ACTA CHEMICA SCANDINAVICA 7 (1953) 913—920

Synthetic High Molecular Weight Enzyme Inhibitors

I. Polymeric Phosphates of Phloretin and Related Compounds *

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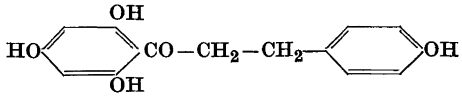
This investigation was started in order to throw some more light upon the mechanism of tubular reabsorption of glucose and the wellknown inhibitory action of phlorizin on this mechanism. According to results reported by Ellinger and Lambrechts [2] phlorizin appears to be absorbed from the tubular fluid by the epithelial cells of the proximal tubules, and the possibility was considered, that in this absorption phlorizin is chemically transformed into a specific agent, acting on glucose reabsorption. In view of the current hypotheses regarding the reabsorption of glucose, phosphorylation of phlorizin may be an obligatory step during its tubular absorption prior to its inhibitory action. Since alkaline phosphatase is generally assumed to be involved in the tubular reabsorption of glucose, some structural analogues of phlorizin were phosphorylated and their action on this enzyme was studied.

While the present study throws little light upon the possible occurrence of a renal phosphorylation of phlorizin, some interesting information concerning a new group of enzyme inhibitors has been obtained.

The phosphorylated substances, reported here, have been found to be the most potent inhibitors of alkaline phosphatase yet described. In addition it has been found, that these substances are very strong inhibitors of both hyaluronidase and urease. The first substance to be phosphorylated was phloretin, the aglucone of phlorizin. According to the structural formula



* A preliminary report [1] of some of these data was presented at the XVIII. Intern. Congress of Physiology, Copenhagen, 1950.

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this substance contains four hydroxy groups. During the phosphorylation of phloretin with phosphorus oxychloride in pyridine at low temperature it was assumed that a mixture of the mono-, di-, tri- and tetra-phosphates would result. However, during this phosphorylation the reaction mixture thickened within a few minutes and suddenly formed a gel, which contained products insoluble in all solvents. It was obvious that a polymerization reaction had occurred, leading to a presumably cross-linked high molecular weight polymer. When using an equimolecular amount of the phosphorylating agent and stopping the reaction just prior to this critical point a product could be isolated, which was soluble in water at a neutral or alkaline reaction, but insoluble in dilute mineral acid. This product did not dialyze through a cellophane membrane and has been shown by ultracentrifugation to have a molecular weight of about 15 000 [3]. If the reaction was stopped earlier, the degree of polymerization was lower. When investigating the polymer as to its effect on alkaline phosphatase and hyaluronidase, a very strong inhibitory effect was found. Therefore a systematic study was made under various experimental conditions of the phosphorylation of phloretin and other related substances. Among these other substances were phlorizin, flavone and flavanone glucosides and their aglucones, *viz.* rutin, quercetin, naringenin and naringin. From all these substances it was possible to obtain high molecular weight condensation polymers with a high antienzymic activity.

EXPERIMENTAL

A. Preparation of inhibitors

I. Polyphloretin phosphate. In a flask fitted with a thermometer, a calcium chloride drying tube and a dropping funnel 11 g of phloretin was dissolved in 50 ml of dry pyridine. The solution was cooled in an ice-salt bath to −10 to −12°. A solution of 4.5 ml of phosphorus oxychloride in 15 ml of dry pyridine was added with shaking at such a rate that the temperature remained at about −10° (about 10 minutes). When the flask was left for a further 3 minutes in the ice-salt bath, the solution gradually became viscous and was then hydrolyzed with crushed ice. The clear or almost clear solution was evaporated *in vacuo* and the thick oil thus obtained dissolved in about 100 ml of 2.5 *N* sodium hydroxide solution. After evaporating *in vacuo* until the smell of pyridine had disappeared, polyphloretin phosphate was precipitated as a viscous mass upon addition of dilute hydrochloric acid. The precipitate was washed with a small quantity of water and then dried *in vacuo* over phosphorus pentoxide and potassium hydroxide, resulting in a hard mass, which after pulverizing yielded a fine powder of a light green or light brown colour. If the moist precipitate was first treated with a mixture of equal parts of dry acetone and ether, a fine powder resulted; yield 10−12 g.

Analysis (from several runs): Moisture (sample dried at 100° *in vacuo*) 2−5 %; Pyridine (determined spectrophotometrically after alkalization and distillation) 5−10 %; Chlorine (combustion): 1−3 %; Phosphorus (calculated on a substance, free from moisture, pyridine and chlorine): 10−11 %.

