Pulmonary Evaluation of Permissible Exposure Limit of Syntroleum S-8 Synthetic Jet Fuel in Mice

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No current studies have systematically examined pulmonary health effects associated with Syntroleum S-8 synthetic jet fuel (S-8). In order to gain an understanding about the threshold concentration in which lung injury is observed, C57BL/6 male mice were nose-only exposed to S-8 for 1 h/day for 7 days at average concentrations of 0 (control), 93, 352, and 616 mg/m³. Evaluation of pulmonary function, airway epithelial barrier integrity, and pathohistology was performed 24 h after the final exposures. Significant decreases were detected in expiratory lung integrity, and pathohistology was performed 24 h after the final exposure. However, morphological examination and morphometric analysis of distal lung tissue, by using transmission electron microscopy, revealed cellular damage in alveolar type II epithelial cells, with significant increases in volume density of lamellar bodies/vacuoles at 352 and 616 S-8 mg/m³. Moreover, terminal bronchiolar Clara injury, as evidenced by apical membrane blebs, was observed at relatively low concentrations, suggesting if this synthetic jet fuel is utilized, the current permissible exposure limit of 350 mg/m³ for hydrocarbon fuels should cautiously be applied.

Key Words: jet fuel; epithelial injury; lung function; permeability; permissible exposure limit (PEL).

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Like JP-8, the broad use of S-8 may pose similar risks to the respiratory system because the USAF wants its aircraft to fly on S-8 in the continental United States by 2016. This study was designed to examine whether adverse effects or lung injury occurred after varied S-8 concentration exposures at the current PEL of 350 mg/m$^3$ through a simulated flightline exposure protocol of JP-8, to establish an understanding of the potential damage the synthetic jet fuel may cause in our well-established mouse model. We hoped to fulfill these aims by relying upon the methods used in our previous JP-8 studies. Physiological levels were investigated utilizing pulmonary function and respiratory permeability tests, whereas cellular levels were measured utilizing histopathology of the distal lung. Morphometric techniques were utilized to quantify cellular damage. Evidence of epithelial injury in distal lung tissue was observed at relatively low concentrations, suggesting if S-8 is to be utilized within USAF and NATO ground-based operations, the PEL should be evaluated and caution should be exercised to limit the risk of human occupational exposures.

MATERIALS AND METHODS

**Animals.** In this study, specific pathogen-free male C57BL/6 mice (~25 g body weight, 6 weeks old, Harlan, Indianapolis, IN) were used. Mice were randomly assigned to either the lowest, middle, or high S-8 jet fuel exposure concentrations ($n=7$) or control group ($n=12$). The choice of the C57BL/6 mouse was determined because many gene knockout mice and a variety of molecular probes for individual proteins are available for the follow-up studies. The age and gender of this strain allows for comparison of results from this study with previous studies that simulated flightline exposure protocol of young-military staff involving jet fuel exposure. All mice were housed in the Association for Accreditation and Assessment of Laboratory Animal Care Institution–approved animal facility at the University of Arizona College of Medicine. Animals were housed two per cage and were fed *ad libitum*.

**S-8 aerosol generation and animal exposure.** Figure 1 is the schematic of S-8 vapor/aerosol generator, mouth-only mouse exposure chamber, and instrumentations. S-8 (CAS No. 437986-4, Synthroleum, Tulsa, OK) vapor-aerosol mixture was generated using a Lovelace jet nebulizer (Model 01-100, In-TOX, Albuquerque, NM). Its physical and chemical properties are summarized in Table 1. Jet fuel vapor and aerosol concentrations at the exposure chamber were monitored using an “in-line real-time” total hydrocarbon (THC) analysis system (Model 20, VIG Industries, Anaheim, CA) and the aerosol spectrometer (Welas System, www.palas.de). The data indicate a nonlinear relationship, whereby the aerosol component accounts for 16% of the total mass at the lower exposure levels but only 10% at the higher exposure levels (Fig. 2). The aerosol sizes were measured by using a 7-stage cascade impactor (0.25–5.0 μm, IN-TOX). The size

