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Effects of some nucleoside antibiotics on morphogenetic development and synthesis of RNA and protein in *Dictyostelium discoideum*

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When toyocamycin, formycin and formycin B, which are all analogues of adenosine, were given to *D. discoideum* amoebae in the early stages of morphogenesis, the former two exhibited inhibitory effects on morphogenetic development, while the latter one caused no inhibition. The results of the incorporation test of radioactive RNA and protein precursors and the sedimentation analysis of RNA showed that formycin and formycin B both prohibit syntheses of all RNA species including polyadenylic acid-containing RNA and are presumably incorporated into RNA chain (toyocamycin not examined because of its similarity to formycin in the mode of action). On the other hand, protein synthesis was not affected within at least 5 hr after the administration of formycin or formycin B. The experimental results with formycin could be interpreted in the light of the assumption proposed previously (MIZUKAMI and IWABUCHI, 1970) as to the relationship between RNA and protein syntheses participating in the morphogenetic development of this organism. However, the reason is unknown at present why formycin B exerted no influence on morphogenesis, in spite of the fact that the agent have the inhibitory activity on total RNA synthesis.

When amoebae of the cellular slime mold *D. discoideum* are nutritionally deprived, they enter to the morphogenetic phase in which the fruiting bodies composed of spore and stalk cells are finally formed through a series of developmental events. In recent years, considerable attention has been focused on the transcription and translation during cellular differentiation and development of this organism (PANBACKER, 1966; PANBACKER and WRIGHT, 1966; SUSSMAN, 1967; LOOMIS, 1970; MIZUKAMI and IWABUCHI, 1970; SOLL and SUSSMAN, 1973; FIRTEL *et al.*, 1973; LODISH *et al.*, 1974). In studies dealing with such problems, several antibiotics have been employed as excellent tools to inhibit the macromolecule biosynthesis. In fact, some experiments using actinomycin D, daunomycin and cycloheximide have af-

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forded available evidence for understanding the relationship between morphogenetic development and RNA and/or protein syntheses (MIZUKAMI and IWABUCHI, 1970; LOOMIS, 1970; FIRTEL *et al.*, 1973).

Previously we have investigated the effects of actinomycin D and cycloheximide on morphogenesis and RNA and protein synthesis in *D. discoideum* and reached a tentative conclusion that protein synthesis in the early stage of morphogenesis may be possibly controlled by messenger RNA (mRNA) synthesized in the prior, not the same stage of development (MIZUKAMI and IWABUCHI, 1970). In the present study, the evidence supporting the above view could be obtained in similar experiments using formycin and toyocamycin which are analogues of adenosine (Fig. 1) and possess inhibitory activity on the growth and RNA synthesis of some animal tumor cells (SUHADOLNIK, 1970).

The experimental results reported here indicate that formycin and toyocamycin, but not formycin B, exert the inhibitory effect on morphogenetic development of *D. discoideum* amoebae. It was also elucidated that formycin and formycin B cause inhibition of syntheses of all RNA species including polyadenylic acid (poly(A))-containing RNA, while not affecting total protein synthesis within at least 5 hr after the administration of the drugs.

Materials and Methods

Culture of amoebae and synchrony of morphogenesis: *D. discoideum* NC-4 amoebae were grown in association with *Escherichia coli* on 2% agar plates containing SM-medium (SUSSMAN, 1966). Synchronization of morphogenesis was done by using the Millipore filter method as previously described (MIZUKAMI and IWABUCHI, 1970; SUSSMAN and LOVGREN, 1965). Proliferating amoebae were harvested in the mid-log phase of growth, freed from bacterial cells by centrifugation ($1,500 \times g$, 3 min) with a solution composed of 0.016 M phosphate buffer (pH 6.5) and 250 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and suspended in the above solution at a cell density of $1 \times 10^8/\text{ml}$.

