Induction of xenobiotic metabolising enzymes in the common brushtail possum, *Trichosurus vulpecula*, by *Eucalyptus* terpenes

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Abstract

The mixed function oxidase (MFO) activity and content was studied in the liver of common brushtail possums fed for 10 days on a diet containing a mixture of terpenes found in *Eucalyptus* leaves on which possums commonly browse. The MFOs were compared to the MFOs in possums fed a control diet of fruits and cereals only. The terpenes chosen were 1,8-cineole, *p*-cymene, *a* -pinene and limonene. The selected terpenes caused induction of P450 enzymes, as shown by a 53% higher cytochrome P450 content and a 45% increase in aminopyrine demethylase activity in the test animals. Aniline hydroxylase activity was significantly increased, with levels of 2.95 and 1.43 nmol min$^{-1}$ mg$^{-1}$ microsomal protein in the test and control animals, respectively. There was also a significant increase in androstenedione 16*α*-hydroxylase activity in the test group, 0.85 as compared to 0.50 nmol mg$^{-1}$ min$^{-1}$ in the control group. Western blot studies using human CYP2E1 and rat CYP2C11 and CYP2C6 antibodies gave CYP2E, CYP2C11 and CYP2C6 immunoreactive bands of greater intensity in the test animals as compared to the control group. This study has shown experimentally that dietary terpenes cause enzyme induction in folivorous marsupials. It also confirms the importance of knowledge of diet when studying xenobiotic metabolising enzymes, particularly in wild animals such as the brushtail possum. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Common brushtail possum; Cytochrome P450; Eucalyptus; Induction; Terpenes

1. Introduction

Terpenes are major constituents of the essential oil in *Eucalyptus* sp. and marsupials, such as the brushtail possum (*Trichosurus vulpecula*) and koala (*Phascolarctos cinereus*), rely on these leaves for their major and only food source, respectively. These animals therefore ingest and absorb significant quantities of terpenes in their diet. Freeland and Janzen [13] hypothesised that these specialist *Eucalyptus* herbivores use highly efficient enzyme systems to metabolise plant secondary metabolites (PSMs), such as terpenes and phenolics, to non-toxic substances, which can then be readily excreted in the urine. Metabolism of monoterpenes such as *p*-cymene [36,41], limonene [20,31] and *α* - and *β*-pinene [18] has been studied in vivo in both vertebrates and invertebrates. The metabolism of 1,8-cineole, a monoterpane that can constitute up to 90% of the essential oils in *Eucalyptus* sp., has been extensively studied in the brushtail possum [5,7,8,10,36]. In comparison to the relatively large amount of information available on the metabolism of these compounds in vivo, very little research has been carried out in vitro. Madyastha and Srivatsan [25] studied myrcene metabolism in vivo and in vitro in rats but did not attempt to identify the enzymes involved. The enzymes used in the detoxification of these compounds remain largely unknown. Very few studies have been carried out in marsupials to examine xenobiotic metabolising enzymes such as cytochrome P450s (CYP450s).

Early work by McManus and co-workers [28–30] on the mixed function oxidase (MFO) activity and content of a number of Australian marsupials provided a starting point for understanding the enzymatic detoxification systems. However, since these initial investigations few studies have investigated the enzyme activity in
Eucalyptus eating herbivores. Bolton and Ahokas [3] recently reported significant levels of a range of MFOs in brushtail possums. Similarly, Olkowski et al. [33] investigated cytochrome P450 enzyme activities in the brushtail possum in comparison with the rat, rabbit, sheep and chicken. They concluded that enzymatic activity and content in the possum was similar to or slightly higher than in the other animals they tested. These studies have provided important insights into the enzymatic metabolic capabilities of the brushtail possum; however, in both studies the possums’ diet was not controlled as the animals used in these studies were captured just prior to organ harvesting.

Dosage of rats with terpenes has been shown to induce liver enzymes. For example, Hiroi and co-workers [16] found that essential oils, extracted from wood and leaves, increased the activities and levels of some P450 isozymes in the rat. In a similar study, De-Oliveira et al. [9] found that oral administration of the terpene, β-myrcene, induced CYP2B1 and CYP2B2 isozymes in the rat liver. However, this induction has not been studied in animals which include terpenes in their normal diet.

