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Inhibitors of Poly(Adenosine Diphosphate–Ribose) Synthesis: Effect on Other Metabolic Processes

Abstract. *3-Aminobenzamide and benzamide, purported to be specific inhibitors of the synthesis of poly(adenosine diphosphate–ribose), were used to elucidate possible functions of this biopolymer. These compounds, at frequently used experimental concentrations, not only inhibited the action of poly(adenosine diphosphate–ribose) synthetase but also affected cell viability, glucose metabolism, and DNA synthesis. Thus, the usefulness of 3-aminobenzamide and benzamide may be severely restricted by the difficulty of finding a dose small enough to inhibit the synthetase without producing additional metabolic effects.*

Mono-ADP-ribosylation (ADP, adenosine diphosphate) and poly-ADP-ribosylation are processes belonging to a class of covalent modifiers of proteins (other such processes are acetylation, phosphorylation, and methylation) that alter enzyme function and structural conformation (*1*). The biological significance of poly-ADP-ribosylation is unclear but appears to be related to regulation of several aspects of DNA metabolism as diverse as DNA synthesis (*2*), sister chromatid exchange (*3*), and differentiation (*4, 5*). There is evidence that repair of DNA damage may depend on the synthesis of poly(ADP-ribose) in the nucleus of the injured cell (*1, 6*), causing conformational changes in the DNA (*7*) and increasing the activity of DNA ligase II (*8*).

Much of the evidence for the cellular roles of poly(ADP-ribose) is based on inhibition of the enzyme poly(ADP-ribose) synthetase by compounds such as 3-aminobenzamide (3AB) and benzamide (*9, 10*). Although these compounds inhibit the synthesis of poly(ADP-ribose), the specificity of this inhibition has not been established. It is generally assumed that there are no other signifiicant metabolic effects in undamaged cells (*11*). Our study shows that 3AB and benzamide in concentrations commonly used to inhibit poly(ADP-ribose) synthetase produce adverse effects on other cellular metabolic processes.

A lymphoid cell line (WIL-2) was maintained in suspension culture in RPMI 1640 medium supplemented with 10 percent fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). In all experiments, the cells were incubated with one of four benzamide analogs for 24 hours at 37°C. In addition to benzamide and 3AB, the metabolic effects of *m*-aminobenzoic acid (*m*-ABOA) and benzoic acid (BOA), two analogs of benzamide that do not inhibit poly(ADP-ribose) synthetase, were examined (*10*).

Cytotoxicity of various concentrations of benzamide, 3AB, *m*-ABOA, and BOA (0 to 20 m*M*) was monitored by cellular release into the medium of the cytoplasmic enzyme lactate dehydrogenase (*12*); none of the four benzamide analogs affected this standard enzyme assay. The rate of glucose oxidation in cells treated with the benzamide analogs (0 to 7 m*M*) was measured by the conversion of D-[^{14}C(U)]glucose (0.5 μCi/ml, 348.2 mCi/mmole) to $^{14}CO_2$ (*13*). DNA synthesis from [*methyl*-^{3}H]thymidine (10 μCi/ml, 74.9 Ci/mmole), [*methyl*-^{3}H]methionine (10 μCi/ml, 80 Ci/mmole) or [^{14}C]glucose (0.5 μCi/ml) in the presence of one of the four benzamide analogs (0 to 5 m*M*) was also determined. DNA was isolated from the cells by extraction with a mixture of

