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IONIZATION OF DEAE-CELLULOSE

DEPENDENCE OF p*K* ON IONIC STRENGTH

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SUMMARY

The salt dependence of the ionization of DEAE-cellulose has been investigated using potentiometric acid–base titrations. Two ionizable groups were identified. The p*K* of the major group was exponentially related to ionic strength. However, the minor group was very much less sensitive to changes in ionic strength. The fraction of total ion-exchange sites represented by the minor group decreased with increasing salt concentrations and was insignificant in back titrations with sodium hydroxide, suggesting the elimination of structural or electrostatic barriers which otherwise sequester a significant fraction of potential ion-exchange sites which resist ionization.

INTRODUCTION

Ion-exchange chromatography continues to be one of the most useful and powerful methods for isolation and purification of polyelectrolytes. The development of recombinant DNA techniques, and commercial exploitation of these techniques for large scale production of biologically important proteins and peptides, has become an important driving force behind technical development of chromatographic separations[1]. This renewed emphasis has led to modifications of previously well established methods[2]. Since the initial introduction of substituted celluloses[3] other materials such as dextrans, polyacrylamide[4] and more recently polyethyleneimine coated silica[5] have been successfully applied to the purification of proteins by gel and high-performance ion-exchange chromatography. It is significant that pellicular supports have the same general mobile phase elution characteristics as conventional gel-type ion exchangers, and that ionic and pH conditions that work well for the latter are usually suitable for high-performance columns[2,6]. Although there has been a significant shift from classical gel-type to high-performance media[6] the former is still commonly used in large scale and preparative procedures. Despite the availability of many types of ion exchangers, diethylaminoethyl and carboxymethyl modified exchangers are perhaps the most commonly used. This is a consequence of improvements in physical and chemical characteristics of these exchangers. In a previous communication[7] we have tested a model, based essentially on the law of mass action as applied by Langmuir to

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surface phenomena, to the ion-exchange elution characteristics of a homologous polylysine series. Although the validity of the model is limited by simplifying assumptions, it does explain many characteristics observed in the ion exchange of polyelectrolytes, such as proteins. The relationship of various process parameters on resolution has also been investigated[2,8,9], and the effect of salt on the capacity[10] of some ion exchangers has been demonstrated, *i.e.*, the salt dependence of the pK[2,11,12]. The importance of local charge distribution on chromatographic behavior of proteins has also been superbly documented[13], as has the observation that very small ionic strength changes can result in large elution volume differences of polyelectrolytes[7,14]. As a correlary to the latter, in this communication we describe changes in the ionic structure of DEAE-cellulose that are exponentially related to salt concentration, and putative changes in local charge distribution, as observed by potentiometric acid–base titrations.

EXPERIMENTAL

Buffers

The following reference buffers were prepared as described[15]: phosphate (0.025 M KH_2PO_4, 0.025 M Na_2HPO_4) pH 6.87 at 23°C, borate buffer (0.01 M $Na_2B_4O_7$) pH 9.205 at 23°C. These buffers were prepared in double distilled, boiled water and stored under argon or nitrogen.

Solutions

Sodium chloride (Mallinckrodt, Analytical Reagent) solutions were prepared in double distilled, boiled water and stored under argon or nitrogen. After bubbling nitrogen, to remove CO_2, solutions of higher salt concentration remained basic and were titrated dropwise to neutrality (pH 6.8–7.2) with 0.001 M HCl.

Titrants

HCl (Baker, Reagent) and NaOH (Mallinckrodt, Anal. Reagent) were prepared in double distilled boiled water, standardized against Tris [tris(hydroxymethyl)amino-methane] (Fisher Scientific) and against potassium biphthalate (Baker, Reagent) respectively, and stored under argon or nitrogen. During titration, titrants were protected with Ascarite-filled caps. NaOH was passed through a Dowex-1 column to remove carbonates.

DEAE-cellulose

Whatman (DE-52 microgranular, preswollen, 1.0 mequiv./g); BioRad (Cellex-D, 0.66 mequiv./g); Mann Research (Mannex-DEAE, 1.1 mequiv./g; Sigma (DEAE-cellulose, 0.89 mequiv./g). Celluloses were processed by washing with 0.5 M HCl (30 min), with water, then 0.5 M NaOH (30 min), then water until effluent was neutral.