We have studied the MFO activity and content in the liver of common brushtail possums that had been fed a mixture of terpenes commonly found in Eucalyptus leaves for 10 days and compared this with the MFOs in possums fed an artificial diet of fruits and cereals only. A number of assays were conducted to measure the activity of a range of CYPs which are known to be subject to induction in rats.

2. Materials and methods

2.1. Chemicals

NADP, isocitrate dehydrogenase, unlabelled androst-4-ene-3,17-dione (androstenedione), isocitrate, aniline, aminopyrine, Tris, Tris–HCl, 1,8-cineole, a-pinen, a-pinene, p-cymene and limonene were purchased from Sigma (St. Louis, MO, USA). Acrylamide, ammonium persulphate, N,N’-methylene-bis-acrylamide, bromophenol blue, 4-chloro-1-naphthol, sodium dodecyl sulphate and Tween-20 were purchased from Biorad (Sydney, Australia). [4-14C]Androstenedione (sp. act. 59 mCi mmol⁻¹) was purchased from Amersham Australia (Sydney). Horseradish peroxidase conjugated donkey anti-sheep and sheep anti-rabbit secondary antibodies were purchased from Gentest (Milwaukee, WI, USA), and CYP2E1 antibody was a kind gift from the Department of Clinical Pharmacology, Flinders Medical Centre, SA, Australia. The human CYP2E antibody was raised against CYP2E1 expressed in Escherichia coli, and produced a single band with SDS-PAGE immunoblotting [40]. The CYP2C11 antibody was a gift from Professor M. Murray (University of New South Wales). The antibody had been raised to purified CYP2C11 [6] and had been preabsorbed with CNBr-Sepharose 4B-bound, isosafrole-induced microsomes from female rat. Solvents and other miscellaneous chemicals were analytical grade.

2.2. Animals and diet

The research was approved by the Animal Experimentation Ethics Committee of the University of Tasmania and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. A total of eight male common brushtail possums (average weight 3.61 ± 0.24 kg) were captured in wire cage traps in the Hobart urban area, under permit from the National Parks and Wildlife Service (Tasmania). The possums were housed in large holding enclosures (6 × 2 × 2 m) in the Central Animal House, University of Tasmania, until required for experimentation. During experiments, four of the animals were randomly chosen for the test group and were transferred in hessian bags to the experimental animal house where they were housed in individual metabolism cages (60 × 45 × 60 cm), under a 12:12 h light:dark regime. The remaining four were kept at the central animal house and were allocated to the control group. Separate housing was required for the terpene-fed animals and the control animals due to the volatility of the terpenes used. If all animals had been housed in the same room, control animals would have been exposed to terpene vapour, which may have affected their enzyme activity.

Animals were maintained on an artificial diet which consisted of (% wet matter): apple (39.6%), silverbeet (29.9%), carrot (8.2%), lucerne (4.5%), brown sugar (3.0%) and water (14.9%). The average dry matter content was 18.2%. The dry matter content of the diet offered was determined by taking a portion (50 g wet weight) of the food offered each afternoon and dried at 60°C to constant weight. Food intake was calculated from food offered less that refused. During the pre-experimental period, 600 g (wet weight) of food was offered each afternoon (~ 16:00 h) and the weights of refusals of food from the previous day were taken each morning (~ 09:00 h). All eight possums were maintained on this basal diet for at least 7 days before allocation to the test or control group. During the experimental period, a pre-calculated amount of terpe-
nes was added to the test animals’ diet and this mixture was presented to the animals as a wet mash. The terpenes chosen to use in the feeding trial were 1,8-cineole, p-cymene, α-pinene and (S)-(−)-limonene. The proportions of each terpene added to the possums’ diet was based on the amounts that have been reported to be extracted from the leaves of *E. melliodora* [2], a food for these animals. The amount of each terpene presented to the possums is shown in Table 1. Test animals were maintained on the terpene diet for a total of 10 days. Possums in this group were fed the terpene mix at 2.13% dry matter offered for the first 5 days of the experimental period. This concentration was then increased to 4.26% dry matter for the final 5 days of the experiments. Animals in the control group were maintained on the basal diet described above over the 10-day period.

