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A REEXAMINATION OF THE EFFECTS INDUCED BY ADENOSINE AND ITS DEGRADATION PRODUCTS ON RAT FAT CELL LIPOLYSIS

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Abstract—The effects of adenosine and of some products of its metabolic degradation on lipolysis were studied in rat fat cells isolated from epididymal adipose tissue. Basal glycerol release was not affected by adenosine and by uric acid, but it was significantly increased by inosine (1–100 μM) and by hypoxanthine (10–100 μM). Adenosine was more effective than inosine in antagonizing the lipolytic response of fat cells to theophylline. Also hypoxanthine and uric acid exerted a very potent, non-competitive antagonism towards theophylline. Norepinephrine-induced lipolysis was inhibited by adenosine, hypoxanthine and uric acid approximately to the same extent, while inosine was ineffective at this level. Adenosine deaminase (0.5 U/ml) increased basal as well as theophylline- and norepinephrine-induced lipolysis. Moreover, adenosine deaminase enhanced the lipolytic rate in cells incubated with low (0.1, 1 μM) and, to a lesser extent, with high (10, 100 μM) inosine concentrations. These results suggest that inosine is the adenosine metabolite that may accumulate in the incubation medium following fat cell treatment with adenosine deaminase, thus contributing to the stimulatory effects of this enzyme on lipolysis.

Adenosine is known to play a primary role as an endogenous modulator of fat cell lipolysis and changes in the amount of adenosine released by the adipocytes incubated *in vitro* appear to be a crucial factor in determining the lipolytic rate under basal conditions as well as in response to stimulatory and inhibitory agents [1–3]. For this reason adenosine deaminase is often added to the incubation medium in order to remove the inhibition caused by endogenous adenosine [1, 2, 4]. It should be stressed, however, that the addition of this enzyme is followed by an accumulation of adenosine metabolites [5], which were previously shown to antagonize the response to lipolytic agents, thus mimicking the effect of adenosine [6]. The ability of adenosine deaminase to potentiate the response of fat cells to catecholamines would suggest that the inhibitory potency of these metabolites is lower as compared to adenosine, while Ebert and Schwabe [6] showed that inosine is as effective as adenosine in inhibiting norepinephrine-induced lipolysis. Moreover, the same authors reported that adenosine markedly stimulates the basal rate of lipolysis, while the stimulation by inosine and by hypoxanthine is much lower [6]. On the basis of these data the ability of adenosine deaminase to increase the basal rate of lipolysis is hard to explain.

In the attempt to clarify these points, the influence of adenosine, inosine, hypoxanthine and uric acid on rat fat cell lipolysis was reexamined, both in the absence and in the presence of norepinephrine and of theophylline.

MATERIALS AND METHODS

Epididymal fat pads were excised from male Wistar rats (180–240 g b.w.) under light ether anaesthesia. The tissue was washed in Krebs–Ringer bicarbonate buffer pH 7.4, containing 3% bovine serum albumin and then minced and suspended in the same buffer. Fat cells were isolated by the addition of crude collagenase (1 mg/ml) according to the method of Rodbell and Krishna [7]. After filtration and repetitive washing of the cells, the final cell suspension in Krebs–Ringer bicarbonate albumin buffer was properly diluted to give 40,000–60,000 cells/ml and the method described by Rodbell and Krishna [7] was used for counting the number of cells. Aliquots of 2 ml of this suspension were preincubated for 15 min at 37° in a metabolic shaker in the absence or in the presence of adenosine, inosine, hypoxanthine, uric acid or adenosine deaminase at the indicated concentrations. Norepinephrine and theophylline were then added and after 30 min of further incubation the reaction was stopped by the addition of 0.2 ml of 50% trichloroacetic acid.

After centrifugation of the samples, 0.5 ml aliquots of the supernatant fluid were used for the colorimetric determination of glycerol according to the method of Lambert and Neish [8] by using the acetyl acetone reagent as proposed by Nash [9]. Adenosine and its metabolites did not interfere with the colorimetric assay.

Norepinephrine, theophylline, adenosine, inosine and adenosine deaminase were dissolved in saline (NaCl 0.9%) and added to the samples in 0.01 ml volumes. Hypoxanthine and uric acid were dissolved and properly diluted in 0.1 M Na_2CO_3. These solutions were added to the incubation medium in 0.01 ml volumes, which did not change the pH of the buffer. The same volume of Na_2CO_3 was added in the control samples, where hypoxanthine and uric acid were not present.

