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GEL CHROMATOGRAPHY OF INOSITOL POLYPHOSPHATES AND THE AVIAN HAEMOGLOBIN–INOSITOL PENTAPHOSPHATE COMPLEX

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SUMMARY

Gel chromatography of inositol polyphosphates has shown that a substantial anion exclusion effect exists which is diminished, but not necessarily eliminated, by concentrations of eluant electrolyte up to 2 *M*. Under the appropriate conditions of ionic strength and pH, gel chromatography provides a useful adjunct to the established fractionation procedures for inositol phosphates. It has been successfully used to demonstrate that myoinositol 1,3,4,5,6-pentaphosphate forms a strong ionic association with pigeon and chicken haemoglobins.

INTRODUCTION

Established methods for the fractionation of inositol polyphosphates include anion-exchange chromatography, paper chromatography, paper electrophoresis and fractional precipitation. These procedures have been reviewed by COSGROVE[1]. In addition moving paper electrophoresis[2] and thin-layer chromatography[3] have now been described.

Separations of a series of compounds by chromatography on a cross-linked dextran gel (Sephadex) have been reported for inorganic polyphosphates[4] and polynucleotides[5]. The present study records the distribution coefficients[6] ($K_d = (V_e - V_0)/V_t$, where V_e is the elution volume of the solute, V_0 is the void volume and V_t the volume of the stationary phase) of various inositol polyphosphates and some other reference compounds on a number of Sephadex gels. The variation of these K_d values with ionic strength and pH is examined. The application of gel chromatography to the elucidation of the ionic nature of the pigeon and chicken haemoglobin-inositol pentaphosphate complex is described.

EXPERIMENTAL

Materials

The Sephadex (Pharmacia AB) gels and columns used are shown in Table I.

TABLE I

SEPHADEX GELS AND COLUMNS

Sephadex grade	*Mesh size* (μ)	*Column parameters*[a]				*Sample* (*ml*)	*Fraction size* (*ml*)
		Length (*cm*)	V_t (*ml*)	V_0 (*ml*)	V_i (*ml*)		
G-200	140–400	36	1020	290	700	10	16.5
G-100	40–120	78	770	260	470	8	14.0
G-50	100–300	58	710	270	410	6	14.0
G-25	100–300	52	500	210	250	5	16.5
G-15	40–120	46	300	120	130	4	7.0

[a] For pH $<$ 13 and eluant salt concentrations $\leqslant$2 *M*.

The reference compounds, their methods of preparation or source and the symbols used in this paper are: myoinositol hexaphosphate[7], IP_6; myoinositol tripyrophosphate[7], $\pi_3\varnothing$; chicken blood myoinositol pentaphosphate[7], IP_5(CB); alkaline hydrolysis myoinositol pentaphosphate[2], IP_5(OH); myoinositol tetraphosphate[8], IP_4; myoinositol triphosphate[8], IP_3; myoinositol diphosphate[8], IP_2; myoinositol monophosphate[9], IP_1; glycerol phosphoryl myoinositol[10], GPI; myoinositol*, I; orthophosphate*, P_i; inorganic pyrophosphate*, PP_i; adenosine monophosphate*, AMP; adenosine triphosphate*, ATP; phosvitin[11], Pv; triethyl phosphate*, Et_3P; and fructose*, Fr.

Washed red blood cells were lysed and the haemolysates obtained by the DRABKIN procedure[12].

Methods

Columns were prepared and packed after equilibration with eluant, in accordance with manufacturers' directions. Lithium chloride was used as the eluant electrolyte to facilitate recovery of the ethanol-insoluble lithium phosphates[13].

The sample (200–300 μg P) was layered direct onto the filter paper or gauze disc that covered the gel surface and suitable fractions were collected. All runs were made at room temperature (20–27°). K_d values were calculated by use of Blue Dextran 2000 (Pharmacia AB) and tritiated water to determine V_0 and V_t, respectively.

Aliquots from each fraction were analysed for total phosphorus[14]. Inorganic phosphorus was determined by the ascorbic acid method[15], fructose was measured by the anthrone method[16] and inositol by periodate oxidation[17]. Haemoglobin absorbance was measured at 577 nm.

