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Free-radical Mechanisms in the Cytotoxic Action of Antitumour Antibiotics

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The results of studies of the free-radical mechanisms of the action of various antitumour antibiotics are surveyed. The data obtained in the investigation by physicochemical methods (EPR, polarographic, and spectrophotometric) as well as quantum-chemical calculations of the biotransformation processes of antitumour antibiotics, namely the formation of chemically and biologically active free-radical forms of antibiotics, are presented. The ways in which they can be transformed, including the transfer of the electron to oxygen molecules with formation of superoxide radicals, as well as covalent binding to biomacromolecules are discussed. The important role of the acid-base properties of the antibiotics in their free-radical reactions and the characteristics of the biological action of the spin-labelled analogues of anthracycline antibiotics are examined.
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I. INTRODUCTION

The present stage of the development of the experimental chemotherapy of malignant neoplasms, including various aspects of the biochemistry of the cancer cell and the chemical pharmacology of anticancer preparations, is characterised by the wide-scale employment of the ideas and methods of the exact sciences. A combined approach to the solution of the problem of the inter-relation between the structure and biological properties of carcinolytic substances makes it possible to develop the fundamental principles which are essential for the creation of new effective anticancer preparations. An important step in the development of these principles is a new classification of antitumour preparations in terms of the types of their reactivity.[1] This classification is general and, having created the preconditions for a rational planning of the synthesis of anticancer preparations, also permits a more detailed analysis and explanation of the chemical and biological mechanisms of their action. Polyfunctional compounds with hybrid structures and reactivities fit within the framework of this classification. In particular, the majority of antibiotics are preparations of this type.

Antibiotics have been finding in recent years increasingly wide-scale applications in the chemotherapy of malignant diseases. The studies by chemists in this field have been directed towards the synthesis of derivatives of already known antibiotics by their chemical modification and also towards the total synthesis of structural analogues.

The antitumour antibiotics known at present consist of organic compounds of several types from the chemical standpoint, the cytotoxic properties of the majority of which have already been known for a long time. For example, the study of the structure of mitomycin C has demonstrated the presence in its structure of well known cytotoxic groups—benzoquinone, urethane, and aziridine. A whole series of anthracycline antibiotics are characterised by the presence as the aglycone of derivatives of hydroxyanthraquinone, while the carbohydrate fragment consists of aminosugars whose antitumour properties have also been known for a long time. Streptozotocin (a carbohydrate derivative of nitrosomethylurea) was discovered after nitrosocarbamides had been found to possess antitumour properties.

As a consequence of the polyfunctional nature of the antibiotics, studies of the role of various chemical mechanisms

(electrophilic substitution, complex formation, and free-radical reactions) in the manifestation of the biological effect are extremely important.

The oxidation-reduction biotransformation processes involving the majority of antitumour antibiotics, accompanied by the intermediate formation of free radicals, have become the subject of numerous investigations during the last decade. To a large extent, this is associated with the important biological role of the endogenic (including metabolically active) and exogenic free radicals and also with the possibility of their detection in various biological media by electron paramagnetic resonance.

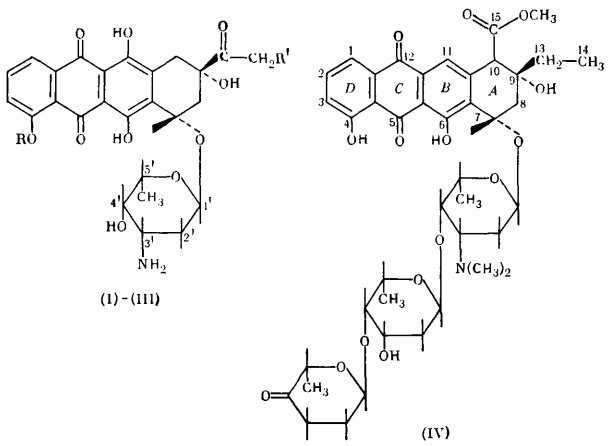
It is noteworthy that ideas concerning the free-radical mechanisms in relation to *in vivo* conditions embrace a wide range of biochemical one-electron transfer processes with participation of paramagnetic species of various types, including free radicals. It is known that, under the influence of many xenobiotics, the content of the radical-ion forms of organic coenzymes and paramagnetic states of metalloprotein molecules increases in organs and tissues and the peroxide oxidation reactions of lipids are activated. Different radical states of xenobiotics, capable of resulting in the formation of active forms of reduced oxygen, are sometimes detected. The action of stable free radicals and other chemical compounds with a paramagnetic centre on living organisms constitues a special case.

Various chemobiological aspects of free-radical mechanisms have also been investigated in connection with the action of antitumour antibiotics. Attention has been concentrated on three types of free-radical species: (1) the radical-ions arising from the antibiotics during their redox reaction; (2) the nitroxy-derivatives of anthracyclines; (3) superoxides. The elucidation of the possible involvement of these radical species in the manifestation of the toxic and antitumour properties of antibiotics is of greatest interest.

II. FREE RADICALS DERIVED FROM ANTITUMOUR ANTIBIOTICS

1. Anthracycline Antibiotics

Antibiotics of the anthracycline series have found wide-scale applications in recent years in the chemotherapy of malignant tumours. The best known representatives of this class of antitumour preparations are adriamycin (I), rubomycin (daunomycin) (II), carminomycin (III), and aclacinomycin A (IV).[2] Despite the large differences in their biological activites, these antiobiotics are close chemical analogues:



(I)-(III)

(IV)

R = CH_3, R' = OH (I); R = CH_3, R' = H (II); R = R' = H (III)

The mechanism of the biological action of antitumour anthracycline antibiotics (AAA) is associated with the suppression of the biosynthesis of nucleic acids and (to a much smaller extent) the biosynthesis of protein. Defects in the structure of chromosomes are then observed in dividing cells (fragmentation of chromosomes and mitotic aberrations, mainly the formation of anaphase bridges). Accordingly, the main morphological changes in the cells are associated with changes in the size and shape of the nucleoli and with the appearance of fine-grain chromatin.[3]

The cytotoxic effect of the AAA depends on their concentration, the duration of their action, and the method by which they are introduced into the organism. Thus it has been shown that their action on tumour cells *in vitro* depends on their extracellular concentration and the time of exposure to these substances[4] and the surviving fraction of tumour cells N is described by the relation

$$N = N_0 e^{-ktc},$$

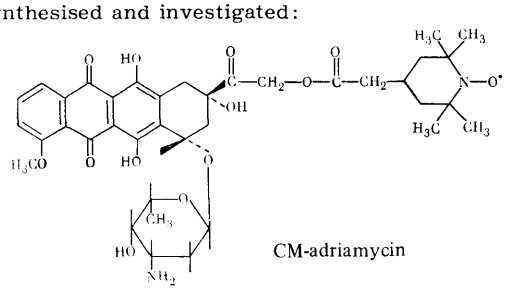
where t is the duration of exposure (min) and c the extracellular concentration of the AAA (μg mol^{-1}). The constant k (mol μg^{-1} min^{-1}) characterises the sensitivity of particular tumour cells to the given preparation. k = 0.045 mol μg^{-1} min^{-1} for the fibroblasts of the Chinese hamster and k = 0.050 mol μg^{-1} min^{-1} for *HeLa* cells. There is a direct correlation between the extracellular and intracellular AAA concentrations. However, there is no proportionality between the duration of exposure and the intracellular concentrations of the preparations.[4]

The differences in the sensitivities to the action of the AAA are also associated with the processes involving their accumulation in the cells. Thus the preparation penetrates readily into L-1210/R leukaemia cells, which are resistant to the action of rubomycin, but it is rapidly metabolised within them; on the other hand, in the L-1210 and P-388 cells, which are sensitive to the preparation, rubomycin persists in the course of 24 h. In chemotherapy the method of introduction and the pharmacokinetic behaviour of the AAA play an important role. The maximum chemotherapeutic effect is attained when the AAA is introduced by methods which ensure direct contact between the preparation and the tumour cells. This fact, which is fairly self-evident from general considerations, has been demonstrated[5] in relation to the B-16 melanoma, which had been grafted intra-abdominally or subcutaneously. A much greater amount of adriamycin penetrates tumour cells and is contained within them for the intra-abdominal growth of the tumour and the intra-abdominal introduction of the preparation.

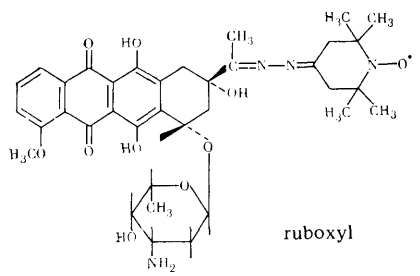
Many AAA derivatives and complexes based on them, differing in their pharmacokinetic behaviour in the organism, have been synthesised in order to increase the efficiency of the chemotherapeutic action. The following complexes have been obtained and investigated: AAA–DNA,[6] AAA–dextran,[7] AAA–aminoacids,[8] AAA-3 × Fe^{3+},[9] AAA incorporated in liposomes,[10] as well as complexes of adriamycin with antibodies,[11] which were distinguished by excellent antitumour properties and a reduced cardiotoxicity.

Among the numerous studies of the chemical modification of the AAA, it is possible to isolate researches[12–14] into the synthesis of nitroxy-derivatives of the AAA which have priority. Such modification of the structure of anthracyclines imparts to them the properties of organic paramagnetic materials where the presence of the free-radical centre alters significantly the oxidation-reduction properties of the preparations and their capacity for involvement in reactions with transfer of one electron. Spin-labelled derivatives of adriamycin (the nitroxy-fragment is linked to the aglycone by an ester bond) and rubomycin (the nitroxy-

fragment is linked to the aglycone via an azine bridge) have been synthesised and investigated:



CM-adriamycin



ruboxyl

The introduction into the antibiotic molecule of fragments containing a free-radical centre entails an appreciable decrease of acute toxicity and an increase of the antitumour activity of the preparations (Table 1). The characteristics of the reduction of nitroxy-fragments and the pharmacokinetic features of the modified AAA have been studied by EPR. The possible chemical causes of the differences in the pharmacokinetic behaviour of nitroxy-derivatives of the AAA can be associated with the differences in the rates and mode of enzymic reduction. Among several possible reduction centres (quinonoid, ketone, and nitroxy-groups), the free-radical fragment undergoes preferential reduction.

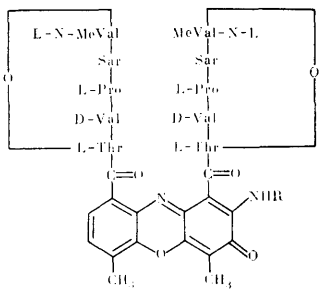
Table 1. Comparative data for the acute toxicities and antileukaemic activities of ruboxyl and rubomycin.[14]

Preparation	LD_{50}, mg kg^{-1}	ILS, %	
		P-388 leukaemia	La leukaemia
Ruboxyl	44.0	155	243
Rubomycin	5.6	68	63

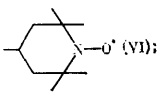
Notation: LD_{50} is a single dose of the preparation inducing the death of 50% of the experimental animals (mice) and ILS is the increase of the average lifespan compared with the control.

