Biochemical Pharmacology, Vol. 23, pp. 3045–3051. Pergamon Press, 1974. Printed in Great Britain.

ACTINOMYCIN D AND PURINE TRANSPORT IN CULTURED RAT HEPATOMA CELLS

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(*Received* 29 *November* 1973; *accepted* 6 *March* 1974)

Abstract—Actinomycin D 10^{-6} M inhibits the uptake of hypoxanthine into the acid-soluble fraction of MH_1C_1 rat hepatoma cells grown in culture to an average of 20 per cent of controls. Cycloheximide and actinomycin D have additive inhibitory effects on hypoxanthine uptake, whereas the uptake of adenine remains unaffected by actinomycin D. Actinocin, the chromophore of actinomycin D, does not inhibit hypoxanthine uptake. The uptake of guanine by the cells is very low compared with that of hypoxanthine or adenine. Actinomycin D does not change the activity of hypoxanthine-guanine phosphoribosyltransferase in homogenates of the cells. Competition experiments with homogenates of the cells support the view that hypoxanthine and guanine are substrates for the same phosphoribosyltransferase, whereas adenine is converted to its corresponding nucleoside phosphate by a separate enzyme. Excess adenine inhibits the cellular uptake of hypoxanthine, whereas excess hypoxanthine does not alter the uptake of adenine.

ACTINOMYCIN D has been shown to stimulate the uptake of the pyrimidine nucleoside thymidine in MH_1C_1 rat hepatoma cells grown in culture,[1] presumably independently of protein and RNA synthesis, and without affecting the activity of thymidine kinase *in vitro*. In contrast the antibiotic inhibited the uptake of the purine base hypoxanthine by the cells.[1] These two effects of actinomycin D on nucleic acid precursor uptake could also be demonstrated in cultured human skin epithelial cells, although less pronounced than in the rat hepatoma cells.[2]

Purine bases are taken up by mammalian cells from an external medium.[3–5] Human erythrocytes are highly permeable to adenine[3] and hypoxanthine;[3,4] rabbit polymorphonuclear leukocytes have been shown to actively take up adenine, guanine and hypoxanthine, but not xanthine.[5]

There are two distinct purine phosphoribosyltransferases in animal cells,[6–8] hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) which catalyzes the conversion of hypoxanthine or guanine to inosine monophosphate (IMP) or guanosine monophosphate (GMP) respectively, and adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) which couples adenine with 5-phosphoribosyl 1-pyrophosphate (PRPP) to form adenosine monophosphate (AMP).

This paper describes the cellular uptake and enzymatic phosphoribosyl transfer of certain purine bases in the MH_1C_1 cells, and the effect of actinomycin D on these processes.

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

(Gibco), 2·5 per cent foetal calf serum (Gibco), and antibiotics (penicillin, streptomycin and nystatin) as described previously.[1]

Cell culture experiments. All experiments were performed in ordinary growth medium with serum. Replicate subcultures were incubated with ^{14}C-hypoxanthine, ^{14}C-adenine or ^{14}C-guanine for 15 min at 37° alone or after preincubation for 2 hr and continuous presence of drugs to be tested. One flask in each of the groups of the different experiments was used to correct for zero time uptake. At the end of the incubations, the medium was poured off and the cells were immediately washed four times with 5·0 ml of an ice-cold barbital–NaCl-buffer pH 7·4. The cells were then suspended in 3·0 ml ice-cold 0·02 % EDTA–barbital–NaCl buffer pH 7·4 and precipitated with 3·0 ml ice-cold 20% trichloroacetic acid (TCA) 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 protein precipitate was washed twice with 10% TCA and twice with ethanol–ether (1 : 1 v/v) and dissolved in 1 N NaOH at 37° overnight for estimation of protein content. Counts were corrected for quenching and related to protein content determined according to Lowry *et al.*,[9] using bovine albumin (Sigma) as standard.

