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SULPHYDRYL ADDITION COMPOUNDS OF SOME QUINONES AND RELATED SUBSTANCES IN THEIR ACTION ON THE GROWTH OF NORMAL CELLS

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We have reported recently (1948) that maleic acid produces in tissue cultures of chick fibroblasts a mitotic inhibition of the same kind and of the same order as 2-methyl-1:4-naphthohydroquinone diphosphate, studied by J. S. Mitchell and I. Simon-Reuss (1947) with the same biological material. This similarity is easily understood if the substituted hydroquinone is degraded in the dividing cell to a quinone, as the quinone molecule contains in its aliphatic part the residue of maleic acid. The question therefore arises whether maleic acid and the quinones have properties in common which may help us to understand the similarity of their antimitotic activity.

Maleic acid and the quinones excel by the ease with which they add other molecules. Amongst the substances which are added the sulphydryl compounds play a prominent part, physiologically as well as chemically. Their role in cell division is well established by the investigations of Shearer (1922), Hammet (1930), Rapkine (1931), Ephrussi (1931), Chalkley (1937), Brachet (1940), and many others. Chemically it has been shown that maleic acid adds thiolacetic acid, cysteine, and glutathione to give well-defined products. Furthermore the reactivity of maleic acid towards –SH compounds permits its use as an inhibitor of –SH enzymes (Morgan and Friedmann, 1938a, b, and c). On the basis of these results the retardation of malignant growth by maleic acid (Brunschwig *et al.*, 1946) has been discussed. The fixation of –SH-containing enzymes by quinone has been suggested by Potter (1942) as an explanation of the growth-inhibiting action of azo dyes. In the naphthoquinone series Fieser and Fieser (1944) emphasize the smooth addition of –SH compounds by naphthoquinones in connection with their physiological activity. Colwell and McCall (1945) suggest that the mode of antibacterial action of 2-methyl-1:4-naphthoquinone is a blocking of essential enzymes or essential bacterial metabolites by its combination with sulphydryl groups.

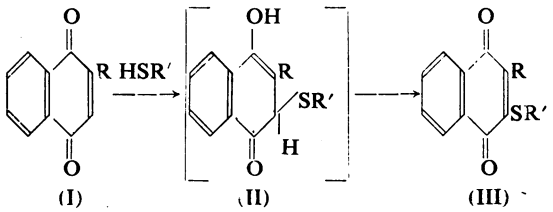
In our own experiments (1948) a parallelism between mitotic inhibition and –SH uptake is apparent. Thus maleic acid adds –SH compounds: it is a strong antimitotic. The *trans*-isomer, fumaric acid, and the methyl derivatives, citraconic acid and mesaconic acid, show no –SH uptake: they are devoid of antimitotic activity. Naphthoquinone and its 2-methyl derivative easily add –SH compounds: they are both strongly antimitotic.

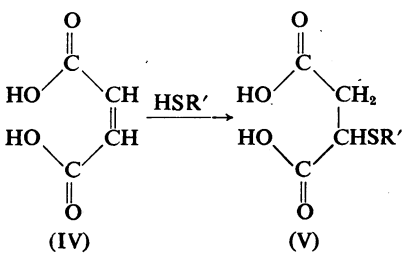
The parallelism between mitotic inhibition and –SH uptake, manifested by maleic acid and the quinones, has been used by us as a starting point for an experimental approach to the underlying problems.

As in tissue cultures the diphosphates of the hydroquinones and not the free quinones were applied, a comparison between maleic acid and the quinones presupposes that the quinones have the same degree of antimitotic activity as the hydroquinones. Lehmann (1942) has shown that this is so. Nevertheless we are fully aware that our choice is an arbitrary one. It compels us to set aside the processes which lead from the diphosphates of hydroquinones to the quinones, reactions which may be associated with physiological processes as important as, if not more important than, those connected with the quinone structure.

The naphthoquinones (I) and maleic acid (IV) behave somewhat differently with –SH compounds: maleic acid forms saturated thio-ethers

of succinic acid (V), but the corresponding naphthalene derivatives (II) are so easily oxidized by unchanged quinone that only thio-ethers of naphthoquinone (III) can be isolated.





