Bull. Chem. Soc. Ethiop. **2010**, 24(1), 1-10. ISSN 1011-3924
Printed in Ethiopia 

# FORMULATION AND CHARACTERIZATION OF A MULTIPLE EMULSION CONTAINING 1% L-ASCORBIC ACID

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(Received December 26, 2008; revised September 1, 2009)

**ABSTRACT.** The purpose of the study was to prepare a stable multiple emulsion containing a skin anti-aging agent and using paraffin oil. Vitamin C, was incorporated into the inner aqueous phase of water-in-oil-in-water (w/o/w) multiple emulsion at a concentration of 1%. Multiple emulsion was prepared by two step method. Stability studies were performed at different accelerated conditions, i.e. 8 $^{o}$C (in refrigerator), 25 $^{o}$C (in oven), 40 $^{o}$C (in oven), and 40 $^{o}$C at 75% RH (in stability cabin) for 28 days to predict the stability of formulations. Different parameters, namely pH, globule size, electrical conductivity and effect of centrifugation (simulating gravity) were determined during stability studies. Data obtained was evaluated statistically using ANOVA two way analyses and LSD tests. Multiple emulsion formulated was found to be stable at lower temperatures (i.e. 8 and 25 $^{o}$C) for 28 days. No phase separation was observed in the samples during stability testing. It was found that there was no significant change ($p > 0.05$) in globule sizes in most of the samples kept at various conditions. Insignificant changes ($p > 0.05$) in both pH and conductivity values were determined for the samples kept at 8, 40, and 40 $^{o}$C at 75% RH, throughout the study period. Further studies are needed to formulate more stable emulsions with other emulsifying agents.

**KEY WORDS:** Multiple emulsion, Vitamin C, Stability

## INTRODUCTION

Multiple emulsions are defined as emulsions in which both types of emulsions, i.e. water-in-oil (w/o) and oil-in-water (o/w) exist simultaneously [1]. They combine the properties of both w/o and o/w emulsions. These have been described as heterogeneous systems of one immiscible liquid dispersed in another in the form of droplets, which usually have diameters greater than 1 μm [1]. These two liquids forming a system are characterized by their low thermodynamic stability [2].

Multiple emulsions are very complex systems as the drops of dispersed phase themselves contain even smaller droplets, which normally consist of a liquid miscible and in most cases identical with the continuous phase [2]. Both hydrophilic and lipophilic emulsifiers are used for the formation of multiple emulsions. Multiple emulsions were determined to be promising in many fields, particularly in pharmaceutics and in separation science. Their potential biopharmaceutical applications [3] include their use as adjuvant vaccines [4], as prolonged drug delivery systems [5-8], as sorbent reservoirs in drug overdose treatments [9] and in mobilization of enzymes [10-11]. Multiple emulsions were also investigated for cosmetics for their potential advantages of prolonged release of active agent, incorporation of incompatible materials and protection of active ingredients by dispersion in internal phase [12-14].

Preparation of multiple emulsions with synthetic oils, e.g. paraffin oil, is a challenging subject due to stability problems. In this study, paraffin oil was used because of its cutaneous cosmetic benefits. Paraffin oil is a mixture of refined saturated aliphatic (C14-C18) and cyclic hydrocarbons obtained from petroleum [15].

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The role of vitamin C as an antioxidant and in the protection of skin against deleterious effects of ultraviolet type B (UVB) light was reported by many workers [16-18]. Vitamin C was also declared to improve the synthesis of collagens increasing the suppleness of the skin [19]. This vitamin was incorporated at a concentration of 1% into the internal aqueous phase of the multiple emulsions prepared in this study.

## EXPERIMENTAL

*Reagents and apparatus*

Abil® EM 90 was purchased from Franken Chemicals (Germany) and Tween® 80, L-ascorbic acid and magnesium sulfate were purchased from Merck (Germany). Double distilled water was prepared by using distillation plant (IM 100-0.43, IRMECO-GMBH, Germany). Digital pH-meter (WTW, Germany), digital conductivity-meter (WTW, Germany), stability chambers (Sanyo, Japan), water bath (HH-S 21-4, China), electrical balance (Precisa, Switzerland), digital humidity-meter (TES Electronic Corp., UK), centrifuge (Hettich, Germany), mechanical mixer (IKA, Germany), refrigerator (Dalwance, Pakistan), microscope (Nikon, Japan) with camera (DCM 35 (350 K, Pixels USB 2, Germany) mounted, microscopic software (MiniSee, Japan) and SPSS 10.0 were used during the study.

