Inhibition of the Secondary Antibody Response in Vitro by Salicylate and Gentisate

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INHIBITION OF THE SECONDARY ANTIBODY RESPONSE IN VITRO BY SALICYLATE AND GENTISATE*

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In recent years chemical suppression of the immune response has been studied mainly with newly isolated antibiotics and other newly synthesized antimetabolites. But the predecessors to these studies began with two products of nineteenth century organic chemistry, mustard gas and salicylic acid. In 1921 Hektoen and Corper (1) described marked inhibition of the immune response by mustard gas (di-(chlorethyl)sulfide). About the same time Swift (2) first observed inhibition of antibody production by salicylates. During World War II the intensive investigations of mustard gas and of nitrogen mustard derivatives yielded other alkylating agents which today are among the more useful immunosuppressive drugs. However, in the several decades following Swift's report salicylate and its acetylated derivative, aspirin, were often found to have only slight or no effect on the immune response (3, 4). Since 1950 this family of aromatic compounds has largely been ignored in immunological investigations. In this report we have examined the effects of salicylic acid (2-hydroxybenzoic acid) and gentisic acid (2,5-dihydroxybenzoic acid) on rabbit lymph node cultures and have observed pronounced inhibition of the secondary antibody response initiated in this system. The inhibitory levels of these compounds in vitro are sufficiently low to invite reexamination of their use in vivo.

Materials and Methods

The general procedures employed in these experiments have been described by Michaelides and Coons (5) and Ambrose (6). They are briefly as follows: Adult male rabbits received their primary stimulation to bovine serum albumin (BSA) and diphtheria toxoid (DT) in vivo by means of a saline solution of both antigens injected intracutaneously into the four footpads and two ears. Three to 12 months later the lymph nodes draining the injection sites were excised and cut into 1 mm cubes. These pooled fragments received a secondary stimulation in vitro by incubation for 2 hr in culture medium containing 0.5 mg/ml BSA and 5 IU/ml DT. Excess antigen was washed from the fragments by several rinses with medium. Fourteen stimulated fragments were aligned in a Leighton tube, and a thin pad of glass wool was inserted on top to hold them in place (see Fig. 1 in reference 7). Each tube received 1 ml of Eagle's Minimum Essential Medium supplemented with 5 additional amino acids (0.1 mM).

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each of alanine, aspartic acid, glycine, proline, and serine), 0.5 μg/ml vitamin B₁₂, 1.0 μM cortisol (Solu-Cortef, Upjohn), 0.5 unit/ml insulin (Iletin, Lilly), 60 units/ml penicillin G, and other substances described in the several experiments. The inclusion of 0.01 mg/ml phenol red in the medium allowed visual estimation of the pH in the culture tube to within 0.1-0.2 unit.

The cultures were kept stationary at 37°C for 18 to 21 days, during which time the culture fluids were replaced every 3 days in most experiments but every 2 days in one described below. The pH of the cultures was recorded before each medium change in all experiments but was also recorded daily in some. The fluids removed from each culture were immediately frozen in the alcohol-dry ice bath and stored at −5°C. Antibody titers to BSA and DT were subsequently measured by the hemagglutination method with tanned sheep erythrocytes (Stavitsky's modification of the Boyden procedure (8)).

Frequent adjustment of the pH of the cultures became necessary in some experiments. Several simple techniques for this adjustment were devised which took advantage of the bicarbonate in the medium. Fine adjustments of the pH were made by blowing into the culture tube for a few seconds a small stream of 5% CO₂ (to lower the pH) or a small stream of air (to flush out CO₂ and thus raise the pH). A more vigorous method for raising the pH involved introducing into the tube a strip of chromatography paper (1.4 × 4 cm) previously moistened with 1 N NaOH but largely dried during autoclaving. This strip was inserted just far enough into the tube so that it could be stoppered, and was never allowed to slip down near the fragments. Within several minutes to an hour, depending on the moistness of the strip, it absorbed sufficient CO₂ from the atmosphere of the tube to raise the pH of the medium by several tenths of a pH unit. The strip was removed when the pH had reached the desired level.

In designing most experiments we assigned groups of 4 culture tubes to each variable. The average titers for each group's 7 medium changes (on days 3, 6, 9, 12, 15, 18, and 21) were used to graph its antibody response and to compare this response with those of other groups. Such comparisons were made as follows: In the control group of cultures, whose medium contained no salicylate or other inhibitor, the sum of the average titers obtained in the 7 medium changes was used as the 100% reference response value. Sums similarly derived from other experimental groups were compared with this sum from the reference group, and the relative responses of these other groups were computed as percentage values.

In many experiments the relative amount of antibody produced after day 9 was determined by comparing the sum of the average titers for the medium changes of days 12, 15, 18, and 21 with the sum of the average titers for all 7 medium changes in that group. Antibody accumulating in the medium during any 3 day period is largely produced during that interval. This conclusion was derived from experiments employing puromycin in this culture system and others involving pulse labeling of the antibody produced with C¹⁴-amino acids (9).