Titration procedure

All titrations were carried out with a Radiometer automatic titrater (pHM26, ABU12, TTT11, SBR2c) and electrode (GK2301c). The pH meter was accurate to ± 0.007 pH units from pH 0 to 12. The buret capacity was 2.5 $\pm$ 0.0025 ml. Titration rates were from 0.03125 to 0.125 ml/min. Results from faster titration rates were not

significantly different from those obtained at slower rates. The pH meter was standardized with phosphate and borate buffers and rechecked before and after each titration. Titrations were carried out in 20.0 ml of water or salt. Nitrogen, scrubbed with Ascarite and washed with sulfuric acid and then water, was bubbled through the solutions, which were adjusted if necessary to neutrality (pH 6.8–7.2) with 0.001 *M* HCl and the normality of initial salt solutions corrected accordingly. Similar corrections were made following addition of DEAE-cellulose (about 0.6 mequiv.). No precipitates were obtained from alkaline extracts of cellulose upon addition of $BaCl_2$. Base titrations were carried out following acid titrations using the resulting hydrochloride form of the exchangers.

Determination of pK values

Equivalence points and p*K* values were determined graphically from tangential lines drawn through inflection points using standard procedures[12] as shown for a typical acid titration curve (Fig. 1). Inflection points of steep portions of the curves were assumed to represent equivalence points. p*K* values at each salt concentration were calculated from several (2–4) independent titrations and uncertainties calculated for a 0.95 probability.

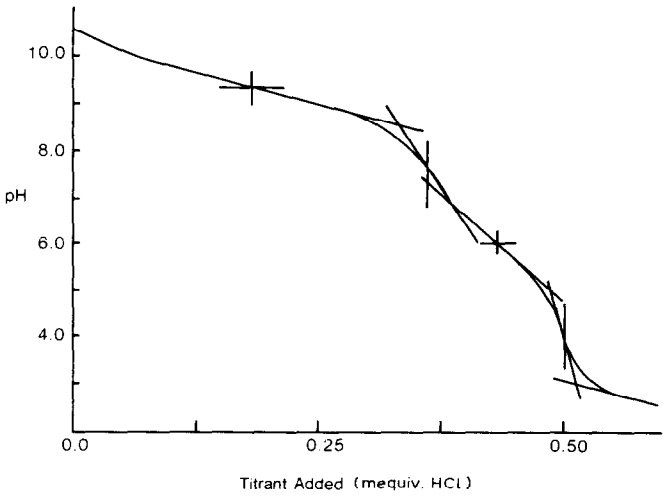

Fig. 1. Typical acid titration curve of DEAE-cellulose. Preswollen DEAE-cellulose (2.0 g, Whatman DE-52) was titrated with HCl (0.241 *M*) in NaCl (0.20 *M*). Standard graphical methods were used to calculate p*K* values and end points, as shown.

Kjeldahl determinations for total nitrogen content were run by standard procedures by the Department of Agronomy and Horticulture of this univerity.

Calorimetric titrations were run on a Tronac Model 450 isoperibol titration calorimeter using standard procedures[20], in cooperation with Dr. Lee D. Hansen.

RESULTS

In the present study microgranular DEAE-celluose was chosen because of its improved matrix and distribution of charged groups[16]. Such ion exchangers purportedly are structurally more reproducible, have faster kinetics, higher capacity, and give maximum resolution (see Whatman Technical Bulletin). Since precycling is necessary to release full capacity and provide optimum interaction with large molecules, all exchangers used in this study were precycled as described in Experimental. Care was also taken to remove CO_2 in order to avoid artifacts caused by the high affinity of carbonate and bicarbonate for DEAE-cellulose.