Assay of HGPRT and APRT. Hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase were assayed *in vitro* by a modification of the procedure described by Krenitsky *et al.*[8] 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 for 2 hr were washed once with ice-cold barbital–NaCl buffer pH 7·4 and suspended in ice-cold 0·02 % EDTA–barbital –NaCl buffer pH 7·4. The cells were spun down, resuspended in KCl 0·15 M, mercaptoethanol 3·0 mM and Tris–HCl 10 mM pH 7·4 and sonicated with a MSE Sonicator for 20 sec. The sonicated cells were centrifugated at 40,000 *g* for 20 min and the supernatant was used for assay. Assay mixtures contained 5-phosphoribosyl 1-pyrophosphate (PRPP) 0·5 mM, $MgSO_4$ 5·0 mM, Tris–HCl 50 mM pH 7·4, 50 μl cell supernatant (30–50 μg protein) and ^{14}C-labelled purine bases 0·3125 μCi/ml, 5×10^{-6} M in the absence or presence of chemicals to be tested in a total volume of 200 μl. Test-tubes were incubated in a shaking water-bath at 37°, and the reactions were stopped after 0, 5 and 10 min with 50 μl 4 N formic acid. 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, by vol.), pH 7·15 for 15 hr at 19°. Unlabelled purines and their corresponding nucleoside phosphates were applied to the same strips. Spots were visualized by u.v. light, the purine base and monophosphate spots were cut out, eluted with 0·5 ml 0·5 N HCl in 15 ml BBOT scintillation fluid for 10 min and counted. Radioactivity in reaction mixtures corresponded to the purine base and its corresponding nucleoside monophosphate, the loss of substrate in the reactions equalling product formed.

Radioisotopes and chemicals. ^{14}C-8-Hypoxanthine (sp. act. 62 mCi/m-mole, Amersham), ^{14}C-8-adenine (sp. act. 59 mCi/m-mole, Amersham) and ^{14}C-8-guanine sulfate (sp.act. 56 mCi/m-mole, Amersham) were purchased through Norsk Atominstitutt. Actinomycin D (Cosmegen Lyovac®) was obtained from Merck, Sharp & Dohme and

5-phosphoribosyl 1-pyrophosphate (PRPP, tetrasodium salt) was purchased from Calbiochem. Unlabelled hypoxanthine, inosine monophosphate (IMP, sodium salt), adenosine monophosphate (AMP, sodium salt), guanosine monophosphate (GMP, sodium salt) and cycloheximide were all obtained from the Sigma Co.; adenine and guanine were obtained from Nutritional Biochemicals Co. Actinocin was a gift from Prof. R. Schwyzer, Institute of Molecular Biology and Biophysics, ETH, Zürich, Switzerland.

RESULTS

The effect of preincubation for 2 hr with actinomycin D and actinocin on the uptake of hypoxanthine, adenine and guanine in the MH_1C_1 cells is shown in Table 1. Hypoxanthine transport was strongly inhibited by actinomycin D, 10^{-6} M gave 16 per cent and $5{\cdot}0 \times 10^{-6}$ M 11 per cent of controls respectively. In contrast, the uptake of adenine was not significantly altered by actinomycin D. Actinocin $5{\cdot}0 \times 10^{-6}$ M, the chromophore of actinomycin D, had no effect on either hypoxanthine or adenine uptake. The cellular accumulation of guanine by the MH_1C_1 cultures was less than 1 per cent compared with that of hypoxanthine or adenine; a time dependent increase in intracellular radioactivity after guanine incubation could be demonstrated, however.

Figure 1 shows the effect of cycloheximide and actinomycin D separately or together on the uptake of hypoxanthine. Actinomycin D 10^{-6} M inhibited hypoxanthine transport to 25 per cent of controls, and cycloheximide 10^{-4} M inhibited the uptake of the base to 53 per cent of controls. When the two drugs were used together, their effects were additive, hypoxanthine uptake being reduced to 16 per cent of controls. In separate experiments 10^{-4} M cycloheximide incubated for 2 hr inhibited ^{14}C-phenylalanine incorporation to 4 per cent of controls. The inhibitory effect of cycloheximide alone on hypoxanthine uptake has been shown to increase with time; however, further attempts to elucidate the mechanism for this effect have not been made.

