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The effects of new local anti-inflammatory steroids on leucocyte migration and prostanoid liberation in rats

JOHN BIRD, JOHN C. LAY AND HENRY J. LEE*

Center for Anti-inflammatory Research, College of Pharmacy, Florida A & M University, Tallahassee, Florida 32307, USA

The local anti-inflammatory potency of steroid-21-oate esters derived from prednisolone was determined by cotton pellet granuloma bioassay. The doses which inhibited granuloma formation by 50% (ID50) were: prednisolone, 0·5 mg/pellet; methyl 20α-dihydroprednisolonate, 5·8 mg/pellet; methyl 20β-dihydroprednisolonate, 1·2 mg/pellet; methyl 17,20α-acetonidodihydroprednisolonate, 6·0 mg/pellet. When administered at these equipotent local anti-inflammatory doses, only prednisolone depressed plasma corticosterone and promoted thymus involution. Prednisolone reduced neutrophil migration into saline-soaked polyester sponges and depressed the levels of 6-keto $PGF_{1\alpha}$, PGE_2 and elastase in the sponge-induced inflammatory exudate. Methyl 20α- and 20β-dihydroprednisolonate had no effect on cell migration, but depressed the levels of 6-keto $PGF_{1\alpha}$ and elastase. The liberation of 6-keto $PGF_{1\alpha}$, PGE_2 and elastase and neutrophil migration were inhibited by methyl 17,20α- and β-acetonidodihydroprednisolonate, but the effect of the β-epimer on cell migration was transient. These data suggest that steroid acid esters which have local and topical anti-inflammatory properties exert their effect in a similar fashion to glucocorticoids with a ketol side-chain (e.g. prednisolone) with respect to liberation of prostaglandins and lysosomal enzymes. However, their effect on neutrophil migration was variable, depending on their structural features. Furthermore, the systemic effects of these new derivatives were drastically reduced indicating that they may be of potential benefit in prolonged treatment.

It has been established that esters of steroid-21-oates with intact ring structures, obtained by modifying the ketol side chain of potent glucocorticoids, retained anti-inflammatory activity, but upon entry into the circulatory system from the site of administration they were hydrolysed to inactive and readily excreted steroid acids (Lee & Soliman 1982). These steroids were shown to have local anti-inflammatory activity approaching that of their parent compound (prednisolone) using the cotton pellet granuloma bioassay, but they were devoid of the major side effects of prednisolone such as pituitary adrenal suppression and thymus involution (Soliman et al 1982; Khalil et al 1985).

Several mechanisms for the inhibition of inflammation by steroids have been proposed, including inhibition of neutrophil migration or chemotaxis (Ward 1966; Wiener et al 1975) and lysosomal enzyme release (Weissmann 1973; Goldstein 1975). The observation that glucocorticoids inhibit the release of arachidonic acid from membrane phospholipids, and thereby suppress its conversion to eicosanoids, has led to the suggestion that this effect also may be largely reponsible for the anti-inflammatory action of these drugs (Nijkamp et al 1976; Flower & Blackwell 1979; Blackwell 1983).

The purpose of the present investigation was to determine the effects of equipotent doses of the steroid-21-oate esters on thymus involution, plasma corticosterone, cell migration into polyester sponges, and on the levels of prostanoids and lysosomal enzymes in the resulting sponge exudates.

MATERIALS AND METHODS

Synthesis of steroid derivatives (Fig. 1)

Prednisolone was purchased from Upjohn (Kalamazoo, Mi) and all other analytical grade reagents from Mallinckrodt (Paris, Ky).

Methyl 20ξ-dihydroprednisolonate was prepared according to Lewbart & Schneider (1969) and was purified by silica gel 60 column chromatography employing hexane–dichloromethane–acetone (40:20:40 by volume) as developing solvent. The column fractions containing the racemic mixture were then concentrated and the individual epimers separated by preparative HPLC on a C_{18} column (250 × 10 mm, Perkin-Elmer, F1). The mobile phase of methanol–water (60:40, v/v) was delivered at

* Correspondence.

