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Mode of Action of Nitrofurazone

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When *Escherichia coli* strain B/r is exposed to 10 to 20 μg of nitrofurazone per ml, mutants with roughly threefold resistance are obtained. Treatment of these mutants with higher concentrations of nitrofurazone yields strains with six- to sevenfold resistance over strain B/r. Each of these steps toward nitrofurazone resistance is accompanied by loss of soluble nitrofurazone reductase activity. When sensitive bacteria are exposed to labeled nitrofurazone or labeled 2-nitrofuran, a considerable amount of radioactivity becomes bound to the cold trichloroacetic acid-insoluble fraction. Very little activity becomes bound in the mutants with six- to seven-fold resistance; mutants with intermediate resistance show intermediate levels of binding. Partially purified nitrofurazone reductase preparations catalyze the conversion of nitrofurazone to compounds which bind to protein and are not removed by prolonged dialysis against 8 M urea or by cold acid. Nitrofurazone reduced by xanthine oxidase or electrolytically reduced also yields compounds which react with protein to form stable derivatives.

The antibacterial properties of nitrofurans were first recognized by groups in the United States (4) and Germany (3) during the 1940's. Since that time, certain derivatives have been widely used in clinical and veterinary medicine. More recently, these and related compounds have been shown to have other biological effects (6, 7, 14, 15, 18-20, 26-29).

Asnis showed some years ago that strains of bacteria which are sensitive to nitrofurazone contain a flavoprotein which catalyzes reduction of the drug (1). Since this reductase was not present in resistant strains, he concluded that it converts nitrofurazone to a compound which damages cells. In this paper we describe the isolation and properties of two types of nitrofurazone-resistant mutants. The first class has threefold resistance and is partially lacking in nitrofurazone reductase. Mutants of the second class are more highly resistant and are almost totally lacking in detectable soluble reductase activity. We also show that although ^{14}C-labeled nitrofurazone becomes bound to the cold-trichloroacetic acid-insoluble fraction of sensitive cells, little binding occurs in the most highly resistant mutants. Reduction of ^{14}C-nitrofurazone by bacterial nitrofurazone reductase or xanthine oxidase produces a compound which reacts with protein. Electrochemical reduction of nitrofurazone under controlled conditions also produces a compound which reacts with protein.

MATERIALS AND METHODS

Chemicals. 2-Nitrofuran was prepared by nitration of furan (5) and 5-nitro-2-furaldehyde from the diacetate (10). Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) was a gift from Norwich Pharmacal, Norwich, N.Y. Glucose-6-phosphate dehydrogenase and xanthine oxidase were obtained from Worthington Biochemical Corp. Pyridine nucleotides and bovine serum albumin were purchased from Sigma Chemical Co. Serum albumin was acetylated by the procedure of Fraenkel-Conrat et al. (8).

Preparation of labeled nitrofurans: ^{14}C-nitrofurazone. Nitrofuraldehyde was condensed with ^{14}C-labeled semicarbazide-hydrochloride (International Chemical and Nuclear Corp.) by using the standard procedure (22). The product had a specific activity of 2 mCi/mmole.

Tritiated 2-nitrofuran. A sample of pure 2-nitrofuran was tritiated by the Radiochemical Centre, Amersham, England. The material was returned in the form of an ethanolic solution which contained several radioactive compounds including a relatively small proportion of 2-nitrofuran. One milliliter of the ethanolic solution was added to 30 ml of benzene and the ethanol removed by fractional distillation. (Nitrofuran has a relatively high-vapor pressure and this procedure minimizes losses.) The benzene solution was applied to a silica gel column which was then eluted with benzene. Nitrofuran is the first major compound to emerge from the column. The benzene was carefully evaporated to yield 2-nitrofuran which appeared to be radiochemically pure when analyzed by thin-layer chromatography in several solvents. The specific activity of the tritiated 2-nitrofuran was approximately 20 mCi/mmole.

An alternative method of purifying labeled 2-nitrofuran was used when only small amounts of material were required. The mixture was chromatographed on a 0.75-mm layer of silica gel GF-240 on a thin-layer plate (20 by 20 cm) by using benzene as the solvent. The ultraviolet (UV)-absorbing band corresponding to nitrofuran was scraped off; the compound was extracted with hot ethanol, concentrated to about 2 ml, and stored as a stock solution.

Electrochemical reduction of nitrofurans. Nitrofurans were electrochemically reduced under nitrogen by means of current from a Sargent Coulombmetric Current Source (model IV). The cathode consisted of a 5-cm square platinum electrode, and the anode was a platinum reference electrode placed in a glass tube with a sintered glass bottom. Cathode potentials were measured against a calomal reference electrode by using a Fisher Accumet *p*H meter (model 210).

Bacteria. *Escherichia coli* B was obtained from the American Type Culture Collection as ATCC 11303. *Escherichia coli* strain B/r was obtained from A. O. Olson, Atomic Energy of Canada Limited, Chalk River, Ontario.