*Preparation of formulation*

For the preparation of primary emulsion, oil phase consisting of paraffin oil (22%) and lipophilic surfactant (Abil® EM 90) (5%) was heated to 75 $^{o}$C ± 1 $^{o}$C [20]. Aqueous phase consisting of ascorbic acid (1%) and magnesium sulfate (0.7%) was also heated to the same temperature. Aqueous phase was added to the oil phase drop by drop while stirring at 2000 rpm. Agitation was continued until cooling to room temperature of 25 $^{o}$C. For obtaining the multiple emulsion, primary emulsion (90%) was added to the aqueous phase containing hydrophilic surfactant (Tween® 80) (1.5%) [20] while agitating at 1000 rpm for 10 min. Emulsion was then homogenized at 800 rpm for 5 min and further at 500 rpm for 5 min more.

*Properties of primary and multiple emulsions*

*Types of emulsions*

Types of emulsions were analyzed by dilution with paraffin oil and water separately and observation under microscope.

*Stability tests*

Stability tests were performed at different storage conditions for both primary and multiple emulsions. The tests were performed on samples kept at 8 ± 0.1 $^{o}$C (in refrigerator), 25 ± 0.1 $^{o}$C (in oven), 40 ± 0.1 $^{o}$C (in oven) and 40 ± 0.1 $^{o}$C at 75% relative humidity (RH) (in stability cabin).

*Organoleptic characteristics*.

Freshly prepared primary and multiple emulsions were investigated organoleptically (color, liquefaction and phase separation). Organoleptic characteristics of both primary and multiple emulsions kept at different storage conditions, i.e. color, liquefaction and phase separation were noted at various intervals, i.e. 0 h, 1 h, 1 day, 3 days, 7 days, 14 days, 21 days and 28 days for 28 days.

*Centrifugation tests*

Centrifugation tests were performed for the primary and multiple emulsions immediately after preparation. The same test was repeated for the multiple emulsions after 24 h, 3 days, 7 days, 14 days and 28 days of preparation. Centrifugation conditions were 25 $^{o}$C and 5000 rpm by placing 10 g of sample in the tube.

*Microscopic tests*

Multiple emulsions were analyzed under the microscope to confirm the multiple characters. A drop of multiple emulsion was placed on the glass slide, diluted with water and covered by a glass cover. A drop of immersion oil was placed on the cover slide and observed under the microscope [21].

*Globules size*

In this study, globule sizes of the multiple emulsions prepared were determined using light microscope fitted with a digital camera for the freshly prepared emulsions and for the emulsions kept at different conditions for 28 days [21].

*pH determination*

The pH value of the freshly prepared emulsions and the emulsions kept at different conditions were determined by a digital pH-meter. pH measurements were repeated for multiple emulsions after 1, 3, 7, 14, 21 and 28 days of preparation.

*Electrical conductivity tests*

Conductivity tests were performed for the multiple emulsion immediately after preparation and for the samples kept at different conditions by using digital conductivity-meter. Conductivity tests were repeated for the multiple emulsions after 1, 3, 7, 14, 21 and 28 days of preparation.

*Statistical evaluation*

The measured values obtained for different parameters (pH, electrical conductivity, globule size) were analyzed using SPSS 10.0 on the PC computer (two-way ANOVA for variation between different time intervals) and the level of significance was 5% confidence level.

## RESULTS AND DISCUSSION

*Organoleptic characteristics*

Organoleptic characteristics of the primary and multiple emulsions formulated are presented in Table 1.

*Centrifugation test*

Results of the centrifugation tests for the primary and multiple emulsions kept at different storage conditions are given in Table 1. For the primary emulsion, no phase separation was seen after centrifugation in any of the samples kept at different storage conditions up to 14th day; however, slight phase separation was determined on the 21st day in the samples kept at 40 $^{o}$C

(in oven) and 40 °C at 75% RH (in stability cabin) and there was no more increase in the phase separation until the end of study period.

