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3-Methyladenine: Specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes

(autophagy/lysosomes)

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ABSTRACT **3-Methyladenine (5 mM) inhibits endogenous protein degradation in isolated rat hepatocytes by about 60%, while having no adverse effect on the degradation of an exogenous protein (asialofetuin), on protein synthesis, or on intracellular ATP levels. 3-Methyladenine appears to act specifically upon the autophagic/lysosomal pathway of degradation, as judged from its lack of effect in the presence of amino acids or a lysosomotropic amine (propylamine). The effect of the purine is not mediated by amino acids because the inhibition of protein degradation is accompanied by a significant depression of intracellular amino acid levels. The ability of 3-methyladenine to suppress the formation of electron microscopically visible autophagosomes suggests that it may be regarded as a specific inhibitor of autophagy.**

Intracellular protein degradation takes place by both lysosomal and nonlysosomal mechanisms (1–3). The lysosomes play a major role in (*i*) the degradation of exogenous proteins (4–7) and (*ii*) the degradation of endogenous proteins under conditions of amino acid starvation (step-down) (3, 8, 9). Amino acids apparently exert their regulatory effect by suppressing the first step in the autophagic/lysosomal pathway—i.e., the formation of autophagosomes (10, 11). Nothing is known, however, about the biochemical mechanisms involved in autophagy.

We have found that, like the amino acids, certain N^6-methylated adenosine derivatives (6-dimethylaminopurine riboside and puromycin aminonucleoside) could specifically inhibit the degradation of endogenous protein by the autophagic/lysosomal pathway in isolated rat hepatocytes (12). In a subsequent screening of a large number of purines and related substances (unpublished data), one compound—3-methyladenine—showed a unique selectivity in being able to inhibit endogenous protein degradation strongly without affecting protein synthesis. In the present report, the effects of 3-methyladenine are described in detail, and it is shown that this purine is capable of suppressing autophagosome formation independently of amino acids.

MATERIALS AND METHODS

Chemicals. 3-Methyladenine (6-amino-3-methyl-purine) was purchased from Fluka, Buchs, Switzerland. 3-Methyladenosine (*p*-toluene sulfonate salt) was a gift from T. Fujii (Kanazawa University, Kanazawa, Japan). The other 3-substituted adenine derivatives (identified by their NSC code numbers) were donated by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Propylamine was purchased from Koch-Light Laboratories (Colnbrook, England), and gentamicin (Garamycin) was from Schering; other biochemicals were from Sigma. [^{14}C]Valine was obtained from the Radiochemical Centre (Amersham, England), and ^{125}I-labeled asialofetuin was a gift from H. Tolleshaug (Institute for Nutrition Research, Oslo, Norway).

Isolation and Incubation of Hepatocytes. Hepatocytes were prepared from the livers of 18-hr starved male Wistar rats (250–300 g) by collagenase perfusion (13). The cells were incubated as 0.4-ml aliquots (50–75 mg wet weight per ml) in 15-ml centrifuge tubes and were kept in suspension by shaking at 37°C. The incubation medium was suspension buffer (13), fortified with pyruvate (20 mM), Mg^{2+} (2 mM), and gentamicin (10 μg/ml). Incubations were terminated by the addition of 0.1 ml of 10% (wt/vol) perchloric acid.

Protein Synthesis. Protein synthesis was measured as the incorporation of 5 mM [^{14}C]valine of constant specific radioactivity (0.25 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) during a 60-min incubation in medium supplemented with amino acids (11). Synthesis rates (percentage per hr) were calculated as described (14).

Protein Degradation. Endogenous protein degradation was measured as the net release of [^{14}C]valine [from protein labeled *in vivo* for 24 hr (3)] between 30 and 90 min of incubation in medium supplemented with 15 mM valine. The protein radioactivity at 30 min was determined, and the degradation rate was expressed as percentage per hr (3).

The degradation of an exogenous protein, ^{125}I-labeled asialofetuin, was measured as the net release of acid-soluble ^{125}I after a 15-min ingestion period, as described by Tolleshaug *et al.* (5).