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Physical and Chemical Properties of S-8 Synthetic Jet Fuel$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flash points (PMCC)</td>
<td>37.8–51.5°C</td>
</tr>
<tr>
<td>Autoignition temperature</td>
<td>210°C</td>
</tr>
<tr>
<td>Appearance</td>
<td>Colorless</td>
</tr>
<tr>
<td>Physical state</td>
<td>Liquid</td>
</tr>
<tr>
<td>Odor</td>
<td>Odorless to mild paraffin</td>
</tr>
<tr>
<td>Vapor pressure</td>
<td>$&lt; 2 \text{ mmHg@20}^\circ\text{C}$</td>
</tr>
<tr>
<td>Vapor density</td>
<td>$&gt; 1$</td>
</tr>
<tr>
<td>Viscosity</td>
<td>1.2–1.9 cSt@40°C</td>
</tr>
<tr>
<td>Boiling range</td>
<td>127–288°C</td>
</tr>
<tr>
<td>Freezing point</td>
<td>$\leq -47^\circ\text{C}$</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>0.76</td>
</tr>
<tr>
<td>Component</td>
<td>C$_{7-18}$-alkane rich</td>
</tr>
</tbody>
</table>

$^a$Data from Synthroleum.
simulate the low levels of intermittent inhalation, calculated by the current PEL of skin exposure-induced confounding effects and minimize oral ingestion of S-8 more closely simulate occupational exposures, but were also utilized to rule out under constant vacuum (25 l/min). Nose-only exposures were not only used to about 3.93 ± 0.34 l, gradually peaking in number at 1.92 ± 0.12 pm, then sharply dropping to nil at about 3.41 ± 0.20 pm (Fig. 2). The size distribution curve of droplet mass (mg/ml) ranged in size from 0.34 ± 0.20 pm, gradually peaking in number at 1.92 ± 0.12 pm, then sharply dropping to nil at about 3.93 ± 0.20 pm (Fig. 2).

Mice were exposed using a nose-only exposure chamber (24-ports, IN-TOX) under constant vacuum (25 l/min). Nose-only exposures were not only used to more closely simulate occupational exposures, but were also utilized to rule out skin exposure-induced confounding effects and minimize oral ingestion of S-8 during postexposure grooming. The exposures in this study was designed to simulate the low levels of intermittent inhalation, calculated by the current PEL of 350 ± 250 mg/m3 for the dosing regimen, for the short period of exposure time. Actually, animals were exposed over a period of seven consecutive days for 1 h/day to average targeted concentrations of 0, 93, 352, and 616 mg/m3. Control mice had the same protocol as S-8 exposure except that ambient air was drawn through an empty inhalation chamber.

Lung function. The procedure to measure pulmonary function was duplicated from the previous study (Wang et al., 2002). Following 24 h after the last S-8 exposure, mice (n = 7 for S-8 exposure groups and n = 12 for control group) were anesthetized with an intramuscular injection mixture of ketamine HCl (80 mg/kg), xylazine (10 mg/kg), and acepromazine maleate (3 mg/kg). Subsequently, a tracheostomy was performed by inserting a Teflon IV catheter (20 gauge, Critikon, Tampa Bay, FL) as an endotracheal tube. The mice were placed under pressure-controlled ventilation from a small animal ventilator (Kent Scientific, Litchfield, CT). A pneumotachograph (Fleisch #0000, Instrumentation Associates, New York, NY) measured airflow while connected to a differential pressure transducer (Validyne, Northridge, CA). A computerized pulmonary function system (PEDS-LAB, Medical Associated Services, Hatfield, PA) was used to measure pulmonary function and record airflow and pressure signals, while normalizing them to each animal’s individual weight.

Respiratory permeability. After recording the pulmonary function characteristics of each animal, respiratory permeability was measured (Wong et al., 2004). The respiratory permeability was calculated by examining the pulmonary clearances of intratracheally instilled 99mTc-labeled diethylenetriaminepentaacetic acid (99mTc-DMT) over a period of 10 min using a gamma counter (Ludlum, Sweetwater, TX). This was expressed as a k value (% clearance/min).