Bacteria were grown in Penassay Broth (Difco) at 37 C with shaking. The sensitivity of bacteria to various nitrofurans was determined by using the gradient plate technique of Szybalski and Bryson (24).

Measurement of reduction of nitrofurazone by intact E. coli. Stationary-phase cells were harvested, washed once with 0.066 M potassium phosphate buffer (*p*H 7.2) and resuspended in the same buffer. An appropriate amount of suspension was shaken in phosphate buffer containing 0.05 M glucose for 30 min at 37 C. Nitrofurazone was then added, and the incubation continued (2). At intervals, 5-ml samples were removed, centrifuged at 15,000 × *g* to remove cells, and the absorbance of the supernatant was determined at 375 nm.

Determination of protein. Protein was determined as described by the Lowry modification (13) of the Folin procedure. The absorbances were converted to milligrams of protein by reference to a standard curve prepared by using bovine serum albumin.

Preparation of cell-free extracts. Cultures (100 ml) of late-log-phase cells were harvested by centrifugation; the cells were washed and resuspended in 5 ml of 0.066 M potassium phosphate buffer (*p*H 7.2). The suspension was then chilled, sonically treated for one period of 30 sec and two periods of 15 sec each with full power from a Branson S 125 sonic oscillator, and then centrifuged for 45 min at about 10,000 × *g*. Extracts of all strains used contained 5 to 6 mg of protein per ml.

Preparation of nitrofurazone reductase. Cells from 4 liters of overnight culture were harvested, suspended in 60 ml of 0.066 M potassium phosphate buffer (*p*H 7.2), and sonicated for six periods of 30 sec each with full power from a Branson S 125 sonic oscillator. By using ammonium sulfate fractionation as described by Asnis (1), a 40 to 60% precipitable fraction containing nitrofurazone reductase activity was prepared. The precipitate was dissolved in 10 ml of phosphate buffer and is referred to as ammonium sulfate-purified enzyme.

Five milliliters of the above preparation was applied to a Sepharose 4B column (1.5 by 80 cm) equilibrated with 0.066 M potassium phosphate buffer (*p*H 7.2) at about 5 C. The column was then eluted with phosphate buffer, and the absorbancy and the nitrofurazone reductase activity of each fraction were determined. The reductase was eluted with 70 to 85 ml of buffer. Enzyme prepared in this way is referred to as Sepharose-purified.

Enzyme assays. Nitrofurazone reductase activity was routinely assayed in 0.066 M potassium phosphate buffer (*p*H 7.2) by determining the decrease in optical density at the absorption maximum of nitrofurazone (375 nm) in the presence of an reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisting of 20 μmoles glucose-6-phosphate, 4 units glucose-6-phosphate dehydrogenase and 0.1 mg of nicotinamide adenine dinucleotide phosphate (NADP) or NADPH. Typically, 30 μg of nitrofurazone was used in a final volume of 3 ml. The initial rates were not increased by doubling the concentration of nitrofurazone. There was a straight-line relationship between enzyme concentration and the rate of reduction up to 0.04 μmoles/min with crude extracts and up to at least 0.12 μmoles/min with ammonium sulfate-purified enzyme.

Determination of radioactivity. To determine the incorporation of radioactivity into bacteria or protein (16), small samples of the culture or reaction mixture were pipetted onto small filter-paper discs which were then placed in ice-cold 5% trichloroacetic acid; washed once in trichloroacetic acid, twice in ethanol, and once in acetone; dried; placed in scintillation vials; and counted in a Nuclear-Chicago Unilux I scintillation counter by using fluid containing 160 ml of Spectrofluor (Amersham/Searle Corp.) per gallon of toluene.

RESULTS AND DISCUSSION

As has been noted by previous workers (14, 28), treatment of *E. coli* B with nitrofurazone or other nitrofuran derivatives yielded mutants with about 10- to 20-fold resistance. These mutants were cross-resistant to UV radiation, having the sensitivity of *E. coli* B/r. Similarly, *E. coli* B/r is resistant to a variety of nitrofurans. This step in the acquisition of nitrofurazone resistance is not associated with the loss of nitrofurazone reductase activity and presumably is a consequence of the ability of B/r strains to survive damage in deoxyribonucleic acid (DNA) (11, 21).

When stationary-phase cultures of *E. coli* B/r were successively plated on media containing increasing concentrations of nitrofurazone, strains resistant to 40 to 50 μg of the drug per ml were easily obtained. We have obtained similar multistep mutants (e.g., *nfr*-40) when cultures mutagenized with nitrosoguanidine were used for the

original plating. Much of our early work was done with such strains, and some of the data thus obtained are included. However, we found that, when cultures of *E. coli* B/r were spread on nutrient agar plates containing 10 to 20 μg of nitrofurazone per ml, the few colonies which appeared were resistant to 12 to 15 μg of nitrofurazone per ml. Twenty-six independent mutants of this type were replated on nutrient agar, and cultures derived from a single colony of each were used for further work. These strains were numbered *nfr*-101 to *nfr*-126. When such strains were streaked on gradient plates containing nitrofurazone, a few individual colonies were found growing well beyond the area of confluent growth. These colonies were picked and tested, and all were found to have acquired resistance to between 35 and 45 μg of nitrofurazone per ml. These strains were numbered *nfr*-201 to *nfr*-226 (*nfr*-201 being derived from *nfr*-101 and so on).