No phase separation after centrifugation was seen in all of the multiple emulsion samples kept at different storage conditions up to 14th day. Slight phase separation was seen in the samples kept at different storage conditions after the 21st day. There was no further increase in phase separation in the samples kept at 8 °C (in refrigerator) and 25 °C (in oven) until the end of 28th day; however, samples kept at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin) showed increase in phase separation until the 28th day.

Table 1. Organoleptic characteristics and centrifugation test results of multiple emulsion at various storage conditions.

| Time | Liquefaction | | | | Color | | | | Phase separation | | | | Centrifugation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| 0 h | - | - | - | - | W | W | W | W | - | - | - | - | - | - | - | - |
| 1 h | - | - | - | - | W | W | W | W | - | - | - | - | - | - | - | - |
| 24 h | - | - | - | - | W | W | W | W | - | - | - | - | - | - | - | - |
| 72 h | - | - | - | - | W | W | W | W | - | - | - | - | - | - | - | - |
| 7 days | - | - | - | - | W | W | W | W | - | - | - | - | - | - | - | - |
| 14 days | - | - | - | - | W | W | W | W | - | - | - | - | - | - | - | - |
| 21 days | - | - | + | + | W | W | YW | YW | - | - | + | + | + | + | + | + |
| 28 days | - | - | + | + | W | W | YW | YW | - | - | + | + | + | + | ++ | ++ |

- = No change; + = slight change; W = white; YW = yellowish-white; ++ = more change A = 8 °C; B = 25 °C; C = 40 °C (in oven); D = 40 °C at 75% RH (in stability cabin) (n = 3).

*Stability*

W/o/w multiple emulsions may deteriorate by several possible mechanisms which include swelling of internal drops due to osmotic pressure leading to passage of water from external phase to internal phase [3]. Other mechanisms are rupture of the oil layer or coalescence of the oil globules or the coalescence of internal water droplets. Presence of electrolytes plays an important role in the stability of multiple emulsions by balancing the osmotic pressure between the internal and external aqueous phases or by forming the rigid interfacial layer between the oil and internal aqueous phase [22]. The osmotic pressure gradient between the aqueous phases may lead to a dynamic change in the volume of internal aqueous phase due to the migration of water through the oil layer [23]. Transfer of active agent to the external aqueous phase can occur either by total breakdown of multiple droplets or by diffusion of unionized active agent through the oil layer [24]. Although diffusion is the most important mechanism of transport in the multiple emulsion systems, there is evidence that ionized materials may pass across the oil layer. Kita *et al.* [25] suggested that ionized drug along with water is mixed with micelles of hydrophilic and hydrophobic surfactants and cross the oil layer. Another possible transport mechanism is carrier mediated transport [26]. The drug is bound to the carrier and this complex is permeable through the oil membrane. After passing through the oil membrane, the carrier is transported back. In this way, the carrier effectively 'pumps' the permeating compound.

In this work, both primary and w/o/w multiple emulsions were divided into four samples separately and these samples were kept at different storage conditions, i.e. at 8 °C in refrigerator, at 25 °C in oven, 40 °C in oven and at 40 °C at 75% relative humidity (RH) in stability chambers. The samples kept at different storage conditions were observed for a period of 28 days. Samples were observed with respect to changes in color, liquefaction and phase separation.

*Color*

Freshly prepared primary emulsion was creamy white in color. There was no change in color at different storage conditions. This shows that primary emulsion was stable at different storage conditions up to 28 days.

Freshly prepared multiple emulsion was white in color. There was little change in color of samples kept at 40 $^{o}$C (in oven) and at 40 $^{o}$C at 75% RH (in stability cabin); the color became yellowish white. The change in color appeared on the 21st day and persisted up to 28 days. The change in color at the end of the observation period may be due to the oily phase separation which is promoted at elevated temperatures.