RESULTS

Dose-Response Relationship for Salicylate Inhibition of the Immune Response in Vitro.—Inhibition of the secondary response by several concentrations of sodium salicylate is shown in Fig. 1. Each of the four curves drawn here represents the average response of a group of quadruplicate cultures maintained for 21 days. Changes of medium every 3rd day exposed each culture anew to a standard concentration of salicylate—0.5, 1.0, or 1.5 mM. In this experiment a level of 0.5 mM (0.08 mg/ml) depressed the average response to 50% of that of the untreated control group of cultures. Higher concentrations reduced the responses proportionately.
The variation in salicylate inhibition encountered in this culture system is
illustrated in Fig. 2, which summarizes anti-BSA and anti-DT responses in
lymph node cultures prepared from 6 rabbits. In each inhibition curve the 21-
day responses permitted by the three standard concentrations of salicylate were
plotted relative to the 100% control (untreated) response of that experiment.
At a concentration of 0.5 mM salicylate there was a moderate spread of the
average responses from 38% to 73%, but at a 1.5 mM concentration all re-
sponses were depressed to 10% or less. This spread of responses at the low
salicylate concentration may reflect errors inherent in the assay procedure but
also suggests secondary influences on the mechanism of this inhibition. Two such

![Graph showing inhibition by salicylate of the secondary antibody response in vitro.](image)

**Fig. 1.** Inhibition by salicylate of the secondary antibody response in vitro. Rabbit lymph
data cultures were maintained for 21 days in medium containing 0, 0.5, 1.0, or 1.5 mM sodium
salicylate. There were 5 cultures in the untreated control group and 4 cultures in each of the
other 3 salicylate-treated groups. HA, hemagglutination.

influences which we have identified are the pH of the medium and its content
of sodium bicarbonate.

*The Effect of pH on the Secondary Response and on Its Inhibition by Salicylate.*
---The effect of pH on the secondary response alone was examined in 4 culture
groups, each consisting of 6 or 7 cultures (Fig. 3 A). The pH of 3 groups was
adjusted daily (or more often) to 6.9, 7.2, or 7.5. The pH of a fourth group was
not adjusted, and averaged 7.4 during the 3-wk experiment; the response of
this untreated group was highest of the 4 groups and was taken as the 100% reference response in Fig. 3 A. While a pH around 7.4 is optimal for this culture system, good secondary responses also occur over the entire pH range of 6.9 to 7.5.

The effect of pH on salicylate inhibition is illustrated in Fig. 3 B. The two
inhibition curves shown here were derived from two sets of cultures, each
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composed of 4 groups of quadruplicate cultures whose pH was adjusted daily (or more often) to 7.0 or 7.5. In each set different groups were incubated in medium containing 0, 0.5, 1.0, or 1.5 mM salicylate. (In the 2 non-salicylate-treated control groups the response of the pH 7.0 group was 89% of that of the pH 7.5 group. However, in computing the data for Fig. 3 B we used the average response of each control group as the 100% reference value for the 3 salicylate-treated groups maintained at the same pH). The two curves show that salicylate inhibition of the secondary response was much greater in those cultures grown in the more acid medium.

Fig. 2. Dose-response curves for salicylate inhibition of the secondary antibody response in vitro. Each curve was derived from 4 groups of quadruplicate cultures maintained for 18 or 21 days in medium containing 0, 0.5, 1.0, or 1.5 mM sodium salicylate. In each experiment the average response of the untreated control group of cultures was regarded as the 100% reference response for the other salicylate-treated groups in that experiment. (Each dose-response curve in this figure summarizes data equivalent to that contained in Fig. 1.) The 11 responses shown here were obtained in cultures prepared from 6 rabbits, 5 of which had been sensitized to both BSA and DT. Curves drawn with open symbols (circles, etc.) represent anti-BSA responses, and those with solid symbols anti-DT responses.
The explanation of this pH effect probably depends on the fact that salicylic acid, like many other weak acids, penetrates cells mainly in its nonionized state (10). Salicylic acid has an ionization constant ($pK_a$) of 3.00, from which it can be calculated that at pH 7.5 only 0.003% is nonionized but at pH 7.0 around 0.01% is nonionized (ca. 3 times more). When the percentage responses

![Graph A](chart.png)

**Fig. 3.** Influence of pH on the secondary response in vitro and on its inhibition by salicylate. Each point is the combined average of the anti-BSA and anti-DT responses produced concurrently by a culture group during this 18-day experiment.

A. The 18-day responses in media maintained at different pH's. Each of the 4 groups was comprised of 6 or 7 cultures. The response at pH 7.4 was maximal and was taken as the 100% reference response in this figure.

B. Dose-response curves for salicylate inhibition in 2 sets of cultures maintained at pH 7.0 or 7.5. Each set consisted of 4 groups of quadruplicate cultures; the medium of each group contained 0, 0.5, 1.0, or 1.5 mM sodium salicylate. The average response of each group is plotted as a function of the total salicylate concentration (in mM) present in the medium of that group.

