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Effect of Nitrophenols and Halogenophenols on the Enzymic Activity of Rat-liver Mitochondria

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Many studies on oxidative phosphorylation involve the use of 2:4-dinitrophenol (DNP) as an inhibitor of that process. Investigations of this kind and of other manifestations of DNP activity are numerous (for a review, see Brody, 1955). In some respects, other nitrophenols and also halogenophenols have similar biological properties to DNP (Clowes, Keltch, Strittmatter & Walters, 1950; De Deken, 1955). In particular, pentachlorophenol has been the subject of a study in parallel with DNP (Weinbach, 1954, 1956), and *p*-nitrophenol has been used by Terner (1954).

In spite of the extensive use of DNP in the study of plant- and animal-tissue metabolism, little is known of the way in which it produces its effects. There is general acceptance of the hypothesis that DNP accelerates the breakdown of some 'high-energy' compound formed during the synthesis of adenosine triphosphate coupled with the oxidation of substrates of the tricarboxylic acid cycle (Lardy, 1956; Slater & Lewis, 1954). The more restricted theory that DNP causes the breakdown of some phosphorylated intermediate was proposed by Hunter (1951), who also suggested the possibility that DNP itself may be phosphorylated with the formation of dinitrophenyl phosphate. The latter hypothesis has been rejected mainly on the grounds of the stability of dinitrophenyl phosphate (Lardy & Wellman, 1953). A similar argument has been advanced by Weinbach (1956) and Bringham, Brinch-Johnsen & Walaas (1956).

It is the object of the present work to record some observations on the similarities and differences of a series of nitro- and halogeno-phenols with respect to their action on the enzymic activity of rat-liver mitochondria *in vitro* and also to record the relative stabilities of some substituted phenyl

phosphates in mitochondrial preparations. For the purpose of this paper, characteristic effects of DNP on liver mitochondria have been compared with effects of the other phenols. There are three well-established biological actions of DNP, namely, the inhibition of oxidative phosphorylation (Loomis & Lipmann, 1948), which is usually demonstrated in the presence of hexokinase and glucose, the stimulation of oxidation of various substrates (Potter & Recknagel, 1951), which is revealed in the absence of hexokinase and glucose, and finally, the stimulation of adenosine triphosphatase activity (Myers & Slater, 1957).

EXPERIMENTAL

Methods

Preparation of mitochondria. The isolation of rat-liver mitochondria and the source and treatment of the reagents used in the enzymic procedures were identical with those described by Aldridge (1957) except in two points of detail. The homogenizer was a Nelco (Measuring and Scientific Equipment, London) used at 5000 rev./min. for 1½ min. and the cell debris and nuclei were removed by centrifuging at 650 g instead of 800 g. By this method mitochondria were produced which satisfied the criterion of normality as defined by Aldridge (1957) and earlier by Potter & Recknagel (1951). That is, in the presence of an appropriate substrate and cofactors these mitochondria are capable of a large increase of oxidative activity if presented with either DNP or hexokinase and glucose, or other substances which increase the rate of utilization of adenosine triphosphate.

Oxidative phosphorylation. The incubations were made in Warburg flasks. Each flask contained 0.5 ml. of mitochondrial suspension equivalent to 250 mg. wet wt. of liver, 0.00115 M-sodium adenosine triphosphate, 0.00115 M-adenosine monophosphate, 0.125 M-KCl, 0.014 M-MgCl₂·6H₂O, 0.001 M-disodium ethylenediaminetetra-acetic acid, 0.0154 M-potassium phosphate, 0.06 M-glucose, 0.0166 M-glycylgly-

cine buffer, pH 6.75, 400 units of hexokinase [modified version of method of Berger, Slein, Colowick & Cori (1946) to about step 3a], 0.01 M-glutamic acid as substrate, 0.1 ml. of an aqueous solution of the phenol or, with phenols of low solubility, a solution in 10% (v/v) ethanol. In the absence of phenol, 0.1 ml. of distilled de-ionized water was used. Final volume was 3 ml.; 0.15 ml. of 20% KOH was present in the centre well. Gas phase, air. Final pH was 6.7–6.8. Temp., 37°. After temperature equilibration for 10 min., O₂ uptake was recorded for the following 10 min. The flasks were removed from the manometers and the reactions stopped by the addition of 0.5 ml. of 60% (w/w) perchloric acid. Initial inorganic phosphate was determined in a zero-time flask to which had been added 0.5 ml. of perchloric acid before the addition of the mitochondria. In each flask inorganic phosphate content was measured. By this method O₂ uptake was measured over a period of 10 min. and phosphate uptake over a period of 20 min., and thus for the purpose of calculation of the P:O ratio the method depends upon the linearity of the O₂ uptake. Such a linear relationship was established for the time which elapsed after the initial O₂ uptake reading at 10 min. and was assumed for the time from 0 to 10 min. As the P:O ratio was required only for comparative purposes, it was felt that the assumption was warranted.

