Regulation of the Secondary Antibody Response in Vitro. Enhancement by Actinomycin D and Inhibition by a Macromolecular Product of Stimulated Lymph Node Cultures

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REGULATION OF THE SECONDARY ANTIBODY RESPONSE IN VITRO

ENHANCEMENT BY ACTINOMYCIN D AND INHIBITION BY A MACROMOLECULAR PRODUCT OF STIMULATED LYMPH NODE CULTURES*

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Because of its interference with the formation of messenger ribonucleic acid, actinomycin D has come to be regarded as a general inhibitor of protein synthesis. Under certain conditions, however, the drug has the opposite net effect and appears to enhance protein synthesis. This paradoxical phenomenon has been examined in bacteria, rats, and many different mammalian culture systems (see DISCUSSION). The proteins most frequently studied have been various induced enzymes. One postulated explanation of this phenomenon is that synthesis of the induced enzyme is modulated by an inhibitor normally appearing soon after enzyme induction and that under certain conditions inhibitor formation may be selectively suppressed by actinomycin. Clearly, isolation of the postulated inhibitor would provide the most convincing evidence for this hypothesis.

Under certain conditions the immune response also may be enhanced by actinomycin. This paper describes both the inhibitory and enhancing effects of the drug on the secondary antibody response in rabbit lymph node cultures. The conditions found critical for enhancement in vitro, such as the timing and level of actinomycin D treatment, are also those theoretically essential for the hypothesis described above. However, greater support for the hypothesis comes from our recovery in used culture medium of a nondialyzable inhibitor of antibody synthesis. Various features of this macromolecule suggest that it is the postulated inhibitor. These studies thus imply a control mechanism for antibody synthesis not widely suspected by immunologists.

Materials and Methods

Michaelides and Coons (1) originated the idea of stimulating and following the secondary antibody response in organ cultures of rabbit lymph node fragments. Briefly, their method involved priming a rabbit by a single set of injections with one or two antigens (e.g. bovine

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serum albumin and diphtheria toxoid). Several months later the popliteal nodes were aseptically removed and cut into small fragments. These were incubated for 2 hr in a dilute solution of the priming antigen(s), rinsed free of excess antigen in Hanks' solution, and then distributed in plasma clots among tubes incubated in a roller drum. Eagle's medium containing 25% normal rabbit serum was added to each tube and replaced every 3–4 days for several weeks. The media removed were refrigerated and subsequently titrated by the hemagglutination method with tanned sheep erythrocytes (Stavitsky's modification of the Boyden procedure).

Our modifications of this technique, used in current experiments and described in previous papers (3–5), include the following: (a) the development of a serum-free, chemically defined medium; (b) the replacement of the plasma clot by a glass wool pad overlaying the fragments in a Leighton tube, thus fixing them in position; (c) the conversion to stationary cultures; (d) the control of pH by gassing individual tubes with 5% CO₂ in air; (e) the utilization of other lymph nodes, such as the axillary, auricular, and cervical nodes; and (f) the use of other antigens, including tetanus toxoid and ovalbumin. The several modifications not previously described but used in experiments of this paper are discussed below in some detail.

It has recently been determined that the osmolarity of the medium considerably influences the course and magnitude of the secondary response. The optimal osmolarity for use with rabbit lymph node fragments is around 280 milliosmols/kg, 10% or so lower than that of conventional media and of those used in earlier experiments. Table I lists the composition of the medium used in the present studies and outlines its preparation.

For experiments with medium replacement every 3 days, 14 fragments/ml medium per Leighton tube sufficed to produce convenient titers. In other experiments, in which the culture fluids were replaced as often as every 2 hr during part of the productive phase, as many as 39 fragments/ml medium per Leighton tube were used. When such frequent changes of medium were made, the media were preheated to 37°C, and the tubes, while outside the incubator, were kept at 37°C by enclosing them in a small heating pad. Still other experiments were performed to isolate an inhibitory material from large volumes of used culture medium. Here large Leighton tubes (Belco Glass, Inc., Vineland, N.J.) were employed, in each of which 100 fragments could be cultured. The volume of medium added was kept around 1 ml/20 fragments. For this work large Leighton tubes (35 X 150 mm) were preferable to Petri dishes, because the rubber-stoppered tubes became infected less frequently and did not require the use of a CO₂ incubator.

In this paper hemagglutination data are presented in one of three graphs. The simplest is the differential response graph, in which the ordinate indicates the average titer of medium removed from a group of cultures at a given time, and the abscissa, the times of the different medium changes. Typical differential response curves are presented in Fig. 1. For certain kinetic studies, integral response curves reveal effects more clearly. On the ordinate of these graphs is plotted the cumulative sum of the average titers of successive medium changes for a group; the abscissa shows the time of the medium replacement. Examples are seen in Fig. 2. The third type of graph involves a dose-response curve like that in Fig. 4. Here the ordinate is a measure of antibody production by each group, and the abscissa, the concentration of some agent whose effect on the antibody response is being measured. Since each point of a dose-response curve is derived from one differential response curve, this graph presents many data in a condensed form.