C. Dose-response curves for salicylate inhibition at pH 7.0 and 7.5. The data of B have been replotted as a function of the concentration of nonionized salicylic acid (in $\mu$M) present in each medium. The horizontal arrows indicate the relative shift in the response curve of the pH 7.5 cultures resulting from this change in the abscissa.

of the two curves drawn in Fig. 3 B were replotted against the concentration of the nonionized salicylic acid present in each medium, a single curve emerged, as can be seen in Fig. 3 C. This result suggests that the intracellular salicylate concentrations in the pH 7.0 cultures were 3 times higher than those in the corresponding pH 7.5 cultures, thus accounting for the greater inhibition at the lower pH.

*The Effect of Sodium Bicarbonate Concentration on the Secondary Response and on Its Inhibition by Salicylate.*—The medium of all the cultures shown in Fig. 3
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contained 10 mM sodium bicarbonate, but in several of the experiments shown in Fig. 2 we had used other levels of bicarbonate. Therefore, the effect of this variable on salicylate inhibition was next examined.

Fig. 4 A shows the effect on the secondary response alone of levels of sodium bicarbonate from 0 to 25 mM. The maximal average response was produced by cultures maintained in 10 mM bicarbonate and was taken as the 100% reference response value here. When no bicarbonate was added to the medium, responses of less than 10% occurred; when 5 mM bicarbonate was added, responses of less than 50% were supported. Levels of bicarbonate higher than 10 mM supported less than maximal but nevertheless substantial responses.

Fig. 4. Influence of NaHCO₃ concentration on the secondary response in vitro and on its inhibition by salicylate. Each point is the combined average of the anti-BSA and anti-DT responses produced concurrently by a group of quadruplicate cultures during this 18-day experiment.

A. The 18-day responses in media having similar pH's but containing different concentrations of NaHCO₃. The response in medium with 10 mM NaHCO₃ was maximal and was taken as the 100% reference response for this figure.

B. Dose-response curves for salicylate inhibition in 4 sets of quadruplicate cultures whose media contained 5, 10, or 20 mM NaHCO₃; the "20 mM (pure)" set of cultures was maintained in media containing EDTA-treated, recrystallized NaHCO₃ (see "Results"). The average response of each group is plotted as a function of the total salicylate concentration (in mM) present in each medium.

C. Dose-response curves for salicylate inhibition in media containing 10 mM or 20 mM (pure) NaHCO₃. The data of B have been plotted in terms of the concentrations of non-ionized salicylic acid (in µM) present in each medium. The horizontal arrows show the relatively small shift in the rightmost curve produced by this change in the abscissa.

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media the pH was adjusted with NaOH freshly dissolved in boiled water; no other special precautions were taken to exclude trace amounts of bicarbonate.

Fig. 4 B shows the effect on salicylate inhibition of three different sodium bicarbonate concentrations: 5, 10, and 20 mM as indicated on the curves. In each set of cultures the response of the non-salicylate-treated control group was taken as the 100% reference response for the 3 salicylate-treated groups supported in medium with the same bicarbonate concentration. Increasing bicarbonate levels in the media diminished the inhibition produced by a given amount of salicylate. For example, 1 mM salicylate permitted only 15% and 21% responses in the 5 mM and 10 mM bicarbonate groups, respectively, but permitted 52% and 62% responses in the 20 mM bicarbonate groups. Three possible explanations for this bicarbonate effect are next considered.

Since salicylate forms stable chelates with Fe++, Al++, and Cu++ (11), we investigated whether increasing levels of bicarbonate effectively inactivated more salicylate through its chelation with heavy metal cations contaminating the impure NaHCO₃. Sodium bicarbonate was treated with a chelating agent (disodium ethylenediaminetetraacetate, or EDTA) to remove trace heavy metals and then was recrystallized from water 3 times. This purified material was used in preparing the medium for cultures designated in Fig. 4 B as "20 mM (pure)." Salicylate inactivation by chelation is not likely according to Fig. 4 B, since the three salicylate levels are even less inhibitory in the medium with the 20 mM purified NaHCO₃ than in medium with the 20 mM regular NaHCO₃.

The second explanation entertained for the bicarbonate effect was the more alkaline pH of media containing the higher bicarbonate levels. We could not keep the pH identical in all sets of cultures, but did determine from a record of their daily pH's that the average 18-day pH values were 7.3-7.4 for the 5 mM NaHCO₃ set of cultures, 7.5 for the 10 mM and 20 mM (pure) NaHCO₃ sets, and 7.6 for the other 20 mM NaHCO₃ set. The divergence of these inhibition curves in Fig. 4 B was not corrected by taking into account the effect of pH on the levels of the nonionized salicylic acid. This is illustrated in Fig. 4 C, which shows the inhibition curves of the 10 mM and 20 mM (pure) NaHCO₃ sets of cultures plotted against the concentration of nonionized salicylic acid in their media. These curves are only slightly less divergent than they are in Fig. 4 B. This suggests that some factor other than mere pH of the culture medium is responsible for the reduced salicylate inhibition accompanying high bicarbonate levels.

