ACTA CHEMICA SCANDINAVICA 12 (1958) 1675—1689

High Molecular Weight Enzyme Inhibitors

III. Polyestradiol Phosphate (P.E.P.), a Long-acting Estrogen

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The preparation and some of the chemical and pharmacological properties of polyestradiol phosphate, a long-acting water soluble estrogen are described. Data are presented indicating that polyestradiol phosphate is a powerful inhibitor of several enzymes, such as acid and alkaline phosphatases and hyaluronidase.

The preparation of a large number of polymeric phosphoric acid esters and amides of polyphenols, aromatic polyamines and aminophenols has been reported previously [1,2]. These compounds have been found to exert a strong inhibitory effect on certain enzymes, *e.g.* hyaluronidase and acid and alkaline phosphatases. The present investigation deals with the preparation of high molecular weight polyesters of estradiol-17β and phosphoric acid as well as with a study of some chemical and biological properties of these polymers.

PREPARATION AND CHEMICAL PROPERTIES OF P.E.P.

In the Experimental Part a typical experiment on the preparation of polyestradiol phosphate (P.E.P. Standard) is described. The phosporylation was carried out in dry pyridine with phosphoryl chloride (1.1 mole) as phosphorylating agent. This product showed a greatly prolonged duration of estrogenic action, when administered parenterally to spayed mice [15].

In the determination of the molecular weight of P.E.P. Standard (ultracentrifuge) a value of 26 000 was obtained (see Experimental Part) indicating that the molecule contains approximately 80 estradiol moieties. The viscomet-

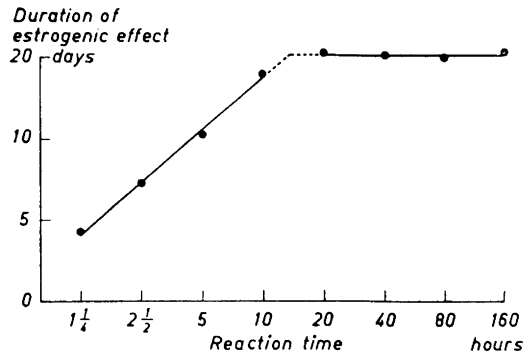

Fig. 1. Correlation between polymerisation time and duration of estrogenic effect of a single subcutaneous injection of 10 μg of polyestradiol phosphate (P.E.P.). Prolonged estrogenic effect was measured by the duration of cornified vaginal smears in spayed mice. The scales are logarithmic but are graduated in true units. Each point corresponds to 30 animals.

ric behavior of this substance is typical for linear polyelectrolytes [3]. The low value of intrinsic viscosity ($[\eta] = 0.04$) may be explained by the hydrophobic character of the estradiol moieties [4].

In order to follow the course of polymerisation, samples were withdrawn at different intervals and the products isolated. After dialysis the non-dialyzable material was investigated. From the data shown in Fig. 1, it appears

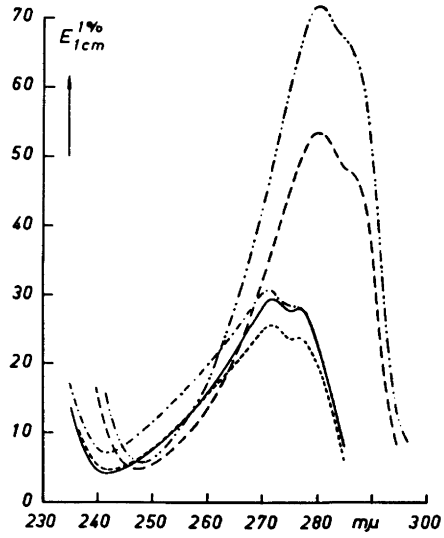

Fig. 2. Ultraviolet absorption spectra of estradiol (—..—..—), estradiol-17-phosphate (— — —), estradiol-3-phosphate (————), estradiol-3,17-diphosphate (. . . . .) and P.E.P. Standard (—.—.—) in 75 % aqueous ethanol.

Table 1. Influence of polymerisation time on the properties of polyestradiol phosphate (P.E.P.).

P.E.P. [a]	Reaction time, hours	% Non-dialyzable $P_{org.}$	% P[b]	% C[b]	% H[bc]	Molar P/Estradiol[d]	% Free hydroxyl groups[e]	λmax.[f] (mμ)
I	1 1/4	37[g]	8.2	—	—	—	26	279
II	2 1/2	42[g]	8.7	—	—	—	22	278
III	5	53[i]	8.8	61.3	7.30	1.00	12	278
III B[h]	5	44[i]	10.9	56.3	6.88	1.35	0	271
IV	10	66	9.3	—	—	—	9	272
V	20	81	9.4	60.4	7.05	1.08	4	272
VI	40	87	9.6	61.7	6.93	1.08	0	271
VII	80	88	9.6	—	—	—	0	271
VIII	160	90	9.5	60.1	6.98	1.10	0	271
P.E.P.-Standard	70	88—90	9.5	62.2	6.88	1.06	0	271

a) In these experiments the samples were hydrolyzed and worked up in the same way as P.E.P. Standard with the exception that the precipitation with HCl was made without ethanol.
b) Calculated on P.E.P., free from moisture, pyridine and chlorine.
c) % H somewhat higher than the value, which can be calculated (from % C, % free hydroxyl groups and % acid groups) possibly due to small amounts of firmly bound water.
d) Estradiol calculated from % C.
e) According to B. Baggett *et al.*[20] Calculated as % of 'estradiol oxygens'.
f) In 75 % ethanol. P.E.P. was dissolved in a small amount of 0.1 N NaOH, ethanol added, and then neutralized with 0.1 N HCl. Immediate reading.
g) Dialyzed in the presence of pyridine in order to avoid precipitation.
h) "Rephosphorylated" with excess $POCl_3$ in pyridine before hydrolysis and dialysis.
i) If % non-dialyzable is calculated on estradiol, the corresponding figures are, 54 % (III), and 52 % (III B).

that a reaction time of 20 h or more will yield polymers exhibiting maximal duration of estrogenic effect. Likewise it appears from Table 1 that after a reaction time of about 20 h the reaction is nearly complete. After this time practically no free hydroxyl groups could be demonstrated in the non-dialyzable material, while for instance after 75 min, 25 % of the hydroxyl groups originally present were free. In an attempt to assess the relative proportions between free 3-hydroxyl and free 17-hydroxyl groups in the polymer, the ultraviolet absorption spectra were studied.

