HALOGEN DERIVATIVES OF THE 1:4-NAPHTHOQUINONE GROUP AND THE MALEIC ACID SERIES IN THEIR ACTION ON THE MITOSIS OF CHICK FIBROBLASTS

by

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INTRODUCTION

In the course of our investigations dealing with the anti-mitotic activity of some quinones and related substances, we have observed various degrees of mitotic inhibition produced by these products, and have drawn attention to a certain qualitative parallelism between mitotic inhibition of a number of these substances and their reactivity towards –SH compounds[1]. In continuation of these researches, the anti-mitotic properties of halogen derivatives belonging to the 1 : 4-naphthoquinone group and to the maleic acid series have been examined. The halogen derivatives of 1:4-naphthoquinone and of 2-methyl-1:4-naphthoquinone gave a picture of activity and inhibition by added –SH groups which corresponded fairly well with similar observations made with the non-halogenated compounds (FRIEDMANN, MARRIAN AND SIMON-REUSS)[2]. Quite different results were obtained in the maleic acid group, results which may appear to reverse the concept gained from the study of the non-halogenated substances, in its main points.[3]

The present paper gives a description of the experiments performed to ascertain the effects of these halogen derivatives on the mitosis of chick fibroblasts.

METHODS

The experiments were carried out in tissue cultures of chick fibroblasts using the *hanging drop method*, as described previously[1].

RESULTS

1. *Action of halogen derivatives of 1:2-naphthohydroquinone and of 2-methyl-1:4-naphthohydroquinone on the mitosis of chick fibroblasts*

The halogen substitution products of 1:4-naphthoquinone and of its 2-methyl derivatives are sparingly soluble in water. We used therefore, in tissue cultures, the

References p. 686.

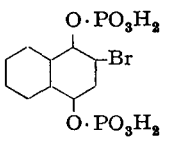
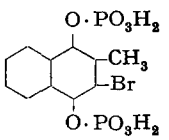
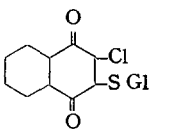
phosphates of the corresponding hydroquinones, as we did in the experiments with the non-halogenated parent substances. For this purpose 2-bromo-1:4-naphthohydroquinone diphosphate and 2-methyl-3-bromo-1:4-naphthhydroquinone diphosphate were prepared.

Table I shows the results obtained with these compounds, as well as the action on mitosis of the glutathione adduct of 2-chloro-1:4-naphthoquinone, synthesised for this experiment.

TABLE I

INHIBITION OF MITOSIS BY SOME HALOGEN DERIVATIVES OF THE 1:4-NAPHTHOQUINONE SERIES

Tissue cultures, chick fibroblasts

Substance	*Activity*
 2-Bromo 1:4-N.H.Q. diphosphate	50% inhibition at $4 \cdot 10^{-6}$ *M*
 2-Methyl-3-Bromo-1:4-N.H.Q.-diphosphate	50% inhibition at $6 \cdot 10^{-6}$ *M*
 S-(2-Chloro-1:4-naphthoquinonyl)-glutathione	inactive at $5 \cdot 10^{-6}$ *M*

It will be seen from Table I that 2-bromo-1:4-naphthohydroquinone diphosphate exerts 50% inhibition of mitosis at $4 \cdot 10^{-6}$ *M* concentration, and that 2-methyl-3-bromo-1:4-naphthohydroquinone diphoshate gives a 50% inhibition at $6 \cdot 10^{-6}$ *M*. The addition of glutathione to 2-chloro-1:4-naphthoquinone produces a compound which is completely inactive at $5 \cdot 10^{-6}$ *M*.

2. *Action of halogen derivatives of the maleic acid group on the mitosis of chick fibroblasts*

We started the investigation of this group by examining the –SH uptake of its members. Chloromaleic acid reacted with thiolacetic acid to the extent of approximately 50% in 5 hours. The *trans*-isomer, chlorofumaric acid showed no –SH uptake. The reaction of chloromaleimide with glutathione was complete in less than one minute and the reaction with thiolacetic acid stopped in one minute after 80% of the –SH compound had been removed.

