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CONDUCTANCE OF B-NICOTINAMIDE ADENINE COENZYMES IN AQUEOUS SOLUTION: APPLICATION ON THE EVALUATION OF DESORPTION OF COENZYMES IMMOBILIZED ON RESINS

FADI ANTOINE TARABOULSI-JR, ESTER JUNKO YORIYAZ AND MICHELE VITOLO*

Department of Pharmaceutical and Biochemical Technology, School of Pharmaceutical Sciences.University of São Paulo. Av. Prof. Lineu Prestes, 580, B.16, São Paulo, SP, Brazil

Author for Correspondence: michenzi@usp.br

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ABSTRACT

The use of conductimetry for determining the concentration of coenzymes (NAD, NADH, NADP and NADPH) in an aqueous solution is poorly described in the literature. Standardized equivalent conductivity (Λ_m; $S.cm^2/mol$) and specific conductance (κ; $S.cm^{-1}$) determinations were made, regarding to coenzyme solutions, whose concentration ranged from $0.5x10^{-3}M$ to $5x10^{-3}M$. Taking into account the values of κ and Λ_m for each coenzyme it was observed the decreasing sequence: NADPH>NADH>NADP>NAD. However, the high equivalent conductivity per negative charge ($60.5\ S.cm^2/mol.c_h^-$) occurred for NADH. The high reliability of conductance measurement enables coupling a conductimetry cell to the outlet reactor pipeline for controlling the leakage of the coenzyme from it.

Key Words: Immobilization, coenzymes, anionic exchange resins, conductance.

INTRODUCTION

β-nicotinamide adenine coenzymes (NAD, NADH, NADP and NADPH) are naturally charged molecules[1] – NAD (one positive and two negative charges), NADP (one positive and four negative charges), NADH (two negative charges) and NADPH (four negative charges) – so it is expected that, when in solution, they can promote the establishment of an ionic current between two electrodes connected to an external source of electricity. In order to explore this possibility, the conductimetry was chosen among all the electrochemical analysis methods known[2].Conductimetry is an analytical method based on the measurement of current intensity generated by applying a difference of voltage between two platinum electrodes inserted in an aqueous solution of an electrolyte. The current results from the moving of ions present in the solution, differently from that current generated, when a metal wire (copper, for example) is submitted to a difference of voltage on its extremities. Here, the current is due to the moving of unpaired electrons. It is also different from electrolysis, in which the current results from the moving of electrons – through an external electric circuit, as Wheatstone bridge for conductance measurement - generated by oxidation and reduction of ionic substances occurring at the anode and cathode, respectively. What really matters on applying conductimetry is its independence on the occurrence of electrode reactions. Thereby, the concentration of complex organic ionized molecules (as coenzymes, for example) present in an aqueous solution could be evaluated without any modification in their molecular structure.

The conductimetry is based on the following general equation:

$$\mathbf{I = E.L} \qquad \text{(Eq. 1)}$$

In which, I is the current in amperes, E the voltage (V) and L the conductance (mho or Siemens, S).
Moreover, L is related to the distance between the electrodes (d), their area (a), the concentration of ions per unit volume of the solution (c_i) and the equivalent ionic conductance (λ_i). We can thus write:

$$L = (\Sigma c_i \lambda_i)/\theta \quad \text{(Eq. 2)}$$

In which, θ (cell constant) = d/a; the summation symbol (Σ) denotes the fact that the contributions to the overall conductance of the various ions present are additive.
Finally, in order to compare the values of conductance obtained with different electrode assemblies, the so-called conductivity or specific conductance (κ) is defined as follows:

