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THE SELECTIVE INHIBITION OF MICROBIAL RNA SYNTHESIS BY SALICYLATE

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Abstract—Salicylic acid inhibited growth when added to logarithmic cultures of *Bacillus cereus* and *Escherichia coli* incubating at pH 6. At 0·25 and 1 mM salicylate, the generation time was increased by about 25 and 100 per cent respectively. The effect was reversible and could not be overcome by the addition of selected vitamins or metals. Growth inhibition varied inversely with pH, and was related to the concentration of undissociated acid.

Cultures grown in the presence or absence of 1 mM salicylate, when compared after similar increases in bacterial turbidity, had identical cell number, and the formation of protein, cell wall and DNA were unchanged. RNA synthesis, on the other hand, was specifically depressed. This effect was associated with all species of RNA examined, and again was quantitatively related to the concentration of undissociated salicylic acid in the growth medium.

The effect on RNA synthesis appeared to be unrelated to the known ability of salicylate to affect oxidation and to uncouple oxidative phosphorylation, since the selective effect on RNA synthesis could also be demonstrated for *E. coli* growing anaerobically in the presence of salicylate. RNA polymerase, though sensitive to higher concentrations of salicylate, was not inhibited by the salicylate concentrations effective on growing cells. Cellular ATP levels were not decreased by growth in the presence of salicylate.

Although the mechanism of the effect on RNA is still unclear, several structurally related compounds, such as benzoate, anthranilate and acetylsalicylate, appeared also to selectively inhibit RNA synthesis though at higher concentrations. The possible relationship of these drug effects to a "shift-down" response is discussed.

DESPITE the fact that numerous metabolic effects of salicylate have been reported,[1] no unifying theory for the major biochemical action of this old and widely-used analgesic and anti-inflammatory drug has been developed. Although a hypothesis for its action as an anti-inflammatory drug relates to its ability to uncouple oxidative phosphorylation,[2] the fundamental mechanisms of its effects remain unclear.

In the present experiments we have examined the effects of salicylate on a variety of biochemical parameters in bacterial systems. Salicylate was found to produce a selective inhibitory action on RNA biosynthesis. This paper describes this drug effect, and attempts to explain its mechanism. Our observations on microorganisms, briefly described previously,[3,4] apparently resemble those in mammalian systems recently reported by Janakidevi and Smith.[5–8]

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EXPERIMENTAL

Bacterial growth and sampling

Bacillus cereus 569H was grown in a medium consisting of 21·09 g KH_2PO_4, 0·51 g $MgSO_4 \cdot 7\ H_2O$, 5 mg Fe $(NH_4)_2(SO_4)_3$; 1·5 g K_2SO_4, 0·5 mg $MnSO_4$ and 10 g Casamino acids (Difco) in 1 l. distilled water. The pH of the medium was adjusted to 6·0 before autoclaving. This medium is patterned after that used previously[9] but contains an increased buffer concentration to maintain the pH at 6 during growth (ionic strength 0·2).

Cells were grown at 37° with aeration by shaking in a New Brunswick Gyrotory shaker. Logarithmic growth was followed by turbidimetry at 540 mμ over two generations, from OD of 0·1–0·4, using a Bausch & Lomb 340 spectrophotometer. Cell numbers and size were verified by use of a Coulter Counter, Model B (settings 1/amplification = 1/4, 1/aperture current = 0·707). A 30-μ aperture was used to count 0·05 ml suspension.

Growth at other pH values

Bacillus cereus was grown in high ionic strength medium titrated previously to pH 6·0, 6·6 or 7·2 with KOH. Cells were pregrown at least two generations in the appropriate medium to acclimatize the cells to the medium.

Anaerobic growth

Anaerobic growth of *Escherichia coli* (ATCC 11303) was followed by addition of 0·1% glucose to the pH 6 medium and shaking of the culture at 37° in a nitrogen atmosphere. The N_2 gas was introduced into the bottom of all flasks. The culture was pregrown in this system for several generations before the incorporation experiment was begun.

Isotope incorporation experiments

Incorporation of radioactive precursors during logarithmic growth was carried out as described by Roodyn and Mandel[10] and Mandel *et al.*[11] Labelled nucleic acid precursors (0·01 μc/ml medium) or amino acids (0·2 μc/ml medium) were added to the cultures which were then subdivided, salicylate was added to one flask, while another served as control, and turbidity was read at 540 mμ during growth. Samples were removed periodically, mixed with an equal volume of cold 10% trichloroacetic acid, and filtered on Schleicher and Schuell B-6 membrane filters. To measure radioactivity contained in the pool fraction, samples were mixed with additional medium at 0° instead of the trichloroacetic acid and were filtered at once; the difference in radioactivity between the two samples was due to the acid-soluble pool fraction.[10] The filters were then air dried and glued onto aluminum planchets for counting in a Nuclear Chicago gas-flow counter. In experiments involving larger volumes, the radioactive supplements were added to the different cultures at a time so that the initial turbidities would be identical throughout. Data from these studies have been presented as radioactivity incorporated into cells per unit of growth, measured turbidimetrically, to select drug effects differing from those due to growth inhibition.

Colorimetric assay for RNA

RNA content was measured by the method of Schneider.[12] Bacterial samples

(1·5 ml) were removed from the culture at intervals and added to 0·5 ml concentrated HCl. One ml of orcinol reagent (1 g purified orcinol in 33·3 ml HCl containing 0·5 g $FeCl_3$) was added to each sample, the mixture boiled for 20 min and read at 660 mμ in the spectrophotometer against a standard curve.