TABLE 1. EFFECT OF ACTINOMYCIN D AND ACTINOCIN ON THE UPTAKE OF PURINE BASES IN MH_1C_1 CELL CULTURES

Purine base	No additions (cpm/mg × 10^2)	With actinomycin D $1{\cdot}0 \times 10^{-6}$M (cpm/mg × 10^2)	With actinomycin D $5{\cdot}0 \times 10^{-6}$M (cpm/mg × 10^2)	With actinocin $5{\cdot}0 \times 10^{-6}$M (cpm/mg × 10^2)
Hypoxanthine	36·50 ± 2·36	5·74 ± 1·46	3·93 ± 1·40	35·33 ± 0·21
Adenine	42·73 ± 1·16	41·71 ± 1·07	39·63 ± 1·29	44·37 ± 1·89
Guanine	0·26 ± 0·02	0·24 ± 0·01	0·23 ± 0·01	—

Replicate subcultures were preincubated without or with actinomycin D 10^{-6} M or 5×10^{-6}M or actinocin 5×10^{-6}M for 2 hr and then incubated with ^{14}C-hypoxanthine, ^{14}C-adenine or ^{14}C-guanine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° without or in the presence of actinomycin D or actinocin. Values are means ± S.D. from three flasks.

The effect of adenine on hypoxanthine uptake and hypoxanthine on adenine uptake in the MH_1C_1 cells can be seen in Table 2. Hypoxanthine in excess did not affect the transport of adenine, whereas adenine at a concentration 12·5 times that of hypoxanthine inhibited its uptake to 59 per cent of controls and at a concentration 125 times that of hypoxanthine reduced its uptake to 16 per cent of controls. The apparent K_m for hypoxanthine uptake has been found to be 5×10^{-6} M and for adenine uptake 8×10^{-6} M.

The effect of actinomycin D 10^{-6} M on the activity of hypoxanthine-guanine phosphoribosyltransferase *in vitro* is shown in Table 3. Preincubation for 2 hr and continuous presence of the antibiotic apparently did not affect the enzymatic formation of the purine nucleoside phosphate.

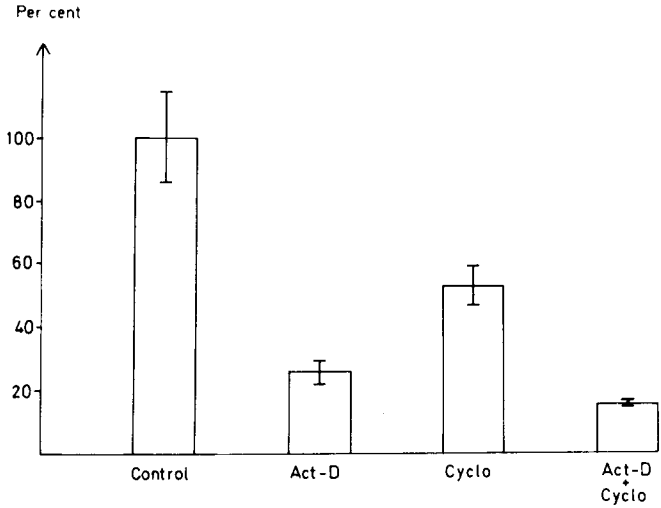

FIG. 1. Effect of actinomycin D and cycloheximide on the uptake of hypoxanthine in MH_1C_1 cell cultures. Replicate subcultures were preincubated without or with actinomycin D 10^{-6} M, cycloheximide 10^{-4} M or actinomycin D plus cycloheximide for 2 hr and then incubated with ^{14}C-hypoxanthine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° without or in the presence of actinomycin D or/and cycloheximide. Values in per cent of controls ± S.D. from three flasks.