Addition products of type (III), where R = H or CH_3, have been prepared from the quinone and thiolacetic acid and glutathione. Addition products of type (V) have been prepared from maleic acid and thiolacetic acid, glutathione and cysteine. The action of these substances on the growth of normal cells has been investigated. The present communication gives the results obtained so far.

EXPERIMENTAL

The experiments were carried out on tissue cultures of chick fibroblasts. The technique used has been described in our first paper (1948), to which we refer for details.

The values for mitotic inhibition and phase distribution obtained with the different substances are collected in the Table. The cytological description will be given by Mrs. I. Simon-Reuss in another paper.

1. S-(1 : 4-naphthoquinonyl-2)-thiolacetic acid (III ; R = H, R′ = .S.CH_2CO_2H)

The Table shows that the addition of thiolacetic acid to 1 : 4-naphthoquinone has abolished the strong antimitotic properties of 1 : 4-naphthoquinone. The phase distribution has also become normal. No abnormal mitoses have been observed.

TABLE

Tissue culture: chicken fibroblasts, hanging drop method, 4th passage, 24 hr. cultures, fixed in Susa, stained in Heidenhain's haematoxylin

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per ent inhibition	Phase distribution in % of mitoses			
				Prophase	Metaphase	Anaphase	Telophase
S-(1 : 4-NAPHTHOQUINONYL-2)-THIOLACETIC ACID (5210 mitotic cells investigated).							
1.	Controls	—	—	14.0	36.9	8.9	40.2
2.	1×10^{-6}	94.6 ± 5.4%	—	14.0	43.1	1.2	36.7
3.	3×10^{-6}	102.8 ± 9.1%	—	13.7	41.6	7.2	37.5
4.	5×10^{-6}	98.6 ± 8.2%	—	14.5	42.3	4.5	38.7
5.	Controls	—	—	14.4	27.9	1.7	56.0
6.	4×10^{-6}	99.3 ± 2.4%	—	15.2	45.1	2.6	37.1
7.	6×10^{-6}	99.6 ± 2.5%	—	15.6	43.6	2.5	38.2
S-(1 : 4-NAPHTHOQUINONYL-2)-GLUTATHIONE (9390 mitotic cells investigated).							
1.	Controls	—	—	16.3	39.2	7.0	37.5
2.	2×10^{-6}	99.3 ± 7.8%	—	17.6	36.5	8.1	37.8
3.	4×10^{-6}	100.9 ± 6.4%	—	17.7	38.8	7.9	35.6
4.	6×10^{-6}	99.8 ± 7.7%	—	17.4	38.9	7.6	36.1
5.	Controls	—	—	18.9	22.1	6.0	53.0
6.	2×10^{-6}	99.8 ± 8.6%	—	11.7	26.3	4.3	57.7
7.	4×10^{-6}	99.8 ± 5.3%	—	16.0	24.3	5.6	53.8
8.	6×10^{-6}	99.0 ± 4.5%	—	13.4	26.1	5.3	55.2
S-(2-METHYL-1 : 4-NAPHTHOQUINONYL-3)-THIOLACETIC ACID (5545 mitotic cells investigated).							
1.	Controls	—	—	17.5	27.0	6.6	48.9
2.	1×10^{-6}	92.1 ± 3.4%	—	17.2	28.1	5.9	48.8
3.	3×10^{-6}	99.7 ± 6.0%	—	14.4	32.8	5.4	47.4
4.	5×10^{-6}	110.5 ± 9.8%	—	13.9	32.4	5.2	48.6
5.	Controls	—	—	14.0	36.9	8.9	40.2
6.	4×10^{-6}	101.4 ± 2.9%	—	17.7	42.9	3.7	35.7
7.	6×10^{-6}	100.7 ± 2.5%	—	16.5	40.0	3.1	40.4

TABLE (*continued*)

