EFFECT OF ACTINOMYCIN D ON RNA SYNTHESIS AND ANTIBODY FORMATION IN THE ANAMNESTIC RESPONSE IN VITRO*, ‡

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The response of antibody-forming cells to antigen provides an example of induced protein synthesis. Ribonucleic acid (RNA) has been implicated in the induction of this reaction in recent experiments. Extracts of macrophages from immunized animals, which could be inactivated by ribonuclease, have been found to initiate antibody formation in normal rabbit lymphoid cells (2, 3). Campbell (4) has reported that small antigen fragments are coupled to RNA, and that these complexes possess antigenic activity. RNA extracted from immunized lymphoid cells has been reported also (5) to confer homotransplantation immunity upon normal lymphoid cells.

The present studies suggest that, in addition to a role in the initiation of the immune response, RNA is required for the production of antibody protein. The secondary immune response, which has been studied in these experiments, is characterized by two phases. Initially, there is lymphoid cellular proliferation and differentiation with synthesis of deoxyribonucleic acid (DNA) (6). Subsequently, cellular proliferation decreases, and antibody synthesis becomes the predominant reaction (7, 8). By analogy to known protein-synthesizing systems, it may be presumed that the DNA formed during the proliferative phase transfers information for the formation of antibody protein to an RNA template. The latter, which has been referred to as messenger RNA (9), has been shown to be relatively stable in the case of hemoglobin synthesis in the reticulocyte (10), or labile in the synthesis of induced enzymes in bacteria (11) and in mammals (12, 13). The participation of messenger RNA in antibody synthesis, and whether it is stable or rapidly turned over have been investigated in the present experiments.

The antibiotic actinomycin D has been shown to block the synthesis of RNA

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by a highly selective binding to the deoxyguanosine residues of DNA required as template for the function of RNA polymerase (14–16). In the present experiments, the effect of actinomycin D and of several other potent inhibitors of nucleic acid and protein synthesis on RNA and antibody formation by anamnestic rabbit lymphoid cells has been studied. Inhibition of the production of antibody to T_2 phage by actinomycin D has recently been described in a preliminary report by Uhr (17).

Materials and Methods

Actinomycin D (Lot No. 1687C-41A) was obtained through the courtesy of the American Cyanamid Company, Research Division, Pearl River, New York. L-Phenylalanine mustard (L-sarcolysin) and hydroxyurea were made available by Dr. Eugene Frenkel of the Southwest Cancer Chemotherapy Group (United States Public Health Service).

Preparation of Cells.—Albino rabbits weighing approximately 2.5 kg were hyperimmunized by 18 triweekly injections of crystalline bovine serum albumin (BSA), given initially in complete Freund's adjuvant and later in alum-precipitated form. After a rest period of 3 to 6 months, these animals were injected with a total of 10 mg of BSA, given in divided doses into each foot-pad and the paravertebral musculature of the neck. One hour prior to injection, 50 mg of promethazine hydrochloride was injected intramuscularly. Three days later (and in two experiments, 5 days later), the anti-BSA titer of the individual sera was checked by the ring precipitation test (18). If the serum demonstrated a trace or more of antibody, the rabbit was considered suitable for use. Sixty-five mg of pentobarbital was administered slowly intravenously, and the animal bled to death by heart puncture. The spleen and lymph nodes were excised and minced in 10 ml of ice-cold Eagle's medium (19). The tissue mince was filtered through a nylon mesh gauze (7) into sterile, chilled conical glass centrifuge tubes. The cell suspension was then centrifuged in the cold at 1000 RPM for 10 minutes, and the supernatant discarded. The packed cells were resuspended in a volume of Eagle's medium sufficient to bring the final cell concentration to approximately 100 million cells per ml. Initial cell viability using the trypan blue method (7) was approximately 90 per cent.

Incubation was carried out at 37°C in a water-saturated atmosphere, made up of 95 per cent air and 5 per cent CO_2 in sterile, disposable, 30 ml plastic tissue culture flasks. The contents of each incubation flask are indicated in Table I.

RNA Extraction Procedures.—Total RNA was isolated at the end of incubation by adding 3 ml of 0.5 per cent sodium dodecyl sulfate in Perry's buffer (20) to each incubation flask, and then extracting three times with water-saturated phenol (21). Ten mg of non-radioactive cytidine monophosphate (CMP) was added to the aqueous extract, and this was then dialyzed against 40 liters of 0.01 M phosphate buffer, pH 7. The dialyzed samples were then lyophilized and redissolved in 0.5 ml of 2 N NaOH. The resulting solution was then heated at 90°C for 30 minutes to fragment the RNA. After cooling, the solution was diluted to 1 ml with water, and added to 10 ml of Bray's phosphor (22) for counting in a Packard tricarb liquid scintillation counter.

