Biochemical Pharmacology, Vol. 23, pp. 395–402. Pergamon Press. 1974. Printed in Great Britain.

EFFECT OF ACTINOMYCIN D ON UPTAKE AND INCORPORATION OF THYMIDINE AND HYPOXANTHINE INTO THE ACID-SOLUBLE AND ACID-INSOLUBLE FRACTIONS OF RAT HEPATOMA CELLS GROWN IN CULTURE

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(*Received* 30 *April* 1973; *accepted* 10 *July* 1973)

Abstract—Preincubation of MH_1C_1 rat hepatoma cell cultures with actinomycin D between 0·01 μg/ml and 10·0 μg/ml gives a dose-dependent increase in the uptake of thymidine into the acid-soluble fraction up to 400 per cent of controls; the same increase is found in the acid-insoluble fraction. The effect is detectable after 5 min incubation, but is only fully developed after 1–2 hr treatment with the drug. The stimulation could not be blocked by cycloheximide. Preincubation with actinomycin D has little effect on uridine uptake compared to that of thymidine; actinomycin D 1·0 μg/ml after 2 hr increases uridine uptake to 131 per cent of controls. In contrast the uptake of hypoxanthine is inhibited by actinomycin D, 50 per cent inhibition is seen at 0·75 μg/ml. The apparent K_m for the thymidine uptake is $5{\cdot}9 \times 10^{-6}$ M; actinomycin D pretreatment altered the V_{max} of the reaction but did not change the apparent K_m. The apparent K_m for the hypoxanthine uptake is $5{\cdot}0 \times 10^{-6}$ M; actinomycin D pretreatment gave an apparently noncompetitive inhibition. Actinomycin D does not change the activity of thymidine kinase in homogenates of the cells.

INTRODUCTION

ACTINOMYCIN D inhibits DNA dependent RNA synthesis by binding to double helical DNA.[1,2] There is a specificity of deoxyguanosine residues for the actinomycin D binding to DNA.[3] A model has been proposed which states that the phenazone ring system of actinomycin D intercalates into the DNA helix, while deoxyguanosine residues interact with both cyclic pentapeptides of actinomycin D through hydrogen bonds.[4]

While studying the inhibitory effects of chlorpromazine and SKF 525-A on the uptake and incorporation of some nucleic acid precursors, it was also found that blockade of nucleic acid synthesis with actinomycin D plus cytosine arabinoside preincubation led to an inhibition of hypoxanthine uptake by 64 per cent into the acid soluble fraction of cultured MH_1C_1 rat hepatoma cells.[5] Previous work has shown that actinomycin D does not affect uridine transport in Novikoff rat hepatoma cells[6] or HeLa cells.[7] The present study was started in order to investigate the underlying mechanism for the inhibitory effect of actinomycin D plus cytosine arabinoside. It led to the observation that actinomycin D alone stimulates thymidine uptake but inhibits hypoxanthine uptake in MH_1C_1 cell cultures. The effect of actinomycin D on the thymidine and hypoxanthine uptake and incorporation is the main topic of the present investigation.

METHODS AND MATERIALS

Methods of cell culture. The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco's modified Eagle's medium supplemented with 15 per cent horse serum, 2·5 per cent fetal calf serum, and antibiotics as described previously.[8]

Cell culture experiments. All experiments were performed in ordinary growth medium with serum. Replicate subcultures were incubated with ^{3}H-thymidine, ^{14}C-hypoxanthine or ^{3}H-uridine alone or after preincubation for various lengths of time and continuous presence of drugs to be tested at 37° for up to 15 min. One or two flasks in each of the groups in the different experiments served as correction for zero time uptake. At the end of the incubations, the medium was poured off and the cells were immediately washed four times with 5 ml of an ice-cold barbital–NaCl-buffer pH 7·4. The cells were then taken off with 3·0 ml ice-cold 0·02% EDTA–barbital–NaCl-buffer pH 7·4, precipitated with 3·0 ml ice-cold perchloric acid (PCA), and allowed to stand on ice for 1 hr. After centrifugation for 10 min at 12,000 *g* and 4°, duplicate aliquots of the supernatant were counted in 15 ml BBOT scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer, Model 3003. The cold PCA-insoluble material was washed twice with 0·5 M PCA, once with 1% potassium acetate, once with ethanol, once with ethanol–ether (1:1 v/v) and once with ether. RNA was solubilized with 0·3 N NaOH at 37° overnight, and the samples were then acidified with a cold mixture of 6 N HCl and 1 M PCA. Aliquots of the supernatant, containing RNA, were counted in 15 ml BBOT in the hypoxanthine and uridine experiments. The pellet was heated with 0·5 M PCA to 90° for 20 min, and aliquots of the supernatant, containing extracted DNA, were counted in 15 ml BBOT in the thymidine experiments. The remaining pellet was dissolved in 1 N NaOH at 37° overnight for estimation of protein content. Counts were corrected for quenching, and related to the total cellular protein content determined according to Lowry *et al.*[9] using bovine albumin as standard.