When determined directly from acid–base titration curves, the pK values of DEAE-cellulose[3] and various polyethyleneimine-coated silica exchangers[2] are known to be dependent on salt concentration. The pK of DEAE-cellulose is about 8.0 in water and 9.5 in NaCl (0.5–1.0 M)[12]. PEI6-LiChrosorb Si 100 anion exchanger used in high-performance ion-exchange chromatography (HPIEC)[5] is similar in this respect. During the last 25 years ionic-strength gradients, at fixed pH values, have been widely used to purify specific components from complex mixtures of proteins and other electrolytes using ion-exchange chromatography[2]. Since the ionic structure or configuration, as expressed by the pK, of numerous ion exchangers is subject to change under such conditions, a more detailed investigation of the characteristics of such salt dependent pK changes is warranted in order to optimize column performance. DEAE-cellulose was specifically selected for a more detailed investigation of the dependence of pK on salt concentration, because of its wide use in large scale purification of proteins and enzymes[2].

Aqueous slurries containing about 0.6 mequiv. of the free amine (of DEAE-cellulose) in 20.0 ml of water, or various concentrations of NaCl, were titrated with HCl (0.24 M). A typical titration curve, obtained in 0.20 M NaCl, is shown in Fig. 1. Two apparent buffering regions are observed, a major one at about pH 9.3 and a minor one at about 6.0. The graphic method used for calculating end points and pK values is also illustrated. Similar titrations were carried out under other ionic conditions, and pK values and end point determined as described above. The major acid titratable group (about 70%) has a salt-dependent pK ranging from 6.92 in water to 9.70 in 2.0 M NaCl, Table I. The pK increased dramatically in dilute salt (0.0–0.2 M NaCl) after

TABLE I

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON THE OBSERVED pK OF THE MAJOR ACID-TITRATABLE GROUP OF DEAE-CELLULOSE (WHATMAN DE-52)

NaCl (M)	*Corrected Cl^- (M)*	*Ave. pK*
0.00	0.00	6.92 ± 0.06
0.01	0.0095	8.35 ± 0.24
0.05	0.048	8.98 ± 0.06
0.10	0.095	9.15 ± 0.09
0.20	0.19	9.28 ± 0.09
0.30	0.29	9.43 ± 0.1
0.50	0.47	9.47 ± 0.1
1.00	0.93	9.59 ± 0.03
2.00	1.82	9.70 ± 0.02
6.24 (satd.)	6.24	9.83 ± 1.0

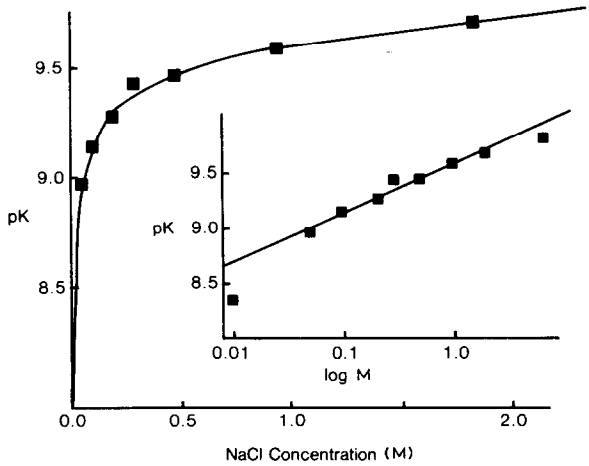

Fig. 2. Effect of increasing salt concentration on the pK of DEAE-cellulose. pK values were determined by acid titration of the free amine in various concentrations of NaCl, as shown in Fig.1. Values were averaged from 2–4 titrations. Normalities were corrected for volume changes resulting from added titrant.

which it asymptotically approached the putative limiting value of aqueous solutions of diethylaminoethanol[3]. As shown in Fig. 2, the pK of this group is, as a first approximation, exponentially related to sodium chloride concentration. A departure from this logarithmic relationship was observed in salt concentrations of 0.01 M or less, where continual pH drifts were encountered and reproducible results were difficult to obtain. This difficulty has previously been reported[3,5], and may be a consequence of poor access of the titrant to the hydrated but insoluble cellulose matrix under these conditions. Reproducible results were also difficult to obtain in saturated NaCl titrations.

