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ANALYSIS OF ANTIMITOTIC ACTION OF CERTAIN QUINONES

BY

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The study of mitotic inhibition in experiments dealing with toxicological and pharmacological problems has been used by Dustin (1934) with great success. He worked with normal tissues and tested antimitotic activities *in vivo*. The very valuable method of tissue cultures has been applied by Ludford (1936) for the same purpose. Other biological materials for the investigation of physiological processes on the same lines have been adopted by Töndury (1941), Lehmann (1942) and Lüscher (1946): Töndury worked with *Triton eggs*, Lehmann (1945, 1946) and later Huber (1947) with the eggs of *Tubifex*; Lüscher investigated regeneration in *Xenopus larvae* under the influence of colchicine. Barber and Callan (1943) studied the effect of cold and colchicine in mitoses of the newt.

From the beginning it was clear that the study of mitotic inhibitors may well pave the way for a chemotherapeutic approach to the cancer problems (Dustin, 1939). The great variety of antimitotic substances and the lack of any chemical connexion between many of them seemed to be a serious obstacle. Furthermore nearly all the potent antimitotics are poisonous to normal cells at concentrations at which they exert antimitotic action on tumour cells, in this way restricting the use of antimitotics to external application on surface tumours. Broderson (1943) has given evidence that it is possible by this method to control the growth of malignant tumours of the skin; he used colchicine, whose strong activity as a mitotic inhibitor was well known.

Nevertheless these difficulties might be overcome. Mitchell (1942) and v. Euler and Hevesy (1942) have shown that therapeutic doses of *x*- and gamma-radiations inhibit the synthesis of thymonucleic acid in proliferating normal and malignant cells. Substances able to interfere with the metabolism of nucleoproteins ought therefore to be endowed with antimitotic properties, and conversely some mitotic poisons should be able to interfere with the metabolism of nucleoproteins; thus the mechanism by which disturbances in the metabolism of nucleoproteins are induced becomes a possible common denominator for the various antimitotic substances even when they are chemically unrelated. To give an example: according to Kopac (1945) stilbamidine blocks mitosis in some neoplastic cells; it dissociates protamine-ribonucleates, releasing the protamine and simultaneously trapping the ribonucleate complex by the formation of an insoluble stilbamidine-ribonucleate. Nucleoproteins, therefore, can be inactivated by low concentrations of stilbamidine. Lettré and his collaborators (1946) found that substances of the type R—Hg—X, where R is an alkyl or an aryl residue, X an inorganic or organic anion or a phenol, are mitotic poisons. They explain the antimitotic activity of these compounds by pointing out that nucleic acids and nucleoproteins are able to combine by salt or complex formation with the organometallic compounds to give insoluble products. The similarity in the mechanism by which stilbamidine and the organometallic compounds disclose their antimitotic properties is easily seen. They belong to the same physiological group of antimitotics, although they are chemically as dissimilar as possible.

As mentioned above, the second great difficulty for the therapeutic utilization of antimitotics is the fact that the concentration at which they display the antimitotic activity is nearly always within the range of their toxicity for normal cells. Two ways of overcoming this difficulty have been investigated. The molecule may be changed chemically until it has the required properties, either by investigating the corresponding homologous series or by introducing new radicals in the hope of improving the therapeutic index. The other way is to

cover the active group by substitution. The work of the cell consists then in releasing the active principle within the cell. This is possible if the cell contains enzymes capable of splitting off the substituent. The choice of the substituent is therefore of primary importance. In view of the role which phosphoric acid plays in the synthesis of nucleic acid and the ease of its transfer in the processes supplying the necessary energy for these and other reactions, the phosphate residue stands out as the most promising substituent. In 1946 Mitchell started to study the therapeutic effects of the diphosphate of 2-methyl-1 : 4-naphthohydroquinone, in combination with radiation, in cancer patients and later gave evidence in collaboration with Simon-Reuss (1947) that this diphosphate produces mitotic inhibition in chick fibroblasts and in some human carcinomata, with potentiation of the mitotic inhibitory effects of *x*-radiation under suitable conditions in the tissue cultures. The mitotic inhibition produced by the unsubstituted 1 : 4-naphthohydroquinone was discovered by Lehmann (1942), who found it active at a concentration of 10^{-7} ; the same degree of activity was displayed by the corresponding quinone. Lehmann worked with *Tubifex eggs*. Meier and Allgöwer (1945) and Meier and Schär (1947) obtained similar results with chick fibroblast cultures.

