XVII. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART III. THE CHEMICAL ACTION OF QUINONE UPON PROTEINS.

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Blyth and Goodban [1907] found that when pure cresylic acid was exposed to light and air until it had become brown its germicidal power was measurably increased. This was probably due to the formation of derivatives of quinone through the aerial oxidation of the cresols.

Thalhimer and Palmer [1911] however could not detect any increase in germicidal power when phenol was exposed to light and air for some time.

Morgan and Cooper [1912] showed that some of the aromatic amines possessed a higher germicidal power when coloured by long standing than when purified by redistillation. It is probable that the increased germicidal power was due to the production of coloured quinone derivatives, which are known to be formed in the oxidation of aromatic amines.

Thalhimer and Palmer [1911] showed that quinone itself was a very efficient disinfectant and was superior to phenol, cresol, quinol, phenoquinone, and formalin in germicidal power.

The author [1912, 1] confirmed some of the latter results and showed further that quinone was more efficient as a germicide than the aliphatic ketone, acetone.

In previous communications [Cooper, 1912, 2; 1913] evidence was set forth which strongly suggested that the germicidal action of the phenols was due, not to a typical chemical union with the bacterial proteins, but to a de-emulsifying effect upon their colloidal suspensions. In view of the high bactericidal power of quinone it was of great interest to investigate the

relations of this ketone to proteins, in order to compare the mechanism of its germicidal action with that of the phenols and to arrive at a conclusion as to the possibility of a relationship between its germicidal efficiency and chemical reactivity.

Würster [1889] showed that when quinone was added to warm solutions of various amino-acids a red coloration was developed.

Raciborski [1907] showed that quinone gave a red coloration not only with amino-acids, but also with peptone and several proteins (egg-albumin, serum-albumin, fibrin, globulin, legumin, and nuclein). Toluquinone reacted similarly to quinone, and xyloquinone also gave a colour-reaction with proteins and peptone, but not with glycine and alanine. Phenanthraquinone and anthraquinone, on the other hand, gave no colour reactions with proteins. The authors put forward no explanation of the above phenomena.

THE EXPERIMENTAL RESULTS.

The investigation described in this paper is divided into three parts.

Part I deals with the relations of quinone to various proteins.

Part II deals with the nature of the chemical action, which quinone was found in Part I to exert upon proteins.

Part III deals with the relation of the chemical reactivity of quinone to its germicidal power.

I. *The relations of quinone to various proteins.*

Gelatin.

When strips of gelatin were immersed in aqueous solutions of quinone (0.1 per cent.) the protein rapidly developed an intense red colour, but retained its transparency. The reaction was irreversible, as the colour was not removed by prolonged boiling with water or absolute alcohol. The red colour was changed to green by the addition of alkali, but was restored by acidification. The altered gelatin was, furthermore, completely insoluble in hot water, and after immersion for 16 hours in quinone solutions the protein was no longer rendered opaque (precipitated) by phenol. Immersion for 30 minutes did not inhibit the precipitation.

Gelatin after immersion in 40 per cent. formalin for 12 hours was also insoluble in hot water and was no longer precipitated by 5 per cent. phenol, although the original gelatin was visibly affected by 2.5 per cent. phenol solutions.

These facts suggest that the quinone was not merely dissolved by the

gelatin, but had reacted chemically with the protein. The colour-reaction did not occur when the gelatin was immersed in solutions of quinone in absolute alcohol, and it only took place to a very small extent in 20 per cent. alcohol. Solutions of quinone in toluene also gave no coloration with gelatin.

Similarly, although Witte's peptone was coloured intensely red when suspended or dissolved in aqueous quinone solutions, no coloration was observed when it was suspended in an alcoholic solution of this substance.

The interpretation of these facts is probably that the quinone is *dissolved* in the gelatin and proteoses before the chemical reaction, so that an efficient external solvent for quinone (such as alcohol) by decreasing the uptake of this substance by the colloids can inhibit the colour-reaction. (See the effect of alcohol upon the distribution of phenol between water and proteins, Cooper [1912, 2].)

Alizarin, like anthraquinone, gave no coloration with proteins. When however gelatin was immersed in strong aqueous solutions of sodium alizarin-sulphonate (Alizarin red, $C_{14}H_5O_4(OH)_2SO_3Na$) it assumed an intense red colour, but, unlike gelatin treated with quinone, it was still readily soluble in hot water and precipitated by 5 per cent. phenol, and the colour was rapidly removed by washing with cold water.

Alizarin and its sulphonic derivative thus differed from quinone in not reacting with proteins, and this inactivity may explain the fact that a saturated solution (0.2 per cent.) of alizarin and a 1 per cent. solution of alizarin-red exerted no measurable germicidal action upon *Staphylococcus py. aur.*

Caseinogen.