References p. 686.

The figures demonstrate that these halogenated derivatives react with –SH groups in a manner corresponding to the reactivity of the non-halogenated compounds. Nevertheless, the anti-mitotic activity of these substances was completely different. Neither chloromaleic acid nor chloromaleimide had any effect on mitosis, while the *trans* compound, chlorofumaric acid, showed a weak but distinct anti-mitotic activity, amounting to 28% at $5 \cdot 10^{-6}$ *M* and rising to 33% at 10^{-5} *M*. This result was repeatedly confirmed, and the investigation of 12,000 cells in mitosis did not change it.

Table II gives the figures obtained in the study of chloromaleic acid, chloromaleimide and chlorofumaric in their action on the mitosis of chick fibroblasts.

TABLE II

TISSUE CULTURES: CHICKEN FIBROBLASTS, HANGING DROP METHOD, 4th PASSAGE, 24-HOUR CULTURES, FIXED IN SUSA, STAINED IN HEIDENHAIN'S HAEMATOXYLIN

Exp.	*Molar conc*	*Mitosis as % of mitosis of controls*	*Per cent inhibition*	*Phase distribution in % of mitosis*			
				Prophase	*Metaphase*	*Anaphase*	*Telophase*
		Chlormaleic acid (5,416 mitotic cells investigated)					
1	Controls	—	—	22.5	33.3	3.6	40.6
2	$1 \cdot 10^{-6}$	91.7 ± 6.1%	8.3%	22.5	34.0	5.9	37.6
3	$2 \cdot 10^{-6}$	98.4 ± 8.4%	0	24.9	33.6	4.1	37.4
4	Controls	—	—	16.5	26.0	4.1	54.0
5	$3 \cdot 10^{-6}$	100.0 ± 4.6%	0	15.0	38.3	6.1	40.5
6	$5 \cdot 10^{-6}$	96.4 ± 5.8%	0	18.4	31.1	7.6	42.9
		Chlormaleimide (4,740 mitotic cells investigated)					
1	Controls	—	—	16.2	38.2	2.8	42.8
2	$6 \cdot 10^{-6}$	100.0 ± 3.9%	0	10.5	58.5	6.2	24.8
3	$3 \cdot 10^{-6}$	100.0 ± 6.1%	0	11.4	44.7	11.4	32.5
5	Controls	—	—	8.5	43.5	3.4	44.6
6	$5 \cdot 10^{-6}$	96.3 ± 3.4%	0	11.2	31.8	3.6	53.4
7	$2 \cdot 10^{-6}$	100.0 ± 2.8%	0	14.0	30.6	4.6	50.8
		Chlorfumaric acid (12,073 mitotic cells investigated)					
1	Controls	—	—	13.7	38.9	5.0	42.4
2	$1 \cdot 10^{-6}$	83.4 ± 7.5%	16.6%	9.0	47.4	8.1	34.6
3	$3 \cdot 10^{-6}$	86.2 ± 5.5%	13.8%	11.3	35.4	7.2	46.1
4	Controls	—	—	16.5	26.0	3.5	54.0
5	$1 \cdot 10^{-6}$	86.8 ± 5.5%	13.2%	16.9	44.7	4.1	34.3
6	$3 \cdot 10^{-6}$	77.7 ± 5.2%	22.3%	20.0	32.0	5.0	43.0
7	$5 \cdot 10^{-6}$	71.8 ± 6.1%	28.2%	21.6	34.6	6.2	37.6
8	Controls	—	—	9.6	42.4	5.8	36.7
9	$1 \cdot 10^{-5}$	67.0 ± 6.7%	33.0%	12.7	44.8	5.8	36.7
10	$3 \cdot 10^{-5}$	82.7 ± 9.0%	17.3%	11.3	53.3	2.7	32.7
11	$5 \cdot 10^{-5}$	70.3 ± 6.3%	29.7%	11.7	46.8	8.1	33.4

References p. 686.