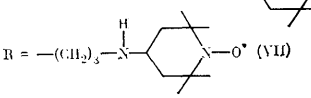
The interaction of a spin-labelled analogue of adriamycin (CM-adriamycin) with DNA has been studied by EPR.[13] In the first place one should note the resistance of the ester linkage in CM-adriamycin to hydrolysis when it interacts with DNA. At neutral pH the EPR spectra of the complex of CM-adriamycin with DNA remain unchanged in the course of several days, which indicates the absence of the hydrolytic scission of the nitroxy-fragment. The form of the EPR spectra of CM-adriamycin does not depend on its relative concentration under conditions where there are between one and four antibiotic molecules for 20 pairs of DNA bases. The subsequent increase of the CM-adriamycin concentration results in the appearance in the high-field region of the EPR spectrum of a narrow line characteristic of CM-adriamycin in solution (Fig.1). Analysis of the EPR spectra established that under these conditions CM-adriamycin forms a complex with DNA as a result of intercalation between the nitrogen-containing bases.[13]

An analogous mechanism of the interaction with DNA is characteristic also of certain other polycyclic compounds, for example, the antibiotics of the actinomycin series. The spin-labelled analogues of actinomycin D have been studied:[15]



(V)-(VII)

R = H (V), actinomycin D; R =  (VI);

R =  (VII)

It is of interest that the nitroxy-derivatives of actinomycin D were bound to DNA to a lesser extent than the initial antibiotic and caused a lower degree of inhibition of the DNA-dependent RNA-polymerase. On the other hand, in experiments on P-388 leukaemia the spin-labelled analogue of actinomycin D showed an antitumour effect which was better by a factor of 1.5.[15] This example indicates the absence of a relation between the degree of intercalation and the antitumour activity of antibiotic derivatives containing a free-radical fragment.

A comparative study of adriamycin, carminomycin, and the 11-methyl ether of carminomycin on the Novikov ascites hepatoma *in vitro* showed that their cytotoxic effects could not be accounted for by their capacity to inhibit the biosynthesis of nucleic acids or to bind on to DNA molecules.[16] Nevertheless the different types of the biological action of anthracyclines (cytotoxic mutagenic, and carcinogenic effects) are explained mainly by the capacity of AAA for intercalation between the DNA bases.

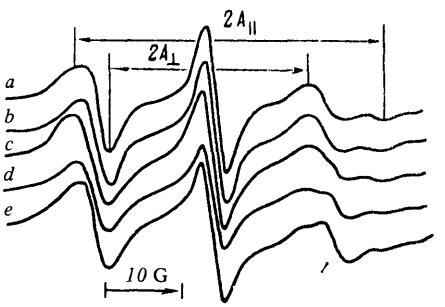
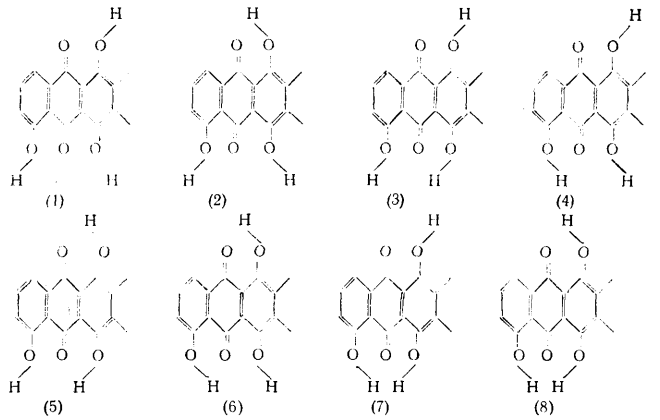


Figure 1. Variation of the EPR spectra of spin-labelled adriamycin in the presence of DNA with its concentration (0.01 M NaCl and 1.2 mg ml^{-1} DNA). Ratio of the antibiotic and DNA (base pairs): *a*) 1:20; *b*) 2:20; *c*) 4:20; *d*) 7:20; *e*) 10:20.[13]

An alternative point of view on the molecular mechanisms of the biological action of AAA, involving the formation of free-radical intermediates, attracts attention in this connection. The characteristics of the chemotherapeutic properties of anthracyclines can be accounted for by the different capacities for donor–acceptor interactions, different permeabilities, and different oxidation-reduction properties of the preparations. The attempts at a rigorous quantitative description of the individual properties of AAA and to establish correlations between the structure and the biological activity of the test substances have now acquired fundamental importance.

Quantum-chemical calculations for the model analogues of the AAA aglycones and the corresponding radical-anions for isolated molecules and their hydrate complexes have demonstrated the necessity to take into account the influence of the solvent on their electronic and conformational properties, since solvation effects can lead to significant conformational rearrangements. The semi-empirical INDO and CNDO/2 methods provide a fairly satisfactory qualitative description of the state of the system, although they yield less realistic solvation energies and hydrogen bond strengths than *ab initio* methods. Calculations of the structures of hydrate complexes incorporating four water molecules have been carried out taking into account the formation of hydrogen bonds involving the oxygen atoms of the anthraquinone fragment.[17]



It has been found that, in terms of the isolated molecule approximation, the formation of intramolecular hydrogen bonds is favoured by energy factors and the appearance of each succeeding bond causes a proportional reduction in the total energy of the system. It has been demonstrated in relation to hydrate complexes that the formation of intermolecular hydrogen bonds is easier than that of the intramolecular hydrogen bonds of hydroxyanthraquinone (Table 2).[17]

Table 2. Calculated formation energies of the intramolecular hydrogen bonds ($\Delta E_b = E_i - E_1$) in isolated molecules and the solvation energies of the tetrahydrate complexes of carminomycin for different conformations of the hydroxy-groups of the anthraquinone ring (ΔE_h).[17]

Structure	ΔE_b, kcal mole^{-1}	ΔE_h, kcal mol^{-1}	Structure	ΔE_b, kcal mole^{-1}	ΔE_h, kcal mol^{-1}
(1)	0	59.8	(5)	15.5	36.2
(2)	7.6	47.2	(6)	17.1	33.2
(3)	8.1	49.0	(7)	17.3	42.5
(4)	9.5	46.4	(8)	24.9	28.2

The distribution of charges and spin densities in the corresponding radical-anions has been investigated taking into account the structure of the isolated neutral molecules and the hydration effects of aglycones. The formation of radical-anions entails the rearrangement of the atomic structure in the chromophoric part of the AAA. The main electronic charge is localised under these conditions at the oxygen atoms of the carbonyl groups: $\Delta q_5 = q_5^a - q_5^n = 8.25 \times 10^{-2}$e and $\Delta q_{12} = 7.41 \times 10^{-2}$e. The unpaired electron is in the lowest unoccupied π_z orbital of the anthraquinone ring, while the spin densities have the maximum value at the oxygen atoms of the carbonyl groups ($\rho^5_{\pi z} = 0.489$; $\rho^{12}_{\pi z} = 0.443$) and at the carbon atoms of the carbonyl groups ($\rho^5_{\pi z} = 0.107$; $\rho^{12}_{\pi z} = 0.128$).

The theoretical spin densities at the hydrogen atoms of the carminomycin aglycone (with and without allowance for hydration) are presented in Table 3. Analysis of these data shows that the experimental splittings in the EPR spectrum ($a_H = 4.1$ G and $a_H = 2.1$ G) can be explained by the interaction of the unpaired electron with the protons of the *A* ring. The HFS constants can be determined from the formula $a(G) = K\rho^S$, where ρ^S is the spin density in the *s* orbital of the proton and *K* a constant equal to 539.86 for hydrogen atoms.[18] The theoretical spin density distributions for the aglycones of other AAA differ little from the data for carminomycin. The splittings by the protons of the *A* ring differ somewhat for the two possible conformations, namely chair and half-chair, but a choice in favour of the existence of a particular conformation in solution cannot be made on the basis of the calculated values.

Table 3. The spin densities in the *s* orbitals of the hydrogen atoms ($10^2\rho^S$) in the radical-anions of carminomycin aglycones calculated for the conformation of the hydroxy-group corresponding to structure (I) for different conformations of the *A* ring [*a*) without allowance for the hydration of anthraquinone; *b*) with allowance for the hydration of anthraquinone[17]].

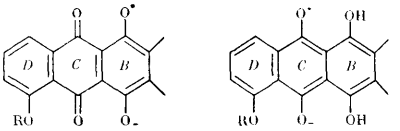
H atom	Chair		Half-chair	
	a	*b*	*a*	*b*
H(1)	0.01	−0.03	0.02	−0.03
H(2)	−0.01	−0.01	−0.02	−0.02
H(3)	−0.10	−0.11	−0.10	−0.11
H(4)	0.01	0.01	0.00	0.01
H(6)	0.01	0.01	0.00	0.00
H(7)^{OH}	0.53	0.50	0.47	0.38
H(7)	−0.09	−0.09	−0.46	−0.44
H(8)	−0.03	−0.03	−0.02	−0.02
H(8)	−0.05	−0.04	−0.01	−0.01
H(10)	0.46	0.41	0.52	0.27
H(10)^a	0.91	0.80	0.74	0.77

Notation: H(7)^{OH} is the hydrogen atom of the hydroxy-group in the 7-position in the aglycone and H(10)^a is the axial hydrogen atom in the 10-position.

An important result of the calculations is the conclusion that the C(7)–O(7) bond is weakened on formation of the radical-anions, which follows from the analysis of Wiberg's indices. Such weakening of the bond, deduced from theoretical calculations for model compounds, shows that the reduction of the AAA can entail the scission of a hydrocarbon fragment (Table 4).[17] Indeed this has been confirmed experimentally. For example, it has been shown[19] that, in the enzymic reduction of rubomycin under the conditions of hypoxia, the consumption of the antibiotic is

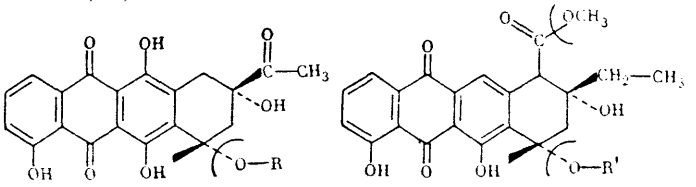
accompanied by an increase of the content of the free aglycone in the system (Fig.2). Anthracycline antibiotics are gradually converted in the cell into deoxyaglycones, which are more lipophilic and possibly more strongly bound to the membranes of microsomes. According to a quantum-chemical calculation, the reduction of aclacinomycin A can be accompanied by the elimination of the 15-methoxy-group as well as the hydrocarbon fragment (Table 6).

These results indicate the potentially important role of the oxidation-reduction reactions with the intermediate formation of free-radical states. One might have expected the formation of free radicals from AAA both as a result of the oxidation of the hydroquinone group (the *B* ring) and as a result of the reduction of anthraquinone (the *C* ring).



