ANTIMITOTIC ACTION OF SOME PHENOLIC COMPOUNDS IN TISSUE CULTURES OF CHICK FIBROBLASTS

by

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INTRODUCTION

In tissue cultures of chick fibroblasts 2-methyl-1:4-naphthohydroquinone diphosphate (Synkavit) is an inhibitor of mitosis, as shown by MITCHELL AND SIMON-REUSS[1]. The same kind and the same order of inhibition is produced by maleic acid as found by the authors[2]. The substituted hydroquinone is derived from a quinone which contains in its aliphatic part the residue of maleic acid. Subsequently the mitotic inhibitions produced by the maleic acid groups have been compared[3]. For tissue cultures it was necessary to use substances which were soluble in water. As many quinones lack this property, the diphosphates of the hydroquinones and not the free quinones were applied. In doing so, we were fully aware that the comparison was based on two assumptions[4]: we had to presuppose that the diphosphates are degraded in tissue cultures to the corresponding hydroquinones, and further, that the quinones have the same degree of antimitotic activity as the hydroquinones. The investigations of MOOG[5,6], showing that embryo extract contains alkaline as well as acid phosphatases, and the observation of LEHMANN[7] that, in *Tubifex* eggs, hydroquinones and quinones are equally active as mitotic inhibitors seemed to justify this approach.

As long as the water solubility of the substances to be examined was the limiting factor of our experiments the evidence obtained by them was bound to remain doubtful or incomplete. Since we succeeded in testing water-insoluble compounds as well by dissolving them in a mixture of cellosolve and gum ghatti (FRIEDMANN AND SIMON-REUSS[8]) it became possible to base the comparison of the antimitotic activities of the phenolic substances, their derivatives, the corresponding quinones and the maleic acid group on direct experimental evidence.

In the present paper we describe the results obtained with some phenolic compounds in their action on the mitosis in tissue cultures of chick fibroblasts. The picture arising from these experiments shows, among other results, that the inhibition of mitosis is produced by phenolic substances and their phosphates in different ways. The assumption that the embryo extract hydrolyses the phosphates of the phenols, giving thereby the active principles, is therefore far too narrow to cope with the observations presented in this paper.

METHODS

The experiments were carried out in tissue cultures of chick fibroblast using the *hanging drop method*, as described previously[2].

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RESULTS

The results obtained with some phenolic substances and their derivatives in the mitosis of chick fibroblasts are summarised in Table I. Table II shows the action of 1:4-naphthohydroquinone and of some of its derivatives on the same biological material.

TABLE I

50% MITOTIC INHIBITION BY SOME PHENOLS AND THEIR DERIVATIVES

Tissue cultures: chicken fibroblasts.
"Inactive" stands for "No activity at 10^{-5} *M* conc. and greater dilutions".

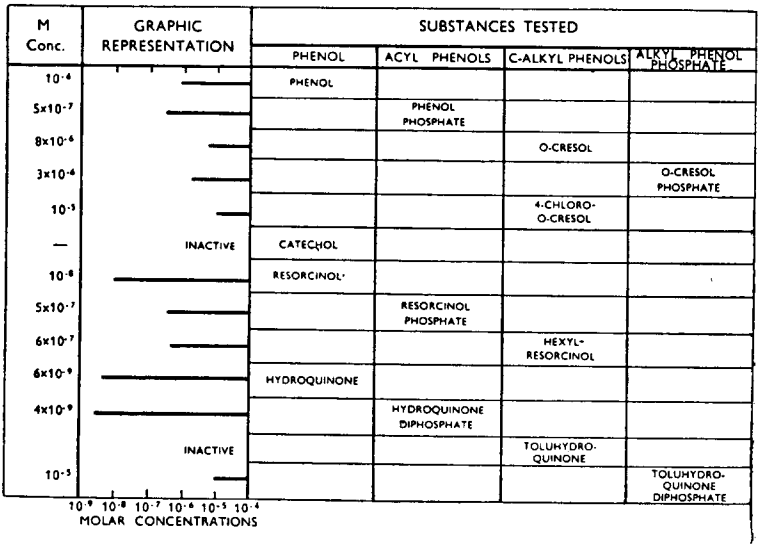
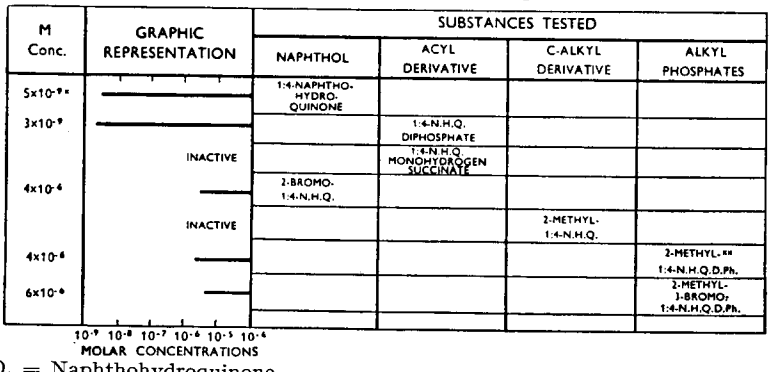


