FIG. 2. Recovery of insulin in the bile at different times after injection of different amounts of bovine insulin. Insulin was injected rapidly into the "abdominal vena cava" at time indicated by arrow. Each curve shows mean values for 3 different animals after insulin injection of 5.6 μg (0.14 IU) △———△; 2.8 μg (0.07 IU) ■———■; and 1.4 μg (0.035 IU) □———□. Insulin concentration expressed as bovine equivalents (potency 25 international units/mg).

nologically active insulin were recovered in the bile of rabbits. Detailed results of these studies will be published elsewhere.

Discussion. The results presented above show that heterologous insulin flows from plasma into the bile even at physiological concentration. In the first 30 minutes after i.v. injection of insulin a rapid increase in the concentration of hormone occurred in the bile and insulin continued to be eliminated through the bile in smaller amounts for several hours. The increased concentration of insulin in the bile coincides in time with the disappearance of plasmatic insulin according to Croughs(5) and also with an increase of the radioactivity when insulin-I^{131} is injected, which may be noted with surface detectors in the hepatic area(6).

These findings are in accordance with the hypothesis that labeled and unlabeled insulin can enter into the liver cell(7).

Summary. Large amounts of insulin appear in the bile of rabbits after i.v. administration of 2 mg (50 IU) of bovine insulin. Insulin was recovered from the bile also after the administration of heterologous insulin in low concentrations. The hormone concentration in the bile reached a peak in the first 30 minutes after i.v. injection of insulin, showing that the bile is avenue for the loss of insulin from the circulation.

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Effect of pH and of Urea on Nitrofurantoin Activity. (32246)

MARY F. PAUL, CATHERINE HARRINGTON, R. C. BENDER,*
ESTHER NOHLE AND MELVIN J. BRYSON†
Eaton Research Laboratories, Norwich Pharmacal Co., Norwich, N. Y.

The clinical effectiveness of nitrofurantoin (Furadantin; 1-[(5-nitro-furfurylidene) amino] hydantoin) in the treatment of various urinary tract infections has been reviewed (1,2). This chemotherapeutic agent has been found useful in the control of both Gram-positive and Gram-negative organisms, including some cases of *Proteus vulgaris* infections. In general, the results of treatment of Pseudomonas infections have not been encouraging(3) although occasional cases(4,5) have responded. It was called to our attention some time ago in a personal communication from Dr. Charles Norfleet that the effectiveness of nitrofurantoin was improved in Pseudomonas infections when an acidifying agent was administered concurrently. Richards *et al*(6) found that the activity of nitrofurantoin *in vitro* against *Escherichia coli* was greatest at pH 5.4 and considerably decreased at pH 8.1. Mou(7), reviewing the effect of urine pH on the antibacterial activity

* Present address: Dept. Surgery, Duke Univ. Medical Center, Durham, N. C.

† Present address: Dept. Obstetrics & Gynecology, Univ. of Utah College of Med., Salt Lake City.

of antibiotics and chemotherapeutic agents, stated that treatment of chronic infections of the urinary tract is most efficient if optimal conditions are provided for the activity of an antibacterial agent. He found nitrofurantoin activity to be significantly enhanced in an acid medium.

This paper is concerned with certain *in vitro* studies to determine the effect of pH and of urea on nitrofurantoin activity. The pH studies led us to investigate the influence of urea on the effectiveness of nitrofurantoin against *Pseudomonas aeruginosa*. Studies in rats were carried out to determine the effect of ammonium chloride ingestion and of high and low protein diets on nitrofurantoin excretion. The high and low protein diets were used as a simple *in vivo* method of producing different urea concentrations in the rat urines.

Methods and materials. Antibacterial activity. A cup plate procedure was used to demonstrate the effect of pH on the activity of nitrofurantoin against *E. coli, Staphylococcus aureus,* and *Pr. vulgaris*. Cup plates were prepared using Penassay seed agar and inoculated with the appropriate organism by streaking. McIlvaine's(8) standard buffer solutions of pH 5, 6, 7 and 8 were prepared. Nitrofurantoin was dissolved in N,N-dimethyl formamide in such concentration that 0.2 ml of the solution added to 25 ml buffer gave the drug concentration desired for cup plate testing. The solutions were transferred in 0.5 ml quantities to the cups or in 0.25 ml quantities when penicylinders were used. Activity was determined by measuring the annular zones of inhibition after 16-18 hours incubation at 37°C.