ATP. The intracellular ATP level was determined in neutralized perchloric acid extracts of the cells by means of a bioluminescence monitor (Luminometer; LKB) by using the luciferin/luciferase assay and the reagents accompanying this instrument.

Electron Microscopy. The hepatocytes were fixed for 1 hr in 1% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4; rinsed overnight at 4°C in 0.1 M sucrose/0.1 M cacodylate buffer; and postfixed for 1 hr at 4°C in 1% OsO_4/0.1 M cacodylate buffer. For each type of treatment, 12 Epon-embedded samples were prepared. Five samples were selected at random, and 10 micrographs (18 × 24 cm) were prepared from each sample at a final magnification of ×12,000. The micrographs were analyzed morphometrically by the point-counting method (15) with a 368-point matrix for autophagosomes (8-point reference matrix) and a 165-point matrix for secondary lysosomes (5-point reference matrix). Final mean values ± SEM were computed from the five sample means.

Amino Acid Analysis. Intracellular amino acid levels were measured in neutralized perchloric acid extracts of the cells by means of an amino acid analyzer (16).

RESULTS AND DISCUSSION

Effect of 3-Substituted Adenine Derivatives on Protein Synthesis and Degradation. Several 3-substituted adenine derivatives were tested for their effect on hepatocytic protein syn-

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Table 1. Effect of various 3-substituted adenine derivatives on hepatocytic protein degradation and synthesis

Test compound	Protein degradation, % change			Protein synthesis, % change		
	0.1 mM	1 mM	10 mM	0.1 mM	1 mM	10 mM
3-Methyladenine (Fluka)	−5	−21	−60	−2	−2	−8
3-Methyladenine (NSC 66389-T)	−4	−22	−65	−1	−2	−14
3-Methyladenosine (Fujii)	−6	−19	−68	0	−5	−46
3-(2-Butyrolactonyl)adenine (NSC 78673-U)	+1	−7	−75	−6	−8	−65
3-Trimethylacetoxymethyladenine (NSC 163552-I)	+1	−12	−69*	−10	−36	−100*
3-Benzyladenine (NSC 211695-M)	−8	−42	−84	−33	−84	−100
3-Benzylideneethyladenine (NSC 78674-W)	−1	−67	−96	−28	−98	−100
3-(5′-Phosphoribosyl)adenine (NSC 270275-L)	−10	−41	−78	−10	−93	−98
3-(5′-Aminophosphoribosyl)adenine (NSC 283836-Z)	−9	−55	−81	−14	−92	−97
3-Ribosyl-8-bromoadenine (NSC 133117-Z)	+2	+2	−12*	−6	+1	−14*

Isolated hepatocytes were incubated at 37°C with the compounds indicated. Protein degradation was measured as the release of [^{14}C]valine from *in vivo* labeled protein between 30 and 90 min of incubation, and protein synthesis was measured as the incorporation of [^{14}C]valine during a 60-min incubation in amino acid-supplemented medium. Each value is the mean of three cell samples; the SEM averaged 6% for protein degradation and 2% for protein synthesis. The source of the compound is given in parentheses; compounds from the National Cancer Institute are identified by their NSC code numbers.
* 5 mM (limited solubility).

thesis and degradation at concentrations of 0.1, 1, and 10 mM (Table 1). 3-Methyladenine from two different sources showed similar activities, with very selective inhibition of protein degradation both at 1 and 10 mM. 3-Methyladenosine inhibited protein degradation to the same extent as did 3-methyladenine, but it also inhibited protein synthesis considerably at 10 mM. All of the other compounds inhibited protein synthesis as well as degradation, with the exception of 3-ribosyl-8-bromoadenine, which displayed very little activity. Thus, 3-methyladenine remains unique among the tested purines in its high selectivity towards degradation.