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of mitoses			
				Prophase	Metaphase	Anaphase	Telophase
S-(2-METHYL-1 : 4-NAPHTHOQUINONYL-3)-GLUTATHIONE (6291 mitotic cells investigated).							
1.	Controls	—	—	17.4	34.5	4.9	43.2
2.	1×10^{-7}	—	—	9.1	62.3	10.0	18.5
3.	3×10^{-7}	—	—	8.4	60.5	9.5	21.7
4.	5×10^{-7}	89.9 ± 3.9%	10.1	17.2	45.0	6.8	30.9
5.	Controls	—	—	18.0	35.0	1.7	45.3
2.	2×10^{-6}	44.1 ± 3.0%	55.9	12.8	36.9	4.1	46.2
4.	4×10^{-6}	47.0 ± 1.5%	53.0	17.1	38.5	8.4	35.9
6.	6×10^{-6}	42.6 ± 2.0%	57.4	12.5	33.9	6.8	46.8
7.	Controls	—	—	18.3	36.5	5.8	39.4
8.	2×10^{-6}	44.5 ± 4.0%	55.5	18.1	33.5	16.0	32.3
9.	4×10^{-6}	41.2 ± 5.8%	58.8	14.7	48.0	3.6	33.7
10.	6×10^{-6}	44.3 ± 3.4%	55.7	11.6	49.2	12.1	27.1
S-THIOLACETO-SUCCINIC ACID (5539 mitotic cells investigated).							
1.	Controls	—	—	20.1	36.4	2.6	40.9
2.	1×10^{-6}	93.9 ± 7.7%	6.0	17.9	38.4	2.0	41.6
3.	3×10^{-6}	71.5 ± 4.8%	28.5	19.7	35.6	3.9	40.8
4.	5×10^{-6}	84.5 ± 6.8%	15.5	22.5	38.6	5.6	33.3
5.	Controls	—	—	17.5	28.2	2.6	51.6
6.	2×10^{-6}	96.0 ± 2.8%	4.0	23.1	21.2	3.1	52.5
7.	4×10^{-6}	70.8 ± 2.4%	29.2	19.0	26.3	2.3	52.4
8.	6×10^{-6}	66.3 ± 3.3%	33.7	18.3	21.9	0.9	58.9
S-CYSTEINO-SUCCINIC ACID (5081 mitotic cells investigated).							
1.	Controls	—	—	22.3	31.3	1.3	45.1
2.	1×10^{-6}	84.7 ± 5.3%	15.3	19.9	32.8	0.6	46.7
3.	3×10^{-6}	81.3 ± 5.4%	18.7	19.6	33.7	0.5	46.2
4.	5×10^{-6}	54.3 ± 3.4%	45.7	14.1	29.8	1.7	54.3
5.	Controls	—	—	19.8	23.0	2.2	55.0
6.	2×10^{-6}	68.3 ± 7.1%	31.7	20.7	21.7	2.5	55.1
7.	4×10^{-6}	68.3 ± 5.9%	31.7	21.4	24.2	3.1	51.4
8.	6×10^{-6}	37.5 ± 3.1%	62.5	19.4	16.5	2.0	62.1
S-GLUTATHIONO-SUCCINIC ACID (7964 mitotic cells investigated).							
1.	Controls	—	—	20.4	19.2	8.4	52.0
2.	1×10^{-6}	86.0 ± 4.8%	14.0	16.1	25.2	9.0	49.7
3.	3×10^{-6}	73.5 ± 5.5%	26.5	16.6	29.1	17.8	36.5
4.	5×10^{-6}	54.9 ± 4.3%	45.3	13.3	35.8	8.1	42.8
5.	Controls	—	—	14.9	34.9	4.6	45.6
6.	2×10^{-6}	70.1 ± 6.6%	29.9	20.7	40.9	11.9	26.7
7.	4×10^{-6}	65.9 ± 6.5%	34.1	16.0	40.6	7.2	36.1
8.	6×10^{-6}	55.7 ± 6.1%	44.8	18.9	28.0	5.3	47.6

2. S-(1 : 4-naphthoquinonyl-2)-glutathione (III ; R=H, R′=.SG)

The substance gives no mitotic inhibition ; the phase distribution is normal. Abnormal cells have not been found.

3. S-(2-methyl-1 : 4-naphthoquinonyl-3)-thiolacetic acid (III ; R=CH_3, R′=$S.CH_2CO_2H$)

Mitoses and phase distribution are normal.

