PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 182, 15–22 (1986)

Adenosine Release from Isolated Rat Adipocytes: Influence of Fat Cell Concentration and Cell Size[1] (42303)

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Abstract. The release of adenosine by isolated rat adipocytes into the incubation medium was studied in relation to fat cell size and concentration. Incubations were carried out for 60 min at 37°C in Krebs–Ringer bicarbonate–albumin medium containing 6 m*M* glucose. 2′-Deoxycoformycin was added to inhibit endogenous adenosine deaminase activity (maximal supression was achieved at 0.8 μM concentration of the inhibitor). The data show that (a) the amount of adenosine released into the medium was similar for the first and second 30-min incubation periods; (b) increasing adipocyte concentration markedly inhibited adenosine release; and (c) large fat cells (volume > 300 pl) released significantly more adenosine (per fat cell) into the medium than smaller fat cells (volume < 180 pl) when incubated at concentrations of ≤350,000 cells/ml. Above this cell concentration, differences between adenosine release and cell size were not noted. Adenosine release by isolated rat adipocytes appears to be a precisely regulated process which is exquisitly sensitive to the number of fat cells in the incubation medium and, to a certain extent, to the adipocyte size.

Adenosine, an ubiquitous nucleoside, is known to have a number of physiological actions. In most vascular beds, adenosine exhibits a vasodilatory effect (1), and in the coronary bed adenosine release has been shown to increase following myocardial ischemia (2–8). In contrast, adenosine produces vasoconstriction in the kidney (1, 9). A neurotransmitter role for adenosine in the central nervous system (10–16), as well as an inhibitory role in the release of noradrenaline from sympathetic nerve terminals in many tissues, including the heart, has been demonstrated (9, 17).

An additional physiological function of adenosine is suggested by its capacity to inhibit insulin release from rat pancreatic islets (18), and by the moderate degree of hyperglycemia produced by adenosine when injected into intact rats (19). The antilipolytic effect of adenosine, shown in adipose tissue (20), isolated adipocytes (21–22), or perifused fat cells (23, 24) challenged by lipolytic hormones, and the enhancement by adenosine of the effect of insulin on glucose metabolism also suggest a local metabolic regulatory role for adenosine in adipose tissue (25–27). Recent studies from our laboratory have shown marked suppression of food intake following subcutaneous or intravenous administration of adenosine (28); these observations have suggested that adenosine could be a physiological signal of peripheral lipid and caloric storage to the hypothalamus.

The present study was directed toward the elucidation of adenosine release by isolated rat adipocytes in order to determine whether factors such as fat cell size or fat cell concentration in the medium may affect the production of adenosine by adipose tissue from animals with different degrees of adiposity.

Materials and Methods. *Animals.* Male Wistar rats (from Royalhart Laboratory, New Hampton, N.Y.) were housed in individual cages, in a temperature-controlled (22°C) room with light–dark intervals of 12 hr. Rats were allowed free access to water and Purina laboratory chow. Three groups of rats were studied: Group A, 6–8 weeks old, weighing 150–230 g; Group B, 3–4 months old, weighing 300–400 g; and Group C, 8–12 months old, weighing 550–670 g.

[1] Supported in part by USPHS Grant AM 17403 and CA 22863.

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0037-9727/86 $1.50

Materials. Collagenase, Type I (lot 47A 144P) was obtained from the Worthington Corporation (Freehold, N.J.). Bovine serum albumin (fraction V powder, No. A-4503), adenosine, inosine, and adenosine deaminase (EC 3.5.4.4) were obtained from Sigma Chemical Company (St. Louis, Mo.). Tri-*n*-octylamine was procured from Aldrich Chemical Company (Milwaukee, Wis.). Freon 113 (1,1,2-trichlorotrifluoroethane) was purchased from Fisher Scientific Company (Atlanta, Ga.). 2′-Deoxycoformycin was a gift from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, (Bethesda, Md.). *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) was kindly provided by Dr. Donald Namm, Burroughs Wellcome (Research Triangle Park, N.C.).