Inhibition of Autophagic/Lysosomal Protein Degradation by 3-Methyladenine. 3-Methyladenine inhibited endogenous protein degradation after a 20-min time lag; its effect was rapidly reversible (Fig. 1A). The purine had no significant additional effect in the presence of an amino acid mixture known to suppress autophagy (11) or in the presence of a lysosomotropic amine, propylamine, which inhibits lysosomal proteolysis (17) (Fig. 1*B*). Therefore, it can be assumed that 3-methyladenine exerts its effect somewhere along the autophagic/lysosomal pathway of protein degradation.

The dose–response curve (Fig. 2) confirms the lack of effect of 3-methyladenine in the presence of amino acids or propylamine. 3-Methyladenine alone inhibited protein degradation by about 60% at 5 mM, with a slight additional inhibition at higher concentrations. The inhibition by 3-methyladenine never attained the level produced by propylamine or amino acids, suggesting that the autophagic/lysosomal pathway was somewhat less than completely inhibited by the purine.

3-Methyladenine Does Not Affect the Degradation of an Exogenous Protein (Asialofetuin). To see if 3-methyladenine might inhibit degradation at a step common to the autophagic

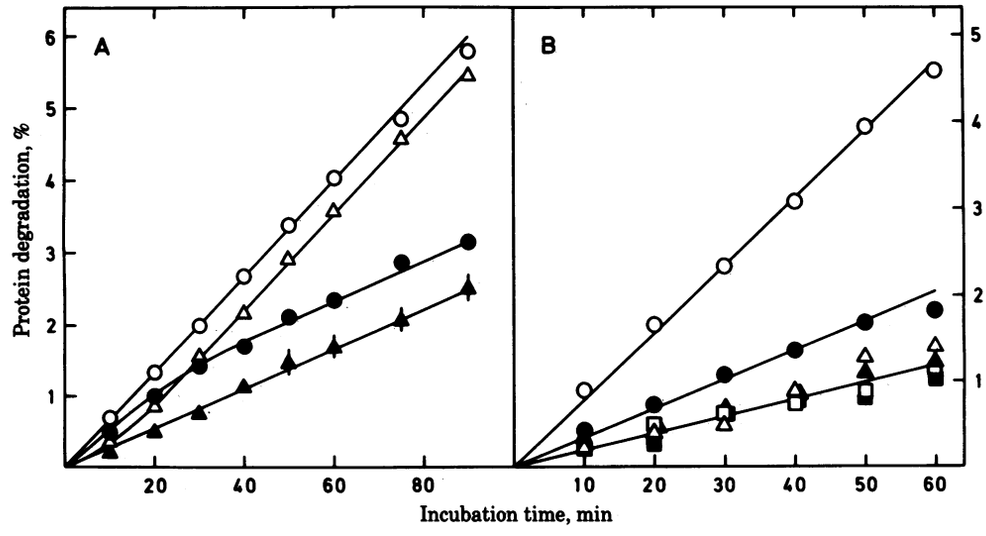

FIG. 1. Inhibition of endogenous protein degradation by 3-methyladenine: time course, reversibility, and lack of effect in the presence of amino acids or a lysosomotropic amine. (*A*) Isolated hepatocytes were incubated at 37°C after a 30-min preincubation in the presence or absence of 3-methyladenine (5 mM): ○, preincubated and incubated without; ●, preincubated without and incubated with; △, preincubated with and incubated without; ▲, preincubated and incubated with 3-methyladenine. (*B*) Hepatocytes were preincubated for 30 min and further incubated for 1 hr without medium change. ○, Control (no additions); ●, 3-methyladenine (5 mM); △, amino acid mixture (11); ▲, amino acids with 3-methyladenine; □, propylamine (10 mM); ■, propylamine with 3-methyladenine. Protein degradation was measured as the net amount of [^{14}C]valine released from 24-hr labeled protein and expressed as the percentage of the initial protein radioactivity. Each value is the mean ± SEM of six cell samples from two different experiments, normalized to the same (average) control value (4.0%/hr in *A*; 4.6%/hr in *B*); most of the standard errors were smaller than the symbols.