The location of the inositol pentaphosphate in haemolysate fractions was determined by precipitation of the acid-soluble phosphorus as the barium salt with subsequent electrophoresis of the anions in 0.1 *M* oxalate at pH 1.5 as previously described[7].

RESULTS AND DISCUSSION

Anion exclusion

The anomalous behaviour of ionic compounds of low molecular weight eluted

* Commercial products.

with water on Sephadex gels has been appreciated since the initial study of GELOTTE[6]. The nature of this effect has been greatly clarified by the work of NEDDERMEYER AND ROGERS[18], who found that irregular elution profiles of anions became symmetrical at 0.01 *M* eluant salt concentration and showed that the asymmetric profiles in distilled water could be attributed to a Donnan anion exclusion effect.

We have investigated the effect of eluant salt concentrations above that which is necessary to produce symmetrical profiles. Fig. 1 shows the results for the eluant salt molarities in the range 0.01 *M*–5 *M* for inorganic phosphate, pyrophosphate, myoinositol hexaphosphate and triethyl phosphate on Sephadex G-25.

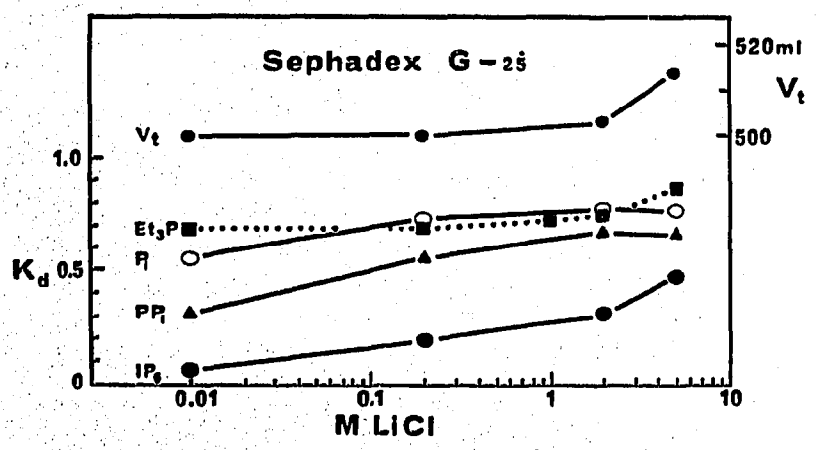

Fig. 1. Variation of the gel column (V_t) and distribution coefficients (K_d) of triethyl phosphate (Et_3P), inorganic phosphate (P_i), inorganic pyrophosphate (PP_i) and myoinositol hexaphosphate (IP_6) with the molarity of the eluant electrolyte (lithium chloride).

The uncharged molecules triethyl phosphate (Fig. 1), fructose (Table II) and myoinositol (Table III) have virtually constant K_d values at eluant electrolyte concentrations of 0.01–0.2 *M*. By contrast the anions of Fig. 1 each show a marked increase in K_d in this range. Iodide and sulphate anions[19] as well as polythymidylates[5] show analogous behaviour. Such results suggest the continued existence of a significant anion exclusion effect above the 0.01 *M* limit suggested by NEDDERMEYER AND ROGERS[18].

With eluant electrolyte concentrations above 2 *M* the situation becomes more complex. Both inorganic phosphate and pyrophosphate have constant K_d values, but the myoinositol hexaphosphate and triethyl phosphate show increased K_d values. It is not possible to decide between the anion exclusion and adsorption mechanisms in the case of the highly charged hexaphosphate but adsorption appears to be the only feasible explanation for the behaviour of the uncharged triethyl phosphate. Interpretation of the results is further complicated by an unexpected swelling of the gel matrix (V_t) above 2 *M* (Fig. 1).

The results from a variety of reference compounds on a range of Sephadex gels are listed in Table II and from this table it is again apparent that, for anionic compounds which are detectably excluded from the gel pores ($K_d < 0.8$) at 0.01 *M* eluant salt concentration, there is invariably an increase in K_d at higher salt concentrations.