TABLE II

50% MITOTIC INHIBITION BY 1:4-NAPHTHOHYDROQUINONE AND BY SOME OF ITS DERIVATIVES

Tissue cultures: chicken fibroblasts.
"Inactive" stands for "No activity at 10^{-5} *M* conc. and greater dilutions".



N.H.Q. = Naphthohydroquinone.
N.H.Q.D.Ph. = Naphthohydroquinone diphosphate.

* Mitotic inhibition at $5 \cdot 10^{-9}$ was 44%.
** J. S. MITCHELL and I. SIMON-REUSS, 1947.

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DISCUSSION

The antimitotic activity of phenolic compounds and their derivatives has been described prior to our investigations by ÖSTERGREN[9], LEVAN[10] and their collaborators. In their extensive and fundamental researches these authors have used the roots of *Allium cepa* as testing material. As we have worked so far only with tissue cultures of chick fibroblasts, we had to use the same biological material for the examination of phenols in order to get comparable results.

With the exception of catechol all the unsubstituted phenolic substances display antimitotic activity. The highest activity is found with the dihydric phenols, 1:4-benzohydroquinone and 1:4-naphthohydroquinone. The order of their activity is 10^{-9} *M*. A lower activity, but still a very high one, 10^{-8} *M*, is found with resorcinol, whilst the antimitotic activity of phenol and cresol is of the order of 10^{-6} *M*.

Phosphorylation of the hydroxylic groups of phenol, *o*-cresol, benzohydroquinone, toluhydroquinone, 1:4-naphthohydroquinone and 2-methyl-1:4-naphthohydroquinone resulted in substances with increased antimitotic activity. In contrast, resorcinol

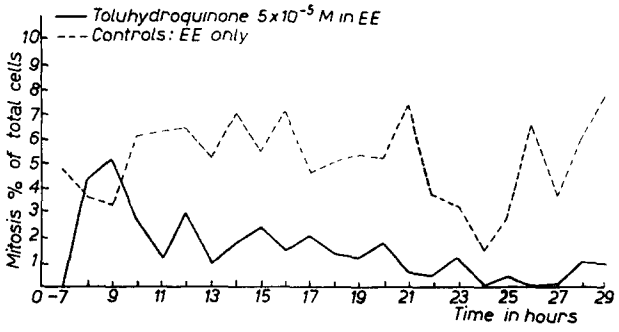

Fig. 1. Cinepicture of toluhydroquinone. Comparison of mitosis after application of toluhydroquinone at $5 \cdot 10^{-8}$ *M* with mitosis in extract alone. Mitoses in % of total cells. Tissue cultures. Chick fibroblasts.

diphosphate has an appreciably lower antimitotic activity, $5 \cdot 10^{-7}$ *M* against 10^{-8} *M* of resorcinol.