The ultraviolet absorption spectra of estradiol, estradiol-3-phosphate, estradiol-17-phosphate, estradiol-3,17-diphosphate as well as that of the P.E.P. Standard are given in Fig. 2. When estradiol-3,17-diphosphate and estradiol-17-phosphate were mixed in such a proportion as to give the same percentage of free hydroxyl groups as found in the different polyestradiol phosphates (Table 1), practically identical ultraviolet absorption curves were obtained, indicating that the free hydroxyl groups are predominantly in the 3-position.

In order to investigate the stability of P.E.P., hydrolysis experiments were carried out in aqueous solution at 98° and at various pH values. The rate of hydrolysis was followed by determining the amount of inorganic phosphorus (P_o) liberated. The formation of P_o was most rapid at pH values between

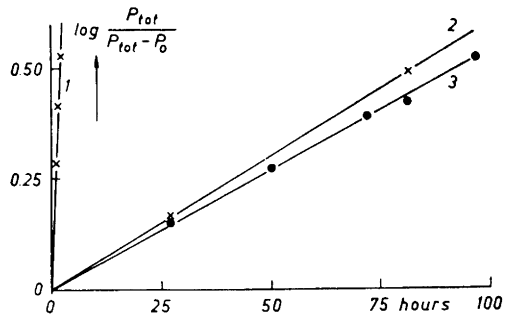

Fig. 3. Hydrolysis of estradiol phosphates in 0.1 M acetate buffer at pH 5.2 (98°). Rates of hydrolysis determined by analysis for liberated phosphoric acid (P_o). 1: Estradiol-3-phosphate ($k = 0.64$ h^{-1}). 2: Estradiol-17-phosphate ($k = 0.014$ h^{-1}). 3: P.E.P. Standard ($k = 0.013$ h^{-1}). As to P.E.P. Standard, 29 % of its phosphorus content is calculated as P_{tot}.

5 and 6; after 50 h 13.5 % of the phosphorus originally present was split off (Fig. 3). In 0.2 N sodium hydroxide solution the formation of P_o took place very slowly (< 1 % P_o, 81 h, 98°).

Furthermore it was found that following hydrolysis at different pH-values (98°, 81 h) the antihyaluronidase activity was practically unchanged within the investigated range (from pH 3.1 to 0.2 N sodium hydroxide solution). In another experiment, using the same conditions (pH 7.1), the duration of estrogenic effect was not changed significantly (potency: 88 % of that of P.E.P. Standard, with fiducial limits ($P = 0.05$) at 75 and 103 %). Under these conditions no free estradiol could be found, not even after a hydrolysis time of 270 h at pH 5.2.

We have also studied the rate of hydrolysis of the different monomeric estradiol phosphates (Fig. 3). In all cases the hydrolysis was found to follow a first order reaction. The period of half life (pH 5.2; 98°) for estradiol-3-phosphate was found to be 1.1 h and for estradiol-17-phosphate 50 h. In case of estradiol-3,17-diphosphate the experimental values were identical with those calculated from the 3- and 17-monophosphate values. The diphosphate is practically stable in 0.1 N sodium hydroxide solution (81 h, 98°) and similarly to the P.E.P. Standard, it has a maximal rate of hydrolysis between pH 5 and 6.

The fact that no free estradiol was found during the hydrolysis of P.E.P. seems to indicate that the liberated P_o is formed from endstanding, primary phosphoric acid esters only.

Even after a very long time (270 h, 98°, pH 5.2) only 28—30 % P_o was found. From Fig. 3 it is evident that the hydrolysis of P.E.P. follows the same course as that of estradiol-17-phosphate. It seems also that the percentage of primary 17-phosphate in P.E.P. lies between 25 and 28 and that the proportion of primary 3-phosphate is small.

From what is said above and from the fact that the free hydroxyl groups found at the beginning of the phosphorylation seem to be in the 3-position,

it seems likely that the first reaction step involves predominantly phosphorylation of the 17-hydroxyl groups yielding estradiol-17-dichlorophosphinate, and thus the polymerisation will take part mainly *via* the 3-hydroxyl groups. In order to confirm this assumption, a phosphorylation was carried out in the same way as described for the P.E.P. Standard, and the reaction mixture hydrolysed after 5 min. The pyridine was removed *in vacuo* (alkalization) and the resulting solution precipitated with hydrochloric acid without dialysis. Analysis of the precipitate and of the filtrate, respectively, showed that 84 % (0.84 mole) of the estradiol had reacted with 79 % (0.87 mole) of the phosphoryl chloride. In titration experiments only primary esters could be demonstrated. The rate of hydrolysis (pH 5.2; 98°) was the same as that of estradiol-17-phosphate. It seems thus very likely that the first step involves a phosphorylation of the 17-hydroxyl group.

