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A RE-INVESTIGATION OF THE PHOSPHORYLATION OF DEXTRAN WITH POLYPHOSPHORIC ACID: EVIDENCE FOR THE FORMATION OF DIFFERENT TYPES OF PHOSPHATE MOIETIES

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ABSTRACT

The products of phosphorylation of dextran with polyphosphoric acid have been re-investigated by gel filtration, potentiometric titration, and ^{31}P-n.m.r. spectroscopy. Mainly (80–88%) alkyl phosphates are formed together with alkyl diphosphates and dialkyl phosphates, the percentages of which depended on the duration of phosphorylation. Mild acid treatment of the crude samples hydrolyzed the diphosphates and gave products with >95% of monophosphate structures.

INTRODUCTION

Dextran sulfate can be regarded[1] as an affinity labeling agent of human hemoglobin (Hb) since it can lower the affinity of the protein for oxygen in the same way as 2,3-diphosphoglycerate, the natural effector of Hb inside the red cell. Dextran polycarboxylates or phosphates also have the same effect on Hb[2].

This type of macromolecular effector could be of interest in the field of blood substitutes[3] since, by coupling them to human Hb, it should be possible to obtain conjugates with relatively high molecular weights and an oxygen affinity lower than that of Hb[2], characteristics which are commonly required for compounds designed for this use.

Various methods for preparing polysaccharide phosphates have been reported[4–7], including one[5] that was claimed to give well-defined monophosphates of dextran. We were not able to confirm these results and, since interpretation of the biological properties of the products necessitates a proper structural analysis, we have re-investigated this method of dextran phosphorylation.

EXPERIMENTAL

Materials. — Dextrans T10 ($\overline{M}_w$ 9900, $\overline{M}_n$ 5700) and T40 ($\overline{M}_w$ 39,000, $\overline{M}_n$

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21,200) were obtained from Pharmacia, and the products were analyzed after dehydration by freeze-drying. Tributylamine was distilled before use. *N,N*-Dimethylformamide (Prolabo) was of Rectapur quality. Dowex 50 X8-400 resin was purchased from Aldrich, and Ultrogel AcA 202 from IBF.

General methods. — Phosphate contents were determined by the method of Ames and Dubin[8]. Potentiometric titration of acidic phosphate-containing compounds was effected with an autotitrator. Sodium was determined with a flame spectrometer. ^{31}P-N.m.r. spectra were recorded for aqueous solutions (internal H_3PO_4), using a Bruker AM 400-MHz spectrometer. High-performance exclusion chromatography was carried out for derivatives of T40 dextran on a column (60 cm) of TSK G 3,000 SW (LKB) with 0.15M phosphate buffer (pH 7.2)–0.2M NaCl as eluent, at 42 mL/h, and refractometric detection. Derivatives of T10 dextran were eluted from a column (25 × 1 cm) of Lichrosorb Diol Si 100 (Merck) with 0.15M phosphate buffer (pH 7.2) at the same flow rate.

Phosphorylation of dextran. — The procedure was derived from that of Whistler and Towle[5]. Generally, polyphosphoric acid was mixed with a solution of tributylamine in *N,N*-dimethylformamide in ratios reported in the Results section. Freeze-dried dextran (1 g) was added slowly, and the mixture was protected from moisture and kept at 90° or 120° for several hours. The viscous solution was dropped slowly into 5 vol. of ethanol and precipitated by the addition of a few drops of saturated aqueous NaCl. The residue was collected by centrifugation, the pH of an aqueous solution was adjusted to 9.5 with M NaOH, the tributylamine was evaporated under reduced pressure, and the residual solution was filtered, dialyzed, concentrated, acidified to pH 2, and finally precipitated with ethanol. The precipitate was then freed from compounds of low molecular weight by chromatography on Ultrogel AcA 202, using 0.2M NaCl.

Part of each crude product was treated with Dowex 50 X8-400 (H^+) resin to give the acid form, and part was titrated with 0.1M NaOH under potentiometric control to give the sodium salt. Each product was immediately freeze-dried and stored dry.