Assay of thymidine kinase. Thymidine kinase activity *in vitro* was assayed as described by Mizel and Wilson[7] with paper chromatography of samples from the reaction mixtures in the system described by Osnes *et al.*[10] Cells from replicate flasks without or after preincubation with actinomycin D 1·0 g/ml for 2 hr were washed once with an ice-cold barbital–NaCl-buffer pH 7·4, and removed with ice-cold 0·02% EDTA–barbital–NaCl-buffer pH 7·4. The cells were spun down, resuspended in 1·0 ml of KCl 0·15 M, mercaptoethanol 3·0 mM, and Tris–HCl 10 mM, pH 8·5 and sonicated with a MSE Sonicator for 20 sec. The sonicate was centrifugated at 10,000 *g* for 10 min and the supernatant was used for assay. Assay mixtures contained ATP 9·0 mM, 3-phosphoglycerate 7·5 mM, $MgCl_2$ 9·0 mM, Tris–HCl 50 mM, pH 8·5, 100 μl of cell supernatant (approx 450 μg protein), and ^{3}H-thymidine 12·5 μCi/ml, $2{\cdot}5 \times 10^{-7}$ M, in the absence or presence of actinomycin D 1·0 μg/ml in a total volume of 400 μl. Glass-stoppered tubes were incubated in a shaking water bath at 37°, and the reactions were stopped after 0, 5 and 10 min in a boiling water bath for 2 min. Particulate material was removed by centrifugation at 1500 g for 5 min. Aliquots (20 μl) were applied to strips (4 × 46 cm) of Whatman No. 1 paper and descending chromatography was carried out in a system containing 96% ethanol:2 M ammonia:2 M acetic acid (100:19:21), pH 7·15, for 15 hr at 19° together with unlabelled thymidine and thymidine-5′-monophosphate (TMP). Spots were visualized

by u.v. light, the thymidine and TMP spots were cut out, eluted with 0·5 ml 0·5 N HCl in 15 ml BBOT scintillation fluid for 10 min and counted in the liquid scintillation spectrometer. The R_f values for thymidine and TMP in this system were 0·71 and 0·33, respectively. The radioactivity of the zones that chromatographed as unlabelled thymidine and TMP consisted of 98·5 per cent of the total radioactivity of the chromatograms.

Radioisotopes and chemicals. ^{3}H-6-thymidine (sp. act. 28 Ci/mmole, Amersham), ^{14}C-8-hypoxanthine (sp. act. 59 mCi/mmole, Amersham) and ^{3}H-6-uridine (sp. act. 30 Ci/mmole, Amersham) were purchased through Norsk Atominstitutt. Actinomycin D was used either as the crystalline form of the drug (Serva) or as Cosmegen Lyovac (Merck, Sharp & Dohme). Unlabelled thymidine, thymidine-5′-monophosphate (TMP, calcium salt), uridine, hypoxanthine, adenosine-5′-triphosphate (ATP, disodium salt), D(−) 3-phosphoglycerate (sodium salt), cytosine arabinoside hydrochloride and cycloheximide were all obtained from the Sigma Co.

RESULTS

Preincubation of MH_1C_1 cells with actinomycin D 1·0 μg/ml for 2 hr affected the uptake and incorporation of thymidine, uridine and hypoxanthine (Table 1).

