A Flow Injection Chemiluminescence Method for the Determination of Retinol in Pharmaceutical Formulations by Using Luminol-Diperiodatoargentate(III) Reaction

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Summary In this paper, a chemiluminescence (CL) method is proposed for retinol determination by combining flow injection (FI) methodology. The CL reaction is based on the oxidation of luminol by diperiodatoargentate(III) (DPA) in the presence of retinol. Under the optimum conditions, the relative CL intensity was linear to the concentration of retinol over the range $5.0 \times 10^{-3}$–$14$ mg L$^{-1}$ ($y=347.26x+2.5944$, $R^2=0.9999$, $n=8$) with limit of detection (LOD) of $1.5 \times 10^{-3}$ mg L$^{-1}$ ($S/N=3$) and limit of quantification (LOQ) of $5.0 \times 10^{-3}$ mg L$^{-1}$ ($S/N=10$). The relative standard deviation (RSD) was from 1.04–3.4% over the range studied and injection throughputs of $150$ h$^{-1}$. The method was satisfactorily applied to retinol in pharmaceutical formulation samples. The samples were saponified and extracted with liquid-liquid extraction using ether as an extractant. The possible CL mechanism is supported by CL and UV-visible spectrophotometric studies.

Key Words vitamin A, analytical applications, CL mechanism, sensitive method, liquid-liquid extraction

Vitamins are organic compounds that are essential in very small amounts for the human body’s usual and normal functions (1). The diet supply containing essential vitamins can have decreased vitamins content after processing, cooking or storage. Therefore, people take multivitamin pharmaceutical formulations; consume vitamin-fortified beverages and milk powder to supplement their diet (2). In 1920, the name vitamin A (VA) was first given while in 1931, its chemical structure was firmly resolved. VA is a group of fat-soluble vitamins, belongs to retinoids which is a sub-class of compounds and most commonly found in animal origin foods. The most useable form of VA is the retinol also known as preformed VA which is easily metabolized in human. In liver tissues, about 90% VA is stored in the form of retinyl ester, which is also accumulated in fat tissues, eyes, kidneys and lungs. Illness and stress decrease the storage of VA unless the intake amount is increased (3).

VA is recognized for its fundamental role in eyesight and contributes in physiological activities associated to the bone metabolisms, brain development, reproduction, embryogenesis, spermatogenesis, gene transcription and cell differentiation (4, 5). It is imperative for eyesight, very essential for growth and an important integral part of rhodopsin (6). VA reduces cardiovascular, cancer and other diseases in high dietary intakes (7). Further, enough quantity of VA and retinoic acid are required for cell differentiation which are considered to increase white blood cells function, and antiviral activity of antibodies to antigens, to sustain lungs normal structure and function as well as the tissues of gastro-intestinal tract and skin of trachea (8). Retinol and retinoic acid play essential role in organogenesis. The former one is necessary for embryonic tissues to be developed properly and the latter one controls all steps of organogenesis (9). Specific promoting genes involved in the development of brain, liver, kidney and laryngeal can be switched on and off by VA, showing the requirement of VA during the development of these organs (10). Retinoids are the synthetic and natural derivatives of VA, which are employed clinically for the treatment of cancer and different types of skin diseases. VA is found to be involved in protein metabolism and in immunoglobulin; low quantities of IgG and IgM have been reported with low VA intake (11).

Numerous analytical approaches have been utilized/ reviewed for VA carotenoids analysis; these include flow-injection (FI)/flow based and manual colorimetry/spectrophotometry (12–14), fluorimetry (15), voltam-
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Flow injection manifold for the determination of retinol. Carrier: ethanol (0.1% v/v), R-I: DPA, R-II: luminol, sample loop volume: 240 μL, PMT (photomultiplier tube).