To determine the effect of pH on the activity of nitrofurantoin against *P. aeruginosa* it was necessary to use the more sensitive broth test, since nitrofurantoin activity could not be demonstrated against this organism in cup plate tests. To attain suitable concentrations of nitrofurantoin, it was dissolved in human urine at pH 8. Solutions were adjusted to lower pH values by addition of 2 N HCl. Sterilization was by Seitz filtration. Nitrofurantoin concentration was further adjusted by addition of similarly sterilized human urine at the same pH. Urine solutions of nitrofurantoin of appropriate pH were added to equal volumes of double strength nutrient broth in matched test tubes (18 × 150 mm). The nutrient broth was made up in double strength buffer (McIlvaine's(8)) at the pH selected. The tubes were inoculated with *P. aeruginosa* (Ps-10) and measurement of growth of organisms was made by determining the transmitted light at various time intervals with the Lumetron colorimeter (filter 580) using uninoculated tubes as controls.

The broth test was used for studies on the effect of urea but urine was omitted. Final urea concentrations of 1 to 4% were selected as being within the range observed in various urine samples. The urea was dissolved in single strength McIlvaine's buffer of pH 6.0 or pH 7.5. Urea was omitted from some tubes for control observations. Nitrofurantoin was added by shaking an excess with the buffer or buffer-urea solutions and then adjusting to 115 mg/l for the pH 6.0 studies, and 115 mg/l and 230 mg/l for the pH 7.5 studies. Sterilization of the buffer solutions was by Seitz filtration. To sterile test tubes (18 × 150 mm) containing 0.4 ml 20 × nutrient broth was added 7.6 ml of buffer-urea or buffer-urea-nitrofurantoin. The tubes were inoculated with *P. aeruginosa* and growth measured with the Lumetron colorimeter as above.

Animal studies. A group of 6 adult female albino rats (mean wt, 239 g) maintained on Animal Foundation ration was used in comparison with a similar group of animals receiving the same ration with 2% NH_4Cl added. After the animals had been on the diet for 3 days, test doses of nitrofurantoin (25 mg/kg) suspended in an aqueous solution of 0.75% sodium carboxymethylcellulose (Hercules) were administered to 3 of the animals in each group by gavage for nitrofurantoin excretion studies. Control animals received the same amount of the suspending agent. The diets were subsequently reversed and after a 2-day period, similar test doses of nitrofurantoin were given to the same animals. The Animal Foundation ration was chosen because we found it to result in a more alkaline urine than other colony diets.

Nitrofurantoin concentrations in urine were determined by a spectrophotometric procedure(9) using control urines for correction purposes and confirming the results by a bacterial cup plate procedure.

Studies with high and low protein diets were made using 6 adult female albino rats in each group. The two diets were as follows: high protein—casein 35, yeast 5, corn oil 3, cottonseed oil 3, salt mix (USP XIV) 4, USP cod liver oil 1, sucrose 49; low protein—same except casein 8, sucrose 75.7, d,l methionine 0.3. On either diet the pH range of the excreted urine was 6.0-6.8. The animals were kept on these diets for a week before the urinary excretion test. It was necessary to give $NaHCO_3$ in order to alkalinize urine for comparative studies. Bicarbonate was given just prior to the nitrofurantoin dose and again 4 hours later. Suitable control groups were kept on similar basal diets to determine the pH of urines in the absence of drug and to obtain control urine for correction use in nitrofurantoin assays(9). Nitrofurantoin was administered by gavage as described above. Urea was determined in duplicate by the microdiffusion method of Conway(10) on 24-hour pooled samples of urine for the 4 groups of animals.