Stimulation of oxidation. These experiments were also performed by the Warburg technique. The constituents and the final concentrations of the contents of each flask were the same as described in the previous method except that glucose and hexokinase were omitted. The mitochondrial suspension was decreased to 0.35 ml., equivalent to 175 mg. wet wt. of liver. The final volume was adjusted to 3 ml. Gas phase, air; pH was 6.8. Temp., 37°. The flasks and contents were equilibrated for 10 min. before the initial reading. Observations of the O₂ uptake were recorded at 10 min. intervals for the subsequent hour.

Adenosine triphosphatase. In these experiments the liberation of inorganic phosphate from adenosine triphosphate was measured in the presence of mitochondria and the conditions of pH and tonicity (0.30–0.33 osmolar) were as nearly as possible identical with those of the previous experiments. Each vessel contained 0.0034 M-sodium adenosine triphosphate, 0.13 M-KCl, 0.014 M-MgCl₂·6H₂O, 0.001 M-disodium ethylenediaminetetra-acetic acid, and 0.0166 M-glycylglycine. The mitochondrial suspension was 0.5 ml. The phenol was added as 0.5 ml. of an aqueous solution and the final volume adjusted to 3 ml., pH 6.8. Temp., 37°. The vessels were placed in a Dubnoff constant-temperature shaker and equilibrated for 10 min. before the addition of the mitochondria. After a further 10 min., the reaction was stopped by the addition of 0.5 ml. of 60% (w/w) perchloric acid. The solutions were made up to 10 ml. with water, centrifuged and phosphate was determined in the supernatant.

Hydrolysis of phenyl phosphates. For these experiments it was necessary to destroy the integrity of the mitochondria as it was found that intact mitochondria hydrolysed the phenyl phosphates only very slowly (cf. Berthet, Berthet, Appelmans & de Duve, 1951). The mitochondria were disrupted by substituting water for the KCl solution in the final suspension, leaving for 0.5 hr. at 0° and then subjecting them to a short treatment by the Nelco homogenizer. Each flask contained 0.0166 M-glycylglycine buffer, pH 6.8, 0.014 M-MgCl₂·6H₂O and 0.0033 M-phenyl

phosphates. Final volume was 3 ml. Temp., 37°. After shaking in the Dubnoff apparatus for 10 min., 0.5 ml. of mitochondrial suspension was added. At intervals of 15 min., flasks were removed and 0.5 ml. of 60% (w/w) perchloric acid was added to each. At the conclusion of the experiment the contents of each vessel were made up to 10 ml. with water, centrifuged and phosphate was determined in the supernatant. Control experiments were performed in vessels without mitochondria to determine the non-enzymic hydrolysis of the phenyl phosphates. With pentachlorophenyl phosphate it was necessary to dilute the mitochondria preparation fivefold before the experiment.

Phosphate determination for all experiments was by the method of Fiske & Subbarow (1925).

The mitochondrial preparations were analysed for protein by a modification of the quantitative biuret reaction of Robinson & Hogden (1940) described by Aldridge (1957).

Dissociation constants. These were taken from the literature as follows: *o*-, *m*-, *p*-nitro-, 2,5-dinitro-, *o*- and *p*-chloro-phenols from Judson & Kilpatrick (1949); 2,4-dinitro-, 2,6-dinitro- and 2,6-dichloro-4-nitro-phenols from Krahll & Clowes (1938); *s*-trichloro- and 2,4-dichloro-phenols from Ketelaar, Gersmann & Beck (1952); and pentachlorophenol from Blackman, Parke & Garton (1955).

