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High Molecular Weight Enzyme Inhibitors

IV. Polymeric Phosphates of Synthetic Estrogens

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The preparation and some of the biochemical and pharmacological properties of polydiethylstilbestrol phosphate and of some other polymeric phosphates of closely related synthetic estrogens are described. These compounds are very potent inhibitors of certain enzymes, such as acid and alkaline phosphatases, hyaluronidase and β-glucuronidase.

When injected systemically to spayed mice, the polymeric phosphates studied, especially polydiethylstilbestrol phosphate, exhibited a prolonged duration of estrogenic activity. When administered by the oral route, their estrogenic effect was negligible.

In the third paper of these series, the synthesis and properties of polyestradiol phosphate (P.E.P.) were described [1]. The greatly prolonged estrogenic activity of this compound [2] suggested a study of similar polymers prepared from synthetic estrogens. The synthesis of one of these polymers has previously been reported from our laboratories [3].

In the present paper the polymeric phosphates of diethylstilbestrol [3,4-bis(*p*-hydroxyphenyl)-3-hexene], dienestrol [3,4-bis(*p*-hydroxyphenyl)-2,4-hexadiene], hexestrol [3,4-bis(*p*-hydroxyphenyl)-hexane] and benzestrol [3-ethyl-2,4-bis(*p*-hydroxyphenyl)-hexane] are described. These polymers were prepared practically in the same way as P.E.P. Some of the properties of these compounds are shown in Table 1. In Table 2 the antienzymic activity and the duration of estrogenic action of these compounds are compared with that of P.E.P.

It appears that among the polymers of synthetic estrogens, polydiethylstilbestrol phosphate has by far the most prolonged duration of activity. Therefore the present investigation will mainly deal with this compound. The polydiethylstilbestrol phosphate preparation obtained following phosphorylation with 1.1 mole phosphoryl chloride for 70 h will hereafter be denoted as P.S.P.

Table 1. Some of the chemical properties of polymeric phosphates prepared from synthetic estrogens.

No. Compound	Molar ratio $POCl_3$: estrogen	Reaction time, h	% Non-dialyzable $P_{org.}$	% P a)	% Free Hydroxyl groups b)	% Hydrolyzable phosphorus c) $[-P(O)(OH)_2]$	% Phosphorus as d) $\rangle PO$	$>P(O)OH$	$-P(O)(OH)_2$
1. P.E.P. Standard	1.1	70	88–90	9.5	0	28–30	10	60	30
2. Polydiethylstilbestrol phosphate	0.8	70	89	7.6	10	3	23	72	5
3. Polydiethylstilbestrol phosphate	0.9	70	85	8.2	10	7	17	75	8
4. Polydiethylstilbestrol phosphate	1.0	70	87	8.9	4	14	11	76	13
5. Polydiethylstilbestrol phosphate	1.1	5	66	9.0	9	30	11	59	30
6. Polydiethylstilbestrol phosphate	1.1	25	79	9.6	—	25	9	65	26
7. Polydiethylstilbestrol phosphate (P.S.P.)	1.1	70	83	9.8	0	25	8	68	24
8. Polydiethylstilbestrol phosphate	1.2	70	79	10.4	—	35	6	61	33
9. Polydienestrol phosphate	1.1	70	75	9.4	—	23	13	63	24
10. Polyhexestrol phosphate	1.1	70	77	9.5	—	24	16	60	24
11. Polybenzestrol phosphate	1.1	70	81	8.4	—	24	—	—	—
12. Diethylstilbestrol diphosphate	—	—	—	14.7	0	100	0	0	100
13. Honvan (R)	—	—	—	—	—	—	—	—	—

a) Calculated on samples free from moisture, pyridine and chlorine.
b) According to B. Baggett *et al.*[14] Calculated as % of 'estrogen oxygens'.
c) Hydrolysis at 100° for 27 h at pH 5.0 (0.1 M acetate buffer).
d) From titration experiments. Results corrected for pyridine and chlorine.

The molecular weight of P.S.P. was found to be about 14 000 (ultracentrifuge) indicating that the polymer contains approximately 40 diethylstilbentrol moieties. The viscometric behavior of P.S.P. was found to be the same as that of P.E.P.[1], that is typical for linear polyelectrolytes.