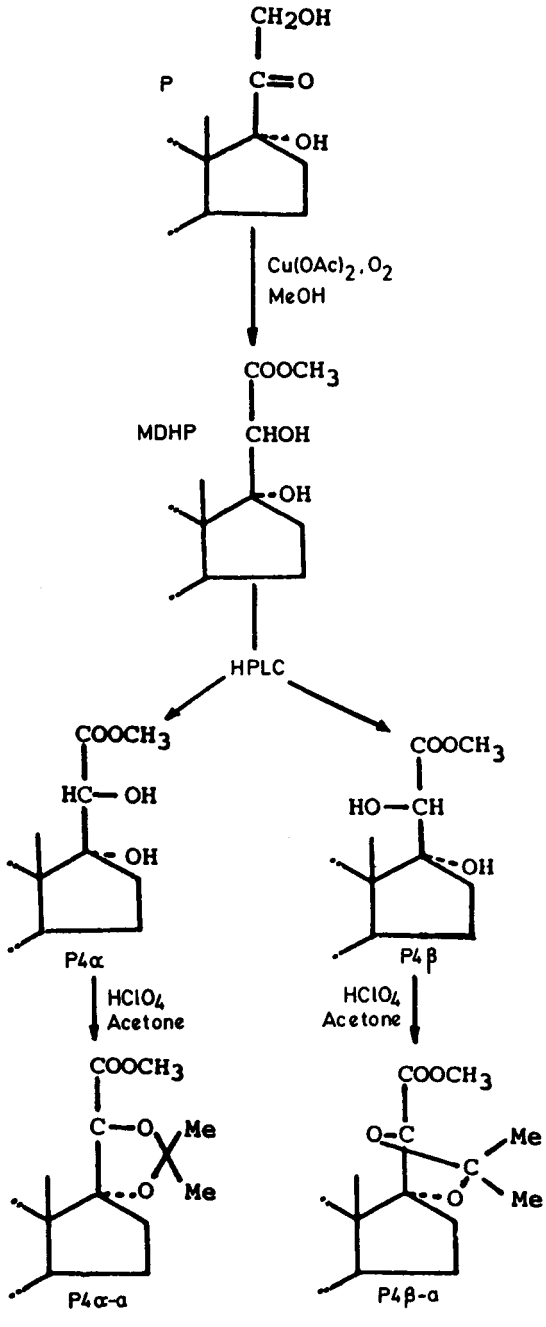

FIG. 1. Scheme for the chemical synthesis of prednisolone derivatives. Steroid nomenclature: Prednisolone (P) 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; methyl 20ξ-dihydroprednisolonate (MDHP); methyl 20α-dihydroprednisolonate (P4α); methyl 20β-dihydroprednisolonate (P4β); methyl 17,20α-acetonidodihydroprednisolonate (P4α-a); methyl 17, 20β-acetonidodihydroprednisolonate (P4β-a).

6 ml min^{-1} and the detector (model 450, Waters Associates, MA) wavelength set at 254 nm. The fractions corresponding to the α- and β-epimer were collected and the methanol evaporated under vacuum at 34 °C. After extraction with ethyl acetate, drying with anhydrous sodium sulphate and recrystallization from acetone–hexane, pure α- and β-epimers were obtained (Lee & Lee 1985).

A racemic mixture of methyl 17,20-acetonidodihydroprednisolonate was prepared from methyl 20ξ-dihydroprednisolonate (1200 mg) with perchloric acid (2·5 ml) in acetone (50 ml) using the method of Lewbart (1969). Separation of the epimers was achieved by column chromatography on a silica gel column (70 × 1·5 cm) eluting with benzene-ethyl acetate (2 : 1 v/v). Fractions corresponding to each epimer were combined, evaporated to dryness and recrystallized from aqueous methanol. The purity and structural confirmations have been described by Lee & Lee (1985).

