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STUDIES ON ANTIBODY PRODUCTION

VIII. THE INHIBITORY EFFECT OF CHLORAMPHENICOL ON THE SYNTHESIS OF ANTIBODY IN TISSUE CULTURE*

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In 1953 Gale and Folkes (1) observed that chloramphenicol inhibited protein synthesis in *Staphylococcus aureus* but permitted substantial nucleic acid synthesis to continue. Subsequently, this drug has served as a powerful tool in studies of protein and nucleic acid metabolism in a wide variety of microbial systems. In sensitive bacterial cultures the addition of 30 to 100 $\mu\text{g}/\text{ml}$ (0.1–0.31 mM) of chloramphenicol results in prompt and nearly complete suppression of protein synthesis (1, 2), while concentrations as low as 2 $\mu\text{g}/\text{ml}$ (0.006 mM) produce 50 per cent inhibition in some bacteria (1). Very low concentrations also inhibit protein synthesis in cell-free bacterial systems (3–7). On the other hand, in mammalian cells these levels of drug have only infrequently been reported to have demonstrable metabolic effects (see Discussion). The pathway for protein synthesis appears to be quite similar in microbial and mammalian cells, but the discrepancy in their sensitivity to chloramphenicol remains a puzzle.

Antibody production *in vitro* became a practical system for studying protein synthesis by mammalian cells following the improvement of tissue culture methods and the development of the hemagglutination assay for antibody. This paper deals with the effect of low levels of chloramphenicol (5 to 50 $\mu\text{g}/\text{ml}$ or 0.015–0.15 mM) on antibody production in cultures of stimulated rabbit lymph node fragments. Such “bacteriostatic” levels of the drug have been found to cause marked suppression of the secondary response induced *in vitro*.

Materials and Methods

The general techniques employed in these experiments have been described by Michaelides and Coons (8); only slight modifications and specific details will be mentioned here. The

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primary immunization of the rabbit popliteal lymph nodes was produced by a single intracutaneous injection into both hind foot-pads of 0.1 ml saline containing 4.0 to 12.5 mg of bovine plasma albumin (BSA) and 0.23 to 0.48 mg (80 to 167 Lf) of diphtheria toxoid. 6 to 20 weeks later these nodes were excised and the fragments prepared from them were incubated with both antigens to stimulate the secondary response *in vitro*. For unstimulated controls a small number of fragments were incubated simultaneously under similar conditions but without the antigens. Twelve fragments were placed along the flat depression of a Leighton tube and held in position by an overlying thin pad of glass wool, which in early experiments was saturated with plasma. Each tube received 1 ml of medium, the composition of which has been described previously (8). Chloramphenicol and puromycin from stock solutions of 1.0 mg/ml were incorporated into the medium of appropriate tubes. Sufficient medium for an entire experiment was prepared, sterilized through Millipore filters (average pore size 0.45 μ), and stored in screw-capped tubes or bottles in the refrigerator. All cultures were incubated at 37°C; in initial experiments they were rotated in a 6 RPM roller drum; in later experiments, when plasma was omitted, they were kept stationary. The medium in all tubes was replaced every 3 days; the fluids removed were immediately frozen in alcohol-dry ice bath and stored for subsequent measurement of antibody titers to BSA and diphtheria toxoid.¹

The chloramphenicol was generously supplied by Mr. C. A. MacDonald of Parke, Davis and Co.

RESULTS

Effect of Continuous Treatment with Several Concentrations of Chloramphenicol.

—The secondary antibody response of immune rabbit lymph node cultures restimulated *in vitro* is shown in Fig. 1. (The average titers plotted in this figure were derived from the individual titers listed in Table I.) When medium did not contain chloramphenicol, cultures produced anti-BSA titers which reached a peak of 1:4800 ($y = 9.9$)² between the 9th and 12th days. When different

¹ Antibody titers were measured by the hemagglutination method with tanned sheep erythrocytes (Stavitsky's modification of the Boyden procedure (38)). During these titrations we occasionally noted false positive agglutinations in random tubes. The film of agglutinated cells on the bottom of these tubes had a very homogeneous appearance compared to the slightly granular character generally seen in sensitized cells agglutinated by antibody. This phenomenon was found in this laboratory by Mrs. Linda Santoro to be caused by trace amounts of saliva accidentally introduced into the tubes during pipetting. The salivas of 10 laboratory workers were all found to contain a factor which agglutinated tanned erythrocytes to titers of 1:640–1:5120. It agglutinated tanned cells sensitized with a variety of proteins and reacted with sheep, rabbit, and human cells similarly tanned and sensitized. These observations will be reported in detail elsewhere.

