II. THE BACTERICIDAL ACTION OF THE NITROSO-COMPOUNDS.

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EARLIER investigations [Cooper, Woodhouse, and Forstner, 1925; Cooper and Forstner, 1926] have shown that the nitrosoanilines are much more effective germicides than the corresponding nitrosophenols. Nitrosoaniline, for example, is inhibitory in concentrations of 1 in 100,000, whilst with nitrosophenol concentrations of 1 in 20,000 are necessary to inhibit bacteria. Notwithstanding this activity it is characteristic of both types of compounds that their high germicidal power is only apparent in long period bactericidal tests, such as the inhibitory method which covers a period of 48 hours. In the ordinary short methods of measuring germicidal power ($\frac{1}{2}$ –3 hours) the nitroso-compounds reveal only a low efficiency.

The present investigation has been concerned with the two following problems.

1. The possible application of the nitrosoanilines as internal disinfectants.
2. The mechanism of their bactericidal activity.

1. The nitrosoanilines as disinfectants.

Nitrosoaniline and nitrosodimethylaniline have the disadvantage of being slightly soluble in water. The hydrochloride is readily soluble, but is much less efficacious. The methyl iodide addition product, on the other hand, is not very soluble, and is also much less effective than the free base.

The results in Table I illustrate these points.

Table I. *Inhibitory concentrations.*

B. coli, 48 hours at 37°

Nitrosodimethylaniline, 1 in 170,000
Nitrosodimethylaniline hydrochloride, 1 in 7,500
Nitrosodimethylaniline methiodide, >1 in 25,000

The loss in activity cannot be explained on the assumption of partition-changes between the phases within the cell caused by differences in solubility, as the readily soluble hydrochloride and the insoluble methiodide, which is, in fact, less soluble than the free base, are both weak germicides. It is concluded therefore that the fall in bactericidal power is associated with the change in

the valency of the amino-nitrogen to the quinquevalent state, and an essential condition for germicidal efficiency in the case of the nitrosoanilines is the presence of nitrogen in the tervalent state as in the amino- or substituted amino-group.

Nitrosodimethylaniline however is easily soluble in glycerol, propylamine, or formamide without significant loss in germicidal power, and the possibility of its clinical application is at present under investigation.

2. *The mechanism of bactericidal action.*

It has already been pointed out that the nitroso-compounds are slowly-acting germicides. In order to understand the nature of their bactericidal action, quantitative studies have been carried out to ascertain the reactivity of these substances towards amino-acids, proteins, lipins, and nucleic acids. These experiments involved the volumetric estimation of *p*-nitrosophenol and *p*-nitrosodimethylaniline in aqueous solution, and this was carried out by titration in acid solution with titanous chloride.

In general 30 cc. of the aqueous solution of the nitroso-compound was placed outside a viscose dialyser contained in a broad stoppered bottle. Inside the dialyser was placed 1 g. of the substance with which the solute was to react, *e.g.* protein, etc., either dissolved or suspended as the case might be in a measured volume of water. Equilibrium, *i.e.* maximum uptake of the nitroso-compound, was found in all cases to be complete within 72 hours. The initial and final concentrations being determined by analysis, the amount of nitroso-compound reacting or absorbed by 1 g. of the substance could be ascertained. The experiments were carried out at 20° (see Table II).

Table II. *Uptake of nitroso-compound % (i.e. % fall in concentration in aqueous phase) by 1 g.*

	(a) Gelatin	(b) Albumin	(c) Edestin	(d) Nucleic acid*
Nitrosophenol	4.9	3.5	4.2	9.7
Nitrosoaniline	5.0	—	0.0	46.0
Nitrosodimethylaniline	0.9	5.6	0.4	22.2

* The nucleic acid was first dissolved in dilute sodium hydroxide solution and the solution neutralised with acetic acid.

Consideration of the results shows that the nitroso-compounds are taken up in large amounts by nucleic acid, but only to a very small extent by proteins. Quantitative experiments showed that there was also no reaction at ordinary temperatures between nitroso-compounds and glycine, diamino-acids or a mixture of amino-acids from autolysed yeast. Furthermore, when solutions of nucleic acid and *p*-nitrosoaniline were mixed together and allowed to stand, a greenish black precipitate was gradually deposited, pointing to a chemical reaction between the two substances. The product was subjected to complete analysis with the following results:

C = 50.8, N = 14.2, H = 5.6 %.

The nucleic acid from which the substance was prepared had the composition:

$$C = 32.8, \quad N = 14.16, \quad H = 5.1 \, \%.$$

It is difficult to draw definite conclusions, but it is possible that the compound is a salt of the nitroso-base with the nucleic acid. The reaction between nitroso-compounds and nucleic acid proceeds with extreme slowness and is scarcely perceptible in a period of three hours, but becomes measurable in periods of from 24 to 72 hours.

There is thus a distinct analogy in the case of nitroso-compounds between the process of germicidal action and the reactivity with nucleic acid, an essential constituent of cell nuclear material and associated with the mechanism of growth. Such cell-constituents as proteins and amino-acids are not appreciably attacked by nitroso-compounds, and the bactericidal action of these slowly acting disinfectants would appear to depend on a gradual but effective interaction with the nuclear constituents. Phenols such as phenol, the cresols, dihydroxybenzenes, and chlorophenols have no precipitating action on the nucleic acids, their germicidal action being more associated with their denaturing action on proteins.

With the object of throwing further light on the problem the influence of concentration on the uptake of *p*-nitrosodimethylaniline by nucleic acid was studied by the dialysis method already described (Table III).