In our own experiments we followed two lines of research. We tried to ascertain the chemical group in the quinone molecule, common to the antimitotics under investigation and responsible for their chemotherapeutic activity. We tried further to get some evidence which would allow us to correlate our results with physiological processes, the disturbance of which may cause interruption of growth, as shown by mitotic inhibition.

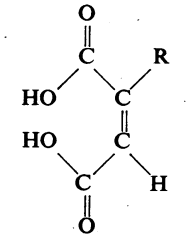
The present communication is the first of a series of papers dealing with these problems.

METHODS

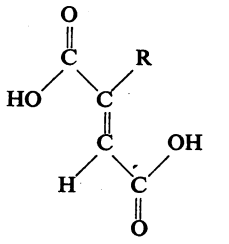
All the experiments were carried out on tissue cultures of chick fibroblasts. The tissues used were taken from the sclerotic and choroid of a 10–11-days-old chick embryo. The medium consisted of equal parts of fowl plasma and of 15 per cent embryo extract. The usual hanging drop technique was applied. Two groups of six cultures of the 4th passage were selected and matched for experimental purposes and controls. The compound to be tested was dissolved in Tyrode solution and the *p*H adjusted to 7.4. The solution was suitably diluted with Tyrode and sterilized by filtration through Seitz filter. Sterilization by boiling in Tyrode was avoided because the boiling of maleic and fumaric acids in Tyrode solution gave unreproduceable results. The diluted solution of the substance was then added to the embryonic extract. The final molar concentrations appear in Table I. The cultures were fixed and stained with Heidenhain's haematoxylin at given intervals and mitotic counts were carried out.

EXPERIMENTAL

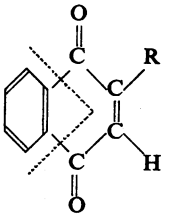
Many chemical properties of the quinones are centred in the non-benzenoid part of the molecule and the same may apply to the physiological activities. Thus the question arises whether the benzene ring of the naphthoquinones (V) is necessary for the mitotic inhibition induced by them. Maleic acid (I), its methyl derivative, citraconic acid (II), and their *trans*-isomers, fumaric acid (III), and mesaconic acid (IV), were therefore investigated.



I, R = H
II, R = CH_3



III, R = H
IV, R = CH_3



V, R = H or CH_3

I. *Maleic acid*

Pure maleic acid was prepared by hydrolysis of maleic anhydride. The results obtained with maleic acid are given in Table I and show that maleic acid has strong antimitotic properties. At a concentration of 0.5×10^{-6} molar the inhibition was 33.6 and 54.2 per cent in two experiments. The mitotic inhibition increased with rising concentration. The increase follows roughly a logarithmic graph, when plotted against the log 10 of the concentration (Fig. 1). The phase distribution calculated in percentage of the total mitoses gives no decisive information. Microscopically the picture common to all substances with antimitotic activity was observed: clumped metaphases and undivided telophases were seen in all phases ; fragmentation of chromosomes occurred in all phases, but mainly in anaphases. At higher concentrations