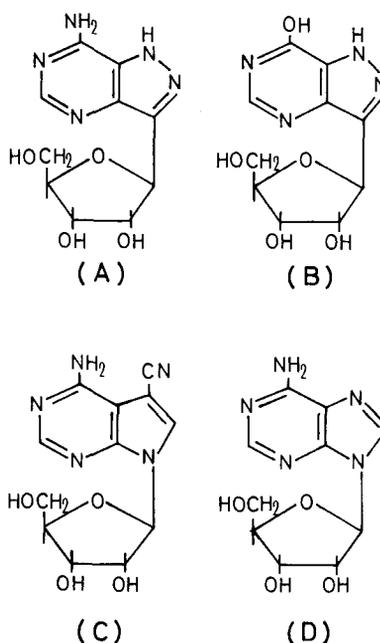


Fig. 1. Chemical structure of formycin (A), formycin B (B), toyocamycin (C) and adenosine (D).

Difinite volumes of the suspension were dispensed on Millipore filter paper on the sponge pad wetted with the above solution and then incubated at 23°C. When the cells at either developmental stage were treated with nucleoside antibiotics, the filters were placed on the pad saturated with the above solution containing the drug (MIZUKAMI and IWABUCHI, 1970). The morphogenetic development of the amoebae in the drug or control experiment was observed with a binocular microscope.

Labeling of cells: For isotopic labeling of RNA or protein synthesized in the cells entering to the morphogenetic phase, the amoebae at interphase which had been suspended in the above phosphate buffer or TKM buffer (0.015 M Tris-HCl, pH 7.6, 0.02 M KCl and 0.005 M MgCl₂) containing 150–250 µg/ml streptomycin at a difinite cell density were exposed to ³H-uracil, ¹⁴C-adenine or ¹⁴C-amino acids at 23°C. Incorporation of ³H-uracil and ¹⁴C-adenine into RNA and of ¹⁴C-amino acids into protein of whole cells was determined by measuring the radioactivity in material precipitated in cold (0°C) and hot (90°C, 20 min) 10% trichloroacetic acid (TCA) containing 5% acetone (MIZUKAMI and IWABUCHI, 1970; IWABUCHI *et al.*, 1971).

Labeled cells from which RNA was extracted were washed in the cold with the same buffer as used in the RNA extraction and stored at –30°C.

Extraction of RNA: Frozen cells were lysed in TE buffer (0.025 M Tris-HCl, pH 9.0 and 0.005 M ethylenediaminetetraacetic acid (EDTA)) containing 2.6% sodium dodecyl sulfate (SDS). The lysate was treated with 1% diethylpyrocarbonate, followed by the addition of two volumes of a solution of phenol-chloroform-isoamyl alcohol (49:49:2) which was saturated with TN buffer and the mixture was then vortexed for 15 min. The aqueous phase separated by centrifugation was deproteinized further four times as done in the above and 0.1 volume of 4 M sodium acetate was added during the last deproteinization. RNA was precipitated from the final aqueous solution by addition of 2 volumes of cold 95% ethanol, following twice ether-treatments.

Sucrose gradient centrifugation: RNA was dissolved into TN buffer (0.01 M Tris-HCl, pH 7.3 and 0.1 M NaCl) containing 0.5% SDS, layered on linear sucrose gradients (10–25% (w/v)) made with the above solution, and centrifuged at 26,000 rpm for 12 hr at 18°C in a Hitachi RPS 27 rotor. Gradients were fractionated into a difinite number of tubes. Aliquots (0.3 ml) of each fraction were used for the determination of both patterns of the absorbance at 260 nm and the radioactivity of TCA-precipitated RNA, and 1 ml of the remaining portion was employed for the assay of binding of RNA to poly(U) filters.