Chemiluminescence (CL) is the light production due to a chemical reaction and the attractive features of CL analytical methods are simple and low-priced equipment, high sample throughput and sensitivity, ruggedness and robustness, which made it enabled for the quantitative analysis of many analytes in different fields (21, 22). Luminol is one of the most widely employed CL reagents (23) and for its oxidation many oxidants such as MnO₄⁻, OCl⁻, IO₄⁻ and H₂O₂ have been employed. As a result of oxidation, the electronically excited product of luminol namely 3-aminophthalate is generated which luminesces blue light with a λmax of 425 nm. Various other stabilized complexes of transition metals in which the central metals exist in higher uncommon oxidation states such as diperiodatonicelate(IV) (Ni⁴⁺ complex, DPN), diperiodatocuprate(III) (Cu³⁺ complex, DPC) and diperiodatargentate(III) (Ag⁺⁺ complex, DPA) have found numerous analytical applications with and without employing CL reagents in either acidic or basic conditions. In flow-mode, DPA coupled with luminol CL reaction has many applications for the determination of different analytes in several samples (21, 24). Several flow injection-CL (FI-CL) and FI-UV/Vis-spectrophotometric methods have been described in the literature for the quantitative measurements of VA (retinol) in different matrices (12, 13, 25–31). The analytical figures of merit, optimum experimental parameters and their applications for all these methods have been described in Table 1.

In this work, a FI-CL method is proposed to determine retinol in pharmaceutical formulations based on its enhancement effect on luminol-DPA CL reaction with a good linearity and a detection limit (S/N= 3) of 1.5 × 10⁻⁷ mg L⁻¹. Retinol from formulations was extracted with ether after saponification with satisfactory results. The proposed CL emission mechanism has been thoroughly explored and discussed.

**EXPERIMENTAL**

Reagents and solution. All reagents used were of analytical-grade reagents and deionized water (Elga, Purelab Option, High Wycombe, Bucks, UK) was used for solution preparations. The retinol stock solution (1.0 g L⁻¹) was prepared by dissolving 10 mg of retinol from its bulk in 10 mL absolute ethanol, sonicated for 10 min and stored in brown bottle at 4˚C. The DPA reagent was synthesized according to the previously reported method (32).

Flow injection manifold. The schematic FI-CL manifold used for retinol assay is shown in Fig. 1. Polytetrafluoroethylene (PTFE) tubings (0.8 mm i.d., Fischer Scientific, Loughborough, UK) were used for connecting all components of FIA manifold. A peristaltic pump (Ismatec, Glattburg-zueich, Switzerland) was used to deliver all solutions at a flow rate of 3.5 mL min⁻¹. Retinol standard/sample solutions (240 μL) were injected via Rheodyne 5020 injection valve (Anachem, Luton, UK) into ethanol 0.1% (v/v) solution used as sample carrier stream which was combined at a T-piece with DPA solution. The mixed stream was then merged with luminol CL reagent and allowed to travel and permits to pass via glass spiral flow cell (1.5 i.d., 18 mm dia) fitted directly in-front of an end window photomultiplier tube (PMT, 9789B, Electron Tubes Ltd., Ruislip, UK) attached with a power supply (Electron Tubes Ltd., PM20SN) operated at 800 V. The CL-intensity was recorded using a strip chart recorder (Kipp & Zonen, BD40, delft, Holland).

Preparation of sample. VA pharmaceutical formulations were collected from local market and analyzed by the proposed and reported (27) FI-CL methods. Most of the samples contain VA in the form of retinyl palmitate and was saponified before analysis. For saponification, known quantity of the sample was added in methanol (3.0 mL) followed by addition of 3.0 mL KOH (60%
The vial containing saponification mixture was protected from light by wrapping with aluminum foil and then incubated for 2 h at 45–50˚C with intermittent mixing and purging of nitrogen gas. The samples were extracted by using liquid-liquid extraction with ether (5.0×5.0 mL). After vigorous shaking, ether portions were recovered and dried under nitrogen stream at 35˚C. The residues were re-dissolved in absolute ethanol and after appropriate dilution and spiking of the analyte were determined by both methods.

RESULTS AND DISCUSSION

Kinetic curve of the CL reaction

Before accomplishing the flow injection procedure, the kinetic characteristics of the proposed CL reaction were studied by static injection mode. Initially, luminol solution (0.5 mL of 0.001 mM prepared in NaOH 0.1 mM) was introduced into the quartz cuvette followed by injection 1.0 mL of DPA (0.2 mM in KOH 1.0 mM) and retinol (0.35 mg L⁻¹) mixture and a significant CL signal was detected in the form of a transient peak recorded on a strip chart recorder shown in Fig. 2. After mixing of the reactants, the CL intensity reached within 0.95 s to its maximum and the CL signal returned to baseline within 3.4 s. These results revealed that retinol had an enhancing effect on the weak CL of luminol-DPA system and the assay of retinol could be achieved in pharmaceutical formulations by combining with flow injection technique.