RESULTS

Inhibition by Actinomycin D Treatment Begun during the Inductive Phase or during the Productive Phase.—The inductive phase of the immune response in

1 C. T. Ambrose, unpublished results.
### TABLE I

**Components, Stock Solutions, and Sequence of Preparing Serum-Free Medium**

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock solutions: concentration, commercial source, and storage temperature</th>
<th>Volume of stock solution added*</th>
<th>Concentration in final medium</th>
<th>Contribution to osmolality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-salt solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double-distilled water</td>
<td></td>
<td>70</td>
<td>5 mmol glucose</td>
<td>250</td>
</tr>
<tr>
<td>Components added in the fol-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lowing order at room tem-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>perature with mixing on a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>magnetic stirrer: 970 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>double-distilled water, 72.6 g NaCl, 9.0 g glucose, 2.98 g KCl, 0.71 g NaHPO₄, 0.17 g NaH₂PO₄·H₂O, 2.03 g MgCl₂·H₂O, and 1.11 g CaCl₂. Final volume is 1000 ml (refrigerate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol red</td>
<td></td>
<td>2.0 mg/ml (refrigerate)</td>
<td>0.01 mg/ml</td>
<td>0</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td></td>
<td>500 mM (room temperature)</td>
<td>10 mM</td>
<td>17</td>
</tr>
<tr>
<td>Eagle’s 12 amino acids</td>
<td></td>
<td>50X MEM amino acids (GIBCo.)§ (refrigerate)</td>
<td>1 X</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td>100X L-glutamine, 200 mM (GIBCo.) (freeze)</td>
<td>2.0</td>
<td>2 mmol</td>
</tr>
<tr>
<td>5 accessory amino acids</td>
<td></td>
<td>10 mM each of alanine, aspartic acid, glycine, proline, and serine (Calbiochem)</td>
<td></td>
<td>(refrigerate)</td>
</tr>
<tr>
<td>NaOH</td>
<td></td>
<td>0.5 N; add volume (x) sufficient to yield final pH of 7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eagle’s “B” vitamins</td>
<td></td>
<td>100X MEM vitamin solution (GIBCo.) (freeze)</td>
<td>1.0</td>
<td>1 X</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td></td>
<td>50 µg/ml (Calbiochem) (freeze)</td>
<td>1.0</td>
<td>0.5 µg/ml</td>
</tr>
<tr>
<td>Penicillin G, potassium</td>
<td></td>
<td>11,800 units/ml = 7.08 mg/ml = 20 mM (Squibb) (freeze)</td>
<td>0.5</td>
<td>59 units/ml = 35 µg/ml = 0.1 mm</td>
</tr>
<tr>
<td>Coristol (Solu-Cortef)</td>
<td></td>
<td>100 µM (Upjohn) (freeze)</td>
<td>1.0</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Insulin, crystalline</td>
<td></td>
<td>2 mg/ml Insulin requires slight acidification for solubilization (Sigma)¶ (freeze)</td>
<td>1.0</td>
<td>0.5 unit/ml = 20 µg/ml</td>
</tr>
<tr>
<td>Final water</td>
<td></td>
<td>Double-distilled water; add (9 − x) ml final water</td>
<td>(9 − x)</td>
<td></td>
</tr>
</tbody>
</table>

* The final volume here is 100 ml.
† Minimal essential medium.
§ Grand Island Biological Co., Grand Island, N.Y.
∥ Calbiochem, Los Angeles, Calif.
¶ Sigma Chemical Co., St. Louis, Mo.
vitro (days 0–9) is nearly 10 times more sensitive to inhibition by actinomycin D than is the productive phase (days 9–21 or later). This differential sensitivity is illustrated by data in Fig. 1 and summarized in Table II. Graph A of Fig. 1 illustrates relative inhibition of the response when different concentrations of the drug were present in the medium, starting on day 3 during the inductive phase. The level of actinomycin D in each treated culture was renewed every 3 days when the medium was replaced. Under these conditions 0.0026 μM (0.0033 μg/ml) depressed the 24 hr response to 33% of the untreated control group of cultures. Higher concentrations of the drug reduced the response proportionately (See column A of Table II).

Graph B of Fig. 1 shows the second part of this experiment, in which other cultures were treated with the same levels of actinomycin D starting 6 days...
later; that is, starting on day 9 early in the productive phase. The lower two levels of the drug appeared to produce enhancement before inhibition finally occurred—a phenomenon explored later in this paper. The two higher drug levels produced prompt inhibition. In this graph are also plotted the responses of the control group (curve A) and of another group treated with 10 µM (5.4 µg/ml) puromycin starting on day 9. After addition of puromycin the response was only 6% that of the control group during the same period. This indicates that little antibody was released from these fragments after synthesis had been stopped. The greater susceptibility of the inductive phase to actinomycin D

TABLE II

Inhibition by Actinomycin D of the Secondary Antibody Response in Vitro; Influence of Drug Concentration and of Drug Treatment Interval*

<table>
<thead>
<tr>
<th>Antibody formation</th>
<th>Days 3-24</th>
<th>Days 9-24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 3-24</td>
<td>Days 9-24</td>
</tr>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td>Drug and level</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Actinomycin D, 0.0026 µM</td>
<td>33</td>
<td>63</td>
</tr>
<tr>
<td>Actinomycin D, 0.008 µM</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td>Actinomycin D, 0.026 µM</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Actinomycin D, 0.08 µM</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>Puromycin, 10.0 µM</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

* These data were derived from cultures whose average responses are graphed in Fig. 1.† The response of untreated (control) cultures from day 3 to 24.‡ The response of these same cultures for the 9-day period from day 3 to 12 (see graph A of Fig. 1).¶ The response of other cultures in this same experiment for the 9-day period from day 9 to 18 (see graph B of Fig. 1).

Inhibition is apparent upon comparing columns B and C of Table II. When treatment began on day 3, 0.008 µM reduced the response to 32% over the ensuing 9 days. But when treatment began on day 9, 0.008 µM actinomycin permitted a 9-day response of 114%. At this time a level of the drug 10 times higher (0.08 µM) was required to depress the response to a similar extent; that is, to 21% of the control group's response.