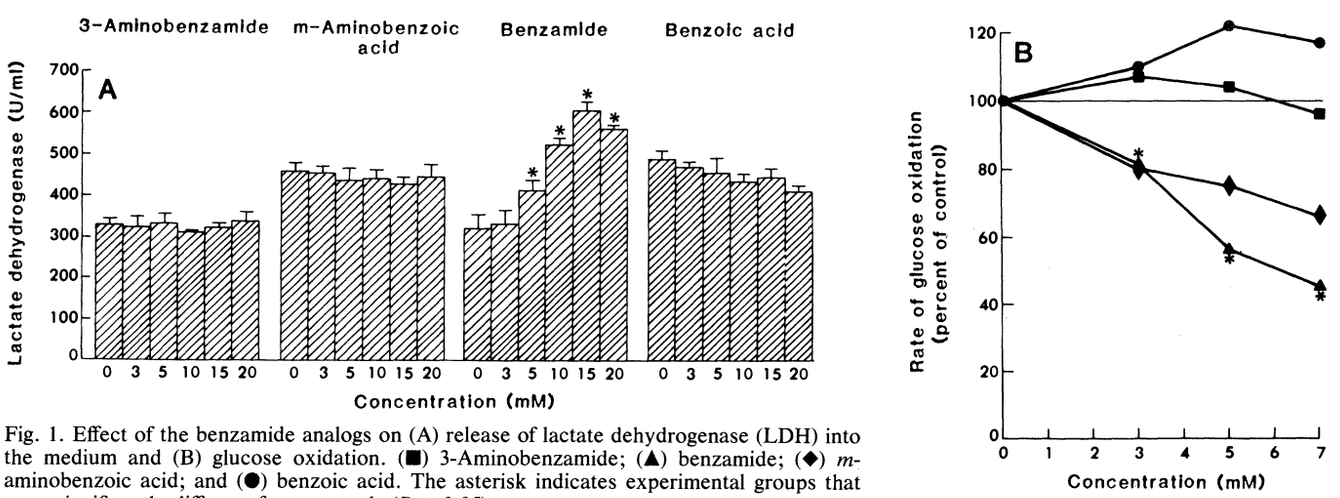

Fig. 1. Effect of the benzamide analogs on (A) release of lactate dehydrogenase (LDH) into the medium and (B) glucose oxidation. (■) 3-Aminobenzamide; (▲) benzamide; (◆) *m*-aminobenzoic acid; and (●) benzoic acid. The asterisk indicates experimental groups that were significantly different from controls ($P < 0.05$).

chloroform and isoamyl alcohol (24:1) and subsequent precipitation with ethanol (*14*), and incorporated radioactivity was determined. All data were analyzed by one-way analysis of variance, and Dunnett's test was used for comparisons of control and experimental means (*15*).

Benzamide, the only chemical that caused a significant increase in the release of lactate dehydrogenase into the medium, was toxic to cells at a concentration as low as 5 m*M* (Fig. 1A). Similarly, glucose oxidation was affected only by benzamide (55 percent reduction caused by 7 m*M* benzamide) (Fig. 1B).

The inhibitors of poly(ADP-ribose) synthetase, 3AB and benzamide, affected DNA synthesis in a similar manner (Fig. 2). The incorporation into DNA of [^{3}H]methionine and [^{14}C]glucose, both of which are involved in de novo synthesis of DNA, was significantly reduced by 3AB and benzamide; for example, 3AB and benzamide at 5 m*M*, a commonly used experimental concentration (*5, 6, 8, 16, 17*), inhibited incorporation of [^{3}H]methionine into DNA by 50 percent. In contrast, [^{3}H]thymidine incorporation into DNA, an index of nucleotide salvage capacity, was unchanged by these chemicals. Results with nicotinamide, another inhibitor of poly(ADP-ribose) synthesis also appears to be nonspecific in that it inhibits DNA synthesis at concentrations above 2 m*M* (*18*).

The effects of *m*-ABOA and BOA, the noninhibitory analogs of benzamide, on the metabolic processes differed from those of the synthetase inhibitors (Fig. 2). Both *m*-ABOA and BOA reduced [^{3}H]thymidine incorporation into DNA. However, *m*-ABOA had no effect on [^{14}C]glucose incorporation, whereas 5 m*M* BOA reduced incorporation of [^{14}C]glucose into DNA by 60 percent. Synthesis of DNA from [^{3}H]methionine was not significantly affected by either chemical.

These results show that 3AB and benzamide are not specific inhibitors of poly(ADP-ribose) synthetase. In addition, they differ from each other in the metabolic processes they affect. Although both 3AB and benzamide reduced glucose and methionine incorporation into DNA, benzamide had a greater effect, probably as a result of its cytotoxicity. In addition, only benzamide was capable of inhibiting glucose oxidation. No clear distinction was found between the poly(ADP-ribose) synthetase inhibitors and their noninhibitory analogs. Although *m*-ABOA and BOA differed from 3AB and benzamide in their effects on thymidine and methionine incorporation, they differed from each other in that BOA, like 3AB and benzamide, inhibited glucose incorporation into DNA, whereas *m*-ABOA did not.

Definitive experimental evidence of a requirement for poly(ADP-ribose) in any cellular process has not been reported.