Hydrolysis of phosphate linkages. — The pH of a solution of each dextran phosphate (3 g) in 0.1M HCl (50 mL) was adjusted to 1.0 and the solution was heated for 40 h at 50°. The resulting mixture was analyzed by high-performance gel filtration, and the contents of inorganic and total phosphates were determined as described above. Dextran and polyphosphoric acid were treated under similar conditions.

RESULTS

Synthesis of dextran phosphates. — With molar ratios r_1 (amine/acid) of 3 (polyphosphoric acid was considered as tetraphosphoric acid) and r_2 (acid/"anhydroglucose") of 1.45, and after heating for 6 h at 120°, the phosphorylated dextran T40 contained 3.5 mmol of P atoms/g of polymer (*i.e.*, 1.2 mol of P

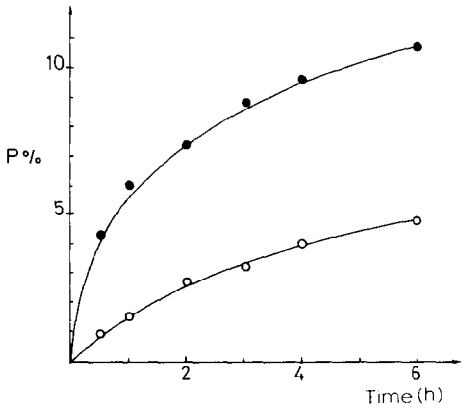


Fig. 1. Rate of phosphorylation of dextran (T40) at 90° (—○—) and 120° (—●—).

atoms/mol of "anhydroglucose" units). Increase of the ratio r_2 up to 3 did not greatly modify this result. The increase of the ratio r_1 up to 5 led to a less substituted polymer (1.5 mmol of P atoms/g of polymer). The influence of reaction time and temperature is shown in Fig. 1. At 120°, a brownish color was often observed and insoluble products were encountered at the longer reaction times.

Characterization of crude dextran phosphates. — Five samples of dextran phosphate, the characteristics of which are shown in Table I, were analyzed by high-performance gel filtration (Fig. 2). Each of the samples was eluted at lower volumes than the initial dextrans (T10 or T40), reflecting an increase in molecular weight, due to the introduction of phosphate groups and/or to cross-linking.

The various acid functions of linear polyphosphoric acids are not equivalent[9–11], each phosphorus atom bears a strongly acidic OH group, and the terminal phosphorus atom bears a weakly acidic OH group. Potentiometric titration thus permits discrimination between monoprotic phosphate (dialkyl esters,

TABLE I

PHOSPHORUS CONTENTS OF DEXTRAN PHOSPHATES[a]

Dextran	*Temperature of reaction (degrees)*	*Time of reaction (h)*	*Phosphorus content (%) of dextran phosphate*	
			Crude	*Acid-treated*
T40	90	5	5.4 (A)	4.8 (A′)
T40	120	1	7.2 (B)	6.6 (B′)
T40	120	3	11.0 (C)	10.3 (C′)
T10	120	1	5.7 (D)	5.1 (D′)
T10	120	3	8.7 (E)	8.1 (E′)

[a]Obtained with $r_1 = 3$ and $r_2 = 1.45$.