DISCUSSION

The chloro-derivatives tested in the naphthoquinone series have been investigated as naphthohydroquinone diphosphates. This seems permissible, as LEHMANN[4] has shown for *Tubifex eggs* that quinones and hydroquinones have apparently the same degree of anti-mitotic activity. The comparison of the physiological activity of the chlorinated diphosphates with the activity of the non-chlorinated products shows that the introduction of a halogen in position 2 of 1:4-naphthohydroquinone diphosphate decreases the 50% mitotic inhibition from $3 \cdot 10^{-9}M$ to $4 \cdot 10^{-6}M$, a decrease of activity which is of the same order as the decrease found after introduction of a methyl group in position 2. In contrast to this considerable change of activity, the introduction of a chloro at 3 in 2-methyl-1:4-naphthohydroquinone diphosphate has diminished the 50% mitotic inhibition from $4 \cdot 10^{-6}M$ to $6 \cdot 10^{-6}M$ only. It may further be noted that the substitution of both hydrogen atoms, the one at 2, and the other at 3, has not abolished the anti-mitotic activity of the resulting hydroquinone.

The inactivity of the glutathione adduct of 2-chloro-1:4-naphthoquinone as mitotic inhibitor corresponds to the inactivity produced with 1:4-naphthoquinone by the addition of glutathione to this compound[2].

The comparison between the results obtained with chlorinated and non-chlorinated derivatives of maleic acid and of fumaric acid gives the following picture:

The *cis* compounds, maleic acid and maleimide on the one side and chloromaleic acid and chloromaleimide on the other side, add –SH groups. Nevertheless, only the non-chlorinated substances, maleic acid and maleimide, show anti-mitotic activity, whilst the chlorinated derivatives, chloromaleic acid and chloromaleimide, are inactive as mitotic inhibitors.

The *trans* acids, fumaric acid as well as chlorofumaric acid, show no –SH uptake. Nevertheless, chlorofumaric acid exerts weak but distinct anti-mitotic activity, whilst fumaric acid is devoid of anti-mitotic activity.

It is of course, possible to formulate the results obtained with chlorinated and non-chlorinated unsaturated acids and their derivatives as being in contrast one with the other, but already the experiments presented by us suggest a close connection between these apparently contradictory observations. This will be seen when the results obtained with chloromaleimide, chloromaleic acid and chlorofumaric acid are analysed separately.

(1) Chloromaleimide is an unsaturated imide. It reacts to the extent of approximately 100% in one minute with glutathione. In tissue cultures the highest concentration at which this imide has been brought into contact with embryo extract and thus into reaction with glutathione has been $6 \cdot 10^{-6}M$. As unsaturated imides react even at this concentration completely and nearly instantaneously with glutathione (FRIEDMANN[5]) there is no reason to assume that chloromaleimide behaves differently. S-glutathiono-chloro-maleimide, formed in this reaction would then fall into line with S-glutathiono-1:4-naphthoquinone and S-glutathiono-2-chloro-1:4-naphthoquinone (this paper), tested as pure compounds, and to be in contrast to maleimide and N-ethyl-maleimide as mitotic inhibitors.

(2) Chloromaleic acid may show no anti-mitotic activity for the reasons given for chloromaleimide, but here the experimental ground is less developed and other factors cannot be excluded.

References p. 686.

(3) Chlorofumaric acid, although unable to take up –SH groups, is a mitotic inhibitor, In this respect the comparison with fumaric acid may be of interest. The cytological investigation of the action of fumaric acid on mitosis, reported previously[1], has shown that numerically no mitotic inhibition can be found. Nevertheless, mitotic disturbances such as chromosome breakages, delayed and clumped metaphases and anaphases did occur, giving microscopical pictures which resembled those obtained with maleic acid. We have drawn attention to the warning conveyed by these observations in interpreting the role of fumaric acid in mitosis. With chlorofumaric acid the mitotic disturbances consisted in the occurrence of a great number of clumped and enlarged metaphases and undivided telophases, and a few multi-polar cells, but here, in addition to the mitotic disturbances, mitotic inhibition has been found. The introduction of a chloro-group into fumaric acid has thus developed the mitotic disturbances of fumaric acid to the full inhibition of chlorofumaric acid, and the differences in the action on mitosis of fumaric acid and chlorofumaric acid are differences of grade and not of kind.

