It is apparent, however, that both the steroid and neural mechanisms described represent long-term regulation, and cannot play a role in short-term adrenal medullary responses to acute stress. The half-life of PNMT in the hypophysectomized rat is quite long, and is probably much longer in the intact rat.[6,7] These findings suggest that the elevation of PNMT in the intact animal, whether mediated by hormonal or neural factors, probably finds its biologic role in chronic stress situations.

Acknowledgements—The authors wish to express their gratitude to Dr. Julius Axelrod and Dr. Jack Barchas, in whose laboratories these studies were performed, for their helpful discussion and encouragement.

Department of Psychiatry,
Stanford University School of Medicine,
Stanford Calif. 94305 *and*
Laboratory of Clinical Science,
National Institute of Mental Health,
Bethesda, Md. 20215, *U.S.A.*

ROLAND D. CIARANELLO
IRA B. BLACK

REFERENCES

1. J. AXELROD, *J. biol. Chem.* **237**, 1657 (1962).
2. R. D. CIARANELLO, R. BARCHAS, G. BYERS, D. W. STEMMLE and J. D. BARCHAS, *Nature, Lond.* **221**, 368 (1969).
3. L. POHORECKY, M. ZIGMOND, H. KARTEN and R. J. WURTMAN, *J. Pharmac. exp. Ther.* **165**, 190 (1969).
4. R. J. WURTMAN and J. AXELROD, *J. biol. Chem.* **241**, 2301 (1966).
5. R. T. SCHIMKE, E. W. SWEENEY and C. M. BERLINER, *Biochem. biophys. Res. Commun.* **15**, 214 (1964).
6. J. VERNIKOS-DANELLIS, R. CIARANELLO and J. BARCHAS, *Endocrinology* **83**, 1357 (1968).
7. R. D. CIARANELLO, J. D. BARCHAS and J. VERNIKOS-DANELLIS, *Life Sci.* **8**, 401 (1969).
8. R. A. MUELLER, H. THOENEN and J. AXELROD, *J. Pharmac. exp. Ther.* **169**, 74 (1969).
9. P. MOLINOFF, S. BRIMIJOIN, R. WEINSHILBOUM and J. AXELROD, *Proc. natn. Acad. Sci. U.S.A.* **66**, 453 (1970).
10. H. THOENEN, R. A. MUELLER and J. AXELROD, *Biochem. Pharmac.* **19**, 669 (1970).
11. R. KVETNANSKY, V. K. WEISE and I. J. KOPIN, *Endocrinology* **87**, 744 (1970).

Biochemical Pharmacology, Vol. 20, pp. 3532–3537. Pergamon Press, 1971. Printed in Great Britain

"Activation" of nitrofurazone in animal tissues

(*Received* 16 *March* 1971; *accepted* 4 *June* 1971)

DERIVATIVES of 5-nitrofuran have been used clinically as antibacterial agents for a number of decades.[1,2] Studies on the mode of action of these compounds have shown that reductive metabolism greatly increases their toxicity to bacteria. Mutants with decreased levels of nitrofurazone-reductase are resistant to the drug[3,4] suggesting that metabolic "activation" is required. Early toxicological studies showed that severe liver and kidney damage was produced in rodents by injection of high but sub-lethal doses of nitrofurazone.[5] Spermatogenic arrest follows administration of several nitrofurans[6,7] and Friedgood and Green[8] reported marked atrophy of testes following oral doses of nitrofurazone. The same workers also observed weak antitumor activity against fibrosarcoma in mice,[8] an observation which led to the experimental treatment of primary and metastatic seminoma with nitrofurazone.[9,10]

More recently it has been discovered that other nitrofuran derivatives are powerful carcinogens.[11–13] Results published within the last year show that nitrofurazone can induce transplantable mammary tumors.[14] Nitrofurantoin does not appear to be carcinogenic.