*Liquefaction*

No liquefaction was observed in the primary emulsion at all storage conditions. For the multiple emulsion, while no liquefaction was observed in the samples kept at 8 $^{o}$C (in refrigerator) and 25 $^{o}$C (in oven) during 28 days, slight liquefaction was observed in the samples kept at 40 $^{o}$C (in oven), 40 $^{o}$C at 75% RH (in stability cabin) on 21st day. Liquefaction is the sign of instability; it may be attributed to the passage of water from the internal phase to external phase as described by many researchers [23, 25].

*Phase separation*

In the case of primary emulsion, no phase separation was observed in any of samples. This indicates that primary emulsion was stable at all storage conditions for 28 days.

For the multiple emulsion, no phase separation was seen in the samples kept at all storage conditions, except slight phase separation beginning on the 21st day.

*Globule size*

Globules sizes of the multiple emulsion kept at different storage conditions are represented graphically in Figure 1 and photographs are shown in Figure 2. Globule sizes of emulsion systems can be determined by light microscope [27], laser diffraction methods [28], electron microscope [29-30] or by coulter counter [31]. Light microscope was used in this study.

The increase or decrease in globule sizes indicates instability [3]. The multiple droplets may coalesce with the other oil drops, internal aqueous droplets may be expelled individually, more than one drop may be expelled, internal globules may coalesce before being expelled out resulting in the shrinkage of internal droplets or water may pass from the external phase to the internal aqueous phase resulting in the swelling of internal droplets followed by complete rupture of droplets.

Mean globule size of the freshly prepared formulation was 14.82 μm. Mean globule sizes of the multiple emulsion samples kept at 8 $^{o}$C (in refrigerator) and 25 $^{o}$C (in oven) were increased beginning from the first 1st hour to the 28th day when the globule sizes were 17.01 μm and 16.08 μm, respectively. This increase may be due to the swelling of globules which is probably because of the water migrating from the external aqueous phase to the internal aqueous phase. This migration was indicated by the decrease in the electrical conductivity of the samples kept at 8 $^{o}$C (in refrigerator) and 25 $^{o}$C (in oven).

Globule sizes of the samples stored at 40 $^{o}$C (in oven) and 40 $^{o}$C at 75% RH (in stability cabin) were decreased beginning from the 1st hour until the 28th day when the mean globule sizes of the samples were 12.98 μm and 13.19 μm, respectively. This may be attributed to the shrinkage of globules which might be due to the expulsion of the internal aqueous droplets to

the external water phase indicated by the increase in the electrical conductivity values of the samples stored at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin).

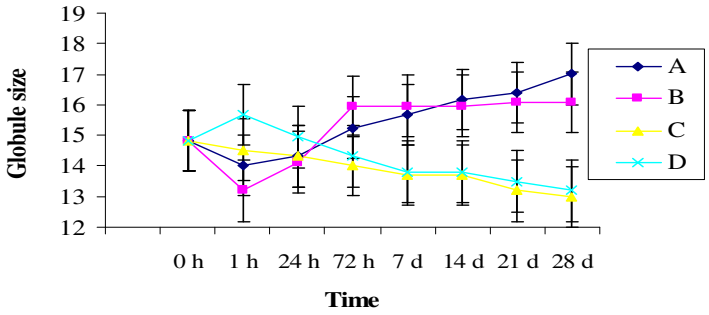


Figure 1. Mean globule sizes of multiple emulsions kept at different conditions; A = 8 °C, B = 25 °C, C = 40 °C (in oven), D = 40 °C at 75% RH (in stability cabin) (n = 3, h = hour, d = days).