Five strains of each series were selected arbitrarily for further study. Strains resistant to nitrofurazone showed cross resistance to other nitrofurans but were just as sensitive as B/r to unrelated agents such as nitrosoguanidine and streptomycin. Figure 1 shows that intact cells of strain B/r rapidly metabolized nitrofurazone, whereas the highly resistant strains metabolized nitrofurazone only about 20% as fast. Mutants with intermediate levels of resistance showed intermediate rates of metabolism of the drug.

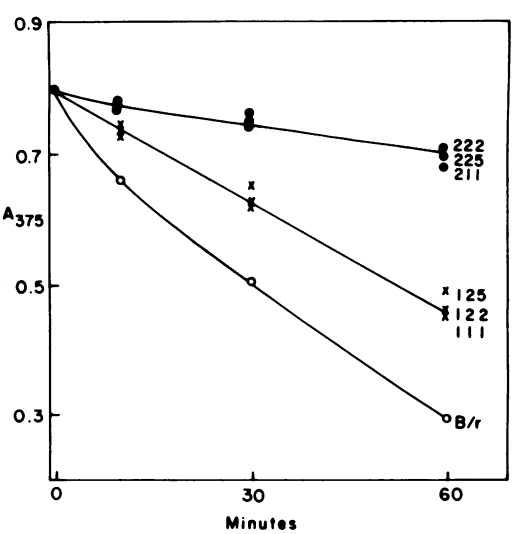

FIG. 1. *Reduction of nitrofurazone by intact cells of various strains of E. coli. Initial nitrofurazone concentration was 10 μg/ml. The concentration of the cell suspensions was adjusted to give a final absorbance (1 cm; 600 nm) of 0.60 (for all strains tested, this represents 0.38 ± 0.04 mg (dry weight)/ml and about 1.2 × 10^9 cells/ml). Reduction of 0.01 μmole of nitrofurazone results in a change in absorbancy at 375 nm (A_{375}) of −0.05.*

Figure 2 shows that crude extracts of *E. coli* strain B/r rapidly reduced nitrofurazone in the presence of NADPH. The rates of reduction of nitrofurazone by extracts of strains of the *nfr*-200 series were about 2% of that found in extracts of strain B/r, whereas strain *nfr*-40 contained about 6% of the B/r activity. Mutants of the *nfr*-100 series gave intermediate rates of reduction (35 to 45% of those obtained with B/r). Similar results were obtained when reduced nicotinamide adenine dinucleotide (NADH)-linked reduction was assayed except that, as previously noted by Asnis (1), the rate of reduction was only about 60% of that observed with NADPH.

When an extract of strain *nfr*-207 was mixed with an extract of strain B/r, the reductase activity was identical to that obtained when the *E. coli* B/r extract was diluted with a comparable volume of buffer. Thus, there is no evidence for the presence of inhibitors, at least in strain *nfr*-207. These observations strongly suggest that in these strains resistance to nitrofurazone is a direct consequence of loss of the reductase.

Table 1 summarizes results obtained during the partial purification of the reductase from *E. coli* B/r. A 10-fold increase in specific activity was obtained with an overall yield of 26%. The purest preparations had a specific activity of 0.33 μmole per min per mg of protein.

Figure 3 shows that preparations of B/r nitrofurazone reductase, partially purified by precipitation with 40 to 60% ammonium sulfate, catalyzed the rapid reduction of nitrofurazone, whereas the corresponding fraction from *nfr*-40 was very much less active. Addition of glucose-6-phosphate dehydrogenase to the partially purified enzyme from B/r increased the rate of reduction by a factor of almost 3, showing that the regeneration of NADPH is the rate-limiting step in such partially purified preparations with NADP or catalytic amounts of NADPH as cofactor. No stimulation was obtained when glucose-6-phosphate dehydrogenase was added to a similar preparation from *nfr*-40. The initial increase in the optical density of the assay mixture with *nfr*-40 extracts supplemented with the dehydrogenase is likely due to the accumulation of NADPH in the reaction mixture since this compound does have some absorption at 375 nm.