The pK of the minor group (about 30%) is relatively insensitive to change in salt concentration and has a value of about 6, Table II. However the percent of this minor

TABLE II

EFFECTS OF SODIUM CHLORIDE CONCENTRATION ON THE OBSERVED pK AND THE AMOUNT OF THE MINOR ACID-TITRATABLE GROUP OF DEAE-CELLULOSE (WHATMAN DE-52)

NaCl (M)	*pK*		*Ave. pK*	*Ave. %*[a]
	Run 1	*Run 2*		
0.01	5.98	–	5.98	43
0.05	5.86	5.87	5.87	34
0.1	6.01	5.93	5.97	27
0.2	6.04	6.05	6.04	27
0.3	6.03	6.14	6.09	26
0.5	5.96	6.18	6.07	24
1.0	6.00	6.17	6.08	23

[a] Calculated as percent of total mequiv. titrated.

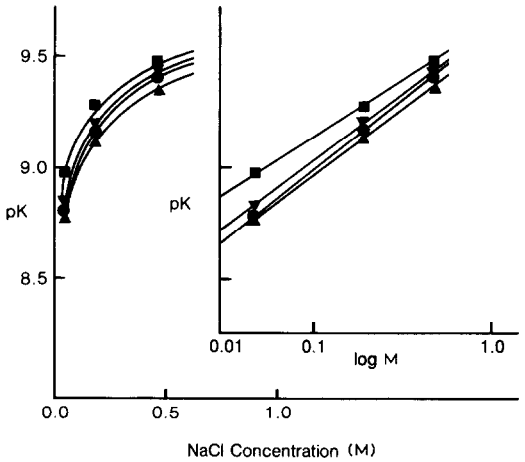

Fig. 3. Effect of increasing salt concentration on the pK values of various commercially available DEAE-celluloses. Values were determined as described in Figs. 1 and 2. pK values were averaged from 2 or 3 titrations. (▲) Sigma; (●) Mannex; (▼) Bio-Rad; (■) Whatman.

group, relative to total acid titratable groups, decreases from about 43% in dilute salt (0.01 M) to 23% in 1.0 M NaCl.

Several commercially available DEAE-cellulose preparations were similarly titrated and compared over a limited range of salt concentrations (Fig. 3). BioRad, Cellex-D, Mannex-DEAE, and Sigma DEAE-celluloses gave similar results. The difference in the slope (log plot) of DE-52 (Whatman) may represent differences in the preparation of this microgranular ion exchanger.

Because of differences in the ionic structure or local charge distribution of the hydrochloride form of DEAE-cellulose relative to the free tertiary amine, basic titrations were also carried out. Calculated pK values at various salt concentrations were very similar to those obtained with acid titrations, Table III. In fact, the relationship between these titration parameters in acid and base, for the major titratable group, was identical, *i.e.*, alkaline titration data were super imposable with those shown in Fig. 2. However the minor buffering group observed in acid titrations, which decreased dramatically with increasing salt concentration, was much less pronounced in basic titrations and disappeared entirely with increasing salt concentrations (data not shown).

TABLE III

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON THE OBSERVED pK OF THE BASE TITRATABLE GROUP OF DEAE CELLULOSE (WHATMAN DE-52)

NaCl (M)	*Corrected Cl^- (M)*	*Ave. pK*
0.05	0.047	9.00 ± 0.25
0.20	0.18	9.25 ± 0.06
0.50	0.44	9.44 ± 0.04

DISCUSSION

Acid–base titration has been used to characterize changes in charge distribution of DEAE-cellulose, as expressed by pK changes with increasing salt concentration. Although this salt dependence is well known[11,12] it has never really been studied in detail. In the majority of experiments described in this communication, Whatman DE-52 microgranular, preswollen ion exchanger (1.0 mequiv./g) was used. The ion exchange capacity calculated from potentiometric (0.96 $\pm$ 0.01 mequiv./g) and calorimetric acid titrations (0.99 $\pm$ 0.09 mequiv./g) agreed with the manufacture's value (1.00 mequiv./g), while Kjeldahl total nitrogen values (average of five separate determinations) were somewhat lower (0.90 $\pm$ 0.03 mequiv./g dry weight). We have consistently observed that the first of a repeated series of potentiometric acid titrations (using prewashing conditions described in Experimental) gives an exchange capacity significantly higher (10–15%) than subsequent titrations (*e.g.* 0.01 M NaCl: 0.472, 0.404, 0.415, 0.405, 0.418 mequiv.; 0.10 M NaCl: 0.530, 0.467, 0.471, 0.456 mequiv.). This phenomenon was observed for all concentrations of NaCl. It might simply be a consequence of residual NaOH contaminating DEAE-cellulose preparations after the final water wash (see Experimental), or perhaps an artifact caused by contaminating quaternary ammonium groups in the hydroxide form.

