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Synthetic High Molecular Weight Enzyme Inhibitors

II. Polymeric Phosphates of Aromatic Hydroxy and Amino Compounds

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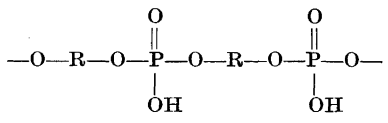
In a previous paper [1] it was shown, that by phosphorylating phloretin and other structurally related polyphenols it is possible to obtain high molecular weight polymers. These polymers are polyanions and they exert a strong inhibitory effect on various enzymes, particularly alkaline phosphatase, hyaluronidase and urease.

From these results it seems possible that the nature of the initial molecules is without significance and that it should be possible to prepare polymers of the same general type and with similar properties from compounds with a different structure, provided that they contain groups, capable of reacting with a phosphorylating agent. *A priori* it should be possible to perform a condensation polymerization between two compounds, if both of them contain at least two reactive groups. The commonest phosphorylating agent is phosphorus oxychloride. This compound has three reactive chlorine atoms, two of which, however, are more reactive than the third [2]. The chlorine atoms of phosphorus oxychloride may react with hydroxy, amino and sulphhydryl groups (hereafter termed reactive groups). The simplest organic aromatic hydroxy compounds, from which it should be possible to prepare polymers of this type are pyrocatechol, resorcinol and hydroquinone. While with pyrocatechol ring formation is known to result [3] it was possible with the other two compounds to prepare non-dialyzable polymers. These polymers may contain elements of the type



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A systematic synthetic study was undertaken with other aromatic compounds containing at least two reactive groups and in practically all cases it was possible to obtain soluble, non-dialyzable polymers of this general type. Some of the compounds from which it was possible to prepare polymers by phosphorylation with phosphorus oxychloride or thiophosphoryl chloride under suitable conditions are listed in Table 1.

Pyrocatechol, *o*-aminophenol and resacetophenone did not form high molecular weight polymers under various experimental conditions. In order to obtain a high degree of polymerization the reaction conditions are of great importance. In most cases the compounds to be phosphorylated were dissolved in pyridine or quinoline, and thereafter at a temperature of −10 to −15° a solution of phosphorus oxychloride was added with cooling and shaking. The reaction velocity depends upon the reactivity of the compound and the number of reactive groups available. In the case of compounds with three or more reactive groups, employing a pyridine solution, the reaction is usually terminated within a few minutes; if, however, the compound contains only two reactive groups, polymerization takes longer. In quinoline the reaction proceeds more slowly than in pyridine. The ratio of the reactants is also of great importance. If the molecules contain two reactive groups, 2/3 mole of the phosphorylating agent was usually employed; if three reactive groups, one mole and so on. If a large excess of phosphorylating agent is used, the degree of polymerization is lowered and an increasing amount of dialyzable material is obtained.

The polymers formed are all very active enzyme inhibitors. Hitherto they have been investigated only as to their effect on hyaluronidase *. It may be assumed, however, that their inhibitory effect on other enzymes is of the same order of magnitude as that of polyphloretin phosphate. As will be seen from the experimental part the degree of inhibition on hyaluronidase may vary to a rather large extent but usually a strong inhibition is observed in concentrations of 5 μg per ml and even less under the test conditions used. It should be pointed out, however, that conditions for the phosphorylation reaction are very specific and even small changes in the conditions of the reaction may give rise to polymers of different properties, *i.e.* either higher or lower antienzymic activities. As to the stability of the polymers in water solution, this varies within a rather wide range from relatively unstable to practically stable products.

From compounds with two reactive groups it might be assumed, that a linear condensation polymer is formed. Some of the third chlorine atoms may also react, however, thus forming a branched polymer. This matter has been studied with hydroquinone and resorcinol. If 2/3 mole of phosphorus oxychloride is used it may be assumed that at first a reaction takes place leading to compounds of the type $HOC_6H_4OP(O)(Cl)OC_6H_4OP(O)(Cl)OC_6H_4OH$. At a later stage of the polymerization also the remaining chlorine atoms may react. It is well known that in triesters of phosphoric acid one ester group is more easily hydrolyzed than the other two [4]. Polymers of this kind are

* Polyphloroglucinol phosphate has an inhibitory effect on alkaline phosphatase of the same order of magnitude as polyphloretin phosphate (E. Diczfalusy; personal communication).