Morphological analysis of the lung. These methods have been described previously from a previous jet fuel study (Robledo et al., 2000). Upon completion of the alveolar permeability testing, three animals per group were randomly assigned for examination of pulmonary morphological characteristics. Animals were anesthetized and then euthanized by exsanguination of the abdominal aorta. Following this, the lungs were removed and cannulated, fixed by intratracheal instillation of half-strength Karnovsky’s fixative (2% paraformaldehyde, 2% glutaraldehyde, and 0.01% picric acid in 0.1M HEPES) at a constant pressure of 20 cm H2O for 1 h. The lungs were then immersed in fixative for 24 h at 4°C. Sagittal sections (2–3 mm) were taken from the midportion of the right and left fixed lungs for light microscopy and were minced into 1-mm3 pieces for electron microscopy. Tissue sections for light microscopy were embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin. The electron microscopy sections (silver to gold interference colors) were prepared by osmication, sectioning, and staining with lead citrate and uranyl acetate. A Philips CM-12 transmission electron microscope (Mahwah, NJ) with both low and high magnifications was used to examine the sections of distal bronchiolar epithelia. Both light and electron microscopy tissue sections were examined using blinded techniques.

Morphometric procedures. The morphometric procedures were duplicated from the previous JP-8 study that utilized standard point counting techniques adapted to the lung (Herrin et al., 2006). Electron micrographs were analyzed through procedures outlined previously (Lantz and Hinton, 1984). Similarly to the recent JP-8 study, electron micrographs were enlarged to 5050 for point counting. The test grid was placed over each micrograph, and was measured to be a 19 × 25 square lattice (520 points per field) with a distance between points of 0.20 and 0.12 µm, respectively. The volume density (Vv) was estimated by examining the number of points falling on structures of interest. In both alveolar type II epithelial cells and terminal bronchiolar epithelial cells, the Vv of the lamellar bodies and vacuoles was determined, respectively.

Statistical analysis. Data were double entered into a database and checked for miscoding of variables. Initially, standard descriptive statistical analyses were run on the data to evaluate distributions, determine transformations needed, and assess potential outliers or discrepancies in the data. Usually, data were tested for homogeneity of variance using Bartlett’s test and normalized as appropriate if not following a Gaussian distribution. Depending upon the experimental designs and the size of samples number, ANOVA was used for comparisons of mean concentrations among groups. Data were expressed as mean ± SEM and p < 0.05 were be considered to be significant. Statistical analyses were performed using SPSS version 15 (Chicago, IL).

RESULTS

Lung Function

No significant pulmonary effects or changes in dynamic compliance were observed in the 93 or 616 mg/m3 group (Table 2), but significant decreases were detected in expiratory lung resistance and significant increases were observed within total lung compliance of the 352 mg/m3 group.
Changes of Lung Function Following Exposure of Mice to S-8 Synthetic Jet Fuel (S-8)

<table>
<thead>
<tr>
<th>S-8 exposure concentrations (mg/m³)</th>
<th>0</th>
<th>93</th>
<th>352</th>
<th>616</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamic lung compliance (ml/cm H₂O/kg)</td>
<td>Inspiratory: 0.074 ± 0.000</td>
<td>0.069 ± 0.000</td>
<td>0.0721 ± 0.000</td>
<td>0.072 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>Expiratory: 0.066 ± 0.000</td>
<td>0.067 ± 0.000</td>
<td>0.0667 ± 0.000</td>
<td>0.067 ± 0.004</td>
</tr>
<tr>
<td>Total</td>
<td>0.066 ± 0.000</td>
<td>0.066 ± 0.000</td>
<td>0.0754 ± 0.001</td>
<td>0.075 ± 0.033</td>
</tr>
<tr>
<td>Lung resistance (cm H₂O kg⁻¹s⁻¹)</td>
<td>Inspiratory: 3.032 ± 0.196</td>
<td>2.814 ± 0.029</td>
<td>3.339 ± 0.111</td>
<td>3.108 ± 0.441</td>
</tr>
<tr>
<td></td>
<td>Expiratory: 2.691 ± 0.079</td>
<td>2.762 ± 0.090</td>
<td>3.008 ± 0.347</td>
<td>2.881 ± 0.168</td>
</tr>
<tr>
<td>Total</td>
<td>2.697 ± 0.062</td>
<td>2.706 ± 0.047</td>
<td>3.032 ± 0.124*</td>
<td>2.855 ± 0.190</td>
</tr>
</tbody>
</table>

Note. n = 7 for S-8 exposure groups and n = 12 for control group. *p < 0.05 when compared with control group.