Several explanations have been given for the observed salt dependence of various ion exchanger materials, including the putative requirement for "bound" counter ions by "insoluble resins"[11]. If this explanation is correct, at pH values near the pK added salt would shift the equilibrium toward ionization, stabilizing the protonated form of the tertiary amine and shifting the pK to higher values. Another explanation is the putative "inaccessibility" of interior groups because of "electrostatic shielding" at low ionic strength[3,12]. The contribution of electrostatic interaction can be estimated using the semi-empirical equation of Kern[17], $\mathrm{pH} = \mathrm{p}K + n \log (1-\alpha)/\alpha$, where α is the degree of ionization and n the putative extent of electrostatic interaction, which should approach unity in dilute aqueous solutions. The potentiometric acid titration data for the major salt dependent ionic group does give a series of straight lines, as predicted by the Kern equation (Fig. 4). Values of n calculated from the slope at each salt concentration are shown in Table IV. This data suggests that in 0.2 M NaCl or above, where n is nearly unity, the DEAE-groups behave much like "soluble" amines with negligible electrostatic interaction. At lower salt concentrations, however, the value of n increases proportionally from 1.0 to about 1.3 or 1.4 in distilled water, suggesting a moderate dependence of pK on the degree of ionization.

Of practical importance is the exponential relationship between salt concentration and pK (Fig. 2). Salt gradient elution techniques common in ion exchange chromatography of proteins, have the potential of generating such pK changes and subsequent ionic strength or pH shifts[12,18,19]. The latter are easily observed in batch experiments by addition of salt to aqueous slurries of DEAE-cellulose at pH values near the pK. With increasing salt the limiting value of the pK is expected to approach that of soluble diethylaminoethanol (9.9) (ref. 3) as observed in Table I. Since elution characteristics of polyelectrolytes, such as many proteins, are very sensitive to small pH or ionic strength changes[7,14], salt dependent increases in pK could generate elution artifacts.

The pK of the minor titratable group (about 6.0, Fig. 1) varied little (less than

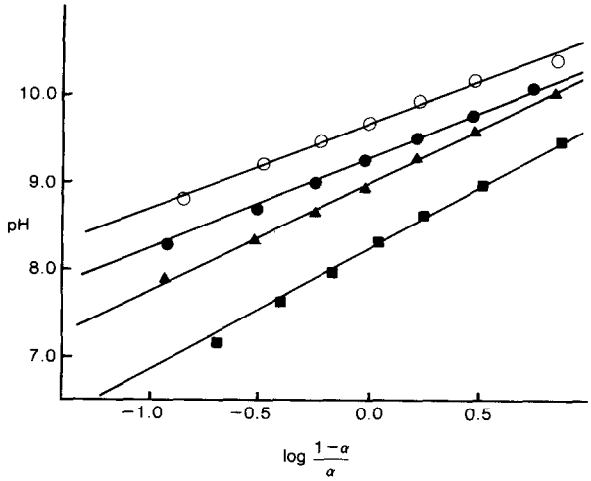

Fig. 4. The relationship between pH and the ionization of DEAE-cellulose, at various concentrations of sodium chloride. The degree of ionization (α) was assumed to be equal to the degree of neutralization or the fraction titrated. The extent of electrostatic interaction (n) was calculated from the slopes at various concentrations of NaCl, and is shown in Table IV[17]. (○) 2.00; (●) 0.20; (▲) 0.05; (■) 0.01 *M* NaCl.

4%) with increasing salt concentration, Table II. Although its fraction of the total potential ion exchange groups is significant in water or dilute salt (43%), this fraction decreases significantly with increasing salt concentration, Table II, and is hardly detectable in alkaline back titrations. This apparent minor group may be an artifact generated by non-equilibrium conditions in low salt. Continuous pH drifts observed with manual titrations at extremely slow flow-rates (one drop at a time), are consistent with this possibility. Such pH drifts were not observed in the absence of DEAE-cellulose. These observations suggest that a significant fraction of potential ion exchange

TABLE IV

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON THE ELECTROSTATIC INTERACTION (n) OF DEAE-CELLULOSE

Values were determined from acid titration data as shown in Fig. 4.