Hybridization assay of RNA with poly(U) filters: Poly(U) filters (polyuridylic acid immobilized to Whatman glassfiber filter, GF/C) were prepared according to the procedure described by SHELDON *et al.* (1972). The RNA sample was bound to poly(U) filters at room temperature in binding buffer (0.01 M Tris-HCl, pH 7.3, 0.12 M NaCl, 0.1 mM EDTA, 0.5% SDS, and 0.1 M sodium phosphate) and then extensively washed with the binding buffer (without containing the sodium phosphate) in order to reduce non-specific binding (FIRTEL *et al.*, 1972). The poly(U) filters were further washed with 0.3 M ammonium acetate in 50% ethanol, dried and counted (FIRTEL *et al.*, 1972)

Measurement of radioactivity: TCA-precipitated material was collected on a glass-fiber filter (Whatman, GF/C), washed with cold 5% TCA and dried. Radioactivity was measured in the Beckman liquid-scintillation spectrometer as previously described (MIZUKAMI and IWABUCHI, 1970; IWABUCHI *et al.*, 1971)

Isotopes and chemicals: ^3H -uracil (44.0 Ci/mmol) and ^{14}C -adenine (54.0 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. ^{14}C -labeled *Chlorella* protein hydrolysates (7.1 mCi/mmol C) was a gift from the Institute of Applied Microbiology, University of Tokyo, through the Japan Isotope Association. Formycin, formycin B and toyocamycin were gifted from the Institute of Microbial Chemistry, Tokyo.

Results

Effect of formycin, toyocamycin and formycin B on morphogenesis: In preliminary experiments, the suitable concentrations of formycin and toyocamycin to show inhibitory activity to morphogenesis were determined to be in the range of 150 $\mu\text{g}/\text{ml}$ to 200 $\mu\text{g}/\text{ml}$. The result of the formycin experiment is illustrated in Fig. 2. When the drug was given to the amoebae at interphase, they still remained in the mid-aggregation stage at 34 hr after beginning of incubation, while the amoebae of the control culture has already constructed normal fruiting bodies. Upon exposure of cells to the drug at the aggregation stage, the morphogenetic development was blocked at the late aggregation to culmination stage. However, when incubated continuously for further 26 hr (60 hr in total) on the drug-containing medium, the amoebae mostly constructed fruiting bodies, though anormous in shape. The removal of the drug from the medium at 34 hr after the onset of incubation resulted in the resumption of inhibited development so that normal fruiting bodies were formed upon the additional incubation for 26 hr. The addition of the drug at the migration and culmination stages exerted no influence on the

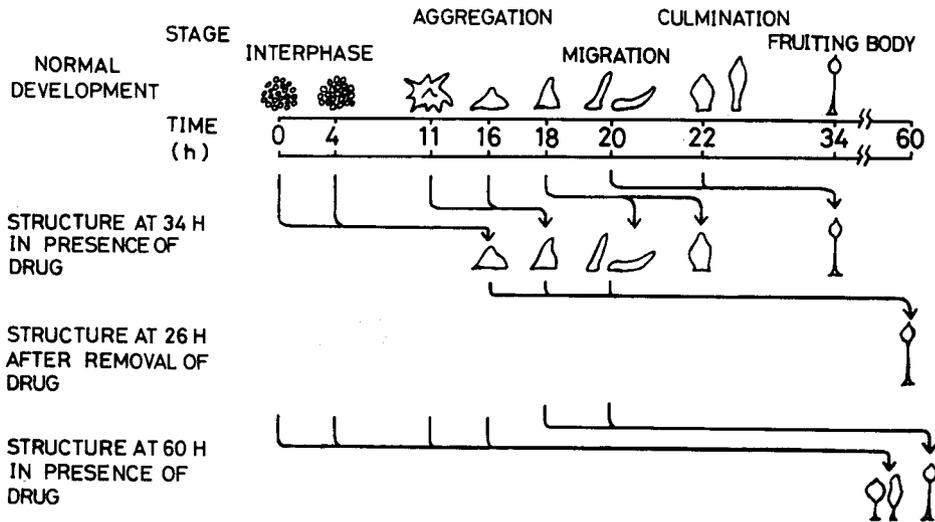


Fig. 2. Effect of formycin on morphogenetic development. For details, see text.