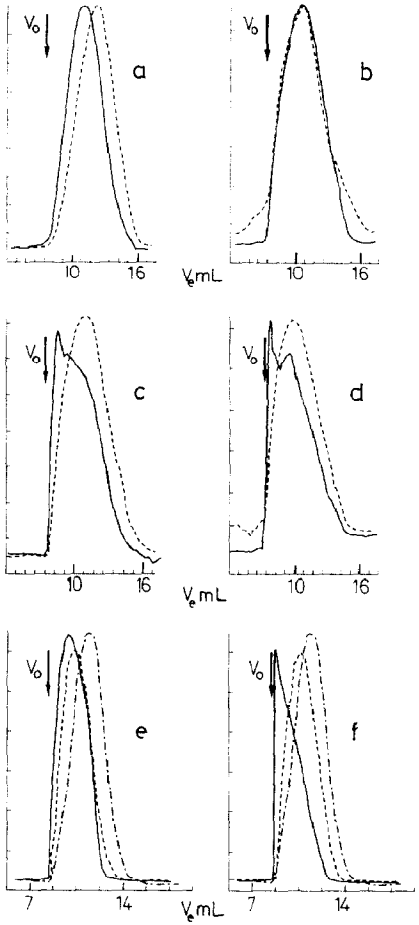


Fig. 2. High-performance gel-filtration chromatograms of dextrans and dextran phosphates before (—) and after (---) acid treatment. A column of TSK G 3,000 SW was used for the dextran T40 derivatives, and a column of Lichrosorb Diol Si 100 was used for the dextran T10 derivatives. (a) T40, (b) A,A', (c) B,B', (d) C,C', (e) D,D', dextran T10 (—·—·—), (f) E,E', dextran T10 (—··—··—); refractometric detection; V_0 is the void volume of the column; other conditions as described in the Experimental.

inner groups of polyphosphates) and diprotic phosphate (monoalkyl esters and end groups of polyphosphates). An example of a titration curve of the acidic form of a dextran phosphate is given in Fig. 3. The samples of dextran phosphate still contained Na^+ ions despite the treatment with the cationic resin, so that the molar ratios R_1 (weakly acidic OH/P) and R_2 (strongly acidic OH + Na^+/P) were taken into account. The data in Table II show that R_1 becomes <1 as the degree of phosphorylation increases, which means that the number of diprotic P atoms decreases in favour of monoprotic P atoms as phosphorylation proceeds; the R_2 values remain approximately constant, near 0.96–0.98.

Each of the dextran phosphates exhibited ^{13}P-n.m.r. spectra resembling that

TABLE II

TITRATION DATA[a] OF THE ACID FORM OF DEXTRAN PHOSPHATES

Dextran phosphate	*P (mmol/g)*	*Na^+ (mmol/g)*	R_1[a]	R_2[a]
A	1.9	0.17	0.93	0.98
A′	1.6	0.16	0.95	0.96
B	2.6	0.20	0.86	0.96
B′	2.3	0.15	0.93	0.95
C	3.9	0.24	0.82	0.96
C′	3.65	0.14	0.93	0.95
D	2	0.08	0.95	0.98
D′	1.8	0.08	0.98	0.97
E	3.2	0.32	0.89	0.98
E′	3.0	0.25	0.97	0.98
Polyphosphoric acid	12.1		0.48	0.96

[a]Precision, ±2%; R_1 = weakly acidic OH/P (mol/mol), R_2 = strongly acidic OH + Na^+/P (mol/mol); symbols as in Table I.

shown in Fig. 4, *i.e.*, a broad and complex band between 6 and 3 p.p.m. two bands centered at −5.4 and −9.4 p.p.m., 2 or 3 peaks between −10 and −11.5 p.p.m., and small peaks at 15.8 and 1 p.p.m. Taking into account that the chemical shifts of main bands (between 6 and 3 p.p.m.) were sensitive to pH (Fig. 5), these bands could be attributed, in agreement with the literature data[12–13], to alkyl phosphates. By comparison with data[12–13] for adenosine diphosphate and polyphosphoric acid, the

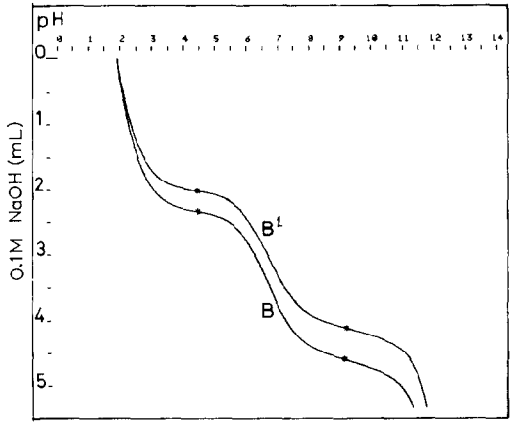


Fig. 3. Titration curves of dextran phosphates B and B′ (respectively, before and after acid treatment).