The introduction of halogen decreases the antimitotic activity of the phenolic compounds.

C-alkylation of the phenolic compounds is accompanied everywhere by a strong decrease of antimitotic activity: in 1:4-benzohydroquinone the 50% inhibition of mitosis drops from $6 \cdot 10^{-9}$ *M* to a value smaller than 10^{-5} *M* in toluhydroquinone and only a cinepicture could reveal the antimitotic activity of this substance, as shown in Fig. 1. This result has some importance as it disposes of the possibility that the lack of antimitotic activity of toluhydroquinone at 10^{-5} *M* may be due to a reaction of this compound with one of the many constituents of the Tyrode fluid, used in preparing the dilutions for tissue cultures.

Similar results are obtained in the naphthohydroquinone group. Here the activity of 1:4-naphthohydroquinone dropped from $5 \cdot 10^{-9}$ *M* to a value smaller than 10^{-5} *M* in the 2-methyl-1:4-naphthohydroquinone. Thus with benzohydroquinone as well as with 1:4-naphthohydroquinone the maximum of activity, exerted by these substances, is changed to a low degree of activity in their 2-methyl derivatives. Less dramatic but

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still considerable is the drop in activity of the corresponding phosphates, following C-alkylation. In benzohydroquinone diphosphate the activity decreased from $6 \cdot 10^{-9}$ M to 10^{-5} M in toluhydroquinone diphosphate, and in 1:4-naphthohydroquinone diphosphate from $3 \cdot 10^{-9}$ M to $4 \cdot 10^{-6}$ M in 2-methyl-1:4-naphthohydroquinone diphosphate[7].

The other phenolic substances investigated gave similar results; with resorcinol, a decrease was produced from 10^{-8} M to $6 \cdot 10^{-7}$ M in hexylresorcinol, and with phenol from 10^{-6} M to $8 \cdot 10^{6}$ M in *o*-cresol.

It has been assumed by some authors that phenols exert their antimitotic activity after being oxidised to quinones. This assumption has no experimental basis, as the direct action of quinones on the mitosis of chick fibroblasts is not sufficiently investigated.

Physical chemical attempts to explain the activity of the phenols are mainly found in the papers of ÖSTERGREN, LEVAN and their collaborators. These authors emphasize the importance of the ratio of water solubility to fat solubility, and have given much experimental evidence which can be explained on the basis of the MEYER, OVERTON theory of narcosis.

Another physical chemical approach has its starting point from the theories and experiments of TRAUBE, HÖBER, WINTERSTEIN AND WARBURG on the surface activity of the cells. TRIM AND ALEXANDER[11] have pointed out in an interesting review that these two concepts do not exclude one other. In this connection the experiments of GALE[12] and his collaborators on the release of important cell constituents through the action of detergent substances have to be mentioned. An observation which suggests a chemical reaction, indicated by volume contraction, has been made by ÖSTERGREN[13] who obtained a shortening of the chromosomes under the influence of phenols.

The phosphates of the phenolic compounds have nearly always a greater antimitotic activity than their parent substances, as shown in a very striking way by the phosphates of toluhydroquinone and of 2-methyl-1:4-naphthohydroquinone. Therefore the high activity of the phosphates cannot be explained by assuming that they are degraded to their less active parent substances, and conversely the lesser activity of the phenols show that phosphorylation cannot play a leading part in their antimitotic activity. Thus in order to exert their antimitotic activities, phenolic substances must follow a pathway which is different from the pathway followed by their phosphates. This recalls similar observations on the pharmacological action of salicylic acid and its acetyl derivative.

Nevertheless there may be a connection—other than phosphorylation—between phosphates, phenols, phenolphosphates and their activities as mitotic inhibitors. In this respect the following points may be remembered. Old experiments of AUTENRIETH[14] and his collaborators have shown that a multiple of the lethal doses of phenol is well tolerated by animals, if phosphate is administered at the same time. Recent experiments of AXELROD[15] have brought to light the faculty of phenolphosphate to allow trans-phosphorylation. Finally it is well known that phosphates penetrate into the cell only with difficulty[16] or not at all.