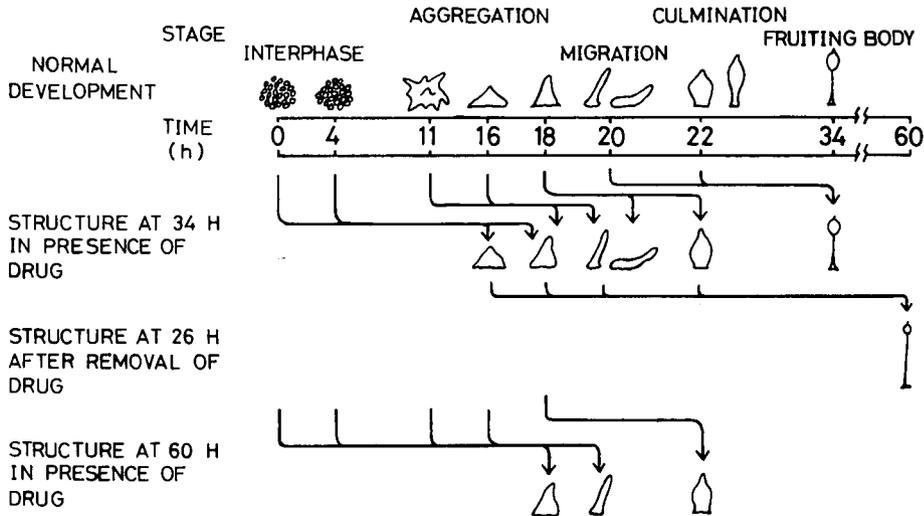


Fig. 3. Effect of toyocamycin on morphogenetic development. For details, see text.

subsequent development.

As indicated in Fig. 3, similar results were obtained in the toyocamycin experiment, except that most of the fruiting bodies formed after the removal of the drug have small sorocarps and slender stalks and that further develop-

ment does not occur during the additional 26-hr incubation in the presence of the drug.

As to the effect of formycin B, the experimental result showed that the drug concentration of 200 $\mu\text{g}/\text{ml}$ exerted no influence on the morphogenetic development.

From the above results it is apparent that the morphogenetic development of *D. discoideum* amoebae is considerably prevented by formycin and toyocamycin but not by formycin B, although the inhibitory action of the drugs is not so immediate as that of actinomycin D and cycloheximide observed in the previous study (MIZUKAMI and IWABUCHI, 1970). In addition, the inhibitory activity of the antibiotic was perfectly abolished by the simultaneous addition of an excess of amount of adenosine (1.5 mg/ml), suggesting that the

TABLE 1. Effect of formycin and formycin B on incorporation of ^3H -uracil, ^{14}C -adenine and ^{14}C -amino acids into TCA-insoluble materials of whole cells at interphase of morphogenesis

Treatment	Inhibition (%) of incorporation of ^3H -uracil		Inhibition (%) of incorporation of ^{14}C -adenine		Inhibition (%) of incorporation of ^{14}C -amino acids	
	2-hr labeling	4-hr labeling	2-hr labeling	4-hr labeling	2-hr labeling	4-hr labeling
Control	0	—	0	0	0	0
Formycin	25	—	59	50	0	5
Formycin B	29	—	63	57	0	0

The suspension of amoebae (1×10^7 cells/ml) at interphase in 0.016 M phosphate buffer (pH 6.5) containing 250 $\mu\text{g}/\text{ml}$ streptomycin were divided into three equal portions of 2.5 ml each and 0.1 ml of formycin or formycin B solution prepared with the above buffer was added to give 200 $\mu\text{g}/\text{ml}$ at the final concentration. To the control culture was added an equal volume of the above buffer. After incubation for 1 hr at 23°C with vigorous shaking in order to avoid the formation of any clumps of amoebae, each of these three batches was further divided into two equal portions of 1 ml each, and one of them received 0.1 ml of the mixture of ^3H -uracil (25 μCi) and ^{14}C -adenine (5 μCi) and other received 0.1 ml of ^{14}C -amino acids (5 μCi). Additional incubation was continued under the same conditions as in the above. At 2 hr and 4 hr after the addition of isotopes, 0.2 ml (2×10^6 cells) of culture was pipetted out and poured into 5 ml of cold 10% TCA containing 5% acetone. Radioactivity was measured as described in Materials and Methods. Each value is the mean of duplicate determinations. Radioactivity of ^3H -uracil incorporated per 2×10^6 cells in the control was 967,700 cpm in 2-hr labeling, and that of ^{14}C -adenine was 53,830 and 74,710 cpm in 2-hr and 4-hr labeling, respectively. Radioactivity of ^{14}C -amino acids incorporated per 2×10^6 cells in the control was 17,820 and 22,450 cpm in 2-hr and 4-hr labeling, respectively.