Messenger activity assay

Polypeptide synthesis was measured according to the method of Nirenberg.[13] RNA was prepared by the cetyl trimethylammonium bromide method[14] as modified by Grünberger and Mandel.[15] The phenol extract was extracted five times with water-saturated ether. RNA, 300–600 μg, from control and 1 mM salicylic acid-treated *B. cereus* was added to the *in vitro* incubation system containing a preincubated S-30 fraction from *E. coli* cells.[15] Incubations were followed for 15 and 30 min at 37° on an Eberbach constant-temperature water-bath shaker.

RNA polymerase from E. coli

This enzyme, corresponding to fraction IV of Chamberlin and Berg,[16] (2000 units/mg protein), was purchased from Biopolymers, Inc., Pinebrook, New Jersey 07058. The Biopolymers modification of the assay method of these authors was used. Reactants were added in the concentrations in μmoles/ml and order as follows: Tris buffer, 40; $MgCl \cdot 6H_2O$, 4; $MnCl_2 \cdot 4H_2O$, 1; 2-mercaptoethanol, 10; GTP, UTP, CTP, ATP, each 0·4; ^{3}H-ATP-tetraLi (10 μc/μmole), $2{\cdot}5 \times 10^{-3}$; DNA, 0·6, based on phosphorus; Na salicylate, 1–40; and enzyme, 2–5 units. Total reaction volume was usually 0·25 ml. All reaction vials were prewarmed and the reaction started at 1-min intervals by addition of enzyme, and stopped after 0, 10 or 20 min by the addition of 1·0 ml of pre-chilled solution containing bovine serum albumin (3·3 mg) and disodium ATP (8 μmoles). This was mixed by swirling, and followed by 1·25 ml of cold 10% trichloroacetic acid. Samples were collected by filtration through membrane filters and were washed twice with 2 ml of 1% TCA. The filters were placed in counting vials, dissolved in 10 ml Bray's solution[17] and counted in a Beckman DPM-100 liquid scintillation counter to 2 per cent error.

ATP assay

(a) *Luciferase solution.* Sigma FLE-50 desiccated firefly tails (50 mg) was added to make a 5-ml aqueous solution of final concentration 0·05 M KH_2AsO_4 and 0·02 M $MgSO_4$, pH 7·4. The mixture was shaken vigorously for 2 min and centrifuged at 14,500 *g* for 35 min in a Sorvall refrigerated centrifuge. The supernatant was decanted and stored in a −10° freezer overnight before use. Insignificant loss of enzyme activity occurred on freezing and thawing of the supernatant, and this procedure provided reproducible readings.

(b) *Arsenate buffer.* Stock Sigma FF-As arsenate buffer (0·1 M) was diluted to 0·006 M with distilled water. This buffer prevents the rapid decay of luminescence.[18]

(c) *Adaptation of spectrometer for assay of ATP.* A Beckman DPM-100 liquid scintillation counter with coincidence circuit disconnected was employed, and the third repetitive 6-sec count after the addition of the enzyme was used for calculations. Luminescence intensity was measured as a variable discriminator module set from 3 to 100 units, and the background was around 3500 counts/min per 6 sec.[19] The method was linear from 1 to 10 nM ATP.

(d) *Assay of bacterial ATP content.* One ml samples of suspension taken from treated or control cultures, and filtrates from such suspensions after membrane filtration, were added to 4 ml of boiling water within 20 sec after removal from the culture. The samples were then boiled for 10 min and cooled in ice to extract ATP. In a partially darkened room with only indirect incandescent light a mixture of 3·8 ml of arsenate buffer and 0·2 ml of enzyme solution at 0° was mixed, and luminescence immediately assayed in the counter; the third repetitive 6-sec count was recorded as the background value. Then 0·2 ml of the ATP standard or bacterial extract was added, and the mixture again mixed and assayed. The ATP concentration was recorded as counts/second and then converted to nanomoles from a standard curve. Salicylate added directly to the ATP assay system had no effect on luminescence.

Preparation of buffers

TMK, Tris 0·01 M, Mg acetate 0·01 M, KCl 0·06 M, pH 7·8. TN, 0·025 M Tris, 0·05 M NaCl, pH as described.

Preparation of ribosomes

The aliquots from control and salicylate-treated cultures were removed and harvested in a refrigerated centrifuge, washed with 10 ml cold TMK buffer, resuspended in 0·3 ml of the same buffer, and sonicated for 5 min in an MSE ultrasonic disintegrator. The sonicate was centrifuged in the cold at 20,000 *g* for 30 min in a Sorvall RC2B automatic refrigerated centrifuge. Suspensions were dialyzed overnight at 4° against a 1000-fold volume of TMK buffer. The dialyzed supernatant was then applied onto a 5–20% sucrose gradient in TMK buffer prepared by use of a Buchler gradient machine, and the tubes were centrifuged for 90 min in a Beckman Model ultracentrifuge at 39,000 rev/min in an SW 39 rotor. The effluent solutions were assayed by an ISCO Model 222 single beam ultraviolet analyzer connected to an ISCO Model 180 density gradient fractionator and recorded by a Model 170 ISCO servographic recorder. Samples of four drops (counted by a Gilford drop counter) were collected, 10 ml Bray's solution added to each tube, and the radioactivity counted in the liquid scintillation counter.