Hydrolysis experiments with P.S.P. performed in the same way as previously described for P.E.P.[1] seem to indicate that also in this case the liberated inorganic phosphorus is formed from primary phosphoric acid esters only.

Titration experiments indicated that a certain degree of branching occurs (Table 1).

EXPERIMENTAL

A. Preparation of polymers

1. Preparation of polydiethylstilbestrol phosphates. This was carried out practically in the same way as previously described for the preparation of P.E.P. Standard[1]. In the

preparation of different batches the molar ratio $POCl_3$/diethylstilbestrol was varied between 0.8 and 1.2. Dialysis was carried out against dist. water containing 1 % NaCl, and the polydiethylstilbestrol phosphate preparations were precipitated by HCl without any addition of ethanol. Using molar ratios of $POCl_3$/diethylstilbestrol between 0.8 and 1.0 it was found that the mixture had changed into a jelly after 10 to 20 h reaction time. After hydrolysis of these jellies with ice it was necessary to heat on a steam bath for 1—2 h with stirring in order to obtain a clear solution (no inorganic phosphorus was liberated during heating). No jelly formation took place when the molar ratio of $POCl_3$/diethylstilbestrol was higher than 1.0. With a reaction time of 70 h the yields from 5 g diethylstilbestrol varied between 5.5 and 5.7 g of crude polymer. The various polydiethylstilbestrol phosphates were analyzed for moisture, *(in vacuo* 100°; found 1—2 %), pyridine (determined spectrophotometrically after alkalization and distillation; found 1—3 %), chlorine (found 0—0.5 %) and phosphorus (Table 1).

The polydiethylstilbestrol phosphate obtained with 1.1 mole $POCl_3$ and with a reaction time of 70 h (P.S.P.) has a melting range of 195—205°.

Analysis of P.S.P.: Moisture: 1.7 %; pyridine: 2.9 %; chlorine: 0.3 %; P, C and H (calculated on sample free from moisture, pyridine and chlorine): 9.8; 63.1 and 5.8 %, respectively. P.S.P. (free from moisture, pyridine and chlorine) should thus contain 78.3 % diethylstilbestrol. Molar ratio P/diethylstilbestrol: 1.08.

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

2. Polydienestrol phosphate, polyhexestrol phosphate and polybenzestrol phosphate. All these polymers were prepared in the same manner as described for P.S.P. (1.1 mole $POCl_3$, 70 h at room temperature). No jelly was formed.

3. Diethylstilbestrol diphosphate. This was prepared by the method of Miescher *et al.*[4] It was purified over the sodium salt. M.p. 213—215°. [Found: P 14.7 %; equiv. wt 108 (as a dibasic acid) and 105 (as a tetrabasic acid). Calc. for $C_{18}H_{22}O_8P_2$: P 14.5 %; equiv. wt 107.]

Hydrolysis experiments. These were run at 100° between pH 3 and 10 (0.1 M buffer solution) and in 0.2 N sodium hydroxide solution. 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 3 and 6, practically all P_o being liberated within 10 h. Practically no P_o was liberated on hydrolysis in 0.2 N NaOH solution.

At pH 5.0 (acetate buffer) the hydrolysis of diethylstilbestrol diphosphate was found to follow a first order reaction with $k = 0.87$ h^{-1}. Under the same conditions when calculated on hydrolyzable phosphorus only (Table 1), the rate of hydrolysis for various polydiethylstilbestrol phosphate preparations was found to be virtually the same. Polydienestrol phosphate, polyhexestrol phosphate and polybenzestrol phosphate were found to have k-values of 0.94, 0.71 and 0.71 h^{-1}, respectively.

Molecular weight, viscosity measurements and potentiometric titration. All these measurements have been carried out in the same manner as previously described for P.E.P. Standard [1]. For P.S.P. the following values were found. In 0.2 M phosphate buffer solution at pH 7.1, $S^\circ_{20} = 1.4 \times 10^{-13}$; $D_{20} = 7.8 \times 10^{-7}$; $V_{20} = 0.69$ *. The molecular weight was calculated to be approximately 14 000. The intrinsic viscosity of the sodium salt was found to be $[\eta] = 0.04$ in 0.25 M NaCl solution at pH 7.3. The amount of primary phosphoric acid esters found in titration experiments corresponded well to the values obtained in hydrolysis experiments (Table 1).