Cotton pellet granuloma bioassay

The anti-inflammatory activity of the steroids was evaluated using a modification of the cotton pellet granuloma bioassay described by Meier et al (1950). Cotton pellets (35 ± 1 mg) were impregnated with 0·2 ml of steroid solution in acetone. After removal of the solvent by evaporation the pellets were injected with 0·2 ml of antibiotic solution (1 mg penicillin G and 1·3 mg of dihydrostreptomycin ml^{-1}) and subsequently implanted subcutaneously, one in each axilla, into male Sprague-Dawley rats (100–125 g, Southern Animal Farms, Al) under light ether anaesthesia. Control rats were implanted with pellets containing only antibiotics. Seven days later the animals were killed, the pellets removed, dried at 60 °C for 48 h and weighed. The increment in pellet dry weight was taken as a measure of granuloma formation. For the determination of the ID50 for each compound four groups of 5 rats were used with doses/pellet ranging from 0·01 to 2 mg for prednisolone, 0·5 to 2·5 mg for methyl 20β-dihydroprednisolonate and methyl 17,20α-acetonidodihydroprednisolonate, and 1 to 7·5 mg for methyl 20α-dihydroprednisolonate and methyl 17,20β-acetonidodihydroprednisolonate. To evaluate the relative systemic effects of the compounds, an additional experiment was performed with groups of rats implanted with pellets containing an ID50 dose of the respective compounds. At the time of death the final body and thymus weights were recorded and a plasma sample obtained by cardiac puncture for later measurement of corticosterone levels by a

spectrofluorometric method (Vernikos-Danellis et al 1966).

Collection of inflammatory exudates

Sterile polyester sponges (35 × 15 mm diameter), impregnated with steroids at doses found to inhibit cotton pellet granuloma formation by 50%, were soaked in 0·9% (w/v) sterile saline and implanted subcutaneously into Sprague-Dawley rats (200–250 g). The rats were killed 5 and 24 h later, the sponges were removed and the inflammatory exudate carefully squeezed into polypropylene tubes containing acetylsalicylic acid (2 × 10 mol litre^{-1} final concentration). After centrifugation for 8 min at 1000*g*, the cell-free exudate was stored in aliquots for subsequent analysis. The cell button, together with that obtained after washing the sponge with 4 ml of saline containing 15 u ml^{-1} heparin, was resuspended in saline and the number of leucocytes determined using a haemocytometer; in some cases a differential count was also performed after staining with Wright's stain.

Analysis of sponge-induced inflammatory exudate

The levels of 6-keto $PGF_{1\alpha}$ and 13,14-dihydro-15-keto PGE_2, the metabolites of PGI_2 and PGE_2, in the cell-free exudate supernatant (diluted 1 : 4) were measured by radioimmunoassay using the protocols and specific assay systems supplied by Amersham Corporation, Arlington Heights, IL. These samples were then counted in 15 ml of Aquasol II (New England Nuclear, Boston, MA).

Elastinolytic activity was measured as described by Saklatvala (1977). Briefly, 0·5 ml of exudate and 0·25 ml of 0·1 M Tris-HCl buffer (pH 8·0) with 5 mM calcium chloride were incubated for 4 h at 37 °C with 0·75 ml of 2 mM succinyl-L-alanyl-L-alanyl-L-alanine-4-nitroanilide (SLAPN, Sigma Chemical Co., St Louis, MO). Following incubation, 0·5 ml of 12% trichloracetic acid was added, the precipitate removed by centrifugation (10 000*g* for 10 min) and 0·1 ml of 1·84 M NaOH added to 1 ml of the supernatant. The absorbance at 410 nm was read against control samples to which substrate was added at the end of the incubation. Enzyme activity was expressed as nmol of substrate hydrolysed h^{-1} ml^{-1} of exudate.

In some experiments aryl sulphatase activity was determined according to Worwood et al (1973) by incubating 0·5 ml 20 mM nitrocatechol sulphate in 0·5 M sodium acetate-acetic acid buffer (pH 5·6) with 0·5 ml of exudate for 30 min at 37 °C. The reaction was terminated by addition of 2 ml of 1 M NaOH and absorbance read at 515 nm. Results are expressed as nmol substrate broken down min^{-1} ml^{-1} of exudate. The activity of collagenase and acid phosphatase in the exudate was determined using assay kits supplied by New England Nuclear and Sigma.

Data were analysed for significance using Student's *t*-test and ID50 values calculated using linear regression analysis.

RESULTS

A summary of the data obtained using the cotton pellet granuloma bioasssay is given in Table 1. The order of anti-inflammatory potency (ID50) of the compounds was prednisolone (0·5 mg/pellet) > methyl 17,20α-acetonidodihydroprednisolonate (1·0 mg/pellet) > methyl 20β-dihydroprednisolonate (1·3 mg/pellet) > methyl 20α-dihydroprednisolonate (5·8 mg/pellet) > methyl 17,20β-acetonido-dihydroprednisolonate (6·0 mg/pellet). Table 1 also illustrates the effects on relative thymus weight and plasma corticosterone at an equipotent dose (ID50) of each compound. Prednisolone significantly decreased relative thymus weight and plasma corticosterone, none of the other steroids did so.

