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# SELECTIVE INHIBITION OF SEPARATED FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM RAT HEART BY SOME PENTASUBSTITUTED QUERCETIN ANALOGS

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**Abstract**—Synthetic analogs of quercetin were evaluated as inhibitors of cyclic nucleotide phosphodiesterase in rat heart preparations. Three main enzymatic forms of cyclic nucleotide phosphodiesterase can be resolved from rat heart cytosol by isoelectric focusing. Inhibition studies were performed with the whole cytosolic and particulate preparations and with the cytosolic separated enzymatic forms. All the compounds studied proved more potent as inhibitors of the particulate preparation than as inhibitors of the cytosolic fraction. With the exception of water-soluble derivatives and quercetin, they showed a better potency to inhibit cyclic AMP than cyclic GMP phosphodiesterase activity of the cytosolic and particulate preparations; likewise, among the three separated enzymatic forms, the cyclic AMP specific form of pI 5.55–6 is the most inhibited by these flavonoid compounds. In all cases, the highest selectivity was observed with pentaethyl quercetin (**2**).

An increasing amount of evidence suggests that the multiple molecular forms of cyclic nucleotide phosphodiesterase found in virtually all mammalian organs are not equally distributed in the tissues [1]. This particularity gives rise to the possibility of an acute control of each cyclic nucleotide concentration in a discrete area through the selective inhibition of the major phosphodiesterase isoenzymes in a given tissue. Thus, beside its theoretical interest, the selective inhibition of the different phosphodiesterase isoenzymes may have important pharmacological and therapeutic implications [2, 3]. As cyclic AMP is involved in cardiac contractile activity [4], heart phosphodiesterase might be a suitable target for several cardioactive drugs. We have recently separated three isoenzymatic forms of phosphodiesterase from rat heart cytosolic preparations by isoelectric focusing [5] and also characterized one enzymatic form by isoelectric focusing of the detergent-treated particulate material [6]. As observed in most tissues [1, 7], these forms differ in their kinetic behaviour, substrate specificity and sensitivity to endogenous calmodulin or exogenous calcium chelators. In order to characterize further these separated forms, we intended to study their sensitivity to various xenobiotic inhibitors such as butenolides [8] and flavonoid compounds. Some of the naturally occurring bioflavonoids which are known to exhibit various pharmacological effects [9] and to inhibit several membrane-bound enzymes [10, 11] or biological systems [12], were recently described as potent inhibitors of cyclic nucleotide phosphodiesterase from bovine heart [13] and lung [14, 15] and from the liver fluke [16]. The flavonoid compounds studied in the present report are quercetin derivatives, pentasubstituted by either lipophilic or polar groups, previously synthesized in our laboratory [17]. Inhibition studies were performed with each separated cytosolic form of cardiac phosphodiesterase and with a purified membrane preparation.

It is accepted that the use of separated forms of phosphodiesterase allows a more straightforward interpretation of the selectivity and nature of inhibition than does the use of crude preparations [3]. However, the patterns of inhibition observed in unseparated systems may be more representative of the composite state that occurs in intact cells [18]. A parallel study of the inhibition on the crude cytosolic phosphodiesterase might bring further insight into the actual functioning of phosphodiesterase.

## MATERIALS AND METHODS

*Chemicals*. [8-$^3$H]Cyclic AMP (20–30 Ci/mmole), [8-$^3$H]cyclic GMP (10–30 Ci/mmole), [U-$^{14}$C]-adenosine (500 mCi/mmole), [U-$^{14}$C]inosine (577 Ci/mmole) and [U-$^{14}$C]guanosine (525 mCi/mmole) were supplied by the Radiochemical Centre (Amersham, U.K.). Snake venom (*Ophiophagus hannah*), bovine serum albumin and unlabelled cyclic nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). AG1X2 resin (200–400 mesh) was from Bio-Rad Laboratories (Richmond, CA). Sephadex G75 was delivered by Pharmacia Fine Chemicals (Uppsala, Sweden). Ampholine carrier ampholytes were obtained from LKB-Produkter (Bromma, Sweden). EGTA (ethyleneglycol 2-(2-aminoethyl) tetra-acetic acid) and all other chemicals were reagent grade.