The free-radical intermediates formed under these conditions are characterised by different reactivities, which may be the reason for the selectivity of the biological action of AAA, in particular it may determine the ratio of the degree of toxicity to the antitumour effect.

Table 4. The Wiberg bond orders in the neutral molecules and radical-anions of carminomycin (III) and aclacinomycin A (IV).[17]



(III) (IV)

State	(III)		(IV)				
	C(7)—O(7)	O(7)—R	C(7)—O(7)	O(7)—R′	C(10)–C(15)	C(15)=O	C(15)–O
Neutral molecule	0.4620	0.4890	0.4790	0.4790	0.4866	0.9274	0.4849
Radical-anion	0.4522	0.4950	0.4631	0.4793	0.4891	0.9319	0.4740

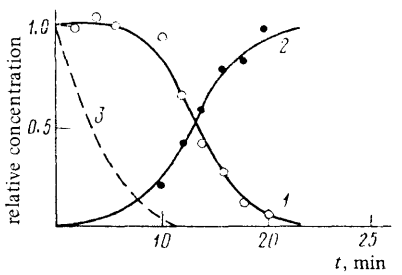


Figure 2. Kinetic curves for the variation of the contents of rubomycin (curve *1*) and its aglycone (curve *2*) and of the partial pressure of oxygen (curve *3*) in the microsomal system in the presence of NADPH (according to the data of Bachur et al.[29]

The capacity of the AAA aglycones for reduction and oxidation has been estimated on the basis of the calculation of the energies of the lowest unoccupied molecular orbital (E_{LUMO}) and the highest occupied molecular orbital (E_{HOMO}).[20] The values of E_{LUMO} and E_{HOMO} for carminomycin, rubomycin, adriamycin, and aclacinomycin A and their O_6^- anions are presented in Table 5. Evidently in the neutral state all the aglycones have similar values of both E_{LUMO} and E_{HOMO}. Consequently the anthracycline antibiotics examined above have approximately the same electron-accepting properties. The extremely high absolute values of E_{HOMO} show that in the neutral state all the aglycones are fairly resistant to oxidation. On the other hand, the AAA anions, characterised by much lower values of E_{HOMO}, exhibit an increased capacity for oxidation. It follows from Table 7 that the aclacinomycin A anion showed the lowest capacity for oxidation, which was in fact to be expected in view of the absence of the hydroquinone fragment from its structure. The most pronounced electron-donating properties are characteristic of the carminomycin aglycone anion.

Table 5. The energies of the HOMO and the LUMO for the neutral molecules and anions in the series of anthracycline antibiotics and their toxicity on intra-abdominal administration.[20,21]

Parameter	Carminomycin	Rubomycin	Adriamycin	Aclacinomycin A
E^n_{HOMO}, eV	−10.306	−10.288	−10.217	−10.342
E^a_{HOMO}, eV	−3.203	−3.209	−3.288	−3.670
E^n_{LUMO}, eV	0.690	0.690	0.719	0.633
E^a_{LUMO}, eV	4.680	4.678	4.810	3.695
LD_{50}, mg kg^{-1}	1.3	5.5	8.5	25.0
pK_a	8.4	9.6	9.6	9.0

Notation: E^n_{HOMO} and E^n_{LUMO} are the energies of the HOMO and the LUMO of the neutral molecules of the AAA aglycones and E^a_{HOMO} and E^a_{LUMO} are the energies of the HOMO and LUMO of the anions of the AAA aglycones.

Table 6. Covalent binding of [^{14}C]adriamycin labelled in the 14-position to microsomal proteins.[13]

Experimental conditions	α, %
Denatured microsomes + adriamycin + NADPH	1.1
Microsomes + adriamycin	0.8
Microsomes + adriamycin + NADPH	4.4
Microsomes + adriamycin + SOD	1.0
Microsomes + adriamycin + NADPH + SOD	4.5

Notation: SOD is superoxide dismutase and α is the fraction of the bound radioactive label.

The oxidation-reduction properties of the AAA at physiological pH can be influenced by the acid–base characteristics of the aglycones. The study of the electronic absorption spectra of the antibiotics in the visible region at different pH of the medium demonstrated the presence of isosbestic points (Fig.3), which indicates the existence of equilibrium

in alkaline solutions between the neutral and ionised forms of the antibiotics. It follows from the data presented in Table 5 that at physiological pH the molecules of the anthracycline antibiotics are in a partly ionised state.

The formation of the ionised form of the AAA is a fairly complex process owing to their polyfunctional character. Apart from the aglycone hydroxy-groups, capable of dissociation, the nucleophilic centres of the molecules, responsible for the possibility of association, also participate in the process. For example, the formation of dimers in aqueous rubomycin solutions (5×10^{-8} M) has been demonstrated spectrophotometrically and by the NMR and circular dichroism methods.[22] At a constant pH a decrease of the degree of dissociation is therefore observed with increase of the AAA concentration.

Table 7. The rate (w) of the microsomal oxidation of NADPH stimulated by various antitumour preparations.[26]

Preparation	w, nmol/mg protein per min	
	air	CO
Control	0	0
Adriamycin	73	84
Mitomycin C	84	81
Bruneomycin	249	209
Lapachol	15	11
Cyclophosphane	0	0
Methotrexate	0	0
N,N'-Di(chloroethyl)-N-nitrosourea	0	0

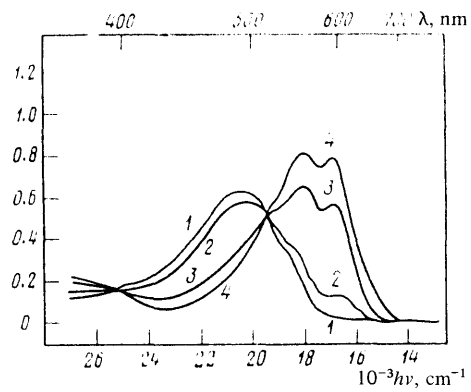


Figure 3. Optical absorption spectra of rubomycin at different pH of the medium: 1) 6.8; 2) 9.2; 3) 10.5; 4) 13.[20]

For a comparative study of the electron-donating properties of the AAA, the reaction involving the oxidation by potassium hexacyanoferrate(III) has been investigated.[20] The dependence of the effective rate constants k_{eff} for their oxidation reaction on the oxidant concentration and the pH of the medium was studied. Fig.4 illustrates the variation of k_{eff} with the oxidation of carminomycin, rubomycin, and adriamycin as a function of the hexacyanoferrate(III) concentration at pH 7.6. Evidently the replacement of the hydrogen atom in the 14-position by the hydroxy-group does not influence the electron-donating properties of the antibiotics. On the other hand, the replacement of the 4-methoxy-fragment by the hydroxy-group decreases sharply the pK_a of the aglycone and enhances its electron-donating properties. The oxidation rate constant depends markedly on the pH of the medium. Thus, in the oxidation of rubomycin and carminomycin (5×10^{-5} M) with potassium hexacyanoferrate (III) (5×10^{-3} M), it was found that k_{eff} is 0.3×10^{-2} s^{-1} and 0.7×10^{-2} s^{-1} respectively at pH 7.0, while 1.3×10^{-2} s^{-1} at pH 8.0 was obtained. The oxidation of the relatively non-toxic aclacinomycin A by the hexacyanoferrate(III) was not observed even at pH 9.5.

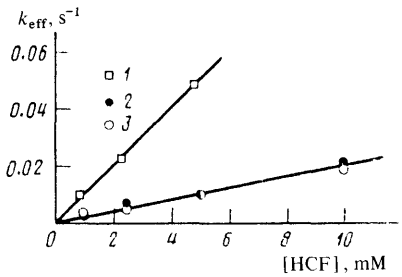


Figure 4. Dependence of the rate constants for the oxidation of different antibiotics on the concentration of hexacyanoferrate(III) (HCF) (pH 7.6, T = 25 °C):[20] 1) carminomycin; 2) rubomycin; 3) adriamycin.

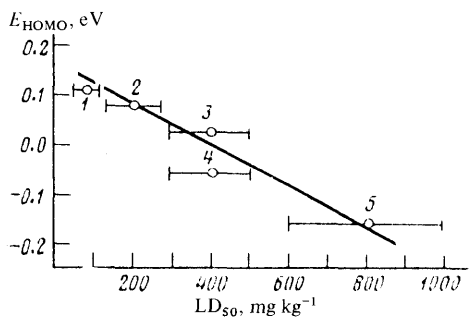


Figure 5. Correlation between the energies of the highest occupied molecular orbitals of the reduced forms (R^-) of derivatives of 2,2,6,6-tetramethyl-4-X-piperidine N-oxides [X: 1) $-(CH_2)_2NH_2$); 2) $-OC(O)NHPh$; 3) $-OH$; 4) $-NHC(O)-NCH_2CH_2$; 5) $=O$] and acute toxicity (LD_{50}).[24]

Thus the electron-donating properties of the AAA are enhanced in the sequence aclacinomycin A < adriamycin ≅ rubomycin < carminomycin; their acute toxicity increases at the same time (LD_{50}) diminishes. When account is taken of these results, the diminution of the cytotoxic properties of the 11-methyl ether of carminomycin can be accounted for by the modification of the structure accompanied by a decrease of the capacity for oxidation (there is no hydroquinone fragment in the structure).

A statistically reliable correlation between the LD_{50} for a series of 4-substituted derivatives of the 2,2,6,6-tetramethylpiperidin-1-yloxy-radical and the E_{HOMO} of the corresponding anions is noteworthy in this connection (Fig.5).[24] The linear regression equation has the following form with a reliability of 95%:

$$LD_{50} = aE_{HOMO} + b,$$

where $a = -2352 \pm 1208$ and $b = 371 \pm 119$. This result shows that the processes involving the oxidation of the reduced forms of the nitroxy-radicals are responsible for their toxicity and not reactions involving the endogenic reduction.

The above correlation between the capacity for oxidation and the toxic properties of antitumour preparations must be taken into account in their chemical modification and in the development of combined polychemotherapy. However, at the present time the formation of free radicals from anthracycline antibiotics has been studied mainly under the conditions of their reduction.

