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On the Biosynthesis of Toluquinones from *Aspergillus fumigatus*

II. Hydroquinone Forms of the Pigments

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By the application of molecular orbital methods the reduction potentials of the toluquinones from *A. fumigatus*, L. S. H. T. M. A 46. were calculated. A determination of the oxidation-reduction potential of the culture medium indicated that the pigments produced would be present predominantly in the hydroquinone form during the first two weeks of cultivation. This was confirmed by titration of the amount of quinones present in the medium. Fumigatin hydroquinone, 3-hydroxy-2,5-toluhydroquinone, 3,4-dihydroxy-2,5-toluhydroquinone, and 3-methoxy-4-hydroxy-2,5-toluhydroquinone were identified, by chromatography, in young cultures of the mould. The pigments seemed to be produced, and released into the culture medium, in the reduced form, oxidation into the corresponding quinones taking place at a late stage in the development of the mould. Endo-cellular enzyme systems appeared not to be involved in the oxidation process.

Six different toluquinones (compounds Nos. 10—15 in Table 2) have been recognized as metabolic products of certain strains of *Aspergillus fumigatus* Fresenius. Two of these pigments, fumigatin [1] and spinulosin,[2] were isolated by Raistrick *et al.* some thirty years ago, while the other four pigments were detected and identified more recently.[3,4] Fumigatin, which was isolated from *A. fumigatus*, L. S. H. T. M. A 46, seemed to be present in the culture medium partly in the oxidized (quinone) form, and partly in the reduced (hydroquinone) form. Both forms were, in fact, isolated from the same metabolism solution. Now that several other quinonoid pigments are known to be produced by the strain mentioned above, one would also expect that these could be present in both the forms.

Quinone-hydroquinone ($Q-H_2Q$) systems are known to give reversible oxidation-reduction potentials (E), which are dependent on the pH of the solution and on the concentration of the oxidized and the reduced forms, in accordance with the expression

$$E = E_0 + \frac{RT}{2F} \, {}^{e}\log \frac{[Q][H^+]^2}{[H_2Q]} \qquad (1)$$

In a solution, such as the culture medium from *A. fumigatus*, containing a mixture of different quinone-hydroquinone pairs, an equilibrium between the different systems will rapidly be attained. The relative proportions of the two forms in each system are, at equilibrium, determined by the corresponding redox potentials:

$$\frac{[Q']}{[H_2Q']} = \frac{[Q'']}{[H_2Q'']} \exp[(2F/RT)(E_0''-E_0')] \quad (2)$$

The redox potentials of the toluquinonoid pigments from *A. fumigatus* are, for practical reasons, difficult to determine experimentally with a desirable accuracy. The hydroquinone forms are most susceptible to air oxidation, and cannot be obtained in a sufficient pure state. Also the quinone forms, especially 3-hydroxy-2,5-toluquinone and 3,4-dihydroxy-2,5-toluquinone, are fairly unstable. Quinone-hydroquinone redox potentials may, however, be estimated by the application of molecular orbital methods. It is well established that the reduction potential of a quinone is proportional to the delocalisation energy change (ΔD. E.) on the conversion into the corresponding hydroquinone.[5,6] It has, further, been shown that the redox potentials are also linearly related to the energy of the lowest unoccupied molecular orbital (E. L. U. M.O.) of the oxidized form (and to the energy of the highest occupied molecular orbital of the reduced form).[7] The energy levels and delocalisation energies were, therefore, computed for both forms of the different pigments, and for a number of quinone-hydroquinone systems with known redox potentials [8,9] (see Table 1). The latter reference systems define the regression lines obtained in a plot of the redox potentials against E. L. U. M. O. (Fig.1) or ΔD. E. (Fig. 2), and the redox potentials of the pigments can be estimated by interpolation. The values (listed in Table 2) obtained by these two methods are in good agreement. As a control of the theoretical calculations the reduction potential of fumigatin (the major pigment in *A. fumigatus*, L. S. H. T. M. A 46) was measured by potentiometric titration with titanous chloride. The experimental determination yielded $E_0 = 0.46$ V, agreeing fairly well with the calculated value (0.44 V).

Table 1. Molecular orbital data and redox potentials (25°; pH 0) of quinone-hydroquinone reference systems.

