Appendix G3

Dataset Received from S.C. Johnson & Son, Inc. in Support of Gran et al. (2003) Poster Presentation
October 13, 2004

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Christina,

Hi! How are you? I am happy to be submitting data on sodium percarbonate, which was discussed in the poster citation listed below:


Included with this submission are the following documents:

1. Cover letter
2. Poster text
3. Histology slides
4. Coded formula spreadsheet

Study Protocols:

The standard Draize protocol was used by the supplier for the in vivo studies. We have been granted permission to share this data on sodium percarbonate for the purpose of this review. Due to the powdered form of the raw material, a bulk density determination was made to determine the weight equivalent of a 100 μL dose. Because significant irritation was observed in the acute dermal study, anesthetic was applied to the rabbit eyes five minutes prior to dosing in the primary eye irritation study.

The standard BCOP protocol for solid test articles was not used for the in vitro work at IIVS. Test articles were tested as 50% (w/w) slurry suspensions in sterile, deionized water. Treated corneas were incubated for 10 and 30 minutes with post-exposure incubation
periods of 4- and 20-24 hours. The details of the protocol are provided in the poster text. Concurrent positive and negative controls were performed with each assay. Negative control corneas were prepared for each post-exposure incubation time.

**Formula Spreadsheet:**

The formulas listed in this spreadsheet are coded similarly to formulas listed in the poster. Test material number is the unique sample number and the group name denotes formula description. Raw materials are listed followed by their percentages in each formula.

**Poster:**

The poster offprint is not included. John Harbell sent it to you previously.

**Poster Text:**

A word document consisting of poster text and tables is included in this submission for ease of reading. This document highlights where the histology slides should be inserted for ease of understanding.

**Histology Slides:**

Histology slides should be referenced on page 8.

**Data Worksheet:**

Since the rabbit study was terminated at 96 hours because of the severe nature of the responses, we must assume that the *in vivo* response fits Category 1 (both GHS and EPA). The 96-hours readings are listed in the table below:

<table>
<thead>
<tr>
<th>Animal</th>
<th>Opacity</th>
<th>Area</th>
<th>Iris</th>
<th>Redness</th>
<th>Chemosis</th>
<th>Discharge</th>
</tr>
</thead>
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<td>1</td>
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<td>3</td>
<td>2</td>
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<td>4</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

a – Iris could not be scored because of severe corneal opacity

**Summary:**

The standard BCOP protocol for solids was not utilized in this investigation of sodium percarbonate. The standard protocol, developed for pharmaceutical intermediates that are relatively insoluble, calls for using a 20% suspension with a 4-hour exposure time. Based on past experience with the BCOP assay, the eye irritancy potential of more aqueous-soluble
solids such as laundry powders using the standard solids protocol is vastly overpredictive of the outcome resulting from accidental human exposure. Furthermore, experience has shown that reactive/oxidizing chemistries (such as bleach, percarbonates and peroxides) have a delayed toxicity response in the assay necessitating increased post-exposure observation time.

The question the investigators faced in this case study of sodium percarbonate was what protocol parameters were needed to model the bolus exposure for an extended period that occurs in the Draize eye irritation protocol as well as what might be expected to be a realistic maximum exposure for humans. The following parameters were chosen: A 50% suspension of the solid with a 30-minute exposure time to model the in vivo exposure and 10-minute exposure time to model maximum accidental human exposure. While post-exposure time in the BCOP is typically 2 hours, times of 4 and 20-24 hours were chosen.

Utilizing the protocol considerations discussed above, the BCOP assay was able to adequately predict the irritancy potential of two different concentrations of sodium percarbonate for both a realistic human exposure scenario and an in vivo exposure scenario. Reduction of sodium percarbonate concentration predictably reduced the irritancy potential of the end-use formulation. Histology as a third endpoint in the BCOP assay was critical in evaluating the depth and degree of injury.