RNA from the incubated samples was also fractionated according to the method of Yamana and Sibatini (23) as modified by Holland (24). Two ml of water-saturated phenol was added to the living cell mixture, and after shaking and centrifugation, the aqueous supernatant and interface material were collected separately. The aqueous supernatant, which has been shown to contain most of the ribosomal and transfer RNA (25), was extracted twice more with an equal volume of water-saturated phenol. The supernatant was then dialyzed following addition of non-radioactive CMP, and prepared for radioactive counting as above.

The precipitated interface material obtained above was resuspended in 2 ml of 0.5 per cent sodium dodecyl sulfate in Perry's buffer (20). Two ml of water-saturated phenol was then added, and the mixture shaken and centrifuged. The aqueous extract of the interface material, which has been shown to contain the rapidly labeled, presumably messenger RNA fractions (26), was then dialyzed in the presence of CMP, lyophilized, dissolved, and counted as above.

Sucrose density gradient separation of RNA fractions was carried out by the method of Scherrer and Darnell (27).

Antibody Isolation.—At the end of a 6 hour incubation period, 5 ml of normal rabbit serum was added to each sample. The contents of the flasks were then frozen and thawed three times by immersion in an alcohol–dry-ice bath, and dialyzed in size 20 Visking tubing for 36 hours in 40 liters of 0.01 M Na_2HPO_4 at 4°C. The samples were then spun at 100,000 *g* for 30 minutes. The supernatant was then transferred to a diethylaminoethyl (DEAE) cellulose column for isolation of γ_2-globulin according to the method of LoSpalluto and coworkers (28). Remaining protein adherent to the column was eluted as a single fraction with 0.15 M NaH_2PO_4 in 0.15 M NaCl (28). The pooled fractions containing γ_2-globulin were lyophilized, dissolved in 3 ml of water, and clarified by centrifugation for 10 minutes at 25,000 *g*. To each sample was added a chromatographically separated preparation of non-radioactive rabbit γ_2-globulin (28) with a previously determined high titer of anti-BSA antibody, and containing approximately 50 mg of protein per ml. An equivalent amount of BSA was then added to give a total antigen-antibody precipitate of approximately 5 mg (29). The samples were incubated at 37°C for 1 hour, cooled overnight, and centrifuged at 25,000 *g* for 10 minutes. The antigen-antibody precipitates were then dissolved in 0.5 N NaOH, heated in a boiling water bath for 10 minutes, and added to 10 ml of Bray's phosphor (22) for counting.

TABLE I

Contents of Incubation Flasks

Material	Concentration per ml*
Lymphoid cells	40–50 million cells
Leucine-1-C^{14}	1.3–3.1 μc
H^3-Cytidine monophosphate	2.1–5.0 μc
Penicillin G	6250 units

* In Eagle's medium (19), made up without leucine for incorporation of leucine-1-C^{14} and complete for incorporation of H^3-cytidine monophosphate. Final volume was 2 ml.

RESULTS

Effect of Actinomycin D on Anti-BSA Synthesis.—Incorporation of radioactivity into newly synthesized antibody by control cell suspensions was observed to be approximately linear up to 6 hours. Following addition of actinomycin D, an abrupt cessation of antibody synthesis occurred after about 2½ hours (Fig. 1).

Synthesis of antibody and RNA by lymphoid cells was studied as a function of the concentration of actinomycin D. The effect of preincubation before addition of radioactive label was also examined. Viability of untreated cells,

as measured by the trypan blue method (7), which was initially approximately 90 per cent, declined to 50 to 60 per cent after 6 hours. Actinomycin D, in concentrations up to 25 μg per ml, produced no difference in cell viability between control and inhibited flasks. Above 1 μg per ml, increasing concentrations of actinomycin D produced a progressive decrease in synthesis of anti-BSA (Fig. 2). Above 25 μg per ml, a further slight decrease in antibody synthesis was accompanied by a decrease in cell viability.

Inhibition was more pronounced when actinomycin D was added 30 minutes prior to addition of radioactive label than when both were added at the same time, as is shown by comparison of the upper with the lower curve in Fig. 2.

