(8) T. Kametani, M. Ihara, K. Fukumolo, and H. Yagi, *J. Chem. Soc. C*, **1969**, 2030.
(9) V. V. Berezhinskaya, *Postep Dziedzinie Leku Rosl.*, **1972**, 164.
(10) D. H. R. Barton, G. W. Kirby, W. Steglich, G. M. Thomas, A. R. Battersby, T. A. Dobson, and H. Ramuz, *J. Chem. Soc.*, **1965**, 2423.
(11) D. M. S. Wheeler, T. H. Kinstler, and K. L. Rhinehart, *J. Amer. Chem. Soc.*, **89**, 4494(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 11, 1974, from the *Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612, and the ‡Department of Pharmacology, School of Medicine, Pahlavi University, Shiraz, Iran.

Accepted for publication July 10, 1975.

ˣ To whom inquiries should be directed.

Effect of Sodium Salicylate on Hamster Cells *In Vitro*

M. RUBENSTEIN* §, D. GIACOMONI *, and L. M. PACHMAN ‡ˣ

Abstract □ Doses of sodium salicylate greater than 100 μg/ml increased the generation time of baby hamster kidney (BHK 21) cells in culture from 16 to 35 hr. Exposure to similar doses of salicylate for 18–44 hr resulted in a marked reduction of RNA synthesis. The species of RNA synthesized in the presence of sodium salicylate appeared to be similar to those synthesized by normal cells in the absence of sodium salicylate. Sodium salicylate did not alter the oxidative phosphorylation of BHK cells.

Keyphrases □ Sodium salicylate—effect on hamster kidney cells in culture, generation time, RNA synthesis □ Cell culture—hamster kidney cells, effect of sodium salicylate on generation and RNA synthesis □ RNA synthesis—hamster kidney cells in culture, effect of sodium salicylate □ Analgesics—sodium salicylate, effect on hamster kidney cells in culture, generation time, RNA synthesis

Aspirin is used to treat various rheumatic diseases. Sodium salicylate has been used as a probe for *in vitro* studies because its effect was similar to that of aspirin on DNA synthesis by stimulated lymphocytes *in vitro* (1). Sodium salicylate inhibited the *in vitro* protein, RNA, and DNA syntheses of human lymphocytes responding to phytohemagglutinin or antigen (1–3). Since great variability has been observed in the suppressive effect of sodium salicylate on human lymphocytes, a genetically defined cell line of baby hamster kidney (BHK 21) cells was selected as a model system to study the effect of salicylate on mammalian cells *in vitro*.

EXPERIMENTAL

Baby hamster kidney cells[1] (BHK 21) were grown as monolayers in a carbon dioxide incubator at 37° using Dulbecco modified Eagle's medium[2] supplemented with 10% fetal calf serum[3] and 10% tryptose phosphate broth[2]. The growth of cells was monitored by harvesting the monolayer after trypsinization and counting the cells using a hemocytometer[4]. Viability was determined by exclusion of 0.1% trypan blue dye.

Total "early labeled" RNA synthesis was determined by exposing the cells to ^{3}H-uridine[5] (2 μCi/ml) for 30 min. At the end of the exposure, sodium azide (0.2%) in saline was added and the cells were harvested and divided into two aliquots. One aliquot was used to measure the radioactivity incorporated in the 10% trichloroacetic acid-insoluble fraction; the other aliquot was used to determine the total DNA by the method of Seibert (4). RNA synthesis was measured as radioactivity incorporated per milligram of DNA.

To determine the effect of sodium salicylate[6] on the type of RNA molecules synthesized, exponentially growing cells were exposed to sodium salicylate (200 μg/ml) for 18 hr. After this incubation time, ^{3}H-uridine (2 μCi/ml) was added for 30 min. Then cells were harvested and mixed with cells that had been grown in the absence of sodium salicylate and similarly exposed to 0.5 μCi/ml of ^{14}C-uridine[5].