(−)Norepinephrine bitartrate, theophylline, bovine serum albumin fraction V, powder (prepared from pasteurized bovine serum and low temperature

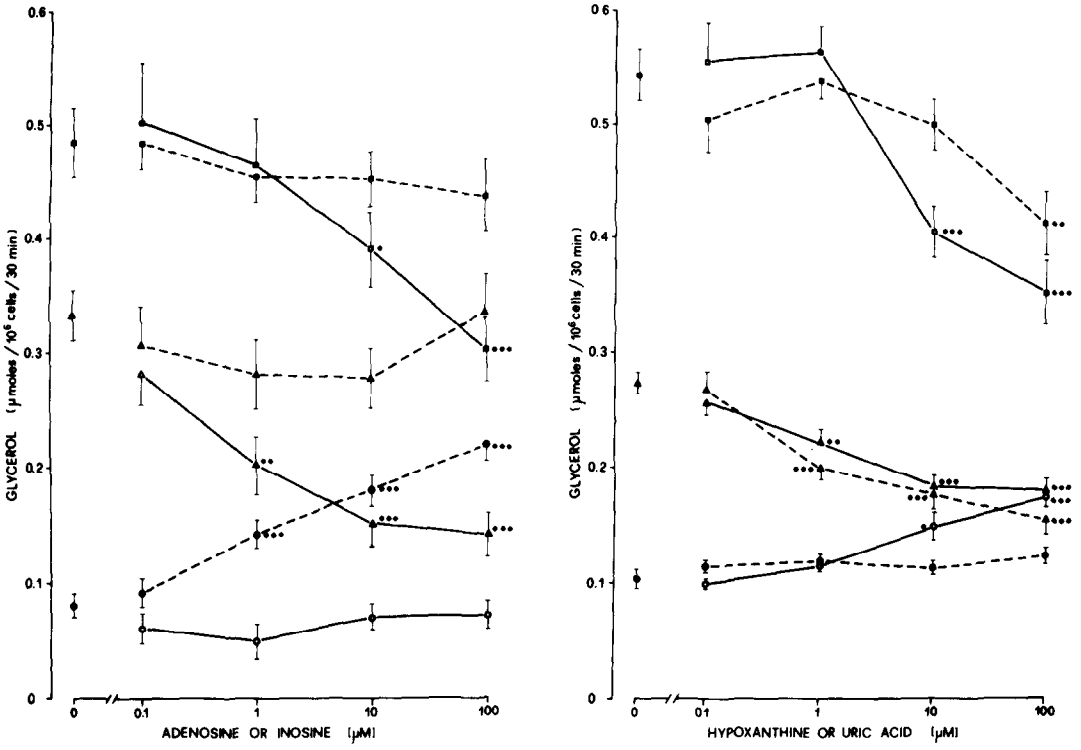

Fig. 1. Influence of different concentrations (0.1–100 μM) of adenosine, inosine, hypoxanthine and uric acid on glycerol release by rat fat cells in the absence (○ ●) and presence of 0.6 mM theophylline (△ ▲) or of 2 μM norepinephrine (□ ■). Left: effect of adenosine (solid lines, open symbols) and inosine (broken lines, closed symbols). Each point represents the mean (±SEM) of the data obtained from 10 to 12 incubations in 4 experiments. Right: effect of hypoxanthine (solid lines, open symbols) and uric acid (broken lines, closed symbols). Each point represents the mean (±SEM) of the data obtained from 12 incubations in 4 experiments. The statistical significance of the changes induced by adenosine, inosine, hypoxanthine and uric acid was calculated by Student's *t*-test. * $P < 0.02$; ** $P < 0.005$; *** $P < 0.001$.

precipitation, Sigma catalogue number A-8022, lot 84F-0099), crude collagenase (from Clostridium venom, Type II), adenosine deaminase (Type I, from calf intestinal mucosa), adenosine, inosine, hypoxanthine and uric acid were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals and reagents were of analytical grade.

The statistical significance of the differences between means was calculated by using the Student's *t*-test.

RESULTS

Concentration-dependent changes induced by adenosine, inosine, hypoxanthine and uric acid on lipolysis

In the first set of experiments the influence of increasing concentrations of adenosine and inosine on lipolysis in the absence and presence of 0.6 mM theophylline or of 2 μM norepinephrine was compared (Fig. 1, left). The effects of hypoxanthine and of uric acid at this level were evaluated in a separate set of experiments (Fig. 1, right).