Titration experiments with P.E.P. Standard (see Experimental Part) indicate the occurrence of 30 % monoester, 60 % diester and 10 % triester. This latter fact also points to a certain branching of the polymer. The value of 30 % monoester agrees well with that (28—30 %) found in the hydrolysis experiments.

EXPERIMENTAL

A. Preparation of estradiol phosphates

1. Preparation of polyestradiol phosphate. Estradiol-17β (27.2 g) was dissolved in dry pyridine (600 ml) in a flask fitted with a mechanical stirrer, a thermometer, a dropping funnel and a $CaCl_2$-tube. The flask was surrounded by an ice and salt mixture and when the temperature reached $-10°$ to $-12°$ a solution of phosphoryl chloride (10.1 ml) in dry pyridine (80 ml) was added in the course of 25 min. The temperature of the reaction mixture was then allowed to rise to room temperature. The stirring was stopped and the reaction mixture kept at room temperature for another 70 h. (After 30 to 50 h standing the solution may change into a jelly.) 250 g of crushed ice was then added with vigorous stirring and the mixture allowed to stand over night. 2 N NaOH (225 ml) was added and most of the pyridine evaporated *in vacuo*. The rest was made up to a volume of 2 000 ml by water and the pH adjusted to 7.0 to 7.5. The solution was dialyzed against dist. water (cellophane tube, several days). To the content of the tube containing 90 % of the phosphorus originally present, the same volume of ethanol was added and the solution poured with stirring into a mixture of 5 N HCl (200 ml) and ethanol (400 ml). The resulting white precipitate was then filtered and washed with 30 % aqueous ethanol and finally with water. Yield (after drying *in vacuo* over phosphorus pentoxide) about 30 g. Melting range 195—202°. A number of different batches, prepared according to the above mentioned procedure were mixed and the resulting mixture is referred to as P.E.P. Standard *.

Analysis: (P.E.P. Standard)

Moisture: (*in vacuo* 100°) 3.9 %.

Pyridine: (determined spectrophotometrically after alkalization and distillation) 0.3 %.

Chlorine: None.

Phosphorus: (calculated on dried and pyridine free sample) 9.5 %. $[[\alpha]_D = +40°$ ($c = 1$, in pyridine-water 1:1).]

* Samples of this have been distributed to different centra as a reference standard for the bioassay of long-acting estrogens. Such standards can be obtained on request from the Steroid Reference Collection, Postgraduate Medical School, London W 12, and from AB Leo, Hälsingborg, Sweden.

P.E.P. is very soluble in aqueous pyridine, soluble in aqueous alkali and very slightly soluble in ethanol, ethanol-water (1:1), water, dioxane, acetone and chloroform.

Molecular weight. This has been determined on the sodium salt of P.E.P. at pH 7.5 in 0.5 M NaCl solution. The sedimentation constant, determined in a Spinco ultracentrifuge model E with a synthetic boundary cell was found to have the same value at P.E.P. concentrations 1 and 0.5 %, $S_{20} = 2.16 \times 10^{-13}$ *.

The diffusion coefficient (moving boundary method using area and height of diffusion curves) was found to be $D_{20} = 5.8 \times 10^{-7}$ and the partial specific volume $V_{20} = 0.65$. The molecular weight was calculated to be about 26 000.

Viscosity measurements. These were made in an Ubbelohde viscometer at 25° ± 0.01°. The intrinsic viscosity $[\eta]$ has been determined in 0.25 M NaCl solution at pH 7.5. The value of $[\eta] = 0.04$ has been obtained by extrapolating η_{sp}/c to infinite dilution (c in g/100 ml).

Potentiometric titration. P.E.P. Standard was dissolved in an excess of 0.1 N NaOH in the presence of 0.1 M NaCl and titrated with 0.1 N HCl. Inflexion points were found at pH 9.4 and 5.5 respectively. The titrations show 0.90 strongly acid groups and a total number of 1.20 acid groups per phosphorus atom.

The different monomeric estradiol phosphates were dissolved in ethanol-water (1:1) and titrated with 0.02 N NaOH.

2. Preparation of estradiol-3-phosphate. Estradiol-17-acetate (1.5 g) was dissolved in dry pyridine and the solution was added drop by drop with shaking to a solution of phosphorus oxychloride (1.3 ml) in dry pyridine at −10°. After standing for one hour at this temperature the reaction mixture was hydrolyzed with crushed ice. Most of the pyridine was evaporated *in vacuo*. In order to hydrolyze the 17-acetate, 2 N NaOH (50 ml) was added. After standing for 1 h at room temperature the remaining pyridine was removed by shaking several times with ether and the solution poured with stirring into 5 N HCl (50 ml). The resulting precipitate was filtered, washed with 0.2 N HCl and finally with a small quantity of water. Yield after drying in vacuo: 1.6 g. M. p. 212–214°. Found: P 8.9; equiv. wt 177 (as a monobasic acid) and 174 (as a dibasic acid). Calc. for $C_{18}H_{25}O_5$ P: P 8.8; equiv. wt 176.]

3. Estradiol-17-phosphate was prepared in the same way as the 3-phosphate from estradiol-3-acetate (1.5 g). Yield 1.6 g. M. p. 181–183°. [Found: P 9.0; equiv. wt 174 (as a monobasic acid) and 170 (as a dibasic acid). Calc. for $C_{18}H_{25}O_5$ P: P 8.8; equiv. wt 176.]

4. Estradiol-3,17-diphosphate was prepared as above from estradiol (2.5 g) and phosphorus oxychloride (7.5 ml). After 1 h at −10° the mixture was maintained at room temperature for another 3 h. Yield 2.7 g. M. p. 179–181°. [Found: P 13.6; equiv. wt 118 (as a dibasic acid) and 115 (as a tetrabasic acid). Calc. for $C_{18}H_{26}O_8P_2$: P 14.3; equiv. wt 108.] Discrepancy probably due to firmly bound water.