Experimental protocol. After the animals were killed (by cervical dislocation in the fed state between 9 and 11 AM), the epididymal fat pads were removed, cut into small pieces (10–20 mg), and incubated at 37°C for 1 hr with collagenase (5–10 mg/g of tissue) in Krebs–Ringer bicarbonate (KRB) with 4% bovine serum albumin and 6 m*M* glucose, at pH 7.4. The isolated fat cells were washed four times with KRB–albumin medium and resuspended. Aliquots of the isolated fat cell suspension were removed and diluted with KRB–albumin medium (in siliconized Erlenmeyer flasks) to give three separate cell concentrations (at an approximate ratio of 1:2:4) corresponding to 0.6, 1.2, and 2.4 $\times$ 10^6 fat cells/ml, respectively, in final incubation volumes of 12 ml.

Six experiments were carried out with pooled epididymal adipose pads obtained from 12 rats in Group A, four experiments with pooled pads from 6 rats in Group B, and five experiments from the pads of animals in Group C.

Incubations were carried out at 37°C in a Dubnoff metabolic shaker (80 strokes/min) under a gas-phase of 95% O_2–5% CO_2. Duplicate samples of 3 ml each were removed within 0.5 min and after 30 and 60 min of the incubation, and then transferred into nitrocellulose test tubes. After centrifugation for 20 sec at 500*g* the bottom of the tube was punctured and the medium was allowed to drip into a plastic centrifuge tube. Adenosine was measured in the medium by the procedure described below. In parallel experiments, aliquots of the various fat cell suspensions were removed, prior to and during the incubation, for determination of fat cell concentration and size as previously described (29). The volume of the medium occupied by the cells was also calculated and taken into account for the estimate of adenosine release. Results are expressed as net (final minus initial) adenosine released into the incubation medium by 10^6 fat cells in 30 or 60 min.

In preliminary experiments, two inhibitors of adenosine deaminase were tested to define the appropriate concentration which would prevent deamination of the adenosine released during the incubation period. The two inhibitors tested, EHNA and 2′-deoxycoformycin, were incubated at various concentrations with isolated fat cells at several cell concentrations and sizes. At the end of 1 hr of incubation, aliquots of the cell suspension were removed and processed as described below. The concentrations of inhibitors capable of producing persistent maximal suppression of adenosine deaminase activity (as determined by the presence of a consistently stable peak for adenosine and the lack of an inosine peak in the liquid chromatogram) were 100 μM for EHNA and 0.8 μM for 2′-deoxycoformycin. No significant differences were observed between the two inhibitors in the rapidity of action or the maximal degree of effectiveness. Because of its longer duration of action (data not shown) and effectiveness at lower concentrations, 2′-deoxycoformycin (0.8 μM) was used in all subsequent experiments.

Analytical techniques. Adenosine was determined according to Hartwick and Brown (30) as follows: An aliquot of the incubation medium was transferred to a centrifuge tube containing cold trichloroacetic acid (TCA; final concentration = 4%). The TCA–medium mixture was vortexed and then centrifuged at 500*g* for 20 min at room temperature. An aliquot of the supernatant fraction was transferred to another centrifuge tube and an equal volume of a solution of 4.42 g of tri-*n*-octylamine in 25 ml of Freon 113 was added. The mixture was vortexed and centrifuged at 500*g* for 10 min. The upper phase was removed and stored overnight at 4°C in a siliconized glass vial to be assayed the following day by high-

pressure liquid chromatography (HPLC). The recovery of added adenosine was more than 95%.

A liquid chromatograph (Waters Assoc., Milford, Mass.) Model ALC-202, equipped with uv detector (254 nm) and a μBondapak C_{18} (30 cm × 4 mm) reversed phase column was used. Numerical integration was carried out on a Spectraphysics System IV Computing Integrator for chromatography. The analysis was carried out at ambient temperature. The mobile phase consisted of a 10:90 v/v mixture of methanol (HPLC grade) and 0.007 *M* KH_2PO_4, pH 5.8 (adjusted with H_3PO_4 prior to the addition of methanol). The flow rate was maintained at 2.5 ml/min at a pressure of 1500–2000 psi. Samples (250 μl) were injected via the UK6 Universal injector (Waters).