4. S-(2-methyl-1 : 4-naphthoquinonyl-3)-glutathione (III ; R=CH_3, R′=SG)

The addition of glutathione to 2-methyl-1 : 4-naphthoquinone gives a product which has definite antimitotic properties. Mitotic inhibition seems to start at 5×10^{-7} *M* with inhibition of 10 per cent. The mitotic inhibition increases with rising concentration: at 2×10^{-6} *M* it is 56 per cent. This value seems to represent the maximum of mitotic inhibition, as further increases in concentration are not followed by greater mitotic inhibition. The phase distribution shows features of interest. Disturbances in phase distribution have been observed at 1×10^{-7} and at 3×10^{-7} *M*, i.e. at concentrations where no mitotic inhibitions are found in the 24-hour tissue cultures. Here phase disturbances precede mitotic inhibition. In contrast, no phase

disturbances are to be seen in the 24-hour cultures when the mitotic inhibition reaches its peak. The phase disturbances consist in an accumulation of metaphases and anaphases and a loss of telophases

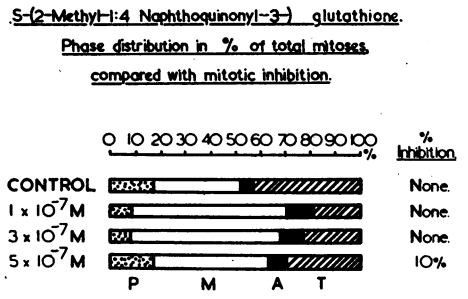


FIG. 1.—Disturbances in phase distribution preceding mitotic inhibition.

(Fig. 1). Abnormal mitoses are present in the whole range of concentrations investigated, even at 1×10^{-7} and 3×10^{-7} *M*.

5. S – thiolaceto-succinic acid (V ; R = $S.CH_2CO_2H$)

The substance has weak antimitotic properties. At 6×10^{-6} *M* the mitotic inhibition is only 33.7 per cent. The phase distribution is apparently not disturbed. Abnormal mitoses are present in all concentrations investigated.

6. S – cysteino-succinic acid (V ; R = $S.CH_2CH(NH_2)CO_2H$)

The mitotic inhibition of S – cysteino-succinic acid is more pronounced than the inhibition by

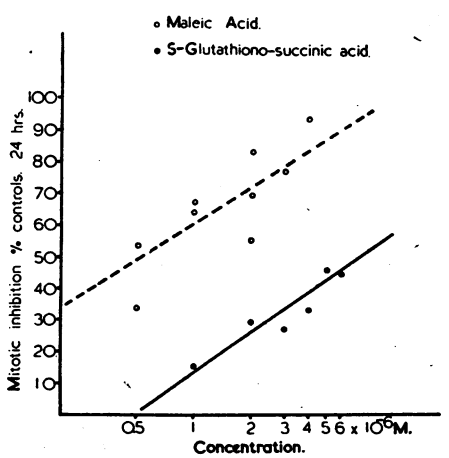


FIG. 2.—Parallelism of the concentration curves showing the mitotic inhibition of maleic acid and of S-glutathiono-succinic acid.

substance 5. At 6×10^{-6} *M* an inhibition of 62.5 per cent has been found. The phase distribution is normal. A few abnormal cells are present at all concentrations. The small outgrowth of the cultures at the higher concentrations (5 and 6×10^{-6} *M*) has to be mentioned.

7. S – glutathiono-succinic acid (V ; R = SG)

The mitotic inhibition of S – glutathiono-succinic acid is weaker than the inhibition produced by the cysteine adduct (substance 6). The inhibition increases with rising concentrations. The concentration curve follows a logarithmic line when plotted against the $\log_{10}$ of the concentrations. The graphic comparison with the corresponding line representing the concentration curve of maleic acid shows that the two lines take parallel courses (Fig. 2). The phase distribution shows accumulation of metaphases and irregular anaphases ; abnormal mitoses are to be seen at all concentrations.

DISCUSSION

The experiments carried out with sulphydryl addition compounds of some quinones and of maleic acid, described in this paper, deal with two groups of adducts which are different in the part of the molecule, apparently connected with their physiological activity. The quinones have given unsaturated addition products (III) which can be regarded as derived from maleic acid, whilst the maleic acid adducts are saturated succinic acid derivatives (V).