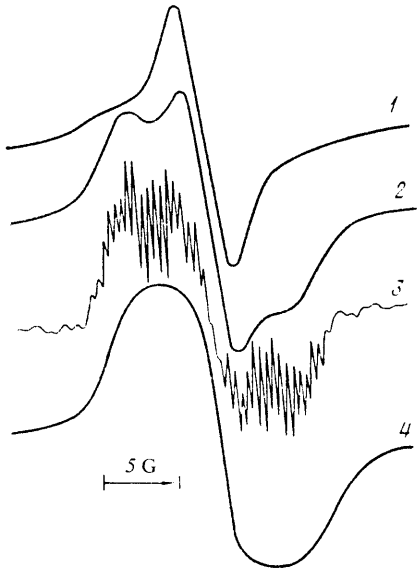


Figure 6. Changes in the EPR spectrum of the rubomycin semiquinone radicals obtained on reduction with KBH_4 after different periods (min): 1) 2; 2) 5; 3) 6; 4) 30.[17]

On reduction of rubomycin in water with potassium tetrahydroborate, an intense signal ($g = 2.004$ and $\Delta H = 4$ G) due to the primary semiquinone radical appears in the EPR spectrum during the first two minutes. After only three minutes, another signal ($g = 2.004$, $\Delta H = 12$ G) with a characteristic hyperfine structure appears against the background of the first. After a further few minutes, both signals become broadened and are recorded as one singlet with a width of 9 G (Fig.6). At the same time a precipitate is formed in the reaction mixture, which may be associated with the elimination of the carbohydrate fragment and the formation of the radical forms of the aglycones, which are sparingly soluble in water. On reduction of the AAA in methanol, stable radicals with a well resolved hyperfine structure are detected.[17]

The spontaneous formation of semiquinone AAA radicals has also been observed at physiological pH values. In this case the rate of formation of the radicals is independent of the oxygen content, but the semiquinone radicals produced are destroyed by oxygen atoms. The rate of formation of the adriamycin semiquinone radicals (20 mg ml^{-1}) varied from 7.0 nmol min^{-1} at pH 7.65 to 48.0 nmol min^{-1} at pH 8.85.[25]

The formation of free radicals has also been demonstrated on enzymic reduction of AAA.[26-30] Semiquinone radicals are detected by EPR when AAA are incubated with cells of the Ehrlich ascites carcinoma[26,27] and also with the microsomal or nuclear fraction of rat liver cells under the conditions of hypoxia in the presence of NADPH.[25,28-30] Electron transfer from the AAA to NADPH is catalysed by one-electron transfer flavoproteins such as NADPH-cytochrome P-450 reductase, nitrate reductase, and NADPH-cytochrome *c* reductase, which indicates the absence of specificity in the reduction of the quinonoid group in the AAA. Reductases of various types are capable of reducing the oxo-group of rubomycin in the 13-position with formation of 13-dihydrorubomycin (daunomycinol). Adriamycin and carminomycin undergo analogous reactions, but are transformed much more slowly. The reduction of the oxo-group can be catalysed by the aldoketoreductase from rat kidneys and liver in the presence of NADPH.[31] On treatment with reductases, the sugar fragment can be split off with formation of free aglycones, but the reduced aglycones are 100 times less active than the initial AAA.[5] This is apparently associated with the lower solubility of the AAA aglycones and the consequent pharmacokinetic characteristics.

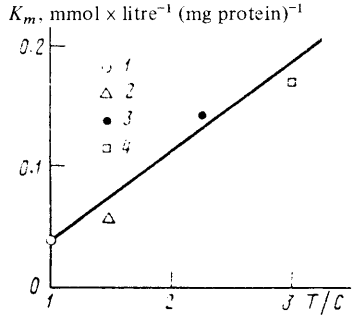


Figure 7. Relation between the antitumour effectiveness (T/C) and the Michaelis constants K_m in the enzymic reduction of rubomycin: 1) L-1210/R; 2) L-1210; 3) P-388; 4) B-16 (T is the lifespan of the animals in the experimental group following the administration of the rubomycin and C the lifespan of control animals) (according to the data of Yesaik et al[5]).

The sensitivity of tumour cells to the AAA depends on the presence in the former of enzyme systems capable of activating and metabolising the AAA. Thus rubomycin is reduced to daunomycinol most vigorously in the cells of the B-16 melanoma—the tumour which is most sensitive to the action of the AAA. This indicates the importance of the enzyme activity of rubomycin in tumours and the possible role of daunomycinol in the manifestation of the antitumour effect (Fig.7). In this connection one can also note that the local

hyperthermia of the tumours increases the effectiveness of adriamycin against the background of the increased intensity of its metabolism.[32]

Two-electron transfer enzymes such as glutathione reductase and lactate dehydrogenase are not involved in the formation of radicals.[30] The kinetics of the reduction of quinones by flavoproteins depend both on the redox potentials of the quinones and on the enzymes catalysing their reduction. Thus the lower limit of the redox potential for the reduction of quinones by the NADPH-cytochrome P-450 reductase is -240 mV, while for NADPH-cytochrome P_5 reductase and NADH-ubiquinone oxidoreductase the limit is -170 mV. Lipophilic properties apparently play a small role in the reduction of these preparations by enzymes.[33]

The EPR spectrum of the semiquinone metabolites of anthracyclines in microsomes does not appear immediately, arising after the consumption of the oxygen dissolved in them and is then observed over a period of several hours.[27] During the incubation of the antibiotics with microsomes, the partial pressure of oxygen diminishes owing to electron transfer from the semiquinone species to oxygen.[26,29,30,34,35] So long as oxygen is present in the microsomes, the signal of the semiquinone radicals is not observed in the EPR spectrum of the antibiotic. As the partial pressure of oxygen diminishes, the EPR signal referring to the semiquinone radical of the anthracycline begins to be detected. The initially symmetrical signal with $g = 2.0036$ then becomes asymmetric and after several hours the spectrum assumes the character of a strongly delayed axially symmetrical signal with $g = 2.0046$ and $g = 2.0021$.[27]

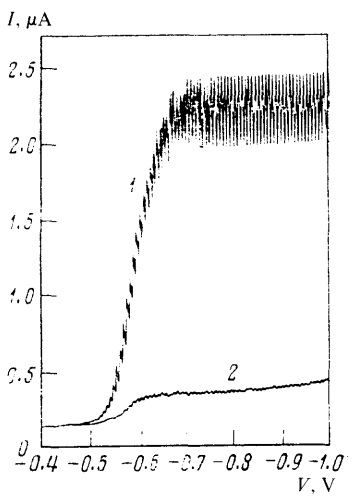


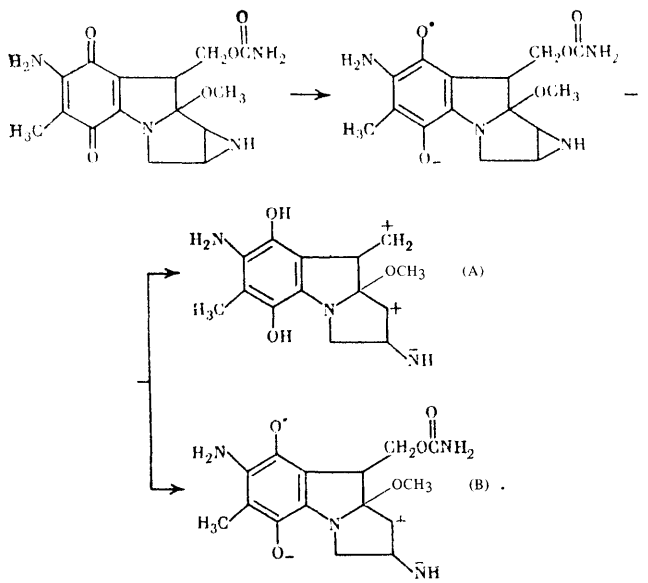
Figure 8. Polarographic reduction wave: 1) rubomycin; 2) rubomycin + DNA (molar ratio DNA : rubomycin = 8.7 : 1).[3]

The anthracycline antibiotics intercalated between the DNA bases are poorly reduced to semiquinone radicals, which agrees with the absence of a polarographic reduction wave of rubomycin after the latter has been incubated with DNA[3] (Fig.8). Antitumour anthracycline antibiotics linked to DNA are poorly reduced by microsomes following the addition of NADPH, so that only a weak signal due to semiquinone radicals is then detected by EPR.[27] All this shows that the AAA intercalated in DNA are "poor" substrates for enzymic reduction.

On chemical and biological activation, AAA molecules are capable of binding covalently to biomacromolecules, which is due to the intermediate formation of semiquinone radicals.[36] The degree of covalent binding of rubomycin to DNA under ultraviolet irradiation increases by a factor of 3 in the presence of oxygen.[36] Covalent binding of adriamycin to microsomal proteins takes place only in the presence of NADPH, which ensures the enzymic reduction of the preparation to a semiquinone. The addition of superoxide dismutase did not affect the binding of adriamycin to microsomal proteins, which rules out the involvement of the superoxide radicals in the process (Table 6).[37]

The generation of semiquinone radicals investigated in relation to anthracyclines can probably be important also for other antibiotics containing quinonoid (or analogous) fragments in their structure. Preparations, in the mechanism of the biological action of which processes involving the intermediate formation of free radicals, can play an important role, are considered below.

Mitomycin C. In 1956 an involved set of substances with antibiotic properties which effectively inhibited the growth of the Ehrlich ascites tumour were isolated from the culture medium of *Streptomyces caspitosus*. Later an individual compound, called mitomycin C, was isolated from this mixture. A rapidly acting bactericidal and cytotoxic antibiotic, mitomycin C is used in the treatment of the cancer of the stomach, the mammary gland, and the pancreas.[38] The possible pathways leading to the chemical activation of mitomycin C, including the formation of carbonium ions and semiquinone radicals, indicate a polyfunctional character of this antibiotic:



The cytotoxic activity of mitomycin C can be accounted for by its capacity both to alkylate monofunctionally the DNA bases (probably as a result of the opening of the aziridine ring) and its capacity to form cross-links between the complementary DNA chains[39-41] in consequence of the bifunctional nature of the mitomycin C intermediates. It has been shown that the DNA incubated with the antibiotic for 1 min

is characterised by the presence of covalent bonds (cross-links) between the complementary chains, which can be readily observed on the basis of the reversibility of its denaturation process.

One of the possible mechanisms of this process involves the formation of intermediates of type (A) with two electrophilic centres arising from the urethane and aziridine groups of mitomycin C. Another pathway involves the formation of the free-radical intermediate (B), which can take place in several stages. The most probable (by a factor of 10–20) monoalkylation process occurs initially and is followed by the generation of semiquinone radicals. This is indicated by the finding that the mitomycin C bound covalently to DNA is also capable of being reduced to a semiquinone radical, whose interaction with oxygen leads to the formation of superoxide radicals.[39] On chemical or enzymic reduction, mitomycin C is converted into a highly reactive bifunctional agent forming covalent bonds with nucleic acids, proteins, and other molecules in the damaged cells. The bifunctionality can therefore be a consequence of the formation from the preparation of semiquinone radicals containing an electrophilic centre consisting of the opened aziridine ring (B). The reduced mitomycin also exhibits a high reactivity and is readily bound to DNA *in vitro*, forming cross-links in the latter. The active form (B) of mitomycin C is apparently extremely unstable since the cross-links arise in DNA only if DNA is added a few seconds after the reduction of the antibiotic. Reduced mitomycin is bound initially to the guanine residues in DNA but can also readily interact with RNA and cell proteins.