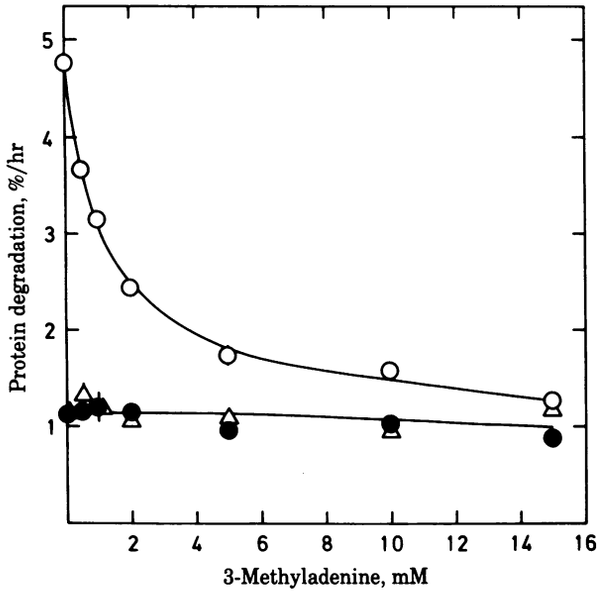

FIG. 2. Dose–response curve for inhibition of endogenous protein degradation by 3-methyladenine. Hepatocytes were incubated at 37°C with various concentrations of 3-methyladenine only (○); with 3-methyladenine plus an amino acid mixture (●), or with 3-methyladenine plus 10 mM propylamine (△). The rate of protein degradation (percentage per hr) was measured as the net release of [^{14}C]valine from 24-hr labeled protein between 30 and 90 min of incubation. Each value is the mean ± SEM of six cell samples from two different experiments; most of the standard errors were smaller than the symbols.

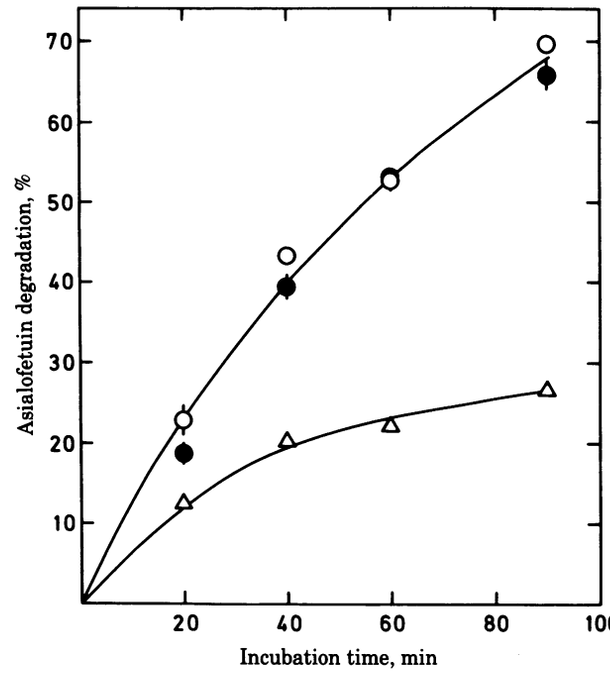

FIG. 3. Effect of 3-methyladenine and propylamine on the degradation of an exogenous protein (asialofetuin). Hepatocytes which had been allowed to ingest ^{125}I-labeled asialofetuin for 15 min were incubated at 37°C with no additions (○), with 5 mM 3-methyladenine (●), or with 10 mM propylamine (△). Asialofetuin degradation was measured as the net release of acid-soluble ^{125}I and was expressed as the percentage of the initial acid-insoluble radioactivity. Each value is the mean ± SEM of six cell samples from two different experiments; some of the standard errors were smaller than the symbols.

and heterophagic pathways, its effect on the degradation of ^{125}I-labeled asialofetuin, a protein ingested by receptor-mediated endocytosis and degraded in lysosomes (5, 6) was tested (Fig. 3). 3-Methyladenine had no effect on asialofetuin degradation, in contrast, for instance, to propylamine, which affects lysosomes directly (17). Therefore, the purine would seem to exert its effect upon a prelysosomal step unique to autophagy.

