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Influence of Sodium Salicylate on Metabolism of Lathyrogen-Treated 3T6 Fibroblasts in Culture

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Abstract □ The effect of sodium salicylate on the metabolism of 3T6 fibroblasts treated with the lathyrogen β-aminopropionitrile was studied. A 5 m*M* concentration of the lathyrogen and/or 0.5 m*M* sodium salicylate was used to study proliferation, collagen synthesis, and mucopolysaccharide synthesis as measured by $^{35}SO_4$ incorporation. Sodium salicylate was able to reverse all lathyrogen-mediated changes to control or near control values. The diversity of these biosynthetic activities suggests that the influence of sodium salicylate on lathyrogen-treated cells is a result of a general metabolic effect rather than a specific one; the exact mechanism of action remains unknown.

Keyphrases □ Sodium salicylate—effect on metabolism of fibroblasts treated with β-aminopropionitrile, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Lathyrogens, β-aminopropionitrile—used to treat fibroblasts, effect of sodium salicylate on fibroblast metabolism □ β-Aminopropionitrile—used to study effects of sodium salicylate on metabolism of lathyrogen-treated fibroblasts □ Fibroblasts (3T6), β-aminopropionitrile treated—effects of sodium salicylate on metabolism, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Metabolism—β-aminopropionitrile-treated fibroblasts, effects of sodium salicylate

The lathyrogen β-aminopropionitrile fumarate in a concentration of 5 m*M* has been shown to depress cell proliferation while enhancing the synthesis of collagen, noncollagenous protein, glycogen, and mucopolysaccharides (1–4). Lesser concentrations of the lathyrogen do not exhibit these changes. Both the higher and lower concentrations, however, do affect collagen maturation, which is thought to be due to an inactivation of lysyl oxidase resulting in a deficiency of lysine-derived aldehydes (5). The discrepancies in data among studies are no doubt due to differences in cell lines used, experimental conditions, and, most important, concentrations of the lathyrogen employed.

Among the antirheumatic drugs, salicylates have been shown to have a beneficial influence on changes in experimental lathyrism by decreasing the elevated proportion of extractable collagen in tissues and the disappearance of the typical histological changes seen in the disease (6–8). Salicylates have also been shown to modify collagen and mucopolysaccharide synthesis to a greater extent than noncollagenous protein (9). Other researchers reported that they stimulate the turnover of collagen in general and its maturation in particular (10) while inhibiting the growth of human embryonic cells (11).

In the present study, an attempt was made to ascertain whether the influence of sodium salicylate was limited primarily to correcting the collagen maturation defect brought about by low concentrations of the lathyrogen (1 or 2 m*M*) or whether sodium salicylate affects other metabolic alterations such as those resulting from a 5 m*M* lathyrogen concentration.

EXPERIMENTAL

Materials and Methods—The 3T6 fibroblasts (12) were grown in the presence of 10% CO_2 in 60 × 15-mm plastic petri dishes in the Dulbecco–Vogt modification of Eagle's medium containing 10% calf serum. Five-day-old cultures were trypsinized and resuspended into a common culture having approximately 100,000 cells/ml. The common culture was then divided into four equal portions: β-aminopropionitrile was added to one at a concentration of 5 m*M*; sodium salicylate was added to the second at a concentration of 0.5 m*M*; β-aminopropionitrile (5 m*M*) and sodium salicylate (0.5 m*M*) were added to the third; and the fourth served as a control. The dosages of the lathyrogen and sodium salicylate used were established by assessing the toxicity of various concentrations. It was found that 5 m*M* β-aminopropionitrile and 0.5 m*M* sodium salicylate produced the maximum response without affecting cell viability.

Replicate cultures were prepared by dispensing 5 ml of cell suspension into each culture plate and incubating them at 37°. The medium was changed three times weekly for the duration of the experiment. Cells were harvested at 2, 4, 7, 9, 11, and 14 days. All media were pooled and saved for analysis, so the data represent total synthesis of material to the day of harvest. For analysis the medium was decanted and centrifuged at 200×*g* for 10 min to remove any dislodged cells or debris, and the cell layer was detached by using a razor blade or by incubation with 0.02% ethylenediaminetetraacetic acid in a phosphate buffer. Quantitation was based on both the cell count and DNA content (13), taking the average of three identically treated plates separately analyzed.