Nitrofurazone reductase preparations also reduce other nitrofuran derivatives of various structural types, including nitrofurantoin, 2-*N*-formyl-amino-(5-nitro-2-furyl)-thiazole, and 3-amino-6-[2-(5-nitro-2-furyl)-vinyl]-1,2,4-triazine

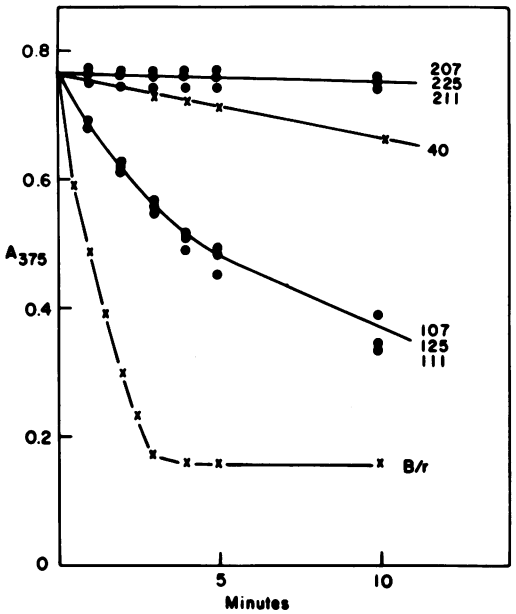

FIG. 2. *Reduction of nitrofurazone by cell-free extracts of various strains of E. coli. Each reaction mixture contained 25 μg of nitrofurazone, 0.1 mg of NADPH, 20 μmoles of glucose-6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, and cell-free extract containing 2.5 mg of protein in 3.2 ml of 0.066 M potassium phosphate buffer (pH 7.2). Reduction of 0.01 μmole of nitrofurazone results in a ΔA_{375} of −0.05.*

TABLE 1. *Purification of nitrofurazone reductase from E. coli B/r*

Stage	Total Activity		Amt of protein	Specific activity[a]	Purification (-fold)
	Units[b]	Percent			
			mg		
Crude extract.....	19.5	100	600	0.033	1.0
NH_4SO_4 precipitate.	17.8	91	100	0.18	5.5
Effluent from Sepharose 4B column.	5.15[c]	26	15.7[c]	0.33	10

[a] Expressed as units per milligram of protein.

[b] Expressed as micromoles of nitrofurazone reduced per minute.

[c] Calculated as to represent the activity and protein content which would have been obtained had the entire ammonium sulfate preparation been chromatographed on Sepharose (i.e., 2.5 times the observed values).

at rates comparable to that for nitrofurazone (Table 2). 2-Nitrofuran, however, is reduced much more slowly.

Intact cells of *nfr*-211, -222, -225, and related strains destroy nitrofurazone at a rate which is considerably higher in relation to the rate found for strain B/r (Fig. 1) than would be expected on the basis of their soluble nitrofurazone reductase activities. One explanation for this might be the presence of a sedimentable NADH-linked nitrofurazone reductase which we found to be present in crude extracts of all of the strains. This activity is solubilized by prolonged sonic oscillation and probably corresponds to the soluble NADH-linked enzyme reported by Asnis (1).

Reaction of ^{14}C-labeled nitrofurazone with intact cells. Figure 4 shows the kinetics of the binding of ^{14}C from nitrofurazone to the trichloroacetic acid-insoluble fraction of intact cells of strains B/r, *nfr*-124 and *nfr*-224. Data on the incorporation of ^{14}C-nitrofurazone into the trichloroacetic acid-insoluble fraction of other strains is given in Table 3. The most resistant strains bound only about 10 to 20% as much radioactivity as did strain B/r. Strains with intermediate levels of resistance were intermediate between the highly resistant and sensitive strains binding roughly half as much activity as did strain B/r. The curves shown in Figure 4 do not extrapolate back to zero counts per minute at zero time, suggesting that there was some

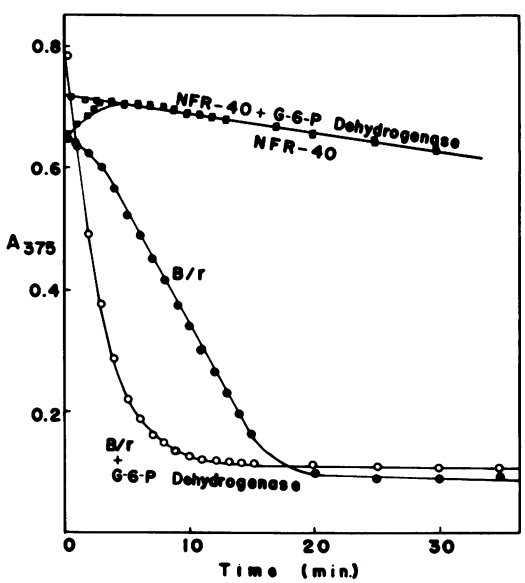

FIG. 3. *Reduction of nitrofurazone by ammonium sulfate-purified enzyme from sensitive and resistant strains of E. coli.* ●, ■, *Basic assay system similar to that described by Asnis (1). (Each reaction mixture contained 25 μg of nitrofurazone, 0.1 mg of NADP, 20 μmoles of glucose-6-phosphate (G-6-P), and 0.1 ml of the ammonium sulfate-purified nitrofurazone-reductase preparation in a final volume of 3 ml of 0.066 M potassium phosphate buffer (pH 7.2).)* ○, □, *Basic system plus four units of glucose-6-phosphate dehydrogenase. Reduction of 0.01 μmole of nitrofurazone results in a ΔA_{375} of −0.05.*