The third explanation considered for the bicarbonate effect was the increased Na⁺ level paralleling the increased NaHCO₃ concentration. Previously, Trowell (12) had observed that rat lymphocytes survive longer in medium containing 102 mM Na⁺ than in medium containing the more conventional
Na⁺ level of 138 mM. Fig. 5 A shows that the secondary response in rabbit lymph node cultures is maximal in medium containing around 123 mM Na⁺ and is less than optimal in our usual medium containing 137 mM Na⁺. The inclusion of 20 mM NaHCO₃ raises the Na⁺ content by 15% to 157 mM. (The Na⁺ added to the medium by 0.5 to 1.3 mM sodium salicylate and by 1.0 mM phosphate buffer was regarded as relatively insignificant and is ignored in this discussion.)

**Fig. 5.** Influence of NaCl concentration on the secondary antibody response in vitro and of Na⁺ concentration on salicylate inhibition of this response. Each point in these figures is the combined average of the anti-BSA and anti-DT responses produced concurrently by quadruplicate cultures during this 18-day experiment.

A. The secondary antibody responses in media containing different concentrations of NaCl. In each of the three separate experiments represented here the 100% reference response was that produced by the group whose medium contained 123 mM NaCl.

B. Dose-response curves for salicylate inhibition of cultures maintained in media with different NaHCO₃ and total Na⁺ concentrations. The media of different sets of cultures contained 5 mM or 20 mM NaHCO₃ and 123 mM or 137 mM NaCl; the total Na⁺ levels in these media were 143, 157, or 142 mM (see 'Results').

Fig. 5 B shows salicylate inhibition curves from 3 sets of cultures whose media differed only as follows: (a) 5 mM NaHCO₃ + 137 mM NaCl = 142 mM total Na⁺; (b) 20 mM NaHCO₃ + 137 mM NaCl = 157 mM total Na⁺; and (c) 20 mM NaHCO₃ + 123 mM NaCl = 143 mM total Na⁺. Inspection of Fig. 5 B will show that reduced salicylate inhibition depends on the NaHCO₃ concentration in the medium and not on the total Na⁺ level.

**The Effect of Cortisol Concentration on Salicylate Inhibition of the Immune Response.**—The similar effects of silicylates and corticosteroids on inflammation and on certain other aspects of metabolism have raised the question whether
part of the clinical response to salicylate is mediated through the pituitary-adrenal system. Indeed, large doses of salicylate have been shown to produce elevated levels of adrenocortical hormones in man and in experimental animals (see review by Done et al. (13)). Therefore, we considered whether inhibition by salicylate of the secondary response in vitro is influenced by the cortisol level in the culture medium.

A previous publication (6) described the requirement for cortisol in supporting the secondary response in vitro. In serum-free medium physiological levels of cortisol, or similar corticosteroids, are essential; good antibody responses are supported over the entire range of 0.01 to 10 μM cortisol. Table I presents data from one experiment in which control cultures supported by 0.01, 1.0, or 10 μM cortisol produced nearly identical 18-day antibody responses (e.g., anti-BSA responses of 98%, 100%, and 100% respectively). In other cultures supported by these three cortisol concentrations, treatment with 1.0 mM sodium salicylate inhibited the average response of each group to very similar degrees (e.g., permitted only 15%, 20%, and 14% of the corresponding maximal anti-BSA responses). Thus salicylate inhibition is not significantly affected by a 1000-fold elevation in the cortisol concentration.

**Inhibition of the Immune Response by Aspirin and Gentisic Acid.**—The effect on this culture system of many compounds structurally related to salicylic acid will be described in a subsequent publication (14). But two compounds warrant preliminary discussion here: (a) acetylsalicylic acid (aspirin) because it was frequently used in early clinical attempts to suppress the immune response, and (b) gentisic acid (5-hydroxysalicylic acid) because it was considered by some investigators to be the active metabolic product of salicylic acid (15, 16).

### Table I

The Failure of Elevated Cortisol Levels to Influence Salicylate Inhibition of the Secondary Antibody Response In Vitro

Groups of quadruplicate cultures were maintained for 18 days in media containing different concentrations of cortisol and salicylate. Concurrent anti-BSA and anti-DT titers were measured and the 18-day responses of each group computed relative to 100% reference responses of the control group supported by 1.0 μM cortisol but not exposed to salicylate.

<table>
<thead>
<tr>
<th>Cortisol concentration (μM):</th>
<th>Anti-BSA response</th>
<th>Anti-DT response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01 1.0 10.0</td>
<td>0.01 1.0 10.0</td>
</tr>
<tr>
<td>Salicylate concentration:</td>
<td>%     %     %</td>
<td>%     %     %</td>
</tr>
<tr>
<td>None</td>
<td>98 100 100</td>
<td>105 100 93</td>
</tr>
<tr>
<td>1.0</td>
<td>15 20 14</td>
<td>9   8 10</td>
</tr>
<tr>
<td>1.5</td>
<td>2     5</td>
<td>1   0 2</td>
</tr>
</tbody>
</table>

1.0, or 10 μM cortisol produced nearly identical 18-day antibody responses (e.g., anti-BSA responses of 98%, 100%, and 100% respectively). In other cultures supported by these three cortisol concentrations, treatment with 1.0 mM sodium salicylate inhibited the average response of each group to very similar degrees (e.g., permitted only 15%, 20%, and 14% of the corresponding maximal anti-BSA responses). Thus salicylate inhibition is not significantly affected by a 1000-fold elevation in the cortisol concentration.
Equimolar concentrations of aspirin and sodium salicylate produce nearly identical inhibition, as shown in Fig. 6 A. Hydrolysis of the acetyl group undoubtedly occurs in the lymph node fragment cultures, converting aspirin to free salicylic acid (see "Discussion").

