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NITROBENZIMIDAZOLES AS INDUCERS OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE IN *ESCHERICHIA COLI*

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Abstract—Extracts of cultures of *Escherichia coli* B grown in the presence of nitrobenzimidazoles contain increased activity of glucose 6-phosphate dehydrogenase. The ratio of activity of glucose 6-phosphate dehydrogenase to the activity of 6-phosphogluconate dehydrogenase may be as high as 8, whereas the ratio for cultures not exposed to drugs is between 1 and 2.

The magnitude and kind of effect of the drug depend on the position of the nitro group on the ring with position 4 more effective than 6. Another substituent on the 6-position modifies the effect of the 4-NO_2 so that the 4-NO_2,6-NH_2 benzimidazole was most effective in decreasing cell division without much effect on other parameters of growth, while the 4-NO_2,6-Cl benzimidazole was more toxic and also the most effective inducer of synthesis of glucose 6-phosphate dehydrogenase.

THE ACTIVITIES of the enzymes D-glucose 6-phosphate: NADP oxidoreductase (1.1.1.49) (G6PD) and 6-phospho-D-gluconate: NADP oxidoreductase (decarboxylating) (1.1.1.44) (PGD) have been determined in extracts of *Escherichia coli*, strain B. Previous studies have shown that the specific activity of the PGD is quite constant under various conditions of growth in various media and in the presence of drugs.[1–3] The activity of G6PD is more variable, so that the ratio between the two activities, G6PD/PGD, is a useful index of the changes in G6PD.[1] If the bacteria were grown in nutrient broth or salts–glucose media enriched with casein hydrolysate and yeast extract, the ratio was approximately 1.[2] Growth in media of inorganic salts with a carbon source such as glucose, gluconate, lactate, or arabinose produced a ratio of approximately 2.[1] Growth in salts–glucose or gluconate medium containing 2,4-dinitrophenol produced significantly high ratios, from 4 to 12, depending on time of exposure and on concentration, from 5×10^{-5} M to 1×10^{-3} M, of the drug.[3]

A series of substituted benzimidazoles[4, 5] which was being tested in a cancer chemotherapy program[6] had shown an interesting pattern of specificity of inhibition of growth of various mutants of *E. coli* and reversal by the required metabolite.[7] Several of these compounds had nitro groups, some in the 4-position and some in the 6-position, and also various other groups substituted on the benzene ring of the benzimidazole. This series presented an opportunity to test the effect of position on the molecule on the biological activity.

MATERIALS AND METHODS

E. coli, strain B, were carried and cultured as described previously.[2] The experimental cultures were incubated in aerated cylinders at 37° in a medium of salts and glucose. When the cultures were in the log phase, solutions of the drugs to give the required concentrations were added to one, two, or three towers, and one tower was left as a control. Turbidity of the cultures was measured by placing the side arm of the cylinder in a Klett–Summerson colorimeter with a 420 filter or with a 660 filter when the compound added to the medium was yellow. The total number of cells was counted in a Petroff–Hauser chamber under the microscope. The comparative size of bacteria and the appearance of filaments which were longer than approximately four times the normal bacteria were also recorded. Counts of viable bacteria were determined by plating.

When the cultures had attained a turbidity indicating at least two doublings of cell substance, the bacteria were harvested by centrifugation and washed. The weighed pellet was ground with Al_2O_3 and extracted with Tris buffer, 0·05 M, pH 7·4. A few cultures were sonicated or agitated in a Nossel disintegrator to determine that the total enzyme activity of the cells was in a nonparticulate fraction. All preparations were centrifuged at 10,000 *g* for 60 min at 4°.

The activities of the enzymes, G6PD and PGD, were determined by reduction of NADP dose, followed in a Beckman spectrophotometer at 36°, as described previously.[8]

Pyruvate was determined in the media by the method of Friedmann and Haugen.[9] The formation of diazotizable amine in the media when the bacteria were treated with nitrobenzimidazoles, was followed by the Bratton–Marshall method.[10] The uptake of glucose from the media was determined by the anthrone method of Mokrash[11] or by glucose oxidase with the "glucostat" supplied by Worthington Chemical Co., Freehold, N.J. The nucleic acid content of whole untreated cells was determined with diphenylamine by the method of Burton[12] for DNA or with orcinol for RNA.[13]

The substituted benzimidazole compounds and the 3,5-dinitro,*p*-aminobenzoic acid (DNPABA), which were synthesized by members of the chemistry department of the University of Pennsylvania, and the sources of other compounds are listed in the paper by Scott *et al.*[6] Ascorbic acid and 2,4-dinitrophenol (DNP) were obtained from Distillation Products Industries, Rochester, N.Y.