### 2.3. Surgical procedures

Anaesthesia was achieved using Zoletil (20 mg kg⁻¹, i.m.). Immediately prior to surgery, animals were euthanased using pentobarbital (325 mg kg⁻¹, i.v.). Livers were excised and immediately placed in ice-cold phosphate buffer (0.1 M, pH 7.4) containing 1.15% KCl. Samples of liver from each possum were then frozen in liquid N₂ and stored in individual plastic sample bags at −70 to −80°C until required.

### 2.4. Preparation of microsomes

All procedures were carried out at 0–4°C. Livers were homogenised in phosphate buffer (0.1 M, pH 7.4) with KCl (1.15%) using a Potter-Elvehjem homogeniser and centrifuged at 9000 × g for 20 min. The resulting supernatant was then centrifuged at 108 000 × g for 60 min. The microsomal pellet was resuspended in the same buffer and centrifuged at 108 000 × g for 60 min. Phosphate buffer (0.1 M, pH 7.4) with 20% glycerol was used to resuspend the final microsomal pellet, and this was stored at −70 to −80°C and thawed only once, at the time of use.

### Table 1

<table>
<thead>
<tr>
<th>Terpene</th>
<th>Terpene offered (mg kg⁻¹ possum)</th>
<th>First 5 days</th>
<th>Final 5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-Cineole</td>
<td>566</td>
<td>1212</td>
<td></td>
</tr>
<tr>
<td>p-Cymene</td>
<td>16</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>(S)-(−)-Limonene</td>
<td>69</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>α-Pinene</td>
<td>214</td>
<td>428</td>
<td></td>
</tr>
</tbody>
</table>

2.5. Assays

Protein concentrations of the microsomes were determined by the method of Lowry et al. [24] for each of the four test and four control possums. Cytochrome P450 content was quantified as previously described [26]. Aminopyrine demethylase activity was measured using a substrate concentration of 25 mM and the formaldehyde that was produced was quantitated by the method of Nash [32]. Aniline hydroxylase activity was measured as previously described [19]. Microsomal androstenedione hydroxylase was assayed by the procedure of Gustafsson and Ingelman-Sundberg [15]. Application to, and development of, TLC plates was carried out as previously described [37]. Zones corresponding to hydroxylated androstenedione standards were visualised under UV light and scraped into vials for scintillation spectrometry (ACS, Amersham, Australia).

2.6. Western blotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [22], using a 4.5% acrylamide stacking gel and a 7.5% separating gel. Samples (5 μg microsomal protein/lane, CYP2C6; 50 μg microsomal protein/lane, CYP2E1 and CYP2C11) were solubilised and heated at 100°C for 5 min. Gels were stacked at 100 V (60 min) then run at 180 V (150 min). The proteins were electroblotted from the gel to a nitrocellulose membrane (25 mM Tris–HCl, 192 mM glycine, 20% methanol; 50 V overnight using a Hoefer TE series transfer electrophoresis unit). Membranes were blocked in 5% skim milk (1 h, 37°C). Immunoblot analysis was performed by incubation at 37°C with 1:1000 dilutions of primary antibody followed by incubation (1 h, room temperature), with horseradish peroxidase conjugated sheep anti-rabbit antibody (CYP2C6 and CYP2C11) and donkey anti-sheep antibody (CYP2E1). Development of membranes was achieved using horseradish peroxidase colour development reagent (2-chloro-1-naphthol).

Quantitation of the immunoblotted microsomal P450s was carried out by laser densitometry (LKB 2222-010 Ultro-Scan XL). Comparison of the possum samples was made to a pooled control rat microsomal sample which was assigned a value of 1. The possum samples were expressed relative to this.

2.7. Data analysis

For each experiment, data were averaged from triplicate observations and the mean was then calculated for each group (*n* = 4). The mean differences between the test and control groups were compared using an independent sample *t*-test (SPSS 7.5; SPSS, Chicago, USA).
microsomal fractions and the results are presented in Table 2. The assay of androstenedione hydroxylase activity also enabled the measurement of 17β-hydroxysteroid oxidoreductase activity, as reflected by the production of testosterone from androstenedione (Table 2).