Table 1. Potency of steroid derivatives in the cotton pellet bioassay and their effects on relative thymus weight and plasma corticosterone.

Treatment[a]	Calculated ID50 (mg/pellet)	Relative thymus weight (mg/100 g body wt)	Plasma corticosterone (μg/10 ml)
Control	—	247·6 ± 14·9[b]	19·1 ± 3·1
P	0·5	162·4 ± 19·9**	8·7 ± 1·3*
P4α	5·8	274·1 ± 22·5	20·6 ± 1·9
P4β	1·2	236·4 ± 16·9	22·8 ± 2·6
P4α-a	1·0	237·6 ± 23·8	17·6 ± 1·4
P4β-a	6·0	260·6 ± 18·1	15·9 ± 2·5

[a] The steroids (see Fig. 1 for abbreviations) were impregnated in the pellets at the indicated ID50 dose.
[b] Data represent the mean ± s.e. of 5 animals. The significance was assessed by Student's *t*-test, where $*P < 0·02$, $**P < 0·005$.

The effect of equipotent doses of the various steroids on the infiltration of neutrophils into saline-soaked sponges was studied. Five hours after sponge implantation, prednisolone and both epimers of acetonide derivatives of methyl 20ξ-dihydroprednisolonate significantly reduced the number of leucocytes in the exudate, whereas 24 h after implantation only methyl 17,20α-acetonidodihydroprednisolonate and prednisolone significantly reduced leucocyte infiltration (Table 2). The migrating leucocytes were almost exclusively neutrophils (92%).

Table 2. Infiltration of neutrophils into inflammatory exudate induced by the s.c. implantation of polyester sponges soaked in 0·9% saline 5 or 24 h previously. For steroid nomenclature and abbreviations see Fig. 1. Data and the mean ± s.e. of 5 animals. The significances, assessed using Student's *t*-test, where $*P < 0·05$, $**P < 0·025$, $***P < 0·005$.

Treatment (mg/sponge)	Leucocyte count × 10^2 ml^{-1} exudate 5 h	24 h
Control	3·0 ± 0·6	6·9 ± 1·0
P(0·5)	1·5 ± 0·3*	1·8 ± 0·4***
P4α(5·8)	1·9 ± 0·4	6·0 ± 1·5
P4β(1·2)	1·9 ± 0·2	6·4 ± 1·3
P4α-a(1·0)	1·4 ± 0·2*	2·3 ± 0·5***
P4β-a(6·0)	1·2 ± 0·2**	6·7 ± 1·3