*Compounds studied*. The pentasubstituted analogs of quercetin were synthesized in our laboratory by Dr Picq [17]. Quercetin dihydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). Compound Ro 20-1724 (4-(3-butoxy-4-methoxy benzyl)-2-imi-

dazolidinone) was supplied by Dr. H. Gutmann Hoffman-La Roche & Cie (CH) and compound M & B 22948 (2-*O*-propoxyphenyl-8-azapurin-6-one) by Dr. R. Broad. May and Baker (GB) Ltd.

*Phosphodiesterase preparation.* Three main enzymatic forms from rat heart cytosolic fraction, prepared as described previously [5], were separated by isoelectric focusing on preparative granular gel plates [5]. The particulate phosphodiesterase preparation was a 105,000 *g* rat heart pellet, washed three times [6].

*Phosphodiesterase assay.* Cyclic nucleotide phosphodiesterase activity was assayed [6], following a modified method based on the original procedure of Thompson *et al.* [19]. $I_{50}$ were determined with 0.25 $\mu$M cyclic AMP and/or cyclic GMP as substrate. $K_i$ determinations were performed with cyclic nucleotide concentrations ranging from 0.25 to 5 $\mu$M. All the test compounds were solubilized in dimethylsulfoxide and brought to the adequate concentration with the incubation buffer. Final $Me_2SO$ concentration did not exceed 5% and did not induce analytical interference under the conditions used. Specially, water soluble compounds, exhibited similar patterns of inhibition with and without dimethylsulfoxide. Optimal pH and $Mg^{2+}$ concentration were previously determined for each cytosolic form and for the particulate enzyme [6–8]. The following assay conditions were used: whole cytosolic preparation: pH 8, 5 mM $Mg^{2+}$, particulate enzyme: pH 8, 7.5 mM $Mg^{2+}$, pI 5.55–6 form: pH 7.5, 5 mM $Mg^{2+}$, pI 5.45 form: pH 7.7, 7.5 mM $Mg^{2+}$, pI 4.9 form: pH 7.5, 5 mM $Mg^{2+}$. All the assays were performed in presence of bovine serum albumin (0.5 mg/ml), at 30°C and in triplicate, with enzyme dilutions adjusted to give linear reaction rates (usually 10–15% substrate hydrolysis in the absence of inhibitor). Adenosine and guanosine recoveries were systematically determined by means of [U-$^{14}$C]nucleosides. The results were corrected for these yields in each sample.

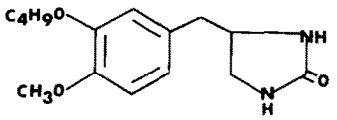
## RESULTS

Beside the flavonoid derivatives, two reference drugs were also examined: compound Ro 20-1724, with an imidazolidinone ring, described as a cyclic AMP phosphodiesterase specific inhibitor and compound M & B 22948 (Fig. 1), an anti-allergic compound related to purines, reported as a cyclic GMP phosphodiesterase specific inhibitor [15, 20].

*Inhibition of phosphodiesterase activities in the unseparated cytosolic and in the particulate fractions from rat heart.* All the flavonoid test compounds proved effective as inhibitors of the crude cytosolic phosphodiesterase preparation (Tables 1–3). Most of them showed a higher selectivity for cyclic AMP hydrolysis as shown by their $I_{50}$ cAMP/$I_{50}$ cGMP ratios being lower than 1. The highest selectivity towards cyclic AMP breakdown was observed with pentaethylquercetin (**2**) which proved quite as selective as the reference compound Ro 20-1724. In contrast with that reported by Ruckstuhl and Landry with bovine lung preparation [15], quercetin exhibited similar potencies for the inhibition of both cyclic AMP and cyclic GMP hydrolysis. It is noteworthy that water-soluble derivatives (**3**, **4**, **6**, **8**, **12**) showed little or no substrate selectivity, as well as the parent compound quercetin.