In this culture system antibody production generally was constant for a week or more during the early productive phase. This feature of the secondary response in vitro is more apparent in Fig. 2, in which the differential responses of Fig. 1 B have been regraphed as integral, or cumulative, responses. Each curve is identified by a letter to facilitate comparison between Fig. 1 B and Fig. 2. In Fig. 2 the linearity of the control group's response between days 6 and 21 is
readily evident (curve A). Treatment with the different levels of actinomycin and of puromycin starting on day 9 (see solid arrow of each curve) stopped antibody synthesis after varying intervals.

The efficiency of inhibition by these drugs could be resolved more finely in cultures whose media were replaced every few hours. In Fig. 3 are graphed cumulative responses between days 10 and 12 for cultures whose medium was replaced every 4 hr. For lack of space in Fig. 3, the average response of the control group of five cultures is shown only for the first 36 hr, but this average response continued in a precisely linear rate for the entire 52 hr studied. The four other curves derived from this experiment have been spaced along the abscissa so that they do not overlap; however, the intervals graphed are the same for all five curves. Different levels of actinomycin D or puromycin were added simultaneously to groups of quadruplicate cultures in the experiment;
the start of treatment is indicated on each response curve by the arrow. Each subsequent medium replacement contained anew the starting level of the drug. From the graph of the average responses, the time required for synthesis of the first 50% of all antibody produced subsequent to the addition of a given level of each drug was estimated. This moment is termed in this paper the

"50% synthesis time." These times were 6, 3.6, and 2.8 hr for actinomycin D levels of 0.4, 2.0, and 10 μM, respectively. In this experiment, the 50% synthesis time for 5 μM (2.7 μg/ml) puromycin was also 2.8 hr.

It is obvious from Figs. 2 and 3 that inhibition of antibody synthesis is faster with higher levels of actinomycin D. A summary of data from seven experiments of this same design has been compiled in Fig. 4. The ordinate indicates the hours required for production of the initial 50% of the antibody response subsequent to addition of a given drug level; the abscissa indicates the different
levels of actinomycin D tested. Low levels of the drug (e.g., 0.016 μM) permitted 50% synthesis times of 30-40 hr; in these experiments the media were replaced only daily or every 12 hr. Higher levels reduced the 50% synthesis time to a few hours; in these experiments the media were replaced more frequently—as

![Diagram of Frequency of Medium Changes and Actinomycin D](image-url)

**Figure 4.** Termination of the productive phase in vitro by actinomycin D. The points plotted here were obtained from seven different experiments like that depicted in Fig. 3. Each point represents the time required for synthesis of the initial 50% of all antibody produced by a group of cultures after addition of a particular concentration of actinomycin D to their medium. The variously shaped points refer to different experiments, whose media were changed with the different frequencies listed in the figure.
often as every 3 hr. In different experiments with low levels of actinomycin D, there was a wide spread in the 50% synthesis times. With increasing levels of the drug, however, this spread decreased, and the values became asymptotic to a line intersecting just below the 3 hr level of the ordinate. Since these concentrations of actinomycin D presumably inhibit synthesis of new messenger RNA, there probably exists in these cultures a family of mRNA's with a half-life of 3 hr that is essential for antibody synthesis. The data do not indicate whether this

mRNA is directly concerned with coding for immunoglobulin chains or is essential for maintaining the antibody-producing cell itself.

*Actinomycin D Enhancement of the Secondary Response In Vitro.*—Treatment with low levels of actinomycin D produced apparent enhancement of antibody synthesis in several early experiments (e.g. curves B and C in Fig. 1 B). This phenomenon was explored systematically in an experiment depicted in Fig. 5. Here the media were replaced every 2 days; actinomycin D at 0.0025 μM (0.0031 μg/ml) was included in the medium of some cultures on day 6 or on day 8 for 2 day or 6 day intervals. The reference response drawn in graph A represents the average of eight cultures not treated with the drug.

In graph B (Fig. 5) is shown the response of cultures treated once with actinomycin D for 2 days, starting on day 6. Enhancement occurred between days 8
and 10, after which time the average response remained at a plateau 50% higher than the average response of the control cultures during the same period.

In graph C is shown the effect of 6 day treatment with the medium containing the drug added successively on days 6, 8, and 10. No significant enhancement occurred. Instead there is evidence of inhibition after day 12, as if too prolonged treatment with actinomycin produced cumulative toxic effects.

Fig. 6. The enhancement of the secondary anti-tetanus toxoid (anti-TT) response in vitro by actinomycin D (AD) added for 2 days at different times. The medium of each culture was replaced every 2 days. The reference response represents the average response of eight cultures not treated with the drug, whereas the other responses represent the average response of four cultures similarly treated. The reference curve has been transposed as a gray background to the other graphs in this figure. The 2 day period of actinomycin treatment coincides with the box at the top of the other graphs; the number beneath each box denotes the day treatment began. The open boxes designate 2 day treatment which yielded no enhancement. The X-marked boxes indicate which responses were enhanced by the 2 day treatment.

In graph D (Fig. 5) is shown the response of cultures treated once with actinomycin D for 2 days, starting on day 8. A striking enhancement occurred during the 2 day treatment period and continued during the following 2 days, reaching a peak average response more than double that of the control group.

In graph E (Fig. 5) is shown the effect of a 6 day treatment with medium containing the drug added on days 8, 10, and 12. Enhancement occurred during the first 2 day treatment period; the response reached a plateau and then fell sharply, again giving the impression that too prolonged treatment with the drug caused a cumulative toxic effect. Not shown in Fig. 6, but also included in
the experiment, was the effect of treatment with actinomycin for 2 days and for 6 days starting on day 10. The responses closely duplicated those shown in Fig. 5 D and E.