TABLE 1. EFFECT OF ACTINOMYCIN D ON THE UPTAKE AND INCORPORATION OF THYMIDINE, URIDINE AND HYPOXANTHINE INTO ACID-SOLUBLE AND ACID-INSOLUBLE FRACTIONS OF MH_1C_1 CELL CULTURES

Precursor added	Control		With actinomycin D	
	PCA soluble (counts/min/mg $\times 10^3$)	PCA insoluble (counts/min/mg $\times 10^2$)	PCA soluble (counts/min/mg $\times 10^3$)	PCA insoluble (counts/min/mg $\times 10^2$)
Thymidine	6·73 ± 0·52	1·89 ± 0·29	18·45 ± 2·53	5·26 ± 0·37
Uridine	14·26 ± 0·56	22·07 ± 3·44	18·61 ± 0·89	2·05 ± 0·18
Hypoxanthine	14·32 ± 0·27	12·00 ± 1·48	5·58 ± 0·67	0·47 ± 0·08

Replicate subcultures were preincubated without or with crystalline actinomycin D 1·0 μg/ml for 2 hr and then incubated with ^{3}H-thymidine 10^{-6} M, 0·2 μCi/ml; ^{3}H-uridine 10^{-6} M, 0·2 μCi/ml; or ^{14}C-hypoxanthine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° without or in the presence of actinomycin D. Values are means ± S.D. from three flasks.

Thymidine uptake into the soluble fraction increased to 274 per cent of controls; this increase was also found in the acid-insoluble fraction (279 per cent). In ten separate experiments actinomycin D 1·0 μg/ml as either Cosmegen or the crystalline compound stimulated thymidine uptake to an average of 238·4 ± 39·3 per cent of controls. Uridine uptake was much less affected than that of thymidine, with an increase of 31 per cent compared to controls, whereas incorporation of uridine under these experimental conditions was inhibited to 9 per cent of controls. Table 1 shows that actinomycin D, in contrast to the stimulated thymidine uptake, inhibited hypoxanthine uptake into the acid-soluble fraction to 39 per cent of controls; hypoxanthine incorporation into acid-insoluble material was 5 per cent of controls. The effect on hypoxanthine uptake noted earlier[5] was achieved by preincubation for 1 hr with both actinomycin D and cytosine arabinoside; in the present investigation experiments with cytosine arabinoside alone reduced hypoxanthine uptake to 80 per

cent of controls after preincubation with the drug at 10 μg/ml for 2 hr. Cytosine arabinoside had little effect on the uptake of thymidine and uridine, both were reduced to 88 per cent of controls.

Figure 1 shows the effect of various periods of preincubation with actinomycin D 1·0 μg/ml on the thymidine and hypoxanthine uptake into acid-soluble fractions, both uptake experiments lasting 15 min. The effect of actinomycin D on both thymidine and hypoxanthine uptake was apparent after the 15 min uptake period without previous preincubation, but was not fully developed until after 1–2 hr preincubation. An experiment measuring uridine uptake showed a 10 per cent increase after 15 min treatment with actinomycin D compared with a 31 per cent increase after 2 hr. After

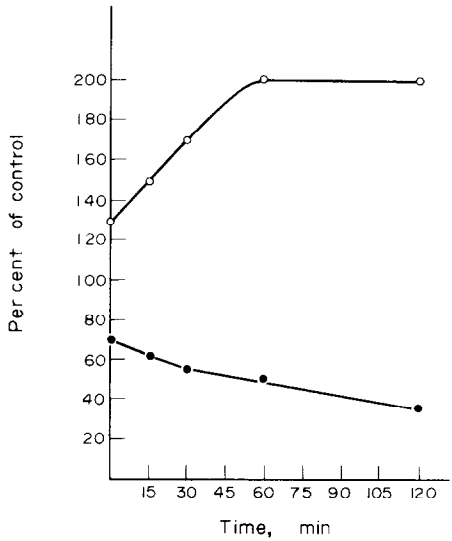

FIG. 1. Time course of the effect of actinomycin D preincubation on the uptake of thymidine and hypoxanthine into the soluble fraction of MH_1C_1 cell cultures. Replicate subcultures were preincubated with actinomycin D (Cosmegen) 1·0 μg/ml for various time intervals and then incubated with ^{3}H-thymidine 10^{-6} M, 0·2 μCi/ml or ^{14}C-hypoxanthine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° in the presence of actinomycin D. Values are means of results from duplicate flasks and are expressed as per cent of values from control flasks to which no actinomycin D had been added. Ordinate per cent of controls, abscissa preincubation time in minutes. (○) Thymidine, (●) hypoxanthine.

replicate cultures had been pretreated with actinomycin D 1·0 μg/ml for 2 hr, three 1-min washes of some of the cultures with medium without actinomycin D followed by incubation with thymidine for 15 min without actinomycin D was compared to uptake in cultures with continuous presence of actinomycin D. Actinomycin D-stimulated thymidine uptake was not reduced by the washing procedure.