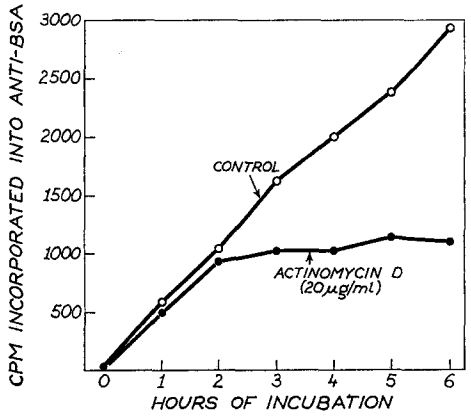

FIG. 1. Incorporation of radioactivity into γ_2–anti-BSA with and without actinomycin D, 20 μg/ml, added 30 minutes before L-leucine-1-C^{14}.

Production of non–anti-BSA γ_2-globulin, and of other protein eluted from the DEAE cellulose column, fell under the influence of actinomycin D in the same manner as that of anti-BSA (Fig. 3). In the non-inhibited control of this experiment, approximately 10 per cent, 30 per cent, and 60 per cent of the total counts were incorporated into γ_2–anti-BSA, other non–anti–BSA γ_2-globulin, and the fraction containing other proteins respectively.

After a 75 minute preincubation with actinomycin D, replacement of the culture fluid with Eagle's medium, free of inhibitor, provided no release from inhibition, suggesting that the binding of actinomycin D, intracellularly, was not reversible.

In the same experiments in which the effect of actinomycin D on antibody synthesis was studied, parallel observations were made on the synthesis of RNA as measured by incorporation of H^3-CMP (Table II). It is seen that, in the absence of inhibitor, antibody-producing lymphoid cells actively synthesized RNA. This was observed in cells removed both 3 days (Table II) and 5 days

after antigen administration. At levels of 10 and 25 μg per ml, actinomycin D produced marked inhibition of antibody synthesis and this was accompanied by an almost complete suppression of RNA synthesis. It should be noted, however, that at a level of 1 μg per ml, marked inhibition of RNA synthesis occurred without a decrease in antibody synthesis.

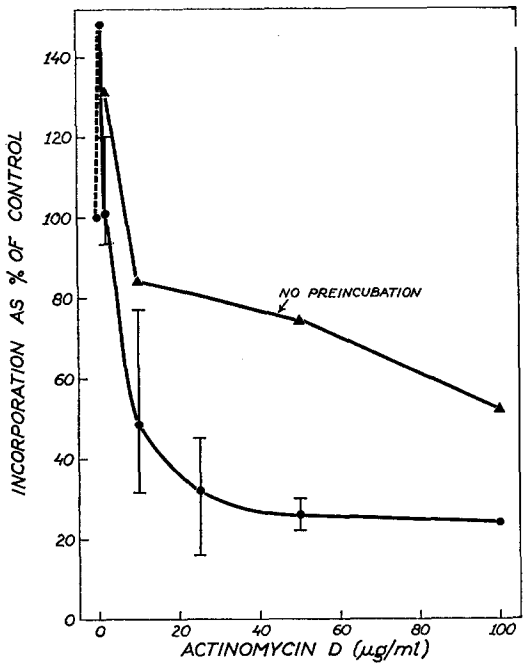

FIG. 2. Effect of actinomycin D on synthesis of anti-BSA. The lower curve represents, at each point, an average of 6 experiments, in which there was a 30 minute period of preincubation before the addition of leucine-1-C^{14}. The dotted line represents a stimulation of synthesis noted at 0.1 μg/ml. The upper curve represents a single experiment in which actinomycin D was added simultaneously with C^{14}-label. Antibody synthesis is expressed as per cent of incorporation observed in the non-inhibited control.

In view of the above finding, it was decided to determine which type of RNA synthesis was sensitive to low concentrations of actinomycin D. This was done by two techniques. The first, illustrated in Fig. 4, shows the effect of actinomycin D on the synthesis of various fractions of RNA separated in a sucrose density gradient (27). The heavier RNA fractions are presumed to be microsomal and messenger RNA, and the lighter fractions transfer RNA (27). It is seen that significant inhibition of the synthesis of all types of RNA was produced.