Optimization of experimental variables

In order to achieve high sensitivity and good analytical performance, a series of univariate approaches were employed. The key variables optimized were luminol, DPA, NaOH, KOH and methanol concentrations, flow rate, sample volume and PMT voltage were investigated. Retinol standard solution (0.1 mg L⁻¹) was used during optimization studies and all the measurements were made in triplicate.

Luminol is one of the most commonly used CL reagents...
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for its oxidation in alkaline condition. The effect of luminol concentration on the CL intensity was examined up to 0.003 mM. The CL emission intensity and background CL both increased with an increase in luminol concentration. As shown in Fig. 3A, the increase was very steep up to 0.001 mM and was then selected as an optimum. Beyond this concentration a slight increase in CL signal intensity but an abrupt increase in background CL was observed. Based on signal to noise ratio, luminol concentration of 0.001 mM was also evaluated as an optimum and used for subsequent experiments.

Luminol always shows efficient CL emission in basic medium and therefore the effect of NaOH concentration on the CL intensity was inevitable to be investigated. The NaOH concentration was examined over the range of 0.01–0.3 M. The CL intensity increased with the increase in NaOH concentration, but the optimum CL intensity with good reproducibility was observed at 0.1 M as illustrated in Fig. 3B and was selected as an optimum for further studies.

DPA is a powerful oxidizing agent in alkaline medium with reduction potential of 1.74 V due to strong versatile nature of the two-electron oxidation. DPA was used as an efficient oxidant in this CL reaction and revealed a great effect on the CL intensity; therefore, the effect of DPA concentration was examined from 0.001–0.4 mM and the maximum and most reproducible CL signals were obtained at when its concentration reached up to 0.2 mM above which the CL intensity decreased due to self-absorption as shown in Fig. 3C. Therefore, the DPA concentration of 0.2 mM was used subsequently.

It is well known that DPA is stable in basic medium. Therefore, the effect of KOH concentration was examined from 0.5–2.5 mM and the maximum and reproducible CL signals were observed at KOH concentration of 1.0 mM as shown in Fig. 3D and therefore was selected as an optimum for further studies.

Retinol is a fat-soluble vitamin and is more soluble in organic solvents such as ethanol, methanol, chloroform, propanol, butanol etc. The stock solution of retinol was prepared in absolute ethanol. Therefore, effect of ethanol concentration, using as a sample carrier stream to increase the retinol solubility and as well as for matrix match, was examined over the range of 0.01–5.0% (v/v). The increase in CL intensity with the increase in ethanol percentage up to 0.1% (v/v) was observed due to increase in solubility and therefore ethanol concentration of 0.1% (v/v) was used as an optimal subsequently.

The effect of physical parameters such as flow rates, sample injection volume and PMT voltage was investigated on the CL intensity of retinol. As kinetic studies showed that the proposed CL reaction is fast and therefore the effect of flow rate was investigated from 0.5–4.0 mL min⁻¹. The CL intensity increased as the flow rate increased up to 3.5 mL min⁻¹ and therefore was selected as an optimal. Sample injection loop volume was investigated from 60–360 µL, the CL intensity increased with the increase in sample loop volume linearly up to 300 µL but 240 µL was opted for subsequent experiment due to economy of sample consumption as well as to increase linear dynamic range. The intensity increased with increase in PMT voltage from 700 to 1,300 V, but 800 V was selected as an optimal due to high signal to noise ratio.

Analytical characteristics

Under the optimized parameters, a linear calibration curve was obtained between the concentration of retinol (mg L⁻¹, taken at abscissa) and CL intensity (mV, taken on ordinate) over the range of 0.005–14 mg L⁻¹ (R²=0.9999, n=8). Figure 4 shows the chart recorder traces for retinol and in the inset is the standard curve. The limit of detection (LOD) and limit of quantification (LOQ) of 0.0015 and 0.005 mg L⁻¹ were calculated as the amount of retinol required to yield CL signals three and ten times of signal to noise ratio respectively. The regression equation was \( y = 347.26x + 2.5944 \), where \( y \) is the CL intensity in mV and \( x \) is the retinol concentration in mg L⁻¹ and the %RSDs over the range were obtained as 0.94–2.85. The injection throughput was 150 h⁻¹.