During the phosphorylation other tertiary amines, *e.g.* quinoline, could be used instead of pyridine. The reaction proceeded more slowly in quinoline than in pyridine. If, for example, one mole of phosphorus oxychloride per mole of phloretin was used, the reaction mixture could be kept at room temperature for more than 24 hours without becoming thick or semi-solid. If a larger amount of phosphorus oxychloride was used (1.5−2 moles) the reaction mixture became semi-solid within one to two hours.

II. Polyquercetin phosphate. Six g of anhydrous quercetin was dissolved in 50 ml of dry pyridine. To this solution, cooled to −10°, a mixture of 2 ml of phosphorus oxychlor-

ide and 5 ml of dry pyridine was added over a period of five minutes. After a further 15—20 minutes at this temperature a thick, brown precipitate was formed. The mixture was then hydrolyzed and treated as above. The product was a yellow to brown powder; yield about 5 g. Phosphorus: 7—8 %. If the reaction time was prolonged a semi-solid product resulted which, however, slowly dissolved in water.

III. Polynaringenin phosphate. To a solution of 8.2 g of naringenin in 50 ml of dry pyridine, cooled to −10°, was added 3 ml of phosphorus oxychloride in 10 ml of dry pyridine over a period of five minutes. After a further 15 minutes at this temperature the solution became turbid. Three hours later the mixture was hydrolyzed and treated as in the previous examples. The product was obtained as a light brown powder; yield about 9 g. Phosphorus: 8.5 %.

IV. Polynaringenin chalcone phosphate. One g of naringenin chalcone (4,2′,4′,6′-tetrahydroxychalcone) was dissolved in 10 ml of dry pyridine. To this solution, cooled to −10°, 0.35 ml of phosphorus oxychloride in 5 ml of dry pyridine was added dropwise. As early as two minutes after the addition the solution became turbid. After a further 15 minutes at the same temperature a precipitate was formed. The mixture was then hydrolyzed and treated as in the previous examples. The product was a deep orange powder; yield about 0.7 g. Phosphorus: 8.7 %.

V. Polyphlorizin phosphate. A solution of 4.4 g of anhydrous phlorizin in 25 ml of dry pyridine was cooled to −10° and 1.5 ml of phosphorus oxychloride in 10 ml of dry pyridine was added with shaking over a period of 3 minutes. After a further 40 minutes the solution became turbid and after an additional 1 hour in the ice-salt bath the mixture was hydrolyzed, the resulting solution evaporated *in vacuo* and precipitated by the addition of dilute hydrochloric acid, saturated with sodium chloride. The semi-solid product was dissolved in a saturated sodium bicarbonate solution. The solution was filtered and the filtrate poured into an equal volume of dilute hydrochloric acid, saturated with sodium chloride. The semi-solid product was dried over phosphorus pentoxide and potassium hydroxide; yield 5.35 g. This product had a very high inhibitory effect on hyaluronidase. If the synthesis was carried out with 1.0 ml of phosphorus oxychloride, the inhibitory effect was similar. If 2 or 3 ml were used however, the inhibition was significantly less and with 5 ml the effect disappeared, presumably due to the formation of products with a relatively low molecular weight.

VI. Polyphlorizin sulphuric acid ester phosphate. To 15 ml of dry pyridine, cooled to −10°, was added 2.7 ml of chlorosulphonic acid. To this mixture a solution of 8.7 g of anhydrous phlorizin in 50 ml of dry pyridine was added slowly with cooling. The mixture was slowly heated on a water bath to 90° and was then left at room temperature for 15 hours. The reaction mixture was cooled to −10° and 2 ml of phosphorus oxychloride in 10 ml of dry pyridine was added and the mixture left at room temperature for 2 days and then hydrolyzed with crushed ice. The clear solution was alkalized with sodium carbonate and dialyzed against distilled water. The inner solution was evaporated *in vacuo* to dryness yielding a colourless powder; yield 11.4 g. Analysis: 4.4 % P; 8.9 % S. Thus the product contained about 2 atoms of sulphur per atom of phosphorus.

VII. Polyphloretin-4′-rhamnoglucoside phosphate. Phloretin-4′-rhamnoglucoside was synthesized by hydrogenation of naringin with Raney nickel catalyst in alkaline solution, m.p. 166—168°. Hydrolysis of this product by boiling with dilute sulphuric acid gave phloretin in good yield.

A solution of 2.2 g of anhydrous phloretin-4′-rhamnoglucoside was treated as above with 1.5 ml of phosphorus oxychloride. After 15 minutes in the ice-salt bath the solution became viscous and turbid. The mixture was hydrolyzed and treated as in V. A colourless precipitate formed with hydrochloric acid; yield 2.2 g. When using 1 ml of phosphorus oxychloride the inhibitory effect was unchanged. With 2.5 ml, however, the effect was significantly lowered.