The interaction of adenine, guanine and hypoxanthine with the purine phosphoribosyltransferases is shown in Table 4. It can be seen that guanine and hypoxanthine do not alter significantly the rate of AMP formation from adenine, and adenine had little or no effect on the conversion of guanine to GMP or hypoxanthine to IMP. In contrast, guanine inhibited the phosphoribosyl transfer of hypoxanthine and hypoxanthine inhibited the enzymatic conversion of guanine, by approximately 50 per cent when substrate and competitor were present in the same concentration and by approximately 90 per cent when competitor was in 10-fold excess of substrate.

TABLE 2. EFFECT OF ADENINE ON HYPOXANTHINE UPTAKE AND HYPOXANTHINE ON ADENINE UPTAKE IN MH_1C_1 CELL CULTURES

Purine base	No additions (cpm/mg × 10^2)	With adenine/ hypoxanthine 5×10^{-6} M (cpm/mg × 10^2)	With adenine/ hypoxanthine 5×10^{-5} M (cpm/mg × 10^2)
Hypoxanthine 4×10^{-7} M	30·90 ± 4·87	18·36 ± 2·53	5·08 ± 1·15
Adenine 4×10^{-7} M	42·07 ± 1·64	40·76 ± 0·82	44·29 ± 1·50

Replicate subcultures were incubated with ^{14}C-hypoxanthine or ^{14}C-adenine 0·025 μCi/ml, 4×10^{-7} M for 15 min at 37° without or in the presence of unlabelled adenine or hypoxanthine 5×10^{-6} M or 5×10^{-5} M respectively. Values are means ± S.D. from three flasks.

TABLE 3. EFFECT OF ACTINOMYCIN D ON THE ACTIVITY OF HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE *in vitro* PREPARED FROM MH_1C_1 CELLS IN CULTURE

Control		With actinomycin D	
5 min (nmoles/μg $\times 10^{-5}$)	10 min (nmoles/μg $\times 10^{-5}$)	5 min (nmoles/μg $\times 10^{-5}$)	10 min (nmoles/μg $\times 10^{-5}$)
41·46 $\pm$ 2·39	74·61 $\pm$ 4·55	40·05 $\pm$ 3·75	76·30 $\pm$ 2·46

Replicate subcultures were preincubated without or with actinomycin D 10^{-6} M for 2 hr and assayed as in methods by incubation with ^{14}C-hypoxanthine 0·3125 μCi/ml, 5×10^{-6} M for 5 and 10 min at 37° without or in the presence of actinomycin D. Values are means $\pm$ S.D. from three parallel estimations.

DISCUSSION

Actinomycin D, as shown earlier,[1] inhibits the uptake of hypoxanthine by the MH_1C_1 rat hepatoma cells. This effect was seen after 5 min incubation but was first fully developed after 1–2 hr preincubation with the drug. It seems that the inhibition is independent of protein and RNA synthesis; when phenylalanine incorporation was blocked almost completely with cycloheximide, actinomycin D still gave an additional effect on the hypoxanthine uptake, and an increase in the dose of actinomycin D above that which completely inhibited uridine incorporation into RNA also produced increased inhibition of hypoxanthine transport.[1] This effect of actinomycin D seems to require the complete antibiotic molecule; actinocin, the chromophore of actinomycin D lacking both cyclic pentapeptide rings, did not affect the uptake of hypoxanthine, nor did it stimulate thymidine uptake (unpublished results). Schwyzer *et al.*[11] found that both actinomycin D and actinocin enhanced the ACTH induced lipolysis in isolated rat lipocytes. The reduced radioactivity in the acid-soluble fraction after incubation with hypoxanthine and actinomycin D is presumably not a result of feedback inhibition by a phosphorylated product, since increased effects were seen at doses above those which completely inhibited hypoxanthine incorporation into the acid-soluble fraction. The effects described here may be explained by actinomycin D interacting with the cell membrane and thus in some way altering the carrier-mediated uptake of hypoxanthine; this is clearly not a result of alteration of the enzymatic coupling of the base with phosphorylribose.