As a general rule, the pentasubstituted analogs of quercetin exhibited a higher potency for the inhibition of the particulate phosphodiesterase than for the cytosolic preparation, with both substrates. Quercetin derivatives which showed a good selectivity for cyclic AMP hydrolysis on the cytosolic preparation (**2**, **7**, **9**, **10**, **11**), also proved selective inhibitors of the particulate cyclic AMP phosphodiesterase (Tables 1–3). In this case, pentaethylquercetin (**2**) still exhibited the highest selectivity (25 times more potent as inhibitor of cyclic AMP hydrolysis than as inhibitor of cyclic GMP hydrolysis). The non-selective hydrosoluble derivatives of quercetin roughly exhibited the same pat-

Ro 20-1724




M&B 22948

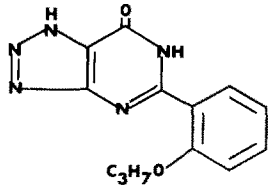
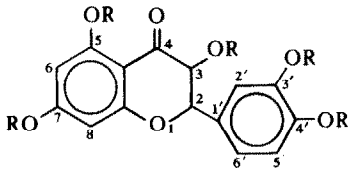



Fig. 1. Structure of reference compounds tested as inhibitors of cyclic nucleotide phosphodiesterase.

Table 1. Inhibition of rat heart cyclic nucleotide phosphodiesterase by penta-*O*-substituted analogs of quercetin



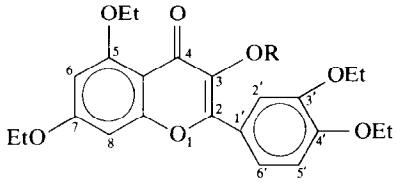

| Compounds | R | $R_f$* | Unseparated cytosolic form $I_{50}$ (μM)† cyclic AMP (A) | cyclic GMP (G) | A/G | Particulate form $I_{50}$ (μM)† cyclic AMP (A) | cyclic GMP (G) | A/G | pI 5.55–6† form $I_{50}$ (μM)† cyclic AMP | pI 5.45‡ form $I_{50}$ (μM)† cyclic GMP | pI 4.9‡ form $I_{50}$ (μM)sd cyclic GMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Quercetin | H | 0.51 | 20 | 27 | 0.74 | 64 | 65 | 1 | 24 | 20 | 66 |
| **2** | $CH_2$–$CH_3$ | 0.67 | 42 | 690 | 0.06 | 4 | 91 | 0.04 | 8.3 | 11 | 800 |
| **3** | $CH_2$–$CH_2OH$ | 0 | 132 | 138 | 0.95 | 84 | 120 | 0.7 | 35 | 66 | 120 |
| **4** | $CH_2$–$CH_2Br$ | 0.05 | 10 | 14.5 | 0.69 | 7 | 12 | 0.58 | 14 | 46 | 80 |
| Reference compounds | | | | | | | | | | | |
| Ro 20-1724 | | | 98 | 4500 | 0.02 | 22 | 400 | 0.06 | 12 | 250 | 500 |
| M & B 22948 | | | 302 | 16 | 19 | 126 | 20 | 6.3 | 79 | 1 | 10 |

* $R_f$ were obtained with a solvent system of acetone–pentane (2:1).

† Substrate concentration was 0.25 μM.

‡ The separate forms were kept at +4° in the following medium: 160 mM Tris–HCl buffer, pH 8, 1 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$.

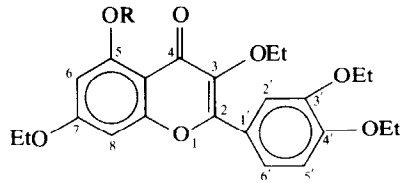
Table 2. Inhibition of rat heart cyclic nucleotide phosphodiesterase by 3′,4′,5,7-tetraethylquercetin analogs differently substituted in the 3 position




| Compounds | R | $R_f$* | Unseparated cytosolic form $I_{50}$ (μM)† cyclic AMP (A) | cyclic GMP (G) | A/G | Particulate form $I_{50}$ (μM)† cyclic AMP (A) | cyclic GMP (G) | A/G | pI 5.55–6‡ form $I_{50}$ (μM)† cyclic AMP | pI 5.45‡ form $I_{50}$ (μM)† cyclic GMP | pI 4.9‡ form $I_{50}$ (μM)† cyclic GMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **5** | $CH_2$–COOEt | 0.69 | 165 | 160 | 1 | 30 | 73 | 0.41 | 240 | 160 | nd§ |
| **6** | $CH_2$–COOH | 0.03 | 38 | 32 | 1 | 14 | 29 | 0.48 | 30 | 20 | 43 |
| **7** | $CH_2$–$CH_2$OH HCl | 0.67 | 170 | >10,000 | <0.1 | 130 | 912 | 0.14 | 110 | 1200 | 10,000 |
| **8** | $(CH_2)_2NEt_2$ | 0.02 | 148 | 45 | 3.3 | 79 | 79 | 1 | 28 | 10 | 31 |