TABLE III

^{31}P-N.M.R. DATA OF DEXTRAN PHOSPHATES: PERCENTAGES OF STRUCTURES **1**, **2**, AND **5**[a]

Dextran phosphate	*P content*[b] *(mmol/g)*	*Structure* **1** *(3–6 p.p.m.)*		*Structure* **2** *(−5 to −9 p.p.m.)*		*Structure* **5** *(−10 to −11 p.p.m.)*	
		%[c]	*Involved P (mmol/g)*	*%*[c]	*Involved P (mmol/g)*	*%*[c]	*Involved P (mmol/g)*
A	1.8	81.6	1.3	14	0.45	1.4	0.02
A′	1.6	94.7	1.5	3	0.1	1.85	0.03
B	2.3	84	1.75	10.6	0.45	2.3	0.05
B′	2.15	95.7	2.05	0.7	0.03	2.7	0.06
C′	3.65	89.3	3.25	0	0	10	0.36
D	1.85	87.9	1.5	7.7	0.25	1.9	0.03
D′	1.6	96.6	1.55	0.8	0.025	1.75	0.03
E	2.8	88.4	2.35	5.6	0.3	5.5	0.15
E′	2.6	92.7	2.4	1.3	0.06	6	0.15

[a] **1**, R–O–P(=O)(OH)–OH; **2**, R–O–P(=O)(OH)–O–P(=O)(OH)–OH; **5**, R–O–P(=O)(OH)–O–R. [b] Refers to the sodium forms. [c] Calculated by assuming that the non-attributed n.m.r. peaks (0–3% of total P) corresponded to monophosphate species.

peaks near −5 and −9 p.p.m. could be attributed to the β and α phosphates, respectively, in structures such as R–O–P$_\alpha$(=O)(OH)–O–P$_\beta$(=O)(OH)–OH. The 2 or 3 peaks at −10 to −11 p.p.m. were not affected after treatment of the dextran phosphate with acid (see below) and were attributed to dialkyl phosphates which are hydrolyzed only under strongly acidic conditions[14–15]. The other peaks, each of which accounted for <3% of the total P, were not assigned.

The percentages of the various forms of phosphate present in the five samples of dextran phosphate are shown in Table III.

Characterization of dextran phosphates after acidic treatment. — Since –P–O–P– linkages are hydrolyzed under mild acidic conditions, the dextran phosphates were treated with 0.1M HCl for 40 h at 50° since, under these conditions, polyphosphoric acid was hydrolyzed completely.

The elution profiles of acid-treated dextran phosphates are shown in Fig. 2 and the P contents in Table I. The treatment with acid leads to a loss in phosphate groups (Table I) and a decrease in the average molecular weight (Fig. 2). For the derivatives of T10 dextran, the backbone of which was not hydrolyzed by the acid treatment, as proved by high-performance gel filtration (result not shown), the shift