No.	Derivative of 1,4-benzoquinone	$\frac{\text{E.L.U.M.O.}-\alpha}{-\beta}$	$\frac{\Delta\text{D.E.}}{-\beta}$	E_0 in V
1	Unsubstituted	0.058	0.304	0.699
2	Methyl-	0.074	0.328	0.645
3	Hydroxy-	0.092	0.362	0.596
4	2,6-Dimethyl-	0.091	0.350	0.593
5	2,5-Dimethyl-	0.092	0.354	0.590
6	Trimethyl-	0.106	0.370	0.527
7	2-Hydroxy-5-methyl-	0.110	0.388	0.523
8	Tetramethyl-	0.121	0.410	0.480
9	2,5-Dihydroxy-	0.130	0.426	0.443

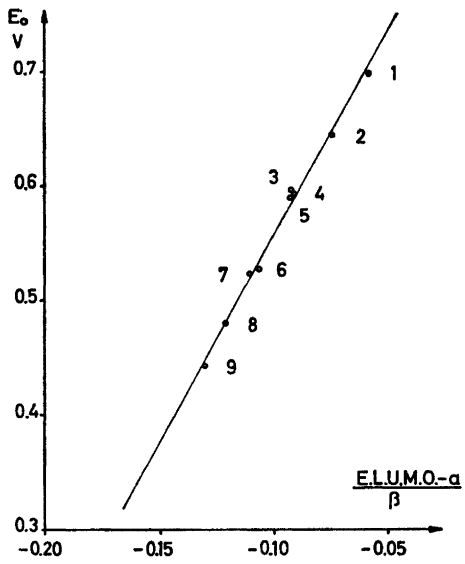

Fig. 1. Regression line of E_2 (25°) on E.L.U.M.O $-\alpha/\beta$. Numbers refer to compounds in Table 1.

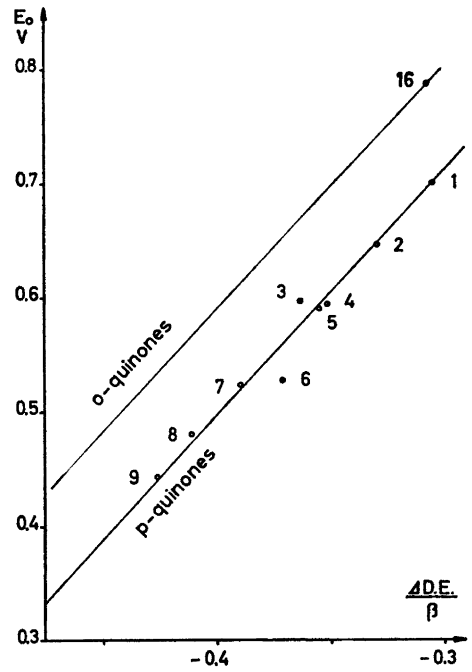

Fig. 2. Regression line of E_0 (25°) on ΔD.E./β. Numbers refer to compounds in Table 1 and 3.

Since all the *p*-quinonoid pigments contain at least one nuclear hydroxyl group, they may exist in at least one, tautomeric, *o*-quinonoid form. This is shown in Fig. 3 for fumigatin and 3,4-dihydroxy-2,5-toluquinone, the pigments that are produced in the largest amounts. The linear correlations between redox potentials and ΔD. E. are known to be different for *o*- and

Table 2. Molecular orbital data and calculated redox potentials (25°; pH 0) of *p*-quinone-hydroquinone systems present in *A. fumigatus*.

No.	Derivative of 2,5-toluquinone	E.L.U.M.O. $-\alpha$ / $-\beta$	ΔD.E. / $-\beta$	E_0 in V E.L.U.M.O.	E_0 in V calculated from ΔD.E.
10	3-Hydroxy-	0.109	0.396	0.532	0.502
11	3,4-Dihydroxy-	0.134	0.418	0.434	0.455
12	3-Methoxy-4-hydroxy-	0.136	0.420	0.426	0.451
13	3-Hydroxy-4-methoxy-*	0.136	0.422	0.426	0.446
14	3,6-Dihydroxy-	0.141	0.440	0.408	0.408
15	3,6-Dihydroxy-4-methoxy-**	0.168	0.476	0.308	0.331