RESULTS

The analyses for the enzyme activities in extracts of bacteria grown in salts–glucose medium, as controls or with different concentrations of substituted benzimidazoles, are listed in Table 1. For comparison, values for cultures grown in different media and in the presence of dinitrophenol or dinitro,*p*-aminobenzoic acid are shown in Table 2. The activity of phosphogluconate dehydrogenase in control cultures was 0·145 with a standard deviation of $\pm$0·03 μmole/min/mg protein in the extracts. In the cultures grown in the presence of drugs, the phosphogluconate dehydrogenase was not significantly different, with mean of 0·156, if we exclude the values for one experiment in which growth of bacteria in the presence of three different concentrations of 4-NO_2,6-Cl benzimidazole produced extracts with values 0·32, 0·35, and 0·36, which are significantly high. Ratios of G6PD to PGD were 1·0 when bacteria were grown in enriched medium and approximately 2 when they were grown in a medium

containing only salts and a source of carbon.[2] Ratios of 3 or above are significantly high and indicate an increased G6PD and were found in extracts of cells grown in the presence of only the nitro-substituted compounds, among the drugs tested. Among the benzimidazoles, the compounds with the nitro group in the 4-position and another

TABLE 1. SPECIFIC ACTIVITIES OF ENZYMES IN EXTRACTS OF *E. coli*

Substitution on benzimidazole	Concentration $\times 10^4$	Time (min)	Enzyme activities*		
			G6PD	PGD	Ratio
Controls, mean	0		0·276	0·145	1·93
Standard deviation			±0·07	±0·03	±0·06
4-NO_2,6-Cl	1·0	130	1·32	0·32	4·1
	1·5	240	1·60	0·19	8·0
	2·0	130	1·93	0·35	5·5
	2·5	180	0·77	0·13	6·3
	2·5†	180	1·25	0·17	7·4
	5·0	200	2·15	0·36	5·9
4-NO_2,6-NH_2	1·0	150	0·51	0·15	3·3
	4·0	220	0·60	0·12	5·0
	5·6	240	0·88	0·13	6·6
4-NO_2,6-SO_2NH_2‡	4·0	270	0·60	0·16	3·9
4-NO_2‡	5·8	270	0·44	0·12	3·7
6-NO_2	6·0	240	0·53	0·16	3·5
	12·0	240	0·83	0·23	3·7
4-Cl,6-NO_2	5·0	200	0·47	0·15	3·2
4-Cl,6-NH_2	5·0	160	0·36	0·17	2·2
4-NH_2,6-Cl	2·0	160	0·34	0·19	1·8
	5·0	160	0·28	0·16	1·8
	10·0	160	0·35	0·18	1·8
4-SH,6-NH_2‡	5·0	200	0·18	0·11	1·6

* As μmoles/min/mg protein of extracts.
† Ascorbic acid also present, 0·6 mM.
‡ Cells disrupted in sonication.

TABLE 2. RATIOS OF ENZYME ACTIVITIES IN EXTRACTS OF CELLS GROWN IN THE PRESENCE OF VARIOUS CHEMICALS

Medium and conditions	Additions	Molarity	G6PD/PGD
Salts–glucose, aerobic	DNP*	2×10^{-4}	10·4
Salts–glucose, anaerobic	DNP	2×10^{-4}	12·8
Salts–gluconate, aerobic	DNP†	1×10^{-3}	11·8
Salts–glucose, aerobic	3,5-Dinitro,*p*-amino-benzoic acid	$8{\cdot}8 \times 10^{-4}$	6·3
Salts–glucose, aerobic	Methylene blue†	1×10^{-5}	8·8
Salts–glucose, aerobic	Phenazine methosulfate†	1×10^{-5}	6·1
Salts–glucose, aerobic	Pyruvate	$1{\cdot}1 \times 10^{-4}$	3·1
Salts–glucose, aerobic	Bilirubin	3×10^{-4}	4·8

* 2,4-Dinitrophenol.
† Higher concentrations inhibited growth.

substituent *meta* to it on the benzene ring were the most potent inducers of increased G6PD.