NaCl (M)	*pK*	*n*	r^{2a}
0.00	7.01	1.28	0.98
0.01	8.25	1.39	1.00
0.05	9.00	1.25	1.00
0.10	9.14	1.13	1.00
0.20	9.28	1.03	1.00
0.30	9.40	1.01	1.00
0.50	9.47	1.04	1.00
1.00	9.58	1.02	0.99
2.00	9.68	0.96	1.00
6.24 (satd.)	9.77	1.14	0.99

[a] r = simple correlation coefficient.

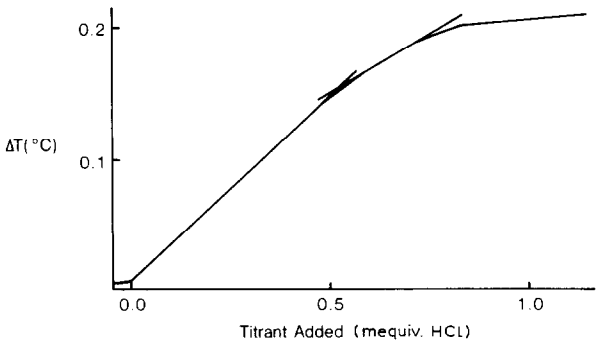

Fig. 5. Thermogram of the acid titration of DEAE-cellulose. DEAE-cellulose (0.84 g, Whatman DE-52) was titrated in water (35 ml) with HCl (0.531 *M*).

groups are sequestered in the cellulose matrix while in the neutral unprotonated form, perhaps stabilized by hydrogen bonding to neighboring glucose residues, and that optimum column performance can only be expected following ionization by an initial acid wash.

Thermograms obtained from preliminary calorimetric acid titration experiments (Fig. 5), also demonstrate the existence of two distinct groups, a major and a minor. However, in contrast to the results described above, sharp end points suggest rapid and complete reaction[20] of all acid titratable groups. The significance of this apparent contradiction is not obvious.

The existence, in some batches of DEAE-cellulose, of a minor ionizable group containing two closely spaced nitrogen atoms has been suggested[12]. The charge resulting from the addition of a proton to the first nitrogen would "oppose" proton addition to the second nitrogen. Such groups purportedly have salt dependent pK values[11], whereas the minor group in the titrations described above is relatively salt independent (Table II). It is significant that pK values calculated from base titration data are practically super-imposable with those from acid titrations shown in Fig. 2, and that the minor titratable group is hardly detectable in base titration curves, especially at higher ionic strengths (data not shown). This suggests that once protonated the putative sequestered groups tend to loose their identity and become indistinguishable from major ionic groups. Perhaps the ionic strength related improved resolution obtain during the first few hours with pellicular exchange packings[5] is also a consequence of unmasking sequestered groups. The disappearance of the minor group with increasing salt suggests structural changes of the ion exchange matrix, and resulting charge distribution changes. This might result in altered chromatographic behavior, as has been observed with changes in protein structure[13].

Preliminary titration experiments in aqueous solutions of multivalent salts ($MgCl_2$, Na_2SO_4) demonstrated that the salt dependence of pK is not a simple function of ionic strength (data not shown). Data was not superimposable with those obtained in NaCl when expressed either as ionic strength or molarity, except in the case of $MgCl_2$ when only chloride concentration was considered in relating pK to salt concentration.

In conclusion, several commercially available batches of DEAE-cellulose were

titrated with acid and base, in solutions containing increasing concentrations of NaCl. A near exponential relationship was found between the pK and the normality of sodium chloride. A major and a minor titratable group was found. The first group accounted for the observed salt dependent pK. The pK of the second group was relatively salt independent and its fraction of the total ionizable groups decreased with increasing concentration (from 43 to 0%). Potentiometric titrations in low salt concentration (0–0.01 M NaCl), and near the pK of the minor ionizable group, were characterized by a gradual pH drift, suggesting sequestered groups and lack of equilibrium. This is however inconsistent with preliminary calorimetric titrations which were characterized by thermograms having sharp end points.

ACKNOWLEDGEMENTS

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