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THE SENSITIVITY OF *PROTEUS* TO NITROFURANTOIN *IN VITRO*

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Dodd and Stillman (1944) described the antibacterial activity of the nitrofurans, and nitrofurantoin was synthesized by the Eaton Laboratories in 1952. Since that time this substance has been given extensive clinical trials in the United States of America in infections of the urinary tract. Limited clinical trials were recently reported in this country (Heffernan, Kippax, and Pamplin, 1955), and arising from this work it was decided to make a more careful study of the sensitivity *in vitro* of *Proteus* strains to nitrofurantoin.

Method

A tube dilution method was used, employing urease activity as an index of growth.

Media and Dilutions.—The first medium tried was "oxoid" urease broth (Maslen, 1952). It was found impossible, however, to determine the solubility of nitrofurantoin in this medium, since precipitation occurred between that substance and some unknown constituent in the medium. At the suggestion of Messrs. Duncan, Flockhart and Co. Ltd. a simple "oxoid" phenol red peptone water with added urea was substituted. This medium was modified as follows: One hundred millilitres of medium were prepared according to manufacturers' instructions. This gave a reddish solution with a *p*H of about 7.4. Since Maslen's modification of Christensen's medium depends for its end-point on a change to alkaline in the medium, the *p*H of the phenol red peptone water was adjusted by adding 2.0 ml. N/10 HCl to each 98.0 ml. This gave an orange yellow colour and a *p*H of 6.5. After autoclaving at 15 lb. for 20 min., 5.0 ml. of sterile 40% urea solution was added to 100 ml. of this acidified solution. Since the resulting medium gave satisfactory results when inoculated with strains of *Proteus*, and since no precipitation was observed when nitrofurantoin was added to it, it was used in all tests described here.

A saturated solution of nitrofurantoin was prepared in the medium (by solution in the autoclave before the addition of urea) and this was used as stock.

A gravimetric estimation of the solubility of nitrofurantoin showed that this stock solution contained 43 mg. per 100 ml. Serial doubling dilutions of this solution were employed in 0.5 ml. amounts, from saturated down to 1/32, and a control row was included using medium without nitrofurantoin.

Inoculum.—Strains of *Proteus* to be tested were grown overnight in peptone water at 37° C. When turbidity fell, as it usually did, between Brown's tubes 2 and 3, the culture was used for preparing dilutions. If the culture was over-turbid, it was adjusted by dilution. Where turbidity fell short of this standard, the culture was not used until subculture had given satisfactory overnight growth. Serial tenfold dilutions of satisfactory cultures, from "neat" to 1 in 1,000,000 were prepared in peptone water.

The Test.—Seven rows of doubling dilutions of nitrofurantoin were prepared in cotton-wool-plugged tubes with 0.5 ml. amounts from saturated to 1 in 32. Each row was inoculated with a different dilution of the *Proteus* culture, starting with 1 in 1,000,000, to avoid carry over, and using 2 drops from a 30-dropper pipette as inoculum.

The entire series was incubated at 37° C. and observed every half-hour for six hours for change to a reddish colour, indicating production of ammonia by urease activity. A further final reading was made after 24 hours' total incubation. A tube of medium made alkaline by the addition of a few drops of ammonia was used as a standard.

Results

Four strains of *Proteus* were tested by the above method. One result is shown in Table I.

TABLE I

Nitrofurantoin dilutions	*Proteus* Dilutions						
	Neat	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000
Saturated	4·0	Negative at 24 hours					
1/2	2·5	24					
1/4	1·0	3·5	24	24	24	24	24
1/8	1·0	2·5	5·5	24	24	24	24
1/16	1·0	2·5	5·5	24	24	24	24
1/32	1·0	2·0	3·5	4·5	5·5	24	24
Control	1·0	2·0	2·5	3·5	4·5	5·0	5·5

Numbers show time in hours to colour change. Where tubes are shown "positive" at 24 hours they were negative at six hours; no readings being made in between.

Twenty-eight further strains of *Proteus* were therefore tested using a shortened series of culture dilutions, "neat," 1/10, and 1/100, and nitrofurantoin dilutions from saturated to 1/16.

The results of the first of these tests, carried out in triplicate on the same culture to check consistency, are shown in Table II At. the end of this test

TABLE II

Nitrofurantoin dilutions	*Proteus* Dilutions								
	Neat	1/10	1/100	Neat	1/10	1/100	Neat	1/10	1/100
Saturated	24	Negative at 24 hours		24	Negative at 24 hours		24	Negative at 24 hours	
1/2	3·5	24		3·5	24		3·5	24	
1/4	2·5	3·5	24	2·5	3·5	24	2·5	3·5	24
1/8	2·0	2·5	4·5	2·0	2·5	4·5	2·0	2·5	4·5
1/16	1·5	2·0	3·5	1·5	2·0	3·5	1·5	2·0	3·5
Control	1·5	2·0	3·0	1·5	2·0	3·0	1·5	2·0	3·0

Readings as in Table I.

(24 hours' incubation) 3.5 ml. phenol red peptone water was added to those tubes containing saturated nitrofurantoin which were negative at 24 hours, thus diluting below the effective level, and the tubes were incubated again for 24 hours and read again. Results are shown in Table III.

TABLE III

Nitrofurantoin Saturated, 0·5 ml.	*Proteus* Dilutions					
	1/10	1/100	1/10	1/100	1/10	1/100
3·5 ml. medium added after 24 hours, reincubated ..	Growth	Growth	Growth	No growth	No growth	No growth

Discussion

Inoculum-size Effect.—Results are believed to confirm the observations of Waisbren and Crowley (1955) that nitrofurantoin *in vitro* shows an inoculum-size effect. This must therefore be taken into account in testing the sensitivity of strains of *Proteus* to this substance.

Nature of Action.—Waisbren and Crowley (*loc. cit*) are of the opinion that nitrofurantoin is bactericidal. It is considered that the results presented here are more in keeping with those of Richards, Riss, Kass, and Finland (1955) and that results such as those shown in Table III (similar results were obtained with 27 other strains of *Proteus* in the course of these experiments) can best be explained by a bacteriostatic effect with random death of some of the smaller inocula during the period of bacteriostasis.

The Sensitivity Test.—A useful sensitivity test which takes into account the inoculum-size effect, gives an easily observed end-point, and obviates the need for dissolving the nitrofurantoin in special vehicles such as polyethylene glycol, can be set up by using a still shorter series of nitrofurantoin dilutions, inoculated only with "neat" *Proteus* culture (standardized between Brown's tubes 2 and 3) and reading after only six hours' incubation. Results to be expected from a test of this type are shown in Table IV, which is extracted from our

TABLE IV

Nitrofurantoin (Dilution from Stock)	Corresponding Concentration (mg./100 ml.)	Sensitive	Resistant
Saturated ..	40	28	Nil
1/2 ..	20	25	3
1/4 ..	10	6	22
1/8 ..	5·0	Nil	28
Control ..			

Numbers show numbers of strains inhibited for more than six hours by concentrations shown. Concentrations in round figures.

series of shortened tests on 28 strains. The extended test (Table I) is not included, but it would appear that the strain used for that test was relatively resistant.

It is suggested that an arbitrarily chosen strain of *Proteus* of known sensitivity should be introduced with each batch of tests as a standard.

There appears to be no reason why the test described should not be applied to other antibacterial agents to give a rapid estimate of the sensitivity of *Proteus*.

Summary

A test for determining the sensitivity of *Proteus* to nitrofurantoin is described.

The substance appears to be bacteriostatic and shows a pronounced inoculum-size effect. Sensitivities of 28 strains of *Proteus* are summarized.

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