XLII. THE CHEMICAL ACTION OF QUINONES ON PROTEINS AND AMINO-ACIDS.

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THE previous work on this subject [Morgan and Cooper, 1924; Cooper and Nicholas, 1927] has shown that whilst *p*-benzoquinone is from 4 to 16 times as active a germicide as toluquinone, yet very little difference exists in their apparent chemical reactivity with such proteins as gelatin and albumin. With certain amino-acids, however, preliminary experiments showed that benzoquinone reacted 5 times as readily as toluquinone, thus suggesting that the germicidal action of the quinones is associated with their reactivity towards the free amino-acids present in the organism rather than with the cell-proteins. Further investigations have since been carried out in connection with this problem in order to substantiate, if possible, the earlier conclusions.

It was found [Cooper and Nicholas, 1927] that the quinones were unstable in aqueous solution when exposed to daylight, but underwent little or no change in the dark. The following results show that the process of decomposition is, furthermore, accompanied by a fall in bactericidal power. The quinones were estimated by the method of Valeur and germicidal power was determined either by the Chick-Martin method or by the inhibitory process.

Table I.

	Period of exposure to daylight	Concentration of solution (g. per litre)	Bactericidal concentration (<i>B. coli communis</i>)
Benzoquinone	Initial	0.1147	1 in 35,000
	2 days	0.1098	—
	13 "	0.0803	1 in 27,500
	27 "	0.0679	1 in 17,500
	47 "	0.0604	1 in 12,500
Toluquinone	Initial	0.0986	1 in 9,000
	1 day	0.0766	1 in 4,500
	4 days	0.0515	1 in 4,500
	7 "	0.0263	1 in 2,000

The results show that the rate of decomposition and fall in germicidal power is more pronounced with toluquinone than with benzoquinone. The chemical changes occurring in solutions of quinones are of great complexity; apparently reduction, oxidation, and polymerisation are involved.

The foregoing observations indicated the importance of careful control work. In fact, when prolonged experiments were in progress, the solutions were stored in darkness to avoid as far as possible loss of quinone.

Reference has already been made to the fact that benzoquinone may be as much as 4-16 times as efficacious as toluquinone in germicidal power. Some further determinations have since been made, using organisms that had not been previously employed. The results are set out in Table II.

Table II.

Organism	Conditions	Carbolic coefficients		
		Benzoquinone	Toluquinone	Ratio
<i>B. typhosus</i>	$\frac{1}{4}$ hr. disinfection at 20°	204	23	9
<i>B. coli</i>	1 " " " 20°	1400	83	17
	$\frac{1}{2}$ " " " 20°	268	52	5
	1 " " " 37°	6650	1710	4
<i>B. pyocyaneus</i>	$\frac{1}{2}$ " " " 20°	15.6	4.3	4
<i>B. mesentericus</i>	$\frac{1}{2}$ " " " 20°	230	21	11
<i>B. prodigiosus</i>	$\frac{1}{2}$ " " " 20°	200	26	8
<i>B. proteus</i>	$\frac{1}{2}$ " " " 20°	200	33	6

It is seen that the ratio of the germicidal activities of the two quinones varies (1) with the organism used from 4 to 17, (2) with the experimental conditions of time and temperature employed.

The *absolute* difference in the bactericidal power of the quinones is probably even greater than represented by the foregoing figures, because it has been found by experiments with viscose membranes that benzoquinone, although by far the more efficacious germicide, diffused somewhat more *slowly* than toluquinone.

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Equal amounts of the protein (0.2 g.) were allowed to react with solutions (20 cc.) of benzo- and tolu-quinone of the same concentration (0.2 %) under the same uniform conditions, and the amounts of the quinones reacting estimated by titration of the solutions at the end of a definite period. The results are expressed in Table III as ratios, representing amount of benzoquinone/amount of toluquinone reacting with the same amount of protein.

Table III.