Fig. 2 shows the effect of pH on K_d values in the presence of 0.1 *M* eluant salt. In the pH range 3–11 where the ionisation of carboxyl groups of the gel matrix and

TABLE II

DISTRIBUTION COEFFICIENTS OF REFERENCE COMPOUNDS ON SEPHADEX GELS

Grade	*Eluant molarity*	*Distribution coefficients (K_d) ± 0.05*						
		Pv	IP_6	PP_i	*ATP*	P_i	*Fr*	*AMP*
G-15	0.01	0.00	0.00	0.04	0.16	0.26	0.63	0.74
	0.20	0.00	0.06	0.25	0.35	0.40	0.65	1.00
G-25	0.01	0.00	0.06	0.31	0.60	0.56	0.75	0.91
	0.20	0.00	0.19	0.56	0.69	0.73	0.75	1.06
	2.00	0.00	0.30	0.67	0.93	0.77	0.81	1.30
	5.00	0.00	0.47	0.65	0.88	0.76	0.79	1.15
G-50	0.01	0.00	0.45	0.81	0.91	0.91	0.89	1.14
	0.10	0.00	0.61	0.83	0.93	0.89	0.93	1.14
G-100	0.01	0.03	0.79	0.97	—	0.97	0.97	1.07
G-200	0.01	0.21	0.90	—	—	0.98	0.98	—

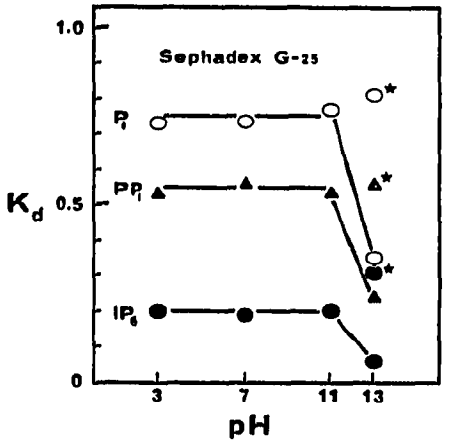

Fig. 2. Variation of the distribution coefficient (K_d) of inorganic phosphate (P_i), inorganic pyrophosphate (PP_i) and myoinositol hexaphosphate (IP_6) with the pH of the eluant electrolyte (0.1 *M* lithium chloride). Values with asterisks at pH 13 refer to an electrolyte concentration of 2 *M*.

the second dissociation of phosphate groups occurs, the incorporation of 0.1 *M* eluant salt is sufficient to suppress the expected fall in K_d values caused by the repulsion of these groups.

However, under strongly alkaline conditions (pH 13), where the ionisation of hydroxyl groups in the carbohydrate gel matrix is significant, a marked anion exclusion effect becomes apparent with a striking reduction in K_d values for the phosphates. If the concentration of lithium chloride in the eluant at pH 13 is now increased to 2 *M*, the K_d values marked by asterisks in Fig. 2 are obtained and the anion exclusion is again substantially repressed.

Molecular size

DETERMANN[20] has comprehensively reviewed the various empirical relationships between K_d values and molecular size parameters. In general there is an orderly

lowering of K_d values as a homologous series is ascended. Table III shows such an inverse relationship between the degree of phosphorylation of the inositol ring and the K_d value on two grades of Sephadex at two eluant salt concentrations. Similar results have been obtained by HOHN AND SCHALLER[5] for oligonucleotides and by OHASHI *et al.*[4] for inorganic phosphates.

TABLE III

DISTRIBUTION COEFFICIENTS OF MYOINOSITOL POLYPHOSPHATES ON SEPHADEX G-50 AND G-25

Grade	*Eluant molarity*	*Distribution coefficients* (K_d) ± 0.05									
		IP_6	$\pi_3\sigma$	$IP_5(OH)$	$IP_5(CB)$	IP_4	IP_3	IP_2	IP_1	*GPI*	*I*
G-25	0.01	0.06	0.06	0.06	0.06	0.12	0.19	0.31	0.44	0.53	0.75
	0.20	0.19	0.19	0.19	0.19	0.25	0.33	0.44	0.59	0.56	0.75
G-50	0.01	0.45	0.48	0.56	0.54	0.57	0.64	0.70	0.86	0.97	0.83
	0.10	0.61	0.61	0.62	0.64	0.68	0.75	0.79	0.86	0.97	0.86

Because both anion exclusion effects and molecular size contribute to the observed K_d values in Table III no attempt has been made to establish a simple logarithmic relationship between K_d and a molecular size parameter alone. Nevertheless by using a suitably calibrated column with appropriate eluant salt concentrations an unknown member of the series could be identified.