The period of optimal enhancement by actinomycin D was explored more thoroughly in the experiment shown in Fig. 6. In these cultures the media were replaced every 2 days. The reference response drawn in the upper left-hand graph depicts the average response of eight cultures not treated with actinomycin D. The other graphs show the average responses of quadruplicate cultures treated at different times for 2 days with 0.01 \( \mu \text{M} \) (0.008 \( \mu \text{g/ml} \)) actinomycin D. The gray shaded area depicting the reference response has been transposed to the graphed responses of each actinomycin-treated group. Before introduction of the drug, the average response of each treated group was very similar to the average response of the untreated reference group. 2 day treatment with actinomycin D starting on day 0, 2, or 4 produced no apparent enhancement or inhibition. Nor was any temporally related enhancement produced by treatment starting on day 16 (the lower right-hand graph). However, treatment for those 2 day periods marked with a \( \times \) in the box at the top of each graph—that is, starting on day 6, 8, 10, 12, or 14—produced an enhancement in each group related precisely to the time when the drug was added to the cultures of that group.

Several experiments already described suggested that a cumulative toxic effect is produced by too prolonged treatment with actinomycin D. Fig. 7 illustrates this observation. The shaded areas in all graphs of this figure represent the average responses of eight untreated cultures. The other curves are average responses of quadruplicate cultures treated with different levels of actinomycin D for 2 or 10 days, starting on day 8. In each graph the drug level tested is listed, and the duration of treatment is indicated by the boxes at the top. The media of these cultures were replaced daily, so that actinomycin D was reintroduced into the culture a second time during the 2 day treatment schedule (top row of graphs) and nine times during the 10 day treatment schedule (bottom row of graphs).

All levels of actinomycin D tested (0.0025–0.01 \( \mu \text{M} \)) produced a similar initial enhancement, with the peak responses being about double that of the untreated group. In all treated groups enhancement continued for 3 days, either as a continually rising response or as an initial high rise with a narrow plateau. The responses then abruptly fell. The terminal descending curves of most groups treated for only 2 days are superimposable on the descending limb of the reference response, while the descending curves of those cultures treated for 10 days with levels above 0.025 \( \mu \text{M} \) soon fall below the corresponding points of the reference response. Thus, in this experiment (as in Fig. 5), continuous treatment with actinomycin D starting on day 8 produced an initial enhancement followed by inhibition.
Fig. 7. Enhancement of the secondary anti-tetanus toxoid (anti-TT) response in vitro by actinomycin D treatment for 2 day vs. 10 day intervals. The reference response was derived from eight untreated cultures; each of the other curves represents the average response of quadruplicate cultures. The reference curve has been transposed as a gray background to the other graphs in this figure. The bar above each graph indicates by its length the duration of treatment, and by its height the concentration of actinomycin used. The latter is also listed on each graph. The media were replaced every day. To facilitate comparison of different curves, the responses on days 8, 12, and 16 are drawn as solid circles.
This is the only experiment of several dozen in which enhancement was produced with a level of actinomycin D as high as 0.01 μg. In contrast are several other experiments which showed no enhancement of antibody synthesis, even when a wide range of actinomycin D levels was used. This variability encountered in different experiments is probably related to the capacity of actinomycin D to bind nonspecifically to glass and specifically to cellular breakdown products, such as effete chromatin and DNA. 14 rabbit lymph node fragments release over 1 million cells into the medium in the first 3 days of culture, half that number in the next 3 days, and proportionately smaller numbers in successive 3 day intervals. The fraction of these cells which die and release nuclear breakdown products doubtless varies from experiment to experiment. Therefore it is not surprising that experiments occasionally fail to show enhancement, since usually successful low levels of actinomycin D may be reduced to an ineffectual level by such extraneous binding.

Recovery of an Inhibitor of the Immune Response from Tissue Culture Fluids.—Several hypotheses have been offered to explain the paradoxical enhancement of protein synthesis by low levels of actinomycin D (see Discussion). One in particular was pursued with the present culture system. This was the hypothesis by Garren et al. (6) of a "repressor" which would affect translation and whose formation would be inhibited preferentially by actinomycin D. Since actinomycin D produced enhancement in the present experiments only during the middle of the secondary response (see Fig. 6), an inhibitor was sought in medium removed from cultures during this middle period of the response. The assay system employed was an in vitro response to a different antigen. Two controls for this experiment were pools removed during the early period of the response and also during the late period of the response. Fig. 8 depicts the scheme and results of this experiment.

Media were saved from 52 tubes of an anti-BSA2 experiment, the secondary response of which is shown in graph A of Fig. 8. As soon as the culture medium had been removed from all cultures on a given day, it was pooled, centrifuged to remove suspended cells lost from the organ cultures, and frozen. At the end of this experiment, the media removed on days 3 and 6 were thawed, pooled together, concentrated by dialysis against sucrose, dialyzed against a balanced salt solution, and incorporated into serum-free culture medium. The same procedure was followed for pools of media removed on days 9 and 12, and also for those removed on days 15 and 18. These three 6 day pools were designated the "early," "mid," and "late" pools. The concentration of the nondialyzable material of these pools after incorporation into each of the three new media was 5.5-fold; that is, 5.5 times that contained in the original used medium pools. The anti-BSA in these pools did not cross-react with the assay system,

2 Abbreviation used in paper: AIM, antibody-inhibitory material; BSA, bovine serum albumin; R-RBC, rat erythrocytes; S-RBC, sheep erythrocytes.
which involved cultures producing an anti-tetanus response. The reference response for these cultures is shown in graph B (Fig. 8); the 21 day antibody synthesis was taken as the 100% response in this assay part of the experiment. Medium containing the early anti-BSA pool was added to cultures shown in graph C for the first 12 days, i.e. on days 0, 3, 6, and 9. No over-all inhibition or enhancement occurred, and the 21 day response was 96% of the reference response. The medium containing the mid-anti-BSA pool was included during the same 12 day interval in cultures whose average response is shown in graph

**Fig. 8. Recovery of an inhibitor of antibody synthesis from “used”, culture medium (see the text). TT is tetanus toxoid.**

D. Here the 21 day response was depressed to 49% of the reference response. Finally, medium containing the late anti-BSA pool was added to cultures whose average response is shown in graph E. There was no effect on the over-all response of these cultures, which yielded 96% of the reference response. Thus the mid-anti-BSA pool contained a nondialyzable substance which was inhibitory to the anti-tetanus toxoid response. The anti-BSA titer was higher in the late pool, which makes it unlikely that the nondialyzable inhibitor found in the mid pool was anti-BSA globulin.