A normal metabolite of salicylic acid is its 5-hydroxy derivative, gentisic acid. Fig. 6 B illustrates the relative inhibition of the secondary response produced in three experiments by equimolar concentrations of gentisic acid and sodium salicylate. Suppression of the response to between 30% and 40% of the untreated control values was produced by 1.0 mM salicylate and by as little as 0.05 mM gentisic acid. For more complete suppression gentisic acid was 3 times more effective than equimolar amounts of salicylate. This greater inhibitory activity of gentisic acid cannot be explained by the level of its nonionized form, since in equimolar solutions at the same pH there is 5 times more nonionized salicylic acid ($pK_a = 3.00$) than gentisic acid ($pK_a = 2.28$). Preliminary experiments suggest that cell penetration by gentisic acid is not limited to its nonionized form but is more nearly a function of the total concentration of the drug in the medium.

![Graph](image-url)
Because of the structural similarity between salicylic and gentisic acids and because of the latter's greater inhibitory effect, we also wondered whether the activity of salicylate in this culture system might be due to its oxidation to gentisic acid. If such activity results entirely from this conversion, then the inhibition produced by 1.0 mM salicylate (as in Fig. 6 B) implies an intracellular level of gentisic acid equivalent to that produced by 0.1 mM gentisic acid in the culture medium. In preliminary experiments we have chromatographed extracts of lymph node fragments incubated with C14-salicylic acid for various times. Extracts of media from such cultures have been examined similarly. As yet we have not found significant radioactivity in the gentisic acid spot of these chromatograms above that due to trailing of the faster moving C14-salicylic acid.

The Effect of Salicylate Treatment at Different Stages of the Secondary Response Initiated in Vitro.—Cultures were incubated with an inhibitory level of salicylate for different intervals during their 21-day life in order to determine whether the drug inhibits some step in the terminal synthesis of antibody (the productive phase) or whether it affects the complex sequence of reactions occurring between the introduction of an antigen to a primed cell and the start of antibody synthesis on ribosomes (the inductive phase).

Typical of many experiments of this design is one illustrated in Fig. 7 A to H, in which the culture medium contained 1.5 mM sodium salicylate only for the intervals indicated by the cross-hatched bar at the top of each figure. In the control group (Fig. 7 A) 92% of the antibody produced during the 21-day experiment was synthesized after day 9. Treatment of other cultures throughout the 21 days with 1.5 mM salicylate (Fig. 7 E) depressed the response to 1% of the control group's average response. In other culture groups salicylate treatment for only the first 3, 6, or 9 days lowered the response to 48%, 27%, and 4%, respectively (see Fig. 7 B, C, and D). On the other hand, treatment with salicylate through day 21 starting on day 3, 6, or 9 depressed the response to 10%, 13%, and 67%, respectively (see Fig. 7 F, G, and H). Thus a substantial response (67%) occurred, although an inhibitory level of salicylate was introduced on day 9 and although most of the antibody in the control group (92%) was produced after day 9.

These data suggest that salicylate affects some step which occurs mainly during the first 9 days of the secondary response initiated in vitro on day 0. This interval corresponds to what we regard as the inductive phase in these cultures. The same pattern of inhibition was obtained when an inhibitory level of gentisic acid was added to other cultures for different intervals during a 21-day experiment. Thus gentisic acid also exerts its inhibitory effect largely during the inductive phase in this culture system.

Chloramphenicol produces a similar pattern of inhibition, illustrated in Fig. 8. In this experiment, treatment for 21 days with 100 ~ (40 ~g/ml) chloramphenicol depressed the response to 7% (Fig. 8 B) and with 1.25 mM salicylate de-
pressed it to 2% (Fig. 8 E). When 100 μM chloramphenicol was added only during days 9 to 21 or days 12 to 21, the responses during these intervals were 64% and 78%, respectively (Fig. 8 C and D). When 1.25 mM salicylate was present only during days 9 to 21 or days 12 to 21, the corresponding responses during these intervals were 47% and 70% (Fig. 8 F and G). These two sets of values should be compared with 96% and 82%, the percentages of the untreated (control) responses produced during days 9 to 21 and days 12 to 21. Thus the addition of either compound to this culture system starting on day 9 or day 12 depressed the subsequent response relatively little in view of the fact that most of the antibody was actually synthesized after day 9 (96%) and after day 12 (82%). Although both compounds appear to inhibit processes occurring mainly during the inductive phase, their modes of action are undoubtedly quite different (17).

*The Temporal Difference between Inhibition by Salicylate and by 2,4-Dinitro-*
One of several reported effects of salicylate on cellular metabolism is the uncoupling of oxidative phosphorylation (18). We assumed that the secondary response would be inhibited rather promptly by agents which interfere with energy generation when added late in the response during the productive phase as well as early during the inductive phase. But we concluded from the experiments described above that salicylate produced disproportionately little inhibition when added on day 9 or later, i.e., when the inductive phase has largely been completed. This discrepancy prompted us to study the standard uncoupling agent, 2,4-dinitrophenol (DNP).

