THE REACTIONS OF ANTIBACTERIAL SUBSTANCES WITH BACTERIA

PART I. METHODS USING NITROFURAZONE AND *AEROBACTER AEROGENES*

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THE interaction of antibacterial substances and bacteria may involve changes in the drug concentration and the production of degradation products. The precise measurements of these substances with nitrofurazone and *Aerobacter aerogenes* as models was attempted. They may be expected to add to our knowledge of the mechanism of action of antibacterial substances[1–4].

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) has been used in the treatment of a variety of localised infections[5] and in veterinary medicine[6]. It has been shown to be metabolised in rats[7], and by tissue slices[8–10], isolated enzymes[11–14] and bacteria[15,16].

EXPERIMENTAL METHODS

Materials

Nitrofurazone. Commercial material was recrystallised from dimethylformamide-ethanol solution to a constant ultra-violet absorption; m.pt. 238° C. with decomposition (Raffauf[17] gave 238° C.). A solution in water gave log ϵ 4·12 at λ max. 260 mμ and log ϵ 4·20 at λ max. 375 mμ. (Raffauf[17] gave log ϵ 4·12 and 4·20 respectively at these wavelengths.)

The culture medium was prepared from Analar reagents and was of the following composition:—KH_2PO_4 0·36, $MgSO_4$ 0·004, $(NH_4)_2SO_4$ 0·10, D-glucose 2·0 per cent. w/v in distilled water. The salts were dissolved in water, the pH was adjusted to 7·0 with sodium hydroxide and the solution sterilised. The glucose was dissolved in water, sterilised and added aseptically immediately before inoculation.

Organism. Aerobacter aerogenes was obtained from Dr. A. M. James; it was originally supplied by the N.C.T.C. (see Lowick and James[18]).

Preparation of the Bacterial Suspensions

A. aerogenes was grown at 40° C. for 16 hours with positive pressure aeration in 250 ml. quantities of medium. The culture was centrifuged at 13,000 r.p.m. for 4 minutes, the cells washed twice and resuspended in the medium either with or without glucose. The final volume of the suspension was adjusted so that, upon twentyfold dilution before exposure to the drug, the optical density at 500 mμ was about 0·65; this dilution corresponded to Brown's opacity tubes No. 3 and approximated to 900 $\times$ 10^6 viable organisms per ml.

When bacteria were to be separated before quantitative examination of the solution, exposure to the drug was in capped nylon centrifuge tubes for small volumes (less than 40 ml.) and in glass stoppered flasks for larger volumes. The test substance dissolved in distilled water was introduced into the exposure container immersed in a water bath at 40·0° C. At timed intervals after addition of the bacteria, aliquot parts of the suspension were withdrawn, centrifuged and the supernatant solution (suitably diluted if necessary) used for ultra-violet absorption and polarographic measurements.

Spectrophotometric Measurements

A Hilger Uvispek spectrophotometer was used. The slit width was opened to double the band width of the incident light and maximum sensitivity was used for measurements involving drug solutions containing suspended bacteria.

Measurements in the presence of bacterial suspensions. 1. In certain experiments, portions of the drug-bacteria suspension were transferred to the cuvettes of the spectrophotometer at intervals; the reference cuvette being filled with a comparable bacterial suspension without drug.

2. In determinations of reaction rates, the drug solution and an equal volume of water, for the reference suspension, were warmed to 40·0° C.; to each was added the same volume of a bacterial suspension and after rapid and thorough mixing the suspensions were transferred to matched cuvettes maintained at 40·0° C. thermostatically. With the constant density of bacterial suspension, matched 1 cm. cuvettes were used for a drug concentration of 10 μg. per ml., 5 mm. cuvettes for 20 μg. per ml. and 1 mm. cuvettes for 50 μg. per ml.

Polarographic Measurements

A Tinsley polarograph in conjunction with twin dropping mercury electrodes and mercury pool anodes was used with the technique previously described[19].

Sufficient Analar potassium chloride was added to give a final concentration of 0·1M in all solutions examined polarographically.

Preliminary Results

Ultra-violet absorption measurements. The ultra-violet absorption curve for nitrofurazone in culture medium diluted twentyfold at pH 7·0 is shown in Figure 1, curve 1; maxima were obtained at 260 mμ and at 375 mμ. Beer's Law was obeyed at 260, 275 and 400 mμ by solutions of the drug containing up to 10 μg. per ml., equivalent to a density of 0·8 at 375 mμ in a 1 cm. cell in distilled water and in diluted culture medium.

Dilute solutions of nitrofurazone slowly decompose. Light absorption measurements show that after 2 days' exposure to light or after 5 days when protected from light there was little decomposition, and that no uptake occurred upon glass surfaces or upon the nylon centrifuge tubes used.

Some of the spectral curves obtained by the measurement of the optical densities of the solutions produced by centrifuging aliquot parts of a bacterial suspension in medium without glucose in contact with 10 μg. per ml. nitrofurazone are recorded in Figure 1. The nitrofurazone peak at 375 mμ disappeared rapidly during the bacteria-drug contact, to be replaced by a peak at 330–335 mμ; the latter peak showed a hypochromic shift on prolonged standing at room temperature. The 260 mμ peak suffered a hypochromic shift and a small bathochromic shift to 270 mμ as the reaction proceeded, but prolonged contact gave a shift back to 260 mμ and a hypochromic effect as the 330 mμ peak became reduced.