A time course study of the uptake of thymidine into the acid-soluble and acid-insoluble fractions of the rat hepatoma cells without or in the presence of actinomycin D 1·0 μg/ml is given in Fig. 2. The increase in the uptake of thymidine into both fractions was evident after 5 min incubation.

The rapid onset of action of actinomycin D on the uptake of thymidine makes the involvement of protein synthesis as the underlying mechanism for the stimulation rather improbable. Table 2 gives the result of preincubation of the cells for 2 hr

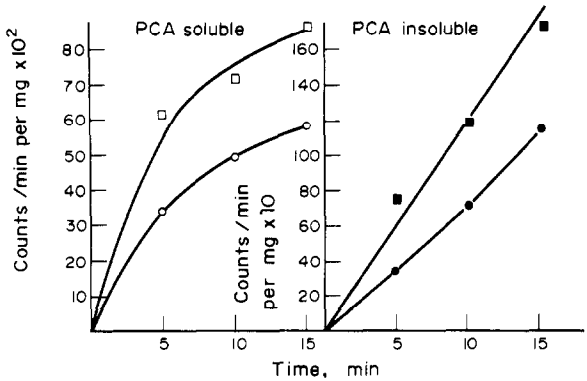

FIG. 2. Time course of the effect of actinomycin D on the thymidine uptake and incorporation into acid-soluble and acid-insoluble fractions of MH_1C_1 cell cultures. Replicate subcultures were incubated with ^{3}H-thymidine 10^{-6} M, 0·2 μCi/ml for 5, 10 and 15 min at 37° without or in the presence of crystalline actinomycin D 1·0 μg/ml. Each point represents the mean of values from duplicate flasks. Ordinate counts/min and mg cell protein, abscissa, time in minutes. Open symbols acid-soluble fraction, filled symbols acid-insoluble fraction. (○) Controls, (□) with actinomycin D.

TABLE 2. EFFECT OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON THE UPTAKE OF THYMIDINE INTO THE ACID-SOLUBLE FRACTION IN MH_1C_1 CELL CULTURES

Control (counts/min/mg × 10^2)	With cycloheximide (counts/min/mg × 10^2)	With actinomycin D (counts/min/mg × 10^2)	With actinomycin D + cycloheximide (counts/min/mg × 10^2)
63·64 ± 8·32	64·41 ± 5·36	110·75 ± 2·71	123·73 ± 9·58

Replicate subcultures were preincubated without or with cycloheximide 15 μg/ml, crystalline actinomycin D 1·0 μg/ml, or cycloheximide plus actinomycin D for 2 hr and then incubated with ^{3}H-thymidine 10^{-6} M, 0·2 μCi/ml for 15 min at 37° without or in the presence of cycloheximide and/or actinomycin D. Values are means ± S.D. from three flasks.

with cycloheximide and actinomycin D separately or together on the uptake of thymidine. Cycloheximide alone did not alter the uptake of thymidine, or reduce the stimulation of thymidine uptake after actinomycin D. Cycloheximide at the concentration used (15 μg/ml) inhibited ^{14}C-alanine incorporation to 13 per cent of controls after 2 hr preincubation and 2 hr incubation together with the radioactive amino-acid, whereas actinomycin D applied for the same length of time reduced alanine incorporation to 81 per cent of controls.

A dose–response curve of the actinomycin D effects on thymidine and hypoxanthine uptake is given in Fig. 3. Doses from 0·01 μg/ml stimulated thymidine uptake and inhibited hypoxanthine uptake with linearity of the LDR plots up to a concentration of 10·0 μg/ml actinomycin D. Higher doses did not significantly increase the uptake of thymidine. Fifty per cent inhibition of hypoxanthine uptake was seen at 0·75 μg/ml actinomycin D.