Fig. 9 A to I compare the effects on antibody production when inhibitory levels of DNP, sodium salicylate, gentisic acid, and puromycin were added to different cultures of the same experiment. These various figures show the cumulative antibody production rather than amounts of antibody produced in each 3 day period (as in Figs. 1, 7, and 8). Fig. 9 A to D show that complete, or nearly complete, suppression of the response occurred when each inhibitor was
included in the medium from day 0 to day 18 at the concentration listed. The level of puromycin used (5 \( \mu \text{g} \), 2.7 \( \mu \text{g/ml} \)) is several times greater than is necessary for complete suppression, but the concentrations of the other three agents are quite near the minimum levels which suppress the response to less than 10%.

Fig. 9 E to I show the control response and the responses when the four inhibitors were added to other cultures starting on day 8 (as indicated by a vertical arrow in each figure). The percentage values under the curves relate to

![Diagram](https://example.com/diagram.png)

**Fig. 9.** Relative inhibition of the secondary antibody response in vitro following the addition of different inhibitors during the productive phase. Each figure represents the cumulative anti-BSA response of a group of 4 cultures whose medium was replaced every 2 days for 18 days. The inhibitors and their concentrations tested are listed above the upper row of figures; the duration of treatment with each inhibitor is indicated by a characteristically shaded bar at the top of each figure.

A to D. Complete, or nearly complete, suppression of the response when the above inhibitors were included in all medium changes throughout the 18-day experiment. The percentage values above these response curves were calculated relative to the 100% produced by the untreated control group of cultures shown in E, the untreated control group of cultures.

F to I. Average responses of cultures treated with one of the inhibitors starting on day 8 (indicated by the vertical arrow) and continuing to day 18. The percentage values under these curves represent the responses produced after day 8 relative to that produced by the control group of cultures after day 8. In the control group (E) 91% of the antibody produced during the 18 days was produced after day 8; this 91% was equated to 100% in computing the percentage values given under the curves in F to I.
the amount of antibody produced by the control cultures from day 8 through day 18. The addition of an excess of puromycin on day 8 caused complete suppression of antibody synthesis within the next 2 days; the 7% response produced after addition of this inhibitor probably represents the small amount of preformed antibody present in the cells of these cultures. The addition of DNP on day 8 caused suppression of the subsequent response to 32% of the control value for days 8 to 18. On the other hand, the addition of equivalent inhibitory levels of salicylate or gentisate permitted 62% and 56%, respectively, of the days 8 to 18 control response. These and similar data from other experiments indicate that DNP has an effect which differs temporally from that of salicylate or gentisate and thus suggest that DNP has a mode of action different from that of the latter two compounds.

DISCUSSION

Reviews concerning salicylate inhibition of the immune response have recently been published by Austen (3) and by Schwartz and Andre (4). In comparing studies on salicylate inhibition in vivo with our investigations in vitro we shall consider initially (a) the significance of total and non-protein-

1 This discussion of experimental results must be prefaced by assurance that salicylate does not interfere with the assay procedure itself, since the drug has been reported to reduce specific precipitation in at least one immune system. For example, Coburn and Kapp (19) added 1.32 mM sodium salicylate to a system of crystalline egg albumin and its antibody and observed that the precipitate was reduced by an amount equivalent to eliminating about half of the total antibody. In an identical system Friend (20) found that 2.0 mM sodium salicylate caused a 54% reduction in the precipitation. However, Jager and Nickerson (21) incubated immune serum with 1.0 mg/ml salicylic acid (7.25 mM) for 18 hours at 37°C and found no decrease in typhoid O and H agglutinin titers. To insure that salicylate present in our culture media did not alter the hemagglutination titer directly, we carried out the following experiments.

Sets of doubling dilutions of an immune serum to BSA were prepared, the final volume of each dilution being 0.5 ml. To each tube was added 0.3 ml of various sodium salicylate solutions such that the final concentration in each tube of a set was 1, 2, 3, or 4 mM. Control sets of dilutions with 0.3 ml normal saline (145 mM NaCl) were also prepared. These sets were incubated at 37°C for 1 hr before 0.05 ml of 2% tanned sheep erythrocytes passively sensitized to BSA was added to each tube. The end points of these titrations were identical in all sets, indicating that salicylate at concentrations of 4 mM or less in all dilutions of the immune serum had no effect on the assay.

In another experiment, samples of the immune serum were diluted with equal volumes of sodium salicylate solutions such that the final concentration was 1, 2, 3, 6, or 25 mM. These salicylate-treated serum samples were incubated at 37°C for 6 days in sterile sealed tubes and then individually titrated. Again the end points were identical in all samples, indicating that the hemagglutination titer of immune serum was not depressed by prior incubation with as much as 25 mM salicylate. Similar experiments with serum-free tissue culture fluids containing antibody showed that incubation with salicylate did not reduce their hemagglutination titers. The level of 25 mM salicylate is 17 times greater than any used in the tissue culture experiments described in this paper.
bound salicylate levels, (b) the inhibitory levels in vitro and in vivo, and (c) the time of effective administration of salicylate in both situations.