B. Antienzymic properties

The enzymes studied were acid phosphatase, alkaline phosphatase and hyaluronidase.

1. *Acid phosphatase* **. Human seminal acid phosphatase was selected for the purposes of this study in view of its high enzymic activity.

Enzyme. A pool of human seminal fluid was used without purification. Semen was used in a dilution of 1:2 000.

Substrates. a. Phenyl phosphate and *b. p*-nitrophenyl phosphate were used.

Analytical methods. a. Phenol was estimated by Folin's reagent as described by Buch and Buch [5]. Inorganic phosphate was determined according to the method of Martin and Doty [6].

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* These measurements have been carried out in the Carlsberg Laboratories, Copenhagen, by the courtesy of Professor K. Linderstrøm-Lang.

** The experiments with acid phosphatase using phenyl phosphate as substrate have been carried out in collaboration with Dr. C.-G. Beling. A detailed report of the kinetics and inhibition of this enzyme by different polymeric phosphates will be published elsewhere.

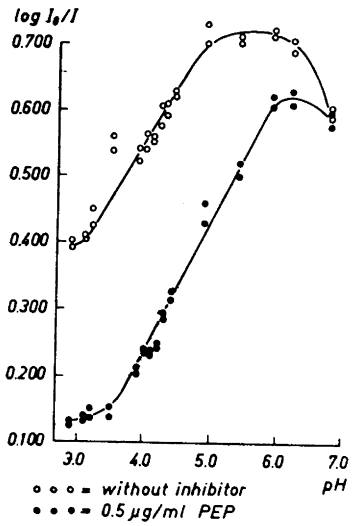


Fig. 4. Relationship between pH and inhibition of human seminal acid phosphatase by 0.5 µg/ml polyestradiol phosphate. 0.15 M acetate buffer, 37°, 8 min. incubation. Substrate: 2×10^{-3} M phenyl phosphate.

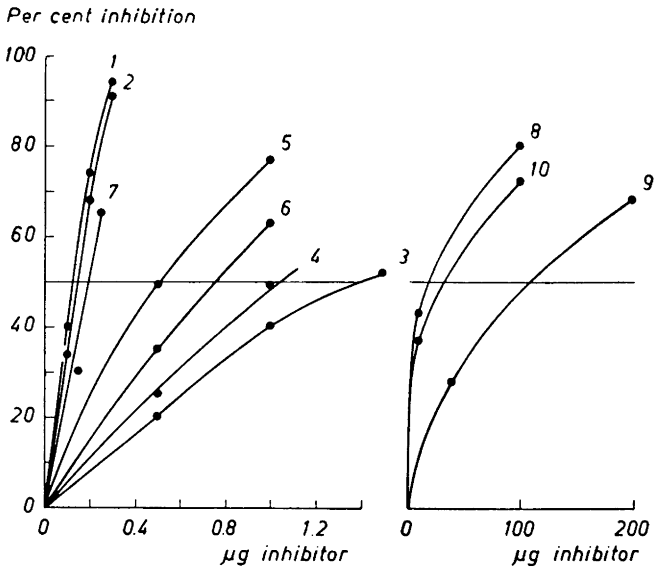


Fig. 5. Inhibition of seminal acid phosphatase by different batches of phosphorylated estradiol at pH 4.5 (acetate buffer). Substrate, *p*-nitrophenyl phosphate. Incubation time 30 min. at 37.5°. 1. P.E.P. Standard, 2. P.E.P. VI + P.E.P. VII (1:1), 3. P.E.P. I, 4. P.E.P. II, 5. P.E.P. III, 6. P.E.P. III B, 7. P.E.P. Standard after hydrolysis (81 h, pH 5.2, 98°), 8. Estradiol-3,17-diphosphate, 9. Estradiol-17-phosphate, 10. Estradiol-3-phosphate.

For further particulars consult Table 1.

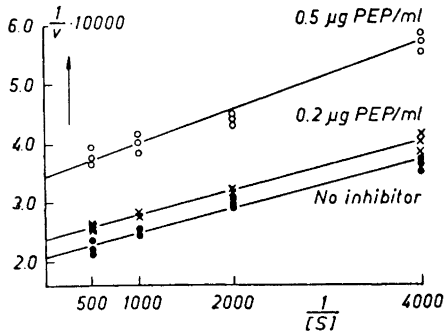


Fig. 6. Relation between acid phosphatase activity and substrate concentration in the presence and absence of polyestradiol phosphate. Activity is expressed as the reciprocal of the amount of phenol liberated in 8 min at 37°. 0.15 M acetate buffer. Substrate: phenyl phosphate, pH 4.5.

b. The method devised by Bessey, Lowry and Brock [7], for the determination of serum alkaline phosphatase was used. The *p*-nitrophenol was determined directly in a spectrophotometer (absorption maximum: 410 mμ).

Conditions of enzyme experiments. a. Enzyme activity was generally estimated within the pH range 3.0 to 6.8, using a 0.15 M acetate buffer, a substrate concentration of 2×10^{-3} M, and a short incubation time (generally 4 or 8 min at 37°).

b. Determinations were carried out at pH 4.5. Reaction volume 1.5 ml (0.5 ml 0.3 M acetate buffer, 0.5 ml 16 mM disodium *p*-nitrophenyl phosphate *, 0.1 ml enzyme). Incubation time 30 min at 37.5°.