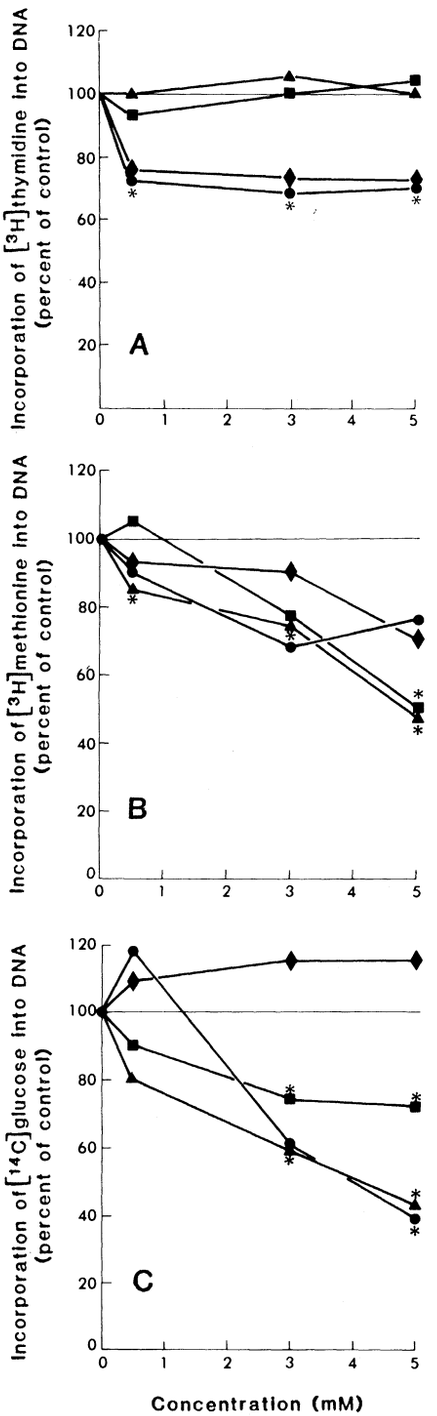

Fig. 2. Effect of the benzamide analogs on incorporation of (A) [^{3}H]thymidine, (B) [^{3}H]methionine, and (C) [^{14}C]glucose into DNA. (■) 3-Aminobenzamide; (▲) benzamide; (◆) *m*-aminobenzoic acid; (●) benzoic acid. The asterisk indicates experimental groups significantly different from controls ($P < 0.05$).

Even the role of poly(ADP-ribose) in DNA repair, one of the most extensively researched areas, has not been unequivocally established. In fact, there are some serious drawbacks and contradictory results in studies of changes in poly(ADP-ribose) concentrations and DNA repair. For instance, it was necessary to use excessively high concentrations of the alkylating agents dimethyl sulfate and *N*-methyl-*N*-nitrosourea to increase poly(ADP-ribose) synthetase activity, even though much lower concentrations depleted cellular pools of nicotinamide adenine dinucleotide (NAD), the precursor of poly(ADP-ribose) (*11, 19*). In another instance, to avoid using poly(ADP-ribose) synthetase inhibitors, investigators reduced cellular NAD to 10 to 20 percent of control values by growing cells in medium devoid of nicotinamide for four generations (*11*). Subsequent impaired DNA repair in these cells may be attributed as easily to nutritional deprivation as to suboptimal poly(ADPribose) concentrations. Furthermore, poly(ADP-ribose) is not consistently involved in the repair of all types of DNA damage. Although repair of some alkylation damage is correlated with increased poly(ADP-ribose) synthetase activity, some is not (*11, 20*). Repair of most strand breaks produced in DNA by x-rays, gamma rays, and ultraviolet radiation does not require poly(ADP-ribose) synthesis (*17*), and inhibition of poly(ADP-ribose) synthesis does not increase the cytotoxicity of x-rays (*21*).

Finally, evidence for an essential role for poly(ADP-ribose) in DNA repair has depended on the use of poly(ADP-ribose) synthetase inhibitors, particularly 3AB. It has been assumed that these compounds specifically inhibit the synthetase and do not affect other basic metabolic functions. Our results reveal adverse effects of both benzamide and 3AB on several important metabolic pathways. These results do not disqualify poly(ADP-ribose) as a key component in a number of metabolic processes, including DNA repair. However, data obtained by the use of these inhibitors should be reevaluated, because changes in cellular processes attributed to reduced poly(ADP-ribose) concentrations may instead be due to the compromised metabolic integrity of cells after treatment. We have found that poly(ADP-ribose) synthesis in permeable cells is more sensitive to 3AB than are strand-break rejoining during DNA repair and the production of sister chromatid exchange in intact cells (*22*). However, the relation between concentrations of exogenous 3AB and the inhibition of

poly(ADP-ribose) synthesis in intact cells versus permeable cells is unknown. Thus, achieving a concentration of exogenous 3AB sufficiently low to inhibit poly(ADP-ribose) synthesis, but to have no nonspecific effects in intact cells, may be difficult.