Standard solutions of adenosine and inosine at concentrations of 5 μg/ml were prepared in distilled water. Calibration was carried out and standard curves were obtained every day prior to sample injections; standards were also run frequently during the adenosine determination. Confirmation of adenosine peaks was achieved by adding known amounts of adenosine to samples prior to injection (superimposition of peak), as well as by the enzymatic peak shift method (disappearance of adenosine peak and appearance of inosine peak) using adenosine deaminase (2 units per sample). This large excess of the enzyme was necessary in the confirmatory tests to overcome the inhibitory effect of the added 2′-deoxycoformycin. The standard curve was linear in the range of 10–1000 ng and day to day variability was less than 3%; the minimum sensitivity of the method was 10 ng of adenosine.

Statistical evaluation of the data. Comparisons of means were done using Student's *t* test (31). Values of $P < 0.05$ were taken to indicate significance. Linear regression utilized the method of least squares: An exponential relationship was discerned by logarithmic transformation (32).

Results. *Rate of release of adenosine.* For the three cell size populations studied, the amount of adenosine produced during the first and the second 30 min of incubation, expressed as ng/10^6 fat cells, is shown in Fig. 1. The plots also depict the data obtained from incubations carried out at different cell concentrations. The results indicate that the net adenosine production by isolated adipocytes was approximately the same for the first and second 30-min incubation periods regardless of cell size and cell concentration; there were no significant differences ($P < 0.05$) in almost all cases (eight of nine panels as shown in Fig. 1).

Effect of cell concentration on adenosine release. Net adenosine production (ng per 10^6 cell) was inversely related to fat cell concentration for the three sizes of adipocytes as shown in Fig. 1. In each set of three vertical panels, net adenosine production, for either the first or second 30 min of incubation, was significantly different ($P < 0.05$) when the values for the higher concentration of cells were compared to the intermediate or lower cell concentrations. The magnitude of the cell concentration effect on adenosine release appeared to be greater in the large fat cells depicted in the set of three vertical panels shown on the right in Fig. 1; this may, however, be due in part to the lower number of cells present in the incubation medium.

Comparison of the cell concentration effect in three types of cells. In Fig. 2, the effect of cell concentration on the suppression of adenosine release is expressed as percentage decrease from the values (arbitrarily set at 100%) observed at the lowest fat cell concentration. This figure not only reemphasizes the marked effect of the cell concentration in adenosine release, but it also indicates that larger fat cells may be more sensitive to the cell concentration effect (by showing a greater percentage decline affected by a lesser change in fat cell density with increasing cell density) than the intermediate or smaller-sized fat cells.

Adenosine release per fat cell in relation to fat cell size. In order to assess whether larger fat cells release more adenosine per cell into the incubation medium than smaller cells, a regression analysis of fat cell number per milliliter and net adenosine release per 10^6 cells was carried out. For this analysis, the cell types encountered in the experiments were subdivided into two major groups: Cells with a mean volume of >300 pl and cells with volume of <180 pl. Figure 3A shows that the relationship between the two variables is exponential. When the fat cell number exceeded 350,000/ml, adenosine release appeared to be