Respiratory Permeability

No significant pulmonary effects or changes in respiratory permeability were observed in all of S-8 groups when compared with controls (Fig. 3), suggesting that there was no loss of epithelial barrier integrity.

Morphological Alteration

Our previous study with transmission electron microscopy (TEM) has demonstrated that S-8 has site-selective and cell-specific toxic effects (Wong et al., 2008). Most obvious alterations occurred in bronchiolar Clara cells following 53 mg/m³ S-8 exposure. Similarly, following morphological alterations were still observed by using TEM, there was no observed alteration by light microscopy after 93–616 mg/m³ S-8 exposure in this study (data not shown).

Bronchiolar Clara cell. In control mice, Clara cells appeared normal for intracellular structures and there were many mitochondria in the columnar profiles distributed throughout the cell cytoplasm (Fig. 4A). Mice at 93 mg/m³ S-8 exposure (Fig. 4B), Clara cells had round mitochondria with few cristae that were distributed most apically in the cell cytoplasm. The apex of the cells also contained circular, electron-dense, membrane-bound secretory granules. The membrane blebbing (Arrow) on apical surface of Clara cells occasionally occurred, an initial sign of cell injury. At mice of 352 mg/m³ (Fig. 4C), many Clara cells had apical membrane blebbing. The blebs were packed with swollen endoplasmic reticulum (ER) and were walled off from the cell by a zone of cytoplasmic filaments. The main body of the cell contained large cytoplasmic spaces, swollen mitochondria with increased granulation in the matrix, and secretory granules. The blebs that contained mitochondria generally had larger clear cytoplasmic spaces than did the blebs that did not contain mitochondria. Mice at 616 mg/m³ exposure level had Clara cells that had widespread loss of cytoplasmic density and pronounced swelling of the ER (Fig. 4D). The majority of the mitochondria, whether swollen or with granular matrices, were clustered above the nucleus. Compared with lower S-8 concentrations, more mitochondria were quite swollen. Cell debris, including apparently intact cells with nuclei as well as small pieces of membrane-bound cytoplasm that lacked nuclei but contained swollen ER, were frequently noted in the lumen of the airway. Cell debris without an associated membrane was frequently found in the airway lumen.

Alveolar type II epithelial cells. In control mice, alveolar area appeared normal for intracellular and extracellular structures (Fig. 5A). There were many typical structures of surfactant-producing lamellar bodies throughout alveolar type II epithelial cells. The majority of mitochondria exhibited a normal shape with obvious cristae in the cytoplasm. S-8 groups had apparent alterations in the content and size of lamellar bodies within alveolar type II epithelial cells (Figs. 5B–D). Under higher magnification, there was the appearance of lamellar inclusion bodies that appeared to be secondary lysosomes containing intracellular debris (not shown). There were not obvious concentration-dependent changes in the content and size of lamellar bodies. However, mice at 616 mg/m³ had severely swollen mitochondria with decreased granulation in the matrix occurred throughout the cytoplasm, indicating necrotic injury (Fig. 5D).

Quantitative Morphometric Findings

Using a morphometric analysis of the electron micrographs of the distal lung tissue of the exposed mice, the morphological
alterations were confirmed and quantified. There were significant differences noted in the volume density of surfactant-producing lamellar bodies in all exposed groups of mice as compared with control animals (Fig. 6A). The group exposed to an average concentration of 93 mg/m\(^3\) experienced a 46% increase in lamellar body volume density in type II cells. Mice exposed to an average concentration of 352 mg/m\(^3\) experienced a 48% increase in lamellar body density in type II cells, as compared with a 39% increase of lamellar body volume density in type II cells with the group exposed to 616 mg/m\(^3\) S-8 jet fuel. The increase in volume density was found not to be dose dependent.