Results. The inhibition of seminal acid phosphatase by P.E.P., is shown in Fig. 4. It appears from this figure that the inhibition is most marked on the acid side of the pH-optimum, and that no inhibition occurs at pH 6.8. The inhibition of acid phosphatase at pH 4.5, by different batches of P.E.P. as well as by estradiol-3-phosphate, estradiol-17-phosphate and estradiol-3,17-diphosphate is presented in Fig. 5. It appears from the data of Fig. 5 that estradiol monophosphates and also the diphosphate are much weaker inhibitors than the different P.E.P. preparations.

Reversibility. The enzyme inhibition by P.E.P. could be reversed by small amounts of basic proteins, *e. g.* protamine sulphate.

Type of inhibition. In order to study the type of inhibition, the concentrations of the substrate and inhibitor (P.E.P.) were varied at constant concentration of the enzyme. The typical Lineweaver plots obtained [8] are shown in Fig. 6. The data indicate that the inhibition is not substrate competitive. The same type of inhibition was obtained when *p*-nitrophenyl phosphate was used as a substrate.

2. *Alkaline phosphatase.* Alkaline phosphatase was measured by the method of Bessey, Lowry and Brock [7], using *p*-nitrophenyl phosphate as substrate.

Enzyme. The enzyme was the purified commercial product of Sigma Chemical Co. (St. Louis).

Reagents. 16 mM disodium *p*-nitrophenyl phosphate (Sigma Chemical Co.) in 0.001 N HCl. 0.1 M glycine buffer **. This was prepared from 7.50 g of glycine, 95 mg (0.001 M) of $MgCl_2$, about 80 ml of 1 N NaOH and water to 1 l. Buffered substrate: This consists of equal volumes of the two reagents.

Conditions of enzyme experiments. Determinations were mainly carried out at pH 9.3. The inhibition by the P.E.P. Standard was also investigated within the pH range 7.0—

* This is a 0.42 % solution of the Sigma 104 phosphatase substrate.

** Since it has been suggested that the use of glycine as buffer inhibits the enzyme activity [22], parallel experiments were run using glycine and 2-amino-2-methyl-1-propanol (1 M), respectively. Under our experimental conditions the use of these two buffers gave identical results.

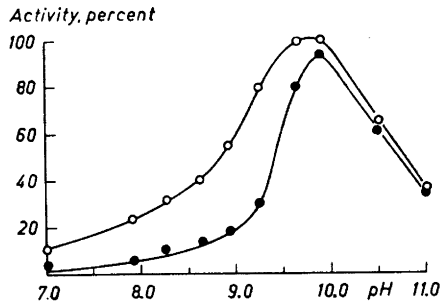


Fig. 7. Relationship between pH and inhibition of the enzymic hydrolysis of *p*-nitrophenyl phosphate by polyestradiol phosphate at 37.5°. 30 min incubation. Substrate concentration 3.3 × 10^{-4} M. Alkaline phosphatase concentration 13.3 μg per ml. O——O——O without inhibitor, ●——●——● 15 μg P.E.P. Standard per ml.

11.0. Reaction volume 1.5 ml (1 ml of the buffered substrate, 0.1 ml enzyme), incubation time 30 min at 37.5°. Enzyme concentration 13.3 μg per ml.

Results. From the relationship between pH and inhibition of the enzymic hydrolysis of *p*-nitrophenyl phosphate by P.E.P., it appears (Fig. 7), that inhibition occurs on the acid side of the pH-optimum only. A similar type of inhibition of alkaline phosphatase by estradiol-3,17-diphosphate, or by polyphloretin phosphate has been reported previously [1,9].

The inhibition of alkaline phosphatase at pH 9.3 by different batches of P.E.P. as well as by estradiol-3-phosphate, estradiol-17-phosphate and estradiol-3,17-diphosphate is presented in Fig. 8. A comparison of the data of Figs. 5 and 8 reveals that estradiol-3,17-diphosphate is a much stronger inhibitor of alkaline phosphatase than of acid phosphatase.

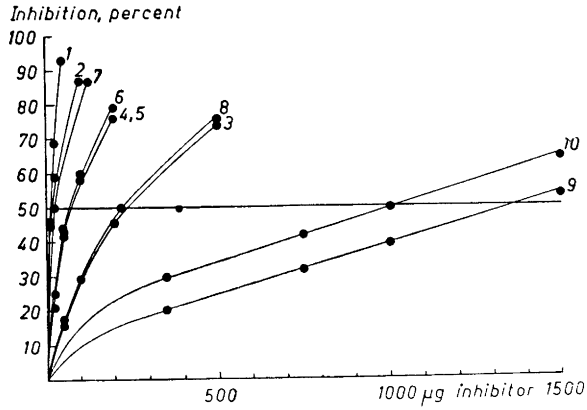


Fig. 8. Per cent inhibition of alkaline phosphatase by different batches of phosphorylated estradiol at pH 9.3. Enzyme concentration 13.3 μg per ml. Substrate, *p*-nitrophenyl phosphate. Glycine buffer with $MgCl_2$, pH 9.3. Incubation time 30 min at 37.5°. 1. P.E.P. Standard. 2. P.E.P. VI + P.E.P. VII (1:1). 3. P.E.P. I. 4. P.E.P. II. 5. P.E.P. III. 6. P.E.P. III B. 7. P.E.P. Standard after hydrolysis. 8. Estradiol-3,17-disphosphate. 9. Estradiol-17-phosphate. 10. Estradiol-3-phosphate.