Chemical Analysis—*Collagen Synthesis*—Hydroxyproline in the medium and cell layer was determined by the method of Prockop and Udenfriend (14). Recoveries by this method averaged 86.8 ± 4.2%. All readings[1] were made at 560 nm. The medium was dialyzed exhaustively and then hydrolyzed in 6 *N* HCl for 16 hr at 104° before analysis. Unused medium served as a blank.

The solubility of the cell layer was determined as follows. Salt-

[1] Hitachi-Perkin-Elmer spectrophotometer.

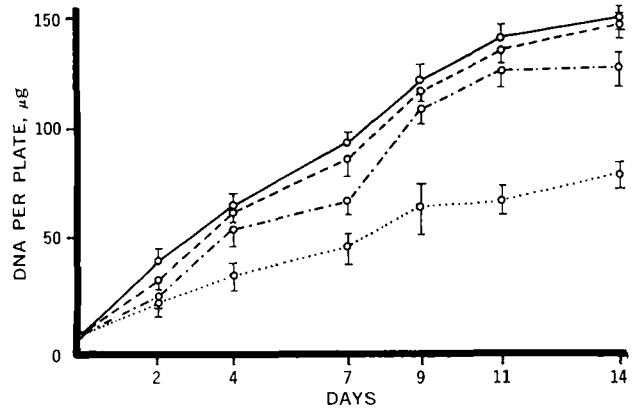

Figure 1—*Influence of 5 mM β-aminopropionitrile and/or 0.5 mM sodium salicylate on cellular proliferation for a 14-day period. Each point represents the average of three identically treated plates separately analyzed. Key: O—O, control; O- - -O, sodium salicylate; O···O, β-aminopropionitrile; and O-·-·O, β-aminopropionitrile and sodium salicylate.*

soluble collagen was extracted by pooling five plates of cells and shaking at 4° in 5 volumes of 1 *M* NaCl (pH 7.6) for 24 hr. Acid-soluble collagen was then extracted by shaking the residue in 5 volumes of 0.1 *N* acetic acid for 24 hr, and the remaining material was designated as insoluble collagen. Each fraction was hydrolyzed and analyzed for hydroxyproline.

$^{35}SO_4$ Uptake—Carrier-free Na_2-$^{35}SO_4$, 1 μCi/ml culture medium, was added to sulfate-free medium. Cells were harvested over a 7-day culture period, and both cells and media were analyzed for $^{35}SO_4$ incorporation. The medium was dialyzed exhaustively while cells were extracted four times with boiling 80% ethanol and then dissolved in 10% sodium hydroxide before counting[2].

RESULTS

Growth—All culture plates were seeded at 1×10^5 cells/ml medium used. Lathyrogen-treated cells showed a depression in cell growth as previously reported (1, 3), while sodium salicylate-treated cells showed very little, if any, change in growth. When both β-aminopropionitrile and sodium salicylate were used together, growth increased to within 85–90% of control values (Fig. 1).

To determine whether the sodium salicylate effect was indeed a true reversal, cells were grown in the presence of β-aminopropionitrile alone for 4 days, at which time the lathyrogen effect is clearly established. At this point, sodium salicylate was added

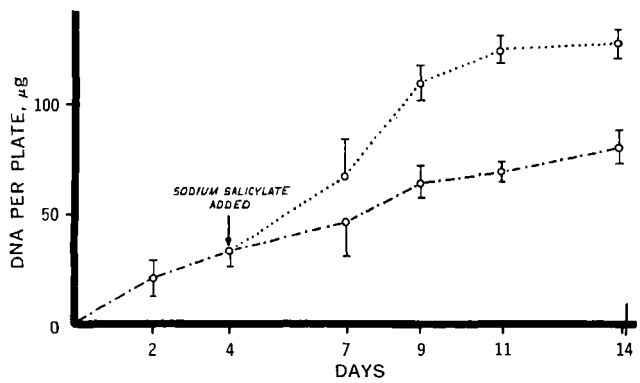

Figure 2—*Modification of cellular proliferation of β-aminopropionitrile-treated cells by 0.5 mM sodium salicylate added on the 4th day of culture. Each point represents the average of three identically treated plates separately analyzed. Key: O-·-O, β-aminopropionitrile; and O···O, β-aminopropionitrile and sodium salicylate.*

[2] Beckman LS-150 scintillation counter.