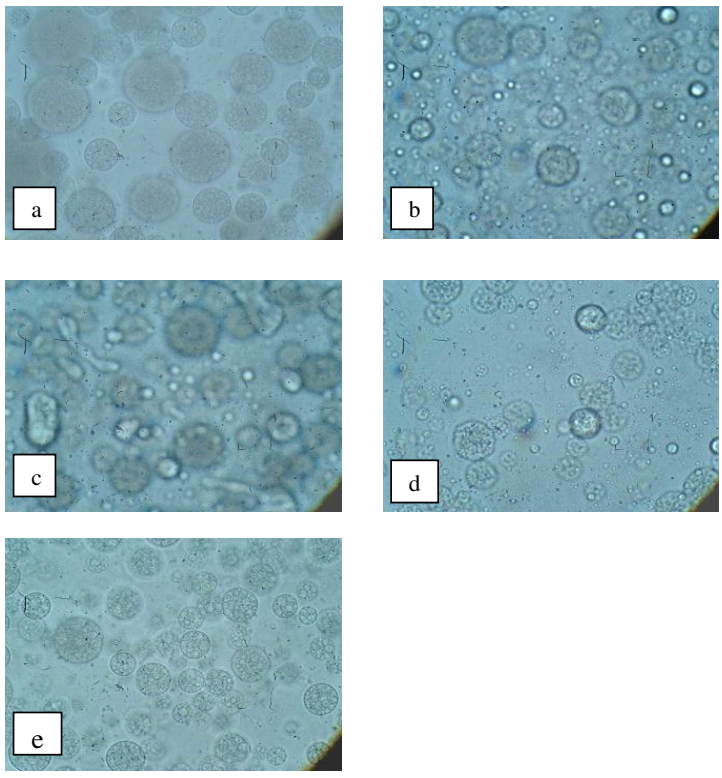


Figure 2. Microscopic image of (a) freshly prepared multiple emulsion, (b) at 8 °C (in refrigerator), (c) at 25 °C (in oven), (d) at 40 °C (in oven) and (e) at 40 °C at 75% RH (in stability cabin).

By using two-way ANOVA test at 5% significance level, it was found that the variation in globule sizes at different time levels was insignificant ($p > 0.05$) while significant ($p < 0.05$) at different temperature levels. LSD test applicable to different temperature levels was performed by taking 25 $^{o}$C as the standard temperature. As a result of this test, it was concluded that the multiple emulsion formulation was stable at the lower temperature of 8 $^{o}$C (in refrigerator) considering the globule sizes; however, there is a possibility of instability at 40 $^{o}$C (in oven) and 40 $^{o}$C at 75% RH (in stability cabin).

*pH values*

pH values of the multiple emulsion kept at different storage conditions are shown graphically in Figure 3. pH values of skin range between 5 and 6, and 5.5 is considered to be the average pH of the skin. Therefore, the formulations intended for dermal application should have a pH value around this range.

In this work, pH of the freshly prepared multiple emulsion formulation was 5.50 which is very close to the skin pH. pH values of the samples kept at 8 $^{o}$C (in refrigerator), 25 $^{o}$C (in oven), 40 $^{o}$C (in oven) and 40 $^{o}$C at 75% RH (in stability cabin) were found to decrease continuously until the 28th day. pH value of the sample stored at 8 $^{o}$C decreased to 3.06 at the end of the 28th day. The decrease ended with a pH value of 3.05 at 25 $^{o}$C on the 28th day. pH values of the samples kept at 40 $^{o}$C and 40 $^{o}$C at 75% RH (in stability cabin) also showed continuous decrease and were 2.86 and 2.80, respectively on the 28th day. These decreases in pH values are probably due to the production of highly acidic by-products of ascorbic acid or any of the acidic ingredients originating from the paraffin oil such as carbon containing compounds.

It was found by the two-way ANOVA test at 5% significance level that the decrease in pH values of different samples was very significant at different levels of time and temperature ($p < 0.05$). LSD test was used to check the average pH values of the samples stored at different temperatures and time intervals separately to get the individual significant values of pH. When the average value of pH at time zero was taken as the standard, significant change ($p < 0.05$) in other levels of time was determined. pH value at the standard temperature of 25 $^{o}$ C was compared to pH values at other temperatures and insignificant results ($p > 0.05$) for 8 $^{o}$C but significant results ($p < 0.05$) for 40 $^{o}$C (in oven) and 40 $^{o}$C at 75% RH (in stability) were obtained. Therefore, it was concluded that the variation in pH values were significant ($p < 0.05$) beginning from the 1st hour after preparation and this significant change continued until the 28th day.