Similar levels of inhibition have been obtained with mid-pool concentrates prepared from both anti-ovalbumin response in vitro and also other anti-BSA responses in vitro. No inhibitory effect has been found using media of cultures
prepared from a primed rabbit which was not stimulated in vitro to give a secondary response. No inhibitory effect was recovered in media from spleen cultures stimulated to give a secondary response, but this failure may be related to the poor response produced by these cultures in vitro. Inhibitory concentrates from mid-anti-BSA pools have also been prepared by lyophilization and by pressure dialysis through ultrafine membranes. The success of these preparations eliminates the possibility that the inhibitory effect resulted from some reaction during dialysis occurring between the culture fluid and the sucrose or a contaminant of it (e.g. ribonuclease).

In another experiment, complete inhibition was produced within 24 hr by a mid-pool concentrate (Fig. 9). Early and late pools from an anti-BSA experiment were combined and concentrated in an ultrafiltration cell (Amicon Corp., Lexington, Mass.) with a UM-2 Diaflo membrane, a synthetic polymeric membrane which retains molecules larger than 1000 mol wt. The mid-pool from the

![Graph](image-url)
same anti-BSA experiment was similarly concentrated. Both concentrates were incorporated into different aliquots of medium, so that the nondialyzable components of each pool were present at a 10-fold concentration. The response of cultures producing tetanus toxoid responses are recorded in Fig. 9 for the 5 day period from day 10 to day 15; the medium of these cultures was replaced every 12 hr. The response of the control group of cultures is shown in graph A of Fig. 9. During this test period average titers between 1:600 and 1:1000 were produced every 12 hr. The cultures of graph B were treated with medium containing the early-late pool concentrate on day 12b and again on day 13a; there was only a gradual, slight inhibition produced by this 24 hr exposure. However, as seen in graph C, treatment of cultures with medium containing the mid-pool concentrate for the same two 12 hr intervals caused prompt inhibition, and the response was completely abolished within 24 hr after initiation of treatment with the mid-pool concentrate. In the same experiment, medium containing the mid-pool at only twice the effective concentration produced a more gradual inhibition of the response.

Rapid Enhancement of Antibody Production by Actinomycin D and Rapid Inhibition by Mid-pool Concentrates.—Although the search for an inhibitor in mid-pools was prompted by the enhancement of antibody synthesis by actinomycin during the second week of the response, no direct evidence relating the enhancement by actinomycin D with inhibition by mid-pool concentrates has been obtained. However, several experiments have shown a rise or fall in antibody synthesis within several hours of the addition of the drug or the concentrate, respectively, indicating a similar rapidity of action for these two opposite net effects. One such experiment is illustrated in Fig. 10. During the productive phase of this experiment, the medium in each tube was replaced every 2 hr; the period graphed in Fig. 10 covers only a 30 hr period during days 10 and 11. The five cultures whose average response is shown in graph A received fresh medium with 0.0025 μg actinomycin D five times during a 10 hr period, indicated by the rectangle at the top of the graph. Within 2 hr the response showed enhancement, and within 6 hr more it reached a peak which was more than twice the pretreatment base line level. The response then began to fall despite another 2 hr of drug treatment, reached the base line level, and remained there for the next 12 hr.

In this same experiment, and during the same 10 hr period, medium containing an early anti-BSA pool was added to three cultures, whose average response is drawn in graph B (Fig. 10). As in previous experiments, there was no effect on the base line response with this “early” medium. The three cultures

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1 The three concentrates tested in this experiment were pooled from the same anti-BSA experiment in the manner described above, concentrated by lyophilization, and then exhaustively dialyzed against normal saline. These pools were finally incorporated into new medium at a concentration of 4-fold.
Fig. 10. The precipitancy of enhancement by actinomycin D and of inhibition by a mid-pool concentrate. The media of anti-tetanus toxoid (anti-TT) cultures were replaced every 2 hr during the early productive phase, and their responses during days 10 and 11 are shown in this figure. During a 10 hr period, which entailed five medium changes, cultures were treated with actinomycin D (AD) or with one of the three anti-BSA pools. This treatment interval is represented by the bar above each graph; the treatment is indicated by the data inside each bar. See the text.
whose response is shown in graph C received medium containing the mid-anti-BSA pool concentrate during the same 10 hr period. A sharp fall in the response occurred within 4 hr; the response eventually fell to less than 50% of the base line and remained at this reduced level for the next 14 hr. Finally, the three cultures of graph D received medium containing the late anti-BSA pool concentrate during the same 10 hr period. This concentrate caused only moderate and gradual inhibition, and the response returned eventually to the base line.

**DISCUSSION**

Enhancement of protein synthesis by actinomycin D is a phenomenon so contrary to the initially expected effect that an isolated report is often met with skepticism. However, this paradox has now been described in so many different areas of biology that collectively the papers cannot be ignored. Indeed, the biological systems exhibiting such enhancement are so varied that the phenomenon appears to be a general one, as illustrated by the following abbreviated survey.