Binding of salicylic acid to macromolecules

DNA. Escherichia coli type VIII or calf thymus DNA (Sigma Chemical Co., St. Louis, Missouri) was diluted to 5 mg/ml with distilled water (final pH 5·8) and sonicated for 2 min. [^{14}C]Carboxyl labelled salicylic acid (Tracerlab lot No. 24-271-3) was dissolved in 0·5 M $NaHCO_3$ and adjusted to pH 6·6 with HCl. The solution was diluted with unlabelled salicylic acid to a final specific activity of 1 μc/mg. The centrifuge tube contained a 1·0-ml cushion of 50% sucrose dissolved in TN buffer (pH 6·0), and a 5–20% sucrose gradient in TN buffer was then added by means of a Buchler gradient machine. A mixture of 0·1 ml [^{14}C]salicylic acid (1 mg/ml), 0·1 ml DNA (5 mg/ml) and 0·2 ml TN buffer was kept in an ice-bath for 90 min, layered on the gradient, centrifuged in a Spinco ultracentrifuge in an SW 39 rotor for 18 hr at 39,000 rev/min, and fractionated in the usual manner. A similar tube containing *E. coli* DNA as a control was run simultaneously. For the RNA binding studies, a mixture of 0·1 ml each of 0·5 mg/ml *B. cereus* RNA and 1 mg/ml [^{14}C]salicylic acid was kept at 0°, layered over a 5–20% sucrose gradient in pH 7·8 TN buffer, and was centrifuged at

39,000 rev/min in a Spinco ultracentrifuge for 5 hr using an SW 39 rotor. To collect *ribosomes*, the cell-free extract from bacterial cells was layered over 4 ml of TMK containing 5% sucrose and centrifuged 2 hr at 39,000 rev/min in an SW 39 rotor. The supernatant was discarded, and the ribosomal pellet was mixed with [^{14}C]salicylic acid in 0·3 ml TMK buffer, layered on a 5–20% sucrose TMK gradient, and centrifuged at 39,000 rev/min for 2 hr in the Spinco ultracentrifuge, using the SW 39 rotor.

Sources of radiochemicals

2-[^{14}C]Guanine and 8-[^{14}C]adenosine, Calbiochem, Inc., Los Angeles, Calif.; [^{14}C]carboxyl-labelled salicylic acid, Tracerlab, Inc., Waltham, Mass.; U-[^{14}C]L-leucine, Volk Radiochemical Corp., Skokie, Ill.; [^{3}H]ATP, Schwarz Bioresearch, Inc., Orangeburg, N.Y.; 1,7-[^{14}C]α, ϵ-diaminopimelic acid, ICN Corp., City of Industry, Calif.; U-[^{14}C]L-phenylalanine, 2-[^{14}C]thymidine, 8-[^{14}C]adenine and 2-[^{14}C]uracil, New England Nuclear Corp., Boston, Mass.

RESULTS

Effect of salicylate on growth and morphology of B. cereus

The characteristics of the salicylate effect on growth in the Casamino acids medium at pH 6 are shown in Fig. 1. Growth was inhibited immediately by salicylate and was dose dependent. Concentrations of 0·25, 0·5 and 1·0 mM salicylate produced 25, 40 and 100 per cent increases in generation time, respectively, compared to control cultures.

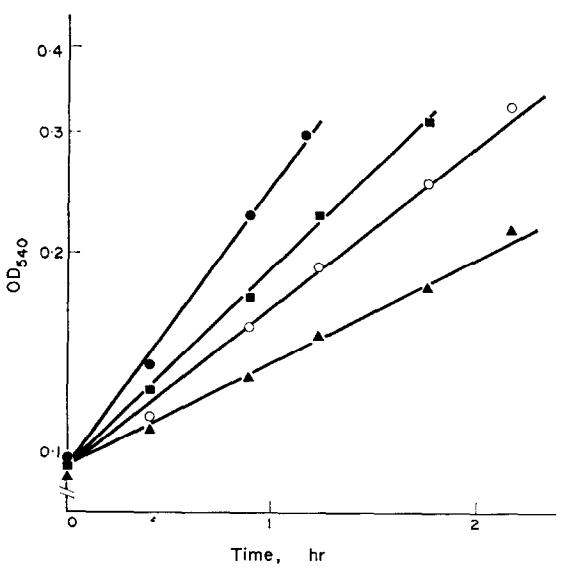

FIG. 1. Effect of salicylate on rate of growth of logarithmic cultures of *B. cereus*, as measured turbidimetrically at 540 mμ. ●, Control; ■, 0·25 mM; ○, 0·5 mM; ▲, 1 mM salicylate.

The salicylate effect was reversible, and drug-treated cultures upon washing and resuspension in fresh salicylate-free medium grew normally. No significant change in size of the microorganisms due to growth in the presence of salicylate could be noted

either by microscopic examination or by Coulter counter analysis, and viability following salicylate treatment was unaltered. A direct relationship between cell number as calculated using a Coulter counter, and turbidity of the suspension as measured at 540 mμ, was established, which was independent of the concentration of salicylate in the bacterial medium. Therefore, bacterial turbidity was used throughout these experiments for comparing equivalent cell populations.