When morphometric techniques were applied to the electron micrographs of the terminal bronchial epithelium, significant differences were observed in the volume density of cytoplasmic vacuoles in the Clara cells at all exposure levels and apparent changes were dose dependent (Fig. 6B). For the group exposed to 93 mg/m\(^3\) of S-8 jet fuel, a 47% increase was noted in volume density of vacuoles seen in Clara cells as compared with the controls. For the group exposed to 352 mg/m\(^3\), a 61% increase was apparent in volume density of vacuoles in the Clara cells as compared with the control animals. Lastly, for the group exposed to 616 mg/m\(^3\) of S-8 jet fuel, a 71% increase was noted in volume density of vacuoles in the Clara cells as compared with the control animals.

**DISCUSSION**

This experiment was designed to verify the toxic effects and signs of lung injury at or below the current PEL of 350 mg/m\(^3\) through simulation of short-term inhalation exposure of S-8 on
male C57BL/6 mice. Data within this study suggest that inhalation exposure to S-8 concentrations around the current established PEL has no consistent adverse effects of lung ventilation function or no loss of epithelial barrier integrity. However, S-8 inhalation obviously induced signs of epithelial damage in the distal lungs, as seen in significant morphological and morphometric alterations in a concentration-dependent manner. These alterations were even apparent among the 93 mg/m³ group, a concentration less than 30% of the current PEL concentration. Along with this evidence found in previous S-8 study at the concentration of 53 mg/m³ (Wong et al., 2008), the vapor based PEL should be reevaluated before S-8 is utilized by USAF personnel, placing them at risk for an occupational exposure.

Although S-8 exposure did not cause a concentration-dependent change in lung function tests, a significant decrease was detected in expiratory lung resistance and a significant increase was observed within total lung compliance of the 352 mg/m³ group. These results would be expected, as physiological changes indicative of lung dysfunction would not be expected at concentrations lower than 352 mg/m³, suggesting that the current the PEL is feasible. However, in our previous study (Wong et al., 2008), a pulmonary function test performed 24 h after the exposure of male C57BL/6 mice to 53 mg/m³ S-8 for 1 h/day for 7 days indicated that there was a significant increase in expiratory lung resistance. The difference to be further demonstrated may possibly be attributed to disposition of the fuel, most likely due to its high volatility and transition between the aerosol and vapor states (Dietzel et al., 2005). Histopathological examination may provide some support for this speculation. When morphological and morphometric analyses were used to further examine the respiratory structures in exposed animal models, Clara cells in the most distal airway were selectively injured at very low dose (53 mg/m³) in the previous study and as the dose increase, injury extended into more proximal alveoli. Signs of cellular alteration and damage of both terminal bronchiolar Clara cells and alveolar type II cells were observed at all exposure concentrations, including those below the PEL. Following lung injury or damage, it is hypothesized that compensatory effects within the lung occur, as seen within morphological evidence and morphometric data. This compensatory mechanism of the lungs increases surfactant in alveolar type II cells to account for hindered respiratory function and decreased pulmonary ability after cellular and tissue-level damage has occurred. Surfactant, an important substance within the pulmonary system secreted by the alveolar type II cells, functions to reduce the surface tension of the alveolar-capillary surface throughout the lungs to prevent them from collapsing.

S-8 exposure resulted in a dose-dependent structural damage to Clara cells involving noted apical membrane blebs and cytoplasmic vacuolization. The cytoplasmic blebs formed in

FIG. 5. Representative transmission electron micrographs of alveolar type II epithelial cells from mice following S-8 jet fuel exposure 1 h/day for 7 days. N: Nucleus. Uranyl acetate and lead citrate. Magnification: ×8000. Note the apparent number increases in surfactant-producing lamellar bodies (arrows) of alveolar type II epithelial cells with swollen mitochondria (arrow heads) in S-8 groups (B–D). At 616 mg/m³ (D), cells had severely swollen mitochondria (arrow heads) and mess lamellar bodies organization (arrows) with decreased granulation (*) in the matrix occurred throughout the cytoplasm, a sign of cell necrosis.
Clara cells are quite similar to blebs formed in naphthalene-induced acute Clara cell toxicity (Van Winkle et al., 1999). Clara cells form blebs that contain dilated SER and mitochondria that are separated from the main cell body by a zone of intermediate filaments. These cells may represent the most severely injured cells within the spectrum of Clara cell injury following S-8 exposure. It is known that bleb formation is an early sign of toxicity that precedes mitochondrial membrane depolarization, disintegration of lysosomes, and loss of cell membrane integrity (Zahrebelski et al., 1995). Our study in S-8 exhibited the pattern of ultrastructural changes preceding loss of cell membrane integrity, as measured by respiratory permeability. Clara cell blebbing in this study may be a uniform response to P-450 bioactivated toxicants in S-8 through one or several possible mechanisms, such as transformation of preexisting microvilli, changes in the cortical cytoskeleton, and disturbances in both thiol and calcium homeostasis within the injured cell (Hinshaw et al., 1986; Van Winkle et al., 1996, 1999). These are injury patterns crucial towards exploring the mechanisms of S-8 associated with characterized epithelial injury of distal lungs around the current PEL.