The Bratton–Marshall[10] test on media in which bacteria had been growing indicated that the nitro groups on either the 4- or 6-position were reduced to amino groups to

the extent of about 50 per cent in 3 or 4 hr, when the drugs were present at concentrations of 1×10^{-4} M.

Analyses of media for pyruvate indicated that more was excreted by bacteria growing in the presence of nitro-substituted compounds. Values as high as 25 or 30 μg/ml were found in comparison with the 2 or 3 μg/ml in control cultures.

Absorption spectra of the supernatant medium in which bacteria had been exposed to 4-NO_2,6-Cl benzimidazole showed a progressive decrease of the maximum at 300 to 350 mμ, and increase from 420 to 460 mμ, which was visible as a deepening of yellow to orange, and an increase from a minimum to a maximum at 260 mμ. These changes were interpreted respectively as uptake of the dye by the bacteria, excretion into the medium of a compound with greater absorption in the visible region, and excretion of nucleic acid derivatives. Similar changes in spectra were found with 4-NO_2,6-NH_2 benzimidazole, slightly with 6-NO_2 benzimidazole, and also with 2,4-dinitrophenol.

Attachment of the nitrobenzimidazoles to the intracellular substance was indicated by the yellow color of the extracts of the washed bacteria.

When the extracts of bacteria grown in the presence and in the absence of DNP were combined, the rates of G6PD activity were additive. The addition of drug to the reaction medium with control extract did not increase the G6PD activity, so there did not seem to be an accelerating effect of the drug on the enzyme in solution. Previous studies[3] have indicated increased synthesis during growth and none when bacteria were maintained without growth in the presence of DNP. Growth of bacteria with high G6PD in medium without drug allowed dilution of the increased activity.

Table 2 lists the ratios of G6PD to PGD for extracts of bacteria grown in the presence of DNP and of pyruvate, methylene blue, phenazine methosulfate, and bilirubin. Anaerobiosis did not change the effect of DNP. The two electron carriers, methylene blue and phenazine methosulfate, increased the enzyme ratios as did another nitro compound, 3,5-dinitro,*p*-aminobenzoic acid. Pyruvate and bilirubin had a slight effect on the ratios.

DISCUSSION

Previous work showed the effect of substituted benzimidazoles on the growth of bacteria. Compounds with NO_2 at the 4-position on the benzimidazole inhibited the growth of *E. coli* 113-3, a mutant requiring either vitamin B_{12} or methionine, and the inhibition was reversed by excess B_{12} and even more by methionine. The 4-NO_2,6-Cl benzimidazole was the most toxic, causing 50 per cent inhibition at 5×10^{-6} M with limiting vitamin B_{12}, and at 6×10^{-4} M with excess methionine. Compounds with SH at the 4-position or NO_2 at the 6-position or 5,6-dimethylbenzimidazole, which occurs naturally in vitamin B_{12}, also inhibited growth of this mutant; the inhibition was reversed by excess vitamin B_{12}, and less by methionine. Substitution by methyl or chloro on 5 or 6 or on both positions gave compounds which at about 1×10^{-5} M produced 50 per cent inhibition of *E. coli* 15 T-, a mutant which requires thymine; excess thymine partially reversed the inhibition. The unsubstituted benzimidazole at 1×10^{-3} M inhibited in this way. The 4-NO_2,6-NH_2 inhibited division of the wild type bacteria so that long filaments were formed in concentrations which did not inhibit growth, as indicated by increase in turbidity.[6] Thus the position of the nitro

group on the benzene ring of the benzimidazole compounds is correlated with the specificity of the effect on growth as well as on enzyme synthesis.

At the same time that the drug affected the bacteria, the bacteria affected the drug, reducing the nitro group to an amino group.