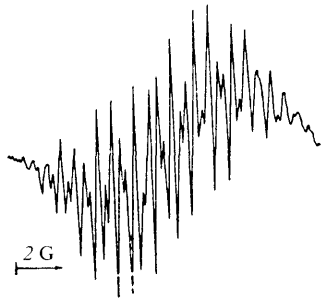
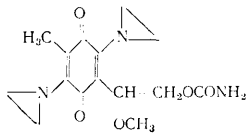


Figure 9. EPR spectrum of the semiquinone radical of mitomycin C reduced with $NaBH_4$ in phosphate buffer at pH 7.0.[39]

The reduction of a solution of mitomycin C in dimethyl sulphoxide (DMSO) by sodium tetrahydroborate leads to the formation of a stable semiquinone radical, whose EPR spectrum contains 36 lines with the splittings $a_{NH_2} = 2.3$ G, $a^N_{ring} = 1.7$ G, $a^H_{CH_3} = 1.0$ G, and $a^H_{CH_2} = 0.65$ G with $g = 2.0046$ (Fig.9).[39] The observation and detection of the semiquinone radicals of mitomycin C in an aqueous medium is complicated by their comparatively short life. When the preparation was incubated with microsomes in the absence of oxygen, the EPR signal of the semiquinone radical of mitomycin C with $g = 2.0035$ and $\Delta H = 9$ g, vanishing in the course of 4 min, was recorded. The signal appears only in the joint presence of mitomycin C, microsomes, and the NADPH-generating system (0.39×10^{-3} M NADH, 5.5×10^{-3} M glucose-6-phosphate, and 0.67 unit ml^{-1} of glucose-6-phosphate dehydrogenase). In terms of its spectroscopic characteristics ($g = 2.0034$), it is identical with the signal recorded on chemical reduction of mitomycin C in an aqueous medium.[27]

It must be emphasised that mitomycin C bound covalently to poly G, poly C also gave rise to a spectrum characteristic of the semiquinone radical following its reduction with $NaBH_4$.[39]

Carboquone, used in the treatment of leukaemia, is a close structural analogue of mitomycin C.



Carboquone has two polarographic reduction waves at −0.23 and −1.6 V relative to the saturated calomel electrode. In liver microsomes in the presence of NADPH carboquone is reduced to a semiquinone radical; its EPR spectrum is characterised by well-resolved hyperfine structures (Fig.10), which can be accounted for by the interaction of the unpaired electron with the three equivalent protons of the methyl group in the 6-position ($a^H_{CH_3} = 0.55$ G) and with the two nuclei of the nitrogen in the aziridine groups in the 2- and 5-positions ($a^N_{N(2)} = 2.78$ G; $a^N_{N(5)} = 3.03$ G). After the incubation of carboquone with Ehrlich ascites tumour cells, the formation of carboquone radicals is also detected. The addition of DNA or glucose under these conditions does not influence the radical formation process.[28]

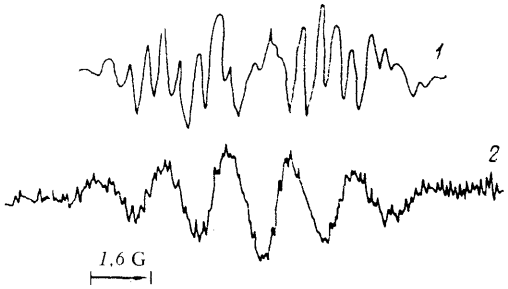
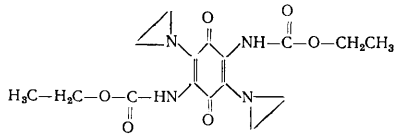


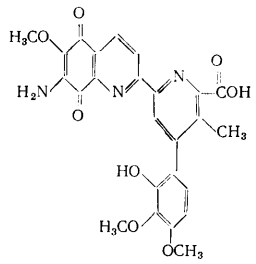
Figure 10. EPR spectra for the semiquinone radicals of carboquone (curve *1*)[28] and AZQ (curve *2*)[42] obtained on microsomal reduction in the presence of NADPH.

Another structural analogue of mitomycin C is the quinone-containing alkylating agent AZQ, which can also be reduced in microsomes to semiquinone radicals:[42]



Bruneomycin. Bruneomycin has been obtained in the USSR from a culture of *Streptomyces albus var bruneomicini* and is identical with the foreign compounds streptonigrin

and rubochromomycin. It is used in the treatment of lymphogranulomatosis, chronic lympholeukaemia, reticulosis and certain other systemic diseases of the blood as well as the kidney adenosarcoma (Wilm's tumour) and neuroblastoma, which are resistant to other antitumour preparations.[43] Bruneomycin suppresses the synthesis of DNA in animal and bacterial cell cultures. It remains bound to DNA isolated from ascites tumour cells of mice.



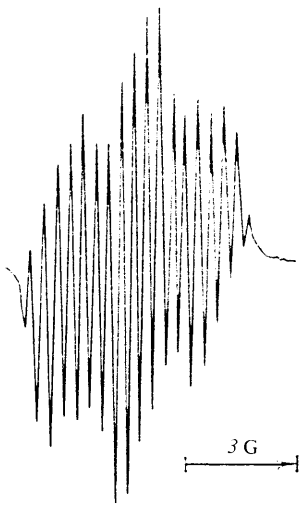
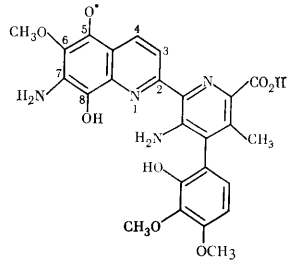


Figure 11. EPR spectrum of the bruneomycin radical (4×10^{-4} M) obtained on reduction with $NaBH_4$ (8×10^{-4} M) in phosphate buffer at pH 7.0.[39]

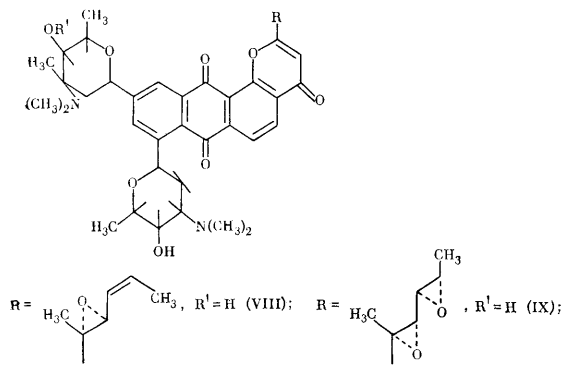
As in the case of mitomycin C, the batericidal action of bruneomycin is accompanied by the degradation of DNA molecules but it does not induce the formation of DNA cross-links.[3] The simultaneous presence of a source of electrons and oxygen is essential for the manifestation of the maximum bactericidal activity.[43] The reduction of bruneomycin by KBH_4 in DMSO in the absence of oxygen led to the formation of a semiquinone radical whose spectrum is characterised by the constants for splitting by the nitrogen atoms in 7- and 1-positions ($a^N_{N(7)} = 0.9$ G and $a^N_{N(1)} = 0.3$ G) and also by the proton in the 4-position (2.1 G) and by the two-equivalent protons of the amino-group (0.3 G).[39]

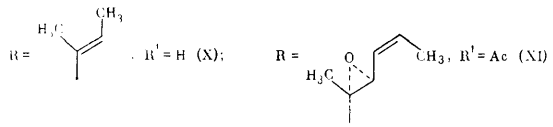


An analogous EPR spectrum has been recorded also on biological reduction of bruneomycin in a suspension of *E.coli* cells, which suggests an important role of free-radical intermediate states in the molecular mechanisms for the biological action of bruneomycin[44] (Fig.11).

The binding of bruneomycin to nucleic acids in the presence of reducing agents and metal ions has been investigated.[45] Incubation of chemically reduced bruneomycin with DNA *in vitro* was accompanied by irreversible binding and led to the formation of a complex containing one mole of bruneomycin for 250 nucleotides. The presence of Zn^{2+} ions greatly increased the degree of binding and the resulting complex contained one molecule of bruneomycin for 80 nucleotides. On the other hand, the presence of Mg^{2+} diminished such binding. A greater amount of the antibiotic was bound to the denatured DNA than to the native DNA. The maximum degree of binding is attained when bruneomycin is reduced in the presence of DNA. A much greater amount of bruneomycin is bound to DNA following its enzymic activation. Studies with synthetic polynucleotides confirmed that in the presence of Zn^{2+}, bruneomycin is capable of binding to both purine and pyrimidine bases.[45]

Rubiflavin and its analogues. The antitumour bactericidal antibiotic rubiflavin (VIII) has been obtained from *Streptomyces* (SC 3728). Rubiflavin analogues such as hedamycin (IX), kidamycin (X), and pluramycin (XI) also possess antitumour properties.[46] Rubiflavin and hedamycin selectively suppress the synthesis of DNA in the cells and affect the synthesis of RNA to a lesser extent. These antibiotics bind strongly (in actual fact irreversibly) to DNA. Both rubiflavin and hedamycin can be reduced by cultures of *E.coli* with formation of free radicals,[44,47] which indicates the importance of the free-radical states of these antibiotics in the manifestation of their biological activity. Fig.12 presents the EPR spectra of the rubiflavin radical-anions obtained on reduction of the preparation in the cells of *E.coli* and on treatment with KBH_4.[44]





Saframycins A and C. The quinone-containing antitumour antibiotics saframycins A and C have been isolated from *Streptomyces lavendulae*[48] (R = H and R′ = CN for saframycin A and R = OCH_3 and R′ = H for saframycin C):

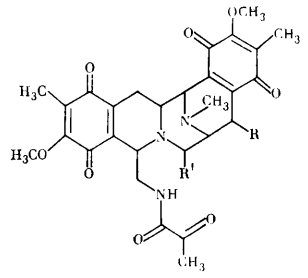




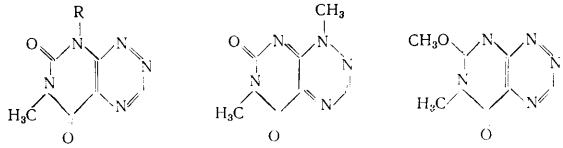
Figure 12. EPR spectra of the rubiflavin radical obtained under different conditions: *a*) on chemical reduction with KBH_4;[44] *b*) on biological reduction in a cell suspension.

Saframycins A and C fully inhibit the growth of L-1210 cells in culture at concentrations of 0.02 and 1.0 μg ml^{-1} respectively. For mice of the C3H/He strain, the LD_{50} of saframycins A and C are 10.5 and 9.7 mg kg^{-1} respectively. The antitumour activity of saframycin A is 50–100 times greater than that of saframycin C. Saframycin A proved to be highly active against P-388 leukaemia and the Ehrlich ascites carcinoma and moderately active against L-1210 leukaemia and B-16 melanoma.[48]

In addition many other natural physiologically active substances contain quinone structures whose action can be associated with the formation of free-radical intermediates. Herbimycin B (XII),[49] javanicin (XIII),[50] the antibiotic WS-5995 A obtained from *Streptomyces auranticolor* (XIV),[51] alkannin (XV),[52] luteoskyrin (XVI),[53] and lapachol (XVII)[54] can be included among preparations of this type.

2. Antibiotics of the Pyrimido [5,6-*e*] -1,2,4-triazine Group

Antibiotics of this series include reumycin, fervenulin, xanthothricin, and 7-methoxyreumycin.