* † ‡ See footnote to Table 1.
§ Not determined.

Table 3. Inhibition of rat heart cyclic nucleotide phosphodiesterase by 3,3′,4′,7-tetraethylquercetin analogs differently substituted in the 5 position




| Compounds | R | $R_f$* | Unseparated cytosolic form $I_{50}$ (μM)† cyclic AMP (A) | cyclic GMP (G) | A/G | Particulate form $I_{50}$ (μM)† cyclic AMP (A) | cyclic GMP (G) | A/G | pI 5.55–6‡ form $I_{50}$ (μM)† cyclic AMP | pI 5.45‡ form $I_{50}$ (μM)† cyclic GMP | pI 4.9‡ form $I_{50}$ (μM)† cyclic GMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **9** | $CH_2$COOEt | 0.59 | 26 | 46 | 0.57 | 7 | 66 | 0.11 | 22 | 18 | 50 |
| **10** | $CH_2$COOH | 0.02 | 12 | 40 | 0.3 | 4 | 40 | 0.1 | 12 | 12 | 48 |
| **11** | $CH_2CH_2$Br HCl | 0.48 | 33 | 60 | 0.55 | 9 | 50 | 0.18 | 3 | 30 | 75 |
| **12** | $(CH_3)_2N(Et)_2$ | 0.02 | 27 | 26 | 1.03 | 34 | 24 | 1.41 | 19 | 38 | 20 |

* † ‡ See footnote to Table 1.

terns of inhibition on both the cytosolic and the particulate preparations.

*Inhibition of the separated enzymatic forms of phosphodiesterase from rat heart cytosol (Tables 1–3).* Inhibition studies with the pI 5.55–6 isoenzymatic form were performed with 0.25 $\mu$M cyclic AMP as substrate. This 'cyclic AMP specific' form was shown to hydrolyze the most part (80%) of cyclic AMP at a low physiological substrate concentration [5]. Quercetin and its related pentasubstituted analogs proved effective inhibitors of this pI 5.55–6 isoenzyme with $I_{50}$ values lower than 35 $\mu$M. The only two derivatives being 5–10 times less potent inhibitors were 3',4',5,7-tetraethylquercetin substituted in the 3 position by an ethoxycarbonylmethyl or an hydroxyethyl group. These compounds (**5**, **7**) were also weak inhibitors of the crude cytosolic preparation. All the 3,3',4',7-tetraethylquercetins, substituted in the 5 position by polar or non-polar groups, were potent inhibitors of this form. Among them, compound **11** (bromoethyl group in 5) proved the most potent inhibitor among all the test compounds ($I_{50}$: 3 $\mu$M). The pI 5.55–6 isoenzymatic form of phosphodiesterase exhibited non-Michaelian kinetics when studied in a broad range of cyclic AMP concentration but linear Lineweaver–Burk plots were obtained in the 0.25–5 $\mu$M cyclic AMP range giving low $K_m$ values of 0.8–1 $\mu$M. With these substrate conditions both pentaethylquercetin (**2**) and compound Ro 20-1724 proved non-competitive inhibitors, as indicated by Lineweaver–Burk plots. Hill plots showed negatively cooperative inhibitions with calculated $K_i$ and Hill coefficient values of 8 $\mu$M ($n = 0.88$) and 6 $\mu$M ($n = 0.86$) for pentaethylquercetin (**2**) and compound Ro 20-1724, respectively (not shown).