In *Bacillus subtilis* low concentrations of actinomycin D were found to double the rate of induced penicillinase formation (7) and to increase ribonuclease synthesis by 80% (8). In rats given corticosteroid injections, the levels of several liver enzymes rise after a few hours, reach a plateau, and then fall to base line values 12 hr or so later (9). In such rats Garren et al. (6) observed that actinomycin D injected several hours after the steroid increased tryptophan pyrrolase activity markedly above that in rats given only the steroid. Similarly, rats treated with phenobarbital by injection show an increased activity in their livers for aminopyrine demethylation; in such rats this activity is enhanced by actinomycin D treatment (10). There are now numerous examples of enzyme levels rising in animals following actinomycin D injection (11, 12) and in tissue cultures after addition of low levels of this drug (13–16). Even in the immunological literature there is a surprising number of references to this phenomenon.

Brown (17) and Wust and Hanna (18, 19) studied the hemagglutinin response of mice treated by injection with sheep erythrocytes. Decreased titers were produced when actinomycin was given after the sheep cell injection, but enhanced titers resulted under certain conditions of administration of the drug before the antigen. Brown observed enhancement when about 150 μg actinomycin C was given 72 hr before 0.2 ml 5% sheep cells. Wust and Hanna obtained enhancement when 12 μg actinomycin D was injected 7 hr before 1.0 ml 10% sheep cells. Speirs (20) also recorded similar increased antitoxin titers in mice given weekly injections of tetanus toxoid plus 2.5 μg actinomycin D; higher doses of the drug were inhibitory. Both Smiley et al. (21) and Stavitsky and Gusdon (22) reported that antibody synthesis in rabbit lymph node cultures was occasionally enhanced by low levels of actinomycin D in the medium but was, of course, always depressed by higher levels.
A perplexing paper by Harris (23) involves plaque-forming cells in spleen cultures prepared from primed rabbits boosted with sheep erythrocytes 2-3 days before death. High levels of actinomycin D (10-100 μg/1.5 ml) or puromycin (50 μg/1.5 ml) added to the culture medium along with sheep erythrocytes produced greatly increased plaque counts compared to controls cultured without either drug or without the sheep cell antigen. "The optimal concentration of actinomycin D which stimulated the appearance of specific PFC's [plaque-forming cells] in vitro was much higher than the level of the antibiotic required ... to inhibit DNA, RNA, and protein synthesis" (p. 680 of ref. 23). This and other features of Harris' study suggest to us that the increased plaque counts may have resulted from (a) weakening of the spleen cell membranes by the high levels of either drug, (b) lysis of these cells in the Jerne plates, and (c) release of preformed antibody.

The common explanations of enhancement of protein synthesis by actinomycin so far proposed will be reviewed in detail elsewhere. Briefly they suggest (a) a diversion of intracellular substrates from the synthetic pathway of these proteins selectively inhibited to the polysomes of those not inhibited by critical levels of this drug (7), (b) a selective inhibition by actinomycin of certain degradative enzymes involved in protein turnover (15, 24, 25), or (c) a selective inhibition of a normally occurring "cytoplasmic repressor" affecting translation (6, 26, 27). The enhancement in antibody synthesis observed in the lymph node cultures treated with low levels of actinomycin in the present study, coupled with the recovery of an antibody-inhibitory material from culture media, favors the third theory. Indeed, the paper by Garren et al. (6) prompted our search for an inhibitor.

Translational "Repressor" Theory.—This explanation for actinomycin D enhancement was originally proposed by McAuslan in 1963 (26), and independently soon afterward by two other groups of investigators (6, 27). Poxvirus infection of HeLa cells causes a marked increase in thymidine kinase activity for 6 hr; treatment of these cultures 2-4 hr after infection with actinomycin D allows kinase synthesis to continue for 18 hr. McAuslan concluded that "repression" of thymidine kinase synthesis normally occurs 6 hr after virus infection, and that actinomycin D prevents this "repression." Similarly, in 1964, Garren et al. (6) proposed that several hours after steroid-induced enzyme synthesis begins in rat liver there appears a "repressor" which prevents further enzyme synthesis. This "repressor" acts by inhibiting the function of the existing mRNA, has a rapid turnover rate, and is itself very sensitive to actinomycin D. Tomkins and coworkers (28) have since duplicated these studies in a hepatoma-culture line and extended their conclusions. Also, in 1964 Scarano et al. (27) postulated inhibition by actinomycin of a "specific repressor" to explain the...
following observation. During embryonic development of the sea urchin there normally occurs a fall in the level of deoxyctydylate aminohydrolase, an enzyme involved in DNA synthesis. Actinomycin D treatment not only prevents this fall but increases the enzyme level above that in the unfertilized egg.

The data presented above raise many questions, but only two can be discussed at any length in this paper: (a) What is the correlation between antibody synthesis augmented by actinomycin and the inhibitory material recovered from used culture medium? (b) What relationship does this antibody-inhibitory material have to certain other factors thought to regulate the immune response?

**Antibody-Inhibitory Material.**—This inhibitory material can be related to the postulated “repressor” substance of these three groups of investigators primarily because the period of AIM production in our cultures (days 6-12, or the “mid-pool” period) coincides with the period of potential actinomycin D enhancement (days 6–15). Also lymph node cultures stimulated to produce a secondary response in vitro yield a potent inhibitory mid-pool concentrate, while other cultures prepared from the same source of fragments, but not stimulated in vitro, produce no antibody and yield no AIM. Thus AIM is produced as a consequence of and concurrent with the immune response. AIM is not, therefore, a property of all used culture medium. Finally, in kinetic experiments enhanced antibody synthesis appears within 2 hr, of adding actinomycin D, while inhibition is evident within 4 hr of adding a potent mid-pool concentrate (see Fig. 10). Since AIM is a macromolecule, it may enter cells more slowly than does actinomycin D (mol wt 1254). Thus these two opposite manifestations develop with a similar speed, which is at least consistent with their representing the same phenomenon.