As shown in Fig. 1, adenosine (0.1–100 μM) did not affect basal glycerol levels while inosine, at concentrations of 1, 10 and 100 μM, increased the rate of glycerol release by 77%, 124% and 172% over the basal value, respectively. Also hypoxanthine stimulated lipolysis, but to a lesser extent as compared to inosine. Hypoxanthine 10 and 100 μM caused a 45% and 69% increase, respectively (Fig. 1, right). Like adenosine, uric acid did not alter the basal release of glycerol (Fig. 1).

In cells incubated with 0.6 mM theophylline the lipolytic effect of this methylxanthine was antagonized by adenosine (Fig. 1, left), hypoxanthine and uric acid (Fig. 1, right). A significant reduction of glycerol levels was obtained at 1 μM of the three compounds and it was almost maximum at 10 μM (32% decrease by hypoxanthine and uric acid and 55% by adenosine) (Fig. 1). In cells incubated with 100 μM hypoxanthine there was no difference between the rate of lipolysis measured in the absence and in the presence of theophylline, due to the ability of this metabolite to increase the basal release of glycerol (Fig. 1, right).

The effect of 0.6 mM theophylline was not influenced by inosine (Fig. 1, left). Similarly, inosine did not cause any significant change in the lipolytic response of fat cells to 2 μM norepinephrine (Fig. 1, left), while glycerol production measured in the presence of the catecholamine was reduced by 10 and 100 μM adenosine (20% and 38% inhibition,

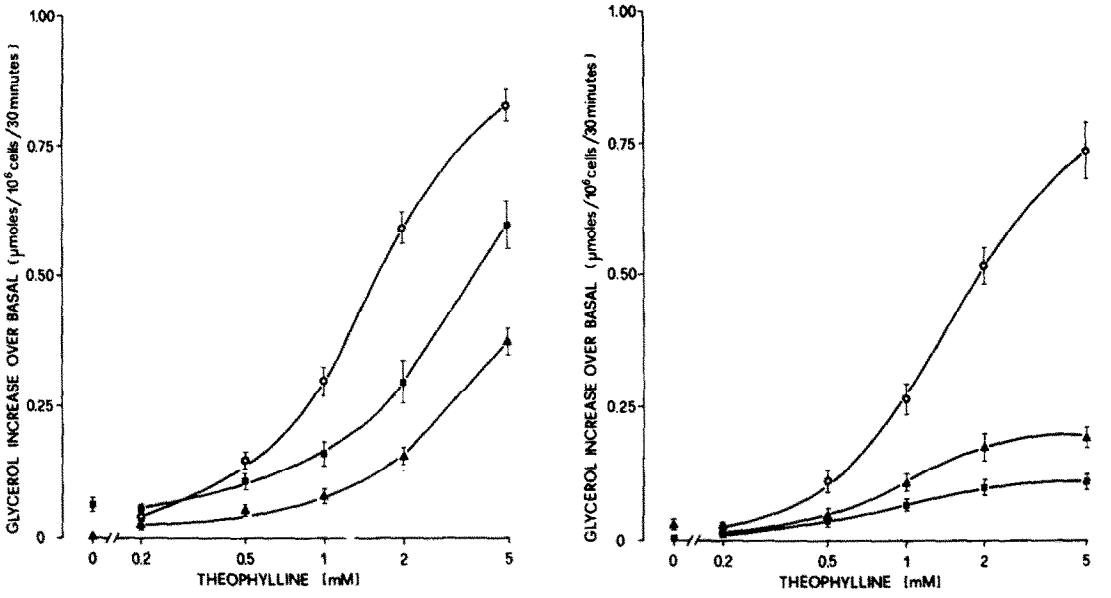

Fig. 2. Left: concentration–response curves for the lipolytic effect of theophylline in the absence (○) and in the presence of 10 μM adenosine (▲) or 10 μM inosine (■). Each point is the mean (±SEM) of the data obtained from 9 incubations in 3 experiments. The basal release of glycerol was 0.051 μmoles/10^6 cells/30 min. Right: concentration–response curves for the lipolytic effect of theophylline in the absence (○) and in the presence of 10 μM hypoxanthine (▲) or 10 μM uric acid (■). Each point is the mean (±SEM) of the data obtained from 11 to 12 incubations in 4 experiments. The basal release of glycerol was 0.044 μmoles/10^6 cells/30 min.

respectively) (Fig. 1, left), by 10 and 100 μM hypoxanthine (25% and 34% inhibition, respectively) and by 100 μM uric acid (23% inhibition) (Fig. 1, right).