TABLE I

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

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of mitoses			
				Prophase	Metaphase	Anaphase	Telophase
MALEIC ACID (10,795 mitotic cells investigated)							
1.	Controls	—	—	11.8	38.1	3.4	46.8
2.	0.5×10^{-6}	45.8 ± 3.8	54.2	17.8	30.4	3.5	51.2
3.	1×10^{-6}	32.6 ± 5.0	67.4	15.7	21.3	3.8	59.2
4.	2×10^{-6}	45.5 ± 8.2	54.5	12.3	46.0	6.1	35.6
5.	Controls	—	—	16.8	29.5	4.4	49.2
6.	0.5×10^{-6}	66.4 ± 5.2	33.6	18.1	39.4	3.0	39.6
7.	2×10^{-6}	15.7 ± 3.4	84.3	24.9	25.8	3.0	46.4
8.	4×10^{-6}	6.0 ± 1.4	94.0	18.6	31.4	3.9	46.1
9.	Controls	—	—	22.5	25.6	5.9	46.0
10.	1×10^{-6}	35.5 ± 2.2	64.5	19.0	44.6	7.8	28.2
11.	2×10^{-6}	32.4 ± 6.8	67.6	18.9	47.6	7.2	26.2
12.	3×10^{-6}	23.4 ± 3.1	76.6	18.4	38.5	4.5	38.5
CITRACONIC ACID (5.449 mitotic cells investigated)							
1.	Controls	—	—	21.1	29.3	7.5	42.1
2.	3×10^{-6}	98.4 ± 2.4	—	21.9	33.7	4.3	40.0
3.	Controls	—	—	13.5	37.5	9.5	39.5
4.	3×10^{-6}	97.3 ± 6.0	—	13.4	38.6	9.9	38.1
FUMARIC ACID (7,841 mitotic cells investigated)							
1.	Controls	—	—	27.1	28.7	1.8	41.8
2.	1×10^{-4}	105.4 ± 1.8	—	26.9	30.9	4.5	37.7
3.	3×10^{-5}	87.3 ± 13.8	—	28.5	22.4	4.7	44.5
4.	Controls	—	—	19.7	29.5	6.1	44.7
5.	3×10^{-6}	84.8 ± 6.9	15.2	21.9	25.9	9.9	42.3
6.	2×10^{-6}	87.9 ± 7.10	—	22.9	36.1	7.7	33.2
7.	1×10^{-6}	99.7 ± 10.0	—	21.0	34.6	5.5	38.9
MESACONIC ACID (3,374 mitotic cells investigated)							
1.	Controls	—	—	18.4	33.9	4.4	43.4
2.	5×10^{-6}	100 ± 3.5	—	30.4	15.7	3.3	50.7
3.	3×10^{-6}	100 ± 6.9	—	19.4	19.9	4.9	55.7
4.	1×10^{-6}	100 ± 3.3	—	16.4	15.4	5.3	62.9
TETRASODIUM 1:4-NAPHTHOHYDROQUINONE DIPHOSPHATE (5,838 mitotic cells investigated)							
1.	Controls	—	—	28.0	34.7	1.2	36.0
2.	1×10^{-6}	21.5 ± 4.8	78.5	3.4	86.2	0.7	9.7
3.	5×10^{-7}	35.7 ± 5.8	64.3	5.2	86.2	0.3	8.3
4.	Controls	—	—	16.9	22.9	1.7	58.8
5.	3×10^{-8}	8.4 ± 2.5	91.6	7.0	69.0	1.4	22.5
6.	1×10^{-9}	19.4 ± 3.5	80.6	10.6	55.9	5.0	28.6
7.	5×10^{-9}	41.0 ± 9.1	59.0	21.9	38.0	5.8	34.2
8.	Controls	—	—	19.8	23.0	2.3	54.9
9.	3×10^{-9}	49.1 ± 6.1	50.9	20.7	24.9	4.2	50.2
10.	1×10^{-9}	77.7 ± 8.9	22.3	16.9	35.8	2.7	44.7
11.	5×10^{-10}	86.2 ± 9.7	13.8	19.5	28.6	3.8	48.1
1:4-NAPHTHOHYDROQUINONE MONO-HYDROGEN SUCCINATE (7,557 mitotic cells investigated)							
1.	Controls	—	—	20.6	38.7	5.7	35.0
2.	1×10^{-9}	103 ± 10.4	—	21.7	30.1	4.7	43.4
3.	3×10^{-9}	105 ± 10.6	—	22.7	29.0	4.9	43.6
4.	5×10^{-9}	104 ± 9.3	—	22.5	30.0	4.4	43.1
5.	Controls	—	—	13.5	36.9	3.9	45.8
6.	1×10^{-6}	103 ± 2.4	—	14.4	43.9	5.7	36.0
7.	3×10^{-6}	102.5 ± 2.1	—	14.7	42.7	6.0	36.6
8.	5×10^{-6}	99.3 ± 2.3	—	14.0	44.9	4.6	36.5

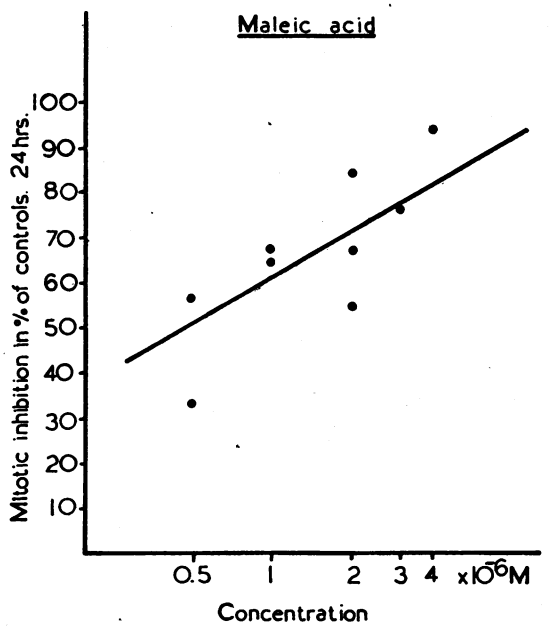


FIG. 1.—Mitotic inhibition plotted as percentage of controls against the logarithm of the concentration of maleic acid.