VIII. Polyrutin phosphate. A solution of 1.2 g of rutin in 25 ml of dry pyridine was treated as above with 0.6 ml of phosphorus oxychloride in 5 ml of dry pyridine. The mixture was hydrolyzed and treated as in V. A precipitate was formed with hydrochloric acid. Yield 1.6 g. When 0.4 and 0.2 ml of phosphorus oxychloride were used in this experiment the antienzymic effect of the product gradually decreased. This decrease was even greater if a larger quantity (1.0 ml) was used.

IX. Polyphlorizin thiophosphate. To 1.1 g of anhydrous phlorizin in 15 ml of dry pyridine was added a solution of 0.6 ml of thiophosphoryl chloride in 10 ml of dry pyridine

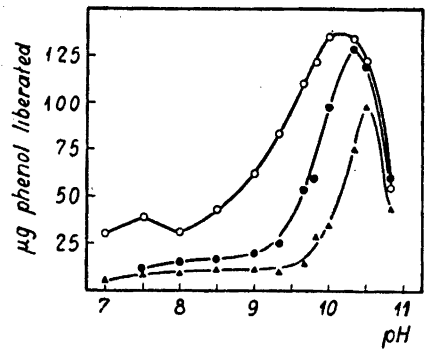


Fig. 1. Relationship between pH and inhibition of the enzymic hydrolysis of phenyl phosphate by polyphloretin phosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate concentration, 0.004 M. Enzyme-protein concentration, 1.2 µg per ml.

—○—○—, *without inhibitor*
—●—●—, *0.025 µg polyphloretin phosphate per ml.*
—▲—▲—, *0.25 µg polyphloretin phosphate per ml.*

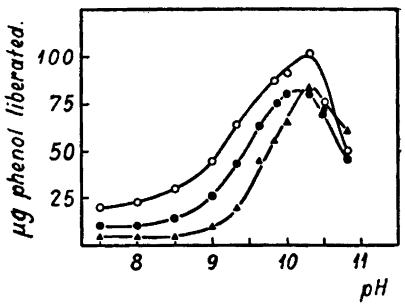


Fig. 2. Relationship between pH and inhibition of phosphatase by polyquercetin phosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate 0.004 M phenyl phosphate. Enzyme protein concentration, 1.2 µg per ml.

—○—○—, *without inhibitor*
—●—●—, *0.25 µg polyquercetin phosphate per ml.*
—▲—▲—, *2.5 µg polyquercetin phosphate per ml.*

with shaking over a period of 1—2 minutes at —10°. The mixture was left in the ice-salt bath for 3 hours and then at room temperature for 15 hours. After hydrolysis the solution was treated as in V; yield 1.25 g.

High molecular weight enzyme inhibitors were also prepared from hesperetin, phloretin-2'-methylether, phloretin-4-methylether, hesperidin and other similar compounds by analogous methods.

B. Enzyme experiments

Alkaline phosphatase.

The enzyme was prepared from the kidneys of young rabbits using the method described by van Thoai, Roche and Sartori [4]. In some experiments an enzyme preparation purified by electrophoresis was used.

Substrates: Phenyl phosphate, β-glycerophosphate and glucose-6-phosphate * were used.

Analytical methods: Phenol was determined with Folin's reagent as described by Buch and Buch [5]. Inorganic phosphate was estimated according to Martin and Doty [6]. The measurements were carried out in a Beckman spectrophotometer.

Conditions of enzyme experiments: Enzyme activity determinations were carried out within the pH range 7.0—11.0, using a 0.04 *M* borate-carbonate-acetate buffer without the addition of magnesium or manganese ions. Reaction volume, generally 2.5 ml; incubation time 10 or 16 min. at 37°. Enzyme concentration generally 1.2 µg of protein per ml.

Results: The inhibition of kidney alkaline phosphatase by polyphloretin phosphate, using phenyl phosphate as substrate is presented in Fig. 1. It appears from Fig. 1 that inhibition occurred on the acid side of the pH optimum only. A similar type of inhibition

* Glucose-6-phosphate was kindly supplied by Dr. O. Lindberg, Wennergren's Institute, Stockholm.