Influence of adenosine, inosine, hypoxanthine and uric acid on the concentration–response curves for theophylline

On the basis of the above illustrated results, 10 μM of adenosine and its metabolites was chosen to test their influence on the concentration–response curve for theophylline. The results reported in Fig. 2 were calculated as theophylline-induced increases of glycerol release over the basal value in the absence and presence of adenosine or inosine (Fig. 2, left) and of hypoxanthine or uric acid (Fig. 2, right). Inosine, that *per se* increased the rate of lipolysis, did not cause relevant changes in the response to 0.2 and 0.5 mM theophylline, but reduced the response to higher concentrations of the methylxanthine. As compared to inosine, adenosine was a more effective antagonist of theophylline-induced stimulation (Fig. 2, left). Hypoxanthine and, even more, uric acid markedly reduced the release of glycerol caused by theophylline and the antagonism was non-competitive (Fig. 2, right).

Influence of adenosine, inosine, hypoxanthine and uric acid on the concentration–response curves for norepinephrine

In these experiments adenosine and its metabolites were used at a concentration of 100 μM. Inosine did not cause any relevant change in the concentration–response curve for norepinephrine, while adenosine reduced the release of glycerol at any catecholamine concentration tested (Fig. 3, left). Also hypoxanthine and uric acid exerted a significant antagonistic effect, that was most evident at submaximum concentrations of norepinephrine (Fig. 3, right).

Norepinephrine was markedly less sensitive than theophylline to the inhibition by all the compounds tested.

Influence of adenosine deaminase on lipolysis

Adenosine deaminase (0.5 U/ml) increased the basal rate of glycerol release and potentiated the lipolytic response to 0.6 and 2 mM theophylline (+70% and +16%, respectively) as well as to 2 and 5 μM norepinephrine (+40% and +22%, respectively) (Table 1).

Treatment of the cells with adenosine deaminase caused an increase of the minimum inosine concentration required for inducing a significant stimulation of lipolysis (10 μM vs 1 μM in the absence of the enzyme). Moreover, at any inosine concentration tested, the rate of glycerol release was higher in the presence than in the absence of adenosine deaminase, with larger differences at the lowest inosine concentrations (Fig. 4).

DISCUSSION

Incubation of rat fat cells with adenosine deaminase causes the disappearance of adenosine from the extracellular fluid and a large accumulation of inosine, which is then converted into hypoxanthine [5]. The latter metabolite undergoes rapid degradation to uric acid so that it can be detected in significant amounts only in the presence of a xanthine