(3 and 4 × 10^{-6} *M*) the cultures showed but little outgrowth, the resting cells were flattened out and some exploded cells could be seen.

II. *Citraconic acid*

The methyl derivative of maleic acid, citraconic acid, showed no mitotic inhibition. There was no difference in the phase distributions compared with the controls (Table I). Microscopically a few clumped metaphases were to be seen but no other abnormalities.

III. *Fumaric acid*

Table I shows that fumaric acid induces no mitotic inhibition. In the phase distribution a slight increase only in anaphase is to be noted. Microscopically all concentrations showed slight mitotic disturbances: clumped metaphases and chromosome breakages in late metaphases and anaphases were seen; only the telophases seemed not to be affected. Microscopical pictures were found which resembled those obtained with maleic acid. Abnormal cells were present, and the proportion in which they were present was very near that observed in normal tissue cultures, viz., 5 per cent. (Found abnormal mitoses in per cent of the mitoses in the experiment at 1 × 10^{-6}*M*: 6.6 ± 0.7 per cent from 377 cells in mitosis; at 2 × 10^{-6}*M*: 9.2 ± 1.72 per cent from 338 cells in mitosis; at 3 × 10^{-6}*M*: 4.6 ± 0.97 per cent from 305 cells in mitosis.)

IV. *Mesaconic acid*

Mesaconic acid (methyl fumaric acid) (Table I) showed no mitotic inhibition and no abnormal cells were found. The phase distribution seemed to indicate an increase in telophase in the lower concentrations which would have to be investigated further.

V. *1 : 4–Naphthohydroquinone diphosphate*

Lehmann (1942) has already shown that 1:4–naphthoquinone is a stronger antimitotic than its 2–methyl derivative. He worked with *Tubifex eggs*. Meier and Allgöwer (1945) and Meier and Schär (1947) tested the substance in tissue cultures and described the cytological pictures obtained.

As quinones and hydroquinones have apparently the same degree of antimitotic activity (Lehmann, 1942) we prepared the previously unknown 1:4–naphthohydroquinone diphosphate in order to ascertain quantitatively its activity in tissue cultures.

Table I gives the results. It will be seen that 1:4–naphthohydroquinone diphosphate is an extremely powerful inhibitor of mitosis. Compared with the results calculated by Huber (1947) for the antimitotic activity of 1:4–naphthoquinone a remarkable agreement is to be noted: Huber found 1:4–naphthoquinone active at 0.7 × 10^{-9}*M* for *Tubifex eggs*, whereas our experiments on chick fibroblasts show threshold activity of 1:4–naphthohydroquinone diphosphate at 0.5 × 10^{-9}*M*. Compared with the 2–methyl derivative, tested by Mitchell and Simon-Reuss (1947), the activity of

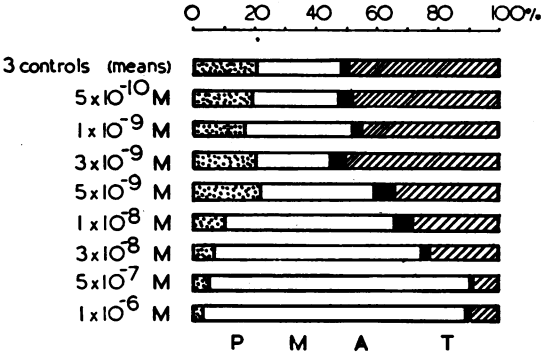


FIG. 2.—Tetrasodium 1:4-naphthohydroquinone diphosphate. Phase distribution as percentage of total mitoses: normal from 5 × 10^{-10} to 3 × 10^{-9}*M*, increase of metaphases from 5 × 10^{-9}*M*.

