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On the precise estimation of R_M values in reversed-phase thin-layer chromatography including aspects of pH dependence

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ABSTRACT

In order to improve the application of reversed-phase thin-layer chromatography (RP-TLC) for the chromatographic estimation of drug lipophilicity, some aspects of measuring true R_M values [log $(1/R_F - 1)$] are considered in the present investigation:

(1) An optimization of experimental conditions, including the importance of temperature and humidity, as well as densitometric evaluation of spots is presented.

(2) The estimation of thermodynamically true R_M values is described; it is shown that in case of high modifier contents preloading effects induce pronounced deviations of R_F from R_F' values. Only the latter allow the calculation of true R_M values.

(3) The influence of solvent pH on R_M values is negligible for pure partition chromatography in the case of low modifier contents; with increasing modifier contents polar adsorption becomes more prominent; under these conditions an influence of pH on R_M in the case of strong bases is detected.

INTRODUCTION

Chromatographic estimation of drug lipophilicity is predominantly undertaken by means of RP-18 HPLC, while reversed-phase TLC is less frequently used. This reduced acceptance of the latter [1] may be because of improperly designed experimental protocols. To the authors' knowledge, investigators almost always neglect relevant experimental factors such as temperature and humidity despite their well-known importance for chromatographic behaviour [2]. Most often running distances are measured "by hand" under UV light and corresponding R_F values —related to the observed visible front— are used to calculate R_M values. In the present investigation densitometric estimation of spot positioning is used, as also applied by Dingenen and Pluym [3], and, in addition, a procedure for estimating the "thermodynamically true" front is presented for RP-18 TLC.

The pH dependence of chromatographic data in normal-phase TLC was demonstrated by Stahl and Dumont [4] a long time ago. It deserves mention here that Stahl and Dumont varied the pH of the stationary phase and not that of the solvent. Several authors assume that this pH dependence is equally valid in reversed-phase chromatography [2,5–7]. This is in contrast to

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reports [8–10] that indicate a lack of influence of solvent pH in RP-18 TLC on R_F or R_M in which pH varied between 2 and 11. In a recently published paper Dross *et al.* [11] reported on investigations using silanized silica gel plates, in which application of the commonly used pK correction [5] yielded poorly plausible results. Accordingly, it was concluded that no, or a different pH/pK correction seems necessary. In continuation of such experiments we investigated the pH dependence of "thermodynamically true" R_M values using RP-18 silica gel.

EXPERIMENTAL

For the chromatographic experiments described here, we used precoated TLC plates RP-18 F_{254S}, 10×20 cm in size, purchased from Merck (Darmstadt, Germany). A 0.5-μl volume of an ethanolic solution of the test compounds (1 mg/ml) was applied to the plates with the aid of a Nanomat II (Camag, Muttenz, Switzerland). Before use, plates were preconditioned by heating on a Thermoplate S (Desaga, Heidelberg, Germany) for 15 min at 120°C. The starting points of the test compounds were positioned 10 mm from the bottom edge of the plate and at least 25 mm from the side of the plate with 5 mm between. As front markers we used potassium bromide, potassium iodide and sodium nitrate; 50 mg of these front markers were dissolved in 10 ml of a water–ethanol mixture (25:75, v/v), 0.5 μl of which were applied to the plate in the middle and at the two lateral positions. The Nanomat II does not allow an exact positioning of starting points; accordingly they were exactly evaluated with the aid of a CD50 densitometer (Desaga, Heidelberg, Germany) in remission mode. Compounds were measured at appropriate wavelengths.

Running of the plates was performed in twin trough chambers (Camag) which were lined with blotting paper in order to guarantee a saturation of the gas phase. Chambers were placed in an incubator adjusted to 30°C; the incubator contained a water-filled glass to ensure constant humidity. In some cases horizontal sandwich chambers (Camag) were used.