It is further suggested that the uptake of adenine and hypoxanthine by the cells are separate events, judging from the lack of effect of actinomycin D on adenine uptake and from the competition experiments. The uptake of adenine was unaffected by high doses of hypoxanthine, whereas the uptake of hypoxanthine was inhibited by high doses of adenine, indicating that the uptake mechanism for hypoxanthine is less specific than that of adenine. Hawkins and Berlin[5] reported that hypoxanthine 10^{-3} M inhibited adenine $9{\cdot}7 \times 10^{-6}$ M uptake by 42 per cent (the adenine K_m here being 7×10^{-6} M) in rabbit polymorphonuclear leukocytes, and that xanthine entered by a transport carrier distinct from that of the adenine system, whereas Larsen and Overgaard-Hansen[12] found that hypoxanthine inhibited uric acid transport through the human erythrocyte membrane in a competitive manner.

The low cellular uptake of guanine compared with the two other purines was interesting, as in the system of rabbit neutrophils[5] guanine is actively taken up to the same extent as hypoxanthine and adenine. Cultured human skin epithelial cells (NCTC

TABLE 4. EFFECT OF PURINES ON PURINE PHOSPHORIBOSYLTRANSFERASES *in vitro* PREPARED FROM MH_1C_1 CELLS IN CULTURE

Substrate (5×10^{-6} M)	No additions (nmoles/μg $\times 10^{-5}$)	With adenine 5×10^{-6} M (nmoles/μg $\times 10^{-5}$)	With adenine 5×10^{-5} M (nmoles/μg $\times 10^{-5}$)	With guanine 5×10^{-6} M (nmoles/μg $\times 10^{-5}$)	With guanine 5×10^{-5} M (nmoles/μg $\times 10^{-5}$)	With hypoxanthine 5×10^{-6} M (nmoles/μg $\times 10^{-5}$)	With hypoxanthine 5×10^{-5} M (nmoles/μg $\times 10^{-5}$)
Adenine	29·13 ± 0·55	—	—	30·91 ± 1·28	31·31 ± 2·36	29·58 ± 0·83	29·11 ± 1·33
Guanine	59·66 ± 2·09	59·49 ± 1·60	57·96 ± 1·04	—	—	30·48 ± 3·28	6·66 ± 0·19
Hypoxanthine	42·10 ± 1·79	38·76 ± 1·40	38·95 ± 1·99	23·44 ± 1·20	4·58 ± 0·48	—	—

Homogenates, prepared as described in methods, were incubated with ^{14}C-hypoxanthine, ^{14}C-adenine or ^{14}C-guanine 0·3125 μCi/ml, 5×10^{-6} M as substrates for 5 min at 37° without or in the presence of unlabelled adenine, guanine or hypoxanthine 5×10^{-6} M or 5×10^{-5} M respectively. Values are means ± S.D. from three parallel estimations.

2544) do not take up guanine (E. Dybing, manuscript in preparation). As homogenates of the cells readily convert guanine to guanosine monophosphate, however, this reaction cannot be controlling the cellular uptake of guanine in the MH_1C_1 cells.

The competition experiments with the purine bases on the purine phosphoribosyltransferases from the MH_1C_1 cells confirm the findings of others,[7,8] namely that guanine and hypoxanthine are substrates for the same enzyme, and that adenine is coupled with PRPP by a separate phosphoribosyltransferase. When the results from the cellular transport experiments are correlated with the phosphoribosyltransferase experiments, it seems feasible to postulate that the cellular uptake of the purines tested are reactions distinct from those of enzymatic conversion to their nucleoside phosphates.

Acknowledgements—This study was supported financially by the Norwegian Research Council for Science and Humanities (NAVF) and the Norwegian Cancer Society. The excellent technical assistance of Mrs. Birgitta Baardsen is gratefully acknowledged. I am indebted to Prof. R. Schwyzer, Zürich, Switzerland, for the generous gift of actinocin.

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