R = H, reumycin
R = CH_3, fervenulin

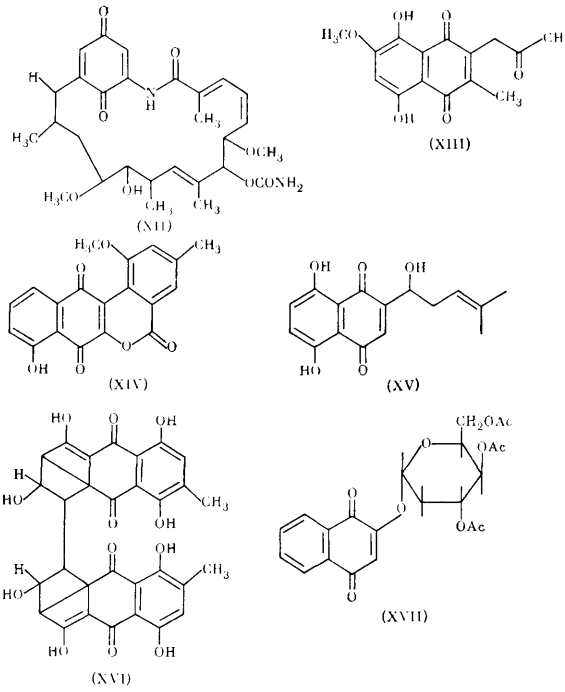
xanthothricin

7-methoxyreumycin

One of the representatives of this group of antibiotics, namely reumycin, has been shown experimentally to exhibit a broad spectrum of antitumour activity.

The molecular mechanisms of the biological action and biotransformations of these compounds can be related to the intermediate formation of active free-radical states. Indeed, the formation of stable radical-anions from pyrimidotriazines during their electrochemical reduction has been demonstrated by EPR.[55] Microsomes, in which the reaction and detoxication systems of the xenobiotics are localised, can be used for the enzymic activation of pyrimidotriazines *in vitro*. It has been shown that the antibiotics of this series are capable of accepting an electron from the NADPH-cytochrome b_5 reductase chain.[56]

The reduction of reumycin in microsomes has been investigated in the presence of NADPH at different pH. When reumycin was reduced in an alkaline medium (the chemical reduction was carried out with potassium tetrahydroborate at pH 9.0, while the microsomal reduction was carried out at pH 8.0), the EPR spectrum consisted of an octet of lines with the constants $a^H = a^N = 5.7$ G for splitting due to the interaction with the three equivalent nitrogen atoms and the hydrogen atom of the triazine ring (Figs.13*a* and 13*b*).



Following the microsomal reduction of the methylated reumycin derivative, an analogous eight-component spectrum was recorded, whence it follows that the electronic structure and the distribution of spin density in the reumycin and 7-methoxyreumycin radical-anions are similar. Since reumycin ($pK_a \approx 6.5$) exists mainly in the anionic form at pH 8, presumably the anion undergoes one-electron reduction in the microsomes to a radical-dianion, which is capable of being rapidly protonated at the O(7) or the N(1) atom. However, one should note that the EPR spectrum of this radical differs from that of the radical-anion obtained on electrochemical reduction of 7-methoxyreumycin in dimethylformamide, which can be attributed to the conditions of its preparation in the aprotic medium.

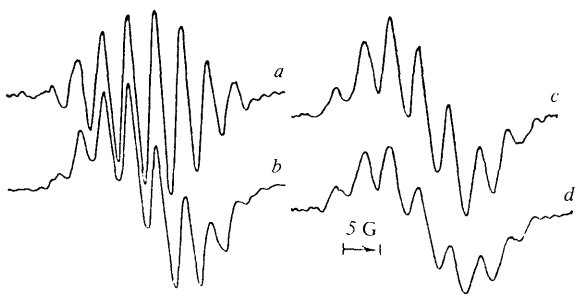


Figure 13. EPR spectra of reumycin radicals obtained under different conditions: *a*) on chemical reduction with KBH_4 at pH 9.0;[35] *b*) on microsomal reduction in the presence of NADPH at pH 8.0; *c*) on chemical reduction with KBH_4 at pH 4.0; *d*) on microsomal reduction in the presence of NADPH at pH 6.0.

In an acid medium, where unionised forms of reumycin are reduced (the chemical reduction is carried out at pH 4.0 and the enzymic reaction at pH 6.0), the character of the measured EPR spectra changes. Thus a seven-component EPR signal with constants for splitting (a = 5.6 G) by the three approximately equivalent nuclei of the nitrogen atoms in the triazine ring was obtained for the radical-anion (Figs.13*c* and 13*d*). On recording the spectra at a lower modulation amplitude, a finer structure due to the further splitting of the above seven lines was observed.

An analogous spectrum was recorded on microsomal reduction of fervenulin. The replacement of the hydrogen atom by the methyl fragment in the 8-position probably does not influence the character of the distribution of spin density in the radical-anions. Following the microsomal reduction of xanthothricin, a ten-component spectrum was obtained: this is also due to the splitting of the signal of the unpaired electron as a result of the interaction with the three equivalent nitrogen atoms and the three hydrogen atoms of the methyl group in the triazine fragment. The spin density in the pyrimidotriazine radical-anion is apparently localised mainly in the triazine ring. Thus in biological systems there is a possibility of the reduction of both reumycin and of its anion depending on the pH of the medium.[35]

In the general case the equation of the oxidation-reduction reactions in protic media can be written in the form

$$A + n e^- + m H^+ \rightleftarrows AH_m^{(n-m)-}.$$

For reversible processes, the potential of this system is described by the following expression according to the Nernst equation:

$$E = E_0^0 + \frac{RT}{nF} \ln \frac{[A]\,[H^+]^m}{[AH_m^{n-m}]}.$$

At a reduction half-wave potential $E_{1/2}$ such that the concentrations of the oxidised and reduced forms on the electrode surface are equal, the above equation can be put in the form

$$E_{1/2} \cong E^0 + \frac{RT}{nF} \ln [H^+]^m \cong E^0 - \frac{2.3mRT}{nF} \text{pH}$$

or

$$\Delta E_{1/2}/\Delta \text{pH} = -2.3\frac{mRT}{nF}.$$

The overall dependence of $E_{1/2}$ on pH is represented by several straight lines having different slopes and the pH values at the break points correspond to the ionisation constants (pK_a) of the oxidised or reduced form. If the absolute value of $\Delta E_{1/2}/\Delta$pH, i.e. the slope of the straight line, beyond the break point decreases with increasing pH, then the break itself is due to the dissociation of the reduced form and the corresponding pK_a refers to the latter.[57]

Thus a comparative study of the rules governing the variation of the half-wave potential $E_{1/2}$ with the pH of the medium for two pyrimidotriazine antibiotics showed (Fig.14) that in the pH range 4–10 the dependence of $E_{1/2}$ on the pH for fervenulin, which is incapable of ionisation, consists of a straight line whose slope is −0.06. For reumycin, which is an acid (pK_a = 6.5), this relation has a more complex form with a characteristic break in the region where the pH approximately equals pK_a. As a result of this, reumycin is reduced less effectively in alkaline media and more effectively in acid media than fervenulin.

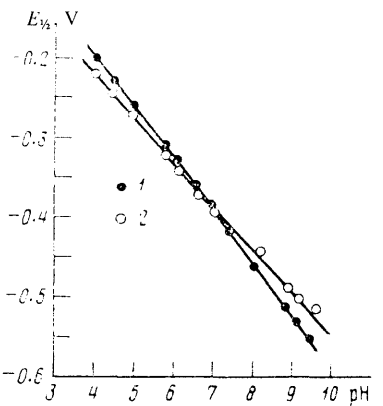


Figure 14. Dependence of $E_{1/2}$ for reumycin (line *1*) and fervenulin (line *2*) on pH.

The overall tendency towards an enhancement of the electron-accepting properties of the antitumour antibiotics with decrease of pH can be one of the causes of their selective action on tumour cells whose pH is lower than the normal value.

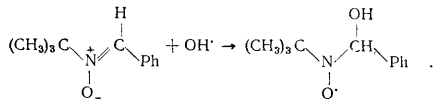
III. THE INTERACTION OF THE SEMIQUINONE RADICALS OF THE ANTIBIOTICS WITH INTRACELLULAR OXYGEN

The semiquinone radicals of antibiotics, formed during enzymic reduction, are destroyed under aerobic conditions on interaction with oxygen, forming the superoxide radical:

$$A^{\cdot-} + O_2 \overset{k}{\rightleftarrows} A + O_2^{\cdot-}$$

The rate constant for the generation of superoxide at pH 7 is higher than 10^8 litre mol^{-1}. This reaction is reversible, the rate of the reverse reaction being lower by two orders of magnitude.[29,58,59] According to the results of Chance et al,[60] the partial oxygen pressure is 7 mmHg (10 μM) in normally functioning cells *in vivo*. Assuming that the oxygen concentration is 10 μM and that $[O_2] \gg [O_2^{\cdot-}]$ (i.e. the reaction is of pseudofirst order), we find that the lifetime of the semiquinone radical *in vivo* is $\ln 2/k[O_2] = 6$ μs. Consequently the semiquinone radicals of antibiotics are unstable in normal cells and their chemical reactions (including the possible reactions with macromolecules) are very rapid. Naturally, in the presence of a reduced oxygen content (for example, in the cells of malignant tumours) the lifetime of semiquinone radicals can increase significantly, which entails an increase of the probability of free-radical reactions in the biotransformation of the antibiotic.

The detection of the formation of the $O_2^{\cdot-}$ and $OH^{\cdot}$ radical-ions is difficult owing to their high reactivity. One of the most convenient ways of detecting the formation of superoxide and hydroxy-radicals is the spin trapping method. N-t-Butyl-α-phenylnitrone (BPN) and 5,5'-dimethyl-1-pyrroline 1-oxide (DMPO) are most often used for this purpose. For example, when an aqueous solution of BPN in a phosphate buffer at pH 7 is added to a solution of the semiquinone radical in DMSO, obtained by the reduction of the AAA with KBH_4, a radical, which is a spin-adduct of BPN and the OH group of the semiquinone radical, is formed:



Its EPR spectrum consists of a doublet (a = 16.0 G) of triplets (a = 3.4 G) with g = 2.0061. When a solution of the semiquinone radicals of antibiotics was mixed with an aqueous solution of BPN containing catalase or superoxide dismutase (SOD), the formation of nitroxy-radicals was not observed.[39] On the other hand, the denatured forms of these enzymes no longer inhibited the formation of the BPN–OH nitroxy-radical spin-adducts. It has been shown for bruneomycin that in the presence of EDTA the spin-adducts are not formed, which indicates an important role of heavy metal ions in the generation of the $OH^{\cdot}$ radical-anions.[39]

Naturally the above spin-adducts are formed only in the presence of oxygen. For example, when adriamycin is incubated with microsomes, the formation of the DMPO–superoxide adduct is observed only when oxygen is present in the microsomes.[27] After the complete consumption of the oxygen dissolved in the incubation medium, the EPR signals of the spin-adducts of $OH^{\cdot}$ and $O_2^{\cdot-}$ disappear and the EPR signal of the semiquinone radical of adriamycin appears. At least two factors influence this phenomenon: firstly, the DMPO–superoxide adduct has a lifetime of 45 s at pH 7.4[27,61] and, since molecular oxygen is consumed, the formation of nitroxy-radicals can cease; secondly the spin-adduct is enzymically reduced, like other nitroxy-radicals.[27,62] On the basis of these features, the following sequence of reactions has been proposed:[33]

$$A + e^- \rightarrow A^{\cdot-},$$

$$A^{\cdot-} + O_2 \rightarrow A + O_2^{\cdot-}$$

$$2H^+ + 2O_2^{\cdot-} \xrightarrow{SOD} H_2O_2 + O_2,$$

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2,$$

$$M(II) + H_2O_2 \rightarrow M(III) + OH^{\cdot} + OH^-,$$

$$M(III) + O_2^{\cdot-} \rightarrow M(II) + O_2,$$

$$H_2O_2 + O_2^{\cdot-} \rightarrow OH^- + OH^{\cdot} + O_2,$$

Another way of detecting $O_2^{\cdot-}$ radical-ions in solution may be to determine the adrenochrome formed from adrenalin.[15] The rate of formation of superoxide radicals is estimated from the rate of oxidation of adrenalin and the formation of adrenochrome, which is determined spectrophotometrically from the absorption at a wavelength of 480 nm. The addition of superoxide dismutase (10 μg ml^{-1}) inhibits the formation of adrenochrome.