The second technique, the phenol fractionation method of Holland (24), was utilized to prepare a supernatant fraction containing primarily transfer

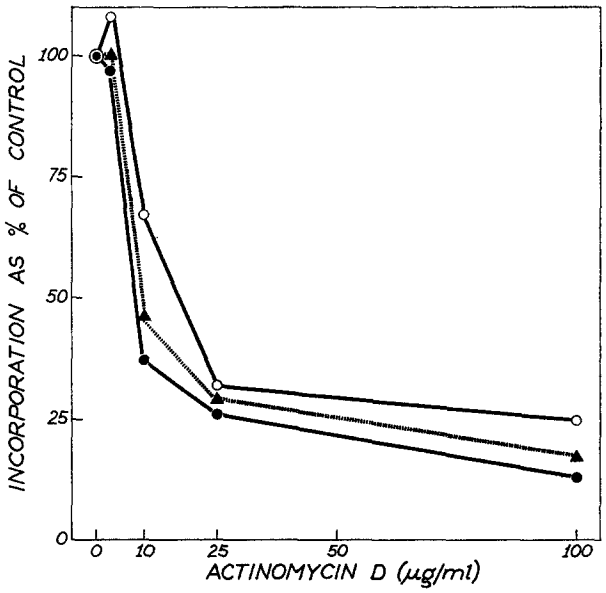

FIG. 3. Effect of actinomycin D on various types of protein synthesized by a lymphoid cell suspension: ○, BSA–anti-BSA precipitate; ▲, non–anti-BSA γ_2-globulin; ●, other protein eluted from DEAE cellulose column.

TABLE II

Effect of Actinomycin D on the Synthesis of RNA and Antibody

Actinomycin D	Ag-Ab ppt	Synthesis anti-BSA, per cent of control	RNA	Synthesis RNA, per cent of control
μg/ml	CPM		CPM	
0	220	100	3432	100
1	232	106	440	13
10	116	53	159	3
25	53	24	22	1

Ag-Ab ppt, antigen-antibody precipitate.

and ribosomal RNA (25), and an interface fraction considered to be messenger RNA (26). As can be seen in Table III, inhibition of the synthesis of all types of RNA again occurred. However, coincidentally with the moderate to marked inhibition of RNA synthesis by actinomycin D observed at levels of 1 μg/ml or less, there was actually an increase in synthesis of antibody. Thus, in both experiments, there was, at low concentrations of actinomycin D, a dichotomy of effect on RNA and antibody synthesis. This consisted of either no change

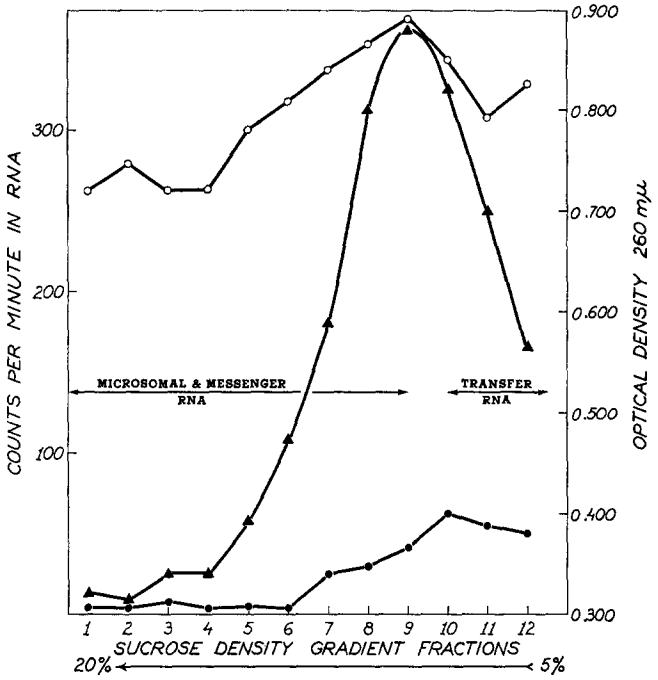

FIG. 4. Effect of actinomycin D on synthesis of various fractions of RNA separated in sucrose density gradient: ○, total RNA as measured spectrophotometrically at 260 mμ, ▲, CPM in RNA of non-inhibited control cells; ●, CPM in RNA of cells exposed to 1 μg/ml of actinomycin D.

TABLE III

Effect of Actinomycin D on the Synthesis of Antibody and RNA Fractions

Actinomycin D	Synthesis as per cent of control		
	Anti-BSA	RNA fractions	
		Supernatant*	Interface‡
μg/ml			
0.1	148	58	71
1.0	128	20	17
5.0	51	8	6
10.0	41	4	2

* Ribosomal and transfer RNA. (These comprise 90 per cent of total RNA.)