Materials

The phenols used were all obtained from commercial sources. The phenyl phosphates were obtained as follows: pentachlorophenyl phosphate and *p*-chlorophenyl phosphate from Roche Products Ltd., *o*-chlorophenyl phosphate and 2,4-dichlorophenyl phosphate from Professor E. J. King; *s*-trichlorophenyl phosphate was prepared by the hydrolysis of *s*-trichlorophenyl phosphorodichloridate (Havenga, de Jongh & Dorst, 1956), which in turn was prepared by treatment of *s*-trichlorophenol with excess of phosphorus oxychloride in the presence of NaCl (Kraft & Katyskhina, 1952); *m*-nitrophenyl phosphate and *p*-nitrophenyl phosphate were prepared by the method of Bessey & Love (1952). The following methods were used in unsuccessful attempts to prepare 2,4-dinitrophenyl phosphate: reaction between DNP and dibenzyl phosphorochloridate, followed by catalytic hydrogenation to remove the benzyl groups; reaction between DNP and bis-dimethylaminochlorophosphine oxide under various conditions, followed by hydrolytic removal of the dimethylamino groups; reaction between DNP and phosphorus oxychloride in the presence of pyridine (Bessey & Love, 1952); reaction between DNP and excess of phosphorus oxychloride with and without organic solvents, followed by various treatments of the dinitrophenyl phosphorodichloridate so formed.

RESULTS

Preliminary experiments confirmed that at least qualitatively substituted nitro- and halogenophenols simulate the action of DNP *in vitro*. Thus they were all capable of stimulating the oxidation of glutamic acid; they could all stimulate or release 'latent' adenosine triphosphatase (ATP-ase) activity of mitochondria, and they would all inhibit the phosphorylation associated with the oxidation

of glutamic acid. The concentration of a given phenol needed to evoke any one of these effects was dependent upon the phenol concerned and, in general, could be correlated with its dissociation constant (Fig. 1). Similar relationships to that depicted in Fig. 1 could be demonstrated for the concentrations required to stimulate oxidation or ATP-ase activity. Phenol and *o*-nitrophenol both exhibit a lower activity than would be expected from a knowledge of their dissociation constants.

Examination of the character of the stimulation and inhibition curves of these phenols shows that they can be divided into two groups on the basis of their resemblance to or diversity from DNP. Fig. 2 presents an illustration of the differences between the two groups. The curves of Fig. 2A (DNP) are characteristic of pentachlorophenol, trichlorophenol and *p*-nitrophenol. The main features are: a maximum in the ATP-ase curve, a large stimulation of oxidation of glutamic acid and an inhibition of oxidative phosphorylation with no effect on the concurrent oxygen uptake except at concentrations which completely inhibit

phosphorylation. The second group of phenols, exemplified in Fig. 2B by *p*-chlorophenol, also includes 2:4-dichlorophenol, *o*-chlorophenol, *m*-nitrophenol, *o*-nitrophenol and phenol. These compounds produce no maximum ATP-ase activity, they stimulate oxidation only slightly and inhibition of oxidative phosphorylation is closely followed by inhibition of the concurrent oxygen uptake. The differences between the two groups are further demonstrated in Table 1, where their activities compared with DNP are listed.

It is noticeable in Fig. 2A that the concentration of DNP producing maximum stimulation of ATP-ase activity coincides with that concentration which produces maximum stimulation of oxidation. This coincidence appears to be a property of the other phenols of this group (Table 2). The concentration of a phenol of this group required to inhibit oxidative phosphorylation by 50% is of the same order as the concentration evoking any one of the other two responses.

The hypothesis that DNP and other substituted phenols act by accepting phosphate from a phosphate donor of the high-energy system requires that the phosphorylated intermediate should be unstable. With intact mitochondria the rates of hydrolysis of the various phenyl phosphates were very slow. This was also the case with adenosine

Fig. 1. Relationship between dissociation constant and concentration of phenol required to inhibit phosphate uptake by 50%. For concentrations and conditions of each experiment see Experimental section. ●, Phenols studied with respect to their effect on oxidative phosphorylation, oxidative stimulation and increase of ATP-ase activity; Δ, phenols studied with respect to their effect upon oxidative phosphorylation only. 1, DNP; 2, *o*-nitrophenol; 3, *p*-nitrophenol; 4, *m*-nitrophenol; 5, pentachlorophenol; 6, *s*-trichlorophenol; 7, 2:4-dichlorophenol; 8, *o*-chlorophenol; 9, *p*-chlorophenol; 10, phenol; 11, 2:5-dinitrophenol; 12, 2:6-dinitrophenol; 13, 2:6-dichloro-4-nitrophenol.