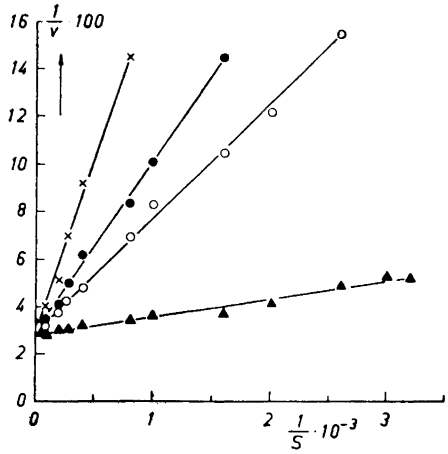


Fig. 9. A double reciprocal plot of the effect of polyestradiol phosphate (P.E.P. Standard) upon the velocity of alkaline phosphatase action at various substrate concentrations. The effect of 3.3 μg (○), 6.6 μg (●), and 10 μg of Polyestradiol phosphate [P.E.P. Standard] (×) upon the action of 13.3 μg alkaline phosphatase at pH 9.3 and 37.5° for 30 min. in the presence of various amounts of *p*-nitrophenyl phosphate. Filled triangles (▲) indicate enzyme activity in the absence of inhibitor.

Reversibility. Similarly to the case of acid phosphatase, the inhibition of alkaline phosphatase could also completely be reversed by basic proteins.

Type of inhibition. 3.3, 6.6 and 10 μg of P.E.P. Standard were added to solutions containing 13.3 μg of enzyme, and 0.27—27 μ moles/ml of *p*-nitrophenyl phosphate. A double reciprocal plot of velocity *versus* substrate concentration is shown in Fig. 9. It appears from the figure that the inhibition is of a competitive nature.

These findings are different from previous observations on polyphloretin phosphate [1], which study, however, was carried out at a lower pH (7.5). Experiments were therefore

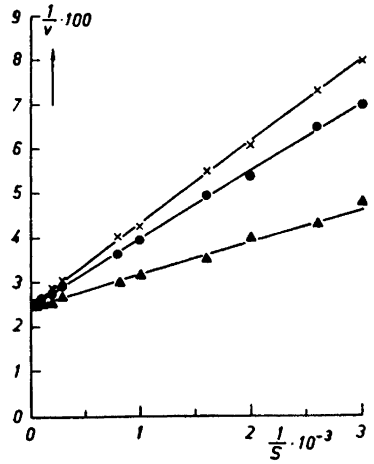


Fig. 10. A double reciprocal plot of the effect of polyphloretin phosphate (P.P.P). upon the velocity of alkaline phosphatase action at various substrate concentrations. The effect of 3.3 μg (●), and 16.7 μg (×) of P.P.P. upon the action of 13.3 μg alkaline phosphatase at pH 9.3 and 37.5° for 30 min. in the presence of various amounts of *p*-nitrophenyl phosphate. Filled triangles (▲) indicate enzyme activity in the absence of inhibitor.

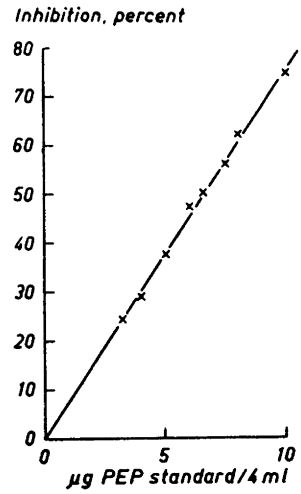


Fig. 11. Inhibition of hyaluronidase by polyestradiol phosphate (P.E.P. Standard). The effect of various amounts of inhibitor upon the action of 5 I.U. hyaluronidase per 4 ml at pH 7.0 and 37.5°.

carried out with polyphloretin phosphate at pH 9.3 and with *p*-nitrophenyl phosphate as substrate. It appears (Fig. 10) that the inhibition under these conditions is substrate-competitive. Further data on this problem will be reported in a forthcoming publication, dealing with polystilboestrol phosphate.

3. *Hyaluronidase.*

Bovine testicular hyaluronidase. The highly purified enzyme was prepared from bull testes according to Högberg [10] and assayed 90 000 I.U./mg nitrogen. Before use the enzyme was dissolved in water and the solution was stabilized with 2 µg methylgelatine (Leo) per I.U. of enzyme. 5 I.U. enzyme was used for the test.

Hyaluronic acid. The substrate, hyaluronic acid, was prepared from human umbilical cords and contained 3.39 % N, 10.3 % acetyl, 42.9 % glucosamine and 46.0 % glucuronic acid. Mc Ilvaine citrate-phosphate buffer, pH 7.0, containing sodium chloride was used

Table 2. The effect of polymerisation time upon the inhibition of phosphatases and of hyaluronidase by different batches of phosphorylated estradiol.

Inhibitor	No. in Figs. 5 and 8	Rel. amount of inhibitor necessary for 50 % inhibition		
		Hyaluronidase	Alkaline phosphatase	Acid phosphatase
P.E.P. Standard	1	1.0	1.0	1.0
P.E.P. Standard *	7	1.0	1.7	1.5
P.E.P. I	3	12	15.5	10.8
P.E.P. II	4	5.0	4.8	8.1
P.E.P. III	5	3.1	4.4	3.8
P.E.P. III B	6	4.0	4.3	5.8
P.E.P. VI+VII (1:1)	2	1.0	1.0	1.2
Estradiol-3,17-diphosphate	8	225	14.5	154
Estradiol-17-phosphate	9	225	90	810
Estradiol-3-phosphate	10	225	67	270

* P.E.P. Standard after hydrolysis (81 h, pH 5.2, 98°).