If you have any questions or comments on this data set, please feel free to contact either Judith Swanson or myself at the following:

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Judith Swanson  
(262) 260-2688  
jeswanso@scj.com

Sincere regards,

/S/

Nicole Cuellar  
Sr. Research Toxicologist
POSTER TEXT FOR S.C. JOHNSON SUBMISSION DATED OCTOBER 13, 2004

TITLE

EVALUATING THE IRRITANCY POTENTIAL OF SODIUM PERCARBONATE: A CASE STUDY USING THE BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) ASSAY.

B.P. Gran¹, J.E. Swanson¹, J.C. Merrill² and J.W. Harbell²
¹S.C. Johnson & Son, Inc. Racine, WI; ²Institute for In Vitro Sciences, Inc., Gaithersburg, MD.

ABSTRACT

Sodium percarbonate (2Na₂CO₃ • 3H₂O₂) is a component in cleaning products but the neat powder has the potential to be highly irritating to the ocular tissue of rabbits (EPA Category I). This injury results from the chemical’s reactivity and dosing method that may trap the powder against the eye. In the BCOP assay, experience has now shown that oxidizing/reactive materials often require a longer post-exposure time to fully manifest cytopathic changes. When testing reactive chemistries, the post-exposure incubation times are increased from 2 hours to 4 and 24 hours. Exposure times of 10, 20, 30 and 60 minutes were used in this study. Sodium percarbonate and percarbonate-based formulations were evaluated as 50% suspensions in water. Abattoir-derived corneas were received, mounted, exposed to test materials, and opacity, permeability and histological endpoints measured as previously reported Curren et al.(2000). Opacity and permeability scores increased with increasing exposure times and concentration of percarbonate. After the 10-minute exposure to percarbonate alone, the 4-hour post-exposure corneas showed focal epithelial layer changes that progressed to a loss of epithelium after 24 hours. Stromal damage included collagen matrix vacuolization and loss of basophilic components in the keratocyte cytoplasm. Exposures of 20 minutes or greater led to rapid destruction of both the epithelial and stromal cells and marked collagen matrix swelling. Tissue lesions declined rapidly with decreasing percarbonate concentration. Thus, the marked ocular damage induced by neat percarbonate in the rabbit, could also be reproduced in the modified BCOP. These data suggest that the modified BCOP assay can be effectively used to evaluate the safety of percarbonate-based formulations and lead to appropriate labeling and packaging decisions.
INTRODUCTION

The sodium percarbonate molecule owes its current popularity in cleaning products to its capability to be a powerful oxygen generator when combined with water. This crystalline solid is a highly reactive molecule that has been shown to be very irritating to rabbit eye tissue. When rabbits were exposed in the standard EPA Guideline eye irritation assay, corneal epithelial peeling, iridial involvement and severe conjunctival irritation occurred. (supplier data)

An initial investigation of the eye irritation potential of sodium percarbonate using the standard BCOP Assay protocol resulted in a relatively benign profile, a strikingly different result from the in vivo study.

This case study of sodium percarbonate presents an effort to understand how a non-animal assay, the BCOP Assay, can be utilized to realistically predict human eye irritancy potential of reactive molecules. The BCOP Assay was chosen for this work as it allows exact control over the exposure times and provides several measures of tissue damage. Exposure times were chosen to encompass the range of effective exposures that might occur in the rabbit. This enabled us to identify the exposure time in the BCOP Assay that resulted in a comparable level of injury to that seen in the rabbit study. Corneal injury was evaluated using the standard BCOP endpoints, opacity and permeability, as well as histological examination.
MATERIALS AND METHODS

Bovine Eyes

The BCOP assay was performed following the methods of Sina et al. (1995). Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals. The eyes were grossly examined for damage and those exhibiting defects were discarded. The corneas were excised such that a 2 to 3 mm rim of sclera was present around the cornea. The corneas were mounted in the holders and the two chambers filled with Minimum Essential Medium Eagle (MEM) without phenol red, supplemented with 1% fetal bovine serum (complete MEM). The corneal holders were incubated at 32 ± 1°C for a minimum of 1 hour.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the medium replaced in both chambers and the opacity was determined for each cornea using a Spectro Designs OP-KIT opacimeter. Three corneas, whose opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The medium was then removed from the anterior chamber and replaced with the test article, positive control, or negative control.