$$\kappa = L.\theta \quad \text{(Eq. 3)}$$

Nowadays, there are a lot of analytical methods based on conductimetry[3-7]. However, nothing was found in the literature regarding the detection and quantification of β-nicotinamide adenine coenzymes through this procedure.
As coenzymes are quite expensive substances, the reason for which the use of coenzyme-dependent dehydrogenases (for example) in catalytic processes has been repressed up to the present, immobilizing them on inert materials was tried aiming their recuperation at the end of the reaction conducted in a batch reactor or their full entrapment in a continuous reactor (mainly the membrane type continuous stirred tank reactor)[8-9]. In this work, DOWEX®-type resins were chosen due to the fact that it is a non- toxic material, plentifully available in the market and inexpensive, among others characteristics[10]. Desorption of the coenzyme from the support during the reaction constitutes a failure regarding the final yield of the process. The coenzyme immobilization follows the same reasoning applied to the immobilization of biocatalysts such as enzymes, cells or organelles[6,11].
The solubility in water of sparingly soluble salts was successfully determined through conductimetry[2]. By assuming that the coenzyme desorption follows a pattern analogous to that presented by sparingly soluble salts (Figure 1), then conductimetry could also become a useful analytical procedure for determining the amount of coenzyme which eventually leaked from the support to the supernatant (batch process) or in the outlet solution (continuous process).

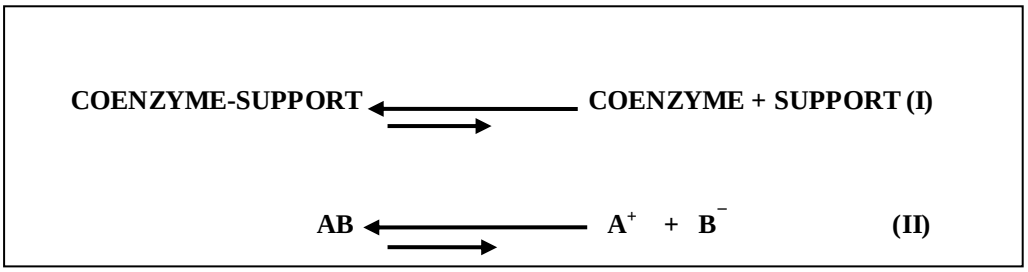

Fig.1.Generic equilibrium equations related to desorption of the coenzyme from the support and the decomposition in water of a sparingly soluble salt.

Equation II could represent, for example, a saturated solution of AgI or AgCl, whose solubility at 25°C is $1.2x10^{-8}$M and $1.0x10^{-5}$M, respectively[12].

Particularly for the continuous process, a conductance cell could be adapted to the exit of the reactor, so that the outlet solution would flow through it and the eventual coenzyme dissolved would be monitored and quantified on line through the evaluation of the ionic current generated. The system should be similar to that commonly used for monitoring the water attained from a deionizer apparatus. It must be understood that both platinum electrodes used in conductimetry can be miniaturized or enlarged according to the application envisaged for the conductance cell, making it easy to adapt to any outlet reactor pipeline. This work deals with the application of conductimetry for estimating the concentration of the β-nicotinamide adenine coenzyme dissolved in deionized water and/or in a diluted buffer. For example, this procedure can be valuable on the detection of coenzymes (NAD, NADP, NADH and NADPH) leaked from an insoluble support, such as an ion exchange resin (DOWEX® type), on which it was immobilized.

MATERIAL AND METHODS

MATERIAL

The coenzymes [NAD(P) and NAD(P)H] and the anionic exchanger resins were purchased from Sigma® (St. Louis, MO, USA). The conductance was measured using a conductance-meter (model TEC-4 MP) purchased from Tecnal (Piracicaba, São Paulo, Brazil). All other chemicals used were of analytical grade.

METHODS

Immobilization of Coenzymes

The anionic exchanger resin (1X2-100 or 1X8-100) weighed 100mg. The beads were rinsed with 25 mL of deionized water (pH 7.5). The suspension was kept in a shaker at 30°C and 100 rpm for 24h. Afterwards, the suspension was centrifuged at 3000xg for 10 min. The supernatant was discharged and the beads were re-suspended with 20 mL of $5x10^{-3}$M solution of coenzyme at pH 7.5. This solution was made by dissolving the coenzyme in deionized water (pH 7.5; adjusted with sodium hydroxide) or in a phosphate buffer (0.1mM; pH 7.5). The new suspension was left under an agitation of 100 rpm at 30°C for 4h. After centrifugation (3000xg/10min), the conductance of the supernatant was measured in order to determine the amount of coenzymes eventually unlinked.

Coenzyme Desorption Determination

The desorption tests were performed by suspending the coenzyme/resin system in 25mL of deionized water (pH adjusted with HCl or NaOH, respectively, to 6.0 or 9.0) or 0.1mM phosphate buffer (pH 6.5 or 7.5), which was left under agitation of 100 rpm at 37°C for 30h. Afterwards, the suspension was centrifuged at 3000xg/10min and an aliquot sample of the supernatant was taken for the determination of the conductance.