TABLE 2. *Reduction of various nitrofurans by Sepharose-purified nitrofurazone reductase*[a]

Compound	λ_{max}	Amt reduced/min	Rate relative to nitrofurazone
		μmoles	
Nitrofurazone	375	0.012	1.0
Nitrofurantoin [*N*-(5-nitro-2-furfurylidine)-1-aminohydantoin]	385	0.015	1.3
3-Amino-6-[2-(5-nitro-2-furyl)vinyl]-1,2,4-triazine	410	0.015	1.3
2-*N*-formylamino(5-nitro-2-furyl)thiazole	390	0.011	0.92
2-Nitrofuran	310	0.0005	0.04

[a] The standard assay mixture was used except that the final concentration of all the nitrofurans was 0.03 mM. Enzyme preparation equivalent to 0.04 mg of protein was used. The results are based on the initial rate of change in the absorbance of the solutions at the absorption maximum of each nitrofuran.

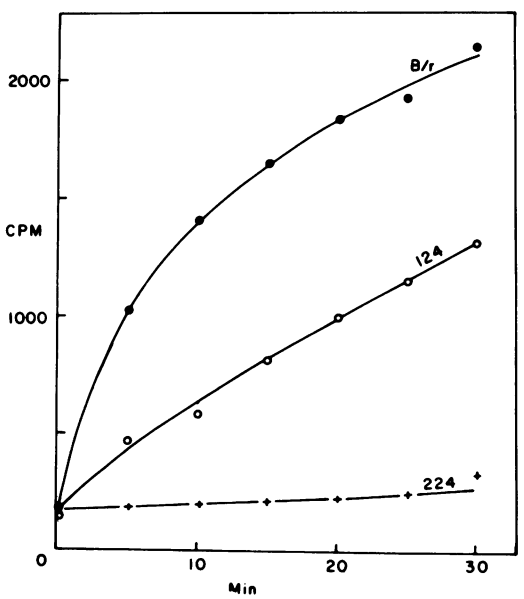

FIG. 4. *Incorporation of ^{14}C-nitrofurazone into the cold trichloroacetic acid-insoluble fraction of intact cells of various strains of E. coli. Cultures were grown to an absorbance of 0.80 (1 cm; 600 nm) representing about 1.6 × 10^8 cells/ml. Culture (2 ml) was added to 45 μg (0.45 μCi) of ^{14}C-nitrofurazone in 1 ml of water. Samples (50 μliters) were taken for counting. CPM = counts per minute.*

TABLE 3. *Binding of ^{14}C-nitrofurazone to the trichloroacetic acid-insoluble fraction of intact E. coli cells*[a]

Expt	Strain	Acid-insoluble material[b]	Per cent of *B/r* value
1	B/r	1,845	
	nfr-124	1,009	55
	nfr-224	230	12
2	B/r	2,510	
	nfr-111	1,139	45
	nfr-125	1,212	48
	nfr-211	452	18
	nfr-225	416	16
3	B/r	3,800	
	nfr-40	420	11

[a] See Fig. 4 for experimental details.
[b] Expressed as counts per minute bound in 20 min.

adsorption of labeled material to cell constituents. The amount of such adsorption was low relative to the amount which became bound by sensitive strains. However, adsorbed radioactivity does appear to account for much of the label associated with cells of the *nfr*-200 series. The amount of ^{14}C bound to strain B/r represents of the order of 50 million molecules of nitrofurazone per cell.

Strain B/r cells labeled with ^{14}C-nitrofurazone as described in Fig. 4 were collected, washed, sonically treated in 0.066 M potassium phosphate buffer (*p*H 7.2); the resulting suspension was centrifuged at 10,000 × *g* for 30 min. The trichloroacetic acid-insoluble radioactivity in samples of the extract treated for 1 hr with 1 mg of Pronase per ml was only 40% of that obtained from control samples (not exposed to Pronase). Treatment with ribonuclease and deoxyribonuclease did not result in a measurable decrease in acid-insoluble counts. This result provides tentative evidence that much of the radioactivity bound to cellular material is associated with protein. This labeling was not merely the result of incorporation of amino acids containing label from degradation products of the nitrofurazone into protein, since the amount of radioactivity which became associated with protein was not decreased by 20 μg of chloramphenicol per ml, a concentration which reduced leucine incorporation to less than 11% of the control rate.