Method for Testing Liquid or Surfactant Materials

The test articles were tested as 50% (w/w) slurry suspension in sterile, deionized water. An aliquot of 750µl of the test article, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. A total of three corneas per treatment group were incubated in the presence of each test article at 32 ± 1°C for 10, 20, or 30 minutes with a post-exposure incubation period of 4, 20, or 24 hours. The negative control was tested, in groups of 3 corneas each, to match the short and long post-exposure incubation periods. The positive control was tested in three corneas at 32 ± 1°C for 10 minutes with a post-exposure incubation period of two hours. After the test or control article exposure, the epithelial side of the corneas was washed at least three times with complete MEM to ensure total removal of the test or control articles. The anterior chamber was refilled with fresh complete MEM and an opacity measurement was performed. After the post-exposure incubation period, a second measure of opacity was obtained. The corneas designated for the post-exposure incubation periods of 2 or 4 hours did not require refeeding with fresh medium prior to the second measure of opacity. The corneas designated for the over night post-exposure incubation periods were refed with fresh medium approximately every 6 hours and immediately prior to the second measure of opacity.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was refilled with complete MEM, and 1 ml of a 4 mg/ml fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1°C. After the incubation, an aliquot of 360 µl from each chamber was placed into the designated well on a 96-well plate. The optical density at 490 nm (OD 490 ) was determined using a Molecular Devices V max kinetic microplate reader.
Opacity Measurement: The change in opacity for each cornea was calculated by subtracting the pre-treatment opacity readings from the final opacity readings. The corrected opacity value of each cornea was calculated by subtracting the average change in opacity of the time-matched negative control corneas from that of each treated cornea. The mean opacity values of each treatment group were then calculated.

Permeability Measurement: The corrected OD\textsubscript{490} was calculated by subtracting the mean OD\textsubscript{490} of the time-matched negative control corneas from the OD\textsubscript{490} value of each treated cornea. The mean OD\textsubscript{490} values of each treatment group were then calculated.

**Histology**

The corneas were placed in individual, prelabelled cassettes and fixed for at least 24 hours in 10% buffered formalin. The fixed corneas were transferred to Pathology Associates - A Charles River Company (Frederick, MD) for embedding, sectioning and staining. Each slide was then stained with hematoxylin and eosin. Slides were returned to the Institute for In Vitro Sciences, Inc. for evaluation. Cornea sections were examined for the presence of changes in the epithelial, stromal, and endothelial areas of the tissue. Treated tissues were compared to concurrent negative and positive control tissues. Photomicrographs and thickness measurements were prepared using a Spot Insight (Spot Diagnostic Instruments) digital camera and associated software.

**Primary Eye Irritation Study of FB Sodium Percarbonate in Rabbits (1982 EPA Guidelines 81-4)**

The primary eye irritation study of sodium percarbonate in six albino rabbits [Hra: (NZW)SPF] was conducted in 1989 according to the 1982 EPA Guidelines for Acute Eye Irritation (81-4). The study was also in accordance with GLP standards of 1983 since the in-life portion of the study was completed before the effective date of the revised standards (9/18/89). A bulk density determination was made to determine the weight equivalent of a 100 µl dose. Due to irritation observed in the acute dermal study, anesthetic was applied to the eyes five minutes prior to dosing. The weight equivalent of 100 µl was placed in the conjunctival sac of rabbit and the eyelids were gently held together for one second. The contralateral eye served as the untreated control. Observations for ocular irritation were made at 1, 24, 48, 72 and 96 hours after treatment. The study was terminated at 96 hours after consultation with Sponsor due to severity of irritation observed. Acute irritation seemed to peak at 48 hours after instillation. (Study information provided by supplier under confidentiality agreement.)
RESULTS