As solvent we used methanol–buffer mixtures of varying composition. For preparation of Tris buffer pH 7.4, purest water from a Milli-Q plus water system (Millipore, Bedford, MA, USA) was used; in some cases commercially available buffers (pH 3, 10 and 12) from Riedel-de Haen (Seelze, Germany) were applied. Plates were run up to 1 cm below the upper end of the plate,

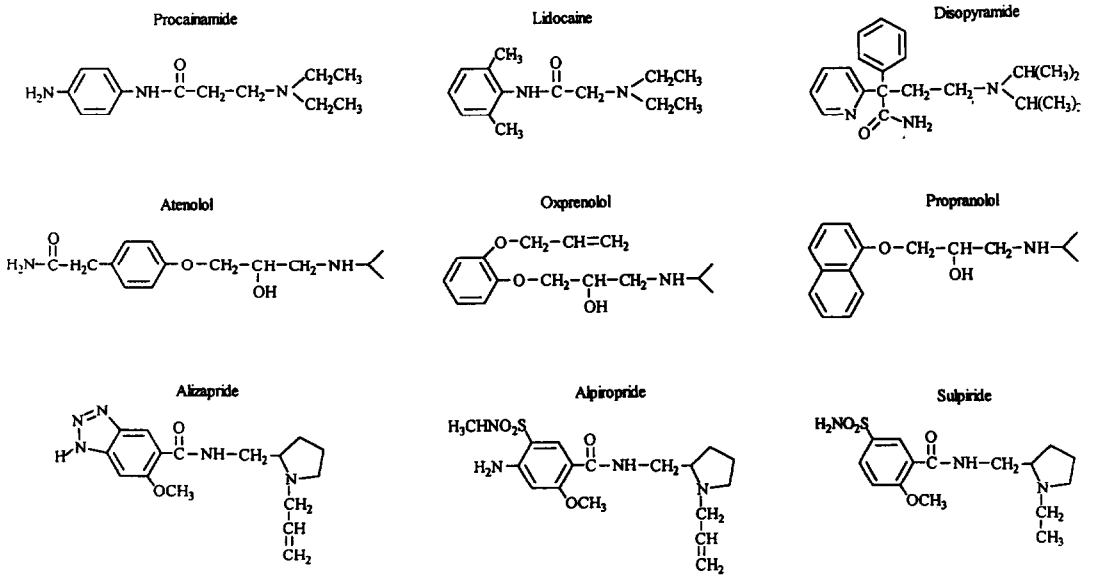

Fig. 1. Structural formulae of tested drug molecules.

which corresponds to a start–front distance of about 8 cm. After the run the plates were dried in a cold stream of air; afterwards the runs were evaluated densitometrically as described above.

Test compounds

Benzoic acid, 4-iodobenzoic acid, biphenyl and 2,6,2′,6′-tetramethylbenzophenone were kindly provided by R.F. Rekker (Department of Pharmacochemistry, Vrije Universiteit, Amsterdam, Netherlands). Resorcin, naphthoresorcin, diphenylamine and N-phenylnaphthylamine were obtained from Aldrich (Milwaukee, WI, USA). The following drugs were kindly supplied by pharmaceutical companies: procainamide, lidocaine, disopyramide, atenolol, oxprenolol, propranolol, alizapride, alpiropride and sulpiride. Their structural formulae are given in Fig. 1. All other chemical compounds, if not indicated otherwise, were analytical-reagent grade and were obtained from Merck.

RESULTS AND DISCUSSION

The importance of the densitometric evaluation of starting and running points

In the case of manual estimations of R_F values, some error (up to ±1 mm) is unavoidable; in contrast, a densitometer is accurate to about ±0.01 mm. Correspondingly, in the range of R_F values lower than 0.1 a significant improvement in the accuracy of R_M calculations is achieved. Nevertheless, this improvement is only valid if at the same time an exact estimation of the starting point position is performed densitometrically. For technical reasons, the Nanomat II is only adjustable to within about 0.5 mm; in addition, the support for the microcapillary pipette contributes position uncertainty of up to 0.3 mm (see Table I).

The experimental conditions described here prevent, in the case of low R_F values resulting in high R_M values, errors in calculating R_M of up to ±0.5.

The estimation of the "thermodynamically true" R_M

"Thermodynamically true" R_M values in TLC can only be obtained if it is possible to determine the thermodynamically true position of the front. It is known [2,12,13] that the visible front is not identical to the "true front". According to Geiss [2,12] two factors in particular have to be considered for correction in conventional TLC: the "preloading effect" and the "front gradient". The "preloading effect" causes the stationary phase to adsorb volatile mobile phase molecules

TABLE I

MEAN POSITION (mm) OF SPOTS ON FOURTEEN RP-TLC PLATES

The second column gives the maximum deviation of 30 spots, applied to each of the fourteen plates with the aid of a Nanomat II applicator, adjusted to about 10 mm.