Depression of Intracellular Amino Acid Levels by 3-Methyladenine. Because 5 mM 3-methyladenine inhibited protein degradation by about 60%, the intracellular amino acid contents would be expected to be reduced to a similar extent (16). As shown in Table 2, the levels of the eight amino acids measured were indeed lowered by 40–80%; the concentration of valine, the amino acid used to estimate protein degradation, was reduced by 65% as expected.

This experiment would seem to rule out the possibility that the degradation-inhibitory effect of 3-methyladenine might be mediated by an elevation of intracellular amino acid levels.

Effect of 3-Methyladenine on Protein Synthesis and ATP Levels. Protein synthesis was not inhibited by 5 mM 3-methyladenine in an amino acid-supplemented medium; in fact, a slight stimulation was indicated (Fig. 4*A*). Because several degradation inhibitors have been found to inhibit protein synthesis in the absence of amino acids, presumably by reducing the availability of degradation-derived amino acids needed for synthesis (18), we also tested 3-methyladenine in an amino acid-free medium. As shown in Fig. 4*B*, there was no inhibitory effect of the purine up to a concentration of 5 mM even under these conditions. This is surprising, considering that 5 mM 3-methyladenine greatly reduces the intracellular amino acid concentrations. One possibility is that the dose–response curve is a composite of stimulatory and inhibitory effects (compare the significant stimulation of protein synthesis by 3-methyladenine in the presence of amino acids). The fact that amino acids by themselves are stimulatory indicates that they are indeed rate limiting for protein synthesis.

Because protein synthesis is very sensitive to interference with energy metabolism (3), 3-methyladenine would not be expected to affect the latter. This could be shown more directly by measuring the intracellular ATP levels, which were unaffected by 3-methyladenine both in the presence and absence of amino acids (Fig. 4*C*).

Inhibition of Autophagosome Formation by 3-Methyladenine. Formation of autophagosomes is the first step in the autophagic/lysosomal pathway and is the step that is inhibited by amino acids (10, 11). Morphometric analysis of electron micrographs (Table 3) indicated a similar inhibition of autophagosome

Table 2. Depression of intracellular amino acid levels by 3-methyladenine

Amino acid	Intracellular amino acid level, nmol/g of cells		
	Control	3-Methyladenine	% change
Ala	298 ± 22	60 ± 3	−80
Glu	315 ± 16	55 ± 3	−83
Gly	430 ± 29	281 ± 12	−35
His	114 ± 9	58 ± 6	−49
Ile	68 ± 7	10 ± 2	−85
Leu	122 ± 7	22 ± 2	−82
Lys	353 ± 24	147 ± 6	−58
Val	123 ± 13	43 ± 1	−65

Isolated hepatocytes were incubated for 1 hr at 37°C with or without 3-methyladenine (5 mM), and intracellular amino acid levels were measured in perchloric acid extracts of the cells. Each value is the mean ± SEM of three cell samples.