the new methyl free derivative is more than 1,000 times greater. With rising concentration the activity of the diphosphate increases. From $5 \times 10^{-10} - 1 \times 10^{-8} M$ the increase follows a logarithmic line. The phase distribution (Fig. 2) is nearly normal from $5 \times 10^{-10} M - 3 \times 10^{-9} M$, but at $5 \times 10^{-9} M$ the metaphases are already increased. This increase grows continuously with rising concentration until at $5 \times 10^{-7} M$ 86 per cent of the total mitoses are in metaphase. In the whole range of $10^{-9} M$, the range of its mitotic inhibition, no toxic effect was observed, although the general picture found in mitotic disturbances was well in evidence: clumped metaphases, in all phases some chromosome breakages, some enlarged metaphases and undivided telophases. Toxic effects began to appear at $10^{-8} M$, vacuolized and exploded cells being found. Abnormal cells were investigated for the concentration range $0.5 \times 10^{-9} - 1 \times 10^{-8} M$.

VI. *1 : 4–Naphthohydroquinone mono-hydrogen succinate*

This substance was prepared in order to see whether the phosphate residue in the 1 : 4–naphthohydroquinone diphosphate could be replaced by another acidic group.

Table I shows that 1 : 4–naphthohydroquinone mono-hydrogen succinate had no effect on mitosis. The phase distribution seemed to be slightly affected, but the figures allowed no clear interpretation. Microscopical investigation showed the absence of abnormal mitoses.

Discussion

In this paper we have used tissue cultures as a method of approach to the chemotherapy of growth inhibition. Mitotic inhibition, phase distribution, and the cytological picture have all been studied as indices of the effects exerted on tissue growth by the different agents. The picture disclosed by the three parts of this analysis may seem to give a reliable basis for the chemotherapeutic evaluation of the substances tested ; this is obviously only true within certain limits, although the results given in the three parts of this analysis have been obtained by quantitative methods.

The study of phase distribution alone for instance gives little reliable information. Maleic acid, a strong inhibitor of mitosis, shows no appreciable change in phase distribution (Table I). Furthermore disturbances in phase distribution may precede mitotic inhibition at lower concentrations (unpublished data) or they may follow mitotic inhibition after the peak of the inhibition has nearly been reached (1 : 4–naphthohydroquinone diphosphate ; Table I, experiment 7), or one set of experiments may display a shift of the phases in one direction, another set in the opposite direction (Table I, 1 : 4–napthohydroquinone mono-hydrogen succinate).

Mitotic disturbances, as shown by the occurrence of abnormal cells, without mitotic inhibition and without alteration in phase distribution have not been prominent with the substances examined in this paper ; but they occur, as shown by v. Möllendorff (1938), and the slight indications of such a possibility, encountered in the study of fumaric acid, convey a warning which cannot be dismissed The quantitative determination of abnormal mitoses in connection with mitotic inhibition may provide useful chemotherapeutic information. A special investigation is planned to elaborate these relations. The figures given for mitotic inhibition seem to have a greater individual importance than those for phase distribution. They are the result of many mitotic processes and represent an average expression of them. Their fluctuations, recorded statistically, are, as a rule, within reasonable limits and allow their use as a quantitative expression of mitotic inhibition. Thus the positive statement of mitotic inhibition of a definite degree for a given concentration is fairly safe. The negative statement, no mitotic inhibition, based only on the absence of mitotic inhibition, may be erroneous.

Taking mitotic inhibition to express our results the experiments can be summarized as follows:

Mitotic inhibition	I. Maleic Acid.	II. Citraconic acid.	III. Fumaric acid.	IV. Mesaconic acid.
Mol/1:	5×10^{-7}	None.	None.	None.

Mitotic inhibition	V. 1 : 4-naphthohydroquinone diphosphate.	VI. 1 : 4-naphthohydroquinone mono-hydrogen succinate.	2-methyl-1 : 4-naphthohydroquinone diphosphate. (Mitchell and Simon-Reuss)
Mol/1:	1×10^{-9}	None.	3×10^{-6}

The results show that the ring structure of the quinones is not necessary for inducing antimitotic activity. Maleic acid (I) which contains the aliphatic part of the quinone molecule is a strong antimitotic. In this connection it may be remembered that Brunschwig *et al.* (1946) were able to show that the growth of cancer in rats can be retarded by maleic acid.

Introducing a methyl group into maleic acid gives citraconic acid (II), which does not show the antimitotic properties of maleic acid.