Like certain other carcinogenic nitro compounds,[15,16] nitrofuran derivatives are known to undergo reductive metabolism in animal tissues.[17] We have recently shown that intermediates formed during the reduction of nitrofurazone react with serum albumin to form derivatives which are stable to dilute acid and to prolonged dialysis against 8 M urea[4] and which have altered electrophoretic mobility.*

* D. R. McCalla, A. Reuvers and C. Kaiser, unpublished results.

The final reduction product, however, does not react with protein. Since protein-bound derivatives were formed whether nitrofurazone was reduced electrolytically, by a bacterial nitrofurazone-reductase or by milk xanthine oxidase, it was of interest to examine the possible "activation" of nitrofurazone by enzymes in animal tissues.

NADPH, NADH, FMN (flavine mononucleotide), nicotinamide, and glucose 6-phosphate were obtained from Sigma Chemical Corp. and glucose 6-phosphate dehydrogenase from Worthington Biochemical Corp. Nitrofurazone (5-nitrofuraldehyde semicarbazone) was a gift from Eaton Laboratories, Norwich, N. Y. Allopurinol (4-hydroxypyrazolo-[3,4-d]pyrimidine) was obtained from Burroughs-Wellcome Laboratories, Tuckahoe, N. Y. Nitrofurazone (4·68 μc/mM) labeled with ^{14}C in the semicarbazone moiety was prepared as described previously.[4]

All tissue samples were from male hooded rats weighing between 250–300 g. The animals were killed by a blow on the head and the tissues immediately removed and washed in ice-cold isotonic potassium chloride. Testes tissue was prepared by teasing the tubules apart and other tissues by slicing to 1–1·5 mm thickness. The 9000 *g* supernatant fraction of rat liver homogenate was prepared as described by Fouts and Brodie.[18] Tissue slices were used as soon as possible and homogenates within 3 days of preparation. All preparations were kept at 0–5° unless otherwise specified.

To measure rates of nitrofurazone metabolism, sliced tissues (0·75 g) were incubated in a shaking water bath at 37° with 4 ml of 0·067 M potassium phosphate buffer, pH 7·2, containing 1 mg of glucose and 50 μg of nitrofurazone per ml. Half-milliliter samples of the incubation fluid were withdrawn after 30 and 60 min and added to 3 ml of 95% ethanol, and the mixture centrifuged. The absorption of the clear supernatant was determined at 375 nm in a Hitachi–Perkin Elmer Model 139 spectrophotometer.

Binding of activated nitrofurazone was examined by incubating sliced tissue (250 mg) in a shaking water bath at 37° with 1 ml, pH 7·2, 0·067 M potassium phosphate buffer containing 1 mg of glucose and $6·8 \times 10^5$ counts/min of ^{14}C nitrofurazone (50 μg). At intervals tissue slices were removed, washed with 5 ml cold 5% TCA, homogenized in 3 ml cold 5% TCA, and the mixture centrifuged. The pellet was resuspended in 5% TCA, washed three times with cold 5% TCA and twice with 95% ethanol. The supernatant after the final wash contained a negligible amount of ^{14}C. The final pellet was resuspended in 3 ml of water. One-milliliter-samples of the suspension were added to 10 ml of a solution consisting of 8 g Omnifluor (New England Nuclear) per liter of dioxane. Samples were counted in a Nuclear Chicago Unilux I scintillation counter at an efficiency of about 65 per cent.

When slices of tissue from various organs were shaken with buffered nitrofurazone solutions under aerobic conditions, nitrofurazone disappeared at a rate characteristic of the tissue used (Table 1)—liver, kidney and testes metabolizing nitrofurazone at higher rates than skeletal or heart muscle, lung and spleen. The rate of disappearance of nitrofurazone was nearly constant so long as the concentration of the drug remained nonlimiting. Addition of the xanthine oxidase inhibitor, allopurinol,[19] had no effect on the rate of disappearance of nitrofurazone (Table 2). Tissue slices incubated with [^{14}C]-nitrofurazone incorporated radioactivity into TCA-insoluble material (Table 3). The extent of incorporation in various tissues correlated well with rates of nitrofurazone metabolism. The maximum

TABLE 1. RATES OF METABOLISM OF NITROFURAZONE BY RAT TISSUE SLICES*

Tissue	Rate of nitrofurazone metabolism†	Standard deviation
Liver	0·50†	0·14
Kidney	0·48	0·12
Testes	0·39	0·065
Muscle	0·16	0·04
Heart	0·15	0·045
Lung	0·15	0·020
Spleen	0·07	0·015

* See text for experimental details.