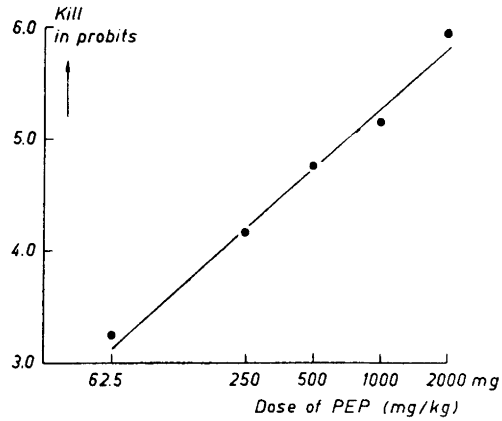


Fig. 12. Relationship between log dose of polyestradiol phosphate and kill (in probits) in a 60-days chronic toxicity test. Each point represents 25 adult male mice.

to dissolve hyaluronic acid (Na_2HPO_4 2 H_2O 16.1 g; citric acid. 2.02 g; NaCl 3.05 g; water to 1 000 ml). The concentration of the substrate was chosen to give a relative viscosity of 4.

Inhibitors. The inhibitors studied were shaken with the buffer and then diluted with buffer to a concentration of 0.1 mg per ml.

Conditions of enzyme experiments. The determination of the antihyaluronidase activity was carried out exactly according to the scheme described previously [1].

Results. Fig. 11 illustrates the inhibition of hyaluronidase at different concentrations of P.E.P. Standard. The influence of polymerisation time on the inhibitory activity of phosphorylated estradiol is demonstrated in Table 2. The inhibition is increased by increased polymerisation time, indicating that the inhibition is depending on the molecular size. An increase in the number of acidic groups (rephosphorylation) or a decrease (hydrolysis) does not change the magnitude of the inhibition (Table 2).

C. Pharmacological properties

Toxicity. The acute toxicity of P.E.P. was estimated in adult male mice. 48 h following a single intravenous injection the L.D.50 was found to be 240 mg/kg, with 95% fiducial limits of error at 212 and 272 mg/kg. It is realized, that in the absence of a toxicity reference standard the calculation of fiducial limits has a rather limited significance.

Chronic toxicity was estimated as follows: groups of 25 adult male mice were injected subcutaneously with P.E.P. in water on every 10th day. On the 60th day of experiment the number of survivors in each group was recorded. No correction has been made for spontaneous mortality. The correlation between the logarithm of dose and mortality in probits [21] is shown in Fig. 12. It appears from the data that under these conditions the L.D.50 is 700 mg/kg. Confidence limits (P = 0.95) were calculated as 350 and 1 400 mg/kg.

Biological activity. The duration of action of P.E.P. depends on the polymerisation time, as demonstrated in Fig. 1. From these data it appears that a polymerisation time less than 20 h will yield P.E.P. preparations with a relatively poor duration of action.

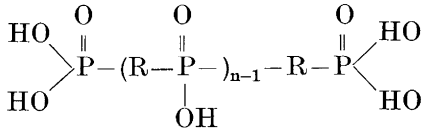
When different preparations of P.E.P. were administered to spayed mice it was found that the logarithm of dose was linearly related to the logarithm of duration of estrogenic action (in days) [11]. On basis of this observation a method of bioassay of long-acting estrogens was worked out [12]. When a mixture of P.E.P. preparations VI and VII (Table 1) was assayed by this method against the P.E.P. Standard, a relative potency of

103 % was obtained (fiducial limits of error: 91 and 117 %, respectively). The index of precision (estimated as Gaddum's λ-criterion [13]) was 0.09, indicating a very satisfactory precision.

It also appears from the data of Table 1 that following "rephosphorylation" with excess $POCl_3$ in pyridine before hydrolysis and dialysis, a P.E.P. preparation (III. B) could be obtained, in which no 'free' hydroxyl groups, but a great number of primary phosphate groups were present. Polymerisation time was only 5 h, which — according to the data of Fig. 1 — is too short for obtaining preparations with an optimal duration of action. When this "rephosphorylated" preparation was bioassayed against the P.E.P. standard, its potency was only 34 % of that of the Standard. This estimate of potency agrees very closely with that (30 %) of another preparation obtained following the same reaction time without "rephosphorylation" (prep. III). It is concluded therefore that the duration of estrogenic effect of P.E.P. does not depend on the relative proportion of acid groups present, but rather on the degree of polymerisation. It is also of interest, that when tested at the dose levels of 7 and 14 μg, respectively, estradiol-3,17-diphosphate did not show any prolonged estrogenic activity. In the same experiment the P.E.P. Standard exhibited a mean duration of 17.9 days (7 μg) and 24.6 days (14 μg), respectively. Finally, when as much as 100 μg of P.E.P. Standard were administered orally to each of 15 spayed mice, in all but one of the animals only negative smears could be obtained, indicating that P.E.P. is inactive when given orally.

DISCUSSION

Assuming that in the P.E.P. molecule the estradiol moieties are linked together by -OPO-bridges and that no free estradiol hydroxyl groups are present, the maximum phosphorus content and some other data can be calculated. The highest possible value of the phosphorus content of a polyestradiol phosphate molecule is dependent on the number of estradiol moieties only and not on possible branchings (one phosphorus being attached to three estradiol moieties). Thus, if the number of estradiol moieties (R) is n, it follows from the formula



that the phosphorus content (per cent) of this molecule is

$$100 \cdot \frac{31\ (n+1)}{334\,n+98} = 9.28 \cdot \frac{n+1}{n+0.29}$$

that the corresponding estradiol (HRH) content is $81.4 \cdot \frac{n}{n+0.29}$

and that the number of acid groups per phosphorus $= \frac{n+3}{n+1}$.

If the molecular weight of P.E.P. Standard is accepted to be 26 000, the n value will be about 80. In the formula above the theoretical percentage of phosphorus will be 9.36 and that of estradiol 81.1. The number of acid groups per phosphorus can be calculated as 1.03. P.E.P. Standard contains 9.5 % phosphorus, 78.2 % estradiol and 1.2 acid groups per phosphorus.