Fig. 2. Characteristic activity and inhibitory curves of two groups of phenols. Type A, e.g. DNP. Type B, e.g. *p*-chlorophenol. Values for each point for the three experiments were obtained as described in the Experimental section. Δ, Uptake of phosphate; ○, uptake of oxygen.

Table 1. *Relative influence of substituted phenols on enzymic activities of mitochondria*

In the third column the stimulation of oxidation by DNP has been equated to 100% and the stimulation by the other phenols expressed on this scale. In the seventh column the presence or absence of a maximum in the ATP-ase activation curves is described as + or - respectively. All concentrations are expressed in molar terms.

Compound	Stimulation of oxidation		Inhibition of O ₂ uptake when phosphate uptake = 50%		Maximum stimulation of ATP-ase	
	Concn. (M)	(%)	Concn. (M)	(%)	Concn. (M)	
DNP	2.0×10^{-5}	100	1.0×10^{-5}	0	2.7×10^{-5}	+
<i>p</i> -Nitrophenol	2.2×10^{-4}	100	1.0×10^{-4}	0	2.2×10^{-4}	+
Pentachlorophenol	9.2×10^{-6}	80	9.0×10^{-6}	0	1.0×10^{-5}	+
Trichlorophenol	1.8×10^{-4}	70	4.0×10^{-5}	0	1.0×10^{-4}	+
<i>o</i> -Nitrophenol	3.3×10^{-3}	16	4.5×10^{-4}	40	2.4×10^{-3}	-
<i>m</i> -Nitrophenol	6.7×10^{-4}	22	1.6×10^{-4}	40	6.3×10^{-4}	-
2:4-Dichlorophenol	1.1×10^{-4}	27	7.4×10^{-5}	39	3.4×10^{-4}	-
<i>o</i> -Chlorophenol	2.4×10^{-3}	15	4.5×10^{-4}	24	2.8×10^{-3}	-
<i>p</i> -Chlorophenol	2.8×10^{-4}	21	2.9×10^{-4}	40	8.0×10^{-4}	-
Phenol	8.0×10^{-3}	7	6.0×10^{-3}	46	1.6×10^{-2}	-

Table 2. *Comparative concentrations of type A phenols affecting enzymic activities of mitochondria*

Row 1, concentrations producing 50% inhibition of the P:O ratio; row 2, concentrations producing maximum stimulation of ATP-ase activity; row 3, concentrations producing maximum stimulation of oxidation. All concentrations are expressed in molar terms.

Compound	DNP (M)	<i>p</i> -Nitrophenol (M)	Trichlorophenol (M)	Pentachlorophenol (M)
Experiment				
50% inhibition of P:O ratio	1×10^{-5}	1×10^{-4}	4.0×10^{-5}	9×10^{-6}
Stimulation of ATP-ase	2.7×10^{-5}	2.2×10^{-4}	1.0×10^{-4}	9.2×10^{-6}
Stimulation of oxidation	2.0×10^{-5}	2.2×10^{-4}	1.8×10^{-4}	1.0×10^{-5}

Table 3. *Relative rates of hydrolysis of nitro- and halogeno-phenyl phosphates*

For composition of medium and concentration of phenyl phosphates or of adenosine triphosphate see Experimental section. For each experiment the rate of hydrolysis by water-treated mitochondria of each compound was compared with that of phenyl phosphate. Actual rates were calculated as μ moles of inorganic phosphate liberated/mg. of mitochondrial protein/10 min.