It remains to discuss the question as to whether –SH groups can play a part in the action of chlorofumaric acid as a mitotic inhibitor. Although *cis* as well as *trans* compounds are able to add –SH containing substances, –SH uptake has been found only with the *cis* acid, maleic acid, and not with the *trans* acid, fumaric acid. So far the addition of –SH groups to these substances has been studied in phosphate buffer of pH 7.4. No information is available as to whether fumaric acid can react with the –SH group, at an acid pH. The interaction of chlorofumaric acid with thiolacetic acid has therefore been investigated, using an acetate buffer of pH 4.6. The reaction has been carried out in evacuated Thunberg tubes at 37° as described by MORGAN AND FRIEDMANN[6], with mixtures of chlorofumaric acid and thiolacetic acid, 1 mol to 1 mol and 4 mol to 1 mol. No –SH uptake has been observed after 5 hour incubation. The experiment shows that the pH of the medium has nothing to do with the failure of chlorofumaric acid to act with –SH groups and gives additional evidence that –SH containing substances play no direct part in the action of chlorofumaric acid as mitotic inhibitor. This conclusion was confirmed by spectroscopical investigations, similar to those described previously[5].

In the group of unsaturated dicarboxylic acids containing 4 carbon atoms *cis* and *trans* derivatives may give mitotic inhibition. In the *cis* series this activity seems to be connected with the property of these substances to react with –SH compounds whilst the *trans* configuration has potentialities for anti-mitotic activity in which the –SH compounds play no direct part.

CHEMICAL SECTION

2-Bromo-1:4-naphthoquinone

It was found most convenient to prepare this compound by an adaptation of the method of ADAMS, GEISSMAN, BAKER, AND TEETER (1941). 1:4-naphthoquinone (4.74 g) was dissolved in warm acetic acid (30 ml) and the solution cooled to incipient crystallisation. Then was added a solution of bromine in acetic acid (42 ml of a 4% v/v solution), followed by powdered anhydrous sodium acetate (7.8 g). The whole was heated on the steam bath for 30 minutes, poured into water and the precipitated quinone filtered off, washed with water, and recrystallised, while still damp, from methyl alcohol (150 ml). Yield 5.44 g yellow needles m.p. 129–130° (76.5%) while a further 0.55 g m.p. 126–129° can be isolated by evaporation of the mother liquors. ZINCKE AND SCHMIDT (1894) give m.p. 130° for 2-bromo-1:4-naphthoquinone.

References p. 686.

2-Bromo-1:4-naphthohydroquinone diphosphate

Using the above quinone (4.0 g) the phosphorylation was carried out exactly as for the 2-methyl-3-bromo-1:4-naphthoquinone. The crude barium salt weighed 4.35 g together with a further amount (1.82 g) which had formed a brown hard resin and was therefore discarded by manual selection. The barium salt in water was converted to the free acid by sulphuric acid as before and the filtered solution put through two ion exchange columns of Dowex 50 resin, the first loaded with sodium ions and the second with hydrogen ions. The final eluate was clarified with Hyflo and allowed to evaporate in a desiccator forming almost colourless prisms having no m.p. up to 320°. (Found (in material dried at room temperature) C, 29.2: H, 2.44: Br, 19.7%; Equiv. 97.2; Loss at 120°, 0.7%. $C_{10}H_9O_8P_2Br$. ½ H_2O requires C, 29.4; H, 2.47; Br, 19.6%; Equiv. 102; Loss of ½ H_2O, 2.2%).