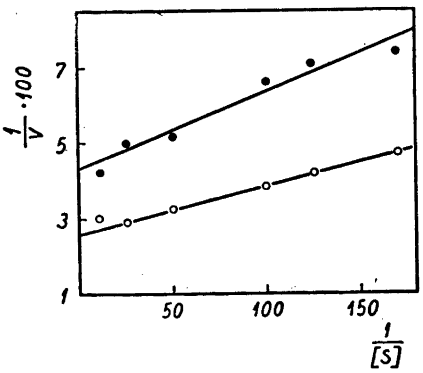


Fig. 3. Relationship between enzyme activity and substrate concentration in the presence and in the absence of polyphloretin phosphate. Activity is expressed as the reciprocal of the amount of P liberated in 10 min. at 37°. Enzyme protein concentration, 1.2 µg per ml. Substrate, phenyl phosphate. Acetate-carbonate-borate buffer pH 7.5.

—○—○—, *without inhibitor*
—●—●—, *0.025 µg polyphloretin phosphate per ml.*

was obtained by the use of other compounds of this group; in Fig. 2 the inhibition of alkaline phosphatase by polyquercetin phosphate is illustrated. As with polyphloretin phosphate, no inhibition could be demonstrated on the alkaline side of the pH optimum.

Substrate specificity: The inhibition of alkaline phosphatase is not substrate specific; using phenyl phosphate, β-glycero-phosphate or glucose-6-phosphate a similar inhibition was apparent.

Reversibility: It was found that the inhibition of alkaline phosphatase could not be reversed by magnesium or manganese ions in a concentration of 10^{-3} *M*. On the other hand, the inhibition could be quantitatively reversed by small amounts of basic proteins such as protamine sulfate or methyl gelatine in a concentration of 0.5 g/l. These two compounds did not activate the enzyme in the concentrations used.

Type of inhibition: To determine, whether the inhibition of alkaline phosphatase by polyphloretin phosphate was competitive or non-competitive, the concentration of phenyl phosphate was varied at a constant concentration of the enzyme in the presence and in the absence of the inhibitor. In Fig. 3 the reciprocal of the rate of enzymic hydrolysis (*v*, expressed as µg of P liberated after 10 min. incubation at 37° C) is plotted as ordinate against the reciprocal of the substrate concentration (*S*), following the procedure of Lineweaver and Burk [7].

It appears from Fig. 3 that the inhibition is not substrate competitive. A similar type of inhibition of kidney alkaline phosphatase by oestradiol-3,17-diphosphate has been reported previously [8].

Hyaluronidase.

Bovine hyaluronidase: The enzyme was prepared from bull testes and contained about 5 000 V.R.U. per mg N. The enzyme should be free from any stabilizer.

Hyaluronic acid: The substrate, hyaluronic acid, was prepared from umbilical cords by the method of Jeanloz and Forchielli [9] and a 0.2 % solution of the substrate in McIlvaine buffer, pH 7, was used for the test.

Inhibitors: The inhibitors studied were shaken with McIlvaine buffer, pH 7 ($Na_2HPO_4 \cdot 2H_2O$ 16.1 g; citric acid 2.02 g; NaCl 3.05 g; water to 1 000 ml) and then diluted with buffer to a concentration of 0.1 mg per ml.

Viscosity reducing test. Calibration of Ostwald viscosimeter: The readings were made in a bath at 37.5° C. 4 ml of the buffer solution was poured into an Ostwald tube and run through several times. The flow time was recorded.

Determination of viscosity of substrate solution: A mixture of 3 ml of the substrate solution and 1 ml of the buffer solution was run through as before. The flow time was recorded.

Determination of viscosity reduction by the enzyme: 0.1 ml of an enzyme solution, containing 2 V.R.U. (one V.R.U. is defined as the amount of enzyme, which will reduce the viscosity of the substrate used by one half in 10 minutes) and 0.9 ml of the buffer solution

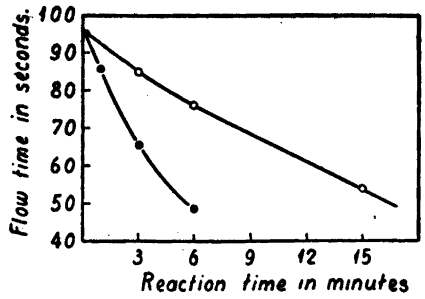


Fig. 4. Determination of anti-hyaluronidase activity. Flow time of buffer and substrate solution 11.5 and 95.5 sec. resp. Half time with and without inhibitor 15.5 and 5 min.

resp. % *inhibition* $\frac{15.5-5}{15.5} \times 100 = 68.$

—●—●—, *without inhibitor*
—○—○—, *with inhibitor.*

was mixed with 3 ml of the substrate solution. The stop watch was started immediately after the addition and the mixture was run several times through the viscosimeter with subsequent readings of the flow time.