² Depiction of data: In each graph in the accompanying figures the abscissa indicates the duration of the culture in days, while the ordinate is a logarithmic function of the antibody titer. Each point on a graph represents the average titer of culture fluids removed simultaneously from several identically treated cultures. Each point was computed as the average of the reciprocal hemagglutination titers of the media removed the same day from all the cultures in the group. In order to plot these average titers, each value was converted into an exponential function of 2 by use of the following formula:

$$y = \log_2 \left(\frac{1}{5(\text{HA titer})} \right) = 3.322 \log_{10} \left(\frac{1}{5(\text{HA titer})} \right).$$

In this formula the range of individual titers obtained in these experiments—1:10, 1:20,

concentrations of chloramphenicol were maintained in medium during a period of 15 days, it was observed that increasing concentrations of the drug (5, 10, 20, 35, and 50 $\mu\text{g}/\text{ml}$) produced greater inhibition of the secondary response. The anti-BSA response was inhibited 95 per cent by 20 $\mu\text{g}/\text{ml}$ and over 99 per cent by 50 $\mu\text{g}/\text{ml}$.

These same cultures were also restimulated with diphtheria toxoid and produced antibodies to this antigen as well as to BSA. The peak anti-diphtheria toxoid titers appeared around the 9th day and averaged 1:600 ($y = 6.9$). The anti-diphtheria toxoid response in most experiments was lower than the simultaneous anti-BSA and in this particular experiment was suppressed 99 per

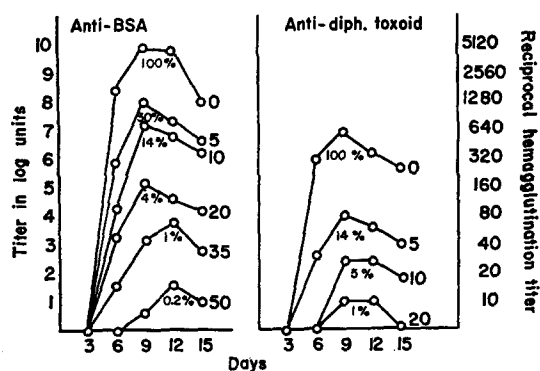


FIG. 1. Inhibition of the *in vitro* secondary response by various concentrations of chloramphenicol (5 to 50 $\mu\text{g}/\text{ml}$ medium). Each per cent value represents the average over-all response of a group of 4 similarly treated cultures compared with the corresponding response of the untreated control (100 per cent) group. The titers of individual tubes are listed in Table II.

cent by only 20 $\mu\text{g}/\text{ml}$ of chloramphenicol in the medium. In subsequent experiments described in this paper chloramphenicol was used at a concentration of 50 $\mu\text{g}/\text{ml}$ (0.15 mM).

Effect of Treatment with Chloramphenicol for Various Intervals.—Šterzl (9) reported that antibody production was reduced by 6-mercaptopurine administered during the "inductive" phase but was not impaired by the drug given anytime during the "productive" phase. This observation suggested a similar study to determine whether chloramphenicol preferentially affects either of these two phases in the secondary response stimulated *in vitro*.

Cultures were treated with 50 $\mu\text{g}/\text{ml}$ of chloramphenicol starting at different times and continuing for various intervals. The different periods of exposure are

1:40, . . . 1:10,240—is represented by a series of whole numbers: 1, 2, 3, . . . 11, respectively. The factor 5 in the denominator of the formula is necessary to compensate for starting with a dilution of 1:10 instead of 1:2 in the twofold dilutions. A table of logarithms to the base 2 has been published by Finney, Hazlewood, and Smith (39).

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indicated by the stippled area on each graph of Fig. 2. These graphs were derived from 3 experiments (Experiments 2, 4, and 5) and are aligned under columns designated *A* through *F*, which coincide with the different periods of exposure.