2-Methyl-3-bromo-1:4-naphthohydroquinone diphosphate

2-Methyl-3-bromo-1:4-naphthoquinone (Adams, *et al.*, 1941) (4.0 g) in ether (200 ml) was reduced by shaking with a solution of sodium hydrosulphite (8.0 g) in water (60 ml) till no further colour changes occurred. The ether solution was then washed twice with saturated sodium chloride solution containing a little sodium hydrosulphite solution and filtered through a funnel containing sodium sulphate in an atmosphere of nitrogen. The dried ethereal solution was then evaporated *in vacuo* at 50° using a nitrogen bubbler. The residual hydroquinone was not purified further but was dissolved in pyridine (90 ml) kept under nitrogen, and added over 30 minutes to an ice-cold stirred solution of phosphorus oxychloride (9 ml) in pyridine (60 ml). After final addition, the whole was stirred for 30 minutes in ice and then evaporated *in vacuo* under nitrogen at 50°. When almost dry, more pyridine (50 ml) was added and the evaporation carried to dryness. The residue was left *in vacuo* overnight over sulphuric acid. The residue was then cooled in ice and treated with ice-water (200 ml), shaken till dissolved and left at room temperature for 3 hours. The solution was filtered after addition of Hyflo supercel and made just alkaline to phenolphthalein with saturated barium hydroxide solution (*ca.* 1200 ml). The solution was filtered with the aid of Hyflo, evaporated *in vacuo* at 50° to about half bulk and poured slowly into an equal volume of alcohol. Next day, the barium salt was filtered off, washed with 50% alcohol, and finally with ether. The salt was dried off in a desiccator giving 4.6 g of a colourless amorphous solid (42% crude yield). The salt was dissolved in water (100 ml) and treated with *N* sulphuric acid till no free barium ions remained in solution (as tested for by sodium rhodizonate solution) (22 ml) required. Were the barium salt pure, 27 ml would be required. Evaporation of the filtered and clarified solution gave a partially crystalline solid (2.0 g, 72%) m.p. 237° (dec.) which was dissolved in water and neutralised by the addition of *N* sodium hydroxide solution and put through an ion exchange column (Dowex 50) loaded with hydrogen ions. The eluate was evaporated in a vacuum at 50° to small bulk, clarified by filtration through Hyflo and allowed to evaporate to dryness in a desiccator. The *compound* crystallised in feathery needles m.p. 239–240° (dec.). (Found (in material dried at room temperature) C, 31.2; H, 3.26; Br. 19.75%; Equiv. 110.6; Loss at 120°, 1.8%. $C_{11}H_{11}O_8P_2Br$. ½ H_2O requires C, 31.3; H, 2.87; Br, 19.0%; Equiv. 105.5; Loss of ½ H_2O, 2.1%).

S-(2-chloro-1:4-naphthoquinonyl)-glutathione

A solution of 2-chloro-1:4-naphthoquinone (195 mg) in alcohol (7 ml) was added to a solution of glutathione (153 mg) in water (2 ml) and alcohol (3 ml). After standing overnight, the orange precipitate was filtered off, washed with alcohol, and dried, giving 150 mg of amorphous material which could not be crystallised. Found (in material dried at room temperature) C, 48.7; H, 4.32; N, 8.15, 8.4% $C_{20}H_{20}O_8N_3SCl$ requires C, 48.25; H, 4.05; N, 8.44%.

Chlormaleic acid and *chlorfumaric acid* were prepared by the method of W. H. PERKIN[9].

Chlormaleimide was prepared by the method of G. L. CIAMICIAN AND P. SILBER[10] and purified by vacuum sublimation.

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SUMMARY

1. In tissue cultures of chickfibroblasts 50% inhibition of mitosis was found with 2-bromo-1:4-naphthohydroquinone-diphosphate at $4 \cdot 10^{-6}$ *M* concentration and with 2-methyl-3-bromo-

References p. 686.