From these slim pieces of information we can begin to characterize AIM. The molecular weight of the inhibitor is above 10,000, since it is retained by dialysis tubing (29). The inhibitor is not specific antibody, since mid-pool concentrates from either anti-BSA or anti-ovalbumin responses inhibit antitetanus and anti-diphtheria response. The inhibitor is probably not a γ-globulin, since there are high titers of this protein in the late pool concentrates, which produce little or no inhibition (see Fig. 8 A and E). Further chemical characterization has been delayed for want of an assay system less consumptive of AIM than the one depicted in Fig. 8. A more economical assay appears possible with the present observation that prompt inhibition is produced when AIM is added for only a short time during the productive phase (Fig. 10).

This preliminary information raises several obvious questions about AIM. We have yet to determine whether or not it is active during the inductive phase. In the earliest experiments (exemplified by Fig. 8 D), AIM was present throughout days 0-12, which includes the early productive phase in vitro. Also nothing is known about the stability of AIM in culture, an important consideration if it is to be used in attempts to suppress the response during the week-long
inductive phase. An even more important question concerns the apparent lack of specificity of AIM, as concluded from the observation that mid-pool concentrates of either anti-BSA or anti-ovalbumin experiments inhibit both anti-tetanus toxoid and anti-diphtheria toxoid responses. Two reports in the immunological literature support the existence of such a nonspecific antibody-inhibiting material produced during the immune response. These are reports dealing primarily with antigenic competition.

Other Postulated Mechanisms Controlling the Immune Response: Antigenic Competition.—Wust and Hanna (18, 19) studied the impact of actinomycin D on the antibody response in mice treated by successive injections with two "immunologically unrelated" antigens: sheep erythrocytes and rat erythrocytes. Injection of either antigen alone in control mice produced comparable hemagglutinin responses, first detectable after a latent period of 1–2 days. Their observations germane to our discussion are summarized below.

1. Mice treated with actinomycin D 7 hr before injection of sheep erythrocytes showed (a) a 3-day latent period, (b) antibody response rates "comparable" to those of control mice, but (c) "significantly higher" peak titers than those of control mice. Having observed that large pyronin-staining cells in the spleen survived the drug’s cytotoxic effect, Wust and Hanna speculated that these cells were "stimulated by residual antigen" and rapidly repopulated the splenic nodules "because of increased volume ... available after clearance of cell debris."

2. Mice treated with actinomycin D 7 hr before injection of rat erythrocytes showed (a) a latent period of 6–7 days, (b) normal rates of synthesis, but (c) no peak titers above those of control mice. The differences between observations 2 and 1 were thought due to rat erythrocytes' being antigenically less foreign for mice than are sheep erythrocytes.

3. Injection of sheep erythrocytes followed 2 days later by rat erythrocytes resulted in a normal anti-S-RBC response but no anti-R-RBC response. Delaying the R-RBC injection until 6 days after the S-RBC stimulation allowed a subnormal anti-R-RBC response, and delaying it until 10 days permitted a normal anti-R-RBC response. Wust and Hanna argued that the anti-S-RBC response produced a 6 day refractory period for the R-RBC because the first antigen "committed most of the potentially competent cells" and pre-empted a response to the second antigen over the next 6 days.

4. In contrast to observation 3, when the reciprocal sequence of injections was tested, there was a normal response to the second antigen (S-RBC). Presumably, the less foreign R-RBC injected first did not completely commit all the potentially competent cells.

5. In mice treated with successive injections of actinomycin D, S-RBC 7 hours later, and R-RBC 2 days later, there were normal responses to both antigens. Thus the refractory period expected for the second antigen was eliminated.
by prior treatment with actinomycin D. Wust and Hanna reasoned that the drug interfered with the cells' becoming "totally committed to the first antigen."

The concept of AIM can provide an alternative single explanation for Wust and Hanna's observations enumerated above. In mice treated with actinomycin D 7 hr before antigen stimulation, the delay in the response obviously involves several factors: the cytotoxicity of the drug, its initially high but soon falling intracellular level, the recovery rate of the immunocompetent cells, and the antigen's relative immunogenicity. The enhanced anti-S-RBC response following treatment with actinomycin D (see observation 1) resulted from a fortuitously optimal residual level of actinomycin D in the cells at the time AIM synthesis would have begun. In the mice treated with actinomycin D and R-RBC (see observation 2), the response was not enhanced because it developed 3–4 days later than in observation 1 and too few molecules of the drug were present in the anti-R-RBC responding cells at the time of their AIM synthesis. Since AIM is secreted during the early productive phase into tissue culture medium, it must also be released into splenic nodules in vivo. Thus the refractory period for R-RBC described under observation 3 reflected production of AIM by those cells responding to the first antigen injected, S-RBC. In the reciprocal injection sequence (see observation 4), the slightly slower anti-R-RBC response did not produce enough AIM soon enough to impede the second response to the more immunogenic S-RBC. In mice treated sequentially with actinomycin D, S-RBC, and R-RBC (see observation 5), the drug reduced the synthesis of AIM during the anti-S-RBC response; this had the dual effect of augmenting the anti-S-RBC response and permitting an otherwise inhibited anti-R-RBC response.