‡ Rapidly labeled, presumably messenger RNA (10 per cent of total).

TABLE IV

Effect of Puromycin, Hydroxyurea, L-Phenylalanine Mustard, and 6-Mercaptopurine on Synthesis of Anti-BSA

Puromycin, *mmoles/ml*	10^{-3}	10^{-4}	10^{-5}
Per cent of control	9	16	82
Hydroxyurea, *mg/ml*	3.0	0.3	0.03
Per cent of control	61	78	92
L-Phenylalanine mustard, *μg/ml*	40	20	2
Per cent of control	37	73	102
6-Mercaptopurine, *μg/ml*	400	200	20
Per cent of control	91	96	91

or even a significant increase in antibody formation in the presence of a marked decrease in synthesis of RNA.

Effect of Other Inhibitors.—Puromycin (Table IV), which is known to affect protein synthesis at the level of transfer RNA (30), caused marked suppression of antibody formation. This is in agreement with a recent report of Ambrose and Coons (31). Hydroxyurea and L-phenylalanine mustard, substances believed to affect synthesis of DNA, suppressed antibody formation only at high concentrations, well above those reached therapeutically in man (32, 33). 6-Mercaptopurine (6-MP) had no significant effect in this system, even at very high concentrations.

DISCUSSION

Actinomycin D produced marked suppression of antibody synthesis, even at moderately low concentrations. This agent has been shown to have a highly selective action, the inhibition of DNA-dependent RNA synthesis, including the synthesis of messenger RNA (14–16). Mitchell and Nossal (34) have reported that plasma cells engaged in the secondary antibody response did not incorporate significant amounts of H^3-uridine, as measured by radioautography, and, therefore, were presumably not synthesizing messenger RNA. They suggested on the basis of this finding that the messenger RNA responsible for continued antibody synthesis in the anamnestic lymphoid cell was completely stable, and perhaps even contributed by a parent cell. The present observation that H^3-CMP was incorporated into chemically isolated RNA both 3 and 5 days after antigenic stimulation indicates that new synthesis of RNA does occur during this period of most rapid antibody formation. However, in the present mixed cell population, it is not certain what fraction of this synthesis is contributed by antibody-producing cells. Recently published observations of

the suppression of antibody synthesis by actinomycin D in an *in vitro* culture of secondarily stimulated lymphoid cells have led Uhr (17) to suggest that this suppression was a consequence of inhibition of the synthesis of messenger RNA.

The abrupt cessation of antibody synthesis observed approximately 2.5 hours after addition of actinomycin D at a concentration of 20 μg/ml (Fig. 1) suggests that the messenger RNA in this system has a finite stability. This observation is analogous to that of Levinthal and coworkers (35) on the effect of actinomycin D on protein synthesis in *Bacillus subtilis*, in which the mean life of messenger RNA was estimated as approximately 2 minutes.

The observation that antibody production may continue when RNA synthesis is markedly inhibited indicates that the relationship of messenger RNA to antibody synthesis is a complex one. Thus, when the synthesis of various RNA fractions, isolated by sucrose density gradient or supernatant-interface phenol fractionation, was measured, it was found that the formation of all fractions of RNA, including the portion believed to contain messenger RNA, was markedly inhibited at actinomycin concentrations which produced no decrease in antibody synthesis. This suggests that the level of messenger RNA may not be the limiting factor in the series of reactions involved in the formation of antibody, and that antibody production is inhibited only when a relatively stable messenger RNA fraction is reduced in concentrations below a critical level by an almost total inhibition of new synthesis.

The simultaneous suppression of protein synthesis other than that of γ_2-anti-BSA by actinomycin D represents inhibition of formation of other antibodies and of non-antibody protein associated with lymphoid cell function. It is reasonable that the synthesis of other γ_2-globulin should be equally susceptible to suppression by actinomycin D. The observed decreased synthesis of non-γ_2-globulin protein has several possible explanations. A portion of this protein may consist of γ_1 A- and γ_1 M-immunoglobulin, the synthesis of which might be susceptible to the effect of actinomycin D in the same manner as γ_2-globulin. It is also possible that protein synthesis generally is suppressed in the antigenically stimulated lymphoid cell in a uniform manner by actinomycin, either by a similar effect on all types of messenger RNA or by a selective effect on the messenger RNA required for the synthesis of a critical cell constituent.