* fumigatin ** spinulosin

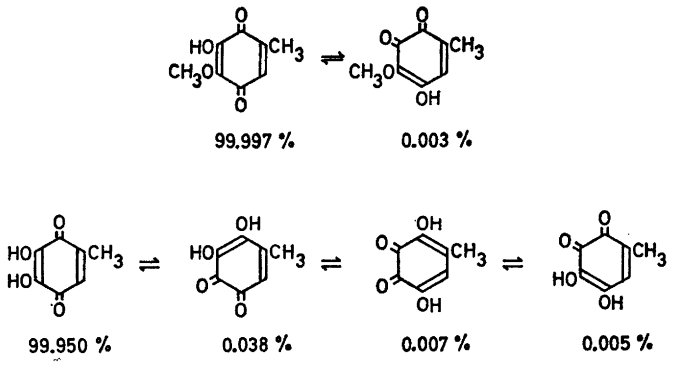

Fig. 3. *o*-Quinonoid tautomers of 3-hydroxy-4-methoxy-2,5-toluquinone (fumigatin) and 3,4-dihydroxy-2,5-toluquinone.

p-quinones, although the slopes of the lines are equal. [5,6] The regression line for *o*-quinones, indicated in Fig. 2, was obtained using *o*-benzoquinone as the reference compound [9] (see Table 3). Interpolation from the line gives the approximate reduction potentials of the different tautomeric *o*-quinonoid forms of the pigments, enabling a theoretical estimation of the ratio *o*-quinone/*p*-quinone (the tautomeric constant) from eqn. (2); the tautomers have a common reduction product. The results, given in Table 3, indicate that the *o*-quinonoid forms of the pigments may be neglected. Only the *p*-quinonoid tautomers will, therefore, be considered in this paper.

With introduction of the more convenient r_H notion eqn. (1) yields (E_0 in V);

$$r_H = 33.807\ E_0 + {}^{10}\log\ ([Q]/[H_2Q]) \qquad (3)$$

Table 3. Molecular orbital data and calculated redox potentials (25°; pH 0) of *o*-quinone-hydroquinone systems present in *A. fumigatus*.

No.	Derivative of 1,2-benzoquinone	$\frac{\Delta D.E.}{-\beta}$	E_0 in V	pK*
16	Unsubstituted**	0.306	0.787	—
17	4-Hydroxy-6-methyl-	0.382	0.625	4.16
18	3,4-Dihydroxy-6-methyl-	0.402	0.582	4.29
19	3,6-Dihydroxy-5-methyl-	0.404	0.578	4.16
20	3,4-Dihydroxy-5-methyl-	0.414	0.556	3.41
21	3-Methoxy-4-hydroxy-5-methyl-	0.410	0.565	3.85
22	3-Methoxy-4-hydroxy-6-methyl-	0.404	0.578	4.46
23	4,5-Dihydroxy-6-methyl-	0.434	0.514	3.58
24	3-Methoxy-4,5-dihydroxy-6-methyl-	0.460	0.458	4.29

* K stands for the tautomeric constant; K = *o*-quinone/*p*-quinone.
** Reference system (not present in the mould.)

The investigation by Raistrick *et al.* of the metabolism solutions from *A. fumigatus*, L. S. H. T. M. A 46, indicated that the ratio [fumigatin]/[fumigatin hydroquinone] was approximately equal to unity. At the corresponding r_H value (about r_H 15) of the solution, those pigments with higher redox potentials than fumigatin would be present predominantly in the reduced form. This is evident from eqn. (3); the calculated percentages of hydroquinone forms of the pigments at different r_H values of the culture medium are listed in Table 4. It will now be shown that this theoretical conclusion is, in fact, supported by actual experiments.

Table 4. Theoretical percentages of hydroquinone forms of the pigments at different r_H values. For each pigment the arithmetic mean of the calculated E_0 values (Table 2) has been used.