drugs might compete with adenosine *in vivo* as its metabolic analogue.

Effect of formycin on incorporation of radioactive RNA and protein precursors into whole cells: The nucleoside antibiotics used here are thought to give some influences to RNA synthesis as metabolic analogues of adenosine. Then, attempts were made to examine the incorporation of radioactive RNA and protein precursors into TCA-precipitated material of whole cells at interphase of morphogenesis under the influences of the drugs. Since the mode of the inhibitory action of toyocamycin has been shown to resemble that of formycin (SUHADOLNIK, 1970), tests were done with formycin and formycin B.

As the results are summarized in Table 1, these two agents showed the analogous effect on RNA and protein synthesis. The incorporation of ^3H -uracil and ^{14}C -adenine in the drug experiments was inhibited 25-29% and 59-63% that of the control, respectively, whereas no incorporation of ^{14}C -amino acids was affected within at least 5 hr after the administration of the drugs. The above results certainly indicate that RNA synthesis in the morphogenetic development is blocked by formycin and formycin B, while protein synthesis is not influenced for some time (at least 5 hr in the presence of the drugs). In addition, larger inhibition of ^{14}C -adenine incorporation relative to ^3H -uracil incorporation will permit us to infer that the nucleoside antibiotics are incorporated into newly synthesized RNA, thereby the synthesis rate of RNA will be reduced.

Sedimentation analysis of RNA synthesized in the presence of formycin or formycin B: In the experiments shown in Fig. 4, RNA was prepared from cells labeled doubly with ^3H -uracil and ^{14}C -adenine for 30 min at interphase of morphogenesis in the presence or absence of formycin or formycin B and subjected to sucrose gradient centrifugation, followed by the examination of poly(A)-containing RNA by the poly(U) filter method (SHELDON *et al.*, 1972; FIRTEL *et al.*, 1972). The radioactive RNA in the control (no drug) experiment exhibits two large peaks of material sedimenting at approximately 30 S and 22 S, a small peak of 5 S and tRNA, and some heterodisperse material sedimenting in the region from 6 S to 20 S (Fig. 3 A). The labeled 30-S and 22-S species are probably rRNA precursor molecules previously reported (IWABUCHI *et al.*, 1971), although the rate of synthesis of rRNA in interphase amoebae has been shown to be considerably low, as compared with that in vegetatively growing amoebae (MIZUKAMI and IWABUCHI, 1970).

Examination of poly(A)-containing RNA showed that about 32-36% of total ^3H - or ^{14}C -labeled RNA was absorbed to poly(U) filters and most of the