The average response of the untreated cultures in each experiment is taken

TABLE I
Inhibition of the Secondary Response by Various Concentrations of Chloramphenicol

| Tube No. | Ag* | Chloramphenicol μg/ml | Anti-BSA | | | | | Anti-diphtheria toxoid | | | | |
|----------|-----|--------------------------|----------|--------|--------|---------|----------|------------------------|--------|--------|---------|----------|
| | | | 0 to 3 | 3 to 6 | 6 to 9 | 9 to 12 | 12 to 15 | 0 to 3 | 3 to 6 | 6 to 9 | 9 to 12 | 12 to 15 |
| 2 | 0 | 0 | 40 | 80 | 640 | 1,280 | 320 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 10 | 80 | 640 | 320 | 160 | 0 | 0 | 0 | 0 | 0 |
| 3 | + | 0 | 0 | 2,560 | 5,120 | 5,120 | 1,280 | 0 | 320 | 1,280 | 640 | 310 |
| 6 | + | 0 | 0 | 2,560 | 10,240 | 5,120 | 1,280 | 0 | 640 | 320 | 320 | 160 |
| 4 | + | 0 | 10 | 1,280 | 2,560 | 5,120 | 1,280 | 0 | 160 | 640 | 320 | 320 |
| 5 | + | 0 | 0 | 640 | 1,280 | 2,560 | 1,280 | 0 | 80 | 160 | 160 | 160 |
| 8 | + | 5 | 0 | 640 | 1,280 | 1,280 | 640 | 0 | 40 | 80 | 80 | 40 |
| 10 | + | 5 | 0 | 320 | 1,280 | 640 | 320 | 0 | 40 | 80 | 40 | 40 |
| 7 | + | 5 | 0 | 160 | 1,280 | 640 | 640 | 0 | 20 | 80 | 40 | 40 |
| 9 | + | 5 | 0 | 80 | 1,280 | 640 | 320 | 0 | 20 | 80 | 80 | 40 |
| 14 | + | 10 | 0 | 160 | 1,280 | 640 | 320 | 0 | 10 | 40 | 40 | 40 |
| 12 | + | 10 | 0 | 80 | 640 | 640 | 640 | 0 | 0 | 20 | 20 | 10 |
| 11 | + | 10 | 0 | 80 | 640 | 640 | 320 | 0 | 0 | 40 | 40 | 20 |
| 13 | + | 10 | 0 | 80 | 320 | 320 | 160 | 0 | 0 | 10 | 10 | 0 |
| 17 | + | 20 | 10 | 80 | 320 | 160 | 160 | 0 | 0 | 20 | 20 | 20 |
| 15 | + | 20 | 0 | 40 | 160 | 160 | 80 | 0 | 0 | 0 | 10 | 0 |
| 16 | + | 20 | 0 | 40 | 160 | 80 | 80 | 0 | 0 | 10 | 0 | 0 |
| 18 | + | 20 | 0 | 40 | 80 | 80 | 40 | 0 | 0 | 10 | 10 | 0 |
| 22 | + | 35 | 0 | 20 | 80 | 80 | 40 | 0 | 0 | 0 | 0 | 0 |
| 19 | + | 35 | 10 | 20 | 40 | 80 | 40 | 0 | 0 | 0 | 0 | 0 |
| 20 | + | 35 | 0 | 10 | 40 | 80 | 40 | 0 | 0 | 0 | 0 | 0 |
| 21 | + | 35 | 0 | 10 | 20 | 40 | 20 | 0 | 0 | 0 | 0 | 0 |
| 24 | + | 50 | 0 | 0 | 10 | 20 | 20 | 0 | 0 | 0 | 0 | 0 |
| 25 | + | 50 | 0 | 0 | 10 | 20 | 10 | 0 | 0 | 0 | 0 | 0 |
| 26 | + | 50 | 0 | 0 | 10 | 20 | 10 | 0 | 0 | 0 | 0 | 0 |
| 23 | + | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Reciprocal anti-BSA and anti-diphtheria toxoid titers from Experiment 97. Rabbit 21-97 received a primary injection in each hind foot-pad of 12.5 mg BSA and 0.5 mg diphtheria toxoid on August 24, 1961. At the time of sacrifice 20 weeks later on January 11, 1962, the rabbit's circulating titer for BSA was 1/320 and for diphtheria toxoid was 1/20.

* Fragments soaked in antigen solution. See text.