RNA was extracted from the cells as previously described (5). The sedimentation profile of the radioactive RNA was determined by layering an aliquot on top of a sucrose linear gradient [5–40% sucrose in 0.01 *M* sodium acetate (pH 5.1), 0.1 *M* NaCl, and 0.001 *M* edetate disodium] in a tube fitting the SW41 rotor of an ultracentrifuge[7].

After 17 hr of centrifugation at 4°, 0.5-ml fractions were collected from the top of the tube using a density gradient fractionator[8]. The fractionator recorded the optical density at 260 nm continuously. To each fraction, 1 ml of cold 20% trichloroacetic acid was added, followed by 5 ml of cold 10% trichloroacetic acid to precipitate RNA. The resulting sediment was collected over a 0.45-μm filter[9], and the acid-precipitable radioactivity was determined.

Determination of the oxidative phosphorylation and the hexose monophosphate shunt was adapted from the method previously described (6). In brief, 3 ml of medium containing 750,000 BHK cells was dispensed into 100-ml serum bottles[10], and sodium salicylate was added to triplicate flasks in a final concentration of 200 μg/ml. Control bottles were prepared containing fewer cells (500,000/3 ml) so that the numbers of cells in each category (sodium salicylate and control) would be equivalent at the end of the 24-hr incubation period.

After 24 hr of incubation, the medium was decanted from all bottles. The cell monolayer was washed with 0.85 *M* phosphate-buffered saline containing 1000 μg/ml of dextrose. To each culture, 3.0 ml of saline–dextrose containing 200 μg/ml of sodium salicylate or saline was added. ^{14}C-Glucose, labeled in the C-1 or C-6 position, was then added (0.5 μCi/ml). The bottles were immediately capped with rubber stoppers, from which were suspended ampuls containing 1.0 ml of a quaternary ammonium hydroxide[11].

[1] Obtained from Dr. C. Basilico.
[2] Grand Island Biological, Grand Island, N.Y.
[3] Flow Laboratories, Rockville, Md.
[4] Newbauer.
[5] New England Nuclear, Boston, Mass.
[6] Merck Chemical Division, Merck & Co., Rahway, N.J.
[7] Spinco L265, Beckman Instrument Co., Fullerton, Calif.
[8] Instrumentation Specialties Co., Lincoln, Neb.
[9] Millipore, Bedford, Mass.
[10] Bellco Glass, Inc., Vineland, N.J.
[11] Hyamine hydroxide, Packard, Downers Grove, Ill.

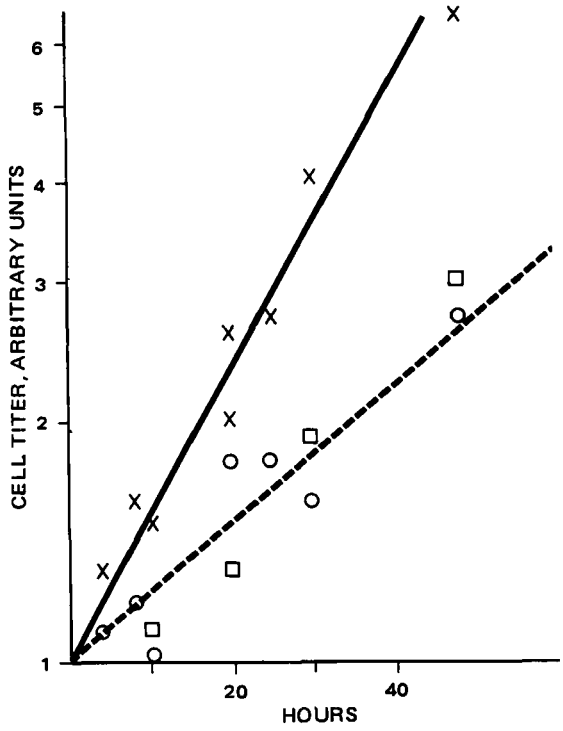

Figure 1—*Effect of sodium salicylate on growth of BHK 21 cells. BHK 21 cells were grown in the absence and in the presence of different amounts of sodium salicylate. The cell number at time 0 was given the value 1. Key: ×—, control (no salicylate added); □ - - - -, 100 μg/ml of sodium salicylate in the medium; and ○ - - - -, 200 μg/ml of sodium salicylate in the medium. Data from two experiments are presented.*

The bottles were incubated for 3 hr in a 37° water bath. Then the ampuls containing adsorbed ^{14}C-carbon dioxide were immediately removed, and their radioactivity was determined in a liquid scintillation counter[12].