9.80 ± 0.20	10.46 ± 0.28
10.09 ± 0.15	10.00 ± 0.20
9.95 ± 0.20	9.98 ± 0.16
9.92 ± 0.16	10.07 ± 0.10
9.59 ± 0.20	9.56 ± 0.16
9.83 ± 0.13	9.88 ± 0.30
9.88 ± 0.25	9.86 ± 0.30

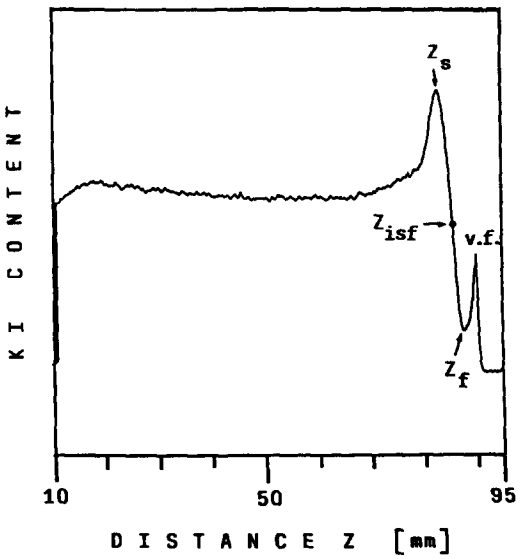

Fig. 2. Densitogram of a TLC plate which runs with buffer–methanol (30:70, v/v) and 0.05% potassium iodide in the buffer. Marked are the visible front (v.f.), the position until which stationary phase is solvent saturated (Z_s) and the mobile phase front of unsaturated flow (Z_f). Half-way between Z_s and Z_f the front of idealized saturated flow (Z_{isf}) is also indicated. According to Bolotov [13] Z_{isf} is the position of the thermodynamically true front. The distance between Z_f and v.f. is the area of preloading.

TABLE II

OBSERVED hR_F VALUES OF THE THREE FRONT MARKERS POTASSIUM BROMIDE, POTASSIUM IODIDE AND SODIUM NITRATE, ON RP-18 TLC USING VARIABLE MIXTURES OF METHANOL AND BUFFER, pH 7.4.

The higher the methanol content, the lower the hR_F due to larger preloading of stationary phase with increasing methanol content in the solvent. Above 65% methanol the hR_F of potassium iodide is the highest of the markers tested.

	Methanol concentration (%)					
	60	65	70	75	80	85
$hR_{F_{obs}}$, KBr	93.8	92.6	91.6	90.6	89.6	88.6
$hR_{F_{obs}}$, KI	93.6	93.0	92.9	91.6	91.1	90.8
$hR_{F_{obs}}$, $NaNO_3$	93.7	92.3	91.0	89.9	88.9	87.7

(*e.g.* methanol molecules from methanol–buffer mixtures) from the gas phase in the chamber before or during chromatographic runs. On the other hand, the mobile phase moves through the porous thin layer as an unsaturated flow, the mobile phase content in the layer varying along the distance from the source to the front and producing in the front region a gradient from fully saturated mobile phase content to zero content (see Fig. 2).

Almost all previous investigators [2,12,13] have tested the importance of these factors on silica gel or aluminium oxide layers. Accordingly, front markers used in these cases were rather lipophilic dyes such as Sudan Red with an anticipated R_F of 1.0.

The "front" in TLC is approximately equivalent to the "dead volume" (t_0) in HPLC. Therefore we investigated inorganic salts such as potassium bromide, potassium iodide and sodium nitrate as putative front markers, which were proposed by Braumann [14] as probes for estimating the dead volume in RP-HPLC. In a horizontal sandwich chamber, with and without water saturation, the three above-mentioned front markers were run in a methanol–buffer mixture of 1:1 (v/v) as well as in pure methanol. In all cases the front markers ran almost identically with the front; the difference amounted to less than 0.3 mm at a running distance of 80 mm ($R_F > 0.996$).