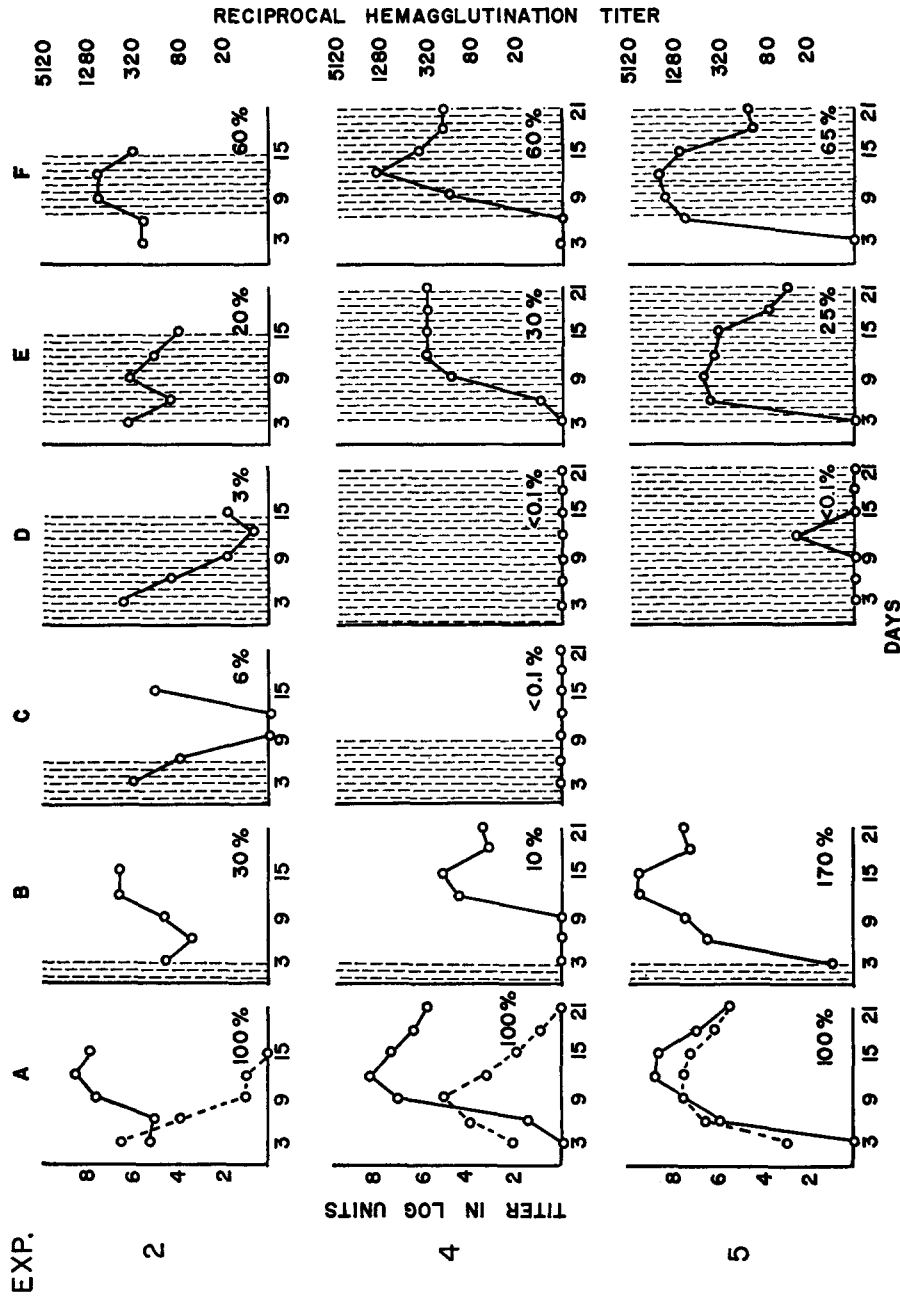


FIG. 2. Effect on the *in vitro* secondary response (anti-BSA) of cultures treated with 50 μ g/ml chloramphenicol during various intervals as indicated by the stippled areas in each graph. Each per cent value represents the average over-all response of a group of similarly treated cultures compared with the corresponding response of the untreated control (100 per cent) group of the same experiment. (See text.)

as the 100 per cent reference standard for calculating the effect of the drug shown in other graphs of the same experiment. The responses depicted by the dotted lines in these graphs are of control cultures not intentionally restimulated with BSA on day 0. In Experiment 2 there was a waning primary response. In the other two experiments the moderate secondary response indicated by the dotted lines was probably due to traces of sequestered BSA released from the capsule when the nodes were cut into fragments (10).

Under column *D* are shown the responses of cultures exposed to chloramphenicol throughout the three experiments. If antibody produced by the waning primary response in experiment 2 is taken into consideration, then it is apparent that 50 $\mu\text{g}/\text{ml}$ of the drug caused virtually complete suppression of the secondary response in these three experiments.