1:4-naphthohydroquinone diphosphate at $6 \cdot 10^{-6}$ *M*. S-(2-chloro-1:4-naphthoquinonyl)-glutathione was inactive at $5 \cdot 10^{-6}$ *M*.

2. Chloromaleic acid and chloromaleimide add –SH compounds in the same way as the non chlorinated parent substances. Chlorofumaric acid shows no –SH uptake, neither at pH 7.4 nor at pH 4.6.

3. Chloromaleic acid and chloromaleimide are inactive as mitotic inhibitors. Chlorofumaric acid has weak, but distinct antimitotic properties.

4. The contribution of these results to our views on the role of –SH compounds in mitosis is discussed.

5. The preparation of 2-bromo-1:4-naphthohydroquinone diphosphate, of 2-methyl-3-(bromo-1:4-naphthohydroquinone diphosphate and of S-(2-chloro-1:4-naphthoquinonyl)-glutathione are described.

RÉSUMÉ

1. Dans les cultures de tissus une inhibition mitotique de 50% a été trouvée avec le diphosphate de la 2-bromo-1:4-naphthohydroquinone, à une concentration moléculaire de $4 \cdot 10^{-6}$ *M*, et avec le diphosphate de la 2-méthyl-3-bromo-1:4-naphthohydroquinone, éprouvée à $5 \cdot 10^{-6}$ *M*. Le S-(2-chloro-1:4-naphthoquinonyl)-glutathion, produit de la réaction entre le glutathion et la 2-chloro-1:4-naphthoquinone, est inactive comme inhibiteur mitotique à $5 \cdot 10^{-6}$ *M*.

2. L'acide chloromaléique et la chloromaléimide réagissent avec des substances sulfhydriques de la même façon que leurs substances mères, non chlorées. L'acide chlorofumarique ne réagit ni à pH 7.4 ni à pH 4.6 avec des groupes sulfhydriques.

3. L'acide chloromaléique et la chloromaléimide sont inactives comme inhibiteurs mitotiques. Par contre, l'acide chlorofumarique déploie des propriétés antimitotiques faibles, mais néanmoins distinctes.

4. La contribution de ces résultats à la conception du rôle, joué par les corps sulfhydriques dans les processus de mitose, est discutée.

5. La préparation du diphosphate de la 2-bromo-1:4-naphthohydroquinone et de la 2-méthyl-3-bromo-1:4-naphthohydroquinone ainsi que la préparation du S-(2-chloro-1:4-naphthoquinonyl)-glutathion a été décrite.

ZUSAMMENFASSUNG

1. 2-Bromo-1:4-naphthohydrochinondiphosphat gab in Gewebekulturen von Hühner-Fibroblasten eine 50%ige Hemmung der Mitose bei einer molaren Konzentration von $4 \cdot 10^{-6}$ *M* und 2-Methyl-3-bromo-1:4-naphthohydrochinondiphosphat bei $6 \cdot 10^{-6}$ *M*. Das Addukt, S-(2-Chloro-1:4-naphthochinonyl)-glutathion war bei $5 \cdot 10^{-6}$ *M* unwirksam.

2. Chlormaleinsäure und Chlormaleinimid reagieren mit Sulfhydrylverbindungen in derselben Weise wie ihre chlorfreien Muttersubstanzen. Chlorfumarsäure zeigt weder bei pH 7.4 noch bei pH 4.6 Aufnahme von –SH Gruppen.

3. Chlormaleinsäure und Chlormaleinimid sind unwirksam als mitotische Hemmungsstoffe. Chlorfumarsäure dagegen hat schwache aber deutliche antimitotische Wirkung.

4. Der Beitrag dieser Resultate zur Beurteilung der Rolle, die Sulfhydrylverbindungen in der Mitose spielen, wird erörtert.

5. Die Darstellung von 2-Bromo-1:4-naphthohydrochinondiphosphat, von 2-Methyl-3-bromo-1:4-naphthohydrochinondiphosphat und des Adduktes, S-(2-Chloro-1:4-naphthochinonyl)-glutathion wird beschrieben.

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