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Control of growth by picolinic acid: Differential response of normal and transformed cells

(cell synchrony/chelating agents/NAD^+/pyridine derivatives/viral transformation)

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ABSTRACT **Picolinic acid reversibly inhibits the growth of cultured cells. Fourteen other pyridine derivatives were ineffective or toxic. Untransformed normal rat kidney (NRK) cells are reversibly arrested in the G_1 stage of the growth cycle as shown by cell counts, mitotic index, [^{3}H]thymidine incorporation, and flow microfluorometry. Flow microfluorometry was used to monitor the effects of picolinic acid on numerous other cell lines. Normal cells are blocked in G_1, whereas transformed cells show responses that are dependent upon the transforming virus and independent of species or origin of the cell line. Kirsten sarcoma virus-transformed cells are blocked in G_1. Simian virus 40-transformed cells progress to a G_2 block. Cells transformed by polyoma or Harvey sarcoma virus with Moloney virus coat have flow microfluorometry profiles that indicate blocks in both G_1 and G_2. Cells transformed with Moloney sarcoma virus are not blocked in a specific phase of the cell cycle. Picolinic acid does not change the levels of NAD^+ plus NADH; however, the growth inhibition by picolinic acid is partially overcome by nicotinamide. These results suggest that picolinic acid interacts with a specific growth control mechanism that may involve NAD^+ and that this control mechanism is altered by different transforming viruses in different manners.**

Picolinic acid is a metal-chelating agent (1) and is also structurally related to nicotinic acid, a precursor in the biosynthesis of NAD^+. On the basis of theoretical considerations on the role of metal ions and NAD^+ in growth control (J. A. Fernandez-Pol, unpublished), picolinic acid was tested and found to be a useful agent to study cell growth regulation. Picolinic acid is of further interest because it is a biological component in at least some cells and could participate in a physiological growth regulatory mechanism.

Here we report on the effects of picolinic acid on the growth of cultured cells. The agent is an inhibitor of cell growth and reversibly arrests cells at a specific point in the cell cycle. A preliminary report on this work has been published (2).

MATERIALS AND METHODS

Materials. Nicotinamide, nicotinic acid, picolinic acid, NAD^+, and 2,6-pyridine dicarboxylic acid were obtained from Sigma; picolinamide, from Matheson, Coleman, and Bell; isonicotinamide, isonicotinic acid, isonicotinic acid hydrazide, 3-aminomethylpyridine, 2,4-lutidine, 2-aminomethylpyridine, 2,3-pyridinedicarboxylic acid, and 3,5-pyridinedicarboxylic acid, from Aldrich. 3-Mercaptopicolinic acid was provided by H. Saunders, Smith, Kline and French, Philadelphia, PA, and Ca^{2+} ionophore A-23187 by R. J. Hosley, Eli Lilly Co., Indianapolis, IN.

Methods. Cells were grown in Dulbecco–Vogt modified Eagle's medium containing 10% (vol/vol) calf serum (Colorado Serum Co.) in 20-cm^2 tissue culture dishes (Falcon Plastics) under 95% air/5% CO_2, humidified atmosphere, at 37°. The cell lines used are listed in Table 1. Unless otherwise indicated, cells were planted at 1.5×10^5 cells per dish. Twenty-four hours later, media were replaced by new media with (treated) or without (control) picolinic acid. All cells lines were treated with picolinic acid at 1.5, 2, 2.5, 3, and 4 mM and analyzed for 4 days with media change every other day.

Cells were analyzed for DNA content by flow microfluorometry (FMF) after trypsinization and suspension in propidium iodide solution (0.05 mg/ml in 0.1% trisodium citrate) at 2×10^6 cells per ml (3). The frequency distribution of fluorescent emission per cell (proportional to DNA content) was measured in 10^5 cells using a cytofluorograph (Biophysics Systems, Inc., Baldwin Place, NY), and analyzed with a multichannel analyzer (Tracor Northern, Inc., Middleton, WI).

NAD^+ plus NADH was assayed as previously described (4). Recovery of a known amount of NAD^+ was greater than 95%; no reactive material was detected in medium, and medium containing 3 mM picolinic acid did not interfere with the assay. Cells were counted with a Coulter counter. Thymidine incorporation was measured (5) with 0.05 μCi of [^{3}H]thymidine per dish.