The importance of the *cis*-configuration of maleic acid is shown by the behaviour of fumaric acid (III) and mesaconic acid (IV), the *trans*-

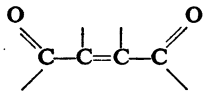
isomers of maleic and citraconic acids. Fumaric acid exerts no mitotic inhibition, in agreement with the results obtained by Pomerat and Wilmer (1939); mesaconic acid was also completely inactive.

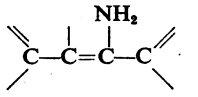
A methyl group introducted into maleic acid has obviously a dystherapeutic effect. The same is true in the 1 : 4–naphthoquinone series. The methyl free 1 : 4–naphthohydroquinone diphosphate belongs to the most powerful antimitotics obtained so far. Compared with colchicine and taking the figures given by Huber (1947) for its antimitotic action on *Tubifex eggs* as basis ($8.41 \times 10^{-9} M$) it is approximately 15 times more active than colchicine.

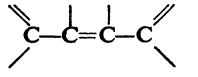
The importance of phosphorylation, inaugurated in this series by Mitchell and Simon-Reuss (1947) with the examination of the diphosphate of 2–methyl–1 : 4–naphthohydroquinone, is again apparent. Introducing succinic acid instead of phosphoric acid in 1 : 4–naphthohydroquinone gave 1 : 4–naphthohydroquinone mono-hydrogen succinate (VI) which was completely inactive. The role of the phosphorylation will be discussed in another paper of this series.

The present investigation discloses clearly the unit which maleic acid and the quinones have in common. We find in both groups the same uninterrupted system of three conjugated double bonds in which the second double bond is activated by the two adjacent C=O groups. The experimental evidence, so far obtained, seems to suggest that the unit O=C–CR=CH–C=O, R=H, in maleic acid and R=H or CH_3 in the quinones, is connected with the antimitotic activity of this group.

This unit is closely related to stilbylamine, which Lettré (1946) has shown to be the active principle in the great colchicine group, basing his deduction on the Windaus formula for colchicine, although not disregarding the alternative structure put forward by Cook *et al.* (1944) and by Dewar (1945). It is also related to stilbene, enclosed in the stilboestrols and investigated in tissue cultures by von Möllendorff (1939) and by Lettré and Albrecht (1941 and 1943).


in quinones,


in the colchicine group,


in the stilboestrols.

CHEMICAL SECTION

Preparation of 1 : 4 - naphthohydroquinone diphosphate and its salts. Dibarium salt.—A stream of nitrogen was passed into a flask containing phosphorous oxychloride (9 c.c.) in pure dry pyridine (60 c.c.). The contents were stirred in ice during the dropwise addition over 45 minutes of an ice-cold solution of 1 : 4-naphthohydroquinone (3.5 g.) in pyridine (90 c.c.). After final addition, the mixture was stirred in ice for 30 minutes and then evaporated *in vacuo* under nitrogen at 50°. When the residue was almost dry, pyridine (50 c.c.) was added and the evaporation repeated. The residue was left overnight *in vacuo* over sulphuric acid. Water (200 c.c.) was then added, the whole well shaken and left at room temperature for 1 hour. Hyflo supercel (*ca.* 1 g.) was added, the liquid filtered under reduced pressure, and the pale yellow filtrate made just alkaline to phenolphthalein by addition of a saturated solution of barium hydroxide (*ca.* 1 litre). The small precipitate was filtered off after addition of Hyflo and the filtrate evaporated *in vacuo* under nitrogen at 70° to about 500 c.c. The concentrate was filtered through a layer of Hyflo on a Buchner funnel and the filtrate added slowly with shaking to alcohol (500 c.c.). The flocculent precipitate was left for 36 hours at room temperature in the dark after displacing air with nitrogen. The product formed a gel which was centrifuged off, washed once with 50 per cent aqueous alcohol, once with alcohol, and finally with ether. The residue was dried *in vacuo* over silica gel. Crude yield 13.3 g. colourless amorphous solid. The salt appears to decompose when heated in solution.

Tetracyclohexylamine salt.—A solution of *cyclo*hexylamine (5 c.c.) in water was titrated with *N.* sulphuric acid till a *p*H of 3.8 was reached (43.5 c.c. 1.006*N.* sulphuric acid required). This solution was diluted to 100 c.c. with distilled water.