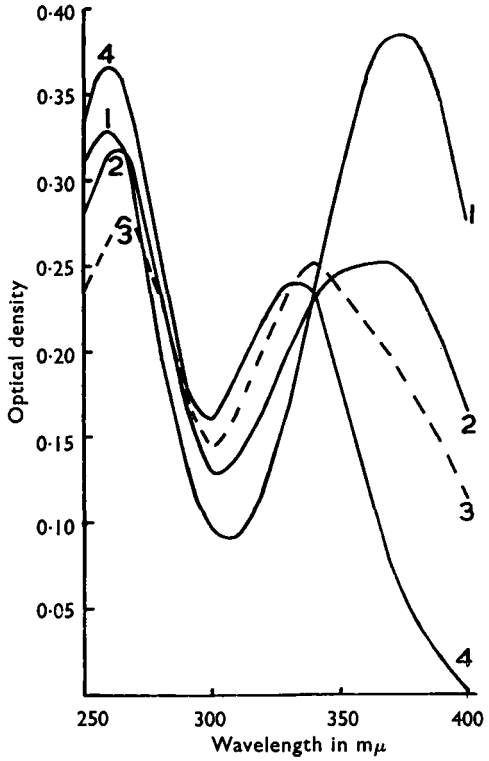


FIG. 1. Ultra-violet absorption curves of supernatant solutions obtained by contact of *A. aerogenes* with 10 μg. per ml. nitrofurazone in presence of culture medium (glucose omitted). The solutions were diluted 1 in 2 and the pH was 7·0 throughout. Curve 1, 5 μg. per ml. nitrofurazone (reference curve). Curves 2, 3 and 4 represent the solutions obtained after ½, 1 and 23½ hours respectively.

These results indicated that nitrofurazone gave a product with maxima at 330–335 mμ and 270 mμ which underwent further change to yield a product with a maximum at 260 mμ. Complete interpretation of these curves was complicated by the presence of unchanged drug as well as metabolic product, or products in some of the solutions and by the possibility of leakage from the cells of materials with absorption in the 260 mμ region[20–24]. Procedures involving separation of bacteria before examination of the solution were inconvenient for the determination of reaction rates, and for the preparation of solutions containing reaction products free from the last trace of nitrofurazone. A combination of ultra-violet and polarographic measurements was therefore adopted.

Polarography. Polarography has been used by Cramer[16] to measure decrease in nitrofurazone concentration upon metabolism. Sasaki[25] reported two reduction steps in aqueous solutions of nitrofurazone the first, reduction of the nitro group and the second, reduction of the

—CH=N⁻ bond in the side chain. Both half wave potentials varied with the pH of the solution, the former being within the range of an aromatic nitro group. Sasaki concluded that a 4 electron irreversible reduction to the hydroxylamino compound was effected. Polarography seemed to offer a suitable method to measure the metabolism if the nitro group only were involved, and also to check whether the semicarbazone chain had been affected.

Ultra-violet absorption measurements in the presence of bacteria. The problems involved in these measurements are discussed in the appendix.

Results

Polarography

Nitrofurazone in diluted medium without glucose at pH 7·0 showed two reduction steps (see Fig. 2). ($E_{\frac{1}{2}}$ — 0·35V. and — 1·35V. against mercury pool anode.) Plots of nitrofurazone concentration between 1–20 μg. against diffusion current gave a straight line calibration employing the first reduction step.

The buffering power of the medium in concentrations equivalent to those used in metabolism experiments was adequate in the absence of glucose.

The polarographic reduction of nitrofurazone was unaffected by the presence of eluate from *A. aerogenes*, or the metabolic product exhibiting an ultra-violet absorption peak at about 330 mμ.

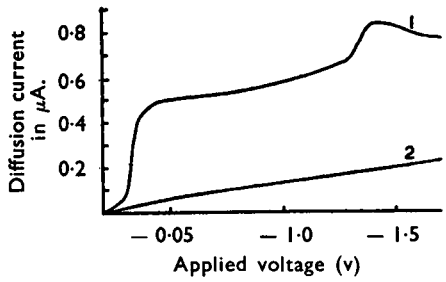


FIG. 2. 1. Polarogram of 10 μg. per ml. nitrofurazone in diluted culture medium (1 in 20) at pH 7·0 in presence of 0·1 M potassium chloride. 2. Residual current.

Using the twin capillaries with their separate calibration curves, and taking the precautions described in a previous paper[19], nitrofurazone could be determined at concentrations of 2–10 μg. per ml. in solutions equivalent to those obtained under biological conditions, with an accuracy of $\pm$ 3 per cent.

Combined Ultra-violet and Polarographic Determinations

The spectrum of the centrifuged solution measured after contact of *A. aerogenes* with 10 μg. per ml. nitrofurazone in presence of glucose-free medium is shown in Figure 3, curve 2. Simultaneously, the nitrofurazone content of a portion of the solution was determined polarographically. A solution of nitrofurazone, of concentration equal to that determined polarographically, was placed in the reference cuvette, and the ultra-violet absorption spectrum of the contact supernatant solution determined (Figure 3, curve 4). The curve of this nitrofurazone concentration is also shown (curve 3). The relatively low optical density at 375 mμ shown in curve 4, and the shape of this curve proves that curve 2, between 310–

400 mμ, represents the summation of the light absorbing properties of the unchanged nitrofurazone and its metabolic product exhibiting a peak (330–335 mμ) and reveals the light density additivities of these substances.