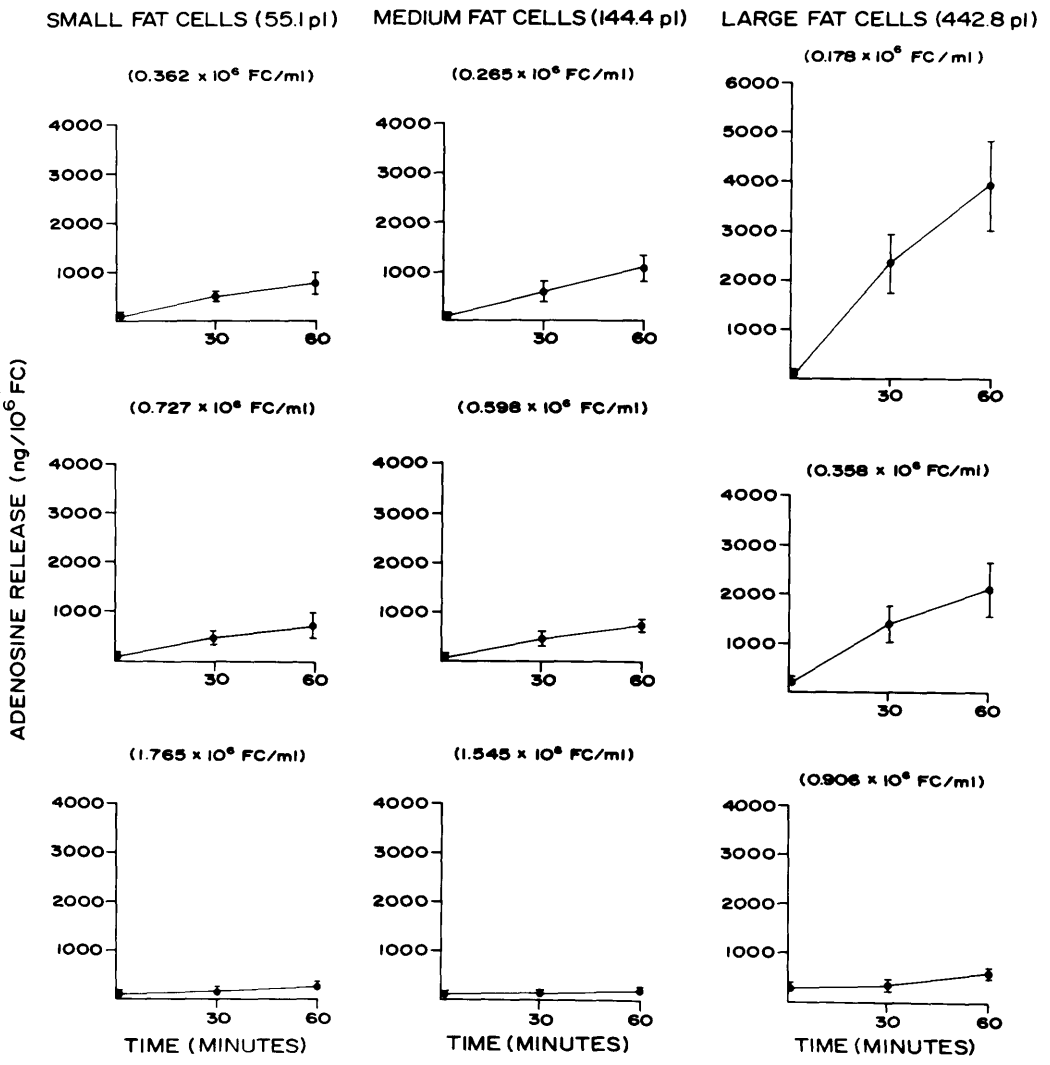

FIG. 1. Comparison of adenosine release into the medium by three types of rat adipocytes (small, mean volume = 55.1 pl; intermediate, mean volume = 144.4 pl; large, mean volume = 442.8 pl), incubated at three cell concentrations (top panel—low concentration; middle panel—intermediate concentration; bottom panel—high concentration). Values are means ± SE of duplicate observations in five experiments for each point. The numbers in parentheses represent fat cell concentration.

suppressed and thus differences among the two types of cells could not be clearly identified. When the cells were incubated at a cell density lower than 350,000/ml, however, the two cell populations, identified by cell volume, appeared to separate in two groups with regard to adenosine release (Fig. 3B). When subjected to group mean comparison by Student's t test, the larger cells released significantly more adenosine than the smaller cells ($P < 0.01$).

Discussion. The present study was carried out to determine the relationship between *in vitro* adenosine release by rat adipocytes and the size and concentration of the fat cells. Our study shows that (a) production of adenosine was approximately the same for the first and second 30-min periods of incubation; (b) adenosine release was correlated with adipocyte concentration; and (c) adipocytes of large size (above 300 pl in volume) released, per