Table 1 reports the comparison of analytical performance of the proposed method with previously reported methods for the assay of VA in different matrices. Among FI-CL methods reported in the literature (25–31), the proposed method has low detection limit, wide linearity and high injection throughput except lucigenin-Brij-35-CL (27), tris(2,2'-bipyridyl)Ru(II)-Ce(IV)-CL (25) and Ce(IV)-Na₂SO₄-CL (29) methods respectively and in most of these methods, high concentration of CL reagents and PMT voltage have been employed comparatively.

Effects of coexisting foreign species

Under the selected optimal experimental conditions, the effect of commonly found excipients in VA pharmaceuticals, possibly coexisting species inorganic ions was examined on the blank (in the absence of retinol) and on
| Detection technique | Samples | Chemical parameters | Physical parameters | LOD (mg/L) | Linear range (mg/L) | $R^2$ | Calibration equation | Injection throughput (h) | Ref. |
|---------------------|---------|---------------------|---------------------|------------|---------------------|-------|----------------------|-------------------------|-----|
| FIA-CL              | Pharmaceutical formulations | Luminol (1 μM in NaOH 0.1 M; DPA, 0.2 mM in KOH 1.0 M; ethanol 0.1% (v/v)) | PMT voltage 800 V; sample volume 240 μL; flow rate 3.5 mL/min | 0.0015 0.005–14 | 0.9999 | $y = 347.26x + 2.5944$ | 150 | This method |
| FIA-CL              | Pharmaceutical formulations | Tris(2,2'-bipyridyl)Ru(II) 1 × 10^{-5} M; Ce(IV) 1 × 10^{-3} M; C_{2}H_{4}OH 5%; H_{2}SO_{4} 0.04 M | PMT voltage 850 V; sample volume 120 μL; flow rate 1.8 mL/min; mixing coil 100 cm | 0.023 0.286–28.645 | 0.9991 | $l = 3.159c + 3.5093$ | 100 | 25) |
| FIA-CL              | Pharmaceutical preparations | KMnO_{4} 2.5 × 10^{-5} M; HCHO 0.5 M; C_{2}H_{5}OH 5%; H_{2}SO_{4} 0.04 M | Sample volume 120 μL; PMT voltage 900 V; flow rate 2.0 mL/min | 0.0016 0.0143–1.43 | 0.9991 | $l = 4.0579c + 1.002$ | 100 | 26) |
| FIA-CL              | Pharmaceutical formulations | Luminol 5 × 10^{-5} M; borate buffer pH 11, 0.1 M; SDS 0.2%; V(V) 1 × 10^{-9} M | Sample volume 300 μL; PMT voltage 1,000 V; flow rate 2.3 mL/min | 0.023 0.03–2.85 | 0.9989 | $l = 0.117c + 2.54$ | 20 | 28) |
| FIA-CL              | Milk and pharmaceutical formulations | Ce(IV) 5 × 10^{-5} M; Na_{2}SO_{3} 1 × 10^{-3} M; H_{2}SO_{4} 0.1 M; CH_{3}OH 0.5% | Sample volume 180 μL; PMT voltage 1,300 V; flow rate 4 mL/min | 0.01 0.1–8.0 | 0.9986 | NR | 180 | 29) |
| FIA-CL              | Milk | Luminol 1 × 10^{-5} M; IO_{4} 2.5 × 10^{-4} M; borate buffer pH 12, 0.1 M; CH_{3}OH 0.5% | Sample volume 120 μL; PMT voltage 9.50 V; flow rate 2.0 mL/min | 0.023 0.0286–2.86 | 0.9996 | $l = 7.3885c + 13.102$ | 90 | 30) |
| FIA-CL              | Pharmaceutical formulations | Rose bengal 1 × 10^{-5} M; Cu^{2+} 5 × 10^{-5} M; luminol 5 × 10^{-5} M; NaOH 0.125 M; pyrophosphate buffer pH 10.25, 0.01 M | Sample volume 60 μL; PMT voltage 1,000 V; flow rate 2.0 mL/min; photoreactor coil length 200 cm; UV lamp 4 W, 254 nm | 0.008 0.05–15 | NR | $l = 67.88c + 2.62$ | 70 | 31) |
| FIA-Spec            | Pharmaceuticals | Glycine buffer 0.05 M, pH 11; NAD^{+} 1 × 10^{-1} M | Flow rate 0.8 mL/min; sample volume 60 μL; immobilized enzyme column length 2 × 80 mm; temperature 37˚C; λ_{max} 340 nm | ≤0.286 0.573–2.865 | 0.9967 | $y = 0.003x - 0.0004$ | 25 | 12) |
| FIA-Spec            | Aqueous solution | Iodine 20 mg/L | Flow rate 1.08 and 1.46 mL/min; sample volume 100 μL; reaction coil 30 cm; λ_{max} 226 nm | 0.053 0.1–50 | 0.999 | $y = 2.242x + 0.096$ | 30 | 13) |
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![Graph A](image1.png)