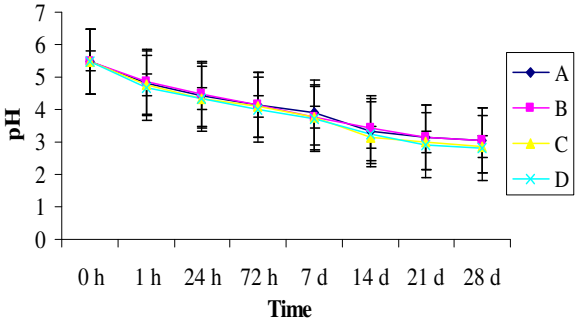


Figure 3. pH values of multiple emulsions kept at: A = 8 $^{o}$C (in refrigerator), B = 25 $^{o}$C (in oven), C = 40 $^{o}$C (in oven), D = 40 $^{o}$C at 75% RH (in stability cabin) (n = 3, h = hour, d = days).

*Electrical conductivity*

Electrical conductivity values of the multiple emulsion kept at different storage conditions are shown graphically in Figure 4. Electrical conductivity of the freshly prepared formulation was found to be 25.2 µS/cm. Electrical conductivities of the samples kept at 8 °C (in refrigerator) and 25 °C (in oven) were found to decrease with time (20.0 and 19.8 µS/cm, respectively); however, electrical conductivity values increased for the samples kept at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin) (31.3 µS/cm and 30.9 µS/cm, respectively). The increase in electrical conductivity values may be due to the transfer of electrolyte $MgSO_4$ entrapped in the internal aqueous phase of the multiple emulsion to the external aqueous phase while the decrease may be attributed to the transfer of $MgSO_4$ lost into external aqueous phase during the process of manufacturing towards the internal aqueous phase.

By using two-way ANOVA test at significance level of 5%, electrical conductivity values were found to be significant ($p < 0.05$) at different levels of temperature, but insignificant ($p > 0.05$) at different time levels. Taking the average electrical conductivity of the sample kept at 25 °C as the standard in the level of significance difference test, it was found that the most significant changes ($p < 0.05$) in electrical conductivities were observed in the samples kept at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin) and changes in the values of electrical conductivity were insignificant ($p > 0.05$). It can be concluded that the multiple emulsion kept at 8 °C (in refrigerator) is the most stable among the formulations kept at other conditions.

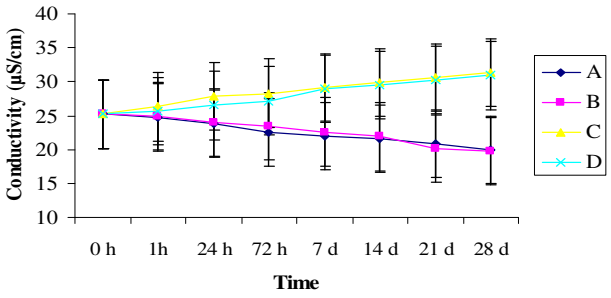


Figure 4. Electrical conductivity values of multiple emulsions kept at: A = 8 °C (in refrigerator), B = 25 °C (in oven), C = 40 °C (in oven), D = 40 °C at 75 % RH (in stability cabin) (n = 3, h = hour, d = days).

## CONCLUSIONS

This study found that a w/o/w emulsion containing ascorbic acid could be formulated using paraffin oil. There was phase separation in the formulations stored at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin) beginning from the 21st day of storage until the 28th day. Liquefaction of the multiple emulsion formulation was increased with time at the storage temperatures of 40 °C (in oven) and 40 °C at 75% RH (in stability cabin). Color of the formulation became yellowish-white with time. Globule sizes of the formulations kept at 8 °C (in refrigerator) and 25 °C (in oven) increased leading to swelling of globules while the globule sizes of the formulations kept at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin) decreased. pH values of the formulations decreased with time at all storage conditions. Electrical conductivity values of the formulations kept at 8 °C (in refrigerator) and 25 °C (in oven) decreased with time while increased for the formulations kept at 40 °C (in oven) and 40 °C at 75% RH (in stability cabin). The formulated multiple emulsion kept at 8 °C (in refrigerator)

seems to be the most stable. However, further studies are needed to obtain more stable multiple emulsions using other emulsifying agents.

## AKNOWLEDGEMENTS

Financial support to this study was given by the Department of Pharmacy, Islamia University, Bahawalpur, Pakistan. The authors thank to Vice Chancellor of The Islamia University, Bahawalpur for his efforts to take this support.

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