† All values in μmoles per hour per gram of tissue calculated from the ΔA_{375} during the first 30 min. These values represent the average of measurements obtained using five different preparations.

incorporation represents about 8 per cent of the nitrofurazone added to the incubation mixture. Thin-layer chromatography showed that little if any unmetabolized nitrofurazone was present in hot ethanol extracts of liver, kidney and testes at the end of the incubation period. Extracts of spleen slices, however, did contain unchanged drug.

Table 4 shows that dialyzed 9000 *g* supernatant reduced nitrofurazone provided that suitable hydrogen donors were added and oxygen excluded. With NADPH the rate of reduction of nitrofurazone was 10–12 μmoles/hr/g tissue and was not appreciably increased by added FMN. Reduction of *p*-nitrobenzoic acid was about 90 times slower but was stimulated 8-fold by 5×10^{-4} M FMN.

TABLE 2. NITROFURAZONE METABOLISM OF RAT LIVER AND KIDNEY SLICES IN THE PRESENCE AND ABSENCE OF ALLOPURINOL*

Tissue	Control	+ Allopurinol
Liver	0·47†	0·50
Kidney	0·48	0·46

* The allopurinol-containing samples were pre-incubated in a shaking water bath at 37° with 2 ml, pH 7·2, 0·067 M potassium phosphate buffer containing 0·73 μmole allopurinol for 15 min; at zero time 2 ml of the same buffer containing 100 μg of nitrofurazone per ml was added to the mixture. See text for further experimental details.

† μmoles per hour per gram of tissue.

TABLE 3. INCORPORATION OF RADIOACTIVITY FROM [^{14}C]NITROFURAZONE INTO TCA-INSOLUBLE MATERIAL IN RAT TISSUE SLICES*

Tissue	Radioactivity (counts/min/0·25 g tissue after)	
	30 min	60 min
Liver	8300	17,600
Kidney	10,600	15,201
Testes	8200	13,600
Spleen	1200	2650

* See text for experimental details.

When hypoxanthine was used as H donor, the rates of reduction were about half those obtained with NADPH. Hypoxanthine-dependent reduction was completely suppressed by allopurinol while the NADPH dependent reduction was not affected. When both NADPH and hypoxanthine were present, the rate of reduction was roughly the sum of the rate with hypoxanthine alone plus that with NADPH alone.

Figure 1 shows that radioactivity from [^{14}C]nitrofurazone became bound to TCA-insoluble material when NADPH is added to dialyzed 9000 *g* supernatant. The high zero time value suggests that not all of the binding is enzyme catalyzed and that some adsorption takes place. When no hydrogen donor is added, similar high zero time values are observed but the amount of bound radioactivity did not increase appreciably with time. Seventy-seven per cent of the TCA-insoluble counts bound to the 9000 *g* fraction in the presence of NADPH was lost when the labeled preparation was incubated with 1 mg of bacterial protease per ml for 1 hr at 37° indicating extensive binding to protein or to components which are solubilized during hydrolysis of protein. There was no loss of TCA-insoluble ^{14}C when the labeled 9000 *g* supernatant was dialyzed against 50 vol. of 8 M urea for 48 hr, indicating that the ^{14}C is very tightly bound to TCA-insoluble material.