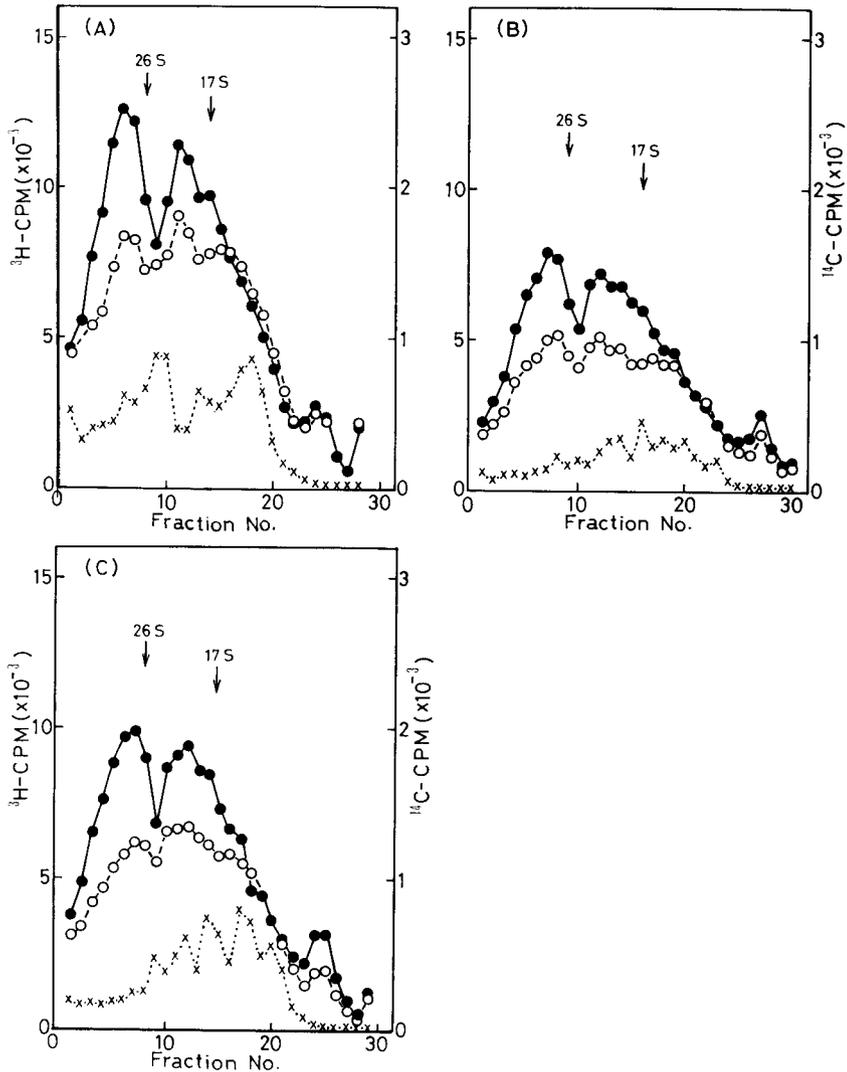


Fig. 4. Effect of formycin and formycin B on RNA synthesis.

The suspension of amoebae (1×10^9 cells/ml) at late interphase in TKM buffer containing $150 \mu\text{g/ml}$ streptomycin was divided into three equal batches of 1.5 ml each. To two batches was added formycin or formycin B at the concentration of $200 \mu\text{g/ml}$ of culture. One batch was used as the control. After incubation for 1 hr at 23°C with a vigorous shaking, each of culture received $200 \mu\text{Ci}$ of ^3H -uracil and $27 \mu\text{Ci}$ of ^{14}C -adenine and was immediately adjusted to 2 ml in total and to $200 \mu\text{g/ml}$ at the final concentration of the drugs. After an additional incubation for 30 min, the cells were harvested and RNA was extracted from whole cells. The RNA was dissolved in 1.5 ml of TN buffer containing

0.5% SDS and analyzed on 34 ml of 10% to 25% SDS-containing sucrose gradients. Centrifugation was at 26,000 rpm for 12 hr at 18°C in a Hitachi RPS 27 rotor. The gradients were fractionated, the A_{260} and acid-precipitable radioactivity for each fraction determined using 0.3 ml portion, and the rest (1 ml) passed through poly(U) filters in binding buffer. The filters were then extensively washed with binding buffer minus the sodium phosphate, followed by 0.3M ammonium acetate in 50% ethanol, dried and counted. Since total A_{260} of the formycin and formycin B experiments was respectively 1.13 and 1.20 that of the control, all of the radioactivity of the gradient fractions was multiplied by 0.89 and 0.83 in the formycin and formycin B experiments, respectively.