No.	Derivative of 2,5-toluquinone	% hydroquinone at r_H 20	18	16	15	14	13.5	13	12
10	3-Hydroxy-	0	77	99	100	100	100	100	100
11	3,4-Dihydroxy-	0	0	10	53	92	97	99	100
12	3-Methoxy-4-hydroxy-	0	0	7	41	88	96	99	100
13	3-Hydroxy-4-methoxy-	0	0	5	36	85	95	98	100
14	3,6-Dihydroxy-	0	0	1	6	38	66	86	99
15	3,6-Dihydroxy-4-methoxy-	0	0	0	0	0	0	0	6

The medium of young cultures of *A. fumigatus*, L. S. H. T. M. A 46 (this strain was used throughout the present investigation) is yellowish-brown, but after about two weeks of growth the culture medium becomes an intense purple colour. The toluquinonoid pigments, which all contain at least one dissociable hydroxyl group ($pK_1 = 4.8-6.0$), are yellow in acid solutions, and red-violet to blue-violet in neutral or slightly alkaline solutions. It, therefore, seemed likely that the change in colour of the culture medium could be due to a pH change of the solution. This was also confirmed by experiments, in which the pH of the medium was determined as a function of the age of the cultures. As indicated in Fig. 4, the pH of the medium rapidly increases from pH 3 to about pH 7 after two weeks of cultivation. At this time the medium (a modified Raulin-Thom solution) was found to be essentially depleted of glucose, indicating that lack of nutrient was the ultimate reason for the change in pH and colour. Confirmatively, after addition of glucose to old cultures (pH above 7) of the mould, the pH immediately began to decrease; the magnitude and duration of the decrease in pH corresponded well to the amount of glucose added.

However, when the medium of young cultures was made alkaline, it remained brown, or showed a faint blue-green colour. When the alkaline solution was shaken with air (or oxygen), it rapidly became the intense purple colour characteristic of the toluquinones. During this process oxygen was taken up, and a rough estimate of the amount of oxygen absorbed suggested that the pigments were present almost exclusively in the hydroquinone forms. Ether extracts of the medium from young cultures of the mould were, there-

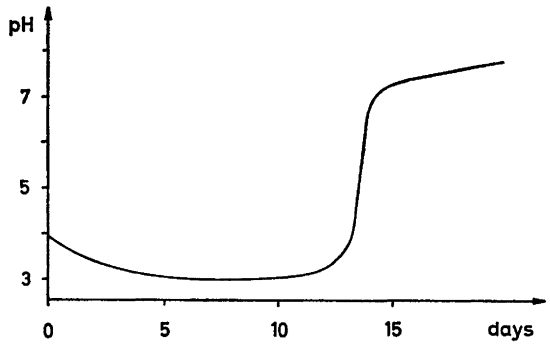

Fig. 4. pH of the medium as a function of the age of the culture.

fore, carefully investigated by chromatography. In this way the reduced forms of four of the six pigments produced could be identified. Spinulosin hydroquinone and 3,6-dihydroxy-2,5-toluhydroquinone were not detected. Great practical difficulties were encountered in the chromatographic analysis. Even though all efforts were made to prevent the autoxidation of the hydroquinones, it was observed that authentic samples of the substances were oxidized to a fairly large extent during the chromatographic procedures. Since spinulosin hydroquinone and 3,6-dihydroxy-2,5-toluhydroquinone seemed to be especially susceptible to air oxidation, the failure to detect the latter two compounds can, at least partly, be assigned to experimental difficulties.

No hydroquinones could be detected in old cultures of the mould. Further studies were, therefore, undertaken, in which the total amount of pigments, and the percentage of quinone forms, were determined titrimetrically as a function of the age of the cultures. The results of this investigation are shown in Fig. 5. After a lag period of four days, during which time the mycelial mat was being established, pigment production began and remained almost linear for about ten days. During this linear phase of production the pigments were found to be present predominantly in the hydroquinone form. At the twelth to fifteenth day of growth an increase in the pH of the medium was accompanied by a fairly slow conversion of the reduced forms of the pigments into the corresponding quinones. After a further three days the pigments were present exclusively in the oxidized form.

Similar results were obtained in electrometric determinations of the oxidation-reduction potential of the culture medium. This is shown in Fig. 6, where the r_H value of the medium is plotted against the time of cultivation. Within a few days of growth the r_H decreased to about r_H 13.5, at which value all the pigments, except for spinulosin, would be present predominantly in the reduced form (see Table 4). This low r_H value was then essentially maintained during the linear phase of pigment production. From the fourteenth day on, the r_H of the medium increased, indicating the gradual oxidation of the hydroquinones (see Table 4) concomitant with the pH change of the solution.