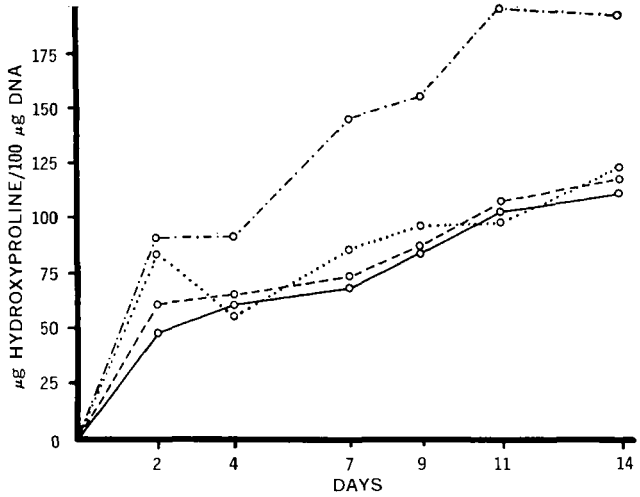

Figure 3—*Medium hydroxyproline measured over a 14-day period. All media were pooled and saved for analysis; therefore, the data represent total synthesis of material to the day of harvest. Each point represents the average of three plates separately analyzed. DNA was measured as previously described. Key: O—O, control; O- - -O, sodium salicylate; O-·-O, β-aminopropionitrile; and O···O, β-aminopropionitrile and sodium salicylate.*

and growth was monitored for an additional 10 days. At the end of 14 days, growth was the same as for those cultures in which both lathyrogen and sodium salicylate were added at the beginning of the experiment (Fig. 2).

Collagen Synthesis—*Medium*—At the end of a 14-day culture period, the lathyrogen produced the previously reported (1) increase in a hydroxyproline-containing macromolecule in the medium. Sodium salicylate alone had no effect on hydroxyproline synthesis, while the addition of sodium salicylate to the lathyrogen-treated cultures reduced hydroxyproline synthesis to control values (Fig. 3).

Cell Layer—There was virtually no significant difference in the total collagen associated with the cell layer. Salt-soluble fractions were similar in both control and sodium salicylate-treated cells, while the lathyrogen produced almost twice the amount of salt-soluble material. The addition of sodium salicylate to lathyrogen-treated cultures lowered the salt-soluble collagen and increased the insoluble fraction to close to control values. The acid-soluble fractions remained relatively unchanged throughout (Fig. 4).

$^{35}SO_4$ Uptake—Lathyrogen-treated cultures showed an increased uptake into a sulfated macromolecule in both the cell

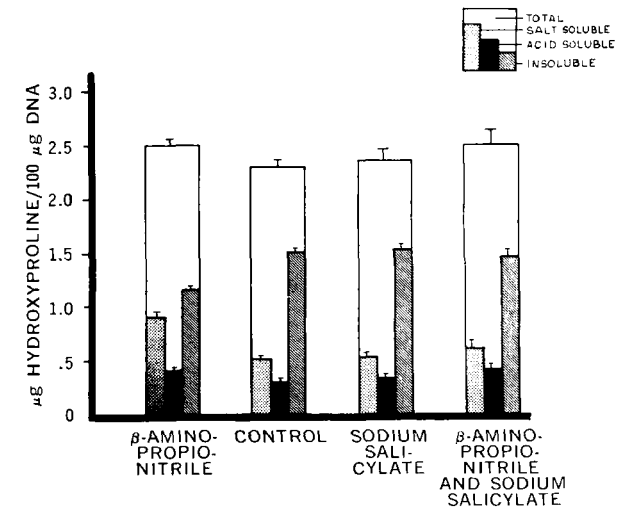

Figure 4—*Cell-associated hydroxyproline at the end of a 7-day culture period showing total, salt-soluble, acid-soluble, and insoluble fractions. Results are the average of three experiments; five plates of cells were pooled for each analysis.*