B. Antienzymic properties

The enzymes studied were acid phosphatase, alkaline phosphatase, hyaluronidase and β-glucuronidase. With the exception of the last mentioned enzyme, the experimental conditions and the preparations used have been described in detail in a previous communication [1].

* The estimation of the sedimentation constant has been carried out in the Carlsberg Laboratories, Copenhagen, through the courtesy of Professor K. Linderstrøm-Lang.

Table 2. Some of the biochemical and pharmacological properties of the compounds studied.

No.	Rel. amount (P.E.P. = 1.0) of inhibitor necessary for 50 % inhibition				Duration of estrogenic effect a)
	Hyaluronidase	Alkaline phosphatase	Acid phosphatase	β-Glucuronidase (bacterial)	
1. (P.E.P.)	1.0	1.0	1.0	1.0	100
2.	1.0	113	1.0	0.36	63
3.	0.7	40	0.6	0.13	100
4.	0.6	27	—	0.12	100
5.	0.7	10	1.0	—	97
6.	0.7	17	0.7	—	104
7. (P.S.P.)	0.6 b)	17 b)	0.6 b)	0.14	102
8.	0.7	15	0.5	0.12	107
9.	0.7	43	1.2	0.20	41 c)
10.	0.7	87	0.6	0.28	24 c)
11.	1.0	57	1.1	0.39	30 c)
12.	800	19	1 000	>12	—
13.	160	18	46	>12	1 c)

a) Potency is expressed as a percentage of the effect of P.E.P. Standard (Preparation No. 1).
b) Practically unchanged after hydrolysis (27 h, pH 5.0, 100°).
c) When assayed against P.S.P. these compounds show a significant deviation from parallelism. Thus these assays are statistically not valid, and the potency estimates are only approximate values calculated from the results obtained at the same dose levels (5 and 10 μg, respectively). These data are included only to demonstrate that the potency of these compounds is much less than that of P.S.P.

β-Glucuronidase. For the measurement of enzyme activity the liberation of phenolphtalein from 0.0005 M phenolphtalein glucuronide in 0.05 M acetate buffer was estimated by a modification [5] of the method of Talalay, Fishman and Huggins [6]. The volume of the incubation mixture was 1.5 ml and after 30 min at 37° the reaction was stopped and the colour developed by the addition of 5 ml of 0.2 M glycine buffer (pH 10.4).

Enzyme. Bacterial β-glucuronidase was a purified commercial product from Sigma Chemical Co., St. Louis, U.S.A. Another enzyme prepared from the common limpet *(Patella vulgata)* according to the method of Levvy, Hay and Marsh [7] was also used in some of the experiments.

Results. The relative amounts of inhibitors necessary for 50 % inhibition of the enzymes studied are shown in Table 2. In all cases the enzyme inhibition by P.S.P. could be reversed by basic proteins, *e. g.* protamine sulphate. The type of inhibition was studied by preparing classical Lineweaver plots [8]. The results are summarized in Table 3.

Table 3. Type of inhibition of different enzymes studied.

Compound	Alkaline phosphatase at pH 9.3	Acid phosphatase at pH 4.5	Bacterial β-glucuronidase at pH 7.0
Polyestradiol phosphate (P.E.P.)	competitive	non-competitive	competitive
Polydiethylstilbestrol phosphate (P.S.P.)	»	non-competitive	»
Polyhexestrol phosphate	»	—	»
Diethylstilbestrol-4,4′-diphosphate	»	competitive	»
Estradiol-3,17-diphosphate	»	»	»