In the case of chamber saturation with methanol instead of water, the difference between the visible front and the marker position increased by up to 4 mm ($R_F = 0.95$). This observation might indicate that in RP-TLC correction of the "front gradient" is of marginal importance, while the "preloading effect" demands correction.

In a further series of experiments performed under the above-described conditions, *i.e.* twin trough chambers with solvent saturation and a temperature of 30°C, the apparent R_F values of the three front markers were estimated. Because of some residual free silanol groups in the stationary phase one has to consider a limited adsorption, which explains the lack of identity of front and marker positioning. As can be seen from Table II, of the three investigated compounds, potassium iodide exhibits the highest R_F values, at high methanol concentrations; thus we consider this salt to be the most convenient marker of the salts tested.

To further characterize the "front gradient"-related corrections, we performed investigations under standard conditions; potassium iodide (0.05%, w/v) was added to the buffers which were used as solvent in varying mixtures with methanol. Fig. 2 exemplifies typical results of such chromatograms.

For calculating the position of Z_{isf} (idealized solvent front, see also Fig. 2) in the gradient a modification of the procedure of Bolotov [13] was used. On standard silica gel plates Bolotov observed a gradient curve that could be described by an elliptical equation, while in case of RP-TLC, as shown in Fig. 2, the gradient exhibits a sigmoidal shape. Therefore, the thermodynamically true front position that equals Z_{isf} is exactly half-way between Z_s and Z_f and can be calculated with the equation

TABLE III

OBSERVED R_F VALUES, RELATED TO THE VISIBLE FRONT, OF POTASSIUM IODIDE AND OF THE FRONT OF IDEALIZED SATURATED FLOW, Z_{isf} (SEE FIG. 2)

From the last row it can be seen that their ratio varies only slightly around 0.99.

	Methanol concentration (%)					
	60	65	70	75	80	85
$R_{F_{obs}}$, KI	0.940	0 926	0.912	0.901	0.889	0.873
$R_{F_{obs}}$, Z_{isf}	0.950	0.936	0.921	0.910	0.897	0.884
R_F, KI/R_F, Z_{isf}	0.989	0.989	0.990	0.990	0.991	0.988

$$Z_{isf} = (Z_s + Z_f)/2 \quad (1)$$

In Table III the apparent R_F values as related to the visible front, of the front gradient ($R_{F_{obs}}$, Z_{isf}) and of potassium iodide ($R_{F_{obs}}$, KI) are listed for varying methanol contents. As can be seen from the data, the front gradient always surmounts the potassium iodide peak. The most accurate estimation of the thermodynamically true R'_F —and thereby of R_M— would obviously be represented by adding potassium iodide to all modifier mixtures and a direct estimation of the values with the thermodynamically true front. However, orientating experiments revealed some limitations in this respect. Many compounds, namely amines, exhibit a rather different chromatographic behaviour in iodide-containing modifiers as compared with iodide-free conditions. This is presumably due to the formation of adducts with iodide. Thus, we used a simplified procedure to calculate the thermodynamically true R_M values. As can be derived from Table III (third row) the ratio of potassium iodide peak to thermodynamically true front amounts to 0.99. Accordingly, using potassium iodide as a front marker, one has to divide the observed potassium iodide migration distance by 0.99. Formally, the observed apparent R_F of the compound X is:

$$R_{F_{obs},X} = (z_X - z_0)/(z_f - z_0) \quad (2)$$

The R_F value of the compound X when the potassium iodide peak is considered to indicate the front is:

$$R_{KI,X} = (z_X - z_0)/(z_{KI} - z_0) \quad (3)$$

The "thermodynamically true" R'_F value is then given by:

$$R'_{F,X} = [(z_X - z_0)/(z_{KI} - z_0)] \times 0.99 \quad (4)$$

R_M values have been calculated according to Bate-Smith and Westall [15]:

$$R_{M,X} = \log(1/R'_{F,X} - 1) \quad (5)$$

All R_M values have been calculated in this manner. R_M calculation with and without front correction causes differences of up to ±0.25, especially at high modifier contents. The differences between estimated true R_M values (*i.e.* related to the idealized solvent front) and uncorrected, apparent R_M values (*i.e.* related to the visible front) can be seen in Fig. 3. True R_M values are always lower than apparent R_M values; this difference increases with increasing modifier content, as also shown in Tables II and III. Consequently, linear regression is improved (see Fig. 3A).