The data in Table 2 indicate that the addition of the terpene mixture to possums’ diet of fruit and cereals produced a 53% increase in the cytochrome P450 content of hepatic microsomes. Similarly, significant differences were found between the test and control groups in aminopyrine demethylase (45% increase), aniline hydroxylase (105% increase) and androstenedione 16α-hydroxylase (71% increase) activities. No significant differences were found with respect to androstenedione 6β-hydroxylase or 17β-hydroxysteroid oxidoreductase activity.

All data was tested for homogeneity of variance using Levene’s test and no significant differences were found between the two groups in all parameters of CYP content or activity except in 17β-hydroxysteroid oxidoreductase activity (Table 2).

Immunoblots of human CYP2E and rat CYP2C11 and CYP2C6 immunoreactive protein in microsomes from test and control possums are shown in Fig. 2. In the immunoblot of CYP2C6 immunoreactive protein (Fig. 2A), differences between the control and test group are obvious. CYP2C6 immunoreactive protein is clearly absent in the control group. CYP2C11 immunoreactive protein was detected in both test and control groups (Fig. 2B). Quantitation by densitometry indicated a 1.8-fold higher amount of CYP2C11 immunoreactive protein in the test as compared to the control group, however a t-test showed no significant difference between the two groups (0.24 ± 0.20 test group, 0.13 ± 0.06 control group, P = 0.211). CYP2E immunoreactive protein was detected in both test and control groups (Fig. 2C). Quantitation by densitometry indicated a 1.5-fold higher amount of CYP2E immunoreactive protein in the test as compared to the

Table 2

<table>
<thead>
<tr>
<th>Content or activity</th>
<th>Terpene group (n = 4)</th>
<th>Control group (n = 4)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450</td>
<td>0.405 ± 0.018</td>
<td>0.265 ± 0.042</td>
<td>0.023</td>
</tr>
<tr>
<td>Aminopyrine demethylase</td>
<td>5.714 ± 0.314</td>
<td>3.950 ± 0.635</td>
<td>0.047</td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td>2.947 ± 0.375</td>
<td>1.431 ± 0.208</td>
<td>0.012</td>
</tr>
<tr>
<td>Androstenedione 6β-hydroxylase</td>
<td>0.199 ± 0.010</td>
<td>0.236 ± 0.017</td>
<td>0.111</td>
</tr>
<tr>
<td>Androstenedione 16β-hydroxylase</td>
<td>0.291 ± 0.039</td>
<td>0.206 ± 0.015</td>
<td>0.088</td>
</tr>
<tr>
<td>Androstenedione 16α-hydroxylase</td>
<td>0.854 ± 0.047</td>
<td>0.501 ± 0.052</td>
<td>0.002</td>
</tr>
<tr>
<td>17β-hydroxysteroid oxidoreductase</td>
<td>1.304 ± 0.276</td>
<td>1.546 ± 0.152</td>
<td>0.480*</td>
</tr>
</tbody>
</table>

* nmol mg⁻¹ microsomal protein.

b nmol product formed min⁻¹ mg⁻¹ microsomal protein.

c t-test.

* Although the variances were not equal (Levene’s test P = 0.034), the means were very close and a t-test showed that the difference was far from significant.

3. Results

Food intake in the terpene treated animals and in the controls remained normal and constant over the experimental period. The percentage of food intake consumed in both groups is shown in Fig. 1. The mean (% S.E.) weight of the terpene fed possums at the beginning and end of the feeding trial was 3.74 ± 0.18 and 3.85 ± 0.16 kg, respectively, indicating that the animals maintained weight throughout the experimental period.