![Graph B](image2.png)

Fig. 5. Interference activities of (A) cations and anion and (B) organic chemical species at 0.1, 1, 10 and 100-fold of retinol 0.1 mg L\(^{-1}\). 1, retinol; 2, glucose; 3, cholesterol; 4, tocopherol; 5, vitamin K\(_2\); 6, retinyl ester; 7, retinyl palmitate; 8, starch; 9, sucrose; 10, methyl cellulose; 11, vitamin C; 12, glycerol; 13, vitamin D\(_3\); 14, stearic acid; 15, gum acacia; 16, mg-stearate.

Table 2. Retinol recovery from pharmaceutical capsules and its comparison with a reported method.

| Sample matrix | Spiked (mg L\(^{-1}\)) | Proposed FI-CL method | Reported FI-CL method (27) |
|---------------|------------------------|------------------------|---------------------------|
|               | Found (mg L\(^{-1}\)) | Recovery (%) | RSD (%) (n=4) | Found (mg L\(^{-1}\)) | Recovery (%) | RSD (%) (n=4) |
| Tablet-I      | 0.000                  | 0.310       | —                | 0.285                  | —            | 3.1            |
|               | 0.200                  | 0.491       | 96.3             | 0.530                  | 109          | 2.8            |
|               | 0.400                  | 0.743       | 105              | 0.690                  | 101          | 1.7            |
|               | 0.600                  | 0.885       | 101              | 0.942                  | 106          | 1.5            |
| Tablet-II     | 0.000                  | 0.292       | —                | 0.293                  | —            | 2.2            |
|               | 0.200                  | 0.534       | 108              | 0.470                  | 95.3         | 1.9            |
|               | 0.400                  | 0.763       | 110              | 0.730                  | 105          | 1.7            |
| Injection     | 0.000                  | 0.325       | —                | 0.321                  | —            | 2.3            |
|               | 0.200                  | 0.552       | 105              | 0.490                  | 94.0         | 2.1            |
|               | 0.400                  | 0.730       | 101              | 0.742                  | 103          | 1.9            |
|               | 0.600                  | 0.892       | 96.4             | 0.878                  | 95.3         | 1.8            |

Student t-test value: \(t=0.735\), \(t\)-distributed (95\%) = 2.20.
the determination of retinol (0.1 mg L⁻¹). The tolerance of each foreign species was taken as the largest concentration giving less than ±5% of the error of the adaptive concentration of retinol. Figure 5A and B shows the interference activity of 0.1, 1.0, 10 and 100 folds of inorganic ions and organic compounds on the determination of retinol (0.1 mg L⁻¹) respectively. The tolerable ratio for foreign species was 100-fold for K⁺, Na⁺, Mg²⁺, Cl⁻, CO₃²⁻, PO₄³⁻, SO₄²⁻, starch, methyl cellulose, retinyl ester, retinyl palmitate, glycerol, vitamin D₃, stearic acid, gum acacia, Mg-stearate sucrose, cholesterol and α-tocopherol which did not show any interference activity. Similarly, 10-fold for Zn²⁺ and Pb²⁺, 1-fold for Fe³⁺, Co²⁺, glucose and vitamin K₂ and 0.1-fold for Cu²⁺, Mn²⁺ and vitamin C did not show either any enhance or inhibition effect on the CL intensity of retinol determination as well as on the blank. However, Cr³⁺ <0.1-fold caused an enhance effect. The interference of chemical species as shown in Fig. 5A and B is essential in luminol based CL reactions because of their catalytic effect on the oxidation of luminol. Chromium(III) catalysed light emission from luminol oxidation by peroxide (33) which can be removed by adsorption on the surface of Fe₂O₃ or formed complex with organics (34) or by precipitation as Cr(OH)₃ (35). However, retinol from the samples was extracted with ether after saponification and thus neither metals nor water soluble organic species extracted into the ether phase during liquid-liquid extraction. Thus, the proposed method could be applied for the determination of retinol in pharmaceutical formulations.