The above results seem to establish that the toluquinones from *A. fumigatus* are biosynthesized *via* the corresponding hydroquinones. The failure to detect

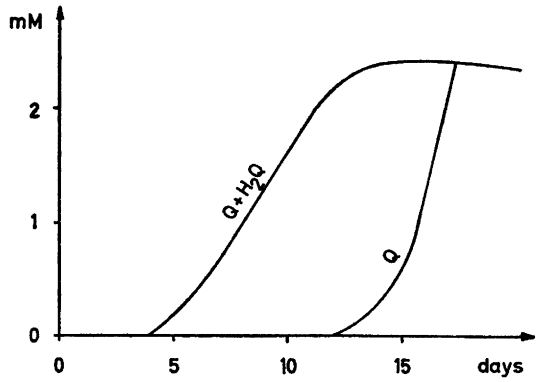

Fig. 5. Total amount of pigments (Q + H_2Q) and amount of quinone forms (Q) as a function of the age of the culture.

3,6-dihydroxy-2,5-toluhydroquinone, which in young cultures (r_H 13.5) would be present in larger amounts than the corresponding quinone, is probably due to experimental difficulties (see above). Similarly, there is little reason to make an exception for spinulosin, even though its hydroquinone form neither was detected, nor would be expected to be present in significant amounts. Spinulosin has, in fact, been shown to be derived from the hydroquinone form of fumigatin, not from the oxidized form.[4]

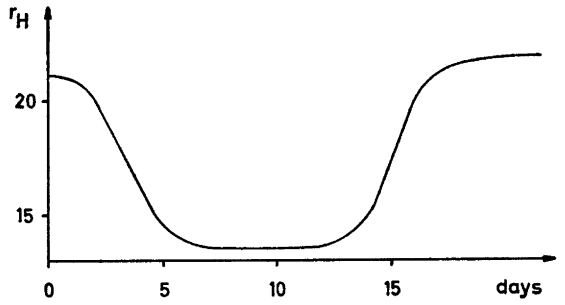

Fig. 6. r_H of the medium as a function of the age of the culture.

The oxidation of the hydroquinones was found to take place in the culture medium, without the participation of endo-cellular enzyme systems.[10] The rate of oxidation was, in fact, considerably increased when the mycelium was removed immediately after the pH change of the medium. Furthermore, the hydroquinones present in young cultures of the mould were quantitatively oxidized within a few hours, after removal of the mycelium. These observations suggest that the mycelium prevents the hydroquinones from oxidation by the air, quinones being formed as soon as oxygen becomes available.

Confirmatively, quinones were rapidly formed in growing young cultures when oxygen was supplied to the medium by aeration. However, when the supply of oxygen was cut off, a reduction process immediately started; the quinones that had been formed by aerating the medium were then reduced within some eight hours.

A similar, active, reduction process was demonstrated in old cultures of the mould, where the hydroquinones had been oxidized in connection with the pH change of the medium. Thus the addition of glucose, which led to a decrease in the pH of the solution, also caused a rapid reduction of the pigments. The process, which was followed titrimetrically and by determinations of the r_H of the medium, seemed not to be due merely to the reducing properties of glucose, since it could also be initiated by the addition of gluconic acid or of a non-reducing sugar like fructose. It, therefore, appears that the mycelium not only prevents the hydroquinones from oxidation by the passive exclusion of air, but also actively keeps the pigments in the reduced state, possibly by the formation of reducing metabolites in the carbohydrate breakdown.

Undoubtedly, the oxidation of the corresponding hydroquinones is the last step in the biosynthesis of the toluquinones from *A. fumigatus*. Further experiments, with the aim of deciding whether this process is enzymatically catalyzed or not, are in progress.

EXPERIMENTAL

Culture conditions. A. fumigatus Fresenius, L. S. H. T. M. A 46, was used throughout this work. The culture conditions [11] and the methods for isolation of the toluquinonoid products [3] have been described previously.