RESULTS

Effect of Picolinic Acid on Growth of NRK Cells. The growth of normal rat kidney (NRK) cells was inhibited by picolinic acid in a dose-dependent manner (Fig. 1); the inhibition was reversible within 24 hr after picolinic acid removal. These results, which were reproduced in four separate experiments, suggested a possible block at a specific point in the cell cycle. To test for such a block, NRK cells were exposed to 3 mM picolinic acid for 36 hr. At this time essentially no mitotic cells were observed. A wave of DNA synthesis began 11 hr after removal of picolinic acid (Fig. 2), followed by a burst of mitosis and a doubling in cell number from 16 to 22 hr. In this particular experiment 5–6 hr were required for the cells to double in number after the first mitotic cells were observed. In other experiments this process was even more rapid, with a complete doubling within 2–3 hr. To quantitate and further verify the G_1 block, FMF analyses were done (Fig. 3). The results clearly show that treated cells were predominately in G_1. Furthermore, when picolinic acid was removed, the number of cells in S and G_2 increased and, following the first wave of mitosis, essentially all of the cells were in G_1. Thus, NRK cells were arrested in G_1 by picolinic acid and they proceeded through the cell cycle after its removal.

Effects of Picolinic Acid on Transformed NRK Cells. The effects of picolinic acid were tested on three different transformed cell lines under conditions that arrested normal NRK cells in G_1. Cells transformed by different viruses responded differently. In all three cell lines there was no further increase in cell number after 40 hr, but FMF analysis showed that the

Abbreviations: NRK, normal rat kidney; FMF, flow microfluorometry; SV40, simian virus 40.

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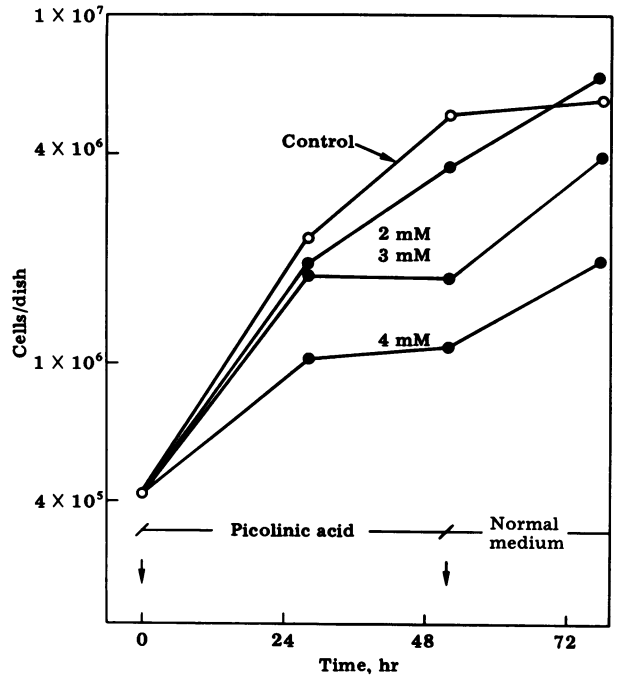

FIG. 1. Dose dependence and reversibility of growth inhibition in NRK cells treated with picolinic acid. Cells were planted at 5×10^4 cells per dish and 66 hr later the medium was removed and new media containing picolinic acid at the indicated concentrations were added. Twenty-four hours later the media were removed, the cultures were rinsed once with fresh medium, and medium without picolinic acid was added. Cell counts were determined at the indicated times; each point is the average of duplicate measurements from two cultures.

point of arrest in the cell cycle was different. K-NRK cells were arrested in G_1 (Fig. 4*B*). This stage of arrest was similar to that observed with the parent NRK cells; however, it has not been established if the two cell lines were blocked at identical points in G_1. Under these conditions simian virus 40 (SV40)-transformed NRK cells were progressing to a G_2 block (Fig. 4*A*). The DNA distribution in treated MSV-NRK cells was the same as that in the control (Fig. 4*C*). This suggests that MSV-NRK cells were not blocked at any specific point in the cycle.