Hepatic cytochrome P450 content, aminopyrine demethylase, aniline hydroxylase, androstenedione 6β-, 16β- and 16α-hydroxylase activities for the terpene fed possums and the control possums were measured in
4. Discussion

Introducing a mixture of terpenes to possums that had been fed a basal diet of fruits and cereals clearly caused the induction of P450 enzymes. Aminopyrine demethylase activity, a general measure of cytochrome P450s, was found to be significantly higher in the test animals than in the controls. This conclusion was confirmed with the finding that cytochrome P450 content was 53% higher in the test animals. This result is consistent with the results of Bolton and Ahokas [3] who found P450 content was higher in possums from non-urban populations than those caught in an urban environment. They concluded that the non-urban animals had higher MFO activity due to the different diets each population would encounter, with the assumption that the diet of the non-urban animals would consist predominantly of Eucalyptus leaves whereas the diet of the urban animals would include fruits and vegetables from gardens and food scraps. The Eucalyptus leaf diet means a greater intake of PSMs such as terpenes, phenolics and tannins and hence the animals are faced with a greater need for detoxification.

The brushtail possum is a resilient species that has adapted well to living in highly populated areas. Since European settlement, the diet of the brushtail possum has changed dramatically from one consisting primarily of Eucalyptus leaves, to that of commercial fruits from orchards and vegetables from urban gardens. The variety of foods now consumed by the brushtail possum makes studies on their enzymatic systems and dietary requirements inconclusive unless the animal’s diet is controlled. The present study has now shown experimentally that dietary terpenes, which can constitute up to 90% of the essential oil in Eucalyptus leaves, cause enzyme induction in folivorous marsupials.

It is important to separate the effects of terpenes from those of other PSMs when investigating the role they play in the enzymatic system of folivorous marsupials. Many studies have been carried out investigating the metabolic fate of terpenes in marsupials (e.g. [11,27]), however, most of these studies have used a leaf diet. Eucalyptus leaf contains an array of plant secondary metabolites such as phenolics, terpenes and tannins. Therefore, the diversity of plant compounds ingested by herbivores feeding on natural plant foods makes it difficult to measure individual effects [12]. By carrying out manipulative experiments using an artificial diet the present study has shown that the enzyme induction in the test animals was due to the introduction of terpenes to their diet.

The cytochrome P450 content of the terpene-fed possums (0.41 ± 0.02 nmol mg⁻¹ microsomal protein) is consistent with the findings of McManus and Ilett who found MFO activity in marsupials was generally lower than the activity of other species [29]. In contrast, the values found in this study were less than half the values (0.90 ± 0.06 nmol mg⁻¹) reported by Bolton and Ahokas [3]. It has been shown that interlaboratory comparisons of the analysis of hepatic microsomes can be somewhat unreliable [4]. Therefore, until methods of analysis are standardised, it is difficult to make conclusions regarding differences between species when the analyses have not been conducted within the same laboratory.

The significant difference in androstenedione 16α-hydroxylase activity between the test and control groups suggests that the selected terpenes were involved in the induction of CYP isozymes which are responsible for
this conversion. Western blot analysis of rat CYP2C6 immunoreactive protein in hepatic microsomes from both groups (Fig. 2A) showed that distinct bands were clearly visible in the terpene fed animals whereas they were not present in the animals fed the basal diet only. There were also CYP2C11 immunoreactive protein bands present in both the terpene fed and the control groups (Fig. 2B) and a non-significant trend for an increase in immunoreactive protein was observed in the terpene fed group. (Fig. 2B) and a non-significant trend for an increase in immunoreactive protein was observed in the terpene fed group. In the rat, androstenedione 16α-hydroxylation is carried out by CYP2C11 [42] which is considered to be a ‘male specific’ (i.e. hormone regulated), but generally non-inducible or constitutive, CYP enzyme. CYP2C6, on the other hand, is considered to be inducible in the rat with a two-fold induction by phenobarbitalone (reviewed in Ref. [23]). Major problems have been encountered when trying to compare enzyme activity between species, particularly CYP2C activity [14]. It is possible that a relationship exists between the 16α-hydroxylation activity and the increase in CYP2C immunoreactive bands. However, at this stage we were able to observe induction, but until cloning and sequencing studies are carried out we are unable to conclude which specific CYP2C enzymes are induced in the possum.