Dibarium 1 : 4-naphthohydroquinone diphosphate (3.2435 g., crude) was dissolved almost completely in water (100 c.c.) and titrated with the above *cyclo*hexylamine sulphate solution till only a trace of free barium ions remained (as tested for by an aqueous solution of sodium rhodizonate). This required 44.5 c.c.; if the barium salt had been pure, 53.5 c.c. would have been needed. Hyflo supercel was added, the precipitated barium sulphate filtered off through a layer of Hyflo and the filtrate evaporated to dryness *in vacuo* over nitrogen at 60°. The gummy residue was dissolved in methyl alcohol (25 c.c.), decolorized with charcoal, and evaporated to about 10 c.c. Ether was added till a slight opalescence appeared. On standing overnight prisms of the *tetracyclohexylamine salt of 1 : 4-naphthohydroquinone diphosphate* separated which were filtered off and washed with methyl alcohol–ether (1 : 1), then with ether, and dried; 2.1 g. colourless prisms (yield 50 per cent) m.p. 193.5–194° (decomp. after sintering at 192°). For analysis, a specimen was recrystallized from methyl alcohol, m.p. unchanged. (Found: C, 54.6; H, 9.0; N, 7.45 per cent. $C_{10}H_{10}O_8P_2.4C_6H_{13}N.2H_2O$ requires C, 54.2; H, 8.8; N, 7.44 per cent).

Tetrasodium salt.—The tetra*cyclo*hexylamine salt of 1:4-naphthohydroquinone diphosphate (1.7330 g.) in water (25 c.c.) was treated with the theoretical amount of sodium hydroxide solution (96.5 c.c. of 0.0956*N.*), the resulting solution filtered through a sintered glass funnel, a little redistilled hexyl alcohol added, and the whole evaporated *in vacuo* over nitrogen at 70°. The colourless oily residue gradually crystallized when left *in vacuo*. The material was ground up with alcohol in an agate mortar, filtered off and dried. The *tetrasodium salt of 1 : 4-naphthohydroquinone diphosphate* crystallized in colourless prisms; 0.8 g. (yield 85 per cent). (Found, in material dried at 50°: C, 24.5; H, 3.2. Loss in weight at 180°, 19.0 $C_{10}H_6O_8P_2Na_4.5H_2O$ requires C, 24.1; H, 3.2. Loss of $5H_2O$ requires 18.1 per cent). The salt is soluble in cold water to form a neutral solution, and forms a gel on addition of organic solvents.

1 : 4-Naphthohydroquinone diphosphate.—(*a*) From the tetrasodium salt. Tetrasodium salt of 1:4-naphthohydroquinone diphosphate (0.45 g.) was treated with *ca.* 5*N.* hydrochloric acid (1.1 c.c.) and water (5 c.c.). The solution was evaporated to dryness under reduced pressure, and the residue well extracted with hot alcohol (total 15 c.c.). The extract was filtered, treated with a little Hyflo and filtered through a little Hyflo. The filtrate was evaporated to dryness *in vacuo* under nitrogen at room temperature and the residue left to solidify.

The resulting solid, admixed with some oil, was dried on a porous tile, ground up with dry ethyl acetate, filtered off and dried. *The compound* formed a colourless partially crystalline solid, m.p. 214–215° (decomp.); 0.2 g. (yield 68 per cent). (Found: C, 34.6; H, 3.3. Equiv. 84.5. Loss in weight at 180° with some decomposition, 9.0. $C_{10}H_{10}O_8P_2.1\frac{1}{2}H_2O$ requires C, 34.6; H, 3.7. Equiv. 86.8. Loss of $1\frac{1}{2}H_2O$ requires 7.4 per cent).

(*b*) From crude dibarium salt. The dibarium salt (7.879 g.) was stirred in water (200 c.c.) till solution was almost complete and *N.* sulphuric acid added till only a trace of free barium ions remained, the precipitated barium sulphate filtered off through a layer of Hyflo, and the colourless filtrate evaporated to dryness under reduced pressure, leaving 1.9 g. of waxy solid. This solid was dissolved in alcohol (150 c.c.), filtered through Hyflo, and allowed to evaporate at room temperature *in vacuo*. The residue crystallized, m.p. 210–212° (decomp.) (equiv. 77.3). The alcohol treatment was repeated and when most of the alcohol had evaporated, a little ethyl acetate was added. The *1 : 4-naphthohydroquinone diphosphate* partially crystallized in colourless prisms on standing, m.p. 214–215° (decomp.). (Found, in material dried at 80°: C, 36.9; H, 3.6 per cent. Equiv. 79.6. $C_{10}H_{10}O_8P_2.\frac{1}{2}H_2O$ requires C, 36.5, H, 3.4 per cent. Equiv. 82.3.)