For the 2-week feeding study, 16 female albino rats 3 months of age were divided into 4 groups of 4 animals each. Animal Foundation ration was the basal diet; additives were incorporated by careful mixing. Ammonium chloride was included in the diet of 2 groups at an amount (2%) which had been predetermined to maintain the urine at pH 6 or below. Nitrofurantoin was added to the basal diet with NH_4Cl for another group to provide about 10 × the human daily dose relative to body weight (4 × 100 mg divided by adult human body weight of 50-70 kg is 5.7 to 8 mg/kg human daily dose). Food consumption of all groups was recorded. Urine samples were collected from each group on 6 separate days at different times of day during the 2-week feeding period. The pH of the urine was recorded and the urine samples carefully examined for the presence of crystals. The animals were sacrificed at the end of 2 weeks and histologic preparations of the kidneys were made and examined for pathologic changes.

TABLE I. Effect of Solution pH on Nitrofurantoin Activity (Cup Plate Test).

pH of solution	Nitrofurantoin conc, mg/l	Zone width (mm) *E. coli*	*S. aureus*	*Pr. vulgaris*
5.0	400	*	*	4¾
	200	*	*	1¼
	100	9½	8¾	0
	50	8	6	0
	20	5¼	n.d.	0
6.0	400	*	*	4¼
	200	*	*	0
	100	9½	7½	0
	50	7½	3½	0
	20	4	0	0
7.0	400	*	*	2½
	200	*	*	0
	100	9	6½	0
	50	8	2	0
	20	2½	0	0
8.0	400	*	*	partial
	200	*	*	0
	100	9	5½	0
	50	8	partial	0
	20	0	0	0

See text for conditions.
* These concentrations are so high that they were not used for the more susceptible organisms.

Nitrofurantoin was the pure compound manufactured by Norwich Pharmacal Co. Nitrofurantoin is an ionizable compound (pKa 7.2) with a water solubility at 37°C of 155 mg/l at pH 5 and 650 mg/l at pH 7.5. It is somewhat more soluble in urine than in water at the same pH.

Results and discussion. In vitro. Table I shows the results of the cup plate study of the effect on nitrofurantoin activity against *E. coli, S. aureus,* and *Pr. vulgaris.* It is apparent that the pH of the solution in which nitrofurantoin was introduced into the cup plate had a demonstrable effect upon its antibacterial activity. Nitrofurantoin was much more active in solutions of lower pH. At pH 5, nitrofurantoin exists almost completely in the non-ionized and more active form, at pH 7 about 40% is ionized and at pH 8 about 86% is in the ionized (less active) state(12). In the case of *E. coli* or *S. aureus* this may not be of great practical importance since the compound is active against these two organisms at concentrations (*E. coli,* 3 mg/l; *S. aureus,* 12 mg/l) well below

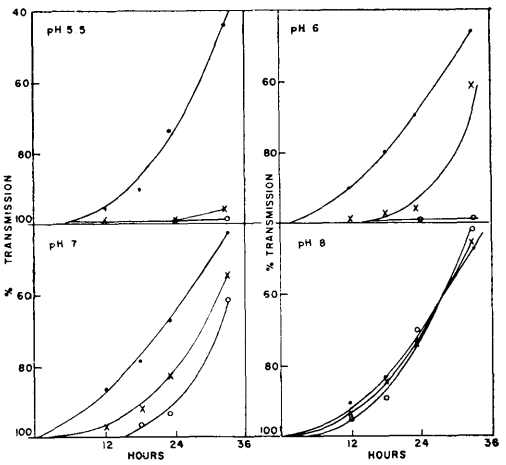

FIG. 1. Effect of pH of medium nitrofurantoin activity against *P. aeruginosa*. Inoculum for all tubes was 2 drops of a 14 hr culture of *P. aeruginosa* (Ps-10). ●———● Control medium - nutrient broth plus urine (1:1). X———X Same medium, nitrofurantoin 115 mg/l. O———O Same medium, nitrofurantoin 230 mg/l.

those attained in human urine. Richards *et al*(6) have reported nitrofurantoin concentrations of 250 to 500 mg/l of urine after 200 mg doses every 6 hours to an 80 kg patient. Beutner *et al*(13) have reported urine concentrations of 200 to 460 mg/l. In our laboratory, using a chemical procedure(14) we found maximum urine concentrations from 158 to 372 mg/l in 9 normal individuals each of whom received a 100 mg tablet of nitrofurantoin at 8 A.M., 12 M., 4 P.M. and 8 P.M. Urine was collected by each individual at 2-hour intervals after the first dose for a period of 12 hours and the 12-24-hour collection was composited.