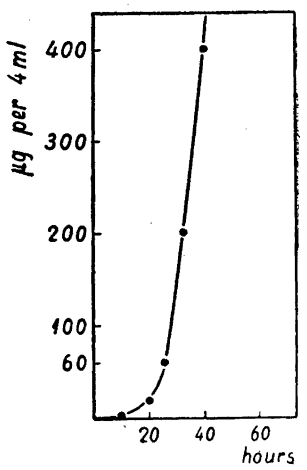

Fig. 1. Hydrolysis of polyphloroglucinol phosphate. Concentration necessary to effect 80 % inhibition is plotted against time in hours.

rather unstable in aqueous solution. As a criterion of stability we have chosen the inhibitory effect on hyaluronidase.

With three or more reactive groups the resulting polymer will be branched. As the degree of branching increases the resulting polymer will be less soluble. If the polymerization is allowed to proceed, an insoluble polymer is formed, as in the case of phloroglucinol. The positions of the reactive groups are, however, of importance. With gallic acid or pyrogallol this stage is not reached, presumably due to ring formation. With phloroglucinol and other similar compounds it is necessary to stop the phosphorylation process at a suitable stage in order to arrive at polymers of the desired degree of polymerization. It is also possible to allow the phosphorylation to proceed to completion and then hydrolyze the resulting insoluble polymer. This may be accomplished by heating or, more conveniently, by boiling with dilute hydrochloric acid. This latter method has been studied in the case of phloroglucinol.

An insoluble polyphloroglucinol phosphate (Example II) was boiled in 0.1 *N* hydrochloric acid until a clear solution was formed. This solution contained soluble polyphloroglucinol phosphates of varying molecular size. 6 % of the organically bound P ($P_{org.}$) dialyzed through a cellophane membrane and 13 % of the total P content was inorganic P.

According to the method of assay used 0.5 µg (corresponding to phloroglucinol) per 4 ml was necessary to effect an inhibition of 80 %. The solution was boiled and portions withdrawn after various length of time. According to Fig. 1 the quantity of material necessary to produce an inhibition of 80 % shows an accelerated increase.

After 20 hours' boiling 20 µg was required and after 100 hours 5 000 µg. During the same time % $P_{inorg.}$ of $P_{tot.}$ increased to 32 and 57 % respectively (Fig. 2). After 24 hours 44 % of $P_{org.}$ dialyzed (Fig. 2). When a solution was tested before and after dialysis all activity remained in the inner solution.

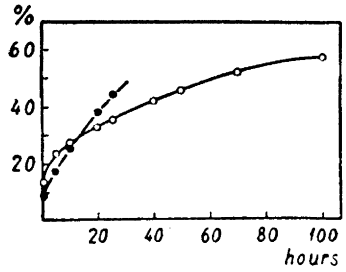

Fig. 2. Hydrolysis of polyphloroglucinol phosphate. Inorganic phosphorus in per cent of total phosphorus (open circles) and dialyzable in per cent of total organically bound phosphorus (filled circles) are plotted against time in hours.

From these and similar experiments it is obvious that the stability of a polymer may be studied by measuring its antienzyme effect and that the antienzymic activity is bound to the non-dialyzable material.

The reactions leading to this type of polymers may be interpreted in the following way. At first a condensation product between the phosphorylating agent and the aromatic compounds is formed and then this product, which, if the initial molecule is a phenol, may be written $ROP(=O)Cl_2$, begins to react with other molecules bearing free OH-groups and condensation polymers are formed. It is impossible, however, to distinguish between these two stages. As the reaction proceeds the phosphorylating agent may well condense with already polymerized molecules. This is especially the case if the phenol or amine in question contains more than two reactive groups. The study of this type of condensation polymerization from a kinetic point of view is thus complicated by the above factors.

EXPERIMENTAL

Preparation of inhibitors

I. *Polyphloroglucinol phosphate* (in quinoline). In a flask fitted with a thermometer, a calcium chloride drying tube and a dropping funnel 10.1 g of phloroglucinol was dissolved in 120 ml of dry quinoline. At −10° a solution of 4 ml of phosphorus oxychloride in 20 ml of dry quinoline was added with stirring (10 minutes). After an additional 10 minutes in the ice-salt bath the temperature was raised to 20° and held there for 30 minutes. After cooling to − 10° a solution of 8 ml of phosphorus oxychloride in 20 ml of dry quinoline was added. After 10 minutes the temperature was again raised to 20° and after 15 minutes the solution became turbid and viscous. The mixture was hydrolyzed with crushed ice, made alkaline with dilute sodium hydroxide solution, extracted several times with ether and then acidified with hydrochloric acid. The resulting precipitate was filtered by suction, washed with dilute hydrochloric acid and dried in a desiccator over phosphorus pentoxide and potassium hydroxide; yield 22.1 g, a light brown powder. Phosphorus: 14.3 %. Inhibition of hyaluronidase 95 % (5 μg per ml).