Determination of Soluble Coenzymes through the Conductance-Meter

The aliquot of each type of coenzyme solution was poured into a 40mL-conductimetric cell and the conductance measured through TEC-4MP conductance-meter (supplier's information: accuracy =$2x10^{-8}$S.cm^{-1};precision =± $1x10^{-8}$S.cm^{-1}; provided with an internal temperature control; cell constant (θ) = 1). The conductance was compared with standard-curves made with coenzymes solutions, whose concentrations for NAD(H) and NADP(H) were varied from $0.50x10^{-3}$M to $5x10^{-3}$M. The conductance-meter was calibrated against the KCl solution, whose concentration varied from $0.5x10^{-3}$M to $10x10^{-3}$M. The measurement was carried out under agitation of 50rpm and at 25°C.

RESULTS

The standard curve at 25°C for the conductance-meter TEC-4MP is presented in Figure 2, in which the conductivity (S/cm) was plotted against the KCl solution, whose concentration varied between $5x10^{-4}$ M and $1x10^{-2}$M. Each point represents the average of five determinations (coefficient of variation = 1.06%). The equation related to the least-square linear regression was:

$$Y_{KCl} = 0.139X - 1.42x10^{-5} \quad (r = 0.99993) \qquad \text{(Eq.4)}$$

Where Y_{KCl} = conductivity (S/cm); X = KCl concentration (M).

Figure 3 shows the variation of conductivity against NAD, NADP, NADH and NADPH solution, whose concentrations changed between $0.5x10^{-3}$M and $5x10^{-3}$M. Each point represents the average of five determinations (coefficient of variation = 0.90%).

The coenzymes (NAD, NADP, NADH and NADPH) were immobilized by adsorption on anionic exchange resins (DOWEX types: 1X2-100 and 1X8-100) at pH 7.5 (adjusted with 0.5M NaOH solution), as described above. The immobilization yields were shown in TABLE 1.

The immobilization yield (IY) was calculated by means of the equation:

$$(IY) = [(ICC - CCS) \div (ICC)].100 \qquad \text{(Eq. 5)}$$

Where ICC = Initial Conductivity of Coenzyme solution (S.cm^{-1}) ; CCS = Conductivity of Coenzyme in the Supernatant (S.cm^{-1}).

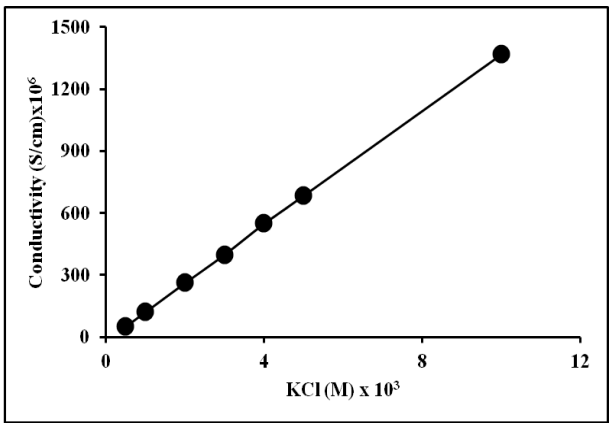

Fig.2.Standard curve for the conductance-meter TEC-4MP

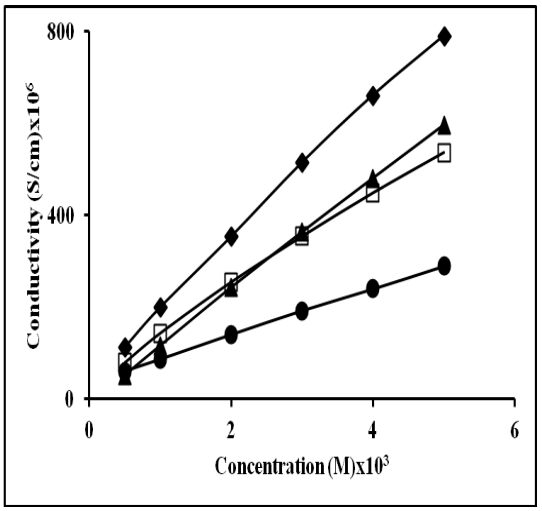

Fig.3.Conductivity-concentration curves for aqueous solutions of NAD(•), NADP(□), NADH(▲) and NADPH(◆).