The generation of superoxide radicals by pyrimidotriazine antibiotics in rat liver microsomes has been investigated.[35] In the absence of NADPH, the rate of formation of $O_2^{\cdot-}$ in the microsomes is very low (w = 0.3 nmol per mg of protein per minute) and does not increase after the addition of reumycin, which is not reduced under these conditions to a radical-anion. In the presence of NADPH, the electron transfer from NADPH to oxygen with formation of superoxide radicals is observed (w = 1.8 nmol per mg of protein per minute). The addition of reumycin to the system increases sharply the rate of formation (w = 6.5 nmol per mg of protein per minute) of superoxide radicals (the formation of adrenochrome) owing to the transfer of an electron from the radical-anions of the antibiotic to oxygen. Under these conditions, the EPR spectra of the reumycin radical-anions were detected only after the complete consumption of oxygen. Xanthothricin, which has the lowest reduction potential,[55] is capable of being reduced in microsomes without the addition of NADPH. The radical-anions formed under these conditions generate superoxide radicals at the highest rate (w = 11.1 nmol per mg of protein per minute). This can probably explain the high toxicity of xanthothricin and its low chemotherapeutic index. This confirms the earlier hypothesis of the role of the stability of the intermediate active free-radical forms of the antitumour preparations in the selectivity of their action.[63]

Comparison of the properties of reumycin with those of other antibiotics of this series makes it possible to explain the higher selectivity of its antitumour action. In tumour cells, where the pH is reduced (6.5), the reumycin–reumycin anion equilibrium is displaced towards the unionised form. Owing to its low reduction potential, the neutral form of reumycin can oxidise a large number of enzyme systems with formation of highly reactive radical-anions and, by virtue of the causes stated above, this can lead to a pronounced cytotoxic activity. In normal cells, whose pH is 7.5, reumycin exists to the extent of 90% in the ionised form, whose capacity for reduction to radical-ions is much lower. This is in fact apparently the cause of the selectivity of the action of reumycin in relation to tumour cells.

The formation of superoxide and hydroxy-radicals as a result of electron transfer from the semiquinone radicals of antibiotics to oxygen has been established also for bruneomycin,[39] actinomycin D,[15] anthracycline antibiotics,[27] and mitomycin C.[27]

The formation of radicals from quinone-containing antibiotics and their subsequent destruction on interaction with oxygen can also be characterised by the degree of microsomal oxidation of NADPH and the consumption of oxygen.

Substances of the benzoanthraquinone class, *N*-heterocyclic quinones, and naphthcquinones stimulate the oxidation of NADPH but at different rates. Cytotoxic preparations (cyclophosphane, methotrexate, etc.) are inactive, which has been established by the quantitative analysis of the microsomal oxidation of NADPH (Table 7).[26]

Another index of the intensity of electron transfer from the antibiotic semiquinone to oxygen is the increase of the rate of consumption of oxygen. It has been observed that the rate of consumption of oxygen is proportional to the concentration of both the quinone-containing antibiotic and that of the enzymes. Antitumour preparations not containing quinone groups and not increasing the rate of microsomal oxidation of NADPH do not influence the consumption of oxygen by microsomes.[26,29] The highest rates of formation of superoxide radicals, estimated from the rate of consumption of oxygen in the presence of rubomycin, was reached at pH 7.8–8.4, although in phosphate buffer a higher rate was noted than in tris buffer. The consumption of oxygen stimulated by AAA was also sensitive to the concentration of the buffer; the maximum rate was attained in 0.2 M buffer.[29]

Table 8. Rates of consumption of oxygen by various subcellular extracts in the presence of rubomycin.[29]

Object	w_1, nmol/mg protein per min	w_2, nmol/mg protein per min
Cytoplasmic liver extract	0.3	1.6
Liver mitochrondria	2.1	4.3
Liver microsomes	25.2	93.0

Notation: w_1 and w_2 are the rates of the intrinsic and rubomycin-catalysed consumption of oxygen.

Various subcellular extracts stimulate the consumption of oxygen to different extents, which is due to the differences between the amounts of enzymes capable of catalysing the electron transfer from NADPH to the quinone-containing antibiotic (Table 8).[29] This process proceeds most vigorously in microsomes. At the same time the difference between the activities of microsomes from different organs is noteworthy. The microsomes of different organs (liver, lungs, and heart) of mice and rats have been investigated and their capacity to increase the consumption of O_2 has been determined using adriamycin as the substrate (Table 9).

It has been found that the microsomes from L-1210 and P-388 tumour cells also catalyse the increase of the consumption of oxygen but to a much lesser extent than the microsomes from the normal organs, which is due to the disturbance of the functional activity of tumour cells and cell organelles (Table 9).[26]

Table 9. Kinetic constants* for the consumption of oxygen stimulated by adriamycin.[26]

Microsomes	$10^4 K_m$, mol litre^{-1}	$10^7 V_{max}$, mol min^{-1}
Rat liver	3.6	0.92
Rat lungs	3.1	0.39
Rat heart	186.0	0.89
Mouse liver	8.0	2.6
Mouse lungs	2.4	0.44
Mouse heart	3.1	0.06
Mouse spleen	28.0	0.14
L-1210 cells	4.4	0.2
P-388 cells	0.6	0.04

*K_m and V_{max} are the parameters of the Michaelis equation.

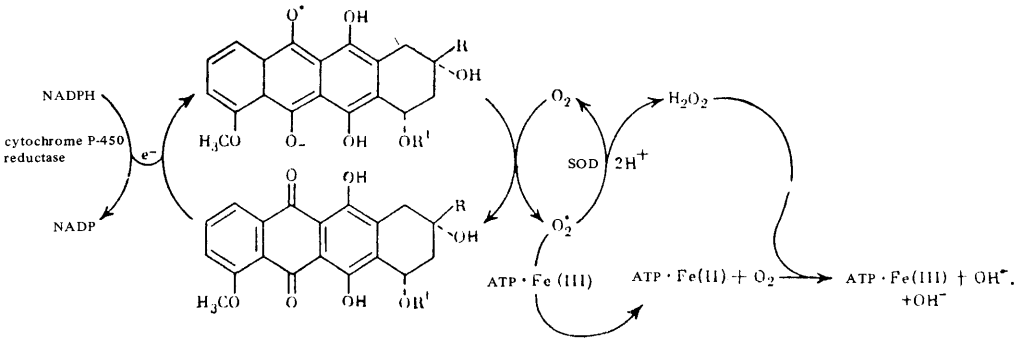
Table 10. Kinetic constants* for the consumption of oxygen stimulated by AAA in microsomes.[29]

Antibiotic	K_m, μmol litre^{-1}	V_{max}, nmol min^{-1}
Rubomycin	390	35
Adriamycin	260	240
Daunorubicinol	250	230
Carminomycin	110	770
Adriamycin Fe^{3+}	250	620

*K_m and V_{max} are the parameters of the Michaelis equation.

All the anthracycline antibiotics are capable of transferring an electron from NADPH to oxygen. Carminomycin is outstanding in this series, catalysing the electron transfer to oxygen at the highest rate; this property is correlated with its high toxicity (Table 10).[29]

In the general case the AAA and their structural analogues with an *N*-quinonoid fragment form semiquinone free radicals on reduction *in vivo*, their autoxidation resulting in the generation of superoxides.



According to the experimentally justified scheme presented above, antibiotics of this series act as competing agents for electron transfer to oxygen in the biological oxidation chain.[64] Naturally, chemical modification of the antibiotics, involving a decrease of their capacity to generate superoxides, should be accompanied by a decrease of toxicity. Indeed the monoxime of bruneomycin, which cannot function as an agent transferring electrons to oxygen, proved to be less toxic in relation to *E. coli* and the magnitude of the toxic dose was independent of the level of the superoxide dismutase activity of the cells.[65]

Various additives to microsomes can alter the consumption of oxygen. Thus coenzyme Q appreciably increases both the intrinsic and the rubomycin-stimulated consumption of oxygen. On the other hand, α-tocopherol, which is a familar natural antioxidant, inhibits the intrinsic consumption of oxygen by 60% and the antibiotic-stimulated consumption by 80%. On the other hand, glutathione and cysteine activate the consumption of oxygen (Table 11).[29]

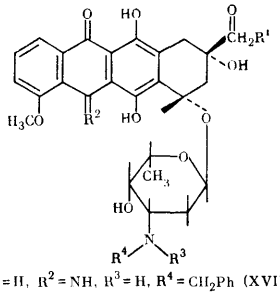
Table 11. The influence of various additives on the rate of consumption of oxygen by microsomes.[29]

additive	w_1, nmol O_2 min^{-1}	w_2, nmol O_2 min^{-1}
Without additive	35	296
Hexobarbital	88	309
Aspirin	54	302
Coenzyme Q	53	447
GSH	53	389
Cysteine	49	388
α-Tocopherol	13	68
EDTA	18	252
Fe^{3+}	49	315

Notation: w_1 and w_2 are the rates of the intrinsic and adriamycin-catalysed consumption of oxygen and GSH is reduced glutathione.

The main secondary effect of the AAA is cardiotoxicity. The relation between the cardiotoxicity in the series of AAA and the inhibition by the latter of the synthesis of cardiac guanylate cyclase has been investigated[66] and it has been observed that synthetic AAA containing hydrazone derivatives in the C(9)-position do not inhibit but actually stimulate the guanylate cyclase activity.

The cardiotoxocity has been explained recently by the formation of the O_2^- and $OH^\cdot$ radical species in the cardiac muscle. There is a correlation between the capacity to form superoxide radicals and the cardiotoxicity of certain anthracyclines[64] (Fig.15).