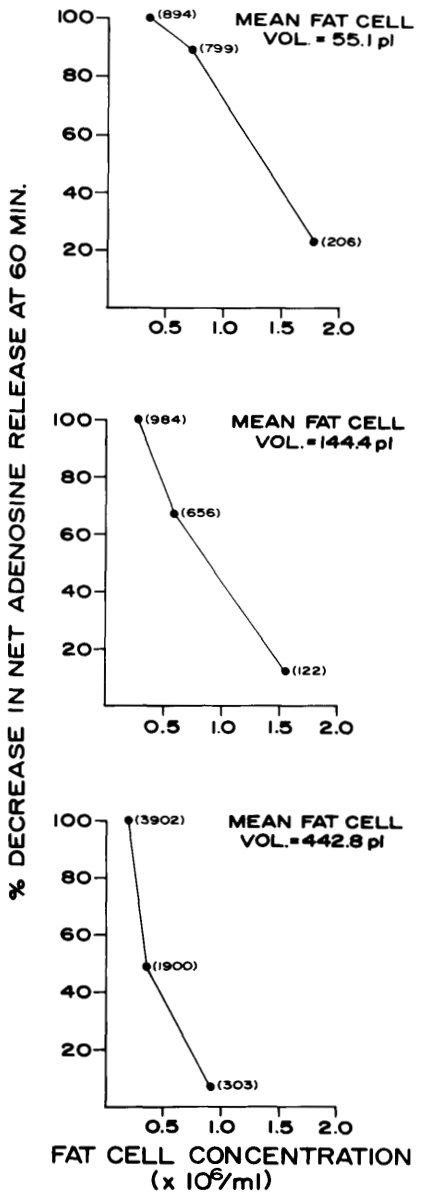

FIG. 2. Relationship between fat cell concentration and relative decrease in net adenosine release at 60 min of incubation for the three sizes of fat cells (as in Fig. 1). Value shown represent means of duplicate observations in five experiments for each cell size type. Values in parentheses are net adenosine released at 60 min, expressed as nanograms per 10^6 fat cells.

unit of time, more adenosine per fat cell than smaller adipocytes.

Certain relevant methodological considerations are in order: (i) The technique of high-pressure liquid chromatography was found to be efficient and reliable. The sensitivity of the method, however, limits the determination of adenosine to 10 ng per sample injected into the chromatograph. This requires a minimum of 100,000–150,000 cells/ml in the incubation medium to collect enough adenosine to be measured. (ii) Since adenosine is deaminated by adenosine deaminase, a rather ubiquitous enzyme, it is essential, in studies such as these, that an adequate concentration of adenosine deaminase inhibitor be employed. (iii) Even though the deamination of adenosine and its subsequent conversion to other metabolic products was prevented, a possible source of imprecision in our determination of net adenosine release might be the continuing removal of adenosine from the medium by the adipocytes themselves. Adenosine is known to be bound and taken up by fat cells (34). In a preliminary series of experiments, we measured the adenosine uptake by adipocytes at various adenosine concentrations (data not shown). Based on our data and those of others (34), the amount of adenosine taken up by the cells after 30 min of incubation approximates one-tenth to one-fifth that released into the medium. Furthermore, since the uptake of adenosine by fat cells is related to its concentration (34), the amount of adenosine removed from the medium may even be lower when cells are incubated at high cell concentrations. The present observations suggest that the extraction of adenosine from the medium by incubated fat cells, although important and sizeable, probably does not significantly alter the overall results and conclusions of this study.

One of the main findings in this study is the suppression of adenosine release by progressively increasing cell concentrations in the medium for all cell sizes studied (shown in Figs. 1 and 2). It appears (Fig. 2) that large fat cells are somewhat more sensitive to the cell concentration effect; large fat cells, however, were incubated at a slightly lower cell concentration than the other two cell types. The cell concentration effect could be the result of a variety of factors that include (a) cell–cell interaction, (b) accumulation of adenosine in the medium with feedback inhibition, and (c) accumulation in the medium of products such as free fatty acids that may independently suppress lipolysis (33). Since Schwabe *et al.*