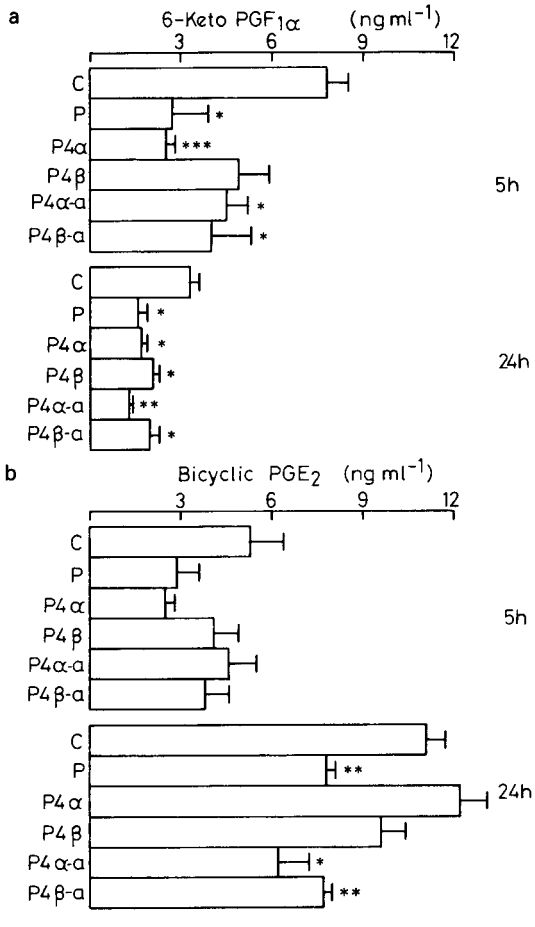

FIG. 2. Effect of equipotent doses (see Table 1) of prednisolone and its derivatives on the concentration of 6-keto $PGF_{1\alpha}$ (a) and PGE_2 (b) in inflammatory exudate obtained 5 and 24 h after s.c. implantation of steroid impregnated saline-soaked polyester sponges. For steroid nomenclature and abbreviations see Fig. 1. Each column is the mean ± s.e. (n = 5) where, $*P < 0·05$, $**P < 0·005$, $***P < 0·001$.

The inflammatory exudate collected from these steroid-impregnated polyester sponges were analysed for its 6-keto $PGF_{1\alpha}$ and PGE_2 content (Fig. 2). Five hours after implantation, the level of 6-keto $PGF_{1\alpha}$ in the exudate was significantly suppressed by all of the steroids except methyl 20α-dihydroprednisolonate, the levels of PGE_2 at this time were not significantly affected. Twenty-four hours after sponge implantation all of the steroids significantly depressed 6-keto $PFG_{1\alpha}$ levels in the exudate. Methyl 17,20α-acetonidodihydroprednisolonate was significantly better than its β-epimer in this respect (Student's *t*-test; $P < 0·05$). The effect on PGE_2 levels was slightly different with prednisolone and both acetonide derivatives significantly reducing the levels while methyl 20α- and 20β-dihydroprednisolonate had no significant effect.

All of the steroids tested significantly depressed the levels of enzyme activity within the 24 h exudate, with the acetonide derivatives being the most effective. No elastase activity was detected in the 5 h exudates (Fig. 3).

Further experiments demonstrated that the steroids did not significantly change the levels of collagenase or acid phosphatase activities in either 5 or 24 h exudates (data not shown). However, when the level of aryl sulphatase activity in the exudates was measured (Table 3) prednisolone significantly reduced this, but only by a small margin (13–19%) while the derivatives of prednisolone had no effect.

Table 3. Level of aryl sulphatase activity in inflammatory exudate induced by the s.c. implantation of saline-soaked polyester sponges 5 or 24 h previously. Data is the mean ± s.e. of 5 observations. The significance assessed by Student's *t*-test was $*P < 0·025$. For steroid nomenclature and abbreviations see legend to Fig. 1.

Treatment	Aryl sulphatase activity (nmol min^{-1} ml^{-1} exudate) 5 h	24 h
Control	4·1 ± 0·1	5·2 ± 0·1
P(0·5)	3·3 ± 0·2*	4·5 ± 0 2*
P4α(5·8)	4·3 ± 0 2	4·8 ± 0 2
P4β(1·2)	4·4 ± 0·2	5·0 ± 0·1
P4α-a 1·0)	4·6 ± 0·2	4·8 ± 0·3
P4β-a(6·0)	4·4 ± 0·1	5·4 ± 0·2

DISCUSSION

From the results obtained, prednisolone was twice as active as methyl 20β-dihydroprednisolonate, and methyl 17,20α-acetonidodihydroprednisolonate and ten times as active as methyl 20α-dihydroprednisolonate and methyl 17,20β-aceto-

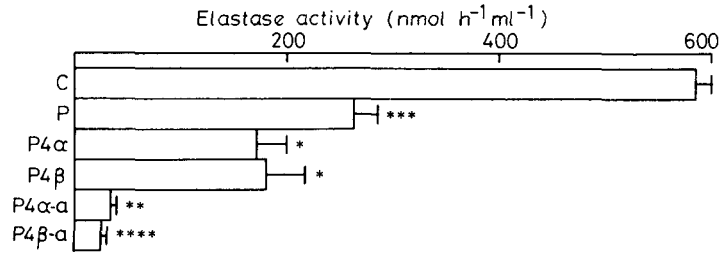

FIG. 3. Effect of equipotent doses (see Table 1) of prednisolone and its derivatives on the level of elastase activity in inflammatory exudate obtained 24 h after s.c. implantation of steroid-impregnated saline-soaked polyester sponges. For steroid nomenclature and abbreviations see Fig. 1. Each column is the mean ± s.e. (n = 5) where $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}025$, $^{***}P < 0{\cdot}005$, $^{****}P < 0{\cdot}001$.