II. *Polyphloroglucinol phosphate.* 500 g of phloroglucinol was dissolved in a mixture of 2 l of dry acetone and 2 l of dry chloroform. 400 ml of phosphorus oxychloride was added at a temperature of − 10°. 400 ml of dry pyridine was then added with stirring and cooling at a rate such that the temperature remained below 0° (about 20 minutes). A further 1 000 ml of dry pyridine was added as rapidly as possible, causing a rise of temperature to about 25°. After a further 10 minutes the mixture became viscous and the

stirring was continued for about 1 hour. 15—20 hours later the precipitate was filtered by suction, washed with acetone and suspended in water. After 24 hours the precipitate was filtered again, washed with acetone and dried at 60—70°; yield 1 060 g. Analysis:

Moisture	6.4 %	
Pyridine	25.0 %	(on dried sample)
Chlorine	3.9 %	» » »
Phosphorus	12.8 %	» » »

This product was insoluble and may be regarded as a very high molecular weight, presumably cross-linked polymer. According to this assumption the product could be partially depolymerized thus yielding water soluble products with a very high inhibitory effect on hyaluronidase. 35 g of the insoluble polymer was boiled with 800 ml of 0.1 *N* hydrochloric acid. After 3 hours a clear, light yellow solution resulted containing polyphloroglucinol phosphates of different degrees of condensation. Inhibition of hyaluronidase 80 % (0.13 μg per ml). By heating the insoluble polymer to 160° for 4 hours a product was formed which gradually dissolved in water. Inhibition of hyaluronidase 70 % (5 μg per ml).

III. *Polyphloroglucinol thiophosphate.* A solution of 2.2 ml of thiophosphoryl chloride was added to 2.6 g of phloroglucinol in 20 ml of dry pyridine at — 10° (2 minutes). After a further 1.5 hour in the ice-salt bath a precipitate was formed and after another 15 hours at room temperature the mixture became semi-solid. After hydrolyzing with crushed ice the mixture was allowed to stand until the precipitate had dissolved (24 hours). On evaporating *in vacuo* a red viscous oil was obtained. Inhibition of hyaluronidase 100 % (5 μg per ml).

IV. *Polyhydroquinone phosphate.* At — 5° a solution of 12.5 ml of phosphorus oxychloride was added to 22 g of hydroquinone in 50 ml of dry pyridine (11 minutes). The mixture was then treated as in III. On evaporating *in vacuo* a yellow oil resulted, which by washing with dilute hydrochloric acid and water became semi-solid; yield after drying in a desiccator 29 g. Analysis:

Moisture	21.7 %	(60° *in vacuo*)
Phosphorus	12.1 %	(dried sample)
Chlorine	0.9 %	» »
Pyridine	14.1 %	» »

The substance was soluble in sodium bicarbonate solution. Inhibition of hyaluronidase 95 % (5 μg per ml). An aqueous solution is, however, not stable. A solution, kept at room temperature for a week showed an inhibition of 40 % and after warming in an autoclave to 120° for 20 minutes the inhibition had decreased to 15 %. No inorganic phosphorus was liberated.

If the synthesis was performed with equimolecular amounts of phosphorus oxychloride and hydroquinone the resulting product had a similar inhibitory effect but the stability was somewhat better. A water solution of this product was dialyzed against running water for 5 days. After this time 90 % of the original phosphorus contents had not dialyzed and the solution had an inhibitory effect of 70 %. A control solution held at the same temperature had after the same time a similar inhibitory effect. Initially all phosphorus was organically bound and no inorganic phosphorus was liberated.

If the synthesis was performed with 1.5 mole of phosphorus oxychloride the inhibition decreased to 20 % (5 μg per ml) while with 2 moles no inhibition could be observed with 50 μg per ml.

Polyhydroquinone phosphate could also be prepared at a higher temperature. If the addition of phosphorus oxychloride was made at 25 to 50° and the mixture hydrolyzed after a further 30 minutes, a product was obtained with similar properties.

V. *Polyresorcinol phosphate.* To a solution of 1.25 ml of phosphorus oxychloride in 10 ml of pyridine there was added 2.2 g of resorcinol in 20 ml of dry pyridine (½ minute, — 10°). The mixture was treated as in III. The resulting oil had an inhibitory effect of 100 % (5 μg per ml). A solution kept at room temperature for a week showed an inhibition of only 20 %.

In another run portions of the mixture were withdrawn 3 and 120 minutes after the addition of phosphorus oxychloride. The samples were worked up as usual and showed

Table 1. The inhibitory effect on hyaluronidase of various inhibitors.