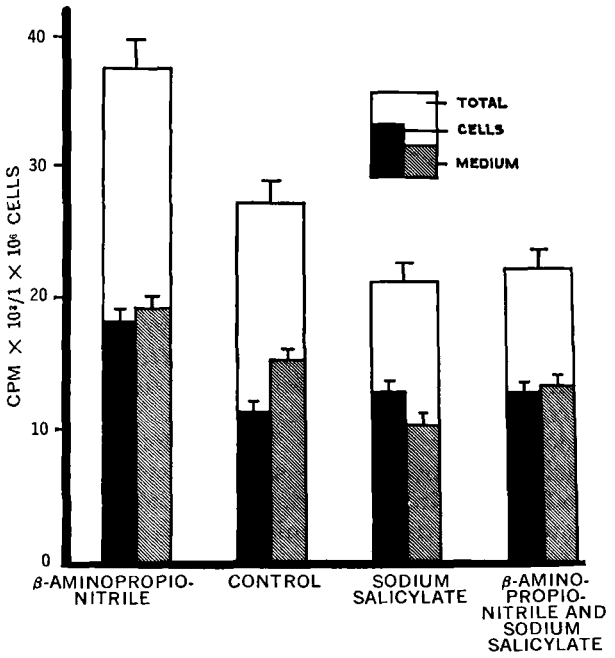

Figure 5—$^{35}SO_4$ *uptake (three plates per point, as previously described) at the end of a 7-day culture period showing total, cellular, and medium uptake.*

layer and the medium, while sodium salicylate lowered the uptake when compared to controls. The addition of sodium salicylate to lathyrogen-treated cultures lowered uptake below control values more closely to those obtained with sodium salicylate alone (Fig. 5).

DISCUSSION AND CONCLUSIONS

There have been conflicting reports in the literature on the *in vivo* effect of lathyrogens on macromolecule synthesis. Polysaccharides, for example, have been shown to exhibit no change, an increase, and a decrease in total synthesis (15–17). Collagen synthesis has also been reported to be normal (18, 19), retarded (20, 21), or accelerated (22). The *in vitro* model system used in this study (5 m*M* β-aminopropionitrile) consistently showed an increase in collagen synthesized into the medium (1), an increase in $^{35}SO_4$ uptake (3), and a decrease in proliferation (1, 3).

At a concentration of 0.5 m*M*, sodium salicylate had little or no effect on cell proliferation. This is a dose-response effect because higher concentrations have a variety of effects, depending on concentration and tissue involved (11, 23). The reversal of growth inhibition produced by the lathyrogen was pronounced whether the sodium salicylate was administered at the beginning of the culture period or on the 4th day when the lathyritic effect was clearly established.

Collagen in the medium was clearly increased by 5 m*M* β-aminopropionitrile, while the cell-associated material showed virtually little change in total amount. The only definitive change in the cell-associated material was an increase in the salt-soluble fractions and a slight decrease in the insoluble fractions of the lathyrogen-treated cells. Sodium salicylate reversed the lathyrogen-produced increase of a hydroxyproline-containing macromolecule in the medium and reduced the changes in the cell layer to near control values. While sodium salicylate by itself did not influence either medium- or cell-associated hydroxyproline, other studies using different model systems and dosages showed that salicylate can and does influence collagen metabolism (6).

Salicylates have been known to inhibit $^{35}SO_4$ incorporation and mucopolysaccharide synthesis in general (23, 24). However, at the dosage used in this study, sodium salicylate alone caused a slight reduction in total $^{35}SO_4$ uptake, while the combination of β-aminopropionitrile and sodium salicylate reduced the total to below control values but did not affect the ratio of cell-bound material to $^{35}SO_4$-containing macromolecule found in the medium.

Aside from the cross-linking defect, lower concentrations of β-aminopropionitrile do not seem to affect cell proliferation or other metabolic processes (25, 26). Higher concentrations, however, limit proliferation while stimulating the general synthetic capabilities of cells in culture (1–4). Experiments in this laboratory designed to test the possibility that limitation of proliferation is related to the phenomena observed demonstrated that these are separate and apparently unrelated events.

The fact that sodium salicylate tends to reverse seemingly diverse metabolic pathways is not surprising. Salicylates seem to act at several possible sites. They have been shown, among other things, to accelerate the metabolic turnover of collagen (6), inhibit the biosynthesis of noncollagenous protein (27) and mucopolysaccharide (24), increase oxygen uptake, and inhibit acetyl-CoA synthetase (28). The diversity of these biosynthetic activities suggests that the influence of the antirheumatic drug, sodium salicylate, on experimental lathyrism is a result of a general metabolic effect rather than a specific one; the exact mechanism of action remains unknown.

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