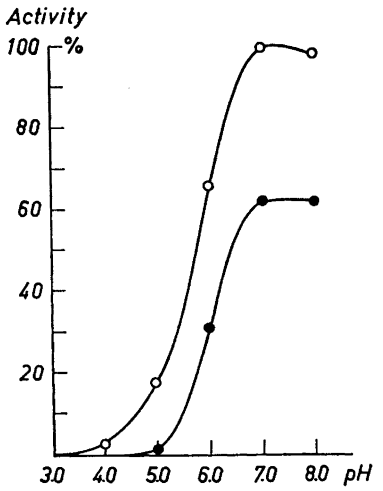

Fig. 1. Hydrolysis of 0.0005 M phenolphtalein glucuronide by bacterial β-glucuronidase (1.15 mg) at various pH values in 0.05 M acetate buffer in the absence (○) and presence (●) of polydiethylstilbestrol phosphate (100 μg P.S.P./1.5 ml).

1. Acid phosphatase. The inhibition of acid phosphatase was studied at pH 4.5 using disodium *p*-nitrophenyl phosphate as a substrate.

It appears from the data of Table 2 that the inhibitory potency of the different polymeric phosphates of synthetic estrogens is of the same order as that of P.E.P. On the other hand, diethylstilbestrol diphosphate is a very poor inhibitor of the enzyme, a fact which is in close agreement with previously reported data on estradiol diphosphate [1]. It is of interest, however, that a commercial diethylstilbestrol diphosphate preparation (Honvan®, Asta-Werke A-G.) exhibited a considerably stronger inhibition than the product synthetized by us.

The inhibition of acid phosphatase by P.S.P. is non-competitive. This is in agreement with previous data on the type of inhibition by P.E.P. On the other hand, diethylstilbestrol diphosphate, and also estradiol-3,17-diphosphate [1] inhibit the enzyme in a substrate-competitive manner.

2. Alkaline phosphatase. The enzyme used was a highly purified commercial product of Sigma Chemical Co., St. Louis, U.S.A., prepared from calf intestinal mucosa. It appears from Table 2 that the polymeric phosphates of synthetic estrogens are much weaker inhibitors of alkaline phosphatase than of acid phosphatase, when compared to P.E.P. It is of interest to note that in contrast to the results obtained with acid phosphatase the inhibition of alkaline phosphatase by diethylstilbestrol diphosphate is of the same order as that by P.S.P. It is also of interest that the use of smaller amounts of $POCl_3$ in the phosphorylation of diethylstilbestrol seems to result in compounds exhibiting a decreased antienzymic activity with regard to alkaline phosphatase, but not to acid phosphatase. The inhibition of alkaline phosphatase by P.S.P. as well as by diethylstilbestrol diphosphate and also by polyhexestrol phosphate is substrate-competitive.

3. Hyaluronidase. It is of interest to note the close correlation between the inhibitory activity of the different compounds on hyaluronidase on the one hand, and on acid phosphatase on the other hand.

4. β-Glucuronidase. The inhibition of bacterial β-glucuronidase by P.S.P. at different pH-values is illustrated in Fig. 1. The same type of curve was obtained when P.E.P. was used as an inhibitor. The schematic curves showing per cent inhibition at pH 7.0 as a function of inhibitor concentration are presented in Fig. 2. Finally Fig. 3 shows the variation in enzymic activity of the limpet β-glucuronidase at different pH-values and the inhibitory effect of P.S.P. and P.E.P. thereupon.

As far as the type of enzyme inhibition is concerned, it was found that all the compounds studied (P.S.P., P.E.P., polyhexestrol phosphate, diethylstilbestrol diphosphate) inhibited the bacterial β-glucuronidase (at pH 7.0) in a competitive manner.