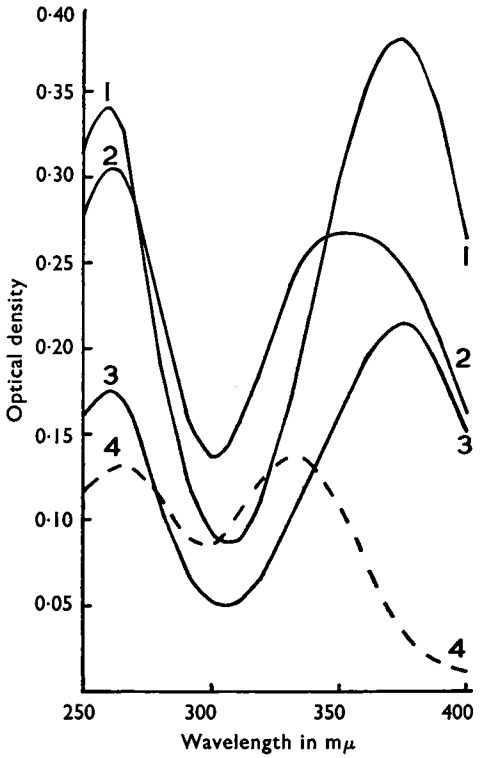


FIG. 3. Ultra-violet absorption curves from combined light absorption and polarographic measurements.

1. 5μg. per ml. nitrofurazone (reference curve).
2. Composite curve obtained after contact of *A. aerogenes* with 10 μg. per ml. nitrofurazone in presence of culture medium (glucose omitted) at pH 7·0 for 2 hours. The supernatant solution was diluted 1 in 2.
3. 2·7 μg. per ml. nitrofurazone, the concentration of drug found polarographically in the diluted (1 in 2) supernatant solution.
4. Absorption curve of the metabolic product obtained by placing 2·7 μg. per ml. nitrofurazone in the reference cuvette and measuring the absorption of the 2 hour supernatant solution described in 2.

The peak in the 260 mμ region in curve 2, probably represents the summation of light absorption of unchanged nitrofurazone, eluate from the cells, metabolic product exhibiting a peak at 330–335 mμ and the product (or products) of decomposition of the metabolite.

The characteristics of curve 4 between 360–400 mμ, especially in the region of 375 mμ illustrate that the concentration of unchanged nitrofurazone has been correctly determined, and that the experimental techniques adopted are valid. Curve 4, between 300–400 mμ, exhibiting an absorption maximum at 333 mμ thus represents the true light absorption in aqueous solution at pH 7·0 of the metabolic product obtained from the interaction of nitrofurazone with *A. aerogenes*.

Ultra-violet Absorption Measurement in the Nitrofurazone—A. aerogenes *System*

When the drug solution was mixed with the bacterial suspension in glucose-free medium and optical densities immediately measured at 375 and 500 mμ against an identical bacterial suspension (see experimental), the readings at 375 mμ were those expected for the concentration of drug present, and those at 500 mμ were zero. The supernatant solution obtained upon centrifuging the drug containing suspension gave identical readings at 375 mμ (water as reference). Consequently, negligible

quantities of drug are absorbed by the bacteria, and the light scattering properties of the latter remain unaltered after a brief duration of contact. Readings at 500 mμ remained virtually zero during 6 hours indicating no change in size, of character of the surface, or in the number of organisms during this period. Suspensions containing drug concentrations of 10, 20 and 50 μg. per ml. behaved similarly. A number of the factors, enumerted in the Appendix, which complicate measurements are therefore absent in the present system.

In the experiments in which measurements of the rate of metabolism of nitrofurazone in presence of bacterial suspensions were involved, measurements at wavelengths of 375, 400 and 500 mμ were made for the following reasons. The wavelength of 375 mμ is the position of the light absorption maximum of nitrofurazone. At pH 7, the metabolic product has negligible absorption in this region (see Fig. 4, curve 2). Reduction of density at this wavelength is a measure of the loss of drug from the solution provided that the density reading at 500 mμ remains zero. Development of acidity in the solution causes a bathochromic shift in the metabolic product which then has significant absorption in this region (Fig. 4, curve 1) and so invalidates the use of reduction in density as a measure of the loss of the drug. The wavelength of 400 mμ, although corresponding to a rather steep portion of the light absorption curve of the drug, proved to be useful for measurement because the metabolic product has negligible absorption even in acid solutions. The wavelength of 500 mμ was chosen as the point at which to check the scattering power of the bacteria—neither drug nor metabolic product absorbs in this region. In all experiments both with and without glucose, readings at 500 mμ remained virtually zero throughout, indicating that neither change in the characteristics of the bacteria nor any possible changes in their number had interfered with the observed optical density readings at 375 and 400 mμ.