Cell counts were done on the monolayer of the triplicate bottles, and the ^{14}C-carbon dioxide evolved was expressed as counts per minute per million cells.

RESULTS

The growth of BHK 21 cells in the presence of sodium salicylate was studied (Fig. 1). The addition of 100 μg/ml of sodium salicylate increased the generation time from 16 (control) to 35 hr. A higher concentration of sodium salicylate (200 μg/ml) did not increase the generation time further. The viability of the cells was more than 95% throughout the experiment.

The effect of sodium salicylate on the incorporation of ^{3}H-uridine by the cells during a 30-min exposure to the label (total early labeled RNA synthesis) was determined. When cells were incubated for 24 hr with 200 μg/ml of sodium salicylate, the incorporation of radiolabeled uridine was reduced by 37–60% with respect to controls. When the cells were incubated for 24 hr in the presence of 350 μg/ml of sodium salicylate, the reduction of incorporation was greater than 90%. After 44 hr of incubation with 200 μg/ml of sodium salicylate, incorporation was reduced by 80%.

When the effect of sodium salicylate on the type of RNA molecules synthesized in cells exposed for 18 hr to the drug was studied, the early labeled RNA was analyzed on a sucrose gradient. ^{3}H-RNA derived from sodium salicylate-treated cells and ^{14}C-RNA from controls were analyzed in the same gradient. This "double label" technique permits the comparison of the sedimentation pattern of the two RNA preparations in the same gradient, thus allowing the detection of even minor differences.

The profiles of the two RNA preparations (Fig. 2) were similar, indicating that there were no major qualitative differences in the early labeled RNA synthesized by cells grown in the presence or absence of salicylate. When early labeled RNA was extracted from BHK 21 cells exposed to sodium salicylate (200 μg/ml) for a longer period (48 hr) and similarly analyzed, a comparable sedimentation pattern was found.

[12] Mark I, Nuclear Chicago, Chicago, Ill.

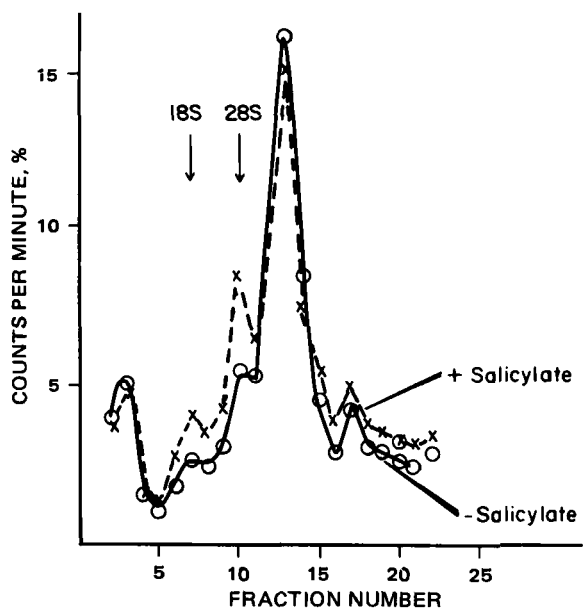

Figure 2—*Sucrose gradient analysis of RNA synthesized in the presence or absence of sodium salicylate. BHK 21 cells grown for 18 hr in the absence or presence of sodium salicylate (200 μg/ml) were exposed for 30 min to ^{14}C- and ^{3}H-labeled uridine, respectively, harvested, and pooled; RNA was extracted and analyzed on a sucrose gradient. The sedimentation positions of 18S and 28S RNA are indicated by arrows. The carbon-14 and tritium radioactivity are indicated as percent of total radioactivity of each isotope. Key: ○—, carbon-14 (RNA from control cultures); and × - - -, tritium (RNA from cells exposed to sodium salicylate).*