TABLE 4. REDUCTION OF NITROFURAZONE AND *p*-NITROBENZOIC ACID BY A DIALYZED 9000 *g* SUPERNATANT FRACTION FROM RAT LIVER*

Expt	H donor	Additions	Rate of reduction of Nitrofurazone†	Rate of reduction of *p*-Nitrobenzoic acid‡
1	NADPH		10·6	0·12
	NADPH	O_2	0·37	0·01
	NADPH	5×10^{-4} M FMN	12·6	0·95
	NADH		4·8	0·05
2	NADPH		12·2	0·12
	NADPH	$1·5 \times 10^{-4}$ M allopurinol	13·0	0·13
	Hypoxanthine		6·0	0·08
	Hypoxanthine	$1·5 \times 10^{-4}$ M allopurinol	0·0	0·00
	Hypoxanthine plus NADPH		18·0	0·19
	Hypoxanthine plus NADPH	$1·5 \times 10^{-4}$ M allopurinol	10·5	0·12

* All experiments were carried out in a total volume of 2·7 ml of pH 7·2, 0·067 M potassium phosphate buffer, containing 0·5 μmole of substrate and 0·5 ml of dialyzed 9000 *g* supernatant under an atmosphere of N_2 (except where otherwise indicated). Where NADPH was the H donor, 0·26 μmoles NADPH, 2 units glucose 6-phosphate dehydrogenase and 40 μmoles glucose 6-phosphate was added. In one experiment, an equivalent amount of NADH was used in place of NADPH.

Where hypoxanthine was used as donor, 1·5 μmoles were added.

In experiment 2 the preparations were preincubated in air for 10 min before flushing with nitrogen and adding substrate. Where allopurinol was used it was present during the preincubation.

After 10 min 0·5-ml samples were removed from the nitrofurazone flasks and added to 3 ml 95% ethanol; the mixture was centrifuged and the nitrofurazone concentration determined from the absorbance at 375 nm. Nitrobenzoic acid samples were incubated for 2 hr, 1-ml samples drawn, made up to 5% TCA, centrifuged, and diazotized and coupled as described by Bratton and Marshall.[20]

† Expressed as μmoles reduced per hour per gram of tissue.

‡ Expressed as μmoles aminobenzoic acid formed per hour per gram of tissue.

Recent experiments in which we have examined the binding of nitrofurazone labeled with ^{14}C in the aldehyde carbon* to tissue slices and to the 9000 *g* supernatant from liver gave results essentially identical with those obtained with the semicarbazone-labeled material. This observation rules out the possibility that the effects observed are due to binding of products derived from the semicarbazone moiety after hydrolysis of the Schiff's base.

Previous workers have reported that a variety of mammalian tissues metabolize nitrofurans.[21,22] We have confirmed these observations and have shown that tissues which are damaged by high doses of nitrofurazone rapidly metabolize this compound to derivatives which are tightly (and probably covalently) bound to TCA-insoluble material. Tissues which do not show histological damage metabolize the drug at a much lower rate.

Several mammalian enzymes that reduce aromatic nitro compounds have been described in the literature. These include xanthine oxidase which is known to reduce nitrofurazone under anaerobic conditions and to catalyze the binding of ^{14}C from radioactive nitrofurazone to protein *in vitro*.[4,23] Early workers, noting a correlation between xanthine oxidase levels and the rates of reduction of nitrofurazone by various tissues, suggested that this enzyme might be important *in vivo*. However, Bender and Paul[21] showed that tissues like muscle which have low levels of xanthine oxidase nevertheless reduce nitrofurans at an appreciable rate. Our data show that when hypoxanthine is added to the 9000 *g* supernatant from rat liver, nitrofurazone becomes reduced. Since allopurinol completely inhibits the hypoxanthine-dependent reduction, we conclude that the xanthine oxidase present can reduce nitrofurazone. However, since allopurinol did not inhibit the metabolism of nitrofurazone by liver or kidney slices, xanthine oxidase cannot be involved in the reduction of nitrofurazone by

*Obtained through the courtesy of Norwich Pharmacal.