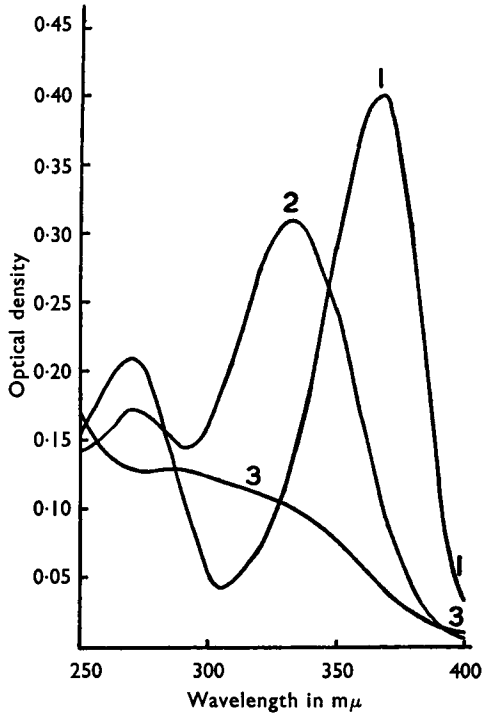


FIG. 4. Effect of pH change on the absorption curve of the metabolic product. 1. pH 3·0. 2. pH 7·0. 3. pH 10·8.

The validity of the method was checked occasionally by taking optical density measurements before and after centrifuging the above suspension, the readings from the supernatant solutions at 375 and 400 mμ (water as reference) were identical with those of the suspensions.

Ultra-violet absorption measurements in the presence of bacteria made possible the determination of the drug content in small samples (1 to 3 ml.) within 1 minute of withdrawal from a bulk suspension. The correct timing of centrifugation of the bulk to yield solutions at the desired stage of metabolism could be readily assessed. The studies of the rates of reaction of the drug and the bacteria under various conditions were facilitated by the use of thermostatically jacketed cuvettes. (Since these measurements can thus be accurately made within 45 seconds of mixing the drug with the suspension, and readings can be taken at 5 second intervals thereafter, a detailed study of the rapid reactions between drugs and bacteria becomes possible. Such studies will be reported in subsequent papers.)

Reactions of A. aerogenes *with Nitrofurazone*

Exposure of viable bacteria to the drug in concentrations of 10 μg. per ml. in glucose-free medium gave the results already described (see Figs. 1 and 3). The rate of metabolism of the drug varied greatly between experiments but in general it was relatively slow. In some, the nitrofurazone was incompletely metabolised even after a 20 hour contact period. With 50 μg. per ml., no trace of the metabolic product could be detected during 20 hour contact periods. A progressive hypochromic shift with increasing time between 300 and 400 mμ indicated that a slow process of adsorption (or absorption) was occurring. The uptake of drug was negligible in the first 10 minutes of contact (in contrast with the rate of adsorption of many drugs).

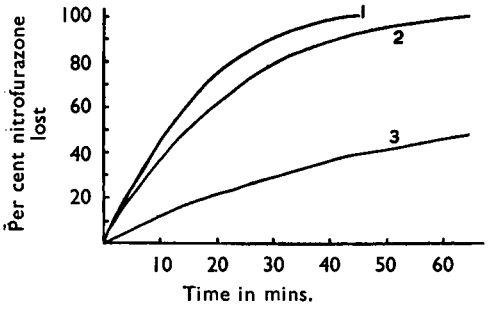


FIG. 5. Rate of loss of nitrofurazone in presence of *A. aerogenes* in culture medium at three drug concentrations:—1. 10 μg. per ml. 2. 20 μg. per ml. 3. 50μg. per ml.

In the presence of culture medium containing glucose, however, the rates of metabolism of nitrofurazone were rapid and much more reproducible, and higher concentrations could be metabolised. Figure 5 shows the rates of metabolism of solutions of nitrofurazone containing 10, 20 and 50 μg. per ml. The times required for the complete metabolism of 10 μg. under these conditions only varied between 50 and 90 minutes using suspensions prepared on different days from different cultures. The increased rate in the presence of glucose is in agreement with the work reported by Asnis and others[15] who showed that the rate of nitrofurazone

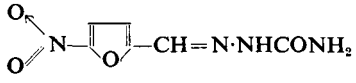
reduction by susceptible strains of *Escherichia coli* and *Staphylococcus aureus* was increased in presence of oxidisable substances.

Cells, after steaming for 20 minutes, produced little effect upon solutions containing 10 and 50 μg. of the drug during exposure periods of 2 hours. At the end of this period, the light absorption curves were identical in shape with those of nitrofurazone and the densities indicated that less than 3 per cent. of the drug had been adsorbed (or absorbed) by the bacteria.

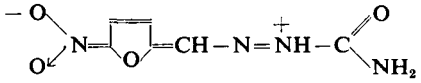
The above results are of a preliminary character to indicate the use of the physical techniques described in this paper. Further work is in progress on the effect of various conditions upon the uptake and metabolism of nitrofurazone. The question of the structure and reactions of the metabolic product, however, have been the subject of more detailed work described below.

The Structure and Reaction of the Metabolic Product

The absorption peak (λ max. 375 mμ log ϵ 4·20) of nitrofurazone (I) can be explained in terms of structure (II), in which there is the equivalent of 5 conjugated double bonds, being the chief contributing structure to the resonance hybrid (see Raffauf[17]).



(I)



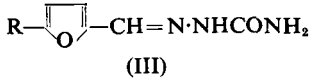
(II)

The ultra-violet absorption spectrum of the metabolic product in a solution in which the metabolism had just been completed (10 μg. per ml.; contact in absence of glucose) is shown in Figure 4, curve 2. It is reasonable to assume from the results of storage experiments of this metabolic product (Fig. 6), that a negligible quantity had decomposed during the time involved in its production. If its molecular weight is presumed to be approximately that of nitrofurazone, then log ϵ will be of the order of 4·1 at λ max. 333 mμ.