The results of a typical experiment on the effect of pH on antibacterial activity of nitrofurantoin against *P. aeruginosa* in nutrient broth with added urine are shown in Fig. 1. Although growth of the organism in control tubes appeared to be best at pH 7, growth was satisfactory in control tubes from pH 5.5 to pH 8. The results could not be extended to pH 5 since organisms would not grow in control tubes buffered to this pH. Using broth at pH 5.5 or 6, growth inhibition by nitrofurantoin at 125 or 250 mg/l was practically complete over a 24-hour period. At pH 7 or 8 nitrofurantoin was much less effective as an antibacterial agent.

When we attempted to extend these studies to *in vivo* demonstrations of the activity of nitrofurantoin excreted in the urine of control rats and in the urine of rats receiving NH_4Cl as an acidifier, it became evident from the variability of our results that some factor in addition to pH was modifying the inhibitory effect of nitrofurantoin. It is known from the work of Krebs and Henseleit(11) that ammonium salts are readily transformed to urea by the liver. Analyses revealed rather wide variations in urea concentrations of different samples of urine.

The effect of urea concentration as well as pH on nitrofurantoin activity was investigated. Urea concentrations of 1 to 4% in nutrient broth (no urine) were selected as being within the range observed in various urine samples. To simplify the experiment the studies on urea were conducted at two pH levels: 6 and 7.5. At pH 6 the solubility of nitrofurantoin limited the attainable concentration to 115 mg/l. At pH 7.5 a higher concentration (230 mg/l) could be used. The results of a typical experiment appear in Fig. 2.

The effect of urea on the growth of *P. aeruginosa* is apparent in these experiments. Although slight inhibition by nitrofurantoin can be shown in the absence of urea, real suppression is obtained only when the pH of the broth is low (pH 6) and urea is present at 1% or more. It is apparent from Fig. 2 that differences in urea content of media could cause wide variations in antibacterial activity of urine samples containing similar amounts of nitrofurantoin and under controlled pH conditions. It will be noted (Fig. 1) that at pH 6 nitrofurantoin appears more effective than in Fig. 2 (no urine) at pH 6. Urine containing urea was present in the experiments depicted in Fig. 1.

Animal studies. Since the solubility of nitrofurantoin is decreased under conditions of lowered pH, the effect was investigated of feeding an acidifier on the attainable concentrations of nitrofurantoin in the urine and on the total excretion of the drug. Laboratory studies(15) had shown that about 30-

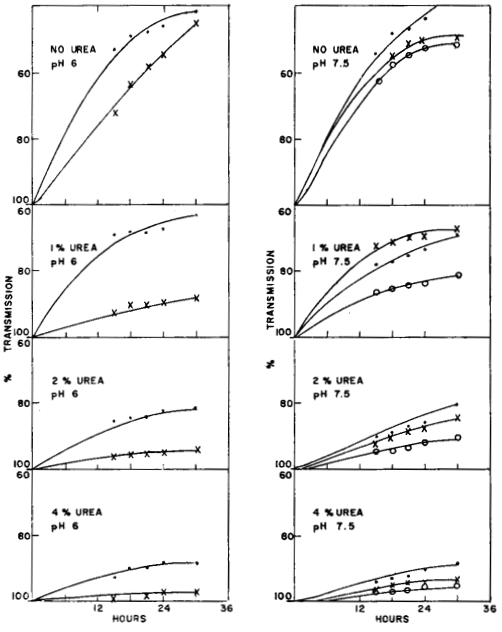

FIG. 2. Effect of urea content in pH 6 and 7.5 nutrient broth on nitrofurantoin activity against *P. aeruginosa*. Inoculum for all tubes was 1 drop of a 9 hr culture of *P. aeruginosa*. ●——● Control medium – nutrient broth – no urine. Urea added as indicated. ×——× Same medium, nitrofurantoin 115 mg/l. ○——○ Same medium, nitrofurantoin 230 mg/l.