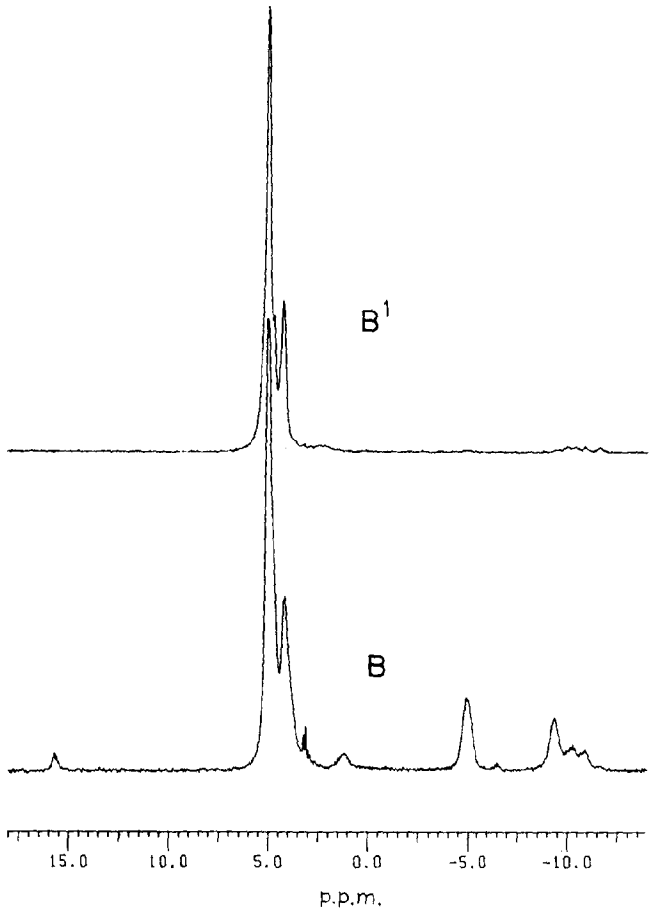


Fig. 4. ^{31}P-N.m.r. spectra of dextran phosphates B and B' (respectively, before and after acid treatment).

in elution volume is related directly to the loss of phosphate. For the derivatives of T40 dextran, the backbone of which was slightly broken by the acid treatment (Fig. 2), the shift in elution volume is the result of both processes.

Table II shows the values of the molar ratios R_1 and R_2 determined from the titration curves of the various acid-treated dextran phosphates. Whereas the R_1 ratios, corresponding to the crude dextran phosphates, were smaller than the R_2 ratios, particularly for the highly phosphorylated derivatives, after the acid treatment, R_1 has almost the same value as R_2, even for the dextrans with high P contents. This means that the acid treatment transformed species containing monoprotic P atoms into species with diprotic P atoms.

Each of the samples exhibited a ^{31}P-n.m.r. spectrum similar to that of sample

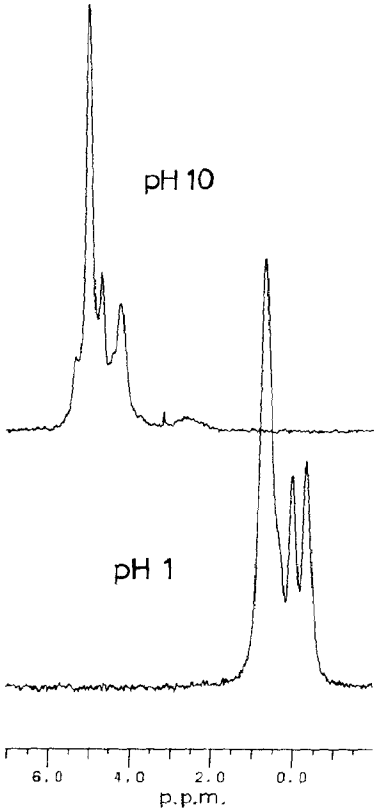
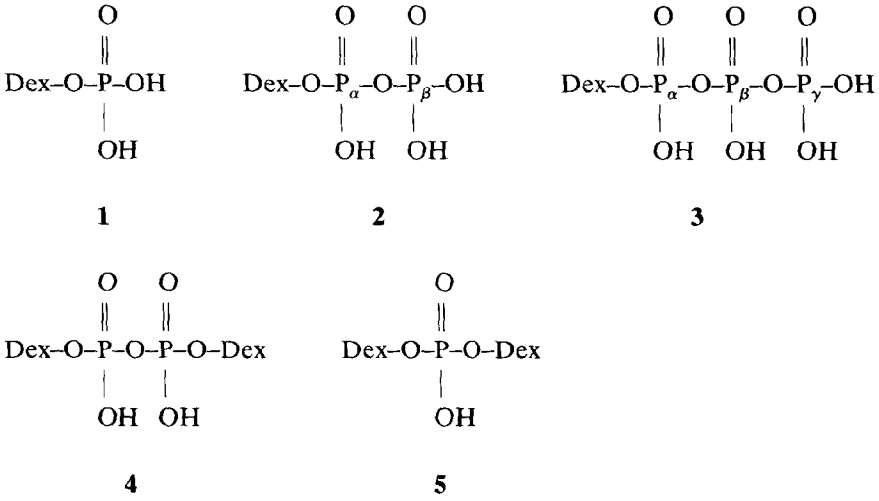