The graphs under columns *B* and *C* reveal that increasingly longer periods of exposure, beginning on Day 0, resulted in ever more complete suppression of antibody production. In Experiment 5 there was no inhibition of the response by an initial 3 day exposure (Graph 5*B*), or by one for the first 2 hours during restimulation, or for the first day of culture (results not shown in Fig. 2 but included in Table II). In other experiments (*e.g.*, Graphs 2*B* and 4*B*), exposure for the first 3 days caused an initial suppression of antibody production; however, antibody production began again 3 to 6 days after removal of chloramphenicol from the medium. More prolonged exposure for the first 6 or 9 days (Graphs 2*C* and 4*C*) suppressed the secondary response for a longer period, but even under these conditions a subsequent rise in antibody titer occurred in several experiments (Table II).

When exposure to chloramphenicol was delayed until day 3 or later and then continued until the end of an experiment, there was progressively less suppression of the secondary response the later the drug was added. Typical responses are shown under columns *E* and *F*.

Table II summarizes the data obtained in 4 experiments and permits the following conclusions: (*a*) The addition of chloramphenicol on day 0 produced greater suppression of the secondary response the longer it was present. (*b*) Addition of chloramphenicol at various intervals after day 0 produced progressively less complete suppression of the secondary response the later the drug was added.

Evidence for de Novo Synthesis and Rapid Secretion of Antibody during Culture.—In order to interpret the preceding data, it is essential to determine the origin of the antibody measured. For example, antibody appearing in the culture fluids might have been synthesized prior to excision of the node and merely secreted during incubation. On the other hand, *de novo* synthesis might account for all or most of this antibody. If the latter origin is correct, some estimate of the kinetics of this synthesis is needed in terms of whether a significant interval separates the actual synthesis *in vitro* and the eventual secretion into

the medium. The experiments which follow have led to the conclusion that most of the antibody which accumulates in freshly added medium during a 3 day incubation is synthesized and secreted during that period.

In 3 experiments cultures were prepared with high initial antibody titers but under conditions which precluded subsequent antibody formation. The replacement of medium every few days for a week or more resulted in a steady and reproducible fall in antibody titer in all the cultures. In the first experiment an initially high antibody titer was obtained by culturing non-immune lymph node

TABLE II
Effect of Chloramphenicol, 50 µg/ml, on the Secondary Response

| Period of chloramphenicol treatment | Antibody production from day 3 to the end of each experiment (expressed as the per cent of the response in the untreated control cultures during the same period) | | | |
|---|---|--------|--------|--------|
| | Exp. 2 | Exp. 3 | Exp. 4 | Exp. 5 |
| None. (untreated controls)..... | 100 | 100 | 100 | 100 |
| 2 hrs. (during antigen stimulation) | — | — | — | 140* |
| Day 0 to 1 (first 21 hrs.)..... | — | — | — | 260* |
| Day 0 to 3..... | 27 | 40 | 10 | 170* |
| Day 0 to 6..... | 6 | 21 | 17 | — |
| Day 0 to 9..... | — | 25 | <0.1 | — |
| Day 0 to end of each experiment.. | 3 | <0.1 | <0.1 | <0.1 |
| Day 3 to end..... | 17 | 26 | 32 | 27 |
| Day 6 to end..... | 62 | 64 | 57 | 65 |
| Day 9 to end..... | 58 | 56 | 21 | — |
| Day 12 to end..... | — | — | 94 | — |
| Per cent of antibody produced in untreated cultures after day 6... | 100 | 55 | 100 | 95 |

* We have no explanation at the present time for these values which are higher than the average response of the untreated control cultures.

fragments for the first 3 days in medium containing antibody; the rate of dilution of the original medium with fresh medium free of antibody is shown in Fig. 3. In the second experiment immune cultures actively producing antibody were killed on day 2 by freezing and thawing 3 times; the diluting effect of successive changes of medium is shown in Fig. 4 and can be compared with the average titer of similar living cultures. In the third experiment immune cultures actively producing antibody were treated on day 6 with puromycin. This antibiotic is known to cause an abrupt cessation of all protein synthesis in microbial and mammalian cultures (11, 12); this effect is clearly indicated in our cultures by the rapid decline in antibody titer of successively replaced medium, as shown in Fig. 5. The continued production of antibody by un-