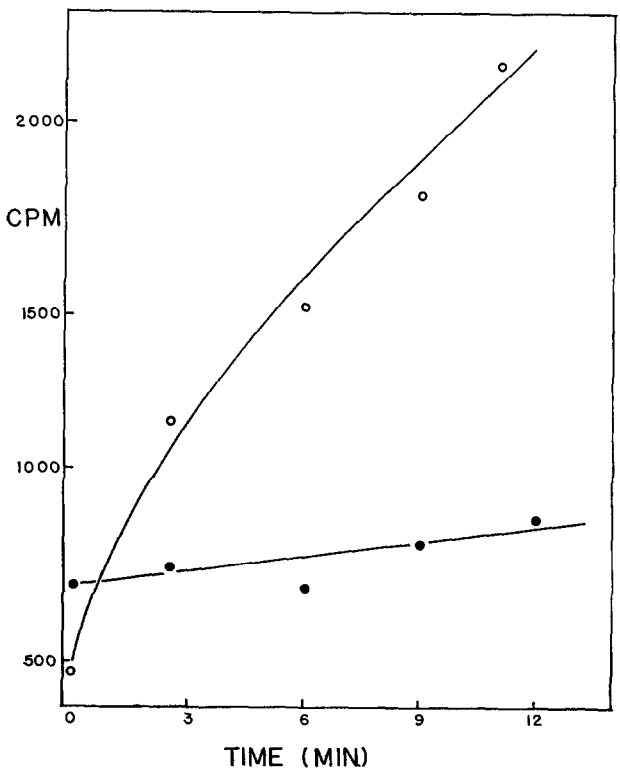

FIG. 1. Incorporation of radioactivity from [^{14}C]nitrofurazone into TCA-insoluble material by a 9000 *g* supernatant of homogenized rat liver. The complete reaction mixture contained: 0·5 ml of the 9000 *g* preparation (see text), 5×10^5 counts/min of [^{14}C]nitrofurazone (0·25 mole), 20 μmoles glucose 6-phosphate, 0·26 μmole NADPH, 2 units glucose 6-phosphate dehydrogenase in a final volume of 1·25 ml, 0·067 M potassium phosphate buffer, pH 7·2. The mixtures were incubated under nitrogen at 37° with shaking. At intervals, 50-μl samples were taken and put on small filter paper disks which were then placed in ice cold 5% TCA, washed three times with cold 5% TCA and three times with 95% ethanol. They were then dried and counted. Open circles, complete mixture; closed circles, complete mixture minus NADPH and the generating system. The data shown represent the counts/min per 50-μl sample.

tissue slices. A second nitrofurazone-reducing system present in the 9000 *g* supernatant uses reduced pyridine nucleotides as the hydrogen donor. This system reduces nitrofurazone at about twice the rate of the xanthine oxidase catalysed reaction. In view of a recent report on the reduction of niridazole,[24] it seems likely that NADPH-cytochrome c reductase may be involved. Our results on the NADPH dependent reduction of nitrofurazone by the 9000 *g* supernatant of liver homogenates are in good agreement with those obtained by Paul and Harrington using other Schiff's base derivatives of 5-nitrofuraldehyde.[25] Like these authors, we find that anaerobic liver extracts supplemented with NADPH show 20-fold higher rates of metabolism of the nitrofuran derivative than does a comparable weight of tissue slices. The lower rate observed with tissue slices may be due to permeability barriers, to partial inhibition of the reductases by oxygen or to auto oxidation of one or more reduction intermediates.

Reduction of the nitro group of 5-nitrofurans has been regarded as a "detoxification" step.[22] However, while the ultimate reduction products may be non-toxic, at least one of the intermediates in the process is an unstable compound which reacts with cellular constituents.[4] Clearly, more work is required to assess the physiological importance of the products observed in this work, especially since some enzymes are apparently inhibited by unreduced nitrofurans.[26,27]

The reactive compounds formed during nitrofurazone reduction are unstable and have not yet been identified. Possible candidates include hydroxylaminofurans or the free radicals described by Stradiņš *et al.*[28]

Acknowledgements—This work was supported by an operating grant from the National Cancer Institute of Canada.