The equations related to the least-square linear regression were: $Y_{NAD} = 0.0512x + 3.6x10^{-5}$ (r = 0.9997);
$Y_{NADP} = 0.101x + 4.1x10^{-5}$ (r = 0.9998);
$Y_{NADH} = 0.121x - 4.5x10^{-6}$ (r = 0.9998); $Y_{NADPH} = 0.152x + 4.8x10^{-5}$ (r = 0.9991).

Table.1.Immobilization yield (IY) for coenzymes immobilized on 1X2-100 and 1X8-100 anionic exchange resins at pH 7.5

Coenzyme Type	1X2-100 (%)	1X8-100 (%)
NAD	95	95
NADP	100	100
NADH	100	100
NADPH	100	100

After submitting the coenzyme/resin system to the desorption conditions described above, the supernatant was collected and the amount of the soluble coenzyme was determined by conductimetry (TABLE 2).

The percent of desorption (PD) was calculated by means of the equation:

$$(PD) = [(SCS) \div (TIC)].100 \qquad \text{(Eq. 6)}$$

Where TIC= Total Immobilized Coenzyme; SCS = Soluble Coenzyme in the Supernatant. The initial coenzyme concentration before the immobilization was $4.75x10^{-3}$M (279.2 $S.cm^{-1}$) for NAD and $5x10^{-3}$M for NADP (546 $S.cm^{-1}$), NADH (600.5 $S.cm^{-1}$) and NADPH (808 $S.cm^{-1}$).
The desorption was estimated under acidic (pH 6.0 and 6.5, respectively for deionized water and 0.1mM phosphate buffer) and alkaline (pH 7.5 and 9.0, respectively, for 0.1mM phosphate buffer and deinozed water) media, because several enzymes require a coenzyme for their catalytic activity – such as the dehydrogenases that catalyse oxi-red reactions at a large pH interval in presence of one kind of β-nicotinamide adenine coenzyme. The phosphate buffer was also tested in order to verify its eventual influence on the conductivity measurement. There are enzymes whose catalytic activity depends strictly on their molecular conformation, which, in general, acquires stability in a buffered solution. By taking NAD as an example, Figure 4 was attained. The variation of conductivity was presented against the concentration of NAD which was dissolved in 0.1mM phosphate buffer (pH 6.5 or 7.5).

Table.2.The percent of desorption of coenzymes from DOWEX resins

Resin	Solvent	pH	NAD	NADP	NADH	NADPH
1X2-100	DW*	6.0	8.06	10.5	12.5	11.0
		9.0	10.7	9.21	14.0	14.8
1X2-100	PB**	6.5	4.81	5.98	11.7	7.50
		7.5	9.68	4.67	14.5	8.00
1X8-100	DW	6.0	8.64	11.8	11.2	12.2
		9.0	11.4	10.6	13.8	14.5
1X8-100	PB	6.5	9.10	9.21	9.93	9.45
		7.5	13.8	7.98	14.4	8.57

DW = Deionized water; **PB = Phosphate buffer (0.1mM)

The percent of desorption of coenzymes from DOWEX resins (1X2-100 and 1X8-100) in deionized water (pH adjusted to 6.0 or 9.0 with 0.5M HCl or 0.5M NaOH, respectively) and 0.1mM phosphate buffer (pH 6.5 or 7.5). The Soluble Coenzyme in the Supernatant (SCS) values presented are those attained by conductimetry.