The BCOP Assay was chosen as a non-whole animal tool for evaluating the potential eye irritancy of sodium percarbonate because it allowed exact control over exposure and observation times, and provided several measures of tissue damage. All assays were performed using a 50% slurry of the percarbonate salt in water to model a concentrated solution of the powder when tearing occurs following accidental exposure.

Exposure times were chosen to encompass the range of what might be a realistic worst possible case in accidental human exposure to an exposure time that would approximate the level of injury found in the in vivo study. Corneal injury was evaluated initially using opacity and permeability endpoints, the standard BCOP Assay measures of irritation. Since the full manifestation of oxidative damage to cells may be delayed for some hours after exposure with some materials, several post-exposure periods were selected to compare the manifestation of damage over time. Table 1 shows the impact of exposure times and post-exposure observations on the quantitative BCOP endpoints and the effects of reducing the concentration of sodium percarbonate in formulation at two exposure times.
Table 1. BCOP Opacity and Permeability Scores from Sodium Percarbonate Exposure: Impact of Exposure and Post-Exposure Time

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Exposure Time</th>
<th>Post-Exposure Incubation Time</th>
<th>Opacity</th>
<th>Permeability</th>
<th>In Vitro Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sodium Percarbonate (500 mg/ml suspension) pH 10.5</td>
<td>10 minutes</td>
<td>4 hours</td>
<td>8.3</td>
<td>0.123</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>30 minutes</td>
<td>4 hours</td>
<td>14.0</td>
<td>2.598</td>
<td>53.0</td>
</tr>
<tr>
<td></td>
<td>60 minutes</td>
<td>4 hours</td>
<td>19.8</td>
<td>4.344</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>10 minutes</td>
<td>24 hours</td>
<td>16.0</td>
<td>0.636</td>
<td>25.5</td>
</tr>
<tr>
<td></td>
<td>30 minutes</td>
<td>24 hours</td>
<td>27.7</td>
<td>1.392</td>
<td>48.5</td>
</tr>
<tr>
<td></td>
<td>60 minutes</td>
<td>24 hours</td>
<td>27.3</td>
<td>1.333</td>
<td>47.3</td>
</tr>
<tr>
<td>2. Sodium Percarbonate (500 mg/ml suspension)</td>
<td>10 minutes</td>
<td>20 hours</td>
<td>11.0</td>
<td>0.025</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>20 minutes</td>
<td>20 hours</td>
<td>14.0</td>
<td>2.810</td>
<td>56.1</td>
</tr>
<tr>
<td>3. Sodium Percarbonate* (300 mg/ml suspension)</td>
<td>10 minutes</td>
<td>20 hours</td>
<td>6.0</td>
<td>0.015</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>20 minutes</td>
<td>20 hours</td>
<td>13.3</td>
<td>0.366</td>
<td>18.8</td>
</tr>
</tbody>
</table>

*Formulation with 60% sodium percarbonate
Additionally, the corneas were sectioned, stained and examined microscopically for depth of injury and histological markers of irritancy for this oxidative material. Table 2 summarizes morphological changes seen in the corneas for different exposure times at both short-and long-term post-exposure times.