Protein	Experimental conditions Temp. 18°	Ratio of reactivity
Edestin	$\frac{1}{2}$ hour	1.9
	1 " "	2.2
	3 hours	1.5
*Wheat gliadin	$\frac{1}{2}$ hour	1.3
	1 " "	0.9
	2 hours	0.6
	16 " "	1.8
Glutenin	3 " "	1.2
	24 " "	1.5
Gelatin	72 " "	1.8
	96 " "	1.8

* Experiments with alcohol-soluble proteins are now of theoretical interest, as it has recently been found that such proteins occur as cell-contents of bacteria [Goadby, 1927].

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In all these experiments the proteins were insoluble in water, and it was only necessary to pipette off the clear quinone solutions for estimations.

In the following experiments colloidal solutions of proteins were employed, viscose dialysers being used, so that the quinone solutions could be titrated at the end of the experiment in the absence of the protein phase. The following results (Table IV) were obtained.

Table IV.

Protein used	Period of experiment	Ratio of reactivity
Gelatin	24 hours	2.7
Edestin (in NaCl solution)	24 "	1.3
Albumin	1 hour	2.2
"	24 hours	1.0-2.4*

* From concentrations of quinones varying from 0.01-0.20 %.

The ratio of the chemical reactivity of benzoquinone to that of toluquinone may thus be slightly higher than in the case of the insoluble proteins, but in no case does the ratio attain the order observed in the bactericidal tests.

The action of the quinones has also been studied comparatively on yeast and *Bacillus xylinus*, the latter being grown on a large scale in glycerol-yeast-water. Weighed amounts of the organisms were placed in viscose dialysers, surrounded by the quinone solutions, as in the previous experiments.

Table V.

Organisms used		Ratio of reactivity of the quinones
1. Yeast	24 hours	1.0
	48 "	1.0
2. Dried yeast	24 "	1.5
	48 "	1.3
3. <i>B. xylinus</i>	24 "	1.1

In all three cases there is thus very little difference in the apparent activity of benzo- and tolu-quinone.

One possible explanation of the low ratios uniformly obtained with proteins and even with micro-organisms is that in addition to chemical action the quinones are *adsorbed* by proteins and other cell-colloids, with the result that the great difference between the reactivity of the quinones indicated by their chemical properties and bactericidal power is obscured. The relative amounts of benzo- and tolu-quinone adsorbed by animal charcoal were therefore next determined.

Table VI.

Final concn. of benzoquinone in water phase %	Amnt of benzoquinone adsorbed by 1 g. charcoal g.	Final concn. of toluquinone in water phase %	Amnt of toluquinone adsorbed by 1 g. charcoal g.
0.140	0.0088	0.176	0.0149
0.068	0.0069	0.082	0.0077
0.055	0.0055	0.037	0.0043
0.019	0.0022	0.015	0.0021
0.009	0.0019	0.010	0.0006

1 g. of animal charcoal was introduced into each of a series of solutions of the two quinones, varying from 0.01 to 0.20 %, adsorption being allowed to continue for 30 minutes. The clear solutions were then pipetted off free from charcoal and titrated. The results are tabulated in Table VI.

At higher concentrations toluquinone is adsorbed somewhat more freely than benzoquinone, but at low concentrations the reverse is the case. The amounts of quinone adsorbed increase with rise in concentration, but not proportionately, a marked falling off in the uptake being observed in the stronger solutions, as is frequently observed in cases of adsorption. When, however, the logarithms of the final concentration and of the amounts adsorbed are plotted against each other, a straight line results. Adsorption is thus evidently a factor in obscuring the greater chemical reactivity of benzoquinone in the case of such colloids as proteins. Toluquinone may be adsorbed to a greater extent than benzoquinone, although only a relatively small amount probably enters into chemical action.

Whether adsorption could be accepted as the principal factor in obscuring the relative chemical reactivity of the quinones could not be decided without further investigations, and experiments were therefore next carried out to compare the action of benzo- and tolu-quinone on the structural units of the proteins, *e.g.* the amino-acids and related substances.