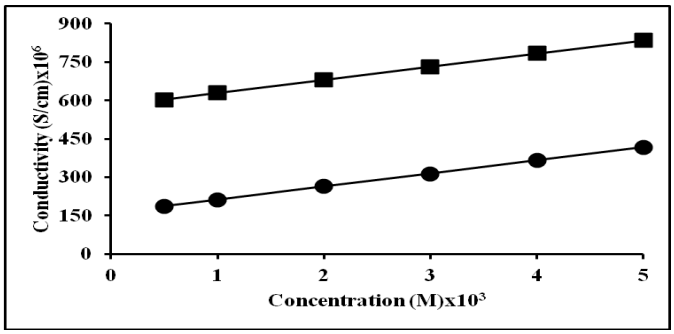

Fig.4.Conductivity-concentration curves for buffered solutions of NAD at pH 6.5 (•) and 7.5 (▪)

The equations related to the least-square linear regression were:
$Y_{NADpH\ 6.5} = 0.0511x + 16x10^{-5}$ (r = 0.99998);
$Y_{NADpH\ 7.5} = 0.0513x + 358x10^{-5}$ (r = 0.99995).

DISCUSSION

As well established to conductimetry, there are two parameters which must be maintained invariant along the entire analytical procedure, i.e., the cell geometry of the conductance-meter (expressed as cell constant), in which the measurements are carried out, and the temperature of the ionic solution. In this work, the temperature was set at 25^0C.

The cell constant can be evaluated by means of the equation:

$$\theta = \kappa/L \qquad \text{(Eq. 7)}$$

Where θ, κ and L are the cell constant, conductivity (S/cm) and conductance (S/cm), respectively.

Setting a KCl concentration of 0.01M, the related conductivity is 0.0014088 S/cm[3,13].

The conductance measured through TEC-4MP at that concentration was 0.0013758S/cm (through Eq. 4), then θ = 1.02 (through Eq.7), a cell constant value near that informed by the manufacturer (θ =1). By using equation 4 for estimating the conductance of the KCl solution at a concentration of 0.02M, and thereby beyond the upper limit of 0.01M set in this work, 0.0027658S/cm, a same value was found in the literature[14]. The use of TEC-4MP for determining conductivities of ionic aqueous solutions is thus reliable. In Table 3, the κ values for some KCl aqueous solutions determined through the approach employed here are presented.

Table.3.Specific conductances of potassium chloride solutions (25°C)

Concentration (M)x10³	κ (S/cm)x10⁶
0.5	53.20
1	125.7
2	270.6
3	412.0
4	566.5
5	706.1
10	1410

The conductivity of aqueous solutions of coenzymes varied linearly with the concentration at an interval from $0.5x10^{-3}$M to $5x10^{-3}$M. The specific conductance regarding each coenzyme presented in Table 4, whereas the equivalent conductivities (Λ), represented by the slopes of the straight lines (Figure 3) multiplied by 1000, to each coenzyme are showed in Table 5.

Table.4.Specific conductances (κ) of NAD, NADP, NADH and NADPH solutions (25°C)

Concentration (M)x10³	κNAD (S/cm)x10⁶	κNADP (S/cm)x10⁶	κNADH (S/cm)x10⁶	κNADPH (S/cm)x10⁶
0.5	61.2	80.7	52.6	115
1	87.7	146	118	204
2	143	260	247	361
3	196	361	371	526
4	245	458	490	675
5	296	548	608	807

The β-nicotinamide adenine coenzymes (NAD, NADP, NADH and NADPH) are naturally ionized molecules (TABLE 5), whose aqueous solutions generate an ionic current – established between two electrodes connected to a source of electricity and immersed into the solution -, which can be measured through a conductance-meter. The intensity of the current will depend on the internal negative/positive charges balance in the coenzyme molecule. This point is clearly observed through the different values on the equivalent conductivities calculated to each coenzyme cited (TABLE 5). In order to attain an idea about the role of electrical charges belonging to the coenzyme molecule on its conductance in solution, $\Lambda_{m'}$ as the ratio $\Lambda_m/(C_h^-)$, was defined in which C_h^- is the number of negative charges of the coenzyme (TABLE 5). Thereby, the ratios $(\Lambda_{m'})_{NAD}/(\Lambda_{m'})_{NADP}$ = 1.0 and $(\Lambda_{m'})_{NADH}/(\Lambda_{m'})_{NADPH}$ = 1.6 indicate some kind of internal neutralization of negative charges, being quite evident in the case of oxidized forms, in which one positive charge neutralizes two of the negative charges in terms of the conductance of the NADP solution. As the mol ratios - $(MW_{NAD})/(MW_{NADP})$ = (663.4/765.4) = 0.86 and $(MW_{NADH})/(MW_{NADPH})$ = (709.4/833.4) = 0.85 – of both coenzyme forms are practically the same, the real contribution to the conductance of coenzyme in a solution is due to the balance among the opposite electric charges present in the molecule.