$R^1 = H$, $R^2 = NH$, $R^3 = H$, $R^4 = CH_2Ph$ (XVIII); $R^1 = H$, $R^2 = O$, $R^3 = R^4 = CH_2Ph$ (XIX); $R^1 = OH$, $R^2 = O$, $R^3 = R^4 = CH_3$ (XX).

The toxic activity of the radicals on cardiac muscle is associated with the reduced content of superoxide dismutase and catalase in the cell (Table 12).[34] It has been demonstrated *in vivo* that the introduction of the natural antioxidant α-tocopherol leads to a decrease of cardiotoxicity and an increase of the lifespan of animals.[67] It has been shown that ascorbate can also decrease the overall toxicity and cardiotoxicity of the preparations due to the AAA. Electron microscope and histological studies have demonstrated that ascorbate prevents the early changes in cardiac muscle under the influence of AAA.[68]

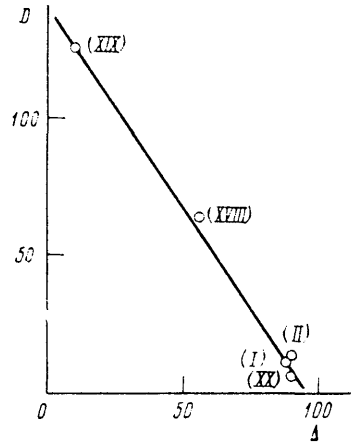


Figure 15. Relation between the dose of anthracyclines giving rise to the same Zbinden cardiotoxic effect (D) and the relative rate of generation of superoxide radicals by various anthracyclines (Δ) (according to the data of Lown et al.[64]

Table 12. The relative activities of catalase (in relative units) in various organs of the rat and rabbit.[34]

Organ	Rabbit	Rat
Blood	20	16.2
Liver	23	45.3
Kidneys	26	19.0
Lungs	2.9	1.9
Heart	1.0	1.0

It has been observed that AAA stimulate the peroxide oxidation of lipids. Incubation of rat liver microsomes with the NADPH-generating system produces approximately 10 nanoequivalents of malonaldehyde per mg of protein in 60 min. The peroxide oxidation was 6 times faster after the addition of 100 μM adriamycin. The AAA-stimulated peroxide oxidation of lipids influences the activity of certain enzyme systems of microsomes.[69] An 85% decrease of the content of cytochrome P-450 reductase and 70% inhibition of the activity of aminopyrine *N*-demethylase have been observed. The peroxide oxidation of lipids did not influence the NADPH-cytochrome *c* reductase activity; the UDP-glucuronyl transferase activity increases by a factor

of 5, while the glucose-6-phosphatase activity decreases by a factor of 6 under these conditions. In the absence of the peroxide oxidation of lipids, adriamycin also influences these enzymes[69] but to a lesser extent (Fig.16).

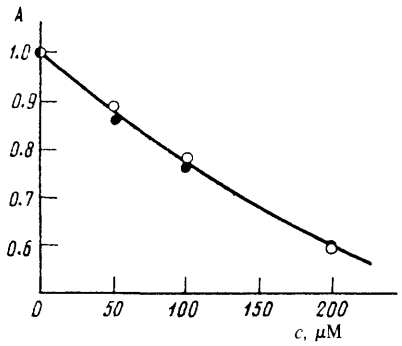


Figure 16. The influence of the concentration of adriamycin (c) on the content of cytochrome P-450 and the activity of aminopurine *N*-demethylase in the absence of the peroxide oxidation of lipids (according to the data of Mimnaugh et al).[69]

From the above, one can reach the following conclusions: both the anions and the radical-anions of the AAA can be oxidised in the organism, the toxic activity of the preparations is due to their capacity for oxidation, and the introduction of special antioxidants competing with the preparations is essential for the reduction of their toxicity.

In order to improve the antitumour index and reduce cardiotoxicity, various analogues of natural AAA have been synthesised. The introduction of the imino-group in the 5-position of the chromophore reduces the capacity for reduction and subsequent oxidation. This leads to a considerable decrease of the rate of generation of superoxide radicals compared with adriamycin.[55]

Changes in the carbohydrate ring can also influence the capacity of AAA for enzymic reduction. Thus *N*-methylation of the sugar group increases the capacity of AAA for reduction, the degree of consumption of oxygen, and the rate of formation of superoxide radicals. The cardiotoxicity of these preparations increases under these conditions.[64] If two *N*-benzyl groups are introduced into the carbohydrate fragment, then the consumption of oxygen and the formation of superoxide radicals diminish sharply. This can be associated with the steric inhibition of the action of the microsomal cytochrome P-450 on the antibiotic.

The activation of medicinal preparations and their subsequent metabolism depend on the functional state of the cells and the degree of their differentiation. The differences in the effectiveness of the action of particular antitumour antibiotics as a function of the degree of aeration of the tumour cells may be associated with this particular factor. In terms of this factor, the series of known carcinolytic substances can be divided into three groups, which are presented in Table 13.[70]

The group of preparations whose cytotoxic effect increases with increase of the concentration of oxygen includes three antibiotics: bleomycin, bruneomycin, and actinomycin D. It is known that bleomycin is characterised by high pulmonary toxicity, which increases significantly with increasing partial pressure of O_2.[71] Bleomycin causes ruptures in DNA molecules and its reactivity depends on the presence of iron ions and molecular oxygen. The highly reactive radical forms of oxygen can be responsible for the breaks in DNA under these conditions. It has been shown that the reaction of bleomycin with [^{3}H]DNA in the presence of iron ions is inhibited when the reaction mixture is bubbled with argon. On aeration, the reaction proceeds via the ternary complex O_2–Fe(II)–bleomycin [O_2–Fe(II)–B], whose properties are analogous to those of haem-containing oxygenases.[72] This complex is formed on direct oxidation with molecular oxygen of Fe(II)–bleomycin [Fe(II)–B], the second-order reaction rate constant amounting to $k = 6.1 \times 10^3$ mol^{-1} s^{-1}:

$$\left.\begin{matrix} O_2 \\ Fe(II)—B \end{matrix}\right\rangle \rightarrow O_2—Fe(II)—B \leftrightarrow O_2^{\cdot-}—Fe(III)—B \left\langle\begin{matrix} \rightarrow O_2^{-} \\ \rightarrow Fe(II)—B \end{matrix}\right.$$

The subsequent stage involving the generation of a superoxide radical with formation of the complex Fe(II)–B is characterised by the first-order rate constant $k = 0.12$ s^{-1}. At pH 7 the oxidation of the complex Fe(II)–B with oxygen leads to the formation of three different paramagnetic forms of Fe(III)–B: one high-spin form ($s = 5/2$) and two low-spin forms ($s = 1/2$). The low-spin form having the spectroscopic parameters $g_{xx} = 2.25$, $g_{yy} = 2.17$, and $g_{zz} = 1.94$ is unstable and disappears rapidly (after 60 s at room temperature). The other two forms, namely the high-spin form ($g = 4.3$) and the other low-spin form ($g_{xx} = 2.40$, $g_{yy} = 2.18$, and $g_{zz} = 1.90$), are stable.[73]

Table 13. Classification of the antitumour agents in terms of the effectiveness of their action as a function of the degree of aeration of the cells.[70]

Agents whose action depends on the degree of aeration		Preparations whose action is independent of the degree of aeration
active under aerobic conditions	active in hypoxia	
Bleomycin	Mitomycin C	5-Fluorouracil
Bruneomycin	Adriamycin	Methotrexate
Actinomycin D	Misonidazole	*cis*-Dichlorodiaminoplatinum
Procarbazine	Metronidazole	*N,N'*-Di(chloroethyl)-*N*-nitrosourea
Vincristine	5-Thio-D-glucose	
Ionising radiation	2-Deoxy-D-glucose	*N*-Chloroethyl-*N'*-cyclohexyl-*N*-nitrosourea

Under aerobic conditions, the reduction of bruneomycin with tetrahydroborate in the presence of DNA is accompanied by a decrease of viscosity and of the average molecular weight of DNA[44] owing to the appearance of single breaks in the DNA molecule.[74] Since superoxide dismutase inhibits this process,[75] presumably active radical forms of oxygen are responsible for the degradation of the DNA. These results make it possible to explain the enhancement of the toxic activity of bruneomycin on *E.coli* in the presence of oxygen or in the presence of a reduced SOD content.

Whereas the action of mitomycin C is determined by the alkylation of biomacromolecules and the formation of cross-links between them, the cytotoxic activity of bruneomycin is due to the active oxygen radicals. The selective action on tumour cells by preparations generating these radicals can be accounted for by the reduction of the SOD content in

tumour cells.[76] The selective increase of the O_2 concentration in tumour cells can apparently be used for the specific chemotherapy of tumours.

Yet another antibiotic of this type is actinomycin D. The mechanism of its action can be accounted for by its capacity for intercalation between the DNA bases.[38] At a concentration less than 0.01 μM, the difference between the cytotoxicities of this antibiotic under aerobic conditions and in hypoxia is small. However, at a concentration of approximately 1 μM, oxygen-rich cells are approximately 100 times more sensitive to the lethal action of actinomycin D than under the conditions of hypoxia.[70] There exists apparently an alternative oxygen-dependent mechanism of the cytotoxic action of actinomycin D where a part of it remains non-intercalated between the DNA bases at fairly high concentrations of the preparation. On incubation with microsomal proteins, actinomycin D forms a semiquinone free radical which stimulates the consumption of oxygen and the electron transfer to oxygen. When actinomycin D and its spin-labelled analogues are incubated with liver microsomes in the presence of NADPH, the rates of consumption of oxygen, of formation of superoxide, and of oxidation of adrenalin increase (Table 14).[15] Evidently the spin-labelled antibiotics stimulate the formation of superoxide radicals to a greater extent than does actinomycin D.

Table 14. The rates of consumption of oxygen (w_1) and formation of andrenochrome (w_2) from adrenalin in microsomes in the presence of actinomycin D and its spin-labelled analogues.[15]

Preparation	w_1, nmol/mg protein per min	w_2, nmol/mg protein per min
(V)	8.17	2.45
(VI)	18.37	4.66
(VII)	99.28	13.96
Control	7.23	1.29

The influence of the binding of the preparations to DNA on the generation of O_2^- has also been studied. After actinomycin D had been incubated with DNA, it lost entirely its capacity to generate superoxide. The incubation of the spin-labelled analogues (VI) and (VII) with DNA reduced the rate of consumption of oxygen by 71 and 50% respectively and also decreased the rate of oxidation of adrenalin. These results indicate yet again that the antibiotics intercalated between DNA bases are "poor" substrates for enzymic reduction. Thus the formation of superoxide, hydrogen peroxide, and hydroxy-radicals can play an important role in the antitumour activity of preparations of the type considered.[15]

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The set of data obtained permit a number of conclusions concerning the molecular mechanisms of the toxic and antitumour activities of the active free-radical forms of antibiotics. The demonstration of the formation of the chemically reactive forms of reduced oxygen and of the covalent binding of the free-radical forms of the antibiotics to biomacromolecules must be regarded as the most important of these.

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