On the other hand Table III also indicates that Sephadex gel chromatography is unlikely to be of much value for separation of mixtures of closely related members of the inositol polyphosphate series which are more satisfactorily resolved by ion-exchange[1,21] and electrophoretic procedures[2].

Application

The major inositol polyphosphate which can be isolated from chicken blood by acidic protein precipitants is now known to be myoinositol 1,3,4,5,6-pentaphosphate[7]. Unsuccessful attempts to isolate the pentaphosphate by ultrafiltration of haemolysed red blood cells suggested that covalent or ionic linkages to some macromolecule may be present. A similar observation has been made for diphosphoglyceric acid in mammalian red blood cell haemolysates by SOLOMON *et al.*[22].

In order to examine whether or not the inositol polyphosphate was covalently or ionically linked to a macromolecule, both pigeon and chicken haemolysates were examined by gel chromatography.

The results for chicken blood haemolysate on Sephadex G-50 are shown in Fig. 3. A similar pattern was obtained for pigeon haemolysate. At 0.1 *M* eluant salt concentration and pH 7 (Fig. 3a), conditions of ionic concentration and pH which approximate to those of the red blood cell, there is a strong association of the inositol pentaphosphate with the pigeon and chicken haemoglobins. At high pH (Fig. 3b) or high eluant salt concentrations (Fig. 3c) the complex is dissociated, which indicates a strong ionic association is present under the conditions of Fig. 3a.

On Sephadex G-50 with 0.1 *M* eluant and pH 7 (Fig. 3a) the haemoglobin–inositol pentaphosphate complex is coincident with the void volume. To confirm that

the polyphosphate was associated with the haemoglobin and not some other protein, the haemolysate was examined on Sephadex G-100 under the same elution conditions. The inositol pentaphosphate and haemoglobin peaks were found to be coincident with $K_d = 0.31$.

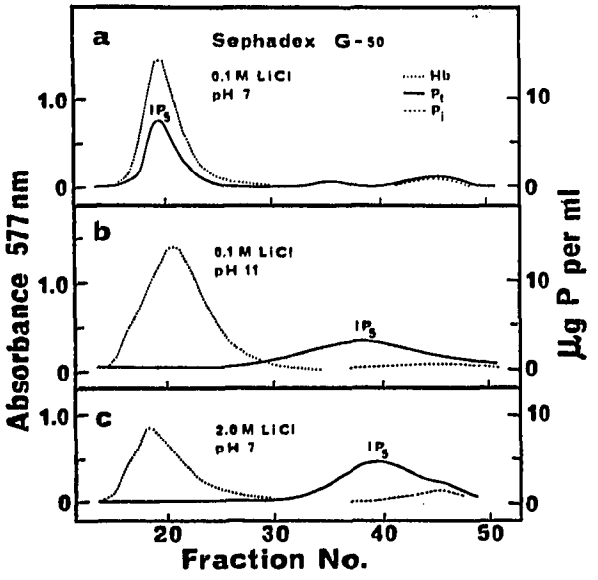

Fig. 3. (a) Assocation of the inositol pentaphosphate and chicken haemoglobin peaks at pH 7 and 0.1 *M* eluant electrolyte concentration. (b) Dissociation of the inositol pentaphosphate and haemoglobin peaks at high pH. (c) Dissociation of the complex by 2 *M* eluant electrolyte at neutral pH.

BENESCH AND BENESCH[23] have shown that the association of organic polyphosphates with both mammalian and avian haemoglobins has a considerable effect upon the oxygen affinity of the haemoglobin. In the case of diphosphoglyceric acid it is only the deoxyhaemoglobin which binds strongly to the phosphate under approximately physiological conditions. However the spectrum of the chicken and pigeon haemoglobin–polyphosphate complexes with pronounced bands at 538 and 577 nm indicates clearly that the inositol pentaphosphate binds strongly to the oxyhaemoglobin, and the binding of oxygen and inositol pentaphosphate to haemoglobin is not a mutually exclusive process as in the case of diphosphoglyceric acid[23].

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