It has been reported that salicylate inhibition of *E. coli* can be reversed competitively by pantoate, apparently because the drug inhibits the synthesis of this compound.[20] Pantoate, as well as pantothenate or biotin (all 10 mM), had no effect in preventing the effect of 0·5 mM salicylate on growth of *B. cereus*. Calcium or magnesium ions, both at 5 mM, did not influence the salicylate effect, suggesting that the drug's chelating properties were unrelated to the inhibition of growth due to salicylate.

Effect of salicylate on incorporation into macromolecules

Bacterial cultures during logarithmic growth in the presence or absence of salicylate received radioactive substrates, and the incorporation of radiocarbon into the cells was measured by collecting aliquots throughout the incubation, filtration and radioassay, as described in Experimental. The incorporation of [^{14}C]leucine exclusively into protein of *B. cereus* has been demonstrated previously.[21] Figure 2 shows that salicylate did not inhibit the incorporation of [^{14}C]leucine into protein over two generations of growth when cell cultures were compared at similar turbidities; experiments with [^{14}C]phenylalanine provided identical results. The incorporation of [^{14}C]diaminopimelic acid, an exclusive precursor of cell wall in *B. cereus*,[21] also was essentially unaffected by salicylate. The incorporation of [^{14}C]thymidine, which is converted entirely into bacterial DNA, also was unaffected by 1 mM salicylate. Since thymidine is known to be degraded to thymine, a product not utilized by the cells,[22] [^{14}C]thymidine was added to control and salicylate-containing cell cultures after equal increases in bacterial turbidity. When the thymidine was added every 15 min to both cultures, actually more radioactivity was incorporated into the slower-growing, drug-treated culture than the control when comparisons were made after equal increases in turbidity.

Figure 2 shows that incorporation of [^{14}C]uracil was inhibited immediately by salicylate. Thus, the effect on the incorporation of uracil was greater than that on growth, and indicated a selective action of salicylate, in contrast to the effects noted with labelled leucine, diaminopimelic acid and thymidine. The incorporation of [^{14}C]-guanine, [^{14}C]adenosine or [^{14}C]adenine was inhibited in a manner similar to that of uracil. These compounds all serve as nucleic acid precursors in *B. cereus*, and more than 90 per cent of the radioactivity is recovered in the RNA.[10] At a concentration of 1·0 mM salicylate, incorporation into nucleic acids was depressed by 50–75 per cent of the amount in control cells grown to the same turbidity.

The acid-soluble pool of radioactivity in cells labelled either with [^{14}C]adenine or [^{14}C]uracil, as measured by the membrane filtration and fractionation techniques,[10] was diminished by salicylate to about the same extent as was the incorporation into RNA.

Effect of salicylate on bacterial RNA content

Figure 3 provides data on the effect of salicylate on RNA content of the micro-

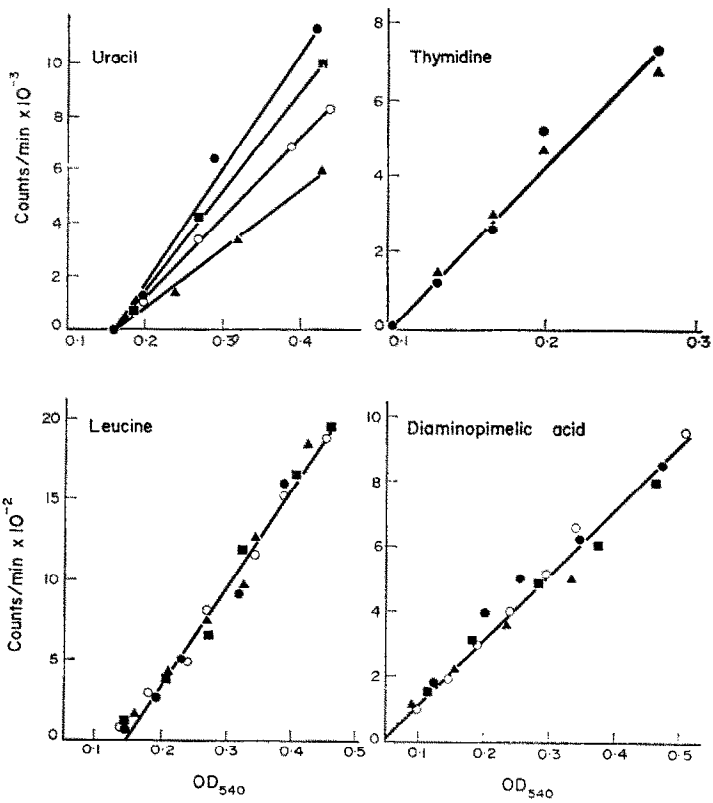

FIG. 2. Effect of salicylate on incorporation of labelled precursors into *B. cereus* cells, as a function of bacterial growth. Cells were grown logarithmically and radioactivity assayed periodically as described in Experimental. [^{14}C]Uracil, -thymidine, -leucine and -diaminopimelate selectively label nucleic acids (almost entirely RNA), DNA, protein and cell wall, respectively. ●, control; ■, 0·25 mM ○, 0·5 mM; ▲, 1 mM salicylate.

organisms, as measured colorimetrically.[12] These data are in good agreement with the results of the incorporation experiments using labelled purines and pyrimidines, and confirm that the salicylate effect involves inhibition of the biosynthesis of RNA. After one doubling of each culture, the cells treated with 1 mM salicylate contained about 50 per cent less *new* RNA than did the control culture.