The pI 5.45 isoenzymatic form hydrolyzed both cyclic nucleotides at high substrate concentrations (25 $\mu$M) but exhibited a marked preference for cyclic GMP at low substrate concentrations [5]. Thus, inhibition studies were performed with 0.25 $\mu$M cyclic GMP as substrate. In preliminary experiments, some butenolidic compounds, showed similar inhibitory potencies with either cyclic AMP or cyclic GMP as substrate. In general, flavonoid compounds proved slightly less potent as inhibitors of the pI 5.45 form than as inhibitors of the 'cyclic AMP specific' form. Beside quercetin, only three compounds (**5**, **6**, **8**) exhibited a marked selectivity for the pI 5.45 isoenzyme. They belonged to the series of the 3-substituted 3',4',5,7-tetraethylquercetins. In contrast, the reference compound M & B 22948 proved the most potent and the most selective as inhibitor of the pI 5.45 form. This isoenzymatic form exhibited linear Lineweaver–Burk plots over a wide range of substrate concentration with both substrates. With cyclic GMP as substrate, pentaethylquercetin and the reference compounds (Ro 20-1724 and M & B 22948) were found to be competitive inhibitors as indicated by Lineweaver–Burk plots. Concave downward Dixon plots and Hill plots showed negatively cooperative inhibitions. The following inhibition constants were calculated from Hill plots: pentaethylquercetin (**2**): $K_i = 13$ $\mu$M, $n = 0.62$; Ro 20-1724: $K_i = 126$ $\mu$M, $n = 0.85$; M & B 22948: $K_i = 0.8$ $\mu$M, $n = 0.55$.

The sensitivity to inhibitors of the 'cyclic GMP specific' enzymatic form of pI 4.9 was investigated with 0.25 $\mu$M cyclic GMP as substrate. All the flavonoid compounds studied proved less effective towards the pI 4.9 form as indicated by their $I_{50}$ values greater than 25–30 $\mu$M. Beside 3',4',5,7-tetraethyl 3-hydroxyethylquercetin (**7**), which proved totally inactive towards peak 4.9 but showed intermediate potencies as an inhibitor of the two other forms, pentaethylquercetin (**2**) was a weak inhibitor of the cyclic GMP specific form while it was a potent inhibitor of the pI 5.55–6 and pI 5.45 forms. Pentaethylquercetin (**2**) proved a non-competitive inhibitor of the pI 4.9 form with a $K_i$ value of 500 $\mu$M as extrapolated from linear Dixon plots. In the same assay conditions, compounds Ro 20-1724 and M & B 22948 were found to be competitive inhibitors with $K_i$ values of 410 and 6 $\mu$M, respectively. In all these cases, Hill plots indicated non-cooperative inhibitions with Hill coefficients $n = 1$. Thus pentaethylquercetin roughly exhibited the same inhibition patterns as did the reference compound Ro 20-1724 on each separated form with a similar high specificity towards the 'cyclic AMP specific form'. The hydrosoluble derivatives of quercetin (**3**, **6**, **8**, **12**) did not exhibit a marked selectivity towards any of the separated enzymatic form, as indicated by $I_{50}$ values in the same range of magnitude for each isoenzyme (Tables 1–3). The most potent inhibitor of the pI 4.9 form in the flavonoid series was 3,3',4',7-tetraethyl 5-diethylaminoethyl quercetin (**12**) with a $I_{50}$ value of 20 $\mu$M.

As described previously [5], the pI 4.9 isoenzyme is fully activated by calcium plus calmodulin in our usual assay conditions, as indicated by the 70% inhibition induced by EGTA (1 mM). In the presence of EGTA, the inhibition produced by compound **12** was markedly enhanced ($I_{50}$: 3–4 $\mu$M).

## DISCUSSION

Although it is difficult to draw clear-cut conclusions from the complex results presented here, some important features have to be underlined. All the quercetin analogs studied proved drastically more potent as inhibitors of the particulate fraction from rat heart than as inhibitors of the cytosolic preparation. With the exception of water soluble compounds which do not exhibit any substrate selectivity, the other quercetin derivatives constantly show a better potency to inhibit the cyclic AMP hydrolyzing activity of crude cytosolic and particulate preparations as well as the 'cyclic AMP specific' separated form, generally referred to as the 'low $K_m$' cyclic AMP or negatively cooperative phosphodiesterase [1, 5]. In this case, inhibition studies on crude tissue extracts reflect well the results obtained on purified isoenzymes. While this selectivity of inhibition with the whole cytosolic enzyme can largely be explained by the heterogenous nature of the preparation, the selectivity observed with the particulate fraction of the heart might appear surprising since only a single enzymatic form could be detected in this preparation [6]. However, the existence of multiple hydrolytic and regulatory sites, differently involved in the breakdown of each cyclic nucleotide, could explain

differences in the sensitivity to inhibitors observed for cyclic AMP and cyclic GMP hydrolysis.