Mitotic inhibition has been found in both groups. Unsaturation of the adducts, therefore, does not seem to be the decisive factor determining their mitotic activities. This result can be supported by an interesting analogy: Lettré and Mohn (1946) have found that the dihydro-derivatives of diethyl stilboestrol have antimitotic properties as well as the unsaturated diethyl stilboestrol. On the other hand, v. Möllendorff's observations (1941) on male sex hormones show clearly that in other groups the unsaturation of the molecule is essential. Only the unsaturated members of this group develop antimitotic properties, whilst the activity as male sex hormones is displayed by the saturated compounds as well. The possible physiological transition of the substances investigated by us from saturation to unsaturation or conversely must be kept in mind for further investigations.

The addition of SH compounds to maleic acid results in the formation of a centre of asymmetry in the adduct when thiolacetic acid is used for this reaction, or of an additional centre when cysteine or glutathione are used. We intend to

supplement our investigations by resolving S–thiolaceto–succinic acid into its optical isomers and investigating their antimitotic activities. S–cysteino–succinic acid seems to be formed under the stereochemically directing influence of L–cysteine (Morgan and Friedmann, 1938c).

The results obtained with the sulphydryl addition compounds of 2-methyl-1:4-naphthoquinone are interesting in several respects. The S–thiolaceto-derivative was completely inactive as an inhibitor of mitosis, whilst the glutathione derivative was active. Here, clearly, the importance of the sulphydryl-carrying reactant comes to light. It has been stressed from the beginning in the maleic acid series that the reactivity of the sulphydryl group with the activated double bond is different from case to case. In the quinone series the interesting results of Walsh and Walsh (1948), showing that liver hexosediphosphatase exhibited a much greater sensitivity towards quinones than did the other phosphatases, point in the same direction.

The activity of the glutathione derivative of 2-methyl-1:4-naphthoquinone as an inhibitor of mitosis, compared with the lack of antimitotic activity of the methyl free S– (1:4-naphthoquinolyl-2)-glutathione, shows a new feature of the methyl group. So far we have seen that the introduction of a methyl group in the quinones as well as in maleic acid has been followed by a decrease of their antimitotic activity. Now the elimination of the methyl group leads to a product which has lost its antimitotic activity. Chemical analogy shows that the 2-methyl group exerts a distinct retarding effect in the introduction of substituents at position 3 by way of 1:4–addition reactions (Fieser and Fieser, 1944, pp. 737 and 743), but there are exceptions where the presence of a 2-methyl group increases the reactivity.

The adducts of thiolacetic acid and of glutathione to 1:4-naphthoquinone were both inactive as antimitotic agents. Other sulphydryl substituents have not been investigated. The different biological results obtained by adding thiolacetic acid or glutathione to 2-methyl-1:4-naphthoquinone show that the possibility cannot be dismissed that other –SH addition products to 1:4-naphthoquinone may display antimitotic activity.

The substances formed by the addition of thiolacetic acid to some quinones are acids in which the sulphur is linked ether-fashion. If one replaces the sulphur by oxygen one comes in the aromatic series to substances related to phenoxyacetic acid. These are known to be differential growth-inhibitors for plants. It is claimed that their action is similar to that of colchicine (Arvy and Lhoste, 1946).

The examination of the reaction products of quinones and maleic acid with –SH compounds has been undertaken in order to see whether their action on mitoses would allow us to explain the parallelism between mitotic inhibition and –SH uptake, established experimentally. The present investigation affords no clear solution of this problem. From the seven adducts examined three have exerted no mitotic inhibition (1, 2, and 3) and four were active as mitotic inhibitors (substances 4, 5, 6, and 7). Dealing with the same problem, Michael (1948) has found unimpaired physiological activity with the quinonoid fuscin after addition of thiolacetic acid. On the other hand Kuhn and Beinert (1945), who investigated the inhibition of carboxylase, have shown that S-cysteino-*p*-benzoquinone, resulting from the addition of cysteine to *p*-benzoquinone, has lost the strong inhibitory activity of *p*-benzoquinone. Furthermore, 2-methyl substitution in 1:4-naphthoquinone decreases the antimitotic activity, whereas the introduction of a methyl group in the *ortho* position in –S–derivatives of 1:4-naphthoquinone increases the antimitotic activity of the new compound as shown in this paper.

Simple thiol addition compounds like those we have prepared and investigated may play no part in the mitotic inhibition induced by quinones or maleic acid. No satisfactory evidence is available to discuss other possibilities.