Effects of Picolinic Acid on Other Normal and Transformed Cells. Numerous normal and transformed lines (Table 1) were tested for 4 days over a wide concentration range (see *Materials and Methods*). In all lines no increase in cell number was detected after 36–48 hr. However, FMF analysis showed differences in the block point. As a function of time the cells progressed into a specific phase(s) of the cycle. A predominance of cells in G_1 (Figs. 3*B* and 4*B*) or G_2 (Figs. 4*A* and 6) indicated a G_1 or G_2 block, or a progression to that phase. A significant percentage of cells in both G_1 and G_2 with no S phase cells showed a block in both phases (Fig. 5). No significant changes in the FMF profile indicated that the cells were not blocked at a specific point (Fig. 4*C*).

Untransformed BALB 3T3, NRK, and WI-38 cells were blocked in G_1 (Table 1). BHK cells were the only normal cell line to show a block in a cell cycle stage other than G_1 (Fig. 5*A*). They ceased growth in G_1 and G_2 as did some transformed cell lines (see below). However, BHK cells show some properties of transformed cells (6); thus it is difficult to interpret the results with this cell line.

Two important facts emerged from studies with transformed lines. Cells transformed by different viruses responded differently to picolinic acid, and cell lines from different species transformed by the same virus were blocked in similar manners

Table 1. Cell lines investigated

Cell lines	Transforming virus	Type of block	Time, hr	Dose, mM
BALB 3T3 ClC/3	None	G_1	48	3
BALB 3T3 Cl31-7	None	G_1	52–72	3–4
NRK Cl5W	None	G_1	48	3
WI-38 ClA-17	None	G_1	72	3–3.5
BHK-21	None	$G_1 + G_2$	48	2.5
$KA_{31}Cl_1$ BALB 3T3	KSV	G_1	43	2.5–3
K-NRK Cl32	KSV	G_1	42–48	3
SV-BALB 3T3 ClSVT2	SV40	G_2	48–72	2.5–3
SV-BALB 3T3 $ClSVTb_3$	SV40	G_2	48	2.5–3
SV-NRK P8 Cl_2T_7	SV40	G_2	72	3
SV-WI-38 ClVA-13	SV40	G_2	96–120	2.5–3
HSV/Mol-BALB 3T3	HSV with MSV coat	$G_1 + G_2$	72	2.5
HSV/Mol-NRK	HSV with MSV coat	$G_1 + G_2$	72	2.5
PY-3T3 Cl11	Polyoma	$G_1 + G_2$	68	2
SV-PY-11 3T3	SV40 and polyoma	$G_1 + G_2$	68	2
MSV-BALB 3T3 Cl31	MSV	Random	43–72	2.5
MSV-BALB 3T3 Cl85	MSV	Random	48–72	2.5
MSV-NRK	MSV	Random	43–72	2.5–4

KSV, Kirsten sarcoma virus; HSV, Harvey sarcoma virus; MSV, Moloney sarcoma virus. BALB cells are mouse; WI-38 cells are human; BHK cells are hamster.

(Table 1). The latter point is clearly illustrated with the SV40-transformed cell lines. Four different lines progressed through the cell cycle into G_2 (Table 1). An example of this progression is shown in Fig. 6. SV40-transformed WI-38 cells (ClVA-13) after 53 hr began to accumulate in S and G_2+M with a concomitant decrease in G_1. The cells then further progressed into G_2, and by 96 hr, most of the cells were in G_2.

3T3 cells transformed by polyoma, another DNA virus, and cells transformed by both SV40 and polyoma (Fig. 5*B*) showed peaks at both G_1 and G_2 but with a significant content of S phase cells after 68 hr. With further incubation in picolinic acid the cells began to show signs of toxicity and no further changes in the FMF profile could be observed. Because some S phase cells were present, it is possible that the cells were not arrested in both G_1 and G_2 but were still progressing through the cell cycle into G_2.

Cells transformed by three different strains of RNA tumor viruses were also tested (Table 1). Both lines transformed by Kirsten sarcoma virus were arrested in G_1. Two lines transformed by Harvey sarcoma virus with the Moloney coat were blocked in both G_1 and G_2, and three lines transformed by Moloney sarcoma virus were not blocked at a specific point.