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Cerebellar Autonomic Function: Direct Hypothalamocerebellar Pathway

Abstract. *A direct hypothalamocerebellar projection in the cat was revealed by means of retrograde transport of wheat germ agglutinin–horseradish peroxidase complex. This appears to be the first demonstration of a significant autonomic input to the cerebellum. The projection has a widespread origin and is bilateral with an ipsilateral preponderance.*

Cerebellar influences on visceral functions, as described by Moruzzi (*1*) more than 30 years ago, have been recently confirmed (*2*). Although a cerebellohypothalamic pathway (from the fastigial nucleus) has been described (*3*), other projections participating in cerebellar autonomic control remain essentially unknown. In particular, information on input to the cerebellum from autonomic centers is lacking. A highly sensitive wheat germ agglutinin–horseradish peroxidase (WGA-HRP) complex, used as a retrograde tracer, revealed a projection from the hypothalamus to the cerebellar cortex.

Seven cats (2.1 to 3.1 kg) were anesthetized with pentobarbital sodium (Mebumal), and 0.2 μl of 1 percent WGA-HRP (Sigma) was injected into the cerebellar cortex through a 1-μl Hamilton syringe. Six cats each received two injections (altogether 0.4 μl of WGA-HRP), and the seventh received four injections in different cortical folia (altogether 0.8 μl of WGA-HRP). After 2 days, the cats, while under deep pentobarbital anesthesia, were killed by intracardiac perfusion with physiological saline; this was followed by perfusion with a solution containing 1.25 percent glutaraldehyde and 1 percent paraformaldehyde in 0.01*M* phosphate buffer at *p*H 7.4, and finally with a solution of 10 percent sucrose. The brain was immediately isolated, removed, and placed in 30 percent sucrose. The cerebellum was cut frontally or sagittally and the brainstem was cut transversally in serial sections at 50 μm on a freezing microtome. Two of five consecutive sections were mounted. The sections were treated with tetramethylbenzidine as a chromogen (*4*). One series was weakly stained with neutral red and the other was left unstained. Several normal animals that served as controls were processed in the same manner as the experimental animals. No labeled hypothalamic neurons were found in the controls.

The sections from the experimental animals (and the controls) were examined with bright-field and interference-contrast microscopy. In five cats the

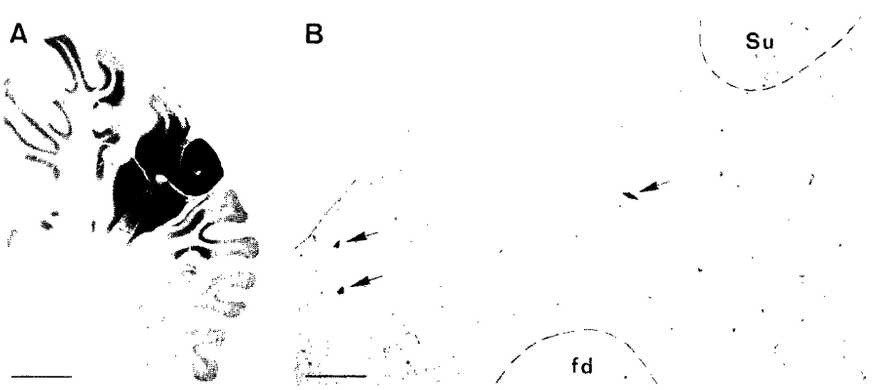

Fig. 1. (A) Photomicrograph showing the cerebellar cortical injection site in cat B.St.L.974 (injected in the anterior lobe). Scale bar, 1.5 mm. Patches of dark staining in the granular layer on both sides of the two injected folia are excess deposits of neutral red. (B) Photomicrograph showing three retrogradely labeled neurons (arrows) in the lateral hypothalamic area in cat B.St.L.977. Scale bar, 150 μm. For abbreviations, see Fig. 2.