Because the labeled nitrofurazone available contained ^{14}C in the semicarbazide moiety and not in the furan ring, it was thought desirable to test the incorporation of some other ring-labeled compound. Since 2-nitrofuran is known to have

antibacterial properties (4), a tritiated sample of this material was prepared. Figure 5 shows that radioactivity from ^{3}H-2-nitrofuran becomes incorporated into intact cells. The difference between the B/r and *nfr*-40 strains was not so marked as with nitrofurazone; nevertheless, however, strain B/r incorporated two to three times as much ^{3}H as did strain *nfr*-40. However, since strain *nfr*-40 is only 1.5 to 2 times as resistant to 2-nitrofuran as strain B/r (*unpublished data*), a large difference in the rates of incorporation might not be expected.

Enzymatic "activation" of nitrofurazone. When ^{14}C-nitrofurazone was incubated with cell-free extracts supplemented with NADPH or NADH, radioactivity became bound to the trichloroacetic acid-insoluble fraction of the extract. In the absence of reduced pyridine nucleotides and the generating system, the amount of radioactivity bound was small and did not increase with time (Table 4). The rate of labeling depended on the strain, being most rapid in B/r, intermediate in the *nfr*-100 series, and low in cells of the *nfr*-200 series and in *nfr*-40 (Tables 4 and 5). Enzyme preparations partially purified by ammonium sulfate precipitation and Sepharose chromatography also catalyzed incorporation of radioactivity from nitrofurazone into protein (Table 6).

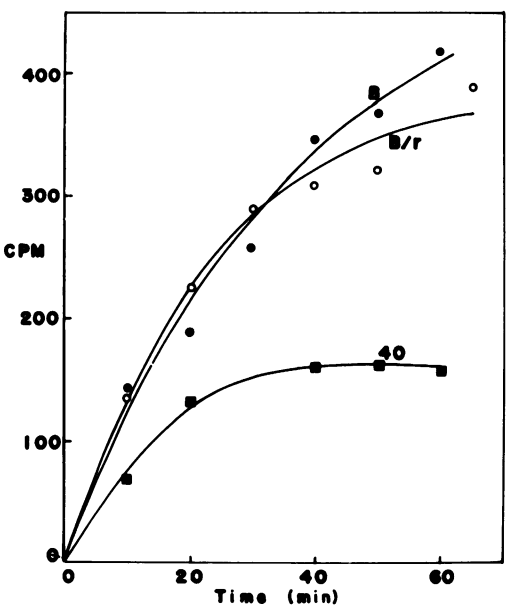

FIG. 5. *Incorporation of [^{3}H]nitrofuran into the cold trichloroacetic acid-insoluble fraction of intact cells. Nitrofuran [17 μg (3 μCi)] was used. For other details, see legend of Fig. 4.*

TABLE 4. *Binding of ^{14}C-nitrofurazone to trichloroacetic acid-insoluble material by cell-free extracts of various strains in the presence and absence of NADH*[a]

Strain	NADH and generating system	Counts/min after		
		1 min	4 min	7 min
B/r	+	633	1,099	1,598
B/r	−	216	233	181
nfr-107	+	458	697	966
nfr-107	−	179	243	196
nfr-207	+	288	355	421
nfr-207	−	223	172	258

[a] Complete reaction mixtures contained 0.1 mg of reduced nicotinamide adenine dinucleotide (NADH), 4 units of glucose-6-phosphate dehydrogenase, 20 μmoles of glucose-6-phosphate, 50 μg (0.5 μCi) of ^{14}C-nitrofurazone, and 0.5 ml of cell-free extract (2.5 mg of protein) in a final volume of 3.2 ml. Samples (50 μliters) were taken for counting.

TABLE 5. *Binding of ^{14}C from ^{14}C-nitrofurazone to trichloroacetic acid-insoluble material by cell-free extracts of E. coli*[a]

Expt	Strain	Acid-insoluble material[b]	Per cent of B/r value
1	B/r	1,012	
	107	378	37
	207	159	15
2	B/r	980	
	122	350	36
	222	130	17
3	B/r	1,206	
	40	200	17
4	B/r	977	
	122	468	48
	222	169	17
5	B/r	1,020	
	107	445	44
	207	175	17

[a] Details as for Table 4 except that NADPH was used instead of NADH.

[b] Expressed as counts per minute bound in 4 min.

Maximum incorporation was dependent on the availability of NADPH, although some counts did become bound to protein in the absence of NADPH or in the absence of the enzyme, again suggesting that some adsorption takes place. When relatively large amounts of reductase at the

TABLE 6. *Labeling of protein by "activated" ^{14}C-nitrofurazone*[a]

Expt	Enzyme	System	Acid-insoluble material[b]	
			10 min	60 min
1	Ammonium sulfate	Complete	1,407	1,682
		Minus enzyme	131	131
		Minus NADPH and glucose-6-phosphate	155	135
		Minus serum albumin	1,257	1,510
2	Sepharose purified	Complete	380	
		Minus serum albumin	115	

[a] In experiment 1 the complete reaction mixture contained: 4 mg of serum albumin, 0.1 mg of NADPH, 20 μmoles of glucose-6-phosphate, 0.5 ml of ammonium sulfate purified enzyme (about 2.5 mg of protein), and 50 μg (0.5 μCi) of ^{14}C-nitrofurazone in a final volume of 2.6 ml. In experiment 2, the complete reaction mixture contained 5 mg of serum albumin, Sepharose-purified enzyme (0.05 mg of protein), 1 mg of NADPH, and 50 μg (0.5 μCi) of ^{14}C-nitrofurazone in a final volume of 2.6 ml. Samples (10 μliters) were taken for counting.