The intensity of the absorption, and the position of this absorption peak of the metabolic product, indicate that the furan ring and the semicarbazone side chain are intact in this compound, and that only the nitro group is implicated in the metabolism of nitrofurazone. This conclusion is supported by the results of the polarographic measurements which demonstrate that the typical reduction step of the nitro group disappears quantitatively in proportion to the disappearance of the 375 mμ absorption peak upon nitrofurazone metabolism, but that the reduction step attributable to reduction of the —CH=N— bond of the side chain is unaffected.

Biological reactions involving the nitro group could possibly lead to the removal of the group, the partial reduction and linking of two of the furan molecules *via* an azo group, or reduction to give a nitroso, hydroxylamino or amino group. That metabolism of nitrofurazone does not involve removal of the nitro group is demonstrated by the occurrence of the peak of furaldehyde semicarbazone at 293 mμ. Formulation of the

metabolic product as involving the azo-link is contra-indicated since such a compound would be expected to give an absorption maximum at wavelengths longer than the nitrofurazone peak and would be reducible polarographically. The formulation as a nitroso group is unacceptable on the polarographic evidence, since nitroso groups attached directly to aromatic systems have been shown to be reduced at lower potentials than corresponding nitro groups[26]. The formulation of the metabolic product as the hydroxylamino compound (III; R = $-NHOH$) or as the amino compound (III; R = $-NH_2$) is consistent with the ultra-violet and the polarographic data.



(III)

The metabolic product underwent a bathochromic and hyperchromic shift during some metabolic studies in the presence of glucose due to the medium becoming increasingly acid. The effect of changes of pH on the light absorption of solutions containing the metabolic product is shown in Figure 4. The spectral shifts affected by changes of pH between 8·0 and 3·2 were reversible, but the rate of reversibility was too rapid to measure.

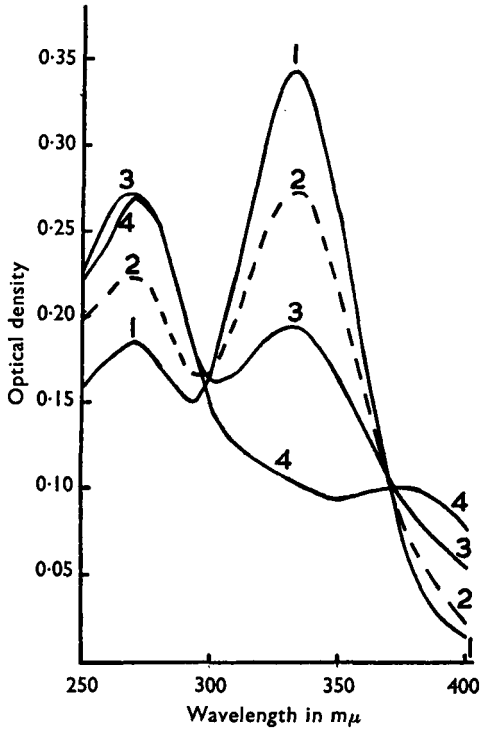
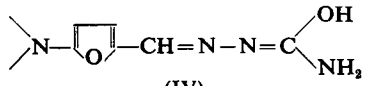


FIG. 6. Effect of storage at room temperature on the ultra-violet absorption curve of the supernatant solution obtained after contact of nitrofurazone with *A. aerogenes* in the presence of culture medium (glucose omitted) at pH 7·0. 1. Immediate readings. 2. 24 hours' storage. 3. 48 hours' storage. 4. 72 hours' storage.

The elimination of the absorption peak at more alkaline pH values (see Fig. 4, curve 3) was irreversible and required about 60 minutes at pH 10·8 at room temperature to complete the change shown. Curves obtained by plotting optical density at 335 and 370 mμ against changes of pH in the range where the effects were reversible, gave the value of approximately pH 4·5 (apparent dissociation constant pKa 4·5) as the mid-point of the change between the two molecular species responsible for the 333 and 370 mμ peaks.

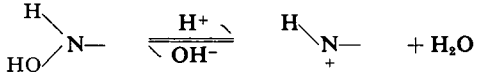
It seems reasonable to assume that the peak of 333 mμ may be attributed to the contribution of the molecular species IV (equivalent to 4 conjugated double bonds) to the resonance hybrid.



(IV)

The bathochromic shift to 370 mμ (presuming ionisation is involved because of the rapid reversibility) seems to indicate the improbability of the metabolic product having the amino structure (III; R = $-NH_2$), since the conversion of unionised amino group conjugated with an aromatic structure to the ionised form upon pH changes is known to give hypsochromic shifts. It is recognised, however, that the semicarbazone side chain attached to the furan ring might possess proton accepting properties although an increase in conjugation upon such a change seemed highly improbable. The absorption peaks of nitrofurazone and of furaldehyde semicarbazone in aqueous solution did not change in wavelength over the pH ranges of 2 to 10 and 2 to 6 respectively. The *p*-nitrobenzaldehyde semicarbazone peak (λ max. 325 mμ in water) was unaffected by pH changes in the range of 1·25 to 5·5 whereas the peak (λ max. 330 mμ in water) of *p*-dimethylaminobenzaldehyde semicarbazone underwent the expected hypsochromic shift of an aromatic amino compound to give λ max. 280 mμ at pH 2·1. If the amino structure (III; R = $-NH_2$) is accepted as the formula for the metabolic product, it must be presumed therefore that the furan nucleus differs from the benzene nucleus in the properties it confers to compounds of comparable structure.

