REVERSIBLE INHIBITION BY METHIONINYL ADENYLATE OF PROTEIN SYNTHESIS AND GROWTH IN CHICK EMBRYO FIBROBLASTS

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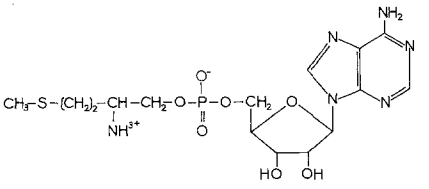
SUMMARY. Methioninyl adenylate (MAMP) inhibits protein synthesis by interacting with the enzyme methionyl-tRNA synthetase. Addition of 1 mM of inhibitor to a growing culture of chick embryo fibroblasts arrests immediately cell growth and incorporation of (^{3}H)leucine into protein. This effect is reversed after up to 2 days of inhibition, by addition of L-methionine or renewal of the medium. The extent and reversibility of the inhibition depend on the concentration of MAMP in the medium and the duration of exposure of the cells to the inhibitor. Growing cells are more sensitive than density-inhibited cells.

Previous studies have shown that aminoalkyl adenylates are very potent and specific inhibitors of aminoacyl-tRNA synthetases in E.Coli cell-free extracts (1). Also, addition of L-methioninyl adenylate (MAMP) (I) to growing cultures of E.Coli has a bacteriostatic effect which is reversible upon adding methionine to the culture. Thus, the in vitro inhibitory effect of MAMP is paralleled by its action in vivo (2). Further investigations have shown that when E.Coli is grown in the presence of MAMP, the enzyme methionyl-tRNA synthetase is inhibited, resulting in an immediate block of acylation of t-RNA met, whereas other tRNAs remain unaffected (3).

The purpose of this work was to investigate whether MAMP is also active in eukaryotic cells, since the availability of a potent inhibitor of protein synthesis and cell growth with a reversible effect and a known



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target enzyme should prove very useful for metabolic studies. We report here the effect of MAMP on cell growth and viability, and protein synthesis in non-confluent, fast growing and density-inhibited cultures of chick embryo fibroblasts.

MATERIALS AND METHODS. Cells and media. Experiments were carried out with secondary cultures of chick embryo fibroblasts (CEF*), prepared as described earlier (4). Culture dishes (35 mm Falcon) were seeded with either 3-5 x 10^5 cells, to obtain nonconfluent fast growing cultures, or 2 x 10^6 cells, to obtain density-inhibited cultures. The medium was Ham F-10 (5) -chosen for its low methionine content- plus 5 % calf serum and antibiotics (penicillin, streptomycin, kanamycin, and fungisone). Incubation temperature was 37°C.

Cell counting. The cells were counted in a haemocytometer, after detachment with 0.12 % Difco trypsin in Ca- and Mg-free PBS.

Cell viability was determined by measuring the cloning efficiency of treated, or untreated cells on cultures of irradiated feeder cells (6). These were hamster BHK21/13 cells irradiated at 4500 R with ^{60}Co-γ rays and seeded at 5 x 10^5/dish, in 50 mm dishes. The medium was Eagle's MEM with double concentration of vitamins and aminoacids, plus 10 % calf serum and antibiotics (vide supra).

Protein synthesis. The rate of protein synthesis was determined by measuring the incorporation of (^{3}H)L-leucine into the hot TCA-insoluble cell fraction (7). Following exposure of the cells to MAMP, (^{3}H)L-leucine (40 Ci/mM) was added to the medium (5-6 μCi/ml), and cultures further incubated 1 hr at 37°C. The cells were then lysed for 20 min, at room temperature, with 0.75 ml of a 0.05 % solution of SDS containing 10 mM EDTA. To 0.5 ml of the lysate, 400 μg of bovine serum albumin was added, as carrier, then 0.5 ml of 10 % TCA. The mixture was incubated 15 min at 90°, in a water bath. The precipitate formed was collected by filtration on a GF/C Whatman glass filter and washed with 5 % TCA containing 0.5 % cold leucine. The filter discs were placed in 10 ml of scintillation solution (8), and the radioactivity measured in a Packard "Tri Carb" scintillation counter. All assays were carried out in duplicate.