Effects of Prolonged Incubation with Picolinic Acid. Normal and transformed cells treated for up to 48 hr showed no toxic effects from the picolinic acid. With longer exposure different responses of normal and transformed cells were observed. NRK-5W cells treated with 3 mM picolinic acid with frequent media changes remained in a quiescent state for 5 weeks (J. A. Fernandez-Pol and G. S. Johnson, unpublished results). In contrast, most transformed cells remained viable only up to 90–120 hr. After this time they began to look granular and to float in the medium. These cells were apparently dead because they would not re-attach to the substratum or increase in number when planted in fresh medium without picolinic acid. Cell death was observed in all transformed cells whether

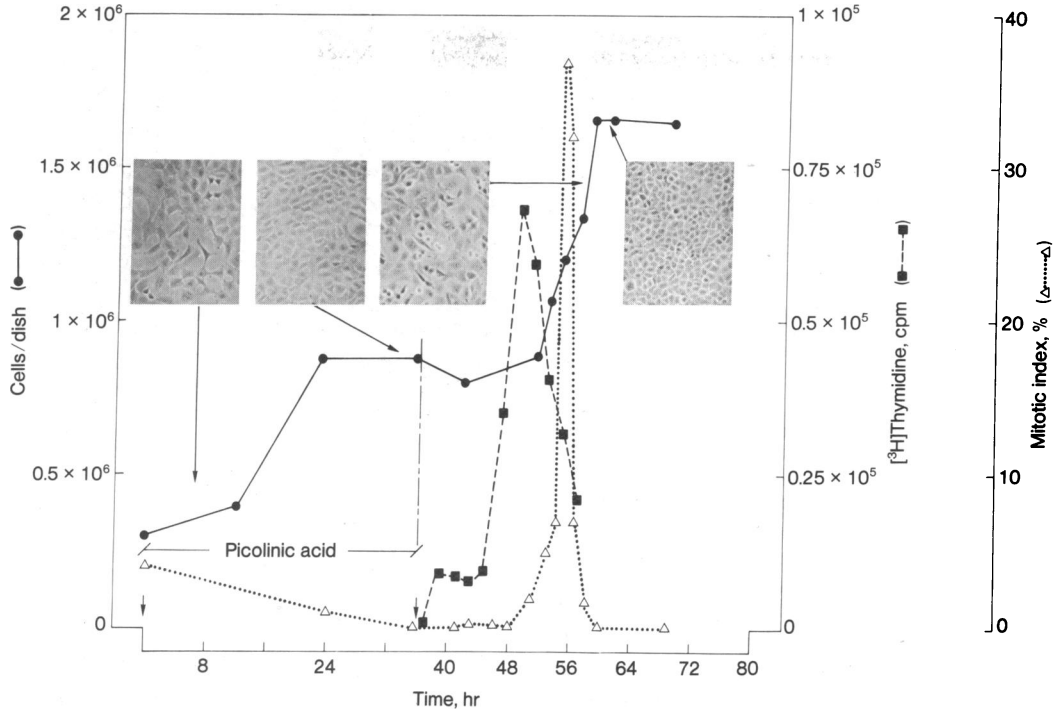

FIG. 2. Synchronization of NRK cell growth by picolinic acid. Cells were planted at 1.6×10^5 cells. After 57 hr the medium was changed and new medium containing 3 mM picolinic acid was added. Thirty-six hours later the medium was removed, the cultures were rinsed, and new medium was added. The cultures were viewed periodically with phase contrast microscopy and the appearances of the cells at the indicated times are shown. Each cell count is the average of duplicate measurements from four cultures. The thymidine incorporation per dish is the average from two cultures.

they were blocked in G_1, G_2, or at random. Further details of this selective cytotoxicity will be published elsewhere.

Effect of Picolinic Acid on Cell Morphology. SVT2 cells treated with picolinic acid became very elongated and flat in appearance (Fig. 7). This morphological change was reversible within 6–10 hr after removal of the agent. Similar effects on morphology were observed in K-NRK cells. The effects on morphology of other cell lines were not as pronounced.