Usually 0.2 g. of the substance (in solution or in suspension according to solubility) was allowed to react with 25 cc. of equimolecular solutions of the two quinones for definite periods, *e.g.* 0.5–48 hours, the solutions then being titrated and the amount of quinone entering into chemical action thus determined. The following results were obtained for the ratio: amount of benzoquinone/amount of toluquinone reacting.

Table VII.

Glycine	...	...	...	1.9–4.9
Alanine	...	...	...	3.9–6.2
<i>r</i> -Leucine	...	...	...	2.7
<i>l</i> -Leucine	...	...	...	0.6–2.6
Tyrosine: solid	...	...	...	0.7–2.1
Tyrosine: dissolved in <i>N</i> /10 HCl	...	...	...	5.8
Phenylalanine	...	...	...	6.0
Glutamic acid	...	...	...	4.3
Asparagine	...	...	...	1.7–6.2
Cystine: solid	...	...	...	1.8
Cystine: in solution	...	...	...	2.4
Mixture of diamino-acids	...	...	...	1.9–7.0
Tryptophan	...	...	...	1.5–3.0
Creatine	...	...	...	0.5–2.4
Creatinine	...	...	...	1.0
Glycylglycine	...	...	...	1.1
Glycine ester-HCl	...	...	...	2.8
Witte's peptone	...	...	...	1.4
Autolysed yeast	...	...	...	1.4–4.6
Sulphanilic acid	...	...	...	8

(Nucleic acid, glucosamine, and amides did not react with aqueous solutions of quinones in a period of 24 hours.)

A consideration of the foregoing observations shows that the highest ratios are obtained with such amino-acids as glycine, alanine, phenylalanine, tyrosine, and diamino-acids, and the marked superiority of benzoquinone as a germicide over toluquinone has a measure of correspondence in the greater readiness with which it reacts with such amino-acids.

The germicidal ratio of benzo- and tolu-quinone varies from 4 to 17 according to experimental conditions, whilst the ratio of their reaction-velocities with certain amino-acids may rise as high as 7, somewhat lower figures of 4–5 being frequently observed. Proteins, on the other hand, give much lower ratios of 1–2, but this is also found to be the case with certain amino-acids, *e.g.* cystine, tryptophan, and also in the case of peptone.

The low ratios obtained with proteins are therefore not entirely due to the complication of adsorption, but must have a structural explanation, and this view is supported by the fact that similarly low ratios are obtained with a protein structural unit, such as the peptide-grouping, *e.g.* glycylglycine, and also with the peptones. Glycine anhydride or diketopiperazine—apparently also a structural component of proteins—furthermore does not react with quinones.

The results suggest that the bactericidal action of the quinones is associated especially with their chemical reactivity towards comparatively simple cell-constituents, and not necessarily with the more complex colloidal substances such as the proteins. Furthermore, such simple amino-acids as alanine seem to be more fundamentally concerned than tryptophan.

The reactivity of the quinones towards amino-acids and proteins has also been studied in alcoholic solution, in view of the fact that the absorption spectra of the quinones are well known to be affected by organic solvents.

The following benzoquinone/toluquinone ratios were observed with glycine in 25 % alcohol.

Table VIII. *Ratios.*

	In 25 % alcohol
$\frac{1}{2}$ hour	14.9
1 ,,	12.6
2 hours	4.5
5 ,,	3.4
24 ,,	1.9

From these observations the interesting result emerges that alcohol very considerably increases the ratio of chemical reactivity of benzoquinone to that of toluquinone in the periods of $\frac{1}{2}$ –1 hour, but the ratio falls rapidly with the progress of the experiment. In the preliminary stages of the reaction benzoquinone is thus strongly activated by the solvent. The ratios of 12–15 are similar in magnitude to the extreme germicidal ratios occasionally observed, and the results suggest that there may be a cell-constituent which possesses the capacity of activating benzoquinone, thus increasing its efficacy to a greater extent than that of toluquinone. The presence of inorganic substances, *e.g.* the constituents

of Ringer's solution, slightly raises the quinone-ratio with glycine, but not sufficiently to be of significance in this matter.