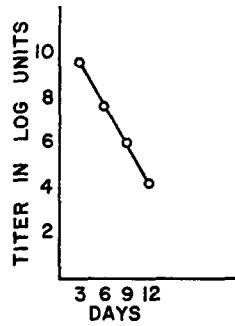


FIG. 3

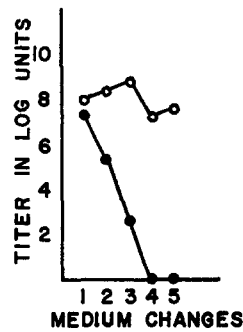


FIG. 4

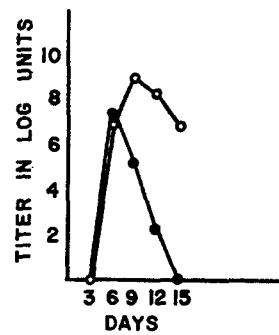


FIG. 5

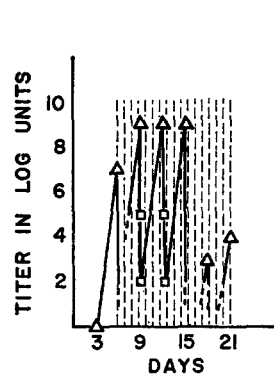


FIG. 6

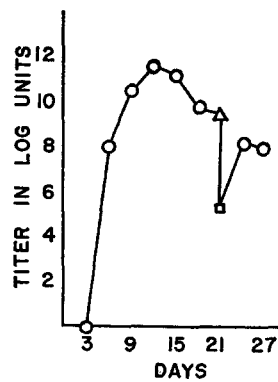


FIG. 7

FIG. 3. On day 0 1 ml of medium containing anti-diphtheria toxoid serum was added to each of 10 non-immune lymph node cultures. The high-titered medium of each culture was replaced with 1 ml of regular antibody-free medium every 3rd day. There was an average fall in titer of 1.8 log units per ml of medium.

FIG. 4. Antiovalbumin titers of lymph node cultures prepared 3 days after a secondary injection of ovalbumin. \circ — \circ average titer from 4 cultures not killed. \bullet — \bullet average titer from 4 cultures killed on day 2 by freezing and thawing $3 \times$ in an ethanol-dry ice bath. The medium changes numbered 1 to 5 were carried out on Day 2, 3, 4, 6, and 8, respectively. There was an average fall in titer of 2.5 log units per ml of medium in the killed cultures.

FIG. 5. Anti-diphtheria toxoid titers of lymph node cultures. \circ — \circ average titer of 4 cultures not treated with puromycin. \bullet — \bullet average titer of 4 cultures treated with medium containing 0.25 mM puromycin from day 6 through day 15. There was an average fall in titer of 2.4 log units per 1 ml medium after day 6 in the puromycin-treated group.

FIG. 6. Anti-BSA titers in media (Δ) and in rinses (\square). The rinses consisted of two 2 ml changes of Hanks' solution before each addition of 1 ml fresh medium. There was an average fall in titer for each 2 ml rinse of 3.5 log units on Day 9 and 12. Chloramphenicol was present at a concentration of 50 $\mu\text{g}/\text{ml}$ starting on day 6, as indicated by the stippled area.

FIG. 7. Average anti-BSA titers (\circ) of media from 8 cultures, which on Day 21 (Δ) were treated with 2 successive 2 hour rinses, each consisting of 1.0 ml medium. The average titer of the second rinses is recorded (\square). There was an average fall in titer per ml of rinse of 2.0 log units.

treated, active cultures is also seen in this figure. In these 3 experiments the steep negative slope of each plot of the titers indicates the rapid dilution of antibody initially present in the cultures. This dilution averages about 2 log units per change of 1 ml. of medium.

Two additional examples demonstrate antibody synthesis during the second and fourth weeks of culture, respectively. Again the diluting effect of several rinses on extracellular antibody titers is followed by the reappearance of high antibody titers in medium subsequently added. In Figs. 6 and 7 are plotted the high titers of the medium preceding the rinses, the low titers of the rinse fluids themselves, and the return of high titers in medium added to the cultures after the rinses. (Fig. 6 is a detailed plot of titers of a single culture which was one of 4 averaged together and plotted in Graph 5F of Fig. 2. Chloramphenicol was present in the medium from day 6 until the end of the experiment; the failure of the drug to prevent the return of high titers in medium added after rinses on days 9 and 12, in particular, merely repeats the observations summarized in the preceding section.)