(Merck's Casein—prepared according to Hammarsten.)

When immersed in aqueous solutions of quinone this protein assumed a purple colour, which was not removed by washing with water or boiling alcohol. Unlike the original caseinogen the coloured product (after being washed with water only) was insoluble in N/5 soda. Contact with the alkali, however, turned it green, but the purple colour was restored by acidification. The altered caseinogen was very slowly soluble in hot concentrated hydrochloric acid yielding a brown solution, and thus again differed from the original protein, which quickly dissolved in this acid forming a violet solution.

Egg-albumin.

As stated by Raciborski [1907] dialysed egg-albumin when mixed with aqueous solutions of quinone soon gave an intense red coloration. The

protein was still coagulated by heat and alcohol, red coagula being formed, which were not decolorised by prolonged washing with water and alcohol. The protein was also precipitated from the red solution by saturated ammonium sulphate. The precipitate was a red flocculent substance, readily soluble in water and becoming dark brown on standing. After this colour change the protein was frequently found to have become almost insoluble in water. The coagula obtained from the original red solution by means of heat, alcohol, and phenol remained permanently red.

Horse-Serum.

When aqueous solutions of quinone were added to horse-serum a red coloration rapidly developed. The serum-proteins were still coagulated by heat and precipitated by alcohol at first reversibly and finally irreversibly. The coagula were red and could not be decolorised by prolonged washing with alcohol or water.

By half-saturation of the red solution with ammonium sulphate a red flocculent precipitate was obtained, which, like the egg-albumin precipitated by ammonium sulphate from solutions containing quinone, became dark brown on standing. The precipitate with magnesium sulphate and the alcohol coagulum, on the other hand, resembled the egg-albumin coagula in remaining permanently red, but they soon became brown when immersed in a solution containing ammonium sulphate and quinone. The cause of these colour-changes could not be discovered.

By acidifying with acetic acid the filtrate from the precipitation with half-saturated ammonium sulphate of the serum containing quinone another red precipitate was obtained, corresponding to the albumin fraction from normal serum. The precipitate quickly redissolved on the addition of water.

It was not found possible to crystallize the quinone derivatives of serum and egg-albumin by the application of the usual methods.

Witte's Peptone.

The observation of Raciborski [1907] that solutions of Witte's peptone gave a red coloration with quinone was confirmed. Experiments were next carried out with a view to the isolation of the coloured products.

Witte's peptone has been separated into five constituents by the method of fractional precipitation with alcohol and salt described by Haslam. The following fractions of proteoses have been isolated in this way:

1. Insoluble in 50 per cent. alcohol and water. Crude hetero-proteose.
2. Insoluble in 50 per cent. alcohol and soluble in water.

- (1) α -proto-proteoses—precipitated by half-saturated $(\text{NH}_4)_2\text{SO}_4$.
- (2) α -deutero-proteoses—precipitated by saturated $(\text{NH}_4)_2\text{SO}_4$.
- 3. Soluble in 50 per cent. alcohol and soluble in water.
 - (1) β -proto-proteoses—precipitated by half-saturated $(\text{NH}_4)_2\text{SO}_4$.
 - (2) β -deutero-proteoses—precipitated by saturated $(\text{NH}_4)_2\text{SO}_4$.

By similarly fractionating the coloured liquid obtained by mixing solutions of quinone and Witte's peptone together it was possible to isolate corresponding precipitates all of which were highly coloured. Some of these products, however, did not differ merely in colour from the fractionated proteoses. Thus, while the alcoholic precipitate (α -proteoses and hetero-proteoses) obtained from an aqueous solution of Witte's peptone was readily soluble in warm anhydrous *m*-cresol, the corresponding fraction obtained from the aqueous solution after treatment with quinone was only slightly soluble.

Again, the α -proteoses after reacting with quinone were no longer precipitated by formaldehyde, although they were still precipitated by alcohol, mercuric chloride, and phosphotungstic acid. The significance of this difference is discussed later. The fractions obtained from Witte's peptone after treatment with quinone corresponding to the α - and β - proteoses gave the characteristic test for proteoses, namely a precipitate with nitric acid, soluble on warming and reappearing on cooling.

The main conclusion to be drawn from these results is that proteins isolated after treatment with quinone are permanently coloured and frequently changed in solubility and precipitability, and thus appear to be chemically altered. In the following pages the nature of this chemical change and the possibility of its relationship to the toxic action of quinone upon bacteria are discussed.

II. *The nature of the chemical action of quinone upon proteins.*

(i) *The colour reactions given by quinone with simple amines.*

A large number of amines readily gave colorations with quinone in aqueous solution at ordinary temperatures.

(ii) *The colour-reactions with imino-compounds.*

In cold aqueous solution quinone gave red colorations with methylaniline and di-amylamine, but not even on warming with succinimide, acetanilide, and uric acid. Under certain conditions quinone could thus react with substances containing the $=\text{NH}$ group.