These apparently divergent data do not allow, for the time being, the development of a coherent picture of the connection between phosphates and phenol activity. But a direct chemical approach to these questions seems to be possible, an approach the more interesting as it deals with reactions taking part in living cells and contrasting hereby to the –SH reactions, proceeding in tissue cultures with the dead matter of embryo extract.

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CHEMICAL SECTION

Phenol phosphate. A solution of phenol (1.9 g) in pyridine (8 ml) was added drop-wise with stirring to an ice-cooled stirred solution of phosphorus oxychloride (3 ml) in pyridine (30 ml). After 15 minutes at room temperature, the pyridine was distilled off *in vacuo* at 50°. The cooled residue was decomposed with ice (50 g) and water (100 ml) and the resulting solution treated with magnesium oxide (7.5 g) suspended in water (200 ml) with stirring. The mixture was filtered (Hyflo) and the filtrate evaporated to dryness *in vacuo*. The residue was dried off *in vacuo* and well extracted with alcohol. Residue 4.0 g.

The magnesium salt was suspended in water, sodium carbonate (2 g) added and the filtered solution brought to pH 7 with 5 *N* hydrochloric acid (3.5 ml). The solution was passed through a column of Zeocarb loaded with hydrogen ion, the eluate evaporated *in vacuo* and the crystalline hydroscopic residue quickly pressed dry on a porous tile. After drying *in vacuo* the residue was 2.05 g, m.p. 89–90°. The hydroscopic nature of the material made further purification difficult. JACOBSEN[17] gives m.p. 97–98° for phenol-phosphate.

o-Cresol phosphate. This substance, m.p. 95°, has been prepared by ASAKAWA[18] by hydrolysis of the corresponding dichlorophosphinate. However it was considered more convenient to prepare this compound as its easily isolated barium salt as follows: a solution of *o*-cresol (3.0 g) in pyridine (30 ml) was added drop-wise to an ice-cooled stirred solution of phosphorus oxychloride (12.2 ml) in pyridine (45 ml) and the whole stirred for a further hour in ice after final addition. The solvent was evaporated *in vacuo* at 50°, the process repeated after addition of more pyridine (30 ml) and the residue dried off overnight in a desiccator. The residue was treated with ice water and made just alkaline to phenolphthalein with barium hydroxide solution. The clarified solution was then poured into an equal volume of alcohol which caused precipitation of the crystalline mono-hydrate of the barium salt of *o*-cresol phosphate (13.5 g, 91% yield). Found (in material dried at room temperature) C, 24.9; H, 2.42; P, 9.0%; $C_7H_7O_4PBa.H_2O$ requires C, 24.6; H, 2.65; P, 9.1%.

The free phosphate, of slightly lower m.p. than that reported above can be prepared from the barium salt in the usual way.

Resorcinol diphosphate. This compound was prepared by MANAKA[19] by hydrolysis of the corresponding tetrachlordiphosphinate as a crystalline solid, m.p. 132°. However, repetition of this method followed by purification on an ion-exchange column gave material of considerably higher m.p. in accordance with that of the material obtained by the method following.

A solution of resorcinol (4.0 g) in pyridine (30 ml) was added drop-wise to an ice-cooled stirred solution of phosphorus oxychloride (14 ml) in pyridine (40 ml) and the whole stirred for 1 hour in ice after final addition. The solvent was then distilled *in vacuo* at 50° under nitrogen, and the evaporation repeated after addition of more pyridine (40 ml) to the residue. After drying off overnight over sulphuric acid, the residue was dissolved in ice-water (150 ml) and the solution made just alkaline to phenolphthalein by addition of a saturated aqueous solution of barium hydroxide. The solution was filtered with the aid of Hyflo supercel and the filtrate evaporated *in vacuo* at 50° till a slight precipitate began to form in the residue. An equal volume of alcohol was then added and the precipitated barium salt filtered off, washed with 50% aqueous alcohol,