It is equally difficult to explain the bathochromic shift exhibited by the metabolic product as the pH changes from neutral to acidic if the hydroxylamino compound (III; R = —NHOH) is accepted as the structure. The possibility was considered of the following reaction proceeding rapidly and reversibly with pH changes to give a free radical ion stabilised by conjugation



with the furan ring and side chain with consequent shift to longer wavelength at acid pH values. The peak (λ max. 230 mμ) of freshly prepared phenylhydroxylamine in water underwent a hypsochromic shift in solution at pH 3 so that its measurement at this pH using the present instrument was precluded. The proton acceptance by the N atom of this compound in acidic solution had reduced the conjugation as expected. Consequently the above formulated change for the hydroxylamine group in a furan compound seems improbable.

All attempts to prepare reference compounds by the chemical reduction of nitrofurazone have so far proved unsuccessful. Attempts to follow the ultra-violet absorption during contact of this compound with reducing agents under aqueous conditions have also failed to yield information of value in the solution of the problem. Until further evidence is available, the structure of the metabolic product remains in doubt, although certain reports favour the formulation of the product of metabolism of nitrofurazone by the xanthine oxidase-hypoxanthine system[11], or by certain bacteria, as the hydroxylamino compound (III; R = —NHOH)[14,15]

Stability of the Metabolic Product

The metabolic product in solution at pH 7 at room temperature gradually decomposed—reduction of the 333 mμ peak occurred with the simultaneous increase in the peak in the 260 mμ region (see Fig. 6). The rate of decomposition increased with rise of temperature. Acid solutions lead to a more rapid decomposition, while in alkaline solutions greater than pH 10, the decomposition occurred almost immediately.

The metabolic product is devoid of antibacterial activity (see also Asnis and others[15] and Cramer).

Appendix

Light Absorption Measurements in Presence of Bacterial Suspensions

Some of the problems involved in attempting to determine the light absorbing characteristics of organic molecules in solutions in which bacteria were suspended are as follows.

1. *The scattering spectrum of bacteria.* The scattering of the incident light by the bacteria will obviously contribute to the optical density. A typical curve obtained by plotting optical density against wavelength between 300 and 1000 mμ of a dilute washed suspension of *A. aerogenes* in distilled water is shown in Figure 7. The optical density of the bacterial suspension will be dependent partly upon the position of the cuvette in relation to the photometer, and partly on the aperture of the latter, since some of the forward scattered light will be included in the transmitted beam. At wavelengths from 220 to 300 mμ it has been shown[27] that the constituents of bacteria e.g., nucleic acids absorb incident light and that the observed densities are the sum of scattering by the cells and absorption by the cell constituents. A typical curve obtained for *A. aerogenes* is shown in Figure 8. The effect of variation of the age of the culture upon the characteristics of the scatter curve was not investigated because all experiments reported in this paper were carried out using a 16-hour culture.

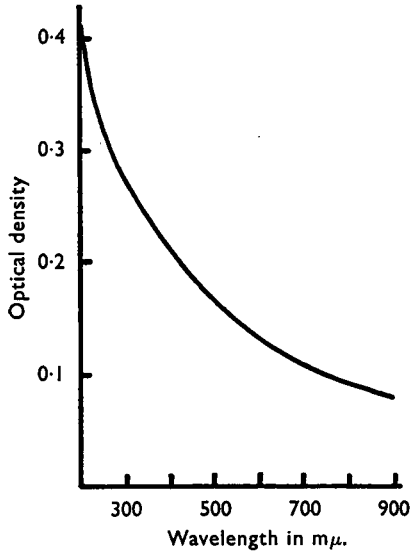


FIG. 7. Scattering spectrum of a 16 hour culture of *A. aerogenes* in distilled water.

The adsorption of, or chemical attack by, a drug upon a bacterial surface may alter the size of the organism or the characteristics of its surface considerably; such changes will be attended by significant

alteration of the scattering spectrum. We have found, for instance, that the adsorption of small amounts of certain phenols doubles the light scattering properties of the suspension immediately. On the other hand, adsorption of relatively large quantities of acridine-type compounds gives only a slight increase in the scattering power[28].

When compounds with negligible light absorption properties at wavelengths longer than 400 mμ are to be determined in bacterial suspensions, check measurements between 500 mμ and 1000 mμ will indicate (*a*) any change in the number of bacteria present in either the test or the reference suspension caused by the presence of either the complete culture medium or the drug or both, (*b*) any change in the light scattering properties caused by the drug. (If the light scattering properties are altered it is possible to extrapolate back from the scattering curves between 1000 mμ and 500 mμ to obtain a measure of scattering density in the region of light absorption by the drug).

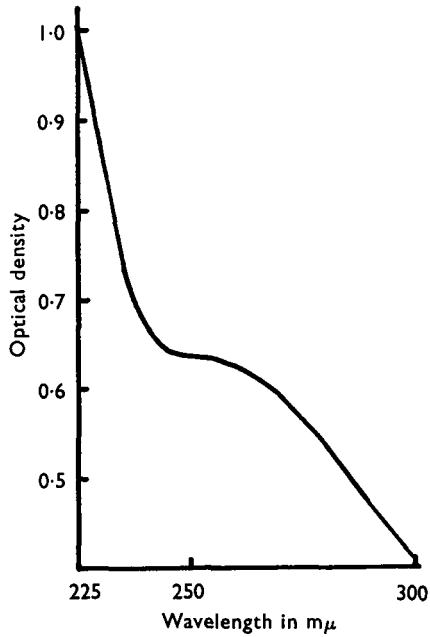


FIG. 8. Scattering and absorption spectrum of a 16 hour culture of *A. aerogenes* in distilled water.