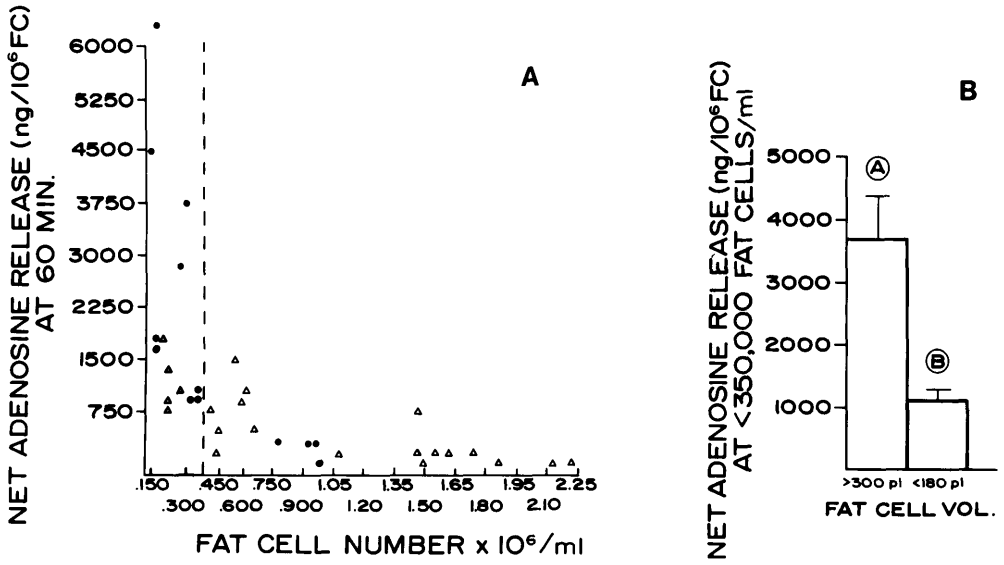

FIG. 3. (A) Relationship between net adenosine release at 60 min of incubation and fat cell concentration for cells in two size groups: Mean volume < 180 pl (△), and >300 pl (●). (B) The adenosine values (A) plotted to the left of the dotted line (reflecting a cell concentration of 350,000 fat cells/ml) were averaged for the two sizes (mean volumes < 180 pl and >300 pl) and replotted. Group mean comparison revealed a significantly greater ($P < 0.001$) adenosine release by the larger fat cells (Group A) than by smaller cells (Group B).

(21, 25) and others (20, 23) have suggested a close link between lipolysis and adenosine release, it is possible that inhibition of lipolysis may alter adenosine metabolism and efflux from fat cells.

Further, adenosine mimics insulin in its antagonism of hormone-stimulated lipolysis (20, 21, 23–25) and promotion of glucose metabolism in fat cells (26); addition of adenosine to incubation mixtures containing fat cells and and insulin appeared to enhance the effect of the hormone. Since large fat cells are less responsive to the effect of insulin on glucose metabolism (29), a greater production of adenosine (by large cells) may not be able to overcome the decrease in insulin effect on glucose transport and metabolism, but may potentiate the antilipolytic effect of insulin. Further studies will be needed to clarify this point.

The data depicted in Fig. 1 showing similar adenosine release in the first and second 30-min periods are at variance with those of Schwabe *et al.* (25), who found diminished release of adenosine with time by isolated fat cells. The absence of an adenosine deaminase inhibitor in Schwabe's study may be responsible for this difference. The present findings also provide evidence (at least for the first 60 min of incubation) against the previously advanced suggestion that adenosine accumulation per se may be a negative feedback signal on adenosine production (25). Indeed, if the adenosine concentration in the medium, found to be somewhat higher during the second 30 min of incubation than during the first 30 min, had been the feedback signal, one would have expected a progressive diminution of adenosine release in the second 30 min of incubation. It appears that factors other than accumulation of adenosine itself into the medium are probably responsible for the inhibition of adenosine release with increasing cell concentration. This observation needs to be investigated further.

A novel finding in this study is the increased production of adenosine by large fat cells, which are also known to present a greater basal lipolytic rate (29). We have previously demonstrated that large adipocytes from older, fatter rats contain greater amounts of ATP than smaller adipocytes (35). It is therefore not surprising that more adenosine appears to be produced in the large fat cells.

In conclusion, the studies reported here in-

dicate that adenosine release by adipocytes is a precisely regulated process which is quite sensitive to the number and concentration of cells in the incubation medium. Adenosine release appears to be linear (in short term studies) with time and related, to a certain extent, to the adipocyte size. We still do not know whether the total contribution to adenosine production by an enlarged adipose organ of an obese animal is greater than that of a lean animal, but our results suggest that this is a viable hypothesis. Studies are now in progress in our laboratory to determine the concentration of adenosine in the blood of lean versus obese animals and thus to explore this possibility.

We gratefully acknowledge the expert help of Dr. Nancy Newton in the preliminary phase of this work concerning the selection of an adenosine deaminase inhibitor. We are particularly grateful to Dr. Steven Moffitt of the Department of Biometry, Emory University, for his valuable contribution to the statistical analysis of the data. We also thank Ms. Cathy Moss and Ms. Sarah Attaway for their help in preparing this manuscript.

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Received June 3, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted January 14, 1986.