(iii) *The inhibitory effect of formaldehyde upon the colour-reactions.*

The fact that quinone gives colorations with amino- and imino- compounds

suggests that the colour-reaction with proteins is due to a condensation of the ketonic groups of the quinone with their $-NH_2$ and $=NH$ groups.

Formaldehyde is known to react with amino- and imino- compounds by condensation with the $-NH_2$ and $=NH$ groups, forming methylene derivatives.

The colour reactions given by quinone with proteins, if due entirely to a similar condensation, should therefore be inhibited by the previous formalisation of these substances.

Substance			Nature of reaction		
Ammonia	...	...	Brown coloration.		
Ammonium sulphate	...	...	Purple	„	
Ethylamine	...	...	Violet	„	
Di-amylamine	...	...	Rose red	„	
Guanidine (carbonate)	...	...	Intense green coloration. (Hot solutions brown.)		
Glucosamine	...	...	Red coloration.		
Amino-antipyrine	...	...	Purple	„	
Tryptophane	...	...	Red	„	
Atoxyl	...	...	Red	„	
Aniline	...	...	Red	„	(followed by brown precipitate).
<i>o</i> -Phenylene-diamine	...	...			
<i>m</i> -Tolylene-diamine	...	...	Violet	„	„ „ „
β -Naphthylamine	...	...	Brown	„	
Tetra-hydro- β -naphthylamine	...	...	Brown oil.		
Pyridine	...	...	Dark yellow coloration passing to red.		
Succinanilamine	...	...	Red coloration (followed by red precipitate).		

It was actually found that in the case of egg-albumin, serum-proteins proteoses, glycocoll, glycyl-*l*-tyrosine, methylaniline, and di-amylamine the colour reactions with quinone were entirely inhibited by adding 40 per cent. formalin to the compounds either previously to or simultaneously with the addition of the quinone. Smaller amounts of formaldehyde (one to ten per cent.) added with the quinone were found to inhibit the colour-reaction partially.

Conversely, the α -proteoses present in Witte's peptone after treatment with quinone were no longer precipitated by formaldehyde, although they were still precipitable by alcohol, phosphotungstic acid, and mercuric chloride.

In the case of gelatin, however, formalisation did not inhibit the colour-reaction with quinone. This was also true in the case of ammonia, and here the absence of any inhibiting effect could not be due to incomplete interaction

2. The replacement of hydrogen attached to the benzene nucleus of quinone by amino-acid residues through the amino- or imino-groups forming compounds of the anilido-type (see above 1, 2, 3).

These two reactions might proceed simultaneously forming compounds analogous to di-anilido-quinone-di-anil.

(iv) *Experiments with quinone-dioxime.*

Attempts were next made to discover to which type of chemical reaction the red colorations given by quinone with proteins were due.

It was thought that experiments with quinone-dioxime could decide this question, because, although substitution in the ketonic groups naturally prevents their condensation with amino- and imino-compounds, it is known not to inhibit the entrance of these substances through their nitrogen atoms into other parts of the quinone nucleus.

Quinone-dioxime was prepared by the action of hydroxylamine hydrochloride upon quinone, the reaction being carried out in acid solution to prevent the reduction of the quinone to quinol.

It was found that aqueous solutions of quinone-dioxime gave no colorations with serum, gelatin, proteoses and alanine. The gelatin after immersion in the quinone-dioxime solution was found to be still soluble in hot water and precipitated by phenol as a white substance, and was thus not chemically altered.

These results strongly suggest that the colour-reaction given by proteins with quinone is due to the condensation of the $-NH_2$ or $=NH$ groups with the ketonic groups of the quinone, compounds similar to quinone-dianil being produced. This conclusion is supported by the fact that no oxime could be obtained by the treatment of the quinone-proteose compounds with hydroxylamine hydrochloride.

The chemical action of quinone upon proteins thus resembles that of formaldehyde.

(v) *The relations of acetone to proteins.*

Since it was found that quinone possessed a germicidal power more than 100 times as great as that of acetone [Cooper, 1912, 1] it was of interest to compare the effects of these two ketones on proteins.

Acetone was found to differ from quinone in exerting a precipitating action upon proteins, but while 0.1 per cent. solutions of quinone gave a colour-reaction with egg-albumin and gelatin, the albumin was not precipitated by aqueous solutions of acetone below 12 per cent. and gelatin was not even affected by immersion in 50 per cent. and 90 per cent. solutions.

The gelatin was still soluble in hot water and precipitable by phenol after this treatment, so that there was no evidence that it was chemically altered by the acetone. This ketone is therefore to be classed with the alcohols and phenols as a protein-precipitant.

