomers might remain trapped in it without being able to elute. The kind of network is influenced by the chemistry of each restorative material.

In the present study, substances were detected only in ethanol 75%. Although saliva is the physiologic medium existing in the oral cavity, and although ethanol 75% can be characterised as aggressive medium, ethanol 75% is used as a storage medium in many studies in order to simulate the oral conditions [10, 21, 24, 25, 35]. According to the guidelines of the Food and Drug Administration (FDA) (1976, 1988), ethanol 75% is a food simulator and aging accelerator and therefore is considered to be clinically relevant [36]. Ethanol 75% has a softening effect on composite materials after attacking their cross-linked network, affecting this way the release of substances. Benetti et al. studied the effect of ethanol on softening and elution of monomers and they concluded that there are negative correlations between softening and elution in ethanol, respectively, and degree of conversion [37]. Schneider et al. showed in their study that one of the main effects of ethanol 75% is the weakening of the mechanical properties of the composite materials [38]. In a previous study [25], we could show that the amounts of TEGDMA und UDMA 2 were released in saliva samples, being in contrast to the present findings, which might be due to the standards used in the present study and the kind of materials studied.

The amount of monomers detected in ethanol after storage of the tested materials was relatively low, although a high amount would have been expected due to the category that the tested materials belong to. Only BisGMA, TEGDMA, and UDMA 2 were found to be released. Additionally, the amount of monomers released from Systemp.c&b was higher to the ones released from Trim. The different composition and chemistry of the tested materials combined with the different ways of mixing art could be the reason of the present findings. Trim contains polyvinyl methacrylate with totally different chemistry compared to Systemp.c&b. The kind of substances used as standards could also have influenced the results. Because of the different composition, MMA was used as standard as a small molecule that might be able to be identified as being an element of bigger molecules contained in the materials tested. According to the present findings, there was great difference among the samples of Systemp.c&b concerning the concentration of UDMA 2 detected, meaning the presence of an inhomogeneous mass of the prepared samples. This might be due to the mixing procedures of the material during the preparation of the samples, suggesting a more careful use of such materials in the clinical praxis. In the present study, the elution of specific substances that are usually claimed to be responsible to harmful effects on the human health was tested. However, further research is
necessary in order to evaluate the possible harmful effect of the provisional materials and their eluates on the oral tissues.

In the present study, beside the analytical procedure concerning the elution of substances from the tested materials, the basic monomers used for manufacturing of the materials and as standards in the present study were mixed together in order to evaluate a further reaction among them that might take place after being eluted. As a result of this mixing, an additional high peak of a molecule with higher mass and lower polarity than the basic monomers was detected suggesting that, during the elution of substances by dental materials, it might come to a kind of interaction among them resulting in additional by-products, which might cause some confusion during the analysis process in case that the not appropriate method is used. These by-products might be parts of the basic monomers produced by their decomposition or new bigger molecules that are composed as a result of their reaction. This might be the reason of the small amount of the tested monomers detected by the materials in the present study. According to these, the second hypothesis made at the beginning of the study, evaluating if the standards of the substances used for manufacturing of composite materials are enough in order to study the elution of substances from composite materials using analytical methods, cannot be accepted. However, the findings in the present study give only an indication for the existence of further substances during the elution besides the used ones for the synthesis of the composite materials, making clear the importance of the selection of the appropriate analytical method. Further research in this field is necessary in order to evaluate the production of other substances from the released monomers under different conditions and by different storage media. Like it has been shown in the past [39], the combination of HPLC with mass spectrometry is very helpful in identifying eluted compounds from composite materials. Because of this, two different substances (UDMA 1 and UDMA 2) were used as urethane dimethacrylate as the selection of the standards can influence the findings of the analysis [39]. Further development of the analytical methods using the combination of HPLC with mass spectrometry could give valuable information on the identification of by-products from composite materials.

5. Conclusions

Within the limitations of the present study, it can be concluded that the elution of substances from materials used for manufacturing of provisional restoration is material dependent, based on the chemistry of each material. In human saliva, none of the tested materials showed any release of substances. Only Systemp.c8b showed elution of monomers through the whole storage time of 28 days. Most of the substances thought to be harmful for the human health were not found to be eluted. However, the detection of additional peaks during the analysis of the ethanol samples suggests the production of by-products due to eluted substances, suggesting a careful use of these materials as they might exert some side effects.

Conflict of Interests

The authors declare that they have no conflict of interests.

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References