The increase in antibody production noted at low levels of actinomycin D is difficult to explain. It may result from suppression of competitive phenomena, which are relatively more sensitive to low concentrations of the drug. Indeed, Perry (20) has shown selective inhibition by very low levels of actinomycin D of nucleolar RNA synthesis, believed to be primarily ribosomal RNA, with very little effect on other nuclear RNA synthesis.

The inhibition produced by actinomycin D could not be reversed by removal of the cells to an inhibitor-free medium, possibly because the drug is irreversibly

bound to the DNA of the cells. This would be in agreement with the findings of Paul and Struthers (36) in L cell fibroblasts inhibited by actinomycin D.

It should be noted that the present experiments have utilized a whole cell suspension to investigate the relationship of RNA synthesis to antibody synthesis in the presence of inhibitors. Although the RNA of the intact cell is associated with the formation of a variety of proteins, it is reasonable to assume that new RNA synthesis in lymphoid cells forming antibody at a maximum rate is primarily related to the production of gamma globulin. Inhibition of antibody production, however, could be the result of the suppression of the synthesis of unrelated messenger RNA necessary for the formation of an essential cell constituent and not antibody. Such a constituent might be an enzyme necessary either for protein synthesis or for the continued viability of the cell. The fact that normal amounts of antibody were produced by cells in which RNA synthesis had been reduced by actinomycin D to 17 per cent of the control value suggests that the cells were still viable at a time when RNA synthesis had been inhibited to a considerable extent. However, at higher levels of inhibition, staining with trypan blue (7), may not be fully satisfactory as a measure of cell viability.

6-Mercaptopurine has been shown to inhibit the conversion of inosinic to xanthylic acid, thus blocking guanine biosynthesis (37). It would be expected, therefore, that this agent would affect both DNA and RNA biosynthesis. In the present studies, no significant effect of 6-MP on the synthesis of either RNA or antibody was observed during the 6 hour period of incubation. This result could reflect a relatively slow onset of inhibition of guanine synthesis, perhaps because of cell permeability barriers to 6-MP, or the presence of a relatively large pool of available purines within the cell which could sustain nucleic acid synthesis during the time period involved. It is also possible that 6-MP affects DNA synthesis primarily, and would, therefore, be relatively ineffective in a resting cell suspension such as the one utilized. The latter possibility is supported by the observations of Šterzl (38) and Schwartz and coworkers (39), who were unable to block antibody synthesis in intact rabbits once the secondary response was underway.

Puromycin has been shown to have no effect on RNA synthesis, but is thought to inhibit the final step in protein synthesis, in which amino acids attached to transfer RNA are condensed to form protein (30). In the present experiments, puromycin strongly inhibited antibody formation, offering further evidence that antibody synthesis follows the classic pathways of protein synthesis. Similar observations have recently been reported by Ambrose and Coons (31). Hydroxyurea and L-phenylalanine mustard inhibited antibody production only in relatively high concentrations, well above those reached in the intact organism (32, 33). Although the exact site of action of these compounds has not been elucidated, they are believed to affect DNA synthesis.

Previous studies, both *in vitro* and *in vivo*, have shown inhibition of antibody formation during the proliferative phase of the secondary response by agents like 5-bromodeoxyuridine (40, 41) and methotrexate (42), which are thought to act on the synthesis of DNA. The present results have demonstrated inhibition of antibody synthesis at two other significant sites, that is, the synthesis of RNA and the coupling of amino acids. The above findings, taken together, are consistent with the view that antibody synthesis, although an induced form of protein synthesis, follows pathways similar to those utilized for other types of protein.

SUMMARY

Antibody synthesis in anamnestic lymphoid cells, measured by incorporation of leucine-C^{14} into specific antibody, was inhibited at moderate concentrations of actinomycin D. This was accompanied by marked inhibition of synthesis of RNA as measured by incorporation of H^3-cytidine monophosphate. However, at low concentrations of actinomycin D, antibody synthesis was unaffected or even increased while RNA synthesis continued to be inhibited. The results obtained suggest that messenger RNA for antibody synthesis, either because it is relatively stable or present in excess, does not become a limiting factor until its synthesis is maximally inhibited.

Puromycin, an inhibitor of amino acid coupling, abolished antibody synthesis in low concentration. 6-Mercaptopurine had no effect on the synthesis of antibody or RNA even at high concentration.

The data obtained support the view that antibody synthesis follows pathways similar to those utilized for the formation of other types of proteins.

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