The small discrepancy in the phosphorus and estradiol values may be due to a relatively small proportion of pyrophosphate linkages (formed from small amounts of water in the reaction mixture). The high ratio acid groups to phosphorus, however, seems to speak in favour of a lower molecular weight, as does the low percentage of tertiary phosphate, which does not agree with the amount of primary phosphoric acid esters found. Assuming the molecular weight to lie between 3 000 and 5 000, the high ratio of acid groups to phosphorus as well as the low percentage of tertiary phosphate are easily explained, and if so the ultracentrifuge values may be due to a strong association.

The enzyme experiments reported in this paper indicate that P.E.P. — like most compounds in these series of polymeric phosphates — is a powerful inhibitor of several enzymes.

The results recorded in Tables 1 and 2 seem to indicate that an increase or a decrease in the number of acidic groups present in the inhibitor does not markedly influence the inhibitory effect. From the results obtained with products of different polymerisation time as well as with the monomeric esters, it appears that the degree of condensation is of greater importance.

Interesting is the relatively strong inhibitory effect of estradiol-3, 17-diphosphate upon alkaline phosphatase, as compared to the weak effect of this compound on the other enzymes studied. These findings are in agreement with the results reported previously [9], and indicate that alkaline phosphatases are inhibited by estradiol-3,17-diphosphate. Thus the previous suspicion [15] that the inhibition of kidney alkaline phosphatase by estradiol-3,17-disphosphate might have been due in part to a contamination of this compound by polymeric estradiol phosphates is not supported by the present data.

P.E.P. as well as polyphloretin phosphate have also been found [14] to exert a strong inhibitory effect on the disruption of mast cells produced by compound 48/80 or phospholipase A.

As far as its biological action is concerned, polyestradiol phosphate is a long-acting estrogen when administered parenterally to spayed mice [15], or to human beings [16, 17]. Since P.E.P. is rather resistant to acid or alkaline hydrolysis, as well as to the effect of different phosphatases, it would seem justifiable to assume, that its prolonged duration of action depends on a slow liberation of estradiol in the organism. In fact, experiments, in which P.E.P. labelled with radioactive phosphorus was administered to spayed mice [11] indicate that a definite relationship exists between circulating blood levels and log dose of P.E.P. administered, as well as between the duration of estrous smears and log dose of P.E.P. These correlations suggest that the circulating amount of P.E.P. may be instrumental in maintaining a prolonged estrogenic activity through its slow degradation into estradiol. Furthermore, evidence is also on record indicating that in the human organism substantial amounts of estradiol are liberated from the administered P.E.P. molecule and that this estradiol is metabolized and excreted in the same way as the body's own estrogen. Following the administration of P.E.P. to surgically castrated women, estrone, estradiol-17β and estriol were isolated and identified in their urine [16].

Finally, it is also of interest to note that P.E.P. has found an application in the treatment of prostatic carcinoma [18,19].

REFERENCES

1. Diczfalusy, E., Fernö, O., Fex, H., Högberg, B., Linderot, T. and Rosenberg, T. *Acta Chem. Scand.* **7** (1953) 913.
2. Fernö, O., Fex, H., Högberg, B., Linderot, T. and Rosenberg, T. *Acta Chem. Scand.* **7** (1953) 921.
3. Strauss, U. and Fouss, R. *Die Physik der Hochpolymeren* vol. II, p. 695 (Edited by Stuart) Berlin Springer 1953.
4. Ferry, J., Vdy, D., Feng Chi Wu, Heckler, G. and Fordyce, D. *J. Colloid Sci.* **6** (1951) 429.
5. Buch, I. and Buch, H. *Acta Med. Scand.* **101** (1939) 211.
6. Martin, J. B. and Doty, D. M. *Anal. Chem.* **21** (1949) 965.
7. Bessey, O. A., Lowry, O. H. and Brock, M. J. *Biol. Chem.* **164** (1946) 321.
8. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
9. Aldman, B., Diczfalusy, E., Högberg, B. and Rosenberg, T. *Biochem. J.* **49** (1951) 218.
10. Högberg, B. *Acta Chem. Scand.* **8** (1954) 1098.
11. Diczfalusy, E., Borell, U., Magnusson, A.-M. and Westman, A. *Acta Endocrinol. Suppl.* **24** (1956) 1.
12. Diczfalusy, E., Magnusson, A.-M., Nilsson, L. and Westman, A. *Endocrinology* **60** (1957) 581.
13. Gaddum, J. H. *Spec. Rep. Ser. med. Res. Council* London **1933** No. 183.
14. Högberg, B. and Uvnäs, B. *Acta Physiol. Scand.* **41** (1957) 345.
15. Diczfalusy, E. *Endocrinology* **54** (1954) 471.
16. Diczfalusy, E. and Westman, A. *Acta Endocrinol.* **21** (1956) 321.
17. Tillinger, K.-G. and Westman, A. *Acta Endocrinol.* **25** (1957) 113.
18. Goodhope, C. D. *J. Urol.* **77** (1957) 312.
19. Jönsson, G., Röhl, L. and Wiegner, K. *Acta Chir. Scand.* **113** (1957) 68.
20. Baggett, B., Engel, L. and Fielding, L. *J. Biol. Chem.* **213** (1955) 87.
21. Finney, D. J. *Probit Analysis: A statistical treatment of the sigmoid response curve,* Cambridge University Press, London 1952.
22. Lowry, H., Roberts, N. R., Wu, M.-L., Hixon, W. S. and Crawford, E. J. *J. Biol. Chem.* **207** (1954) 19.

Received March 13, 1958.