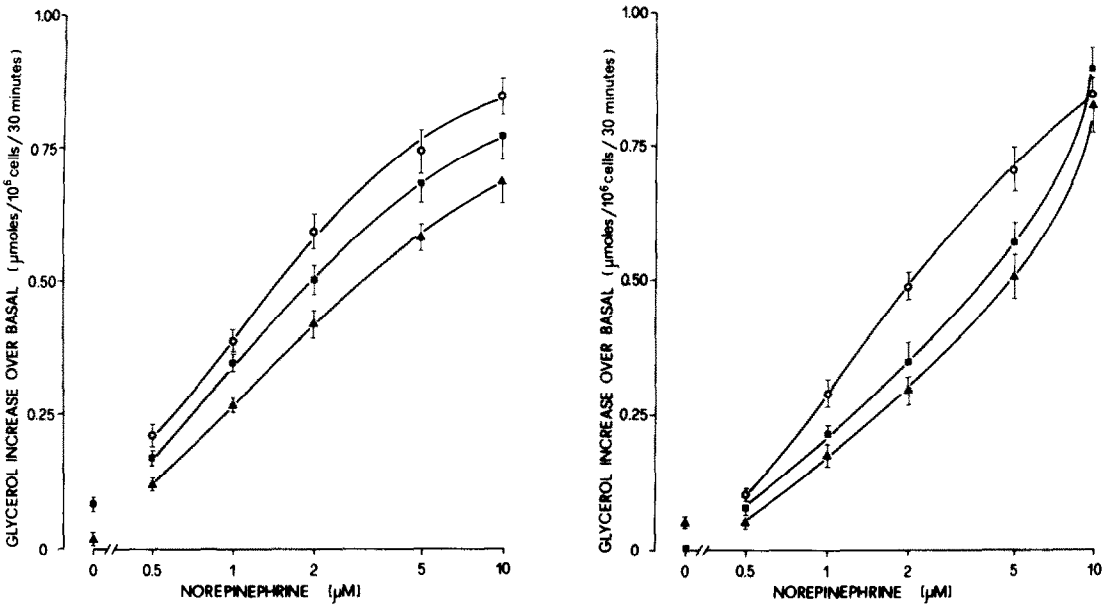

Fig. 3. Left: concentration–response curves for the lipolytic effect of norepinephrine in the absence (○) and presence of 100 μM adenosine (▲) or 100 μM inosine (■). Each point represents the mean (±SEM) of the data obtained from 12 incubations in 4 experiments. The basal release of glycerol was 0.060 μmoles/10^6 cells/30 min. Right: concentration–response curves for the lipolytic effect of norepinephrine in the absence (○) and in the presence of 100 μM hypoxanthine (▲) or 100 μM uric acid (■). Each point is the mean (±SEM) of the data obtained from 10 to 12 incubations in 4 experiments. The basal release of glycerol was 0.068 μmoles/10^6 cells/30 min.

dehydrogenase inhibitor [5]. According to the present results, exogenously added inosine and hypoxanthine are capable of increasing the basal rate of lipolysis in isolated rat fat cells, inosine being the most effective. By contrast, adenosine and uric acid did not influence this process to any significant extent. Thus, if exogenously added molecules mimick the effects of the respective endogenous metabolites, the conversion of adenosine into lipolytic compounds, i.e. inosine and hypoxanthine, may account for the ability of adenosine deaminase to activate basal lipolysis [4, 10, 11], an effect that was confirmed in this study. The lack of adenosine influence at this level is in contrast with the data reported by Ebert and Schwabe [6] showing that adenosine is more effective than inosine in increasing the release of glycerol from fat cells. This discrepancy is not easy to explain, since the experimental conditions were very similar to the ones used in the present experiments, except for the lower albumin concentration in the medium (2%), the larger number of cells in the samples (120,000 ± 20,000 per ml) and the longer incubation time (60 min) used by these authors [6, 12].

Theophylline activates lipolysis by competing with adenosine at P_1-purinergic receptors [4, 6, 13–16]. Inosine is much weaker than adenosine as an inhibitor of the lipolytic response to this methylxanthine [6], in accordance with its low affinity for adenosine receptors [13, 14, 17]. The present data are in full agreement with these findings and degradation of adenosine to inosine by adenosine deaminase may thus explain the potentiation of theophylline-induced lipolysis by this enzyme. This potentiation was orig-

Table 1. Stimulation of lipolysis by adenosine deaminase

Drugs	Glycerol (μmoles/10^6 cells/30 min)		
	Control	ADA (0.5 U/ml)	P
—	0.079 ± 0.009	0.127 ± 0.011	<0.005
Theophylline 0.6 mM	0.253 ± 0.018	0.428 ± 0.021	<0.001
Theophylline 2 mM	0.702 ± 0.043	0.816 ± 0.032	<0.05
Norepinephrine 2 μM	0.560 ± 0.028	0.784 ± 0.041	<0.001
Norepinephrine 5 μM	0.743 ± 0.036	0.904 ± 0.043	<0.01

Fat cells were preincubated for 15 min in the absence or presence of adenosine deaminase (ADA). Theophylline and norepinephrine were then added at the indicated concentrations and the reaction was stopped 30 min later. Each value is the mean (±SEM) of the data obtained from 10 to 12 incubations in 4 experiments. The statistical significance of the changes induced by adenosine deaminase is indicated in the last column (P).