Effect of salicylate on subcellular components of B. cereus

In order to localize any salicylate effect with respect to a particular macromolecular component, extracts were prepared from cells grown in the presence and absence of 1 mM salicylate, labelled as before with [^{14}C]adenine. The extracts were then fractionated as described in Experimental, and radioactivity in ribosomes, ribosomal subunits and soluble RNA assayed. Figure 4 indicates that the drug effect was not specific for a major cellular component and that the depressed incorporation of [^{14}C]adenine was characteristic of all RNA-containing fractions. In most experiments, however, extracts from drug-treated cells appeared to contain somewhat less radioactivity in ribosomes than in soluble RNA, in comparison with control cells.

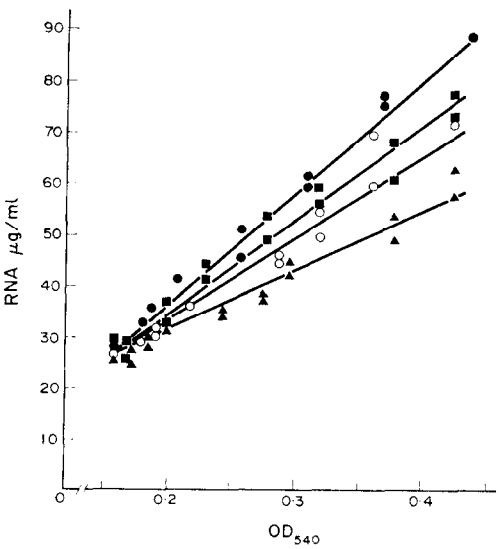

FIG. 3. Effect of salicylate on RNA content of *B. cereus* cells, measured colorimetrically as described in Experimental. ●, control; ■, 0·25 mM; ○, 0·5 mM; ▲, 1 mM salicylate.

An attempt was made to determine the effect of salicylate on messenger RNA activity. For this purpose, RNA extracted from *B. cereus* grown in the presence or absence of 0·5 mM salicylate was used as a source of mRNA, using a preincubated amino acid incorporation system from *E. coli* which is deficient in mRNA.[13] This system, in the presence of poly U, incorporates phenylalanine into polypeptides.[15] Table 1 indicates that at 15 min after the addition of varying quantities of RNA isolated from *B. cereus* cells which had been grown in the presence of salicylate, RNA

TABLE 1. STIMULANT EFFECT OF RNA FROM CONTROL AND SALICYLATE-TREATED *B. cereus* ON POLYPEPTIDE SYNTHESIS IN *E. coli* INCORPORATION SYSTEM

B. cereus RNA (μg)	Period of incubation (min)	Radioactive polypeptide formed (counts/min)		% of control incorporation
		RNA from control cells	RNA from salicylate treated cells	
300	15	1755	1550	89
	30	1900	1750	92
500	15	2160	1480	69
	30	2450	2050	84
600	15	3270	2180	67
	30	5480	5100	94

Each value is the average of two determinations. At zero time, total incorporation of [^{14}C]phenylalanine was about 400 counts/min. Incorporation with 20 μg poly U, at 15 min, about 28,000 counts/min; at 30 min, 32,000 counts/min. For details, see Experimental.

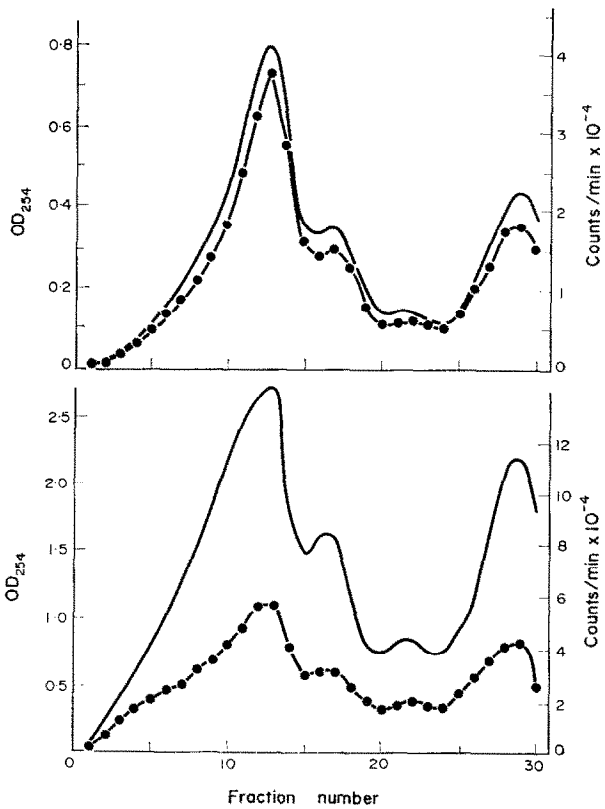

FIG. 4. Effect of salicylate on formation of subcellular RNA fractions of *B. cereus*. Growing cells were labelled with [^{14}C]adenine to allow one doubling of bacterial turbidity, inthe presence and absence of 1 mM salicylate. Cell-free preparations were then fractionated, as described in Experimental, and absorbance at 254 mμ (———) and radioactivity (●———●) measured for the various fractions. 70S ribosomes, 50S ribosomal subunits, 30S ribosomal subunits and sRNA located at fractions 12, 17, 22 and 28, respectively. Top, control experiment; bottom, 1 mM salicylate experiment.

possessed about 30 per cent less messenger activity than the control. At 30 min this effect was less pronounced, probably because by that time the *in vitro* polypeptide condensation reaction was no longer linear. Thus, messenger activity also appeared to be diminished because of the treatment with salicylate.