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alcohol, finally with ether, and then dried off in a desiccator. This salt (9.28 g, 47% crude yield) was dissolved in water (30 ml) and treated with *N* sulphuric acid solution till no free barium ions remained (56.8 ml required). The filtered solution of the free acid was neutralised to phenolphthalein with 2 *N* sodium hydroxide solution and the salt clarified and put on an ion-exchange column of Dowex 50 loaded with hydrogen ions. The eluate was clarified and allowed to evaporate *in vacuo* giving almost colourless prisms of the diphosphate dihydrate, m.p. 175–7°, which resisted further attempts at purification. Found (in material dried at room temperature) C, 23.7, 23.8; H, 3.02, 3.08%; loss at 100°, 5.4%; $C_6H_8O_8P_2 \cdot 2H_2O$ requires C, 23.5; H, 3.94%; loss of 1 H_2O, 5.9%.

Hydroquinone diphosphate. A solution of hydroquinone (1.1 g) in pyridine (8 ml) was added drop-wise to an ice-cooled stirred solution of phosphorus oxychloride (3 ml) in pyridine (30 ml). After stirring at room temperature for a further 30 mins after final addition, the pyridine was distilled off *in vacuo* at 50° and the cooled residue decomposed with ice-water (*ca.* 150 ml). The filtered solution was treated gradually with calcium hydroxide (10 g) in water (80 ml), the whole stirred 15 minutes and filtered. The filtrate was evaporated to dryness, finally in a desiccator, and the residue extracted with boiling alcohol (300 ml) in three portions. The residual calcium salt was a colourless crystalline solid (2.05 g, 59% yield). This was decomposed with the theoretical amount of aqueous sodium carbonate solution by shaking, and the solution of the sodium salt filtered and passed through a Zeocarb column loaded with hydrogen ions. The eluate was concentrated to small bulk *in vacuo* when 0.95 g of almost colourless material separated. The acid was purified by dissolving in a little water, clarifying with charcoal and Hyflo, and evaporating slowly, giving 0.55 g needles, m.p. 203–204°. Found C, 25.9; H, 2.9%; $C_6H_8O_8P_2.\frac{1}{2}H_2O$ requires C, 25.8; H, 3.2%. This phosphate was prepared previously by GENVRESSE[20] by the action of phosphorous pentoxide on hydroquinone followed by hydrolysis and was reported as having m.p. 168–169°. The calcium salt, which crystallised on standing, was prepared from the free acid. Found C, 20.3; H, 2.0%; $C_6H_4O_8P_2Ca_2.\frac{1}{2}H_2O$ requires C, 20.3; H, 1.4%.

Toluhydroquinone diphosphate. A solution of toluhydroquinone (1.3 g) in pyridine (5 ml) was added drop-wise to an ice-cooled stirred solution of phosphorus oxychloride (2.5 ml) in pyridine (25 ml). The mixture was stirred 1 hour in ice after final addition, the solvent distilled off *in vacuo* at 50° under nitrogen, and the residue dried off in a desiccator over sulphuric acid. After addition of crushed ice to the cooled material the solution was treated with magnesium oxide (7.5 g) in water (total 275 ml) with stirring. The solution was filtered and evaporated *in vacuo* to small bulk. The dried residue was extracted with alcohol (300 ml) in three portions, leaving a dried residue (4.9 g). This salt was dissolved in water (250 ml), clarified with charcoal and Hyflo, and the solution passed through a column of Zeocarb loaded with hydrogen ions. The eluate was concentrated *in vacuo* giving a hydroscopic solid residue (2.7 g).

The material so obtained was clarified with charcoal and Hyflo and dried off *in vacuo* giving a colourless slightly sticky solid, m.p. 126–130°, which resisted further attempts at purification. Found (in material dried at room temperature) C, 28.65; H, 3.88; loss at 100°, 3.74%; equiv., 73.5. $C_7H_{10}O_8P_2.\frac{1}{2}H_2O$ requires C, 28.7; H, 3.76; loss of $\frac{1}{2}H_2O$, 3.08%; equiv., 73.3.