In connection with these results it was of interest to study the influence of alcohol and acetone in dilutions of 25 % on the germicidal action of the quinones. The following results were thus obtained.

Table IX. *Germicidal concentrations.*

	<i>B. coli.</i> 30 minutes at 20°.		
	In water	In 25 % alcohol	In 25 % acetone
Phenol	1 in 115	1 in 300	1 in 140
Benzoquinone	1 in 35,000	1 in 400,000	1 in 125,000
Toluquinone	1 in 7,000	1 in 27,500	1 in 17,500
Xyloquinone	Insoluble	1 in 8,500	1 in 6,000
Thymoquinone		1 in 6,000	1 in 4,000
<i>m</i> -Dichloroquinone	1 in 45,000	1 in 300,000	—

It is seen that whilst the germicidal power of phenol is only increased approximately three times in the presence of alcohol, the action of the quinones is increased to a much greater extent, the efficacy of benzoquinone especially being enhanced, so that it is now germicidal in concentrations of 1 in 400,000. The results with acetone are similar, but less marked.

Dichloroquinone is exceptional to the general rule that the substituted quinones are less active than benzoquinone. It is, in fact, slightly more germicidal than the parent quinone, and also exhibits a very marked accession in bactericidal power in presence of alcohol.

It was subsequently found that this halogenated quinone reacts as readily as benzoquinone with glycine, and more readily than toluquinone. The halogen atom is thus favourable to high germicidal power and chemical reactivity.

Experiments were also put in hand to ascertain the influence of alcohol on the uptake of the quinones by proteins. Alcohol is known to diminish the adsorption of dissolved substances by colloids, and it was hoped therefore that it would be possible to exclude the interfering factor of adsorption and thus reveal the true relative reactivity of benzo- and tolu-quinone with proteins.

Weighed amounts of gelatin were allowed to react with solutions of varying concentrations of the two quinones (1) in water, and (2) in absolute alcohol for a period of 4 weeks in the dark at room temperature. A long period was allowed, because preliminary experiments showed that proteins continued to take up benzoquinone for at least 3 months. 1 g. of gelatin at the end of this period had combined with or adsorbed as much as 1.55 g. of benzoquinone.

The tabulated results (Table X) show that in aqueous solution benzoquinone is consistently taken up about twice as readily as toluquinone by gelatin. In alcohol, this is also true at low concentration, but at higher concentrations toluquinone tends to be taken up to a somewhat greater extent than benzoquinone. Furthermore, in aqueous solution the amount of benzoquinone adsorbed by the protein steadily rises with increasing concentration, whilst with toluquinone there is a tendency for the uptake to reach a maximum. In alcoholic solution, however, the uptake of both quinones reaches a limit.

Table X.

In water		In alcohol	
Initial concn. in water phase	Amnt of quinone taken up by 1 g. gelatin	Initial concn. in alcohol phase	Amnt of quinone taken up by 1 g. gelatin
Benzoquinone %		Benzoquinone %	
0.292	0.0892 g.	1.880	0.0220 g.
0.166	0.0425	1.535	0.0139
0.140	0.0329	1.050	0.0147
0.065	0.0219	0.554	0.0129
0.024	0.0111	0.339	0.0162
—	—	0.139	0.0077
Toluquinone %		Toluquinone %	
0.173	0.0268	3.066	0.0213
0.134	0.0233	1.006	0.0213
0.082	0.0166	0.507	0.0162
0.039	0.0061	0.289	0.0071
0.030	0.0045	0.071	0.0006

The interpretation of the results is difficult, and subsequently it occurred to the authors that the separation of the factors of adsorption and chemical action might be better attempted by studying the action of the quinones on desamino-proteins, in which the reacting NH_2 and NH groups have either been removed or substituted by nitroso-groups.