Table III. *Final concentration of p-nitrosodimethylaniline.*

(a) Nucleic acid g. per g.	(b) Water-phase g. per cc.	Distribution ratio <i>a/b</i>
0.01139	0.000797	14.3
0.01577	0.001078	14.5
0.02330	0.001594	14.5
0.02612	0.001885	13.8

The distribution ratio tends to approximate to a partition-coefficient, the base being as much as 14 times as soluble in nucleic acid as in water. The constancy of this ratio suggests that a considerable proportion of the base is merely dissolved in the nucleic acid and the amount in actual chemical union is relatively small.

In order that the *p*-nitrosodimethylaniline may gain access to the nuclear substance of the cells, ready solubility in the intervening cell-constituents is essential, and a series of experiments was therefore next carried out to determine the solubility of the base in a lipin, *e.g.* lecithin.

A stable lecithin emulsion was prepared by dissolving the lipin in ether and pouring the solution into excess of water, the ether then being removed by means of a current of air.

A 5 % emulsion could thus be prepared. The influence of concentration on the absorption of nitroso-compounds, and also of quinones and phenol, was investigated, the dialysing method being again employed (Table IV).

Table IV. *Final concentrations.*

(a) Lecithin g. per g.	(b) Water-phase g. per cc.	Distribution ratio <i>a/b</i>
Benzoquinone		
0.01101	0.005119	2.2
0.01061	0.004255	2.5
0.00901	0.003386	2.7
0.00793	0.002538	3.1
0.00619	0.001684	3.7
Toluquinone		
0.03060	0.005136	6.0
0.02487	0.004303	5.8
0.01592	0.003440	4.6
0.01194	0.002720	4.4
0.00448	0.001684	2.7
Nitrosophenol		
0.04106	0.003599	11.4
0.03367	0.003092	10.9
0.02590	0.002546	10.1
0.02122	0.001713	12.4
0.01246	0.001182	10.5
0.00646	0.000576	11.2
Nitrosodimethylaniline		
0.01778	0.001301	13.7
0.01119	0.000952	11.8
0.00901	0.000796	11.3
0.00659	0.000583	11.4
0.00428	0.000398	10.8
0.00165	0.000194	9.0
Phenol		
0.5141	0.05501	9.3
0.3758	0.04709	7.8
0.1821	0.03558	5.1
0.1741	0.02369	7.4
0.0910	0.01184	7.7
0.0396	0.00595	6.7

Inspection of the results indicates that the nitroso-compounds amongst the substances examined are most readily absorbed by the lecithin. In the case of *p*-nitrosophenol the distribution-ratio is almost constant over a wide range of concentration and the process is one of simple solution in the lipin, *p*-nitrosophenol being about 11 times as soluble in lecithin as in water.

The distribution-ratio of *p*-nitrosodimethylaniline increases somewhat with rise in concentration of 9.0–13.7, suggesting again a process of solution complicated by molecular association of the solute in the lipin. Similar results are obtained with phenol and toluquinone. With benzoquinone, however, the distribution-ratio falls with increasing concentration, suggesting that adsorption or surface-concentration predominates (Fig. 1). The constancy of the partition-coefficient in the case of nitrosophenol is of interest in view of the colloidal nature of the lipin phase.

Evidently the solubility of the substance in the lecithin is sufficiently high to obscure the surface-factor characteristic of colloidal systems. The results are analogous to those obtained in the case of the system water-phenol-protein [Cooper, *et al.*, 1912, 1923, 1927].

Fig. 1.

Fig. 2.

The nitroso-compounds and quinones had no precipitating action on the lecithin, but with phenol precipitation of the lipin as a viscous mass occurred at a phenol concentration of 1-2 %, and it is at this point that a change in the distribution-ratio took place, as in the case of proteins (Fig. 2).

The results with *p*-nitrosodimethylaniline and toluquinone suggest that these compounds undergo molecular association when dissolved in lecithin, and in Table V the distribution-ratios obtained by dividing the square-root of the concentration in the lipin phase are tabulated.

Table V.
Concentration in

Water phase (A) (g. per litre)	Lipin phase (B) (g. per 1000 g.)	Square-root of $B = C$	Distribution-ratio C/A
Toluquinone			
5.136	30.60	5.532	1.07
4.303	24.87	4.990	1.16
3.440	15.92	3.987	1.16
2.720	11.94	3.450	1.26
1.684	4.48	2.012	1.19
<i>p</i> -Nitrosodimethylaniline			
1.301	17.78	4.219	3.24
0.952	11.19	3.347	3.51
0.796	9.01	3.000	3.89
0.583	6.59	2.567	4.40
0.398	4.28	2.069	5.20
0.194	1.65	1.285	6.63

In the case of toluquinone, the new distribution-ratio is almost constant over a wide range of concentration, showing that toluquinone is bimolecular when dissolved in lecithin. On the other hand, this constancy is not observed with *p*-nitrosodimethylaniline, the distribution-ratio being abnormally large at low concentration. It is probable that the nitroso-compound undergoes molecular association in lecithin, but is at the same time adsorbed to a certain extent, thus causing the distribution-ratio to vary.

SUMMARY.

1. An essential condition for the bactericidal action of the nitrosoanilines is the maintenance of the amino-nitrogen in the tervalent state. The hydrochloride and methiodide are weak germicides.

2. The nitroso-compounds are slowly-acting disinfectants, exerting only a small bactericidal action in short periods, but this increases greatly in intensity in periods of 24-48 hours.

3. The nitroso-compounds have little or no action on amino-acids and proteins, but react gradually with nucleic acid forming a dark green insoluble product.

It is concluded therefore that the nitroso-compounds owe their slow germicidal action and marked inhibitory power to their gradual chemical interaction with the nuclear constituents of the cell, thus interfering with and retarding the biochemical mechanism of growth.

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