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SUMMARY

The examination of phenolic compounds in tissue cultures has given the following results:

1. With the exception of catechol all the unsubstituted phenols tested display antimitotic activity. Benzohydroquinone and 1:4-naphthohydroquinone are active at 10^{-9} *M*, resorcinol at 10^{-8} *M*, phenol and *o*-cresol at 10^{-6} *M*.
2. Phosphorylation of the phenolic substances increases their antimitotic activity. However, resorcinol-diphosphate is less active than resorcinol.
3. Introduction of halogen decreases the antimitotic activity.
4. C-Alkylation decreases considerably the antimitotic activity of the phenols.
5. Some physico-chemical attempts to explain the activity of the phenols are discussed.
6. The inhibition of mitosis by phenolic substances and by their phosphates is produced in different ways.
7. A possible connection of these substances in their activities as mitotic inhibitors is pointed out.
8. The preparation of phenol phosphate, *o*-cresol phosphate, resorcinol diphosphate, hydroquinone diphosphate and toluhydroquinone diphosphate is described.

RÉSUMÉ

L'examen de l'influence des substances à fonction phénolique sur la croissance des cultures de tissus a donné les résultats suivants:

1. Tous les phénols non-substitués, à l'exception de la catéchine, sont doués de propriétés antimitotiques. L'hydroquinone et la 1:4-naphthohydroquinone sont actives à une concentration moléculaire de 10^{-9} *M*, le résorcine à 10^{-8} *M*, le phénol et l'*o*-crésol à 10^{-6} *M*.
2. L'introduction du groupe phosphorique dans les phénols augmente leur activité antimitotique.
3. L'introduction des halogènes dans les phénols diminue leur activité antimitotique.
4. La C-alcylation diminue considérablement le pouvoir antimitotique des phénols.
5. L'attention a été tirée sur quelques conceptions physico-chimiques tendant à expliquer l'activité antimitotique des phénols.
6. L'inhibition des mitoses par les substances phénoliques et celle produite par leurs phosphates se produit par deux routes différentes.
7. Il semble possible d'envisager une conception qui embrasse les différents faits trouvés et mentionnés sur un point de vue commun.
8. La préparation des phosphates du phénol, du l'*o*-crésol et celle des diphosphates de la résorcine, de l'hydroquinone et de la toluhydroquinone a été décrite.

ZUSAMMENFASSUNG

Die Untersuchung der antimitotischen Wirkung von Phenolen in Gewebekulturen hat die folgenden Resultate ergeben.

1. Mit Ausnahme von Brenzkatechien zeigen alle unsubstituierten Phenole antimitotische Wirksamkeit.

Benzohydrochinon und 1:4-Naphthohydrochinon sind bei einer molaren Konzentration von 10^{-9} *M* wirksam, Resorcin bei 10^{-8} *M*, Phenol und *o*-Cresol bei 10^{-6} *M*.

2. Phosphorylierung der Phenole erhöht ihre antimitotische Wirksamkeit. Eine Ausnahme bildet Resorcin, dessen Diphosphat schwächer wirksam ist als Resorcin.
3. Einführung von Halogen vermindert die antimitotischen. Wirksamkeit.
4. C-Alkylierung setzt die antimitotische Wirkung der Phenole erheblich herab.
5. Einige physikalisch-chemische Erklärungsversuche der Phenolwirkung werden besprochen.
6. Die antimitotische Wirkung der Phenole und ihrer Phosphate erfolgt auf verschiedenen Wegen.
7. Auf die Möglichkeit eines Zusammenhanges dieser verschiedenen Gruppen in Bezug auf ihre antimitotische Wirkung wird aufmerksam gemacht.
8. Die Darstellung von Phenolphosphat, *o*-Cresolphosphat, Resorcindiphosphat, Hydrochinondiphosphat und Tolyhydrochinon-diphosphat wird beschrieben.

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