Several conclusions can be derived from these simple experiments. When new medium is added to cultures, it is not significantly contaminated with antibody from residual traces of old medium. When active antibody synthesis is suddenly terminated either physically by freezing or chemically by puromycin, the rapid decline in titer in successive changes of medium indicates an absence of a significant intracellular store of antibody which can be secreted subsequently. Therefore, when successive medium changes show a rise in antibody titer or maintain the same titer or even suffer a gradual fall in titer, there is continued *de novo* synthesis of antibody. This is the basis for the use of the sum of the reciprocal titers of the fluids during an interval of time as a measure of the total antibody production during the interval.³ The preceding experi-

³ In comparing responses under different conditions as depicted in the graphs, it is necessary to keep in mind the logarithmic nature of the ordinate. Small differences in the heights of points near the top of the semi-log graphs represent large differences in amounts of antibody, while large differences in the levels of points in the lower half of the graphs represent relatively small amounts of antibody. If a titer of 1:5120 ($y = 10$) is taken as a maximum response (100 per cent) then a titer of 1:512 ($y = 6.7$) is 10 per cent and 1:51 ($y = 3.3$) is only 1 per cent.

The antibody response of cultures treated with chloramphenicol for different intervals has been compared to the response of untreated cultures by taking advantage of the following observations: In these experiments most of the antibody preformed *in vivo* or produced *in vitro* by a waning primary response was secreted by the fragments during the first 3 days and thus was largely eliminated with the culture fluid removed on day 3. On the other hand, little or no antibody from the secondary response appeared in the fluids until after the third day. Thus, for a particular culture the *individual* over-all secondary response has been represented by the sum of the reciprocal titers of all the fluids removed from that culture after Day 3. For a group of similarly treated cultures the *average* over-all response is simply the arithmetical average of the individual over-all responses in that group. This number, repre-

ments and observations support the belief that chloramphenicol inhibits the actual synthesis of antibody and not some mechanism regulating its secretion.

DISCUSSION

Protein synthesis in mammalian cells has been previously reported to be inhibited by chloramphenicol (13-20). However, in most instances the effective concentration was found to be many times greater than levels producing a corresponding inhibition in bacteria. For example, in reticulocyte suspensions 313 $\mu\text{g}/\text{ml}$ of chloramphenicol reduced hemoglobin synthesis 13 to 21 per cent (13-15). In other reports involving Ehrlich ascites tumor cells (16), isolated calf thymus nuclei (17, 18), and a variety of cell-free mammalian systems (15, 19, 20) only levels of 1160 $\mu\text{g}/\text{ml}$ or greater produced more than 10 per cent inhibition of amino acid incorporation. But high levels of chloramphenicol may produce other effects which only secondarily impair protein synthesis. For example, in various mammalian cell cultures concentrations ranging from 200 $\mu\text{g}/\text{ml}$ to more than 1000 $\mu\text{g}/\text{ml}$ have been reported to produce marked reduction of cellular respiration (21), rapid development of toxic cytological changes (22), and even death of proliferating cells (23). In our cultures of lymph node fragments 500 $\mu\text{g}/\text{ml}$ not only reduced aerobic glycolysis, as indicated by diminished acid production, but also caused morphological deterioration, as evidenced by the absence of cellular outgrowth from the fragments and by the presence of pycnotic cells in stained sections. Since it is clearly difficult to analyze the action of chloramphenicol on protein synthesis alone at such high concentrations, this discussion will be limited to data collected at concentrations of 50 $\mu\text{g}/\text{ml}$ or less.

The secondary antibody response in our lymph node cultures was completely inhibited by the same low levels of chloramphenicol that suppress protein synthesis in bacteria (less than 50 $\mu\text{g}/\text{ml}$). This similarity in effective concentration suggests that the mechanism of inhibition may be the same in the two cell types. Equally low levels of chloramphenicol have also been used by Djordjevic and Szybalski (24) to inhibit protein synthesis in cultures of human sternal marrow. Treatment with 40 $\mu\text{g}/\text{ml}$ for 6 days reduced protein synthesis 65 per cent for the period between the 4th and 6th days in their cultures without causing "appreciable cell loss due to detachment from the glass or cell death." Our present study and that of Djordjevic and Szybalski are the only two examples we know of in which low levels of chloramphenicol have caused marked inhibition of protein synthesis.