Preparation of cell-free extract and enzyme activity measurements. The whole extraction was carried out at 4°C. Monolayers were washed with PBS and scraped with a rubber policeman in 5 ml of buffer containing 50 mM Tris-HCl, pH 7.0, 25 mM KCl, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol, and 250 mM sucrose. Cells were homogenized in a Dounce homogenizer, and the nuclei, mitochondria, and membranes removed by centrifugation for 15 min at 12,000 x g. The supernatant fraction was dialyzed overnight against the extraction buffer and tested for methioninyl-tRNA synthetase activity, as follows : enzyme activity was estimated by the methionine-dependent ATP-PPi exchange reaction, as previously described (9). The assay for aminoacyl t-RNA formation was carried out with the standard mixture : imidazole buffer, pH 7.5, 2 mM, EDTA 0.1 mM, 2-mercaptoethanol 1 mM, KCl 150 mM, $MgCl_2$ 5 mM, ATP 2 mM, (^{14}C)L-methionine (50 mCi/mM) 0.1 mM, yeast tRNA 170 μg, and a limiting amount of dialyzed cell free extract. Otherwise, the assay conditions were as described earlier (1).

*Other abbreviations used : (1) PBS : phosphate buffered saline, pH 7.4.
(2) SDS : sodium dodecyl sulfate.
(3) EDTA : ethylene diamine tetracetic acid.
(4) TCA : trichloracetic acid.

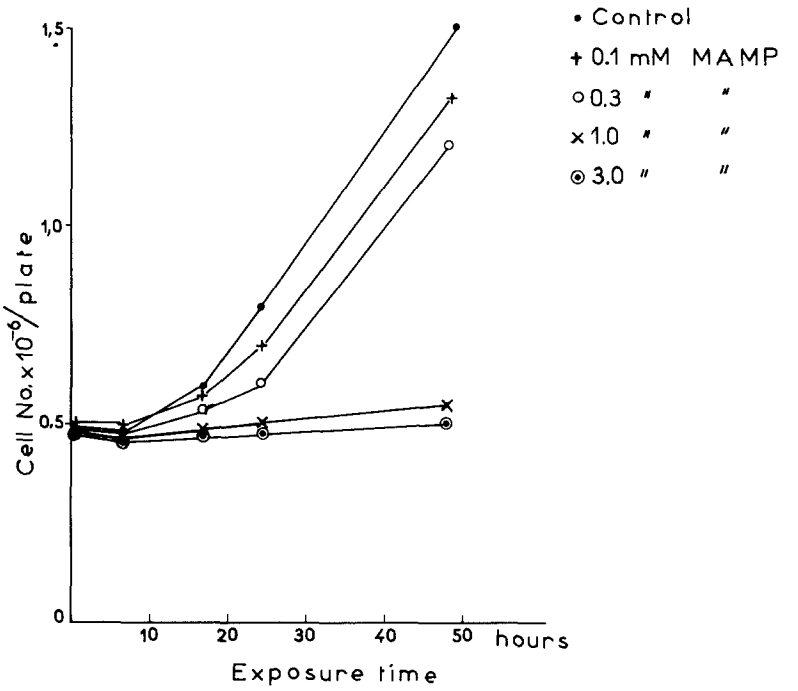

Fig. 1 - Effect of MAMP on cell growth. One day after plating (time 0), nonconfluent cultures of CEF (3 x 10^5 cells/35 mm dish) were incubated in medium containing MAMP, and cells counted at different times. Each point corresponds to the mean of counts of 2 replicate dishes.

All chemicals used were of the highest purity available, and MAMP synthesized as described earlier (1). Protein was determined by the method of Lowry et al. (10), with bovine serum albumin as standard.

RESULTS AND DISCUSSION. Effect of MAMP on cell growth. As is shown in Fig. 1, 1-3 mM MAMP completely arrested cell growth in nonconfluent, fast growing CEF cultures. Lower concentrations (0.1-0.3 mM) produced only a slight inhibition of growth. No inhibition was observed with AMP or methioninol, alone or in combination, at 3 mM.

As is shown in Fig. 2, addition of 3 mM L-methionine to nonconfluent CEF cultures arrested for 24 hr by 3 mM MAMP reversed immediately and completely the growth inhibition (curve b). Reversion was also complete, but delayed about 24 hr, when cells were arrested for 48 hr (curve c). Arrest for 3 days caused some cell death, and the surviving cells began to grow only after almost 2 days (curve d). Removal of MAMP by medium renewal had the same effect as addition of methionine.