The double-reciprocal plot of the 5-min uptake of thymidine into the acid-soluble fraction of MH_1C_1 cells was linear (Fig. 4), giving an apparent K_m of 5·9 × 10^{-6} M and a V_{max} of 6·7 nmoles/mg protein an hr. Preincubation with actinomycin D 1·0 μg/ml for 2 hr increased the maximal velocity of the reaction, but the apparent

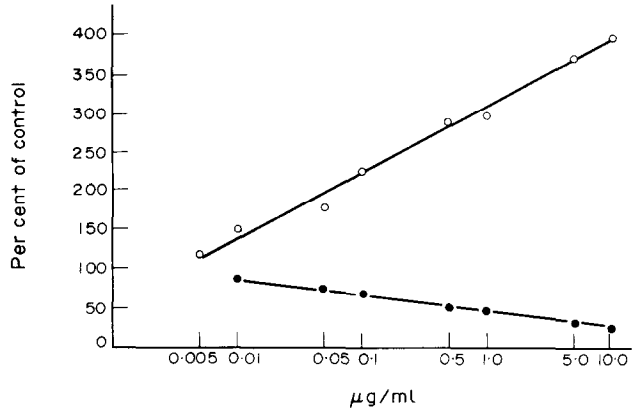

FIG. 3. Log dose–response plot of the actinomycin D effect on the uptake of thymidine and hypoxanthine into acid-soluble fraction of MH_1C_1 cell cultures. Replicate subcultures were preincubated with crystalline actinomycin D in doses from 0·005 to 10·0 μg/ml for 2 hr and then incubated with ^{3}H-thymidine 10^{-6} M, 0·2 μCi/ml or ^{14}C-hypoxanthine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° in the presence of actinomycin D. Values are means of results from duplicate flasks for each concentration of actinomycin D and are expressed as per cent of values from control flasks to which no actinomycin D has been added. Ordinate per cent of controls, abscissa log dose in μg/ml. (○) Thymidine, (●) hypoxanthine.

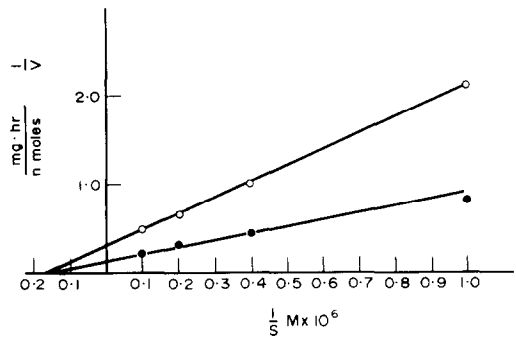

FIG. 4. Double-reciprocal plot of the actinomycin D effect on the uptake of thymidine into the acid-soluble fraction in MH_1C_1 cell cultures. Replicate subcultures were incubated with ^{3}H-thymidine 1·0, 2·5, 5·0 or 10·0 μM, 84 counts/min pmole, for 15 min at 37° without or after preincubation with crystalline actinomycin D 1·0 μg/ml for 2 hr. Each point represents the mean of values from three flasks. Ordinate inverse of the velocity in mg and hr per nmoles, abscissa inverse of the substrate concentration in 10^6 M. (○) Control, (●) with actinomycin D.

K_m remained unchanged. Uptake of hypoxanthine also gave a linear double-reciprocal plot (Fig. 5), the apparent K_m for this reaction was $5{\cdot}0 \times 10^{-6}$ M; whereas the V_{max} (0·15 nmoles/mg protein an hour) was considerably less than that for thymidine uptake. The inhibition of hypoxanthine uptake by actinomycin D pre-treatment followed a non-competitive pattern yielding the same K_m as without the drug.

Table 3 shows the effect of actinomycin D on the activity of thymidine kinase *in vitro*. The phosphorylation of ^{3}H-thymidine was unaffected by actinomycin D 1·0 μg/ml. At the thymidine concentration used 43·4 per cent of the substrate was phosphorylated after 5 min and 63·5 per cent after 10 min.

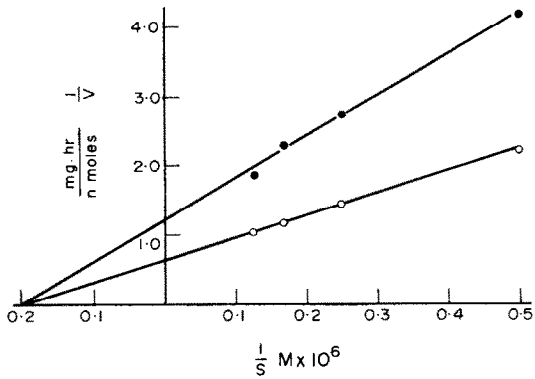

FIG. 5. Double-reciprocal plot of the actinomycin D effect on the uptake of hypoxanthine into the acid-soluble fraction of MH_1C_1 cell cultures. Replicate subcultures were incubated with ^{14}C-hypoxanthine 2·0, 4·0, 6·0 or 8·0 μM, 621 counts/min per pmole, for 15 min at 37° without or after preincubation with crystalline actinomycin D 1·0 μg/ml for 2 hr. Each point represent the mean of values from three flasks. Ordinate inverse of the velocity in mg and hour per nmoles, abscissa inverse of the substrate concentration in 10^6 M. (○) Control, (●) with actinomycin D.