Effects of Structurally Similar Compounds. We tested other pyridine derivatives over a wide concentration range in SVT2 cells for 24–48 hr to determine specificity. This cell line was used because changes in morphology were easily detectable. Nicotinic acid, nicotinamide, isonicotinic acid, isonicotinamide, and picolinamide at 1–5 mM, 3-mercaptopicolinic acid and 2,4-lutidine at 3 mM, and 2,3-pyridinedicarboxylic acid and 3,5-pyridinedicarboxylic acid at 2.5 mM caused flattening and elongation of the cells but no significant change in growth. Isonicotinic acid hydrazide, 5-butylpicolinic acid, and 2,6-pyridinedicarboxylic acid inhibited growth at 0.25–2.5 mM; however, the cells did not resume growth within 24 hr after removal of the chemical and some cells appeared granular and probably were dead. 3-Aminomethylpyridine and 2-picolylamine were highly toxic and caused extensive cell death at 0.1 mM. Thus in all, 15 structurally similar compounds were tested, and from this group, only picolinic acid reversibly inhibited cell growth.

Effects of Other Chelating Agents. Picolinic acid is a metal-chelating agent (1) that could interfere with specific metal requirements for cell growth. We thus determined the effects of other chelating agents on SVT2 cells. Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) had no

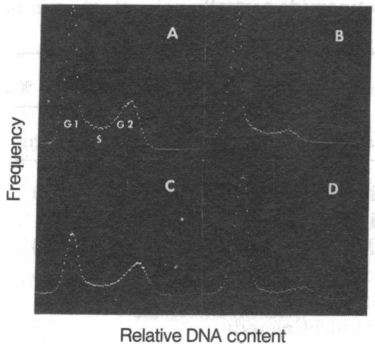

FIG. 3. Sequential DNA distribution profiles of NRK cells treated with picolinic acid. Cells were grown and treated as described in the legend to Fig. 2. (*A*) Control, logarithmically growing cells. (*B*) Cells exposed to 3 mM picolinic acid for 39 hr. (*C*) Seventeen hours after removal of picolinic acid, the movement through the cell cycle is shown by an increase in G_2. (*D*) Twenty-two hours after removal of picolinic acid, and following the first wave of mitosis, essentially all cells are in G_1.

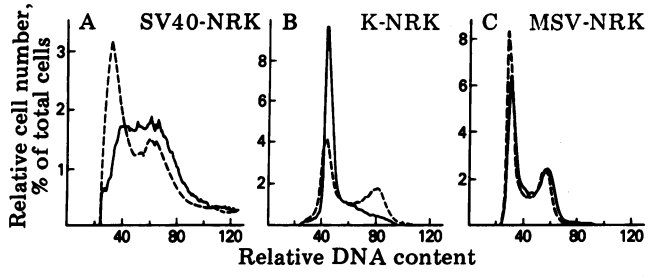

FIG. 4. DNA distribution profiles of three transformed NRK cell lines exposed to 3 mM picolinic acid: (*A*) SV40-transformed cells exposed for 48 hr; (*B*) Kirsten sarcoma virus-transformed cells exposed for 48 hr; and (*C*) Moloney sarcoma virus-transformed cells exposed for 72 hr. Treated cells (—); untreated controls (- - -). The left and right peaks of the control cell profiles represent the position of G_1 and G_2 cells, respectively.

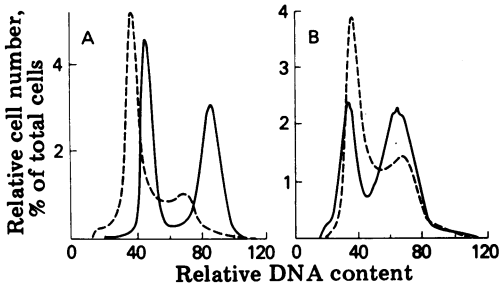

FIG. 5. FMF analysis of cell lines treated with picolinic acid. Picolinic acid (—); logarithmically growing cells (control) (- - -). (*A*) BHK cells treated with 2 mM picolinic acid for 68 hr show two blocks (G_1 and G_2) with greatly reduced S phase. A shift of both G_1 and G_2 peaks to the right in the treated cells is apparent. This shift is presumably a result of an increased stainability of the DNA with propidium iodide. The reasons for this increase are not known. (*B*) SV-PY-11 3T3 cells apparently accumulate in G_1 and G_2 but a considerable S phase is observed under the same conditions as in *A*.

effect on growth or morphology at 1 mM, whereas other chelating agents—ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), quinaldic acid, or 1,10-phenanthroline—caused extensive cell death at this concentration. EGTA at 3 mM was toxic. These results are in agreement with the results of Rubin (7) in chick embryo fibroblasts. Penicillamine had no effect at 1 or 2.5 mM.