Finely divided egg-albumin was treated with nitrous acid by the method of Nakashima [1925] which effects complete deamination of the protein. Kjeldahl estimation showed that the product contained 13.8 % of nitrogen, as compared with 12 % in the original protein, showing a slight fixation of nitrogen, probably as nitroso-groups. The desamino-albumin failed to give a coloration with quinones. The uptake of benzoquinone and toluquinone by this product was then determined by the method already described.

Table XI.

Ratio:	$\frac{\text{Amount of benzoquinone taken up by 1 g. protein}}{\text{Amount of toluquinone similarly taken up}}$	
	Initial quinone concentration varying from 0.01 to 0.15 %.	
	Desamino-albumin	Albumin (control)
$\frac{1}{2}$ hour	0.54	—
24 hours	0.71-1.0	1.0-1.4

The ratio is now seen to vary from 0.5 to 1.0, toluquinone thus being taken up to a somewhat greater extent than benzoquinone by desamino-albumin; whilst with albumin itself the reverse is the case, the ratio varying from 1 to 1.4.

The results with the desamino-product are similar to those obtained with animal charcoal, which at certain concentrations adsorbed toluquinone more freely than benzoquinone.

The suppression of the chemical factor in the case of albumin by deamination thus results in a lowering of the quinone-ratio from on the average 1.2 to about 0.8. This change, however, is insufficient to account for the low combination ratios of the quinones with proteins. The results do not warrant the conclusion that proteins would yield a quinone-ratio of a similar numerical value

to that obtained with amino-acids or in the germicidal tests, if the chemical action were not masked by the physical process of adsorption. Adsorption must therefore be considered a secondary factor, and the low quinone-ratios observed with proteins must be due to an important extent to structural reasons, viz. the presence of (1) certain amino-acid radicles, of (2) the peptide structure, and (3) the diketopiperazine structure, as has been indicated earlier in this paper.

The conclusion is therefore drawn that the simpler cell-constituents, such as certain of the amino-acids, are mainly concerned in the mechanism of disinfection by the quinones.

According to Fischer and Schrader [1910] the general reaction is of the following type:

Three quinone molecules are thus utilised for every two nitrogen atoms.

Since 1 g. of gelatin used up 1.55 g. of benzoquinone, on the above equation the percentage of nitrogen in gelatin would be 13.6 %. Experimental determination showed the sample to contain 14.8 %. However, it is unlikely that every nitrogen atom present in the protein would enter into the reaction, and the result rather points to the conclusion that of the 1.55 g. of benzoquinone, an appreciable amount is adsorbed, and not chemically combined.

SUMMARY.

1. Quinones readily undergo decomposition in aqueous solution in the presence of light, and the change is accompanied by a fall in bactericidal power.
2. Benzoquinone is from 4 to 17 times more active as a germicide than toluquinone, the exact ratio depending on experimental conditions. Notwithstanding, toluquinone is somewhat more diffusible through a membrane and more readily adsorbed by colloids than benzoquinone.
3. Alcohol increases the germicidal power of benzoquinone to a much greater extent than that of phenol and toluquinone. The interaction of benzoquinone and glycine is also greatly accelerated in the initial stages by alcohol.
4. Whilst there is relatively only a small difference in the *apparent* reactivity of the quinones towards yeast, bacteria, proteins, peptones, peptides, and even certain amino-acids, benzoquinone reacts up to 7 times as readily as toluquinone with other amino-acids.
5. Toluquinone is adsorbed to a somewhat greater extent than benzoquinone by charcoal and desamino-albumin. The difference, however, is not sufficiently great to justify the conclusion that adsorption is the primary factor in the protein experiments, and the evidence points to the conclusion that the

small differences in the uptake of benzoquinone and toluquinone by proteins have a structural explanation.

6. The germicidal power of the quinones is not necessarily due to their chemical interaction with the cell-proteins, but is to an important degree associated with their reactivity towards the simpler cell-constituents, such as certain of the amino-acids.

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