TABLE 3. EFFECT OF ACTINOMYCIN D ON THE ACTIVITY OF THYMIDINE KINASE *in vitro* PREPARED FROM MH_1C_1 CELLS IN CULTURE

Control		With actinomycin D	
5 min (nmoles/μg protein × 10^{-5})	10 min (nmoles/μg protein × 10^{-5})	5 min (nmoles/μg protein × 10^{-5})	10 min (nmoles/μg protein × 10^{-5})
5·49 ± 0·17	8·04 ± 0·18	5·47 ± 0·12	8·73 ± 0·36

Replicate subcultures were preincubated without or with actinomycin D 1·0 μg/ml for 2 hr and assayed as in methods with incubation with ^{3}H-thymidine 12·5 μCi/ml, 2·5 × 10^{-7} M for 5 and 10 min at 37° without or in the presence of actinomycin D. Values are means ± S.D. from three parallel estimations.

DISCUSSION

The stimulation of thymidine uptake and inhibition of hypoxanthine uptake brought about by actinomycin D preincubation reported here seems to be independent of its well known action as inhibitor of RNA synthesis, as its effect increased at doses above those which almost completely blocked uridine incorporation. These effects of actinomycin D are also independent of protein synthesis. Schwyzer *et al.*[11] have recently reported that actinomycin D enhances the lipolytic response of isolated rat lipocytes towards ACTH, an effect independent of the antibiotic properties of actinomycin D. This enhancement was roughly paralleled by an increase in the number of ACTH binding sites in the cell sample. This lipolytic effect could also be seen after treatment with actinocin, the chromophore of actinomycin D that has no antibiotic properties. A possible membrane effect of actinomycin D on the rat intestine has been reported,[12] where it prevents the decrease in activity of liver tyrosine aminotransferase after a glucose load, most likely secondary to inhibition of glucose absorption. It was suggested, however, that this could be due to damage of the intestinal epithelial cells brought about by the actinomycin D treatment. The effect of actinomycin D on thymidine uptake in the rat hepatoma cell cultures could

not readily be reversed by washing, as could the inhibition of alanine incorporation caused by chlorpromazine and SKF 525-A.[13]

Plagemann and Roth[14] have shown that the rate of phosphorylation of uridine and choline by Novikoff rat hepatoma cells was limited by the rate of transport of the substrates into the cells, and these transport reactions seemed to be processes distinct from the phosphorylating reactions. Uridine kinase and choline kinase seemed to be present in excess, and upon entry uridine and choline were rapidly phosphorylated and effectively trapped in the cells. In the present study the uptake of thymidine and hypoxanthine into the acid soluble fraction of MH_1C_1 cells followed Michaelis–Menten kinetics. Actinomycin D pretreatment did not alter the apparent K_m of the respective reactions, nor did actinomycin D have any effect on the activity of thymidine kinase *in vitro*.

The present findings presumably are not related to the "pleiotypic response" defined by Hershko *et al.*[15] They did not include the uptake of thymidine as part of the pleiotypic response; they hypothesized that cycloheximide should inhibit the synthesis of the pleiotypic mediator; and they found that actinomycin D had no effect on the altered growth conditions.

Actinomycin D is being extensively used as an experimental tool for its effect as an inhibitor of RNA synthesis. The results reported here indicate that actinomycin D has effects on other cellular mechanisms, a fact that should be considered when interpreting results from experiments using the inhibitor. Altered rates of entry of precursors of nucleic acids may alter the rates of their subsequent incorporation into macromolecules, without necessarily indicating a direct change in the rates of synthesis of the latter.

Acknowledgements—This study was supported by a grant from the Norwegian Research Council for Science and Humanities (NAVF). The excellent technical assistance of Mrs. Birgitta Baardsen is gratefully acknowledged. I am indebted to Drs. Osnes, Christoffersen and Rugstad for valuable discussions.

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