The effect of sodium salicylate (200 μg/ml) on the hexose monophosphate shunt and on the oxidative phosphorylation of BHK 21 cells was studied. The amount of ^{14}C-carbon dioxide evolved from 1-^{14}C-glucose (*via* both the mitochondria and the hexose monophosphate shunt) or 6-^{14}C-glucose (the mitochondria alone) was measured, and the C-1/C-6 ratio was compared with that of the controls (Table I). In sodium salicylate-treated cells, a decrease in carbon dioxide evolved from glucose labeled in C-1 and C-6 was observed, suggesting a lower metabolic activity. The ratio of C-1/C-6 remained the same (12.5), suggesting that there was no change in the way glucose was utilized.

DISCUSSION

BHK 21 cells (a genetically homogeneous mammalian cell line) were selected to avoid the differences in susceptibility to sodium salicylate of lymphocytes from different human donors. The data presented here show that the inhibition of synthesis of total early labeled RNA by sodium salicylate in these rapidly dividing cells is similar to the inhibition by salicylate of RNA synthesis in phytohemagglutinin-stimulated lymphocytes during a 4-hr period (1).

In the present studies, early labeled RNA synthesized by BHK 21 cells in the presence or absence of sodium salicylate had a simi-

Table I—Effect of Sodium Salicylate on Oxidation of Glucose by BHK 21 Cells

Sodium Salicylate, μg/ml	Carbon Diosicle from 1-^{14}C-Glucose[a] (C-1), cpm	Carbon Dioxide from 6-^{14}C-Glucose[a] (C-6), cpm	C-1/C-6[b]
0	6450 ± 400	517 ± 25	12.5
200	4200 ± 500	337 ± 80	12.5

[a] Average of triplicate determinations ± 95% confidence limits. [b] Ratio of the means.

lar sedimentation pattern. These two sets of experiments suggest that sodium salicylate, while reducing the RNA synthesizing ability of BHK 21 cells, affects the synthesis of all species of RNA to a similar degree. A marked decrease also was observed in the growth rate of these cells when exposed to sodium salicylate (100–200 μg/ml); this decrease could be the result of diminished RNA synthesis. Paine and Nagington (7) reported that similar doses of sodium salicylate decreased the growth rate of human embryonic kidney, lung, and heart cells but not of embryonic skin fibroblasts.

The observed decrease in RNA synthesis as well as the decrease in macromolecular synthesis by cells of different origins in the presence of salicylate (2) lends support to the hypothesis that salicylate generally interferes with cellular growth (1, 7, 8). However, the effect of sodium salicylate on the function of the specific cell type may be variable. Data presented in this paper suggest that sodium salicylate (200 μg/ml) does not affect mitochondrial activity of BHK 21 cells, since there is no increase in the ^{14}C-carbon dioxide evolved from 6-^{14}C-glucose.

Sodium salicylate (300 μg/ml) minimally increased both C-1- and C-6-derived carbon dioxide production by human polymorphonuclear cells but did not affect phagocytosis and bacterial killing, a phenomenon greatly dependent on hexose monophosphate shunt activity (9). The same concentration of sodium salicylate uncoupled oxidative phosphorylation of the lymphocytes and markedly impaired synthesis of DNA and RNA (1) as well as immunoglobulins (10). Therefore, it is speculated that the function of cell types more dependent on mitochondrial activity may be more susceptible to the inhibitory effects of sodium salicylate.

REFERENCES

(1) L. M. Pachman, N. B. Esterly, and R. D. A. Peterson, *J. Clin. Invest.*, **50**, 226(1971).

(2) M. T. H. Smith and P. D. Dawkins, *J. Pharm. Pharmacol.*, **23**, 729(1971).

(3) G. Opelz, P. I. Terasaki, and A. A. Hirata, *Lancet*, **2**, 478(1973).