The Relationship between in Vivo and in Vitro Levels.—The therapeutic activity of salicylate is more accurately a function not of the total plasma concentration but of the non–protein-bound (free) salicylate level (22). Salicylate is 50 to 80% bound to human plasma proteins, the percentage decreasing with rising drug levels in the blood (23). In man the therapeutic range of total salicylate is 30 to 50 mg/100 ml and is thus represented by levels of free salicylate between 7.5 and 25 mg/100 ml (see Fig. 1 in reference 24). The tissue culture medium used in our experiments contained no serum or other proteins except for 0.5 unit/ml insulin (ca. 22 μg/ml); and so the salicylate levels used in these cultures should properly be compared with the free salicylate levels in blood. Thus the tissue culture range of 0.5 to 1.5 mM salicylate is equivalent to plasma levels of 7 to 21 mg/100 ml free salicylate, or 30 to 50 mg/100 ml total salicylate.

Inhibitory Levels in Vivo.—Jager and Nickerson (21) found that salicylate blood levels of 30 to 40 mg/100 ml reduced antibody production in human beings immunized with typhoid vaccine. In rabbits serum levels between 20–30 mg/100 ml depressed precipitin production in experimentally induced serum disease (25). Similar results in rabbits were obtained by Smull et al. (26). The highest level of salicylate which can safely be maintained in rabbits is reported to be 30 mg/100 ml (25, 26), or 7 mg/100 ml of free salicylate, if we assume that binding in rabbit serum is quantitatively similar to that in human serum. If comparable degrees of immune inhibition are produced in vitro and in vivo by similar levels of free salicylate, our data indicate that only partial suppression (20 to 60%) of the immune response would be obtained in rabbits. The further extrapolation of our data from rabbit lymph node cultures to human clinical studies suggests that more complete suppression of the immune response might be obtained in clinical situations, since higher salicylate blood levels are apparently tolerated in man than in rabbits. This conclusion is in keeping with the inhibition described in Jager and Nickerson’s well quantitated study (see Fig. 1 in reference 21).

Time of Effective Salicylate Administration in Vivo.—Our in vitro studies show that salicylate inhibition is most effective when the drug is present in the culture medium during the inductive phase of the secondary response. Many of the reported failures to inhibit the immune response in vivo with salicylate probably resulted from inadequate levels of the drug in the antibody-producing tissues during the inductive phase. For example, in several negative clinical studies the antigenic stimulus clearly occurred long before salicylate treatment was begun, e.g., stimulation by Rh antigen during pregnancy (27) or stimulation by streptolysin during streptococcal pharyngitis (28). Analogously, Perry (29) inoculated volunteers with typhoid vaccine who then took aspirin for the next 10 days; all developed agglutinins, as might have been predicted. In con-
trast, Jager and Nickerson (21) administered salicylate to patients for 7 days or more before injecting typhoid vaccine; high plasma salicylate levels were maintained for the next 2 wk with the resulting suppression of antibody formation to typhoid H and O antigens. Studies of immune suppression in experimental animals with various other inhibitors (30, 31) suggest the probable importance of priming animals or man with salicylate several days before antigen stimulation as well as maintaining maximally tolerated blood levels of the drug for a week or more afterwards.

**Inhibition by Aspirin.**—Aspirin has been used in several studies on immune suppression in man (29, 32). After its absorption from the internal tract aspirin is hydrolyzed in the tissues to salicylic and acetic acids (33). Such hydrolysis has been demonstrated in liver, kidney, and brain slices of rats (34) and upon incubation of aspirin with rat blood or thoracic duct lymph (35). Fig. 6 A shows that equimolar concentrations of aspirin and sodium salicylate produce nearly identical inhibition of the secondary response in vitro, suggesting that hydrolysis of aspirin occurs also in rabbit lymph node cultures.

**Inhibition by Gentisic Acid.**—Gentisic acid is more effective than equimolar concentrations of salicylic acid by 3 to 20 times, depending on the level of inhibition of the secondary response chosen for comparison (see Fig. 6 B). (No analogous studies on immune suppression with gentisic acid in animals have been published.) The antirheumatic, antipyretic, and analgesic effects of gentisic acid have been reported as equal, and sometimes superior, to those of salicylic acid (15, 36, 16). Because of their similar metabolic effects and related chemical structure and the fact that gentisic acid is a normal excretion product of salicylate (37), investigators have postulated that gentisic acid is the active agent during salicylate treatment. But this is doubtful for the several reasons discussed below.