(A), control; (B), formycin (200 $\mu\text{g/ml}$); (C), formycin B (200 $\mu\text{g/ml}$). ●—●, Acid-precipitable ^3H -radioactivity; ○—○, acid-precipitable ^{14}C -radioactivity; ×-----×, poly(U) binding ^3H -radioactivity.

RNA bound to poly(U) filters sedimented in the regions from 8 S to 20 S and from 24 S to 30 S. It has been reported in vegetative *Dictyostelium* amoebae that most of the heterodisperse RNA with the sedimentation velocity between 6 S and 16 S (mean S_{20} , 13–14 S) contains a sequence of poly(A) and is certainly mRNA and HnRNA (FIRTEL *et al.*, 1972; FIRTEL and LODISH, 1973; JACOBSON *et al.*, 1974). Therefore, the above 8–20-S RNA bound to poly(U) filters may be presumably poly(A)-containing mRNA and/or HnRNA. It is unknown, however, whether poly(A)-containing material sedimenting between 24 S and 30 S is mRNA or HnRNA synthesized specifically in interphase amoebae or if it is artifactual products.

As shown in Figs. 4 B and 4 C, the sedimentation profiles of RNA labeled in the presence of formycin or formycin B essentially resemble that of the control but total ^3H -radioactivity of the RNA decreased about 29% (in the formycin experiment) and 14% (in the formycin B experiment) that of the control. Of total labeled RNA, about 22–23% and 28–35%

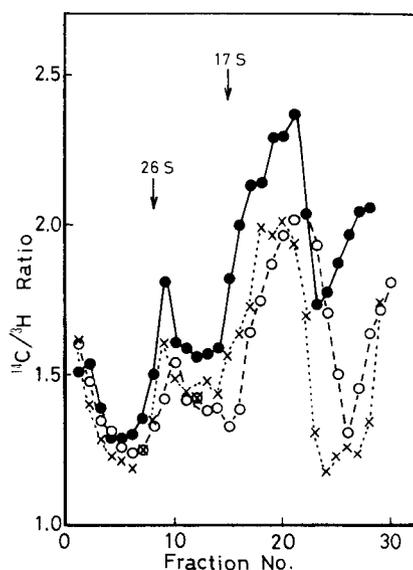


Fig. 5. $^{14}\text{C}/^3\text{H}$ ratio of TCA-precipitable radioactivity in the gradient fractions after sucrose gradient centrifugation of RNA labeled doubly with ^{14}C -adenine and ^3H -uracil in the presence or absence of formycin or formycin B. The $^{14}\text{C}/^3\text{H}$ ratio was obtained from the sedimentation patterns shown in Fig. 4. The details of the experiment are described in the legend to Fig. 4.

●—●, control; ○—○, formycin; ×-----×, formycin B.

were bound to poly(U) filters in the formycin and formycin B experiments, respectively. In the drug experiments, the sedimentation pattern of the RNA capable of binding to poly(U) filters was generally similar to that obtained in the control except for the lack of material sedimenting in the 24-30-S region. These results seem to suggest apparently that both formycin and formycin B arrest syntheses of all classes of RNA species in similar proportion.

The ratio of radioactivity between ^{14}C -adenine and ^3H -uracil incorporated into TCA-precipitated material was computed for every fractions in the sedimentation patterns shown in Fig. 4 (Fig. 5). Comparatively high values of the $^{14}\text{C}/^3\text{H}$ ratio were obtained in the region between 6 S and 17 S, this being presumably due to the existence of poly(A)-containing RNA in this region (FIRTEL *et al.*, 1972; FIRTEL and LODISH, 1973; JACOBSON *et al.*, 1974). In the drug experiments, there is a general tendency for the $^{14}\text{C}/^3\text{H}$ ratio to reduce. In conformity with the evidence obtained in the incorporation test of ^3H -uracil and ^{14}C -adenine into whole cells, the above result certainly indicates that these nucleoside antibiotics are incorporated into RNA chain, as reported by some investigators (SUHADOLNIK *et al.*, 1967; IKEHARA *et al.*, 1968; WARD *et al.*, 1969).