In our previous study, exposure of mice to S-8 at 53 mg/m³ (80 ppm THC) 1 h/day for 7 days showed specific pattern of targeted bronchiolar, rather than alveolar terminal epithelial cells (Wong et al., 2008). The changes include swollen mitochondria and vacuolization of endoplasmic reticulum in Clara cells, disrupted epithelial cilia, and sloughing mucus lining, but there was no observed membrane bleb damage. In the current study, we demonstrated that these cellular alterations in bronchiolar epithelia were not only deteriorated, but also occurred in alveolar epithelial type II cells with increased levels of S-8 exposure. Taken together, it is noted that a broad pattern of targeted epithelium from bronchioles at the low level to alveoli at the higher level may mostly illustrate the increased or changed disposition and/or accumulation of vapor S-8. Therefore, exposure of the high concentration S-8 may lead to more fuel into terminals of lung, critically affecting alveolar function (Robledo et al., 2000; Wong et al., 2008).

This study was not designed to compare the toxicity of S-8 with the current primary jet fuel JP-8 that induced significant physiological, cellular, and biochemical changes (Herrin et al., 2006; Robledo et al., 2000; Wong et al., 2008). As we know, S-8, an aliphatic hydrocarbon (HC) fuel, is synthesized using the FT synthetic fuel process. Synthetic gas produced from natural gas, coal, or biomass is converted using heat and pressure into a clean burning liquid fuel made up of aliphatic HC (Inman et al., 2008). Its toxic effect may differ with JP-8 due to its molecular weight, specific gravities, and compositions, with S-8 jet fuel containing alkane-rich hydrocarbons in the C7–C18 range and JP-8 jet fuel with aromatic hydrocarbons in the C9–C16 range. Considering that S-8 is devoid of aromatic hydrocarbons, S-8 may have the higher threshold level at which damage occurs when compared with that of JP-8 jet fuel. Two recent dermal exposure studies have provided solid support for this speculation (Ramos et al., 2007; Inman et al., 2008). One applied S-8 alone to the skin of mice did not upregulate the expression of epidermal cyclooxygenase-2 (COX-2) nor does it induce immune suppression (Ramos et al., 2007). Adding back a cocktail of seven of the most prevalent aromatic hydrocarbons found in JP-8 (benzene, toluene, ethylbenzene, xylene, 1,2,4-trimethylbenzene, cyclohexylbenzene, and dimethylnaphthalene) to S-8 significantly upregulated epidermal COX-2 expression and suppressed a delayed-type hypersensitivity reaction. Collectively, it appears that S-8 has less toxicity than JP-8, as evidenced by the observation that JP-8.
but not S-8, induces consistent dysfunction of lung ventilation and loss of epithelial barrier integrity around the current PEL (Herrin et al., 2006; Robledo et al., 2000).

Additional chronic toxicological studies are needed to further evaluate the hazards and injury associated with use of S-8 synthetic jet fuel. By relying upon sensitive analysis techniques like morphometric analysis, cellular damage from inhalation of jet fuel can be evidenced, aiding researchers in determining the threshold limit at which damage occurs. Based on the results of the previous and this current S-8 study, we suggest that the current PEL of 350 mg/m³ be re-evaluated to a lower, more accurate level that would fully limit the risk of human occupational exposures.

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**REFERENCES**