Table 2. Morphological Changes in Corneas Treated with Sodium Percarbonate: Impact of Exposure and Post-Exposure Time

<table>
<thead>
<tr>
<th>Test Article Exposure time</th>
<th>Post Exposure time</th>
<th>4 hours</th>
<th>20 to 24</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Epithelium</td>
<td>Stromal Collagen</td>
<td>Keratocytes</td>
</tr>
<tr>
<td>10 min</td>
<td>Surface cells lost but deeper layers remained. Marked focal lesions observed</td>
<td>Increased stromal thickness and moderate CMV* to 40% depth</td>
<td>Moderate increase in cytoplasmic eosinophilia to 40% depth</td>
</tr>
<tr>
<td>20 min**</td>
<td>Epithelium completely lost</td>
<td>Stromal thickness and CMV past 50% depth</td>
<td>Marked nuclear pyknosis and cytoplasmic eosinophilia – full depth</td>
</tr>
<tr>
<td>30 min</td>
<td>Surface cells lost, remaining cells in place but damaged</td>
<td>Marked increase in stromal thickness</td>
<td>Marked nuclear pyknosis and cytoplasmic eosinophilia – full depth</td>
</tr>
<tr>
<td>60 min</td>
<td>Epithelium present but not viable</td>
<td>Marked increase in stromal thickness, gas pockets visible</td>
<td>Marked nuclear pyknosis and cytoplasmic eosinophilia – full depth</td>
</tr>
</tbody>
</table>

* CMV = Collagen Matrix Vacuolization
** 20-hour post-exposure time
See attached FIGURES for specific histology slides.

The following Figures illustrate the qualitative changes in corneal tissue that are summarized in Table 2.

- Figures 1 and 2, A, B, & C show normal untreated corneal tissue to afford a basis for comparison with the tissues that have been exposed to percarbonate slurries.
- Figures 3-7, A, B & C show injury to corneal structures at different time periods.
- Figures 8, A, B & C shows the effects of a reduced concentration end-use formulation compared to full-strength percarbonate in Figures 4, A, B & C.
DISCUSSION

- With reactive molecules like sodium percarbonate, reliance on the traditional 2-hour post-exposure incubation in the BCOP assay can be misleading. The delayed manifestation of toxicity requires an increased post-exposure incubation time (see Table 1).
- The opacity and permeability endpoints may underestimate the toxicity where the epithelium remains physically intact. The focal lesions do not lead to an appreciable increase in permeability scores (see for example Table 1, 10-minute exposures).
- The pattern of lesions in the corneal epithelium suggests that focal lesions develop which breach the epithelial barrier and allow subsequent penetration into the stroma. This pattern of damage is different from what is observed with exposure to surfactants or solvents where the lesions tend to be more uniformly progressive across the epithelial surface of the cornea.
- Loss of the corneal epithelium leads to extensive fluorescein permeability while the corneal stroma is in the process of swelling (Table 1, 30- and 60-minute exposures at 4 hours post-exposure). However, once the corneal stroma has swollen, the relative fluorescein permeability decreases (Table 1, 30- and 60-minute exposures at 24 hours post-exposure). Note the stromal thickness in Figures 5C and 6C.
- The degree and depth of injury to the stromal keratocytes has been shown to be predictive of the degree and duration of ocular injury in vivo (Maurer et al., 2002). Histological evaluation of the bovine corneas, treated in vitro, provides data on keratocyte damage. This damage may not be fully reflected in the opacity and permeability measurements.
- The Draize Test protocol leads to an overestimation of the irritancy of powders. The effects resulting from the Draize methodology greatly exceed what could realistically be expected from accidental human exposure. The differences in exposure include: the quantity and location of material instilled, the occlusion and pressure of the crystalline material against the cornea, mechanical abrasion and a different tearing response. (see Bruner’s discussion of ocular irritation in Frazier’s In Vitro Toxicity Testing, pp.160-161, Wilkie and Wyman’s chapter in Hobson’s Dermal and Ocular Toxicology, p.487. and Maurer et al., 2002)
CONCLUSION