In a clearly reasoned paper Radovich and Talmage (30) examined antigenic competition between sheep and horse erythrocytes injected sequentially into mice. They assayed for hemolytic plaque-forming cells in mouse spleen by the method of Jerne and Nordin. In their normal mice the spleen plaque count reached a peak 4–5 days after injection of either antigen and then rapidly declined. Simultaneous injection of both antigens caused "no significant inhibition of either response," but the response to the second antigen was reduced when it was injected at various times after the first antigen. Maximal reduction (13-fold) occurred when an interval of 4 days separated the two injections. The authors doubted "that the phenomenon of antigenic competition is in fact competition for anything." For this to be so they would have expected maximal competition when the two antigens were injected simultaneously. In adoptive transfer experiments they found a greater reduction in the second antigen's response when 50 x 10⁶ spleen cells were transferred to sublethally irradiated recipient mice than when only 10 x 10⁶ cells were injected. From these data they hypothesized that a humoral factor was produced during the first response and that this factor acted "as a feedback repressor of the response to the second..."
antigen.” This is a clear statement of the “repressor” hypothesis, which our data support.

Cell-Extracted Inhibitors of Antibody Synthesis.—The immune response has been depressed in animals given extracts of various organs by injection (31), isolated serum fractions (e.g. bovine \( \gamma \)-globulin) (32), and chemically derived ribonuclease complexes (33). It is doubtful that the substances listed above are identical with or similar to the inhibitory material found in our tissue culture media (AIM), since these substances are inhibitory only when injected into a test animal 10–24 hr before the antigen. In contrast, AIM is clearly inhibitory when added to cultures during the early productive phase. Because these various protein substances must precede the antigen, some authors (34) have speculated that their inhibitory effect may be due partly to antigenic competition. If this is true, these substances are indirectly related to AIM by virtue of eliciting its production.

Another reason for doubting an identity or similarity to AIM is that these substances were not derived from immunized animals. Thompson and Fishel (31) prepared their extracts from spleen and thymus but did not compare extracts from normal and immune animals. Such a comparison was made by Gurvich et al. (35), who obtained extracts from several rabbit organs: normal spleen and liver and immunized spleen. The extracts were added to suspension cultures of rabbit spleen cells in the early productive phase. Incorporation of labeled amino acids into newly synthesized antibody was depressed by the liver and normal spleen extracts but was slightly stimulated by the spleen extracts from immunized rabbits. The observations of Gurvich et al. involve factors different from those under study in this report, since even normal liver extracts inhibited their antibody system. In contrast, the present investigation has shown production of an inhibitor during only a certain period of the immune response, and never with nonresponding lymph node cultures.

Interferon and Antibody Synthesis.—In an article entitled “The Effect of Newcastle Disease Virus on Antibody Synthesis,” Medzon and Vas (36) reported that this virus not only inhibited antibody formation in immune rabbit spleen cultures but also produced a general arrest of protein synthesis. The title of this paper in particular raised the question whether interferon could inhibit the immune response.

De Somer et al. (37) found that poliovirus-neutralizing antibody titers were reduced in rats or mice when Sindbis virus or \textit{Escherichia coli} was injected 24 hr before poliovirus, but not when either was given 48 hr after the poliovirus. The authors stated that Sindbis virus was a more potent interferon inducer than \textit{E. coli}, but that the virus was the less effective inhibitor. Indeed, small amounts of \textit{E. coli} insufficient to induce demonstrable interferon could still inhibit antibody synthesis. These data may well represent another instance of antigenic competition and thus, possibly, of AIM involvement. De Somer et al. were also
unsuccessful in depressing antibody synthesis by injecting exogenously produced interferon into animals. A similar set of negative experiments has been reported (38).

Mazzur and Paucker (39) preserved mouse spleen cells in cultures by incubating spleen suspensions on rabbit kidney feeder layers. During this incubation the cells could be exposed to interferon, then shaken off the feeder layer, and assayed for antibody production in one of three immune systems, depending on how the donor mice were first immunized—by the Jerne plaque method or by adoptive transfer of spleen cells from mice given sheep erythrocytes or Shigella dysenteriae. In none of the systems they tested did high levels of interferon (up to 600 units) exert any inhibitory effect.

Analogous experiments, using the present culture system, also exhibited no inhibitory effect by interferon. From Sindbis-infected rabbits sera were obtained which contained high titers of interferon; they failed to inhibit the productive phase of the secondary response in vitro.6

**Summary**

Two opposite effects of actinomycin D on antibody synthesis have been studied in organ cultures of rabbit lymph node fragments. These cultures were prepared from previously primed rabbits and stimulated with antigen(s) on day 0 to yield a secondary response, whose inductive phase extended to about day 9 and whose productive phase may last for several months in the serum-free medium described here.

Concentrations of actinomycin D above 0.01 μM (0.012 μg/ml) produce inhibition of antibody synthesis during both phases of the response. However, antibody synthesis is about 10 times more sensitive to inhibition by this drug when it is added during the inductive phase than during the productive phase. During the latter phase, synthesis is more rapidly terminated as the drug level approaches 10 μM (12.5 μg/ml). At this level the 50% synthesis time is about 2.8 hr, which is identical with that found when 5–10 μM puromycin is added to the medium of parallel cultures.

Transient enhancement of antibody synthesis is frequently produced by a brief exposure to low levels of actinomycin D (generally less than 0.01 μM). Enhancement appears in precise temporal association with actinomycin pulses added for 2 days or less only between days 6 and 16. This apparent enhancement of antibody synthesis resembles the increased enzyme synthesis described by Garren et al. (6) and led to a search for an antibody-inhibitory material (AIM) whose synthesis might be stopped preferentially by low levels of the drug.

Stimulated lymph node cultures produce between days 6 and 15 a nondialyz-

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able material which inhibits antibody synthesis during the productive phase of heterologous antigen-antibody culture systems. Just as enhancement with low levels of actinomycin D appears within 2 hr after the drug has been added to cultures, so inhibition occurs within 4 hr of adding AIM to cultures during their productive phase. These observations suggest that AIM is analogous to the translational "repressor" postulated by Garren et al. (6). AIM has relevance in two areas of immunology: (a) it may be the explanation for many examples of antigenic competition, and (b) it may represent a normal control mechanism for the productive phase.

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