These two examples differ strikingly in one respect from those reports cited senting the average response of a treated group, is compared to the corresponding average response of the untreated group in the same experiment to determine the *per cent* responses of the treated group. In this way the effect on the secondary response of treatment with chloramphenicol for various intervals can be compared in different experiments.

above in which low levels did not significantly reduce protein synthesis. This difference lies in the duration of treatment with the drug before its effect on protein synthesis or amino acid incorporation was measured. In the cell-free mammalian systems, thymus nuclei, ascites cells, and reticulocytes the effect was assayed after only 10 minutes to 4 hours of exposure to the drug. Longer exposures could not be carried out in most of these studies, since cell-free systems incorporate amino acids rapidly for only about 30 minutes (5, 25, 26), while thymocyte nuclei maintain a linear uptake of labeled amino acids for only 90 minutes (17), and reticulocytes synthesize significant amounts of hemoglobin *in vitro* for only 4 hours (27). However, in our lymph node cultures and in the sternal marrow cultures of Djordjevic and Szybalski chloramphenicol was present for at least 3 days before antibody or protein determinations were made. In one of our experiments the secondary response was not inhibited by treatment with chloramphenicol for the first 3 days but was markedly inhibited by treatment for the first 6 days or more. These various results suggest that suppression of protein synthesis in mammalian cells requires treatment with chloramphenicol for more than several hours, and possibly for several days in certain cells.

Some hint as to how chloramphenicol may act to inhibit the secondary response may be gained from our experiments in which the drug was present in the medium for limited periods. When chloramphenicol at a concentration of 50 $\mu\text{g}/\text{ml}$ was present in cultures for the first 6 days only, the secondary response was reduced to about 10 per cent of the normal response. However, when the same amount of drug was not added until day 6 but then continued in the cultures until the end of the experiment, the response was about 40 per cent of normal. Treatment of these latter cultures included the terminal 9 days in 2 experiments and the terminal 15 days in 2 others. Important in the interpretation of these data is the fact that in 3 of 4 such experiments over 95 per cent of the antibody of the secondary response appeared in the culture medium after day 6. Thus chloramphenicol affected some process occurring mainly during the initial 6 days after stimulation, a period preceding most of the actual synthesis of antibody.

The initial period following antigen restimulation is characterized histologically by cellular multiplication and differentiation, particularly of plasma cells (28). Low levels of chloramphenicol probably affect some phase of these complex cellular changes. More specifically, during this initial period, there is a great increase in the RNA content of plasma cells (28). Chloramphenicol has long been thought to inhibit protein synthesis through some interaction with RNA (29). More recently, various suggestions have been made concerning the interaction of chloramphenicol with messenger RNA in bacteria: that the drug inhibits its formation (30), or its function (31), or its breakdown (32). However, other investigators have concluded that chloramphenicol does not

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inhibit messenger RNA breakdown (33). As yet, similar studies have not been reported in mammalian cells, probably because low levels of the drug have only recently been shown to inhibit protein synthesis in animal cells (24, 34). However, the hypothesis that chloramphenicol inhibits antibody synthesis through interference with messenger RNA function is consistent with our data. Indeed, this hypothesis also explains the prompt action of chloramphenicol in bacteria and its apparently much slower action in mammalian cells, since the rate of turnover of messenger RNA is probably faster in bacteria than in animal cells (35-37).

SUMMARY

When lymph node fragments from previously immunized rabbits were stimulated *in vitro* to produce a secondary response, the continuous presence of 50 $\mu\text{g}/\text{ml}$ (0.15 mM) of chloramphenicol in the medium during the entire incubation period of 15 to 21 days produced nearly complete suppression of the response. Concentrations as low as 5 $\mu\text{g}/\text{ml}$ (0.015 mM) produced approximately 80 per cent suppression of the response.

When 50 $\mu\text{g}/\text{ml}$ of chloramphenicol was present during only the first 6 days of culture, the secondary response was reduced 90 per cent. When it was absent for the first 6 days but present for the next 9 to 15 days, the response was reduced only 40 per cent. Since over 95 per cent of the antibody of the secondary response in most experiments appeared in the medium after the 6th day, chloramphenicol apparently inhibits antibody production by interfering with some early phase of the response. It is suggested that this interference involves messenger RNA and that animal cells have appeared resistant to this drug only because their complement of messenger RNA present when the drug has been added is stable over the short periods during which protein synthesis has usually been studied.

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