50% of the administered dose of nitrofurantoin could be recovered in the urine of rats, dogs, or humans.

In Table II are shown the results of administration of single doses of nitrofurantoin in animals receiving control diet (Animal Foundation) or the same diet with 2% NH_4Cl added. Urine from the control animals was used for correction purposes(9) in the determination of nitrofurantoin. The nitrofurantoin assays were confirmed by a bacterial cup plate procedure. Such an assay would not show differences in antibacterial activity of the urine due to pH since the urines were all adjusted to pH 6.8 before assay.

Inspection of Table II reveals that the pH of the excreted urine of the rats receiving 2% NH_4Cl in the diet was maintained near 6, while on the control diet it ranged from 7.5 to 7.7. Although the amount of nitrofurantoin recovered in the urine is not much lower on the acidic diet than on the control diet, it probably reflects the finding that nitrofurantoin is more readily reabsorbed by the kidney tubules in the free acid form(16,17,18) than in the ionized salt form.

The major end product of protein metabolism is urea. Since the *in vitro* results had indicated that increased urea concentration in the urine might be beneficial in conjunc-

TABLE II. Effect of Dietary Ammonium Chloride on pH of Urine and Excretion of Nitrofurantoin by Albino Rats.

Group	Dietary addition	Dose nitrofurantoin, mg/kg*	Urine vol 0-4 hr, ml/rat	Urine pH 0-4 hr	Nitrofurantoin excreted,† mg/rat			Nitrofurantoin excreted 0-24 hr, % of dose
					0-4 hr	4-8 hr	8-24 hr	
Control	—	—	2.0	7.5	—	—	—	—
Nitrofurantoin	—	25	3.7	7.7	1.93 ± .24	.62 ± .11	.06	44
"	2% NH_4Cl	25	3.3	6.1	1.66 ± .11	.50 ± .09	.05	37
Control	2% NH_4Cl	—	2.7	5.9	—	—	—	—

* 5.95 mg/rat.

† These values are averages of nitrofurantoin analyses on individual urine collections from 6 rats for the 0-4 hr period and the 4-8 hr period with the standard errors. The value for the 8-24 hr period is the average value on 2 pooled collections from 3 rats.

A t value of 1.01 was obtained for the 0-4 hr period for the 2 groups compared. The t value required for significance is 2.228.

TABLE III. Effect of High and Low Protein Diets and of pH of Excreted Urine on Nitrofurantoin Excretion and on Urea Content of Urine.

Group	$NaHCO_3$ dose, mg/rat	Avg urine vol 0-4 hr, ml/rat	Urine pH 0-4 hr	Nitrofurantoin excreted,† mg/rat 0-4 hr	4-8 hr	8-24 hr	Urea conc in urine, g/100 ml	Nitrofurantoin excreted 0-24 hr, % of dose
I Low protein	—	2.0	6.5	1.83 ± .22	.70 ± .10	.41	2.2	47
II " "	2 × 60	2.4	7.5	2.22 ± .23	.57 ± .16	.12	1.0	46
III High "	—	2.3	6.3	1.92 ± .27	.60 ± .13	.24	4.1	44
IV " "	2 × 80	2.4	7.3	2.88 ± .29	.32 ± .05	.12	4.2	53

See *Methods* for diets and conditions. Six rats in each group, each rat received a dose of 25 mg/kg of nitrofurantoin (6.3 mg/rat) at zero time.

† See footnote to Table II for meaning of values.

The t value for Group I and Group III (low protein *vs* high protein) was .27 for the 0-4 hr period. The t value for Group II and Group IV was 1.756. The t value required for significance is 2.228.

tion with the antibacterial activity of nitrofurantoin, the effects of high and low protein diets on the urinary excretion of urea and of nitrofurantoin were studied. The results of this study appear in Table III. The high protein diet resulted in a higher urea concentration in the urine. Whether the diet was high in protein (about 37%) or low (about 9%), the nitrofurantoin excreted was not significantly different for Groups I and III or for Groups II and IV of Table III.