[b] Counts per minute bound during indicated intervals.

ammonium sulfate stage of purification were used, the amount of trichloroacetic acid-insoluble radioactivity was relatively high even when no albumin was added (Table 6). This was undoubtedly a consequence of the high protein content of the reaction mixture. When small amounts of more highly purified preparations were used, there was a considerable increase in the acid-insoluble counts when serum albumin was added. Since the rate of reduction is not influenced by the addition of serum albumin to the reaction mixture, it is unlikely that the observed increase in counts is due to stabilization of the enzyme or to other indirect effects. This conclusion is strengthened by the results of labeling experiments (e.g., Table 7) in which the ^{14}C-nitrofurazone was completely reduced in the first 20 min of incubation. Under these conditions, there was four times as much trichloroacetic acid-insoluble radioactivity in samples from reaction mixtures which contained albumin during reduction of the nitrofurazone as in samples to which albumin was added only after reduction had been completed. The maximum amount of radioactivity bound to protein in these experiments represents on the order of 0.5 to 1 molecule of nitrofurazone per molecule of protein (assuming an average molecular weight of approximately 68,000).

Sephadex G-100 fractionation of a reation mixture, in which 15 μg (0.15 μCi) of ^{14}C-nitrofurazone was completely reduced by 0.007 unit of nitrofurazone reductase (0.02 mg of protein) in the presence of 10 mg of albumin, showed that 13% of the total activity coincided with the albumin fractions. The corresponding fractions from experiments in which no albumin was added contained no detectable radioactivity. When the combined albumin fractions were dialyzed against 8 M urea for 16 hr, only about 10% of the counts were lost, showing that most of the ^{14}C was firmly bound to protein. The protein-bound ^{14}C was not affected by 16 hr of treatment with 5% trichloroacetic acid at room temperature but was decreased by exposure to dilute alkali at 5 C. Addition of 0.5 mg of cysteine to the reaction mixtures decreased the radioactivity incorporated into protein by about 60%. Acetylation of the protein resulted in a marked decrease in the counts bound (Fig. 6). These phenomena suggest that nitrofurazone is reduced to a metabolite which reacts with the functional groups of protein to form stable linkages. This metabolite itself, however, is not stable in the reaction mixture and if it does not react with protein, it is rapidly inactivated (Table 7). Table 8 shows that nitrofurazone reductase also "activates" 2-nitrofuran so that it reacts with protein.

Taylor et al. have reported that xanthine oxidase also reduces nitrofurazone (25). Since about 1 mole of hypoxanthine is converted to uric acid during the reduction of 1 mole of nitrofurazone, they suggest that the reduction product is the corresponding hydroxylaminofuran. Table 9 shows that purified xanthine oxidase also "activates" nitrofurazone to produce compounds

TABLE 7. *Incorporation of ^{14}C from ^{14}C-nitrofurazone into trichloroacetic acid-insoluble material under various conditions*[a]

System	Counts/min after	
	40 min	70 min
No added protein (1 ml of buffer added after 20 min)	139	164
Serum albumin added 20 min after the start of the reduction	171	203
Serum albumin added at zero time (i.e., present during the reduction)	589	540

[a] Each reaction mixture contained 1 mg of NADPH and 13 μg (0.13 μCi) of ^{14}C-nitrofurazone in 0.4 ml of buffer. At zero time, 0.05 ml of Sepharose-purified nitrofurazone reductase was added. Buffer or serum albumin (5 mg in 1 ml of buffer) was added as indicated. Samples (10 μliters) were taken for counting.

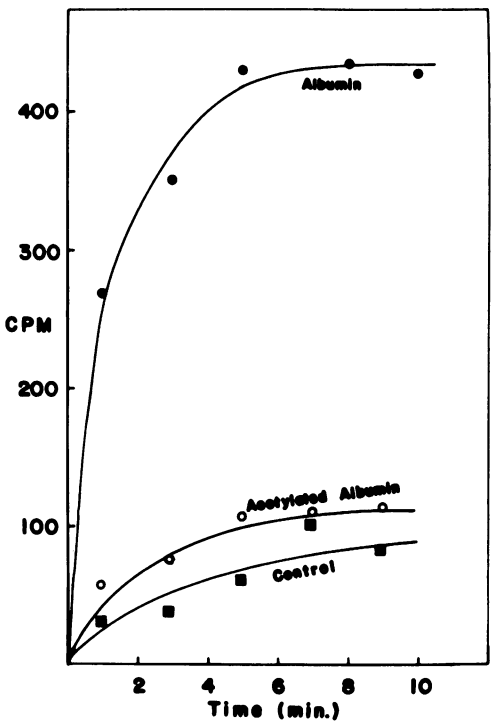

FIG. 6. *Incorporation of radioactivity from ^{14}C-nitrofurazone into serum albumin and acetylated serum albumin. Reaction mixtures contained purified enzyme (0.05 mg of protein), 1 mg of NADPH, 15 μg (0.15 μCi) of ^{14}C-nitrofurazone, and 5 mg of protein in a total volume of 1.4 ml. Samples (10 μliters) were taken for counting. The control contained enzyme, NADPH, and ^{14}C-nitrofurazone, but no additional protein.*

which react with serum albumin but not with acetylated albumin.