The influence of solvent pH on R_M values

In recent investigations [11] it was shown that the commonly applied pH/pK correction of R_M leads to poorly plausible results. The above-mentioned investigations were performed on silanized silica gel plates and acetonitrile was used as modifier. We felt it necessary to prove the importance of these corrections also under the experimental conditions used in the present paper. For this purpose, investigations were

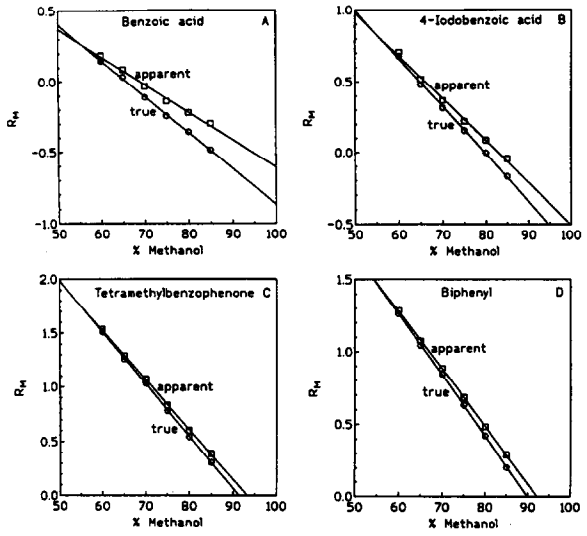

Fig. 3. Plots of linear regression analyses of true (○) and apparent (□) R_M values *versus* methanol concentration (v/v, %); buffer pH is 7.4.

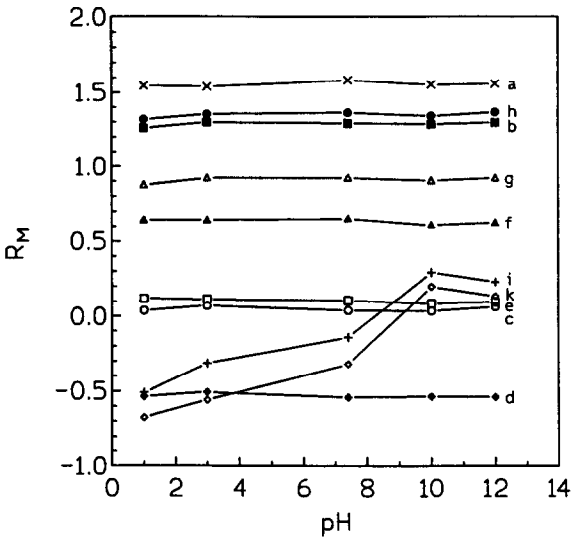

Fig. 4. Plot of R_M *versus* pH of some compounds on RP-18 silica gel plates using buffer–methanol mixtures (40:60, v/v). Only stronger amines such as atenolol (k, ◇) and procainamide (i, +) show variation between pH 1 and pH 10, but no further increase to pH 12. All other compounds show no variation: biphenyl (b, ■), tetramethylbenzophenone (a, ×), resorcin (d, ◆), naphthoresorcin (c, ○), benzoic acid (e, □), iodobenzoic acid (f, ▲), diphenylamine (g, △), N-phenylnapthylamine (h, ●).

carried out with a constant methanol:buffer ratio of 60:40; buffer pH was varied between 1 and 12.

The following ten test compounds were included: the non-polar biphenyl; the polar, non-ionizable tetramethylbenzophenone; the weak acids resorcin and naphthoresorcin (pK_a *ca.* 9.5); the strong acids benzoic and iodobenzoic acid (pK_a 4.2 and 4.0, respectively); the two weak bases diphenylamine and naphthylphenylamine (pK_a *ca.* 0.85) and the two strong bases procainamide (pK_a 9.4) and atenolol (pK_a 9.6).