If the 0-4 hour nitrofurantoin excretion of the 18 rats with urine pH 7.3 to 7.7 from Table II and Table III are combined and compared with the nitrofurantoin excretion of 18 rats with urine pH 6.1-6.5, the average value and standard error for nitrofurantoin excreted by the rats with the alkaline urine is 2.34 mg ± .17 and that for the rats with the more acidic urine is 1.80 ± .12. The combined results are significant (t—2.62) and this confirms the observation that the ionized form of nitrofurantoin is less readily reabsorbed by the kidney tubules(16,17,18). However, the total percent excreted for the 3 groups (Table II and Table III) with the more alkaline urine was 48% of the administered nitrofurantoin and that for the 3 groups with the more acid urine was 43% of the administered nitrofurantoin.

The data on body weights, food consumption, nitrofurantoin and NH_4Cl intake (both calculated from food consumption records), and urine pH range of the rats and controls on the 2-week feeding study appear in Table IV. No loss in weight occurred over the feeding period in the animals receiving NH_4Cl or nitrofurantoin, although the weight gain was not as great as that of the controls. No crystals were observed in the urine samples of any group. The pathologist reported no significant differences between the kidney sections from the rats of any of the groups and no pathologic changes. Some slight changes in the groups receiving the NH_4Cl were attributed to a diuretic effect.

Summary and conclusions. 1. The apparent antibacterial activity of nitrofurantoin (pKa 7.2) against *E. coli, S. aureus,* and *Pr. vulgaris* is increased as the pH of the medium is lowered. 2. *P. aeruginosa* becomes susceptible to nitrofurantoin in the presence of 1%

TABLE IV. Effect of Feeding Nitrofurantoin and NH_4Cl to Albino Rats for Two Weeks.

Diet	Food consumed per rat per day, g	Nitrofurantoin per kg rat per day, mg	Ammonium chloride per kg rat per day, mg	Urine pH range	Wt at start, g	Wt at 2 wk, g
Basal	17.0	—	—	6.8–8.2	208	227
Basal + 2% NH_4Cl	19.6	—	1815	5.5–5.7	209	222
Basal + 0.1% nitrofurantoin	15.4	72	—	6.2–8.1	208	221
Basal + 2% NH_4Cl + 0.1% nitrofurantoin	18.1	87	1732	5.5–5.9	206	212

or more urea and in an acidic medium (pH 6). 3. Incorporation of 2% NH_4Cl as a urinary acidifier in the diet of rats did not have a major effect on the nitrofurantoin concentrations attained in the urine or on the total amount of nitrofurantoin excreted. 4. The ingestion of a high protein diet by albino rats to increase urea excretion did not adversely affect the urinary concentration nor the total amount of nitrofurantoin excreted. 5. Albino rats receiving 0.1% nitrofurantoin (about 10 × calculated human dose) and 2% NH_4Cl in the diet for a 2-week period showed no evidence of crystalluria or kidney pathology.

We wish to thank Dr. Meredith Gardiner for supervising the preparation of the kidney sections and examining them for pathology. We also wish to thank Dr. Paul Newland of the Medical Department for supervising the study in which the four 100 mg tablets of nitrofurantoin were administered to each of 9 normal individuals.

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Effect of Renal Ischemia on Anaerobic CO_2 Production by Dog Kidney *in vitro*.* (32247)

HOWARD M. RANDALL, JR.,† AND JULIUS J. COHEN
Department of Physiology, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

The studies of Trueta, Barclay, Daniel, Franklin and Pritchard(1) demonstrated that renal cortical ischemia may be produced by a variety of humoral and neurogenic phenomena such as: hemorrhage, catecholamine injection, tourniquet compression of a limb and electrical stimulation of the renal nerves (1,2). More recently, associated changes in specific functional and metabolic activities of the kidney have been identified: Gomori(3) reported that, in dogs subjected to electrical stimulation of the renal hilus while undergoing an osmotic diuresis, there were marked

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† Pre-Doctoral Fellow of USPHS-NIH. This report is part of the thesis submitted to the Graduate School of The University of Rochester in partial fulfillment of the requirements for the Ph. D. in Physiology. Present address: Dept. of Physiology, Louisiana State Univ., School of Med., New Orleans.