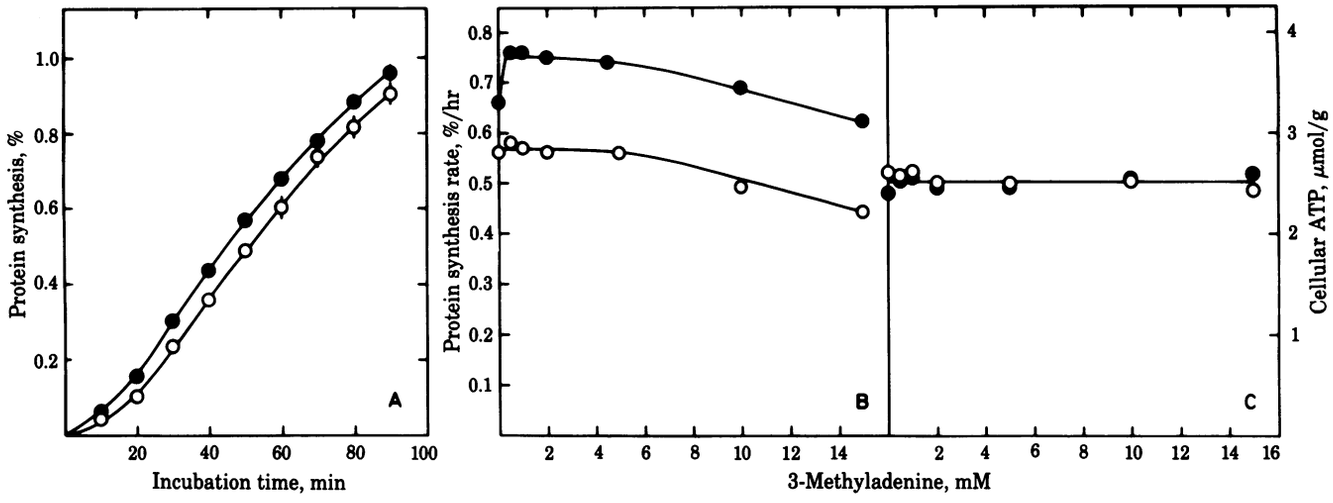

FIG. 4. Effect of 3-methyladenine on protein synthesis and cellular ATP content. (*A*) Time course of protein synthesis (incorporation of [^{14}C]valine), expressed as percentage, in hepatocytes incubated at 37°C in the absence (○) or presence (●) of 5 mM 3-methyladenine. Each value is the mean ± SEM of six cell samples from two different experiments. (*B*) Dose–response curve for the effect of 3-methyladenine on the rate of protein synthesis, expressed as percentage per hr, in hepatocytes incubated for 60 min at 37°C in the absence (○) or presence (●) of an amino acid mixture (11). Each value is the mean of three cell samples; the SEM averaged 1%. (*C*) Dose–response curve for the effect of 3-methyladenine on the ATP content of cells incubated for 90 min at 37°C in the absence (○) or presence (●) of an amino acid mixture. Each value is the mean ± SEM of three cell samples; the SEM averaged 1%. The ATP content did not change significantly between 5 and 90 min of incubation.

formation by 3-methyladenine. The 80% reduction in autophagosome contents and the smaller secondary reduction in lysosome contents were somewhat less with 3-methyladenine than with amino acids, in accordance with the relative effects of the two types of treatment on overall protein degradation (cf. Fig. 2).

Thus, our results indicate that 3-methyladenine is a specific inhibitor of autophagy in hepatocytes. Whether other cell types are similarly affected remains to be seen. 3-Methyladenine may be useful in defining the role of autophagy under various physiological conditions, and knowledge about its mechanism of action should contribute to the understanding of the biochemistry of autophagy. The ability to specifically suppress autophagy also may have practical consequences—e.g., for the maintenance and growth of cultured cells or *in vivo* under conditions where autophagy contributes to excessive tissue wasting.

Table 3. Reduction in hepatocytic autophagosome content by 3-methyladenine and amino acids

Additions	Cytoplasmic volume fraction, mm^3/cm^3	
	Autophagosomes	Secondary lysosomes
None	12.1 ± 0.7	39.3 ± 1.6
Amino acid mixture	1.6 ± 0.4	20.0 ± 5.5
3-Methyladenine	2.6 ± 0.7	29.0 ± 2.0

Isolated hepatocytes were incubated for 2 hr at 37°C with no additions, with an amino acid mixture (11), or with 3-methyladenine (5 mM). Samples were fixed and prepared for electron microscopy, and analyzed morphometrically. Autophagosomes correspond to the vacuoles classified as type I in our previous analysis (11), whereas secondary lysosomes comprise types II and III (i.e., including dense bodies). Each value is the mean ± SEM of five electron microscopic preparations.

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