Attempts to explain the selective effect of salicylate on RNA synthesis

Several approaches were used to understand the mechanism of the action of salicylate on RNA biosynthesis. These involved examination of various possible actions of the drug which might be responsible for the RNA effect: (1) inhibition of ATP synthesis, stimulation of the breakdown of ATP, or the leakage of ATP from the cells; (2) effects on oxidation or uncoupling of oxidative phosphorylation; (3) inhibition of RNA polymerase; and (4) binding of salicylate to DNA or other subcellular components, thus preventing normal replication of RNA. In addition, (5) the effects of compounds structurally related to salicylate that might produce similar actions were

investigated to establish the specificity of the effect in the hope of associating it with a specific portion of the ortho-hydroxybenzoic acid molecule.

1. *ATP content.* The effect of salicylate on total ATP levels of bacteria is shown in Fig. 5. The ATP contents of salicylate-treated and control suspensions were indistinguishable. Furthermore, salicylate did not alter the relative distribution of ATP between cells and medium, indicating that the cellular content of ATP was unchanged by drug treatment, and that no leakage of intracellular ATP had occurred. Thus, the possibility of a deficiency in ATP cannot explain the decrease in RNA synthesis.

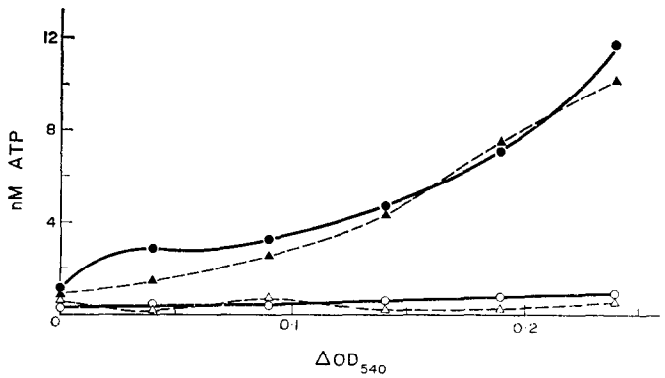

FIG. 5. Effect of salicylate on ATP content per ml of bacterial suspension or the cell-free filtrate of that suspension. Almost all of the ATP was found to be present in the cells. Comparisons made at similar bacterial turbidities of control and 1 mM salicylate cultures of *B. cereus*. For control cultures. values for suspensions (●) and filtrate (○); salicylate cultures, suspensions (▲), filtrate (△).

2. *Oxidative phosphorylation.* The salicylate effect was re-examined in microorganisms grown in the absence of oxygen so that phosphorylation could not be coupled to oxygen utilization. This experiment could not be carried out with *B. cereus*, since this microorganism did not grow anaerobically even in the presence of glucose. *Escherichia coli*, on the other hand, could be cultivated under anaerobic conditions providing that glucose was available, and had a generation time of 50 min. Furthermore, results of aerobic experiments with *E. coli* on the effects of salicylate on growth, and the incorporation of [^{14}C]leucine and [^{14}C]adenine into protein and nucleic acids, respectively, were identical with those in corresponding studies with *B. cereus* (shown in Figs. 1 and 2).

The inhibition of anaerobic growth of *E. coli* by salicylate was immediate and resembled the results shown in Fig. 1. The per cent increases in generation time were 25, 60 and 110 per cent, respectively, for 0·25, 0·5 and 1 mM salicylate. Figure 6 shows the selective inhibitory effect of salicylate on incorporation of [^{14}C]guanine into nucleic acids of *E. coli* growing anaerobically. The conversion of [^{14}C]uracil into nucleic acids also was inhibited,whereas the incorporation of [^{14}C]leucine onto protein was not specifically affected. Thus it was concluded that the inhibitory actions of salicylate on growth inhibition and on RNA synthesis were independent of (1) availability of oxygen, and (2) uncoupling of oxidative phosphorylation.

3. *RNA polymerase.* Since salicylate produced inhibition of all RNA species, a

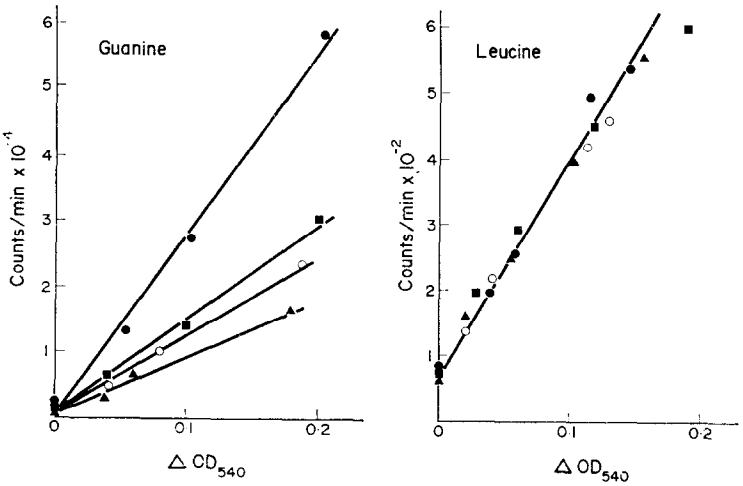

FIG. 6. Effect of salicylate on incorporation of [^{14}C]guanine and -leucine into nucleic acids and protein, respectively, of anaerobically growing *E. coli*. Procedure described in Experimental, presentation as in Fig. 2. ●, control; ■, 0·25 mM; ○, 0·5 mM; ▲, 1 mM salicylate.