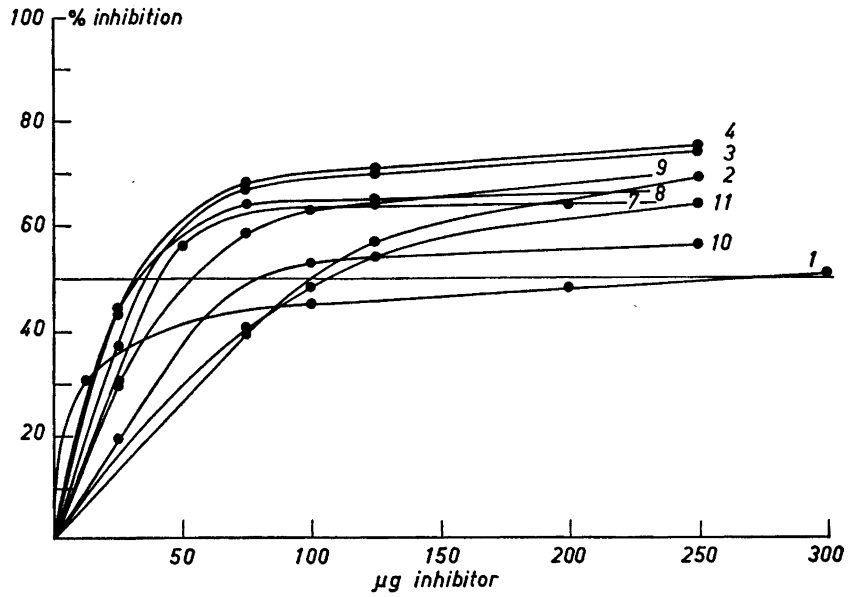

Fig. 2. Per cent inhibition of bacterial β-glucuronidase (1.5 mg) by the substances listed in Table 1. 0.05 M acetate buffer pH 7.0. Substrate 0.0005 M phenolphtalein glucuronide. Incubation time 30 min at 37°.

C. Pharmacological properties

Toxicity. The acute toxicity of P.S.P. was estimated in spayed female mice 48 h following a single intravenous injection. LD 50 was found to be 252 mg/kg, with 95 % fiducial limits of error at 232 and 273 mg/kg, respectively.

When male albino rats were injected intramuscularly with doses up to 10 mg/kg of P.S.P., and sacrificed 10 weeks after the injection, no gross pathological changes could be seen either at autopsy or on microscopical examination, except those attributable to the typical action of an efficient and prolonged estrogen such as decrease in the weight of accessory reproductive organs and gynaecomastia in the highest dosages administered. Thus, the toxicity of P.S.P. seems to be of the same order as that of P.E.P.[1] The low toxicity of P.S.P. suggests that it can probably be administered also to human beings without any major harmful effects.

Biological activity. The duration of action of the various polymeric phosphates was estimated by the use of a previously described method based on the duration of vaginal cornification in spayed mice [9]. The relative potencies of the different compounds when compared to the activity of P.E.P. are summarized in Table 2. It appears from the data of Table 2 that polymeric phosphates prepared from dienestrol, hexestrol or benzestrol exhibit a much shorter duration of estrogenic activity than similar compounds prepared from diethylstilbestrol. It also appears that the commercial preparation of diethylstilbestrol diphosphate (Honvan ®) is virtually devoid of prolonged estrogenic activity. When P.S.P. was hydrolyzed at 100° for 27 h at pH 7.6 (21.2 % P_o liberated), its biological potency remained unchanged. It is also of interest that the polymeric phosphates prepared from dienestrol, hexestrol or benzestrol are much weaker long-acting estrogens than P.S.P. These compounds also exhibit a greatly reduced *anti*-alkaline-phosphatase activity, and a somewhat reduced *anti*-β-glucuronidase activity when compared to P.S.P. However, their effect on hyaluronidase and acid phosphatase appears to be of the same order. It also appears from Table 2 that polyestradiol phosphate and polydiethylstilbestrol phosphate are equipotent long-acting estrogens.

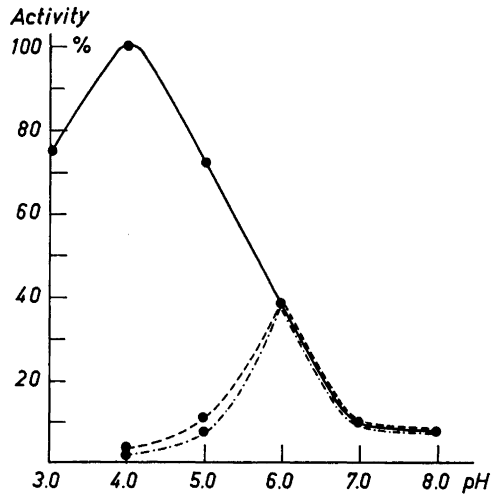

Fig. 3. Hydrolysis of 0.0005 M phenolphtalein glucuronide by limpet β-glucuronidase (112 Fishman units) at various pH values in 0.05 M acetate buffer in the absence and presence of inhibitors; ●——● no inhibitor; ● - - - ● P.E.P. Standard 100 μg/1.5 ml, ● — . — ● P.S.P. 100 μg/1.5 ml. Incubation time 30 min.