Chemical Section

1. *S-(1 : 4-naphthoquinonyl-2)-thiolacetic acid*

The substance was prepared following the directions given by Fieser and Turner (1947) for the preparation of S-(2-methyl-1:4-naphthoquinonyl-3)-thiolacetic acid. 1:4-Naphthoquinone (2.6 g.) was dissolved in warm alcohol (120 c.c.), cooled to room temperature, and mixed with a solution of thiolacetic acid (1.5 g., 1.13 c.c., 1 mol. quantity) in alcohol (4 c.c.). After standing overnight the dark solution was evaporated *in vacuo*, when yellow plates appeared.

Yield 0.8 g., m.p. 173–5° (decomp.) after softening at 164° (20 per cent yield of crude product). A portion (0.5 g.) was dissolved in a solution of sodium bicarbonate (0.5 g.) in water (25 c.c.) and well extracted with ether. The aqueous phase was acidified to Congo red with 5*N* sulphuric acid and the precipitated yellow gel was extracted with a large volume (500 c.c. in all) of ether. The extract was washed twice with water, dried over sodium sulphate, and evaporated to dryness. The crystalline residue was recrystallized from alcohol giving 0.35 g. yellow plates m.p. 183.5° (decomp.). For analysis, the *compound* was purified on a column of alumina, when

washing with methanol removed traces of a dark-coloured impurity. *S-(1 : 4-naphthoquinonyl-2)-thiol-acetic acid* was eluted from the column with aqueous bicarbonate, isolated after acidification and recrystallized twice from alcohol to m.p. 183.5° (decomp.). Found (in material dried at 80° *in vacuo*): C, 58.0; H, 3.4. $C_{12}H_8O_4S$ requires C, 58.1; H, 3.3 per cent.

2. *S-(1 : 4-naphthoquinonyl-2)-glutathione*

The method described by Fieser and Fieser (1944) was used for the preparation of this substance. 1:4-Naphthoquinone (316 mg., 1/500 mol.) was dissolved in warm alcohol (10 c.c.) and cooled to room temperature. The solution was added at once to a solution of glutathione (307 mg., 1/1,000 mol.) in water (4 c.c.) and alcohol (6 c.c.). An immediate darkening occurred with the separation of a yellow solid, which was left overnight at room temperature, filtered off, well washed with alcohol, and dried.

Yield 440 mg. yellow amorphous solid (95 per cent yield). Found (in material dried *in vacuo* at room temperature): C, 51.6; H, 4.9; N, 9.2. $C_{20}H_{21}O_8N_3S$ requires C, 51.8; H, 4.6; N, 9.1 per cent.

3. *S-(2-methyl-1 : 4-naphthoquinonyl-3)-thiolacetic acid*

Prepared by the method of Fieser and Turner (1947), except that after reduction by aqueous hydrosulphite it was found more convenient to re-oxidize to the quinone by means of 10 per cent aqueous ferric chloride containing 1/5 of its volume of 5*N* sulphuric acid.

4. *S-(2-methyl-1 : 4-naphthoquinonyl-3)-glutathione*

Prepared by the method of Fieser and Fieser (1944) in a yield of 86 per cent.

5, 6, *and* 7. *S-thiolaceto-succinic acid* (5), *S-cysteino-succinic acid* (6), *and S-glutathiono-succinic acid* (7) have been prepared by Morgan and Friedmann (1938a). The original substances analysed by these authors have been used for our experiments.

SUMMARY

1. Evidence is given that the mitotic inhibition produced by some quinones and by maleic acid goes parallel with the –SH uptake of these substances.

2. S–(1 : 4-naphthoquinonyl-2)-thiolacetic acid (1), S–(1 : 4-naphthoquinonyl-2)-glutathione (2), S–(2-methyl-1 : 4-naphthoquinonyl-3)-thiol-acetic acid (3), S–(2-methyl-1 : 4-naphthoquinonyl-3)-glutathione (4), S–thiolaceto-succinic acid (5), S–cysteino-succinic acid (6), S–glutathiono-succinic acid (7), have been prepared and investigated in tissue cultures of chick fibroblasts. Substances (1), (2), and (3) give no mitotic inhibition, substances (4), (5), (6), and (7) were inhibitors of mitosis.

3. The biological results are discussed.

4. Simple thiol addition compounds to quinones and maleic acid like those mentioned above seem to play no part in the mitotic inhibition induced by some quinones and by maleic acid.

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