Table.5.The equivalent conductivities calculated for β-nicotinamide adenine coenzymes (NAD, NADP, NADH and NADPH)

Coenzyme type	Λ_m (S.cm²/mol)	Charges Positive	Charges Negative	$*\Lambda_{m'}$ (S.cm²/mol. C_h^-)
NAD	51.2	+1	-2	25.6
NADP	101	+1	-4	25.3
NADH	121	0	-2	60.5
NADPH	152	0	-4	38.0

Moreover, the electric charges of each molecule and the equivalent conductivities per number of negative charge (C_h^-) are also presented.
$*\Lambda_{m'}$ = Equivalent conductivity/negative charge.

As can be seen from TABLE 1, the coefficient of immobilization of the coenzymes on 1X2-100 and 1X8-100 resins was 100%, except for NAD. This result corroborates the data already described[15].The slopes of the conductivity profiles obtained – 0.0511 S/cm.M and 0.0513 S/cm.M for pH 6.5 and 7.5, respectively (Figure 4) - were similar to the coenzyme solution prepared in deionized water (Figure 3). The same was observed for NADH, NADP and NADPH dissolved in 0.1mM phosphate buffer (data not shown). Thereby, the conductimetry is also useful when the enzyme reaction is carried out in a buffered medium. However, it must be clear that, when a more concentrated buffer is employed, the conductivity regarding it must be subtracted from the whole conductivity measured. The case in which the occurrence of the catalyzed reaction did not promote pH changing of the medium, such as observed for dehydrogenases in general, no buffer or a diluted one – only envisaging the stability of the conformation of the enzyme, if needed - could be used. Moreover, deionized water itself is a very poor conductor, its specific conductance being about $5x10^{-8}$S/cm at 25°C[3].

Table.6.Differences on the conductivity of NADP solution present both in the supernatant from immobilization and in desorption solution (pH 6.0 and 9.0)

Procedure	Resins	*C_F, (S.cm^{-1})x10^6	**C_m (S.cm^{-1})x10^6	(C_m/C_F) (%)
Immobilization	1x2	610.0	591.8	3.0
	1x8	602.4	588.6	2.3
Desorption at pH 6.0	1x2	180.2	176.8	2.0
	1x8	173.5	168.4	3.0
Desorption at pH 9.0	1x2	692.3	685.0	1.1
	1x8	690.0	689.4	0.01

*C_F is the conductivity calculated through the equation attained previously (Figure 3):
Y_{NADP} = 0.101x + $4.1x10^{-5}$; **C_m is the conductivity read directly from the conductance-meter.

As an example, the absorption/desorption of NADP on resins (1X2-100 and 1X8-100) was chosen in order to illustrate the difference between values of the conductivity measured (C_m) and foreseen (C_F) through the correspondent equation (Y_{NADP} = 0.101.x + $4.1x10^{-5}$) for the NADP solubilized either in the supernatant from the immobilization procedure or in the desorption solution (TABLE 6). As can be seen from the cited table, the percent difference did not surpass 3%, a good correlation considering that the coefficient of the variation related to the conductance determinations was about 1.1% (see above). As a result, the use of conductivity as a means to identify a possible leak of the coenzyme from the resins under the studied conditions showed to be feasible for bioconversion processes.

CONCLUSION

The immobilization of the coenzymes on 1X2-100 and 1X8-100 had a yield over 95% and desorption was minimal, except at pH 9.0 in which it was about 13%. The conductimetry presented a good performance as analytical method for determining the concentration of the coenzymes in an aqueous solution. The conductimetry, in particular, has been a reliable and sensible procedure. The specific conductance (κ) and the equivalent conductivity (Λ_m) of the coenzymes varied according to the sequence NADPH > NADH > NADP > NAD, although the equivalent conductivity per negative charge (60.5S.cm^2/mol. C_h^-) was the highest for NADH. Finally, the enhanced reliability of the conductance measurement allows envisaging the possibility of using the conductance cell coupled to the outlet reactor pipeline for controlling the leakage of the coenzyme from it.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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