Care must also be taken to ensure that sedimentation of the organisms does not occur during the course of an experiment.

2. *Adsorption of the drug.* If the drug is adsorbed upon the bacterial surface in significant amounts, shifts in the wavelengths of the absorption maxima or changes in the intensity of light absorption may occur. We have found, for instance, that there is a considerable reduction in the light absorbing properties of acridine molecules when they are "bound" upon bacterial surfaces.

3. *Measurements between* 200 *and* 300 *mμ.* The scattering of light by suspensions increases rapidly with decreasing wavelength, especially below 350 mμ. Consequently, only relatively dilute bacterial suspensions can be employed if measurements between 220 to 300 mμ are involved (see later, however). Secondly, if the uptake or metabolism of a drug is dependent upon the viability of the bacteria, it may be unwise to carry out repeated readings, since bacteria are known to be particularly sensitive to ultra-violet radiation at 260 to 270 mμ. Thirdly, it is known that many

agents in contact with bacteria cause a "leak" from the cells of substances exhibiting absorption maxima *ca.* 260 mμ[20–24].

4. *pH changes during experiments.* The absorption spectra of many organic molecules are affected by pH changes. Using live bacteria, pH changes may occur during an experiment with consequent complications in the composite light absorption pattern. Although buffered solutions are usually employed, metabolism in presence of growth media may yield acids in sufficient quantity to produce pH changes, e.g., in some experiments involving nitrofurazone and *A. aerogenes* in the presence of the growth media, the absorption peak of *ca.* 330 mμ underwent a gradual bathochromic shift with increasing time of drug-bacteria contact due to pH changes.

5. *Scattering density in relation to solution absorption density.* It has already been stated that only dilute bacterial suspensions may be used for readings at wavelengths lower than 350 mμ. Even under these conditions, the scattering spectrum may constitute such a high proportion of the observed light absorption under normal working conditions of the instrument, that accurate measurements of drug or metabolite concentrations become difficult. The use of an identical suspension of bacteria (omitting the drug) in the reference cuvette allows the use of higher drug concentrations and more accurate determination of their values. It was found, for instance that, in the 350 mμ region, densities equivalent to 1·5 against water could be used in the reference cell in this relative method when the slit width was opened to double the bandwidth of the incident light and maximum instrument sensitivity was employed.

It was found, under these conditions, using a heat killed suspension of *A. aerogenes* exhibiting a light density reading of 1·2 at 350 mμ (against water) in the reference cell, that Beer's Law was obeyed by nitrofurazone in concentrations equivalent to optical density readings of up to 0·8.

6. *Choice of wavelength for measurement of optical density in rate experiments.* Ideal wavelengths at which to take measurements would be (*a*) a few positions in the region in which incident light is scattered by the bacteria but not absorbed by the drug or metabolic product(s); (*b*) positions of light absorption maxima of the drug and of its metabolic product (or products); (*c*) the 260 mμ region in which light absorption by certain cell constituents released by the bacteria is exhibited. In practice, a compromise is usually necessary especially since there may be much overlapping of the spectra of the drug and its metabolic products. In rate experiments, the choice of wavelengths may also be influenced by the instrument working conditions, e.g., lamp and photocell changes must be avoided if the light absorbing systems are changing rapidly.

Summary

1. The application of spectrophotometric and polarographic methods of determination to the investigation of the reactions of nitrofurazone with *A. aerogenes* is described.

2. A method for the determination of organic molecules in bacterial suspensions is outlined, and some of the problems associated with such determinations are discussed.

3. The production of a nitrofurazone metabolic product by *A. aerogenes* is described and its structure investigated.

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DISCUSSION

The paper was presented by MISS A. E. ROBINSON.

MR. S. G. E. STEVENS (London) said that the authors had chosen a difficult chemical system, and it was not surprising that they had experienced trouble in isolating and identifying some of the reduction compounds. They quoted a shift of the peak from 375 to 335 mμ, and it might be of interest to record that by using dithionite or hydrazine the peak could be shifted to 305 mμ. There was no doubt that nitrofurazone was photosensitive and if a weak alcoholic solution of the compound was allowed to stand in the light, the 375 mμ peak was completely eliminated leaving the pattern shown in Figure 6. He found difficulty in accepting the statement that the absorption peaks of nitrofurazone and of furaldehyde semicarbazone in aqueous solution did not change over the pH ranges of 2 to 10 and 2 to 6 respectively, and suggested that there might have been a transposition of the values. Nitrofurazone developed an intense red colour in alkaline solution, and he felt that this was accompanied by a shift of the peak from 375 mμ. He wished to amplify the statement that nitrofurazone was stable in solution as there could be some misunderstanding. It was true that under the conditions of the authors'

test the solution could be considered stable, but one application of nitrofurazone in solution, namely, the treatment of coccidiosis in poultry where the method of administration was in the drinking water could, however, produce a different picture. Aqueous solutions were stable over the period during which the birds received their drink, provided that the drinking troughs were of good quality galvanised ware. If, however, old chipped and rusty tanks were used these would act as an electrolytic cell and there would be a very rapid breakdown of the nitrofurazone.