Determination of the inhibition: 0.1 nl of an enzyme solution and 0.7 ml of the buffer solution were added to a mixture of 0.2 ml of the inhibitor solution and 3 ml of the substrate solution. The test was performed as above. In Fig. 4 a typical result is given.

Results. As a rule the inhibitory effect was measured at an inhibitor concentration of 5 μg per ml. The total amount of inhibitor used in each test was thus 20 μg.

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

Polymer prepared from	Inhibitory effect on hyaluronidase, %
Phloretin	100
Phloretin-2′-methylether	85
Phloretin-4-methylether	85
4,2′,4′-trihydroxy-3-methoxy-chalcone	70
Naringenin	65
Naringenin chalcone	95
Hesperetin	20
Quercetin	55
Phlorizin	95
Phlorizin sulphuric acid ester	85
Phloretin-4′-rhamnoglucoside	95
Naringin	40
Hesperidin *	70
Rutin	90
Phlorizin and $PSCl_3$	100

Suramin showed no inhibitory effect in this test at a concentration of 10 μg per ml. With 40 μg the inhibitory effect was 42 %.

With 1 mg of protamine or methylated gelatine the inhibitory effect was reversed. With 0.1 mg the effect was decreased by about 50 %. Polyphloretin phosphate was also tested on a bacterial hyaluronidase *(Staphylococcus pyogenes)*. The effect was of the same order of magnitude, but it was necessary to perform the test at pH 6. At pH 7 the inhibitory action was insignificant at a concentration of 5 μg per ml.

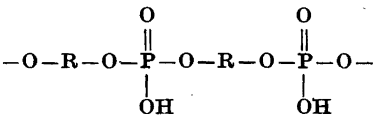
* Beiler and Martin [10] claim to have obtained a mixture of the tetra- and penta-phosphates of hesperidin by phosphorylating hesperidin with a large excess of phosphorus oxychloride.

Other enzymes.

A preliminary trial has been made on the inhibitory effect on several other enzymes. A very strong inhibition took place with prostate acid phosphatase, urease, β-amylase and brain hexokinase. With the last mentioned three enzymes a contact period between inhibitor and enzyme was, however, necessary. A detailed study on the inhibition of these and some other enzymes by the compounds described is in progress.

DISCUSSION

In the present paper, some properties and reactions of a new class of enzyme inhibitors are described and methods of their synthesis are reported. The substances in question are all polymeric and may be obtained by a condensation polymerization reaction between phloretin or other related aromatic polyphenols and a suitable phosphorylating agent. The degree of condensation depends on the particular conditions of synthesis. The polymers with a high degree of condensation are insoluble resinous substances. The members with a lower molecular weight are soluble at a neutral or alkaline reaction. The structure may contain elements of the type



Thus, the substances concerned are polyanions, and this structural property may be of significance as to their effect on enzymes. In this context, the inhibitory effect on enzymes of suramin [11], likewise a rather large anion, may be mentioned. The specificity of inhibition towards different enzymes seems quite similar, though the activity of the present substances surpasses that of suramin by several orders of magnitude. The inhibitory effect, however, does not seem to depend on electrical properties only. Combination between enzyme and inhibitor may also occur on the alkaline side of the isoelectric point of the enzyme, where both enzyme and inhibitor have negative charges. On the other hand, all inhibitions observed, could be reversed by the positively charged protamine, indicating the possibility of an electrostatic interaction. The ability of combining with alkaline proteins seems to be non-specific, since the enzyme inhibition is reversed besides protamine by various alkaline proteins among them gelatine, rendered basic by methylation.

With regard to the effect on enzymes, the difference between the various inhibitors described appears to be quantitative only, the ratio of the inhibitory effect on two enzymes being the same for two inhibitors of this class. Large differences exist, however, as to the effect on different enzymes. In the present work, experiments have been made with alkaline phosphatase, hyaluronidase, urease, brain hexokinase, β-amylase and prostate acid phosphatase. Very strong inhibitions were found with alkaline phosphatase, hyaluronidase and urease. The substances described here are the strongest inhibitors hitherto known of these enzymes.

As regards the mode of the inhibitory action, experiments with alkaline phosphatase revealed that the inhibition in this case was non-competitive, so that the site of combination with the inhibitor and with the substrate may be different. In the case of hyaluronidase the nature of the reaction and that of the substrate do not allow the performance of similar experiments. Since in this case both the substrate and the inhibitor are polyanions, the possibility of a competitive inhibition has to be considered.

SUMMARY

High molecular weight polyesters of phosphoric acid and phloretin and related polyphenols have been prepared. These polyesters have been shown to possess a very high inhibitory effect on several enzymes, above all alkaline phosphatase and hyaluronidase.

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Received February 20, 1953.