Molecular orbital calculations. The secular equations of the different compounds were set up according to the molecular orbital method with the LCAO-approximation. The following parameters were used: Coulomb integrals for carbon = α, for carbonyl oxygen = $\alpha + \beta$, for hydroxyl oxygen = $\alpha + 2\beta$; Resonance integrals between neighbouring carbon atoms = β, between neighbouring carbon and carbonyl oxygen atoms = β, between neighbouring carbon and hydroxyl oxygen atoms = 0.8β, between all other pairs of atoms = 0. The constituent atomic orbitals were assumed to be orthogonal (overlap integrals = 0). Nuclear methyl groups were treated by the heteroatom model of hyperconjugation (Coulomb integral = $\alpha + 3\beta$; bond integral = 0.7β).[5]

The calculations were performed by an electronic digital computer with one single program, and were carried out according to the procedure of Nagakura;[12] the charges and bond orders (p_{rs}) obtained from the zero-order approximation were used to calculate revised values for the Coulomb and resonance integrals, and the calculations were repeated till the results finally became self-consistent.[13] Coulomb integrals were modified by the ω-technique [5] ($\omega = 1.4$) and resonance integrals (β_{rs})according to eqn. (4):

$$\beta_{rs}' = \beta_{rs}(1 + 0.5p_{rs}) \quad (4)$$

The iterations were continued until $|p_m - p_{m-1}| < 4 \times 10^{-4}$ and $|q_m - q_{m-1}| < 2 \times 10^{-4}$, where p_m and q_m stand for the bond order and charge in the m-th iteration. The solution of the final secular determinant yielded the energies of the molecular orbitals, and hence the total energy of the quinone (E_Q) and the hydroquinone (E_{H_2Q}) form.The delocalisation energy change is defined by eqn. (5);

$$\Delta D.\ E. = E_{H_2Q} - E_Q - 2\alpha - 4\beta \quad (5)$$

This theoretical quantity may be used as a measure of the difference in resonance energy between the reduced and the oxidized forms.

The E_0 values listed in Table 2 and 3 were calculated from the energy levels of the undissociated molecules and are, therefore, strictly valid only below pH 4. In more alkaline solutions significant amounts of the pigments (at least of the quinone forms) are present in ionized states; the first dissociation constants of the hydroxytoluquinones range from pK_1 4.8 to pK_1 6.0. The ionisation leads to a decrease in the redox potentials ($E_0 = 0.34$ V for the fumigatin ion).

Electrometric measurements. The pH of the solutions was measured with a calibrated glass-electrode. The corresponding oxidation-reduction potential was determined at 25° with a platinum electrode, using the pH-meter coupled as a potentiometer. In both the cases a calomel electrode was used as the reference.

The reduction potential of fumigatin was determined potentiometrically by titrating a 0.001 M solution of the oxidized form in dilute hydrochloric acid with titanous chloride. The mid-point of the titration curve was obtained graphically. The measurements were performed at pH 1.0, 1.6, and 2.1, yielding closely agreeing E_0 values (0.470, 0.460, and 0.455 V, respectively).

Identification of the pigments in the reduced form. The metabolism solutions from 7 days old cultures of the mould were acidified with conc. hydrochloric acid and extracted with ether. After removal of the ether in vacuum the syrupy residue was chromatographed on thin-layer plates in an atmosphere of nitrogen, with chloroform-methanol-acetic acid [3] as the solvent. All these operations were performed quickly, and as far as possible in the absence of air. In the solvent system used the oxidized form of most of the pigments moves with or near the solvent front, whereas the reduced forms become well separated, yielding R_F values ranging from 0.10 to 0.60. The pigments were detected by exposing the plates to ammonia vapour in the air, by which treatment the hydroquinones were oxidized to the corresponding, strongly coloured, quinones. The identity of the hydroquinones was established by simultaneously running authentic samples, obtained by treatment of the quinones with sodium dithionite, and by two-dimensional chromatography. In the latter case alkaline solvent systems suitable for the quinone forms of the pigments [14] were used for the second dimension. The chromatographic technique could only be used for qualitative analysis of the hydroquinones.

Determination of the total amount of pigments. The culture medium was made slightly alkaline (pH 8.0) by the addition of 0.01 M sodium hydroxide. After a vigorous aeration for 30 sec, giving a quantitative oxidation of the hydroquinones present, the solution was strongly acidified with conc. hydrochloric acid and treated with potassium iodide. By this treatment the toluquinones were reduced, and a corresponding amount of iodine liberated, which was then determined by titration with sodium thiosulphate.

Determination of the amount of quinone forms. Freshly separated metabolism solutions were immediately acidified with conc. hydrochloric acid. The amount of quinones present was then determined titrimetrically, as described above.

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