unitary mechanism was believed to be responsible for this drug effect. The DNA-dependent RNA polymerase reaction was therefore examined by studying the effect of exogenous salicylate on the enzyme *in vitro*. The data of Table 2 show that salicylate only in relatively high concentrations (e.g. 4 mM) inhibited RNA polymerase. This inhibition was not seen at those concentrations of salicylate sufficient to inhibit RNA

TABLE 2. EFFECT OF SALICYLATE ON RNA POLYMERASE

pH of reaction mixture	Incubation period (min)	Salicylate 0	1 mM	4 mM	40 mM
			(counts/min)		
6·5	0	478	414	358	—
	10	557	744	729	
		665	777	590	
	20	869	863	870	
		846	932	890	
8·1	0	577	—	—	—
	10	1450		1105	1201
	20	1802		1450	1195
9·1	0	1187	—	—	—
	20	3630		3863	2530
		4635			2453
		4339			2017
		4039			2476

Incorporation of [^{3}H]ATP into polynucleotides, mediated by *E. coli* RNA polymerase in presence of salicylate. For details see Experimental.

synthesis in microorganisms. The reaction was carried out at three pH values to permit its evaluation under conditions of optimal enzyme activity (pH 8) and of greater drug action in whole cells; in all cases, however, no evidence for a direct inhibition of the enzyme by 1 mM salicylate could be demonstrated.

4. *Binding to polymers.* An association of [^{14}C]salicylate to DNA from *E. coli* or calf thymus during sucrose density gradient centrifugation at pH 6 could not be demonstrated. Similarly, binding of the labelled drug to ribosomes or to RNA could not be shown.

5. *Experiments with compounds structurally related to salicylate.* Several compounds were tested in the *B. cereus* system at pH 6 at concentrations which produced a doubling of bacterial turbidity at about twice the normal generation time. With phenol it was not possible to reach the desired magnitude of growth inhibition even at 6 mM. Drugs were dissolved in dimethyl sulfoxide (final conc. 1%) since they were insufficiently soluble in water and since the solvent did not affect growth or the incorporation experiments. The effects of the drugs on the incorporation of [^{14}C]-guanine or [^{14}C]leucine are shown in Fig. 7. All experiments were repeated at least twice, but no attempts were made to localize the mechanisms of the observed effects. Acetylsalicylate, anthranilate and benzoate produced incorporation patterns closely resembling those of salicylate, although four times the concentration of drug was required. The effects of salicylamide and phenol also were similar, and the somewhat diminished drug effect on [^{14}C]guanine incorporation may be a reflection of the even lower potency of these compounds in this system.

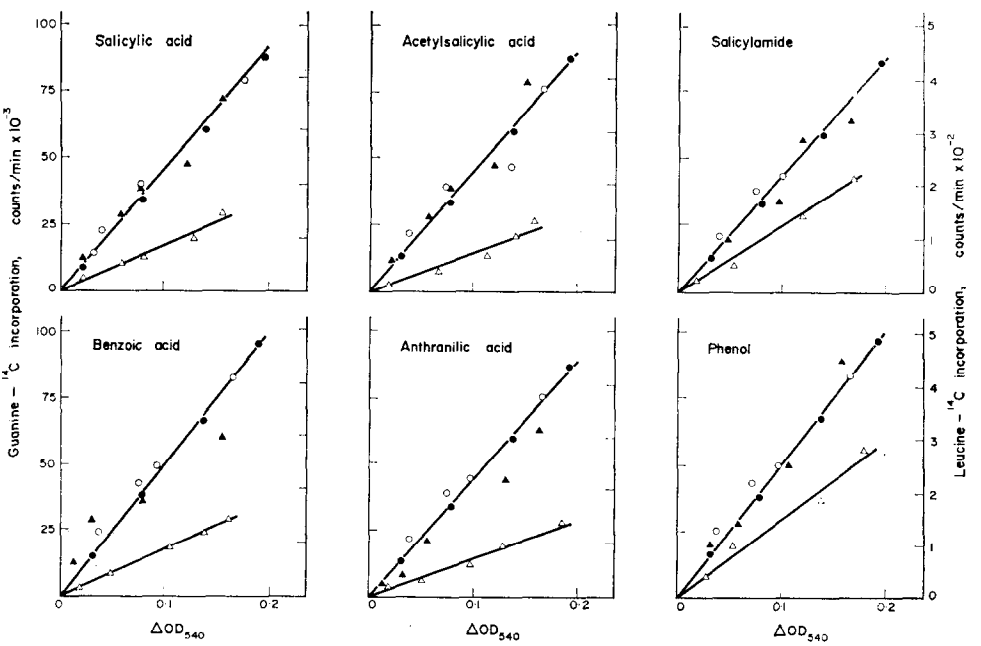

FIG. 7. Effects of various drugs on incorporation of [^{14}C]guanine and -leucine into nucleic acids and proteins of *B. cereus*, respectively, as a function of bacterial growth. Concentrations of drugs chosen: salicylate, 1 mM; benzoate, anthranilate, acetylsalicylate and salicylamide, 4 mM; phenol, 6 mM. Guanine incorporation: ○, control culture; △, drug-treated culture. Leucine incorporation: ●, control culture; ▲, drug-treated culture.