III. *The relation of the chemical reactivity of quinone towards proteins to its germicidal power.*

The fact that as a germicide quinone is greatly superior to many other para-di-substitution products of benzene is seen from the following table [Cooper, 1912, 1 and Morgan and Cooper, 1912].

Substance	Organism	Carbolic coefficient
Quinone	<i>Staphylococcus py. aur.</i>	10
Benzaldehyde ...	<i>B. typhosus</i>	10
Quinol	"	1
<i>p</i> Cresol	<i>Staphylococcus py. aur.</i>	2·4
"	<i>B. typhosus</i>	2·6
Aniline	<i>Staphylococcus py. aur.</i>	0·5
"	<i>B. typhosus</i>	0·57
<i>p</i> -Toluidine ...	"	1·25
<i>p</i> -Phenylene-diamine	"	Under 0·3
<i>p</i> -Nitrophenol ...	<i>Staphylococcus py. aur.</i>	2·3

This itself suggests that the high germicidal power of quinone is associated with its chemical reactivity.

This conclusion is supported by certain other facts.

1. Not only is quinone superior to phenol, *p*-cresol, quinol, *p*-nitrophenol and acetone in germicidal power, but it can exert a chemical action upon proteins in concentrations (e.g. 0·1 per cent.) much lower than those in which the above substances can induce protein precipitation [Cooper, 1912, 2; 1913].

2. Benzaldehyde, which resembles quinone in its chemical action upon proteins, is also approximately equal to quinone in germicidal power, its carbolic coefficient with *B. typhosus* being 10.

There is thus some evidence that the mechanism of the germicidal action of quinone consists in a chemical interaction with some constituent protein or proteins of the bacteria essential for vitality, and not, as seems to be the case with the phenols, in a precipitating effect upon the colloidal suspension. The superiority of quinone as a germicide to various phenols and to acetone is sufficiently explained by the fact that it reacts with proteins in concentrations much lower than those in which the phenols and acetone exert a precipitating action.

SUMMARY.

1. The observations of Würster and Raciborski that quinone solutions gave a red coloration with various proteins and amino-acids have been confirmed.

2. The proteins (egg-albumin, proteins of horse serum, gelatin, Witte's peptone) could be isolated in the coloured condition from the red solutions by means of various precipitants and could not be decolorised by prolonged washing with water or alcohol. Other physical properties of the proteins, e.g. solubility, precipitability, were frequently changed as a result of the treatment with quinone, as is also the case when proteins react with formaldehyde. From these results it appeared that the proteins had become chemically altered by the quinone.

3. The colour-reaction did not occur when gelatin and proteoses were immersed in solutions of quinone in absolute alcohol. It would appear that the quinone was dissolved by the colloids before the chemical reaction, so that an efficient solvent for this ketone such as alcohol, by decreasing the uptake, could inhibit the colour-reaction.

4. The addition of sufficient formaldehyde to proteins, proteoses, amino-acids, and imino-compounds either before or simultaneously with the addition of the quinone completely inhibited the colour-reactions. Smaller amounts of formalin decreased the intensity of the red colorations. Gelatin, aniline, and ammonia however behaved exceptionally inasmuch as they still gave the colorations with the quinone after the addition of formalin. The positive results appeared not to be due to incomplete formalisation, since the isolated compounds of aniline and ammonia with formalin gave colour-reactions with quinone.

The inhibitory effect of formalin upon the colour-reactions given by quinone with certain proteins, with proteoses and amino-acids indicates that the latter react with quinone through their $-NH_2$ or $=NH$ groups.

5. Proteins, proteoses, and alanine gave no colour-reaction with quinone-dioxime, and no oxime could be prepared from the quinone-proteose compounds. This is presumptive evidence that the constituent $-NH_2$ or $=NH$ groups of the proteins and their hydrolytic products condense with the ketonic groups of the quinone. The chemical action of the latter upon proteins thus resembles that of formaldehyde.

6. Acetone differed from quinone in acting as a protein-precipitant.

7. There is some evidence that the germicidal power of quinone is due

to its chemical action upon some constituent protein or proteins of the bacterium essential for vitality and that the superiority of quinone as a germicide to phenol, quinol, and acetone is explained by its reactivity towards proteins in much lower concentration.

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REFERENCES.

- Blyth and Goodban (1907), *The Analyst*, May.
Cooper (1912, 1), *British Medical J.* **1**, 1234, 1293, 1359.
— (1912, 2), *Biochem. J.* **6**, 362.
— (1913), *Biochem. J.* **7**, 175.
Morgan and Cooper (1912), *8th Internat. Congr. Applied Chem.* **19**, 243.
Raciborski (1907), *Chem. Zentr.* **i**, 1595.
Thalhimer and Palmer (1911), *J. Infect. Diseases*, **9**, 172.
Würster (1889), *Chem. Zentr.* **i**, 392.