Electrochemical "activation" of nitrofurans. Electrochemical reduction of nitrofurazone at cathode potentials of between about −0.3 v and −1.0 v in neutral media yields the corresponding hydroxylamino compound (23). When 250 μg of ^{14}C-nitrofurazone was reduced at a cathode potential of −0.8 v for 10 min in 2.5 ml of 0.066 M buffer (*p*H 7.0) containing 10 mg of serum albumin, about 10% of the total radioactivity was rendered trichloroacetic acid-insoluble. Chromatography of the reaction mixture on Sephadex G-25 showed two radioactive components, one of which contained about 10% of the total radioactivity and corresponded to the albumin peak. No ^{14}C was lost when the albumin fraction was made 8 M in urea and dialyzed for 24 hr against 50 volumes of 8 M urea.

Concluding remarks. From these experiments it appears that the reactive compound is likely 5-hydroxylamino-2-furaldehyde semicarbazone, the product of electrochemical reduction and the inferred product of reduction by xanthine oxidase. Although there is no definitive information as to the identity of the active product formed by the bacterial enzyme, it seems reasonable to conclude that the hydroxylamino derivative may be involved since it is undoubtedly formed as an intermediate in the reduction process (9). Hydroxylamino derivatives of other aromatic compounds are known to react with protein and nucleic acid and to have a wide range of biological effects (12, 17).

The possible physiological relevance of the reaction described above is shown by the observation that there is very much less incorporation of ^{14}C from nitrofurazone into cells of the resistant strain than into cells of sensitive strains, a difference which can also be demonstrated in cell-free extracts. This is not, of course, to say that the reaction with protein is necessarily the

TABLE 8. *Reaction of ^{3}H-2-nitrofuran with protein in the presence of E. coli nitrofurazone reductase*[a]

System	Counts/min in 10 μliters
Complete	302
Minus NADPH	96
Minus reductase	0

[a] The complete reaction mixture contained 4 mg of serum albumin, 20 μmoles of glucose-6-phosphate, 0.5 mg of NADPH, 45 μg of ^{3}H-nitrofuran (1.7×10^{6} counts/min) and 0.5 ml of reductase purified to the ammonium sulfate precipitate stage (2.5 mg of protein). The total volume was 2 ml. After 1.5 hr, samples (10 μliters) were taken for counting.

TABLE 9. *Reaction of ^{14}C-nitrofurazone with protein in the presence of xanthine oxidase*[a]

Addition	Trichloroacetic acid-insoluble material	
	5 min	10 min
Serum albumin, 3 mg	330	415
Acetylated serum albumin, 3 mg	80	115
None	80	90

[a] Reaction mixtures contained 20 μg of hypoxanthine, 20 μg (0.2 μCi) of ^{14}C-nitrofurazone, 0.04 units of xanthine oxidase, and the additions shown above. The final volume was 2.2 ml; *p*H 7.2 (0.033 M phosphate buffer). Incubation was at 37 C under nitrogen. Samples (50 μliters) were taken for counting.

[b] Counts per minute after indicated intervals.

basis of the antibacterial action of nitrofurazone. However, it is clear that this reaction could have important biological consequences since we have shown in preliminary experiments that reduced nitrofurazone is a powerful inhibitor of yeast alcohol dehydrogenase, whereas the unreduced compound has no effect.

It seems likely that other macromolecules, such as nucleic acids, may also react with reduced nitrofurazone. The fact that *E. coli* B/r is more resistant to nitrofurazone and other nitrofurans than is strain B suggests that DNA may be an important site of reaction of "activated" nitrofurans (11, 21). It seems reasonable to conclude that whatever the molecular target of the nitrofurans in sensitive bacteria, nitrofurazone reductase is responsible for "activating" these compounds.

It is of some interest that the most highly nitrofurazone-resistant strains of bacteria that have been obtained are only about 10 times more resistant than strain B/r. Since we have demonstrated that strains with this level of resistance contain essentially no soluble nitrofurazone reductase activity, it appears that either nitrofurazone must be activated by other enzymes, or that nitrofurans per se have some activity. This latter possibility is by no means unlikely since there is ample literature documenting the inhibitory effect of unmetabolized nitrofurans on certain enzyme systems (see reference 19).

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