The alterations in cell shape may be due to changes in Ca^{2+} flux because the ionophore A-23187 (8) at 0.1–1 μM prevented the elongation. This ionophore has previously been shown to prevent the butyrate-induced morphological changes in HeLa cells (9). The effect of the ionophore on growth inhibition was also tested. At 0.1–1 μM it did not overcome the growth inhibition by picolinic acid; however, at these concentrations the ionophore itself inhibited growth.

Effects of Picolinic Acid on NAD^+ Metabolism. The amounts of NAD^+ plus NADH in SVT2 and NRK cells were unchanged after 4-, 24-, or 48-hr treatment with 3 mM picolinic acid. At 24 hr, the mean and standard error of the mean from 2 experiments with 9 assays per experiment were 1.20 ± 0.03 and 1.27 ± 0.05 nmol/10^6 cells for control and treated SVT2 cells; 1.34 ± 0.02 and 1.20 ± 0.03 nmol/10^6 cells for control and treated NRK cells.

Nicotinamide has previously been shown to protect against the effects of certain agents that alter NAD^+ metabolism (10). Thus, we tested its effects on the inhibition of growth by picolinic acid. Nicotinamide partially prevented the growth inhibition in SVT2 cells (Table 2) and NRK cells (data not shown). This suggests that picolinic acid could act by altering NAD^+ metabolism. Nicotinic acid did not prevent the growth inhibition.

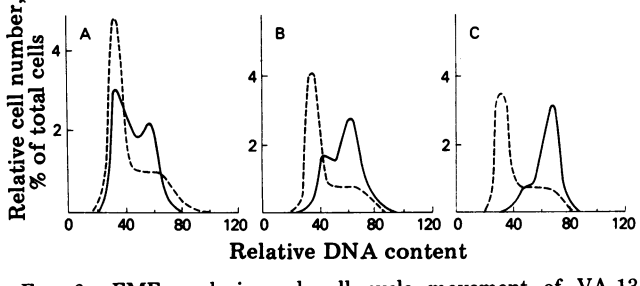

FIG. 6. FMF analysis and cell cycle movement of VA-13 (SV40-transformed WI-38) cells after exposure to picolinic acid (3 mM) (—). All control histograms correspond to logarithmically growing cells (- - -). (*A*) After 53 hr of exposure to picolinic acid, a decrease in G_1 with a concomitant increase in S and G_2 phases of the cell cycle is observed. (*B*) At 72 hr, the G_1 peak is virtually absent. (*C*) At 96 hr, most of the cells are in G_2 while a small percentage remains in S phase.

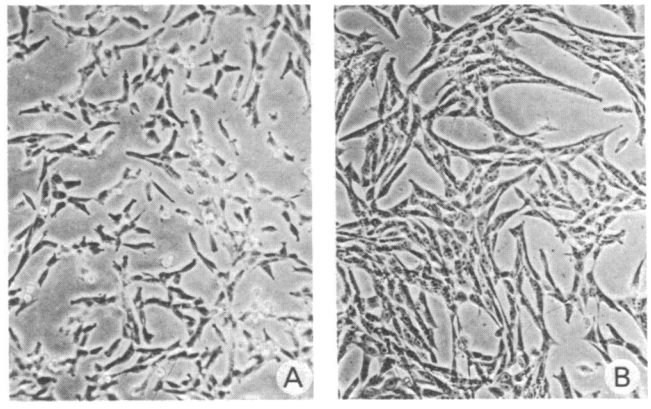

FIG. 7. Effects of picolinic acid on morphology of SVT2 cells. Twenty-four hours after planting, new medium (*A*) or new medium containing 3 mM picolinic acid (*B*) was added. Pictures were taken after another 24 hr. (Phase contrast, ×125.)

DISCUSSION

Picolinic acid arrests the growth of cultured cells, and as a function of time the cells progress to a specific phase(s) in the cell cycle. Normal cells are arrested in G_1. Transformed cells are blocked in different phases; the location of the block point is dependent upon the transforming virus. These observations suggest that picolinic acid interacts with a cellular growth control mechanism and that transforming viruses alter cell growth in different manners. Holley *et al.* (11) reported evidence that benzo[*a*]pyrene and SV40 transform 3T3 cells by different mechanisms. However, definitive data showing differences in transformation by various viruses have not been previously reported. Further studies with picolinic acid should be useful in understanding growth control and possible different mechanisms of transformation.