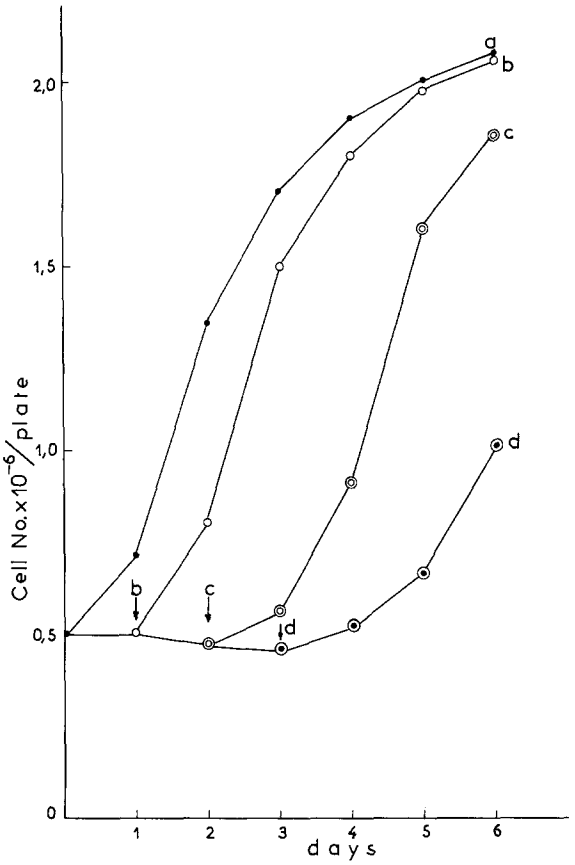

Fig. 2 - Reversal by methionine of growth inhibition by MAMP. Nonconfluent CEF cultures (cf. Fig. 1) were exposed to MAMP (3 mM) one day after plating (time 0). Inhibition of growth was reversed at intervals of 24 hr by addition of L-methionine (3 mM). The medium was renewed at 3 days ; a : untreated controls ; b-d : treated cells. Arrows : time of addition of L-methionine.

Cell viability following inhibition by MAMP. Cell viability following exposure to MAMP was also determined by measuring the cloning efficiency of treated cells (cf. Materials and Methods). In the case of nonconfluent, fast growing cultures, 0.1-0.5 mM MAMP did not alter cell viability after a 48 hr exposure. Only a negligible reduction was caused by a 24 hr contact with 1 or 3 mM of inhibitor, and 80 % and 70 % of the treated cells remained viable after a 48 hr exposure to these concentrations. Parallel exposure of density-inhibited cells did not affect significantly their viability.

Effect of MAMP on cytoplasmic methionyl-tRNA synthetase. In a cellular ex-

Table I. km and ki values obtained in the aminoacyl-tRNA formation assay in cell-free extracts of fast growing (Fg) and density-inhibited (Di) cells.

Cells	km methionine μM	km ATP μM	ki μM
Di	56	-	0.39
	-	1150	0.20
Fg	50	-	0.26
	-	1000	0.30

Table II. Inhibition of (^{3}H)leucine incorporation by different concentrations of MAMP in fast growing (Fg) and in density-inhibited (Di) cells.

Exposure time	% inhibition									
	3 mM		1 mM		0.6 mM		0.3 mM		0.1 mM	
	Fg	Di	Fg	Di	Fg	Di	Fg	Di	Fg	Di
30'	75	81	58	46	41	20	24	0	25	3
60'	82	84	68	65	62	30	46	15	3	11
120'	90	90	82	74	70	40	58	14	20	3
180'	90	88	81	76	75	50	54	12	15	1

tract free of nuclei, mitochondria, and membranes, the two reactions catalysed by methionyl-tRNA synthetase, i.e., the methionine-dependent ATP-PPi exchange reaction and the formation of methionyl tRNA, were both inhibited by MAMP. The inhibition was competitive with respect to ATP and methionine. Also, no significant difference in kinetic parameters was observed between fast growing and density-inhibited cells (cf. Table I).