The biological oxidation of salicylate to gentisate was first studied by Lutwak-Mann (34), who reported that rats poisoned with white phosphorus or carbon tetrachloride and then given salicylate did not excrete gentisic acid. She also found that incubation of liver pulp with salicylate produced a substance giving a color reaction typical of gentisic acid. Although these observations suggested that oxidation occurs in the liver, gentisic acid has not been reported in the blood of salicylate-treated human subjects or experimental animals. Meyer and Ragan (15) did not detect gentisic acid in the blood even of patients treated with sodium gentisate; their assay could measure as little as 5 μg/ml (0.005 mM). This failure may indicate an extremely rapid renal clearance of gentisic acid from the circulation, since Batterman and Sommer (38) recovered in urine up to 84% of the gentisic acid administered orally to patients. Crabtree et al. (39) administered C14-salicylic acid and C14-acetylsalicylic acid to normal and fevered rats and recovered C14-gentisic acid from kidney extracts and the urine of all rats and from the pituitary gland of the fevered rats. These authors related
the latter finding to the antipyretic action of salicylate, but concluded that "the peripheral actions of salicylate are probably due to salicylate itself," since no widespread body distribution of any salicylate metabolite was found.

To date we have failed to detect significant conversion of C14-labeled salicylic acid to gentisic acid in our lymph node cultures. But the identification of any newly synthesized gentisic acid by conventional chromatography or electrophoresis is complicated by the fact that in both methods of separation the Rf values for gentisic acid are smaller than the corresponding values for salicylic acid (40, 39). Thus the gentisic acid spot in either system of separation is frequently contaminated with trace amounts of the faster-migrating radioactive salicylic acid which trail behind.

Arguments against Salicylate's Acting as an Uncoupling Agent of Oxidative Phosphorylation during Immune Suppression.—Experiments were described above in which the effect of an inhibitory level of 2,4-dinitrophenol (12 µM) was compared with that of comparable levels of salicylate (1.25 mM) and gentisate (0.5 mM). These levels are "comparable" in the sense that they are the minimum values which when present from the start of the inductive phase suppress almost completely the in vitro response (i.e., permit less than 10% of the control response). When these same levels were added on day 8 after the inductive phase was largely completed (Fig. 9 G and I), DNP inhibited antibody synthesis to a substantial degree, while salicylate and gentisate exerted considerably less inhibition. This quantitative difference in inhibition permits two possible interpretations: (a) that salicylate and gentisate are weak uncouplers but that this culture system is more sensitive to their action during the inductive phase than during the later productive phase, or (b) that salicylate and gentisate inhibit the immune response by some mechanism other than uncoupling oxidative phosphorylation.

There are several additional reasons for rejecting the possibility that an uncoupling action is salicylate's mode of inhibiting the immune response. Brody (41) observed that gentisic acid did not uncouple oxidative phosphorylation in rat liver mitochondria under conditions where salicylate apparently did. Yet gentisic acid is an even more potent inhibitor of the immune response in vitro than is salicylate. Since these drugs are structurally similar and exert inhibitory effects on the immune response which are temporally alike, it seems unnecessary to postulate two different modes of action. And since gentisic acid fails to uncouple oxidative phosphorylation in mitochondrial preparations, some other mechanism common to the two drugs is possibly responsible for immune suppression in lymph node cultures.

A final argument along the above lines stems from our observations that a 5 mM concentration of sodium salicylate inhibits protein synthesis in _Escherichia coli_ grown anaerobically as well as aerobically (42). Since oxidative phosphorylation (respiration) is not part of the energy source for bacteria under anaerobic
conditions, salicylate must inhibit them by some mechanism other than uncoupling.

Comparison of the Effective Salicylate Level with that of other Inhibitors.—Because relatively high levels of salicylate (500 to 1500 µM) are necessary to suppress the immune response in vitro, the mechanism of its action might appear less specific, and therefore less interesting, than that of other metabolic inhibitors which are active at much lower levels. For example, the secondary response in our culture system is suppressed 90% or more by 0.1 µM actinomycin D; by 1.0 µM puromycin, cycloheximide, or mitomycin C; by 10 µM proflavine, acridine orange, 5-bromo-2'-deoxyuridine, or 2,4-dinitrophenol; and by 100 µM chloramphenicol or oxytetracycline (42). If the active form of salicylate is the nonionized acid and thus the effect of 1.5 mM total salicylate is due to the 0.06 µM salicylic acid present at pH 7.4, then this drug could be considered a relatively potent inhibitor of the immune response in vitro.

**SUMMARY**

Salicylate inhibition of the secondary antibody response initiated in vitro on day 0 has been studied in cultures of rabbit lymph node fragments. Levels of 1.25 to 1.5 mM (0.20 to 0.24 mg/ml) sodium salicylate present in serum-free medium throughout an 18- or 21-day culture period completely inhibit the secondary response. This inhibition is largely accomplished by the drug's action during the first 9 days, which corresponds to the inductive phase for this culture system. Relatively little inhibition is produced by adding the drug only after day 9, although over 90% of the antibody produced during a 21-day experiment is synthesized after day 9. Studies with media of different pH's show that this inhibition is more correctly a function of the nonionized salicylic acid concentration in the medium than of the total salicylate concentration. Arguments are presented against the possibility that salicylate at the levels used here inhibits antibody synthesis by uncoupling oxidative phosphorylation.

Acetylsalicylic acid (aspirin) produces the same degree of inhibition in vitro as do equimolar concentrations of sodium salicylate. Gentisate (5-hydroxy-2-salicylate) is 15-fold more effective in producing 50% inhibition than salicylate; its temporal pattern of inhibition is similar to that of salicylate.

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