One of the most notable findings to emerge from this study was the degree of aniline hydroxylase activity in both terpene fed possums and controls. The activity of the CYP2E1 isozyme has been determined by aniline hydroxylase activity [21]. In this study, the levels of aniline hydroxylase activity were found to be elevated (Table 2) compared to the activity Stupans and co-workers found in the rat [39]. The activity found in the control group was approximately five times higher than the activity commonly found in the rat [39] and that in the test possums were twice as high again. Western blot analysis of human CYP2E1 immunoreactive protein also showed stronger bands in the terpene treated compared to the control animals (Fig. 2C), suggesting the presence of a similar isozyme.

It must be noted that aniline hydroxylase is not always the best measure of CYP2E1. Although the rate of aniline hydroxylation is commonly used as a measure of the activity of CYP2E1 in rats, rabbits and humans, it cannot be assumed that the putative possum CYP2E1 is selective for this substrate. In monkey liver [1], although aniline hydroxylase activity reflects CYP2E1 content, it has been found that CYPs other than CYP2E1 contribute to aniline hydroxylase activity, albeit to a minor degree. Therefore the activity of aniline hydroxylase in the possum may be associated in some degree with other, as yet unidentified, CYPs.

It has been well established that a wide variety of compounds induce CYP2E1 in rats and rabbits [35,43] and humans [34]. The terpenes used in this study were all simple C-10 compounds and were shown to induce CYP2E1 associated activity (i.e. aniline hydroxylase) and CYP2E immunoreactive protein. This suggests that terpenes can act as inducers of CYP2E1 in the possum, however, as with the CYP2C results, until sequencing and cloning studies are carried out we are unable to conclude that CYP2E1 is being induced by terpenes in the possum.

Dosage of rats with terpenes has been shown to induce liver enzymes [9,16]. De-Oliveira et al. [9] found that oral administration of the terpene, β-myrcene, induced CYP2B1 and CYP2B2 isozymes in the rat liver. However, this induction has not been studied in animals which include terpenes in their normal diet. In our study androstenedione 16β-hydroxylase activity, a commonly used measure of CYP2B1 and CYP2B2 activity [42], was not significantly increased.

Our study also examined androstenedione 6β-hydroxylase activity, a marker of CYP3A activity. This activity was shown to be low in comparison to other species (typical androstenedione 6β-hydroxylase activity in rats being in the order of 1 nmol mg⁻¹ min⁻¹ [39]) and did not appear to be induced by terpenes. Thus our results are consistent with other studies which have examined hepatic metabolism in the possum and have noted a low CYP3A activity [17]. Similarly, no induction of CYP1A1 was observed in the test group as assessed by determination of ethoxyresorufin activity (results not shown).

Due to the low levels of androstenedione 16β- and 6β-hydroxylation activity and the insignificant difference between test and control groups, further characterisation of CYP2B and CYP3A isozymes with Western blots was not carried out.

Other studies of hepatic metabolism in marsupials [38] have shown that 17β-hydroxysteroid oxidoreductase is very high in another folivorous animal, the koala. It has been speculated that this high activity has a physiological role in the adaptation of the koala to its diet. From our study it would appear that the 17β-hydroxysteroid oxidoreductase enzyme is not induced by terpenes in the brushtail possum. Likewise, no change of Δ⁴-3-oxo-steroid 5α-oxidoreductase activity was observed as assessed by formation of 5α-androstan-3,17-dione from androstenedione, indicating no induction of the non-P450 steroid 5α-reductase (results not shown) [39].

Arboreal marsupials eat substantial amounts of terpenes in whatever wild foods they encounter (e.g. man-groves, eucalypts, and rainforest vegetation all contain substantial amounts of terpenes). In spite of the emphasis that has been put on terpenes in their importance in plant-mammal interactions, there has been no previous study on a wild mammal that shows how terpenes directly affect the animal in terms of its ability to eliminate these highly lipophilic compounds. This study is the first to show experimentally how naturally occur-
ring plant compounds act to induce enzymes in common brushtail possums. It also confirms the importance of knowledge of diet when studying xenobiotic metabolising enzymes, particularly in wild animals such as the brushtail possum. Although this study has shown that dietary terpenes cause the induction of cytochrome P450s, it has not established whether the enzymes that have been induced by selected terpenes are also responsible for the metabolism of the same terpenes. This could be determined by using chemical inhibitors and molecular studies. In order to understand the detoxification system of these animals, this is an area which requires further investigation.

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References