(4) F. G. Seibert, *J. Biol. Chem.*, **133**, 593(1940).

(5) F. Amaldi, D. Giacomoni, and R. Zito-Bignami, *Eur. J. Biochem.*, **11**, 419(1969).

(6) L. M. Pachman, P. Jayanetra, and R. M. Rothberg, *J. Pediat.*, **52**, 823(1973).

(7) T. F. Paine, and J. Nagington, *Nature New Biol.*, **233**, 108(1971).

(8) T. M. Brody, *J. Pharmacol. Exp. Ther.*, **39**, 117(1956).

(9) L. M. Pachman, *Fed. Proc.*, **29**, 1392(1970).

(10) C. V. Alm and L. M. Pachman, *Experientia*, **27**, 924(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 3, 1974, from the *Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL 60612 and the ‡Department of Pediatrics, Northwestern University, Children's Memorial Hospital, Chicago, IL 60614

Accepted for publication July 14, 1975.

Supported in part by a grant from the Illinois Chapter of the Arthritis Foundation, the Apfelbaum Foundation, and the Leukemia Research Foundation, Chicago, Ill.

The authors thank Sandra M. Baldwin for technical assistance.

§ Supported by U.S. Public Health Service Grant PHS A1 00335 in partial fulfillment of the Doctor of Philosophy degree requirements at the University of Illinois at the Medical Center.

ˣ To whom inquiries should be directed.

Antihemolytic and Antiproteolytic Properties of Substituted Thiosemicarbazidophenothiazines and Thiazolidonylphenothiazines

ANSHUMALI CHAUDHARI *, SUSHIL KUMAR *, SHIVA P. SINGH *‡, SURENDRA S. PARMAR *§ˣ, and VIRGIL I. STENBERG ‡

Abstract □ Antihemolytic and antiproteolytic properties of several 10-(1-acetyl-4-arylthiosemicarbazido)phenothiazines and their corresponding cyclized 10-(2-arylimino-3-acetylamino-4-thiazolidonyl)phenothiazines were investigated. *In vitro* protection of hypoosmotic hemolysis of human red blood cells by substituted thiosemicarbazidophenothiazines and substituted thiazolidonylphenothiazines was concentration dependent; the degree of protection ranged from 19 to 32 and 26 to 42%, respectively, at a final concentration of 0.1 m*M*. All phenothiazines exhibited antiproteolytic activity. The *in vitro* inhibition of trypsin-induced hydrolysis of bovine serum albumin by these phenothiazines was concentration dependent and competitive in nature; the degree of inhibition ranged from 30 to 50 and 32 to 79% for substituted thiosemicarbazidophenothiazines and substituted thiazolidonylphenothiazines, respectively, at a concentration of 1 m*M*. Cyclization of substituted thiosemicarbazidophenothiazines into the corresponding cyclized substituted thiazolidonylphenothiazines increased the antihemolytic and antiproteolytic effectiveness of these phenothiazines.

Keyphrases □ Phenothiazines, substituted—antihemolytic and antiproteolytic activities investigated □ Antihemolytic activity—substituted phenothiazines evaluated □ Antiproteolytic activity—substituted phenothiazines evaluated □ Structure–activity relationships—substituted phenothiazines evaluated for antihemolytic and antiproteolytic activities

It has been shown that the membrane-stabilizing effects of phenothiazine derivatives are reflected by delayed spontaneous hemolysis of red blood cells (1, 2). Phenothiazine derivatives also have been reported to exhibit anti-inflammatory effectiveness against formaldehyde-induced arthritis (3, 4). Recent studies indicated correlation between the antiproteolytic activity of amyrin acetates (5) and their anti-inflammatory activity (6). Furthermore, numerous psychotropic agents have been proposed to affect the physicochemical properties of cell membranes, accounting for the basis of their mechanism of action (7).

These observations prompted studies of the antihemolytic and antiproteolytic properties of some substituted thiosemicarbazidophenothiazines and their corresponding cyclized substituted thiazoli-