The results, shown in Fig. 4, allow the conclusion that there is no need for a pK correction except for the strong bases. For example, for benzoic acid the above-described commonly used scheme for correction [5] leaves the measured value at pH 1 unaltered, while at pH 12 the measured value is to be corrected by +7.7 units. In contrast, the difference in the values obtained at pH 1 and 12 amounts to only 0.02 units. With a total of four hydroxybenzoic acids (pK_a about 3.0) Wilson [8] also did not detect variations in R_M within a pH range of 2–11. Similarly, for strong bases corrections that are far too high are calculated with the commonly used correction procedure. In this case, corrections at pH 12 should be negligible, while for atenolol at experimental pH 1, for example, as much as 8.6 units should be added for correction. In contrast to this theoretical consideration, the measured difference amounts to 0.81 units. The corresponding values for procainamide are 8.4 (theoretical) and 0.74 (experimentally obtained).

From our point of view, these examples convincingly prove the failure of the commonly used pK correction procedure. On the other hand, reasons for the observed weak pH dependence of strong bases remain to be clarified. One might speculate that R_M variation in these cases is a function of the modifier concentration. As shown by Horváth and co-workers [16–18] in RP-HPLC, the so-called silanophilic effect becomes prominent in cases of high modifier content. El Tayar *et al.* [19] have demonstrated the pH dependence of this silanophilic effect.

With these data in mind, we performed investigations with nine strong bases (pK_a values between 8.0 and 10.7) including procainamide

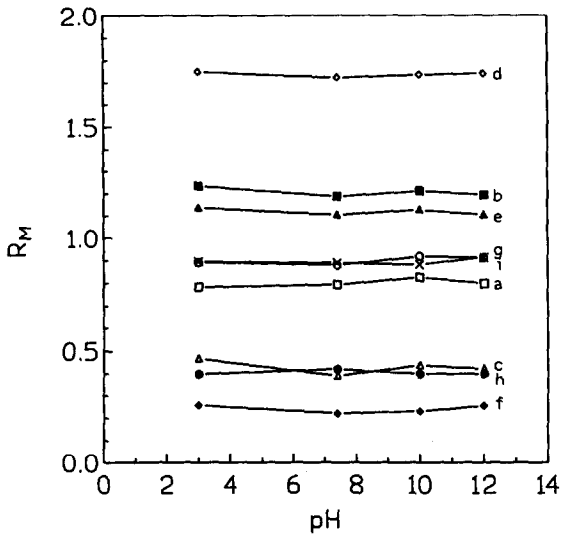

Fig. 5. Plot of R_M *versus* pH using solvent buffer–methanol (75:25, v/v) on RP-18 TLC plates showing the absence of any variation of the stronger organic bases (pK_a: 8.0–10.7) in low modifier mixtures. Key: lidocaine (a, □), disopyramide (b, ■), procainamide (c, △), propranolol (d, ◇), oxprenolol (e, ▲), atenolol (f, ◆), alizapride (g, ○), sulpiride (h, ●), alpiropride (i, ×).

and atenolol; modifier content was reduced to 25%.

As shown in Fig. 5, at this modifier concentration pH dependence of R_M is no longer observed; data differ only within the range of experimental variance. At this modifier content the chromatographic process is almost exclusively determined by partitioning. Obviously, the dissociation of solutes plays a negligible role in the partitioning process in RP-TLC. From similar observations in RPLC [20], Taylor [1] concluded that dissociated organic molecules will also be distributed into the stationary lipid phase. In our opinion this is because the dissociation velocity is much higher than compared to the distribution velocity (it takes time to reach a steady-state equilibrium in the shake-flask method).

With increasing methanol content the chromatographic process is, beyond lipophilic partitioning, increasingly due to polar adsorption, when the importance of ionization becomes more prominent. The pH dependence of this process was shown by Cserháti and Szögyi [21], while Dingenen and Pluym [3] speculated on the influence of silanol dissociation in this context. Our data with respect to atenolol and procainamide (Fig. 4) are not in conflict with this view. The small but continuous increase in R_M of these two amines between pH 1 and pH 10 and the lack of further increase to pH 12 may well reflect dissociation of silicon hydroxide. On the other hand the lack of pH dependence of benzoic acids (pK_a 4–4.2), as shown in Fig. 4, and salicylic acid [8] (pK_a 3.0), even at high modifier content, may well substantiate our interpretation. As mentioned above, the classic study of Stahl and Dumont [4] was performed by varying the pH of the stationary phase. Thus, our findings and those of Dingenen and Pluym [3] closely correspond to those in ref. 4.

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