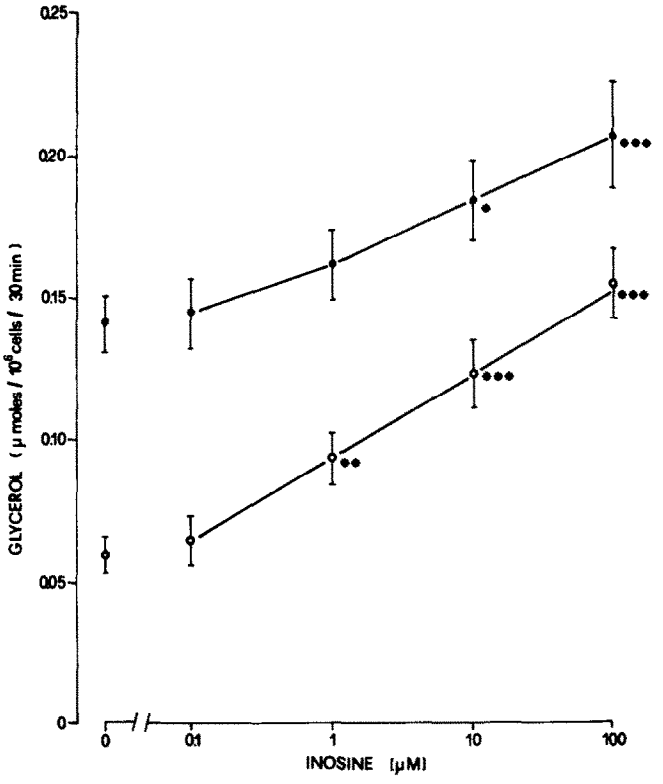

Fig. 4. Influence of adenosine deaminase (0.5 U/ml) on the concentration–response curve for the lipolytic effect of inosine. Fat cells were incubated with various concentrations of inosine in the absence (○) or in the presence (●) of adenosine deaminase. Each point represents the mean (±SEM) of the data obtained from 9 to 12 incubations in 4 experiments. The significance of the changes induced by inosine was calculated by the Student's *t*-test. * $P < 0.05$, ** $P < 0.02$; *** $P < 0.001$.

inally shown by Schwabe and Ebert [4] using submaximally effective concentrations of theophylline, while Fain and Wieser [11] were unable to detect any change by adenosine deaminase, possibly due to the short incubation time they used.

Hypoxanthine, that does not bind to adenosine receptors in fat cells [14], and uric acid were more effective than adenosine in inhibiting the lipolytic response to theophylline through a non-competitive type of antagonism, suggesting that they are not involved in the lipolytic action of adenosine deaminase under these conditions. This view is also supported by the data of Fain [5] showing that the accumulation of these metabolites during incubation of rat fat cells with adenosine deaminase is much lower as compared to the increase in inosine levels.

As previously demonstrated [6], norepinephrine-induced lipolysis was less susceptible to inhibition by adenosine than the theophylline-activated process. However, in contrast with the findings of Ebert and Schwabe [6], hypoxanthine and uric acid caused a reduction of the lipolytic action of norepinephrine that was quantitatively similar to the inhibition caused by adenosine, while inosine, up to 100 μM, was ineffective. These results further stress the possibility that inosine is the adenosine metabolite that largely accumulates in the incubation medium during fat cell treatment with adenosine deaminase, accounting for the effects of this enzyme on lipolysis.

Finally, the ability of adenosine deaminase to increase lipolysis in the presence of low (0.1, 1 μM) and, to a lesser extent, of high (10, 100 μM) inosine concentrations, can be explained by a very large accumulation of this metabolite, mimicking the effect of 10–100 μM of exogenous inosine. However, these concentrations far exceed physiological limits, that are in the range of 0.1–1 μM [1, 18–20]. Thus an alternative explanation can be proposed. The lack of adenosine influence on spontaneous lipolysis may indicate that the endogenous nucleoside is sufficient for maximally inhibiting this process, as previously suggested by Turpin *et al.* [21]. If this is the case, the loss of adenosine-induced inhibition would fully account for the ability of adenosine deaminase to increase the lipolytic response to high inosine concentrations, while the effect of accumulated inosine may further contribute to the influence of the enzyme measured in the presence of inosine concentrations that are in the physiological range [1, 18–20].

While this appears to be a reasonable interpretation of the present and other authors' findings, it is necessary to stress that their physiological significance is limited by various factors, mainly the interference of endogenous metabolites with the response to exogenously added compounds, their possible conversion by enzymes present in fat cells or contaminating albumin preparations [22], and the need for a direct measurement of adenosine and its

metabolites in the incubation medium along with glycerol levels. Fain [5] used fat cells loaded with [8-^{14}C]adenine and followed the incorporation of the ^{14}C label into adenosine, inosine, hypoxanthine and uric acid. We are now developing a very sensitive HPLC method for the direct determination of these metabolites and in a preliminary assay in which normal and adenosine deaminase-treated cells (215,000 cells/ml) were compared, the large increase of the inosine peak (approximately 0.8 μM) accounted for the disappearance of adenosine from the incubation medium (Laura Caparrotta, personal communication). This method, however, still needs some improvements to allow the separation of uric acid.

In summary, while adenosine deaminase is commonly considered to act simply by removing the inhibition of lipolysis caused by endogenous adenosine, the present results offer indirect evidence for a more complex effect, involving adenosine metabolites and mainly inosine.

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