Department of Biochemistry,
McMaster University,
Hamilton, Ontario, Canada

D. R. McCalla
Antoon Reuvers
Christel Kaiser

REFERENCES

1. H. E. Paul and M. F. Paul, in *Experimental Chemotherapy* (Eds. R. J. Schnitzer and F. Hawking), Vol. II, Part I, p. 307 Academic Press, New York (1964).
2. H. E. Paul and M. F. Paul, in *Experimental Chemotherapy* (Eds. R. J. Schnitzer and F. Hawking), Vol. IV, p. 521. Academic Press, New York (1966).
3. R. E. Asnis, *Archs Biochem. Biophys.* **66,** 208 (1957).
4. D. R. McCalla, A. Reuvers and C. Kaiser, *J. Bact.* **104,** 1126 (1970).
5. M. C. Dodd, *J. Pharmac. exp. Ther.* **86,** 311 (1946).
6. W. O. Nelson and D. J. Patanelli, *Fedn Proc.* **20,** 418 (1961).
7. J. T. Prior and J. H. Ferguson, *Cancer* **3,** 1062 (1950).
8. C. E. Friedgood and M. N. Green, *Cancer Res.* **10,** 613 (1950).
9. B. L. Hayllar, A. H. O'Neal, Jr. and J. A. Dotterer, *J. Urol.* **84,** 565 (1960).
10. M. J. Szczukowski, A. L. Daywitt and H. Elrick, *J. Am. med. Ass.* 1066 (1958).
11. E. Ertürk, S. M. Cohen and G. T. Bryan, *Cancer Res.* **30,** 936 (1970).
12. E. Ertürk, J. M. Price, J. E. Morris, S. Cohen, R. S. Leith, A. M. Von Esch and A. J. Crovetti, *Cancer Res.* **27,** 1998 (1967).
13. J. E. Morris, J. M. Price, J. J. Lalich and R. J. Stein, *Cancer Res.* **29,** 2145 (1969).
14. E. Ertürk, J. E. Morris, S. M. Cohen, J. M. Price and G. T. Bryan, *Cancer Res.* **30,** 1409 (1970).
15. S. Laham, *Can. J. Biochem. Physiol.* **38,** 1383 (1960).
16. T. Sugimura, K. Okabe and H. Endo, *Gann* **56,** 489 (1965).
17. H. E. Paul, V. R. Ells, F. Kopko and R. C. Bender, *J. mednl pharm. Chem.* **2,** 563 (1960).
18. J. R. Fouts and B. B. Brodie, *J. Pharmac. exp. Ther.* **119,** 197 (1957).
19. G. B. Elion, S. Callahan, H. Nathan, S. Bieber, W. Rundles and G. H. Hitchings, *Biochem. Pharmac.* **12,** 85 (1963).
20. A. C. Bratton and E. K. Marshall, Jr., *J. biol. Chem.* **128,** 537 (1939).
21. R. C. Bender and H. E. Paul, *J. biol. Chem.* **191,** 217 (1951).
22. M. F. Paul, H. E. Paul, R. C. Bender, F. Kopko, C. M. Harrington, V. R. Ells and J. A. Buzard, *Antibiot. Chemother.* **10,** 287 (1960).
23. J. D. Taylor, H. E. Paul and M. F. Paul, *J. biol. Chem.* **191,** 223 (1951).
24. D. R. Feller, M. Morita and J. R. Gillette, *Biochem. Pharmac.* **20,** 203 (1971).
25. M. F. Paul and C. Harrington, *Biochem. Pharmac.* **16,** 2457 (1967).
26. J. A. Buzard, F. Kopko and M. F. Paul, *J. Lab. clin. Med.* **56,** 884 (1960).
27. M. F. Paul, *Third National Symposium on Nitrofurans in Agriculture*, September (1960).
28. J. Stradiņš, R. Gavars, G. Reichmanis and S. Hiller, *Abh. dt. Akad. Wiss. Berl. Kl. Med.* **4.** 601 (1966).