The tetra*cyclo*hexylamine salt can also be prepared from the free acid in water by addition of excess *cyclo*hexylamine. The residue after evaporation crystallizes from methyl alcohol, m.p. 193.5–194° (decomp.) identical with the material prepared from the dibarium salt.

1 : 4-Naphthohydroquinone mono-hydrogen succinate.—1:4-Naphthohydroquinone (1 g.) and maleic anhydride (6 g.) were carefully mixed and the mixture heated under nitrogen for two hours at 160°. The molten mass was collected, powdered, and extracted with hot ether (400 c.c.) in portions of 50 c.c. The ether extract was filtered after 24 hours from undissolved anhydride (3.3 g.) and extracted with 1 per cent sodium carbonate (45 c.c.) in portions of 4×10 c.c. and 1×5 c.c. The first extracts were acid, the last extract was amphoteric against litmus. The combined 1 per cent sodium carbonate extracts remained clear on acidifying and were discarded. A second extraction with 5 per cent sodium carbonate followed (90 c.c. in portions of 20, 20, and 5×10 c.c.). The second extract, at once acidified with 5 *N.* sulphuric acid (15 c.c.), gave an oily, reddish precipitate which solidified quickly. The precipitate was dissolved in ether. The red brownish ethereal solution was washed with water, dried over anhydrous sodium sulphate, decolorised with charcoal, and brought to dryness. The slightly coloured, crystalline residue (0.7 g.) was extracted with hot benzene (20 c.c.) which removed the coloured impurities completely. Yield 0.6 g., m.p. 175–176° (decomp.). Recrystallization from ether/*n*-hexane gave a product melting at 171°, which was again recrystallized from methanol/water. It was finally obtained in transparent, irregularly shaped plates, single or arranged in rosettes of tilted plates, m.p. 167° (decomp.). Dissolved in dilute alcohol, the substance gives a purple colour reaction with aq. ferric chloride. (Found: C, 64.5; H, 4.35. Equiv. 251. $C_{14}H_{12}O_5$ requires: C, 64.6; H, 4.65 per cent. Equiv. 260.)

1 :4-Naphthohydroquinone bis-hydrogen succinate.—1:4-Naphthohydroquinone (0.5 g.) and maleic anhydride (3 g.) were brought to reaction as described above. The molten mass was poured into water (50 c.c.). The undissolved material was powdered and re-extracted with water (50 c.c.) for one hour. The residue was collected, washed and dried *in vacuo*. The dried substance (0.5 g.) was dissolved in ether (75 c.c.) by heating. The slightly cloudy solution became clear after being gently treated with norite. The norite was removed by filtration and the filtrate concentrated until crystals began to appear. The crystallization was completed by adding *n*-hexane in the usual way. The collected crystals (0.4 g., m.p. 137°) were recrystallized from hot ether (180 c.c.), discarding small amounts of ether insoluble material. The ethereal solution was concentrated to *ca.* 20 c.c. and allowed to crystallize for 24 hours at room temperature. The colourless crystals (0.18 g.) so obtained gave no colour reaction with aqueous ferric chloride and seemed to be pure; m.p. 144.5°. They were analysed, since preliminary experiments had shown that the substance is decomposed by repeated recrystallization. (Found: C, 60.5; H, 4.6. Equiv. 173. $C_{18}H_{16}O_8$ requires: C, 60.0; H, 4.5 per cent. Equiv. 180.)

Summary

The antimitotic properties of maleic acid, citraconic acid, fumaric acid and mesaconic acid have been examined in cultures of chick fibroblasts *in vitro*. Maleic acid proved to be a strong antimitotic.

The connection of this group of substances with the 1:4–quinones has been discussed.

In the hydroquinone series 1:4–naphthohydroquinone diphosphate has been prepared and its antimitotic properties have been examined. It was found to be 1,000 times as active as 2–methyl–1:4–naphthohydroquinone diphosphate. 1:4–Napthoquinone mono-hydrogen succinate was completely inactive.

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