**Analytical applications**

According to the proposed method, retinol in the pharmaceutical formulations was determined. As shown in Table 2, the %recoveries for the proposed FI-CL method and a reported FI-CL method (27) which were 96–110% (%RSD=1.5–3.5, n=4) and 94–110% (%RSD=1.5–3.1, n=4) respectively. For 0.5, 5.0 and 14.0 mg L⁻¹ retinol, the intraday and interday %RSDs were 3.4, 1.12 and 1.04, and 4.32, 2.21 and 1.91 respectively. In addition, Table 2 reports the results obtained for the determination of retinol in un-spiked and spiked pharmaceutical samples both by the proposed and the reported FI-CL methods (27). Paired Student t-test was applied on the results of both methods and tcalc was obtained as 0.735 which was lower than ttab 2.20 at 95% confidence level. Therefore, it can be concluded that the two methods were not different significantly.

**Mechanistic studies of the CL reaction**

For the CL reaction mechanism schemes between retinol and luminol-DPA in basic condition, batch UV-visible spectrophotometric and CL studies in flow mode were...
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performed. Figure 6A shows spectrophotometric studies in which the absorbance intensity of alkaline DPA was progressively reduced by retinol with time (curve B–E). The colour of DPA almost disappeared after 12 min of retinol addition into reaction cell. The phenomenon can be interpreted that retinol undergoes through a redox reaction with DPA, in which retinol acts as a reductant and DPA as an oxidant. Figure 6B shows the transient peaks for the CL reaction of luminol-DPA-retinol under flow-mode. Luminol in the absence of DPA or retinol did not emit any CL light (curve A), but in the presence of DPA emitted strong CL (curve B), which was further enhanced by retinol (curve C).

The anionic complex of Ag(III) i.e. [Ag(H2IO3) (OH)]+ has been widely employed in conjunction with luminol CL for the determination of various analytes (36–39). The CL intensity of luminol-DPA reaction was appreciably increased by the reducing activity of retinol. The retinol acts as a reducing agent and undergoes through a redox reaction with DPA. As a result, Ag in lower oxidation state, which may be Ag(I) or Ag(II), generated. The generated Ag(I) or Ag(II) can react with dissolved oxygen producing superoxide anion or hydroxyl radicals (40). These radicals could further oxidize the luminol molecules into electronically excited state which thus produced enhanced CL signals. The intensity of which is in direct relation with retinol concentration. Excited 3-aminophthalate is the oxidized product of luminol, which on de-excitation emits light centered at 425 nm (38). Based on the above experiments and discussion, the possible CL reaction could be written as Formula 1.

CONCLUSIONS

In this study, a sensitive FI-CL method for the determination of retinol was proposed based on the enhancement effect of retinol in the oxidation reaction of DPA with luminol. The method developed is an attractive procedure due to its reasonable sensitivity, wider linear ranges and lower LOD. Retinol from the samples was extracted with ether after saponification and thus neither metal nor water soluble organic species can be extracted into the ether phase during liquid-liquid extraction and their interfering activity can be ignored. The main advantage of the proposed FI-CL method is the consumption of less amount of CL reagent and wide linearity in comparison to the previously reported FI-CL methods. In addition, the sample throughputs and reproducibility (%RSD) are also better than most of the reported methods.

Disclosure of state of COI
No conflicts of interest to be declared.

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