Discussion

It has been reported that formycin (HORI *et al.*, 1964; TAKEUCHI *et al.*, 1966; ISHIZUKA *et al.*, 1968a) and toyocamycin (MATSUOKA, 1960; SANEYOSHI *et al.*, 1965) have growth-inhibitory effects on various animal tumors, fungi and viruses. The antibiotics have also been shown to be incorporated in limited amounts into RNA and to cause inhibition of RNA synthesis (SUHADOLNIK *et al.*, 1967; IKEHARA *et al.*, 1968; WARD *et al.*, 1969). DALIX *et al.* (1971) showed *in vitro* RNA synthesizing system using formycin triphosphate and *E. coli* RNA polymerase that the drug derivative decreases the rate of chain initiation and blocks the release of newly synthesized RNA chain from DNA template.

The results obtained in the present study indicated that formycin and toyocamycin, but not formycin B, inhibit the morphogenetic development of *D. discoideum* amoebae, although their inhibitory effect does not appear immediately after treatment with the agents. Examinations of incorporation of radioactive RNA and protein precursors into whole cells suggested that formycin and formycin B have also inhibitory effects on total RNA synthesis, while not affecting total protein synthesis at least 5 hr after the administration of the drugs. In addition, the sedimentation analysis and poly(U)-binding assay of the RNA synthesized under the influences of formycin or formycin

B could afford evidence that these nucleoside antibiotics inhibit syntheses of all RNA species including mRNA.

Previously we showed in *D. discoideum* that protein synthesis occurs during overall process of morphogenesis and the protein synthesis required for either stage of the morphogenetic development will be directed by mRNA synthesized in the preceding but not the same stage (MIZUKAMI and IWA-BUCHI, 1970). Thus, the results obtained in the formycin experiment will probably be explained in the light of the above assumption; some of proteins synthesized at interphase of morphogenesis are directed by mRNA formed in the stage prior to interphase. Similar interpretation will be given to the result of the toyocamycin experiment, though the effect of the drug on RNA and protein synthesis was not tested, because toyocamycin has been shown to closely resemble formycin in respect to not only the molecular structure but also the mechanism of its inhibitory action on RNA synthesis (SUHADOLNIK, 1970)

As mentioned above, formycin did not cause complete inhibition of morphogenesis. A possible explanation for this might be that a substantial quantity of formycin incorporated into cells is probably converted to formycin B which was ineffective for inhibiting morphogenesis. In fact, evidence has been presented in Ehrlich carcinoma and *E. coli* that formycin is converted to formycin B by its deamination (UMEZAWA *et al.*, 1967) which has been shown not to prevent the growth of bacteria, fungi and some animal tumors (UMEZAWA *et al.*, 1965). It remains obscure, however, why formycin B gave no influence on morphogenesis, despite the fact that the drug blocked synthesis of RNA. Very recently, BRACKENBURY *et al.*, (1974) reported in *D. discoideum* strain DdB that formycin B does not inhibit morphogenetic development but is capable of changing normal program of morphogenesis under certain conditions. It should be noted here that formycin B is easily converted to biologically inert oxyformycin B which is assumed to be an end product in the detoxification mechanism of formycin (ISHIZUKA *et al.*, 1968 b).

The chemical structure of the nucleoside antibiotics used here is similar to that of cordycepin (3'-deoxyadenosine) which has been recently demonstrated to inhibit the synthesis of poly(A) present in the 3'-end of HnRNA and mRNA (PENMAN *et al.*, 1970; DARNELL *et al.*, 1971; MENDECKI *et al.*, 1972; NAKAZATO *et al.*, 1974). Formycin or formycin B, like cordycepin, appears to be certainly incorporated into newly synthesized RNA. However, it seems unlikely that the drugs selectively block synthesis of poly(A), since the proportion of poly(U)-binding material in total labeled RNA was not greatly

affected by the drug treatment. Our results were different from those obtained by ABELSON and PENMAN (1973), who observed with HeLa cells that formycin selectively inhibits synthesis of 4-S and 5-S RNA.

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