Effect of MAMP on protein synthesis. As is seen from Table II, a 30 min exposure to 3 mM MAMP decreased by 75-80 % the incorporation of (^{3}H)leucine in both fast growing and density-inhibited cells, and the inhibition was at its maximum (90 %) already after 2 hr. The inhibition by 1 mM MAMP was somewhat weaker (75-80 % after 2 hr). Lower concentrations of inhibitor were definitely more active on fast growing than on density-inhibited cells ; after a 2 hr exposure to 0.3 mM MAMP, inhibition exceeded 50 % in fast growing cells and was only about 15 % in density-inhibited cells.

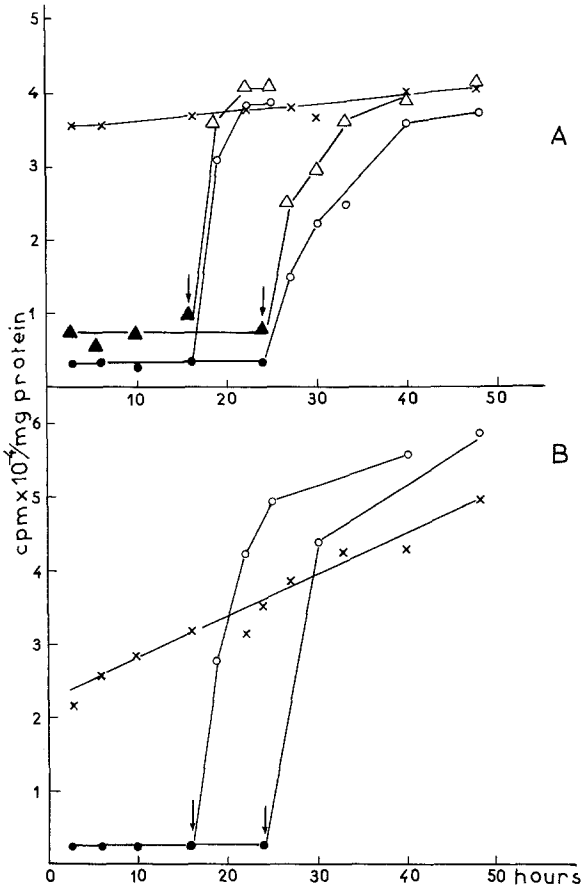

Fig. 3 - Inhibition by MAMP of incorporation of (^{3}H)leucine into cell protein and reversal of the inhibition by medium renewal. Nonconfluent (A) or density-inhibited (B) CEF cultures seeded respectively with 3 x 10^5 or 2 x 10^6 cells, were incubated, one day after plating, first with 1 or 3 mM MAMP for 16 or 24 hr, then in MAMP-free medium. Labelling with (^{3}H)L-leucine was carried out at various times during and after contact with MAMP. Full circles and triangles : treated cultures in contact with MAMP (● 3 mM, ▲ 1 mM). Empty circles and triangles : treated cultures after medium renewal (indicated by arrows). Crosses : untreated controls.

As is shown in Fig. 3, removal of MAMP by medium renewal completely reversed the inhibition of leucine incorporation within 3 hr. Reversion was also complete, but slower (10 hr for 1 mM and 16 hr for 3 mM) following a 24 hr exposure. On the other hand, it was more rapid in density-inhibited than in nonconfluent cells and, in the former cells, leucine incorporation even exceeded that in controls after a very short lag. A similar stimulation was already reported, with HeLa cell extracts, after inhibition of protein syn-

thesis by either cycloheximide (11) or deprivation of essential aminoacids (12).

To summarize, MAMP-treated cells appear to be in a condition of suspended growth, due to the arrest of protein biosynthesis and offer, thus, a unique opportunity for the study of metabolic regulation. The fact that methionine could reverse the action of MAMP indicates the site specificity of the inhibitor. It is also interesting that lower concentrations of MAMP (0.1-0.6 mM) affect much more the nonconfluent, fast growing cells than the density-inhibited cells, although the affinity of MAMP for methionyl-tRNA synthetase is the same in both cases. The difference observed may be due to a different intracellular concentration of L-methionine or a different level of the internal pool of methionyl-tRNA depending on cell density. Prolonged exposure to MAMP could also affect secondarily to a different degree DNA and/or RNA synthesis. These possibilities are being investigated.

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