The blockage of cells in G_1 or G_2 indicates the existence of two domains, one in G_1, the other in G_2, which are important for growth control. Pardee (12) suggested the existence of a restriction point, R_1, in G_1. Our results suggest the existence of a second restriction point, R_2, in G_2. Both R_1 and R_2 may exist *in vivo*. Most non-growing cells *in vivo* are blocked in G_1;

Table 2. Nicotinamide partially prevents the inhibition of SVT2 cell growth by picolinic acid

Agent	% growth 26 hr	% growth 48 hr
2.5 mM picolinic acid	29	17
2.5 mM picolinic acid +8.7 mM nicotinamide	55	35
3 mM picolinic acid	14	2
3 mM picolinic acid +8.7 mM nicotinamide	36	34
8.7 mM nicotinamide	91	91

SVT2 cells were planted at 2.5 × 10^5 cells per dish. The medium was removed 24 hr later and new media containing the indicated compounds were added. Cell counts were determined at the indicated times. The percent growth was calculated by dividing the increase in cell number of the treated cells by the increase in control cultures at the indicated times. The values are expressed as the average from four experiments.

however, a considerable percentage of myocardial, Purkinje, skin, and liver cells are blocked in G_2 under physiological conditions (reviewed in ref. 13). The factors that determine whether cells are blocked in G_1 or G_2 are not known. However, the fact that one agent, picolinic acid, can arrest cells in R_1 or R_2 suggests that the two restriction points may have some common control factors.

Picolinic acid is a naturally occurring compound, but it has no known functions. In the liver it can be synthesized from tryptophan (14). Labeled picolinic acid injected into rats is rapidly excreted in the urine as a glycine conjugate and no other metabolites can be detected (14). Certain derivatives of picolinic acid are active when added to mammalian cells. Fusaric acid (5-butylpicolinic acid) inhibits dopamine β-hydroxylase (15), and 3-mercaptopicolinic acid is an antigluconeogenic agent that inhibits phospho*enol*pyruvate carboxykinase (16). In *Bacillus subtilis*, picolinic acid will inhibit spore formation (17), and *N*-methylpicolinic acid has been found in certain marine organisms (18).

Picolinic acid could act by metal ion chelation (1) or by interference with NAD^+ metabolism or NAD^+ functions. The inhibition of growth is not due to chelation of certain ions such as Ca^{2+} or Mg^{2+} in the medium because other chelating agents do not elicit similar responses. Picolinic acid could bind to and activate a specific protein–metal ion complex in a manner similar to the binding of nicotinic acid to leghemoglobin (19). Numerous pyridine derivatives undergo an exchange reaction with the nicotinamide moiety of NAD^+ to form NAD^+ analogs (20). Possibly picolinic acid could be incorporated into a similar molecule that is the active species. This idea is supported by the fact that the actions of picolinic acid are partially overcome by nicotinamide, a compound that inhibits the exchange reaction with other pyridine derivatives (20). Also, one or two doublings are required before growth cessation (Fig. 1). Thus some metabolic conversion may precede the block. Picolinic acid could alter the activity of poly(ADP-ribose) polymerase, the enzyme that catalyzes the polymerization of the ADP-ribose moiety of NAD^+ (21). In collaboration with W. Kidwell we have found that NRK and SV40-transformed NRK cells contain about three times more poly(ADP-ribose) than do untreated cells. The relevance of this observation to growth inhibition is not clear.

Picolinic acid could inhibit growth by altering the response to growth factors in serum. We have found that prostaglandin E_1 is two to five times more effective in elevating cyclic AMP levels in NRK cells arrested in G_1 by picolinic acid than in control cells (22). This suggests that picolinic acid can modulate hormonal response.

The results of this study clearly show that picolinic acid has effects on the cell cycle that are transformation dependent and may involve NAD^+ metabolism. Because it is a naturally occurring compound, the possibility should be considered that picolinic acid may be part of a normal physiological regulatory mechanism that is altered by transformation.

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