Compound	Relative rate of hydrolysis
Phenyl phosphate	1.00
<i>o</i> -Chlorophenyl phosphate	0.75
<i>m</i> -Nitrophenyl phosphate	0.95
<i>p</i> -Chlorophenyl phosphate	1.40
2:4-Dichlorophenyl phosphate	1.45
<i>p</i> -Nitrophenyl phosphate	1.70
Trichlorophenyl phosphate	2.40
Pentachlorophenyl phosphate	3.20
Adenosine triphosphate	5.50

triphosphate when incubated with mitochondria in the absence of substrate and inorganic phosphate. Nevertheless, rates of hydrolysis comparable with phenyl phosphate could be obtained when the mitochondria had been subjected to some disruptive influence such as water. The results (Table 3) show that nitro- and halogeno-phenyl phosphates derived from phenols whose activity towards mitochondrial systems *in vitro* most resemble DNP are relatively less stable in such mitochondrial pre-

parations than phenyl phosphates derived from phenols of the second group. Thus, of the phenyl phosphates examined, pentachlorophenyl phosphate is hydrolysed at a greater rate than any of the other phenyl phosphates examined and is derived from a parent phenol which is more active towards mitochondrial systems than any other phenol. For comparison, the stability of adenosine triphosphate determined under the same conditions is included in Table 3.

The release of latent ATP-ase from intact mitochondria by treatment with DNP is a well-established phenomenon (Myers & Slater, 1957). An experiment was performed in which the rate of hydrolysis of *p*-nitrophenyl phosphate relative to the rate of hydrolysis of phenyl phosphate was measured in the presence of 10^{-5} M-DNP. The hydrolysis rate was then not increased above the low rate of non-enzymic hydrolysis.

DISCUSSION

It is a common feature of many of the compounds which have been reported to behave as DNP that they possess a phenolic hydroxyl group, e.g. thyroxine, aureomycin, stilboestrol, usnic acid, salicylic acid, nitro- and halogeno-phenols. It has also been shown that the hydroxyl group must be unsubstituted for full activity in mitochondrial

systems *in vitro* (Cross, Taggart, Covo & Green, 1949). In the present study of nitro- and halogenophenols it is seen that there is a general adherence to DNP-like properties, but closer examination reveals the existence of two distinct groups. Of the second group of phenols, here exemplified by *p*-chlorophenol, a failure to produce marked stimulation of oxidation might be due to low solubility of the compound or its presence in the undissociated form. The most important finding, however, is that during their action on oxidative phosphorylation oxygen uptake is also greatly inhibited. This would appear to be the clearest practical distinction between phenols of the two groups. It is of interest that thyroxine (Hoch & Lipmann, 1954) and aureomycin (van Meter, Spector, Oleson & Williams, 1952) behave in a similar fashion. With these two the level of magnesium low concentration is the major factor which determines their departure from the type of activity shown by DNP. This point has not been investigated among the phenols under discussion because it was considered desirable to compare their effects in a medium which, with respect at least to the major ionic species, resembled closely that of the intracellular fluid (Aldridge, 1957).

That the degree of dissociation of a compound is of importance with respect to its biological activity has been stressed by Simon & Beevers (1952) and by Blackman *et al.* (1955). Furthermore, in a study of the effects of a series of nitro- and halogenophenols on yeast metabolism, De Deken (1955) concluded that the active agent was the undissociated phenol. From the present work the opposite conclusion can be drawn for, at the experimental pH 6.8, it is those phenols which are highly dissociated which are the most potent inhibitors of oxidative phosphorylation. On the other hand, those phenols which are poor inhibitors are, in general, in the undissociated form. A reconciliation between the two points of view may be made by consideration of the intracellular pH of the yeast cell. The consensus of opinion (Caldwell, 1956) is that this value is about pH 5.9. When it is borne in mind that the most active phenols used by De Deken have *pK* values of 4.6 or less it is evident that these compounds were largely in the dissociated form in the interior of the yeast cell. A further observation by De Deken (1955), namely that the inhibitory power of a phenol is weakened by the presence of an ortho substituent capable of hydrogen-bonding, is confirmed. Both *o*-chloro- and *o*-nitro-phenol exhibit lower activity than would be expected from a knowledge of their dissociation constants, although the discrepancy with *o*-chlorophenol is the lesser of the two. The decrease in activity due to the proximity of nitro groups to the hydroxyl group is seen in DNP, with one such

group, and 2,6-dinitrophenol with two *o*-nitro groups, whereas the dissociation constants are not very different. In addition, the change from *o*-nitro to *o*-chloro substitution, here exhibited between 2,6-dinitrophenol and 2,6-dichloro-4-nitrophenol, increases the biological activity in favour of the latter compound. Once again the dissociation constants are almost the same. In the two compounds each with *o*-nitro substituents but with differing dissociation constants, the one with the lower *pK* value is the more active, e.g. DNP (*pK* 4.0) is more active than 2,5-dinitrophenol (*pK* 5.1). The marked effect of a second *o*-nitro group was demonstrated in a single experiment with picric and picramic acid on oxidative phosphorylation. Picric acid (*pK* 1) at the highest concentration obtainable (10^{-2} M) was a poor inhibitor, whereas picramic acid (*pK* 4.2) had an inhibitory power similar to that of 2,5-dinitrophenol, although not as great as its dissociation constant would lead one to expect.