- Testing reactive molecules, such as sodium percarbonate, requires a modification of the BCOP protocol to fully evaluate the potential for delayed effects on corneal tissue.
- Because important changes may come at the cellular rather than tissue level (see Jester et al. [1998] and Maurer et al. [2001]), histology evaluation is critical as a third end-point in the BCOP Assay for this type of molecule. These combined endpoints allow for the determination of depth and degree of injury that is required to predict irritation potential (see Maurer et al [2002]).
- In the BCOP assay, exposures of greater than 10 minutes to a 50% suspension of sodium percarbonate are required to achieve tissue damage consistent with the damage reported for the rabbit eyes in the Draize test. These data suggest that trapping of the powder against the cornea in the conjunctival sac may appreciably impact its toxicity in the rabbit.
- Reduction of sodium percarbonate concentration greatly reduced the irritancy potential of the test formulation, even in the more exaggerated 20-minute exposure.
REFERENCES


J.E. Swanson, B.T. White, B.P. Gran, J.C. Merrill and J.W. Harbell; 42nd Annual SOT, Poster #1068.
Proposed figure legends (1066)

Figure 1. Negative Control, 4-hour post-exposure
(A) Epithelium (magnification 230x)

(B) Stroma directly below the Bowman’s Layer (magnification 430x)
Figure 2. Negative Control, 20-hour post-exposure
(A) Epithelium (magnification 230x)
(B) Stroma directly below the Bowman’s Layer (magnification 430x)

(C) Full thickness (magnification 45x)
Figure 3. Sodium Percarbonate 50% (w/v) suspension, 10-minute exposure, 4-hour post-exposure
(A) Epithelium (magnification 230x)

(B) Stroma at mid-depth (magnification 230x)
Figure 4. Sodium Percarbonate 50% (w/v) suspension, 10-minute exposure, 24-hour post-exposure
(A) Epithelium showing marked cell loss (magnification 230x)
(B) Stroma at mid depth showing increased collagen matrix vacuolization (magnification 230x)

(C) Full thickness (magnification 45x)
Figure 5. Sodium Percarbonate, 50% (w/v) suspension, 30-minute exposure, 4-hour post-exposure
(A) Epithelium separated from the basal lamina (magnification 230x)

(B) Stroma at mid depth showing marked nuclear pyknosis (magnification 430x)
(C) Full thickness (magnification 45x)

Figure 6. Sodium Percarbonate, 50% (w/v) suspension, 30-minute exposure, 24-hour post-exposure
(A) Epithelium (lost) (magnification 430x)
(B) Stroma at mid depth showing marked collagen matrix vacuolization and dead keratocytes (magnification 430x)
Figure 7. Sodium Percarbonate, 50% (w/v) suspension, 60-minute exposure, 4-hour post-exposure
(A) Epithelium (nonviable) (magnification 430x)

(C) Full thickness (magnification 45x)
(C) Full thickness showing collagen delamination (magnification 45x)

Figure 8. Sodium Percarbonate (60% in formulation), 50% (w/v) suspension, 10-minute exposure, 20-hour post-exposure
(A) Epithelium (magnification 230x)
(B) Stroma directly below Bowman’s Layer showing the slight increase in collagen matrix vacuolization (magnification 430x)

(C) Full thickness (magnification 45x)
### FORMULAS

<table>
<thead>
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<th>Test Material #</th>
<th>Group</th>
<th>Raw Material</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
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<td>Reactive Chemical Mixture</td>
<td>40-45</td>
</tr>
<tr>
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<td>Sodium carbonate (CAS #497-19-8)</td>
<td>Reactive Chemical Mixture</td>
<td>5-10</td>
</tr>
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<td></td>
<td></td>
<td>Water</td>
<td>45-50</td>
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<tr>
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<td>Sodium Percarbonate (CAS #15630-89-4)</td>
<td>Reactive Chemical Mixture</td>
<td>40-45</td>
</tr>
<tr>
<td></td>
<td>Sodium carbonate (CAS #497-19-8)</td>
<td>Reactive Chemical Mixture</td>
<td>5-10</td>
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<td>45-50</td>
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