nidodihydroprednisolonate using the cotton pellet granuloma bioassay. These data serve to illustrate the importance of the stereochemical configuration of these compounds to biological potency. This phenomenon was previously observed by Ryrfeldt et al (1984) using the asymmetric acetonide budesonide and was stated to be an important factor also in the activity of ester derivatives of steroid 21-oic acids (Lee et al 1984). Administration of the compounds used in this study at doses which inhibited granuloma formation by 50% (ID50) demonstrated that only prednisolone significantly depressed relative thymus weight and plasma corticosterone. Thus, these new compounds have significantly fewer systemic effects than their parent prednisolone. It has been suggested previously (Lee & Trottier 1980; Lee & Soliman 1982) that the absence of systemic activity of steroids of this class was due to their rapid hydrolysis to inactive metabolites.

The effect of equipotent doses of these steroids on the infiltration of neutrophils into saline-soaked polyester sponges was investigated. Five hours after implantation, only the two acetonide derivatives significantly reduced the numbers of leucocytes in the sponges, while 24 h after implantation both prednisolone and methyl 17.20α-acetonidodihydroprednisolonate significantly reduced cell infiltration. Whether or not steroids affect neutrophil migration has been a matter of controversy for over a decade. Some studies have shown that normal pharmacological concentrations of glucocorticoids have little or no effect when using in-vitro chemotactic chambers (Mowat & Baum 1971; Majeski & Alexander 1976). However, cell migration is the result of many different processes including adhesion and cell movement, and steroids have been shown to decrease neutrophil adherence (Clark et al 1979). In this investigation only two of the steroids affected cell migration (prednisolone, methyl 17,20α-acetonidodihydroprednisolonate) while the others had no effect. This divergence of activity has been observed with other steroids, for example, prednisone decreased cell infiltration while dexamethasone increased it (Peters et al 1972).

Another anti-inflammatory action of corticosteroids is their ability to inhibit the production of prostaglandins. The novel steroids described herein decreased, to varying degrees, the liberation of 6-keto $PGF_{1\alpha}$ and PGE_2 into inflammatory exudate. This is in accord with the proposed mechanism of action of glucocorticoids which are indirectly able to inhibit phospholipase A_2 and thereby prevent the biosynthesis of a number of pro-inflammatory mediators including prostaglandins, thromboxanes and leukotrienes (Nijkamp et al 1976; Flower & Blackwell 1979; Blackwell 1983).

In addition to inhibiting the release of prostaglandins into the inflammatory exudate, prednisolone and its derivatives inhibited the release of elastase. It has been frequently postulated that tissue destruction in such chronic disorders such as arthritis and emphysema may be caused by lysosomal proteinases including elastase released from neutrophils (Werb et al 1982; Saklatvala 1977). Thus, the inhibition of lysosomal enzyme release has been suggested as a possible mechanism of action of corticosteroids (Weissmann 1973; Goldstein 1975). These in-vivo results confirm those obtained using an in-vitro system and methyl 20ξ-dihydroprednisolonate (Heiman & Lee 1981).

This study has compared the effect of prednisolone derivatives on granuloma formation seven days post-implantation, and on neutrophil infiltration and prostanoid/enzyme liberation 5 and 24 h post-implantation. This time difference may account for some of the differences found between compounds since based on their in-vitro hydrolysis rates (Kumari & Lee 1985) we would expect a disparate rate of metabolism.

In conclusion, this study has demonstrated that

esters of prednisolonic acid when used at a dose which inhibits granuloma formation to the same extent, do not cause thymus involution or depress plasma corticosterone levels. Thus, use of these new steroids either locally or topically would have significant advantages over conventional steroids such as prednisolone particularly during chronic administration. The anti-inflammatory action of these novel prednisolone derivatives would appear to be substantially similar to their parent compound and other glucocorticoids since these new steroids can suppress prostaglandin release, liberation of lysosomal enzymes and to some degree cell migration. Therefore, these new steroids may prove to be more beneficial in the treatment of topical and local conditions than those currently in use.

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