Fig. 5. Influence of pH on the chemical shift of the alkyl phosphate bands (compound E).

B′ (Fig. 4), *i.e.*, with the broad band between 6 and 3 p.p.m. corresponding to alkyl phosphates, no peak at −5 and −9 p.p.m., which proved that the –P–O–P– linkages have been hydrolyzed, and several peaks around −10 to −11 p.p.m. attributed to dialkyl phosphates. Table III shows the percentages of various forms of phosphate.

DISCUSSION

Following the mechanism of alcoholysis of polyphosphoric acid proposed by Clarke and Lyons[10], which involves a random cleavage of –P–O–P– bonds, two classes of species are expected to be formed by the reaction of this acid with dextran, namely, non-crosslinked chains such as **1–3** and intra- or inter-crosslinked species such as **4** and **5**.



The diprotic structure **1** always represented at least 80% of the total phosphates (Table III). However, contrary to the results published by Whistler and Towle[5] and according to the titration and n.m.r. data, monoprotic phosphates were produced, particularly **2** which represented up to 14% of the total phosphates (sample A). Structure **3** was not found since there was no ^{31}P resonance at ~ -20 p.p.m. Structures **4** and **5** could not be identified with certainty but, from the n.m.r. peaks around -10 to -11 p.p.m. for the products obtained before and after acid treatment, it was assumed that structures of type **5** were present, which are known to be resistant to acid, and the proportion increased with increase in the time of reaction. Moreover, the formation of such structures could account for the insolubility observed after long reaction times. When dextran phosphate C, which was highly phosphorylated (11% P) and was insoluble, was treated with acid, the ^{31}P-n.m.r. spectrum of the products contained peaks at -10 to -11 p.p.m. representing $\sim 10\%$ of the total phosphates (Table III). This finding further confirms the mechanism proposed by Clark and Lyons[10] since it demonstrates that, in the final stage of phosphorylation, the product must contain only alkyl phosphates and a small proportion of dialkyl phosphates. Treatment of crude dextran phosphates with acid hydrolysed the –P–O–P– linkages, as described by Crowther and Westman[16], as was proved by n.m.r. analysis. Monoprotic α phosphates were transformed into diprotic phosphates, which was confirmed by the titration results. However, it is possible that some monophosphates **1** were also hydrolyzed, as reported for alkyl phosphates[17]; the data in Table III suggest that this hydrolysis was very limited.

Thus, the phosphorylation of dextran by polyphosphoric acid yields a mixture of phosphate esters, the percentages of which depend on the reaction conditions, particularly the time of heating. According to the mechanism proposed[10], with short times of heating, alkyl phosphates and alkyl diphosphates were obtained, whereas,

for longer times, cross-linked structures, such as dialkyl phosphates, were assumed to be formed, leading to insolubility. As expected, the influence of this type of dextran phosphate on the oxygen-binding properties of Hb was found to depend on the nature of the ester phosphates present in the polymer. Thus, each sample caused a decrease in the oxygen affinity of Hb, which was always more important with the crude than with the acid-treated samples[18] for the same P/Hb molar ratio, due to the presence of polyphosphate moieties in the former samples. Such findings will have to be considered in interpreting the biological properties of covalent conjugates prepared for transfusion purposes[3] from Hb and dextran phosphates.

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