Kielley[14] has found specificity of position of aromatic nitro groups as electron acceptors for the system of liver xanthine oxidase oxidizing NADH. For dinitrophenols the order of decreasing effectiveness was 2,5; 2,4; and 2,6; that is, the most effective were the nitro groups in *para* position to each other. With aminonitrophenol the nitro group *para* to the OH was more effective than the nitro group in *ortho* position. But the *p*-nitrophenol was less effective than a *p*-nitro disubstituted phenol. More than twice as reactive as any nitro-substituted compound was *p*-nitrosophenol. It was concluded that xanthine oxidase catalyzed the reduction: $-NO_2 \rightarrow -NO \rightarrow -NHOH \rightarrow -NH_2$. Tewfik and Evans[15] discussed the different pathways of enzymic degradation of 3,5-dinitrocresol by various species of microorganisms. All require NADH, and some are stimulated by added FADH. Slater[16] reported that livers of rats treated with azaserine or dimethylnitrosamine were depleted of NAD, probably because of increased turnover of coenzymes of degrading enzymes. He also found depletion of NADP and NADPH, which he attributed to decreased formation by NAD kinase because of low substrate concentrations. It is possible that the increased synthesis of glucose 6-phosphate dehydrogenase could be explained similarly. The low levels of NADPH and NAD may allow increased concentration of G6P, which could induce synthesis of dehydrogenase. Greenbaum *et al.*[17] found that conditions which depleted rat liver of pyridine nucleotides also increased the glucose 6-phosphate dehydrogenase activity.

The phenazine methosulfate and methylene blue act as electron carriers between FADH and oxygen. The resulting increased NADP/NADPH ratio might have induced glucose 6-phosphate dehydrogenase synthesis as well as increasing the rate of reaction by availability of NADP.

The nitrobenzimidazoles have thus two effects on the bacteria. One is the effect as a benzimidazole, acting as an analog of the benzimidazole of vitamin B_{12}. The other is the effect of the aromatic nitro group which can be reduced by enzymes of the bacteria as are the nitro groups of dinitrophenol. However the second ring—the imidazole—changes the benzene ring and allows great specificity of position for reactivity.

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REFERENCES

1. D. B. M. Scott, *Biochem. J.* **63**, 587 (1956).
2. D. B. M. Scott and E. Chu, *Biochem. J.* **72**, 426 (1959).
3. D. B. M. Scott, *Biochem. J.* **63**, 593 (1956).
4. J. Hoover and A. Day, *J. Am. chem. Soc.* **76**, 4144 (1954).
5. J. Hoover and A. Day, *J. Am. chem. Soc.* **77**, 4324, 5652 (1955).
6. D. B. M. Scott, M. L. R. Batcheler, E. C. Lesher and A. M. Pakoskey, *Cancer Res.* **23**, Part 2, p. 1235 (1963).
7. D. B. M. Scott, M. L. Rogers and C. Rose, *J. Am. chem. Soc.* **80**, 2165 (1958).
8. D. B. M. Scott and S. S. Cohen, *Biochem. J.* **55**, 23 (1953).
9. T. E. Friedmann and G. E. Haugen, *J. biol. Chem.* **147**, 415 (1943).

10. A. C. BRATTON and E. K. MARSHALL, JR., *J. biol. Chem.* **128**, 537 (1939).
11. L. S. MOKRASH, *J. biol. Chem.* **208**, 55 (1954).
12. K. BURTON, *Biochem. J.* **62**, 315 (1956).
13. G. L. MILLER, R. H. GOLDER and E. E. MILLER, *Analyt. Chem.* **23**, 903 (1951).
14. R. KIELLEY, in *Inorganic Nitrogen Metabolism* (Eds. W. D. MCELROY and B. GLASS), p. 207. Johns Hopkins University Press, Baltimore (1956).
15. M. S. TEWFIK and W. C. EVANS, *Biochem. J.* **99**, 31 (1966).
16. T. F. SLATER, *Biochem. J.* **99**, 3 (1966).
17. A. L. GREENBAUM, J. B. CLARK and P. MCLEAN, *Biochem. J.* **95**, 167 (1965).