DR. J. C. PARKINSON (Brighton) said that on page 1073 the authors referred to comparable bacterial suspension made at time zero; but after reaction had taken place the comparable suspension would surely have grown, and if in the test suspension there were bacteriostasis, it would not be the same. The authors stated that the effect of variation of the age of culture upon the characteristics of the scatter curve was not investigated. Surely after a short lapse of time there would be a change, and then the test cuvette would not be the same as the reference cuvette? Would not that affect the measurements? It was stated that the metabolic product was devoid of antibacterial activity. It was rather a hypothetical substance and it would be interesting to know whether the authors meant that it was devoid of activity against *A. aerogenes* or whether it was tested against a large number of bacteria.

MR. K. A. LEES (London) asked the authors what motivated their choice of the culture medium used for growing the inoculum, and also the conditions, i.e., the high concentration of glucose used. It would also be of interest to have more details of the technique used for washing bacteria, and whether medium, saline or sterile water was used. The washing of bacteria could in general be regarded as a technique which left the bacteria in a state of shock, and that was possibly connected with the statement in the paper that the uptake of drug was negligible in the first 10 minutes of contact. How long were the washed bacteria allowed to remain before the addition of nitrofurazone, and was there any period of adaptation? Was the nitrofurazone reduced by non-sensitive bacteria?

MR. A. R. ROGERS (Brighton) referred to the structure of the metabolite and asked whether the authors considered that it might be a hydrazo compound. Chemical reduction methods had been tried, but the metabolite might be amenable to electrolytic reduction. It might be possible to isolate the metabolic product either by the cation exchange method, or by paper chromatography.

DR. K. R. CAPPER (London) said it would be interesting to know what was really happening to the organisms during the experiment. It was stated on page 1077 that the readings indicated no change in size, character or number of organisms; but did they remain viable, and was there any change which was not being detected? It would seem that with 50 μg./ml. no trace of metabolite could be detected, but one was dealing not with the antibacterial activity of nitrofurazone, but with the mechanisms of resistance. It would appear that there were susceptible strains and resistant strains. Did resistant strains show greater activity in metabolising

nitrofurazone than susceptible strains? Had the authors obtained exactly the same result when glucose was present and did their readings indicate that there was any change in the number of organisms during the six hour period?

MISS A. E. ROBINSON, in reply, said that it is known that chemical and bacterial reductions may proceed by different mechanisms, and care must be taken in the choice of an analogous chemical reduction, both routes being explicable in terms of accepted chemical theories. With regard to the effect of pH 10 on nitrofurazone solution, they used a phosphate buffer and no distinct red colour was observed. There was a slight colour change but no difference in the location of the peak of nitrofurazone. The readings were taken immediately, the solution not being allowed to stand. Spross, carrying out research on the breakdown of nitrofurazone in aqueous solution, found that it was affected by the presence of metals. With regard to the use of comparable bacterial suspension at time zero, the suspension would be similar in both cuvettes. The effect of incubation would possibly lead to growth, although the concentration of the culture medium was fairly weak; however, the fact that no difference in the ultra-violet absorption readings over a longer wavelength was observed seemed to indicate that there could have been no difference between the two systems after the various contact times. No growth could have occurred, nor could there have been any leakage of cell constituents. Tests had not been made on a range of bacteria, but earlier work had indicated that once the drug was reduced no further antibacterial activity was observed. The culture medium chosen was a simple one and was that used by Hinshelwood in his work. The concentration of glucose was considerably higher than one would perhaps expect, and there was a possibility of the organisms capsulating. The technique for washing bacteria was described in the paper. It was a centrifuging technique and the washing fluid was either the complete medium or the medium without glucose. No lapse of time occurred before the addition of the drug. With regard to the reduction by non-sensitive bacteria, reference should be made to the work of Asnis and others who used sensitive and resistant strains of bacteria and showed that a different rate of reduction occurred between organisms which were resistant and those which were susceptible. The possibility of the formation of hydrazo compound had not been considered. On looking at the possibility it was rather difficult to explain the effect of pH on the compound. If a proton were added to one of the two –NH– groups and two furan molecules were linked, one would expect the formation of two peaks beyond 300 mμ in the ultra-violet absorption spectra. In fact only one peak had been observed, and it seemed to contraindicate the possibility of that compound. Electrolytic reduction had been considered but had so far met with no success. With reference to Sasaki's work, the system was in fact irreversible, which prevented calculation of the electron change by normal means. They had obtained similar results to those of Sasaki. Isolation of the metabolite did not seem likely because of its instability, as was indicated in Figure 6. The rate of decomposition was

too rapid to permit the use of ion exchange and chromatographic methods at present. As to whether the bacteria were still viable, one might refer to Cramer's work, in which once the drug was reduced, growth did ensue in the normal way. The question on results obtained in the presence and absence of glucose could best be answered by the fact that in the presence of glucose the reaction was faster than if it were absent, and probably a reduction of the drug occurred before any growth was effected.

DR. A. H. BECKETT, in reply, emphasised that the authors were always checking the scattering spectrum at higher wavelengths between 500–1000 mμ. It had been shown that if constant checks were made throughout this range, the absence of development and of growth could be guaranteed. In every experiment quoted, such checks were made.