Attempts have also been made to estimate the ED 50 of P.S.P. when given orally. The ED 50 of P.S.P. by the oral route was found to be 3.9 mg/kg, that of diethylstilbestrol diphosphate 0.36 mg/kg, whereas that of diethylstilbestrol was 0.1 mg/kg. When P.S.P. was submitted to hydrolysis at 100° for 74 h at pH 6.7 (24.3 % P_o liberated), its ED 50 by the oral route was still 3.6 mg/kg, indicating that previous hydrolysis has very little, if any, effect on the absorption of the P.S.P. molecule.

In other experiments P.S.P. was administered to spayed mice by the oral route in dosages of 5 mg/kg and 10 mg/kg, respectively. Vaginal smears taken from these animals either could not reveal any estrogenic activity, or showed just a very short, transient estrogenic reaction with a mean duration of 0.8 and 1.1 days, respectively. It seems therefore safe to conclude that orally administered P.S.P. is not absorbed to any appreciable extent. These results have also been confirmed in rats using tritium-labelled P.S.P.[10]

DISCUSSION

In a previous communication[1] where the preparation of P.E.P. was described the molar ratio between $POCl_3$ and estradl-1-io7β was kept constant at 1.1. Under those conditions it was found necessary to employ a reaction time of 20 h or more, in order to obtain compounds exhibiting maximally prolonged estrogenic activity. It appears from the data of the present study that a reaction time of 5 h is already sufficient to obtain polydiethylstilbestrol phosphate preparations of maximal activity. It was also found that preparations with maximally prolonged activity could be obtained in all experiments, where the $POCl_3$/diethylstilbestrol ratio was kept between 0.9 and 1.2. Ratios below 0.9 resulted in compounds with diminished activity. On the other hand, highly active non-dialyzable preparations could be obtained even by the use of $POCl_3$/diethylstilbestrol ratios much higher than 1.2.

As to P.E.P. we have already suggested[1] the possibility that the molecular weight of the compound might be considerably lower than indicated by the

results of ultracentrifugation. A similar analysis of the present data seems to speak very much in favour of the possibility that also P.S.P. might occur in form of an association of smaller molecules.

A comparison between polydiethylstilbestrol phosphate and polyestradiol phosphate reveals marked differences as far as the antienzymatic properties are concerned. Whereas the antihyaluronidase or *anti*-acid-phosphatase activities appear to be approximately equal, polydiethylstilbestrol phosphate is a much weaker inhibitor of alkaline phosphatase than polyestradiol phosphate. As far as the inhibition of β-glucuronidase is concerned, the opposite relationship can be observed, polydiethylstilbestrol phosphate being a much stronger inhibitor than polyestradiol phosphate.

The inhibition of acid phosphatase by P.S.P. was found to be non-competitive. It is of interest that Hummel *et al.* found that the inhibition of this enzyme by polyxenyl phosphate is also of the non-competitive type [11].

As to the type of enzyme inhibition by the various compounds the inhibition of alkaline phosphatase by all compounds studied is competitive. This seems to be at variance with previously reported data from our laboratories [12], where the inhibition of kidney alkaline phosphatase by estradiol diphosphate was described as non-competitive. It should be noted. however, that neither the source of enzyme, nor the experimental conditions were identical in the present investigation with those published by Aldman *et al.*[12]

It also appears from the data of the present investigation that the majority of compounds studied are very potent inhibitors of β-glucuronidase. The inhibition is of the substrate-competitive type. This inhibition seems to be of interest in view of the fact that higher amounts of β-glucuronidase have been found in human cancer tissue than in normal tissues [13].

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