Among the possible modes of action of DNP, Hunter (1951) mentions the intermediate formation and spontaneous or enzymic decomposition of dinitrophenyl phosphate. Since then the hypothesis has been examined and in general rejected mainly on the grounds of stability of the phosphorylated intermediate. It is stated in a footnote to the paper of Lardy & Wellman (1953), for instance, that dinitrophenyl phosphate is a relatively stable compound. Again, for pentachlorophenol, Weinbach (1956) considers the hypothesis as unlikely and states that pentachlorophenyl phosphate is inert in intact or damaged mitochondrial systems. Brigham *et al.* (1956) consider this theory in their studies on the action of DNP but, using *p*-nitrophenol, they were unable to demonstrate the formation of *p*-nitrophenyl phosphate.

The data of this paper suggest that the inhibition of uptake of phosphate, the stimulation of oxidation and the stimulation of adenosine triphosphatase by DNP are manifestations of a single phenomenon. If this is so and if it be that dinitrophenyl phosphate is an intermediate in this reaction it follows that the compound should undergo rapid resolution into dinitrophenol and phosphate. It would therefore be difficult to detect the formation of such a compound. It was thought, nevertheless, that labelling the compound with ^{32}P would increase the chance of such detection. But it was calculated, with *p*-nitrophenol for instance, that under the most favourable conditions of negligible hydrolysis the maximum radioactivity of *p*-nitrophenyl phosphate would not be distinguished from the background activity (determined with an M.G. 20th Century liquid counter). This was in fact the result of an experiment in which carrier *p*-nitro-

phenyl phosphate was added to mitochondria subjected to the inhibitory effect of 10^{-4} M-*p*-nitrophenol in the presence of about $1 \mu\text{C}$ of ^{32}P .

Although the rates of hydrolysis of the nitro- and halogeno-phenyl phosphates in damaged mitochondria may bear no relationship to the rates in intact mitochondria, they at least demonstrate that mitochondria do contain enzymes capable of this action. The reservation must be made that one enzyme which may be responsible for this action is acid phosphatase, an enzyme which de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) have demonstrated to be associated with the particle termed by them 'lysosome'. In this connexion two aspects of the present work are significant. One is that the activity induced by DNP, whilst accelerating the breakdown of adenosine triphosphate, at the same time has no effect on the phenyl phosphates. Secondly, the type of mitochondrial preparation used may possibly contain some of these lysosomes.

It is admitted that this paper presents no evidence that DNP acts by accepting phosphate from some high-energy phosphorylated intermediate formed during the process of oxidative phosphorylation. However, it can be said that, inasmuch as this hypothesis has previously been rejected on the grounds of stability of dinitrophenyl phosphate, this work indicates that such an argument is capable of revision. Evidence for the stability of dinitrophenyl phosphate itself is lacking because of the difficulty of preparing this compound, but this very difficulty provides some indication of the chemical instability of this substance. Also by analogy with the phenyl phosphates described here it is likely that the hydrolysis of this compound in the presence of mitochondrial enzymes would be rapid.

SUMMARY

1. Of a series of nitro- and halogeno-phenols, it is shown that qualitatively there is a resemblance to 2:4-dinitrophenol with respect to the action towards rat-liver mitochondria.

2. Quantitatively, it is further demonstrated that these compounds can be divided into two groups based upon the characteristics of their behaviour towards oxidative phosphorylation, oxidation of glutamic acid and release or activation of latent adenosine triphosphatase.

3. There is a correlation between activity of these compounds *in vitro* and their dissociation constants which is modified by nitro groups ortho to the hydroxyl group.

4. The hydrolysis of some of the corresponding nitro- and halogeno-phenyl phosphates is described.

5. The results are discussed.

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