Polymer prepared from	Tertiary amine, pyridine (Py) or quinoline (Qu)	Concentration of inhibitor μg per ml	Inhibitory effect on hyaluronidase, %
Resorcinol	Py	5	100
» + $PSCl_3$	Py	5	80
Orcinol	Py	5	75
4-Hexylresorcinol	Py	8	90
2-Nitroresorcinol	Py	5	95
4,6-Dibromo-2-nitroresorcinol	Py	1.3	100
4-Nitroresorcinol	Py	50	100
Resorcinol-4-sulphonic acid	Py	50	50
4-Nitrobenzeneazoresorcinol	Py	5	45
Hydroquinone	Py	5	95
Gentisic acid	Py	5	80
Phloroglucinol	Py	0.13	80
»	Qu	5	95
» + $PSCl_3$	Py	5	100
Phloracetophenone	Py	5	60
2,4,6-Trihydroxybenzaldehyde	Py	50	80
Hydroquinone + phloroglucinol	Py	5	100
Pyrogallol	Py	50	100
Gallic acid	Py	8	75
m-Aminophenol	Py	5	40
p-Aminophenol	Py	5	75
p-Methylaminophenol	Py	50	45
p-Aminothiophenol	Py	5	90
5-Aminoresorcinol	Py	0.5	75
p-Phenylenediamine	Qu	5	55
p,p'-Dihydroxybibenzyl	Py	5	95
4,4'-Dihydroxydiphenyldimethyl-methane	Py	0.05	80
4,4'-Dihydroxy-γ,δ-diphenyl-β,δ-hexadiene	Py	5	95
2,2',4,4'-Tetrahydroxydiphenyl-methane	Py	5	85
2,4,4',6-Tetrahydroxybenzophenone	Py	0.5	40
Benzidine	Qu	25	50
4,4'-Diaminodiphenylmethane	Qu	40	90
p,p'-Diaminobibenzyl	Py	5	75
p-Aminodiphenylamine	Qu	50	50
4,4'-Diaminodiphenylsulphone	Py	5	80
1,3-Naphtalenediol	Py	5	80
1,5- »	Py	5	100
2-Methyl-1,4-naphtalenediol	Py	5	100
1,2,7-Trihydroxyanthraquinone	Py	5	95
1,2,5,8-Tetrahydroxyanthraquinone	Py	10	80

As has already been pointed out even small variations in the experimental conditions may give rise to products with varying activities. A study of the effect on other enzymes is in progress.

an inhibition of 0 % (50 μg per ml) and 40 % (5 μg per ml) respectively. After a further 15 hours at room temperature the inhibition was the same as above.

VI. *Polyhexylresorcinol phosphate.* A solution of 0.65 ml of phosphorus oxychloride in 10 ml of dry pyridine was added to 1.95 g of hexylresorcinol in 20 ml of dry pyridine

at −10° (2 minutes). The mixture was treated as in III. The resulting oil was dissolved in a mixture of 25 ml of acetone and 40 ml of 2 *N* sodium hydroxide solution. Upon acidifying with 60 ml of 2 *N* hydrochloric acid the solution became turbid. It was extracted several times with ether, the combined ether extracts were washed once with saturated brine and then evaporated *in vacuo*, yielding a viscous oil; yield after drying 2 g, inhibition 90 % (8 μg per ml). The inhibition did not decrease if an aqueous solution was kept for 7 days at room temperature. After dialyzing (cellophane membrane) for 5 days against running water all the activity remained in the inner solution.

VII. *Poly-4,4'-dihydroxydiphenyldimethylmethane phosphate.* A solution of 0.62 ml of phosphorus oxychloride in 5 ml of dry pyridine was added to 2.28 g of 4,4'-dihydroxydiphenyldimethylmethane in 20 ml of dry pyridine at −15° (5 minutes). After a further 15 hours in the ice-salt bath the viscous mixture was hydrolyzed with crushed ice. Upon addition of 75 ml of 5 *N* hydrochloric acid a powder was obtained, which was filtered by suction and dissolved in sodium hydroxide solution. The solution was washed several times with ether and then acidified with hydrochloric acid, thus yielding a fine white precipitate, which was filtered by suction, washed with water and dried, yield 3.2 g; inhibition 80 % (0.05 μg per ml).

Enzyme experiments

As mentioned above the polymers, with one exception, have been investigated only as to their inhibitory effect on hyaluronidase. The same method was employed as in the previous paper [1]. The results are summarized in Table 1.

SUMMARY

High molecular weight polyesters and polyamides of phosphoric acid and aromatic hydroxy and amino compounds, containing at least two groups, capable of reacting with phosphorus oxychloride, have been prepared. They have been shown to exert a very high inhibitory action on hyaluronidase.

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