Some apparent discrepancies appear between the results of Ruckstuhl *et al.* [14, 15] on lung phosphodiesterase and our own results. Indeed, these authors observed a higher potency of quercetin and related compounds for the inhibition of cyclic GMP hydrolysis with respect to cyclic AMP hydrolysis with bovine lung preparation. Several facts may account for such differences. First of all, synthetic derivatives of quercetin might exhibit quite different patterns of inhibition from that shown by naturally occurring bioflavonoids. However quercetin itself exhibits a slight but non-negligible selectivity for cyclic AMP hydrolysis with heart preparations. Differences in tissues and species provide a probable explanation. In fact, the difference in the nature of the enzymatic forms present in the preparations used for inhibition studies most likely seems to be involved. Indeed, Ruckstuhl *et al.* used a cyclic GMP phosphodiesterase form insensitive to calmodulin plus calcium while the rat heart 'cyclic GMP specific isoenzyme' of pI 4.9 is markedly calmodulin sensitive and proves quite similar to the 'high $K_m$' enzyme described by Wells and Hardman [1, 5]. The cyclic GMP enzymatic form described in the 'washed lung supernatant' most probably resembles the pI 5.45 heart isoenzyme which is only weakly sensitive to calmodulin plus calcium and, in spite of notable discrepancies [5], could be compared to the so-called positively cooperative phosphodiesterase [1]. Indeed, quercetin and some hydrosoluble derivatives show a slightly better potency towards cyclic GMP hydrolysis by the pI 5.45 form. Furthermore, compound M & B 22948 exhibits a drastic selectivity for the pI 5.45 form with inhibition constants quite similar to those reported in the lung [14, 15].

The pI 4.9 enzymatic form, when fully activated by calmodulin, clearly appears to be the least sensitive to flavonoid inhibition. Whereas xanthine derivatives show similar inhibition of the cyclic GMP specific form from pig coronary arteries, with and without calmodulin [18, 21], flavonoid compounds seem to inhibit preferentially the non-activated state of the isoenzyme. Neuroleptic compounds, on the contrary, are more potent as inhibitors of the calmodulin activated forms than as inhibitors of basal phosphodiesterase activity [22]. This original way of inhibition which seems to be characteristic of flavonoid compounds is presently under more extensive investigation.

The apparently identical sensitivity of pI 5.55–6 and pI 5.45 isoenzymes to flavonoid compounds is the last surprising point to be emphasized. Indeed, these two isoenzymes exhibit markedly different inhibition patterns with other chemical families of compounds as butenolides or the reference derivatives M & B 22948 and Ro 20-1724.

In conclusion, we hypothesize that flavonoid compounds exert two distinct types of action upon cyclic nucleotide phosphodiesterase. Firstly, as it was very precisely shown by Ferrell *et al.* [16], flavonoid compounds which exhibit structural similarities with the purine ring of cyclic nucleotides and may compete at the nucleotide binding sites [23] could act by competition with the natural substrates of the enzyme. The competitive nature of inhibition of the pI 5.45 form found with pentaethylquercetin is in good agreement with this proposal. On the other hand, flavonoid compounds could interact with the lipidic environment and hydrophobic regions of the enzymatic protein. Such phenomena are largely involved with other enzymes such as ATPases [10, 11, 23] and with various biological transport systems [12]. With cyclic nucleotide phosphodiesterase, non-specific interactions with lipidic environment or membrane, most likely module the inhibitory potency of flavonoid compounds and particularly enhance the inhibition towards particulate material and the 'cyclic AMP specific' form which is thought to be of particulate origin by many authors [1].

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