Role of unionized form of salicylic acid in drug's effect on growth and RNA synthesis

In order to determine whether the drug effect observed in these experiments was associated with the total concentration of salicylate or that of the undissociated form, growth and incorporation experiments were carried out at different pH values. Whereas control cells grew at identical rates between pH values of 6 and 7·2, salicylic acid produced much greater growth inhibition at lower pH. However, the pH effect became negligible when only the concentration of undissociated salicylic acid was considered, as previously observed for yeast cells by Bosund.[23]

Quite similarly, the effect of the drug on the incorporation of [^{14}C]uracil into nucleic acids was a function of the concentration of the undissociated salicylic acid rather than that of total salicylate (Fig. 8). Identical results were obtained from corresponding experiments with [^{14}C]guanine.

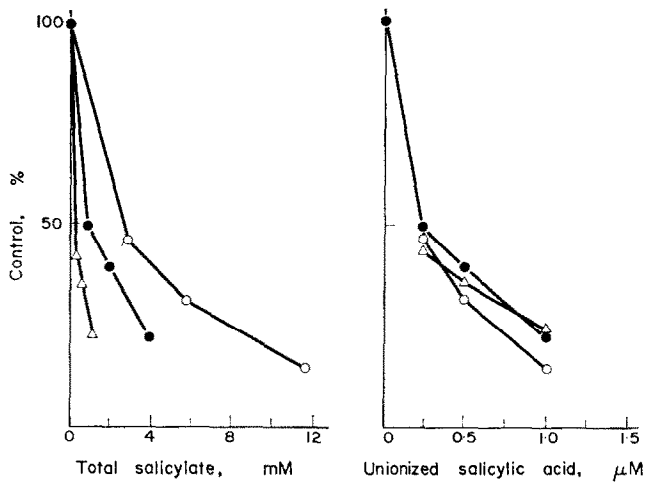

FIG. 8. Effect of salicylate on incorporation of labelled uracil into nucleic acids of *B. cereus*, in terms of total drug concentration (left) and calculated concentration of undissociated salicylic acid (right). Incorporation experiments carried out at pH 6 (△), 6·6 (●) and 7·2 (○). Comparisons made at equal bacterial turbidities.

DISCUSSION

The selective inhibitory action of salicylate on RNA synthesis of microorganisms, specifically *B. cereus*, is of particular interest because of the independent reports of Janakidevi and Smith[5–8] who have observed inhibition of RNA polymerase by salicylate in the rodent. These authors have used drug concentrations between 2–4 mM to produce their effects on RNA formation. In the bacterial system RNA polymerase was also inhibited at such concentrations, but the effect on RNA synthesis could be demonstrated at concentrations as low as 0·25 mM. The lack of an effect at these low concentrations suggests a different mechanism for the inhibition of RNA synthesis in bacteria. Although in the present investigation it has not been possible to explain precisely the drug action, it did not appear to be related to the drug's known effects on oxidation,[24] uncoupling of oxidative phosphorylation[25] or ATP levels, or the drug's binding to subcellular constituents. It is possible that the preferential loss, in the presence of salicylate, of potassium ion, as reported previously for *B. cereus*[4] and

mammalian systems,[26,27] may reflect increased membrane permeability and leakage of some essential cell constituent needed for the synthesis of RNA.

Alternatively, the salicylate effect in microorganisms could be related to a "shift-down" induced by a less nutritional environment or various drugs.[28] For example, levorphanol,[29-30] dinitrophenol[31] and cyanide[32] selectively reduce RNA synthesis in *E. coli*, presumably by interfering with energy metabolism. Certain drugs related to salicylate produced effects superficially similar to that in salicylate-treated *B. cereus*, even though much higher concentrations were required. Thus, even though no generalized effect on energy metabolism of salicylate could be demonstrated, the drug might interfere at a stage of intermediary metabolism most readily expressed by a slowing of growth, secondarily resulting in the specific inhibition of RNA synthesis. The possible site of action of the "shift-down" effect is still unclear.

It is difficult to extrapolate the results of experiments with drugs on microorganisms to the therapeutic action of these agents in man. The similarity in the biochemical observations of the present work with those of Janakidevi and Smith[6] undoubtedly rests on the fundamental uniformity of biochemical pathways which may be sensitive to a particular drug at specific sites. Although it is hard to visualize at present how the effect of salicylate on RNA synthesis can explain the drug's analgesic effect, interference with nucleotide metabolism is believed to account for the drug's anti-inflammatory actions.[2] It is quite likely that the effect on RNA synthesis is related to the teratogenic actions of salicylate,[8] as described for the rodent, and probably also accounts for the preservative action of the agent. Bacterial studies may therefore serve as useful models for uncovering biochemical actions of drugs responsible for pharmacological actions. Such model systems are readily reproducible, allow remarkable quantitation, and permit evaluation of many biochemical parameters simultaneously. Ultimately it should be possible to explain the actions of many drugs by the use of suitable, simple, single-cell model systems.

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