Implantation of Exogenous Enzymatic Activity in Isolated Alveolar Macrophages

Abstract. *Alveolar macrophages acquired the capacity to oxidize uric acid after exposure to uricase. Presumably this resulted from intracellular incorporation of the enzyme by endocytosis. This approach may provide a general method for (i) testing regulatory biochemical mechanisms, (ii) therapeutic replacement of deficient regulatory macromolecules, and (iii) studying environmental factors that produce genetic alterations.*

The uptake of macromolecules by endocytosis is a well-known property of macrophages (*1*). Previous studies in our laboratory demonstrated the uptake of ferritin (molecular weight 450,000) by isolated alveolar macrophages without functional impairment of the cells (*2*). This observation raised the possibility that exposure of alveolar macrophages to biologically active foreign macromolecules, such as enzymes, might result in the cellular uptake of such molecules, thus endowing the cell with new biochemical functions. This has been accomplished. Alveolar macrophages acquired the capacity to oxidize uric acid after exposure to the enzyme uricase (E.C. 1.7.3.3).

Alveolar macrophages were obtained from healthy New Zealand white rabbits (2 to 3 kg) by pulmonary lavage (*2, 3*). Following removal, the lungs were lavaged three times through the trachea with a modified Ringer-phosphate solution (NaCl, 110 m*M*; Na_2HPO_4, 0.42 m*M*; KH_2PO_4, 0.35 m*M*; Na_2SO_4, 19.5 m*M*; K_2SO_4, 2.57 m*M*; $CaCl_2 \cdot 2H_2O$, 1.24 m*M*; and glucose, 10 m*M*; *p*H 7.40). The lavage fluid was collected and centrifuged at 850*g* for 10 minutes at 4°C. Approximately 100 mg of cellular material was obtained from each animal. Material from eight to ten animals was pooled to obtain an adequate number of cells, rewashed with Ringer solution, and recentrifuged to obtain the final cell suspension. The suspension contained less than 2 percent white blood cells and a small number of red cells. As judged by the eosin Y viability test (*4*), more than 98 percent of the macrophages were viable.

Uricase activity was determined by measuring the decrease in uric acid in the incubation medium. Six experiments were performed as follows. Approximately 800 to 1000 mg of cells were incubated in 5 ml of Ringer-tris solution (same composition as Ringer-phosphate, plus 0.05*M* tris(hydroxymethyl)aminomethane, *p*H 7.40) containing 4 units (*5*) of uricase (Sigma) for 1 hour at 37°C in a Dubinoff metabolic shaker. Control cells were incubated without uricase during this time. The cells were then separated from the medium and washed until there was no measurable uricase activity in supernatant fluid. The washed cells were resuspended in 4 ml of Ringer-tris solution containing uric acid (50 μg/ml), *p*H 7.40. This suspension was mixed for 10 minutes in a Dubinoff metabolic shaker at 37°C, and 1 ml was obtained to determine the initial concentrations of uric acid and lactic acid in the supernatant and the protein concentration in the suspension (T_0 samples). The suspension was then incubated for 1 hour at 37°C in the shaker, and these measurements were repeated (T_1 samples). The mixture was then centrifuged, the supernatant was incubated for another hour at 37°C, and another measurement of uric acid concentration was made. The supernatant from the T_0 sample was incubated for 2 hours at 25°C, and uric acid concentration was remeasured. The amounts of uric acid oxidized and lactic acid generated during the incubation period were determined from differences in concentration in the T_0 and T_1 supernatants.

More than 85 percent of cells were viable at the end of the experiments, as shown by the eosin Y viability test. Essentially no uricase activity was found in homogenates of cells not exposed to uricase. Uric acid concentrations were measured spectrophotometrically at 293 nm by the enzymatic method of Praetorius (*6*). Lactate concentrations were determined by the enzymatic method of Scholtz *et al.* (*7*). Protein concentrations were determined in the cell suspension by the method of Lowry *et al.* (*8*).

Enzymatic activity in untreated and uricase-treated cells is compared in Table 1. Although there was wide variation in acquired uricase activity, treated cells invariably developed the capacity to oxidize uric acid after exposure to uricase. Uricase activity in the isolated supernatants was sometimes too low to be detected, and was always significantly less than the activity found when the cell fraction was present. This indicates that the uricase activity was associated with the cell fraction and did not reflect leak of uricase into the extracellular phase.

No significant difference was found between lactate generation in control and uricase-treated cells ($P > .7$, $n = 6$). Lactate generated (micromoles per milligram of protein per hour) was 0.18 ± 0.08 in control cells and 0.20 ± 0.10 in uricase-treated cells. The fact that lactate generation remained unaltered in uricase-treated cells supports the results of the eosin Y viability test in demonstrating that the enzymatic oxidation of uric acid occurred in living cells.

A major question is whether the demonstrated uricase activity was the result of specific intracellular uptake, or whether the activity represented nonspecific adsorption of uricase to the cell surface. In the latter circumstance, the oxidation of uric acid could occur by the exposure of extracellular uric acid to membrane-bound uricase. Nonspecific adsorption of protein has been reported for various cell types, such as Ehrlich ascites tumor cells. Repeated washings did not lead to total removal of the adsorbed protein (*9*). Two types of studies were performed to test this possibility. (i) Alveolar macrophages were exposed to a metabolic inhibitor, and uricase activities in exposed and unexposed cells were compared. (ii) Uricase activity was studied in human red blood cells, which lack the property of endocytosis.

Alveolar macrophages exposed to antimycin A ($10^{-6}M$) during the incubation with uricase retained significantly less uricase activity than did cells not exposed to antimycin A (Table 2). The methods described earlier were used, except that the incubation with uricase also contained $10^{-6}M$ antimycin A (experimental) or 50 μl of 50 percent ethanol (control).

Antimycin A, an inhibitor of mitochondrial oxidative phosphorylation, specifically affects cytochrome b of the cytochrome electron transport chain. The drug ($10^{-6}M$) produced an 82 percent reduction in oxygen consumption of alveolar macrophages during a 1-hour incubation. Cohn (*10*) reported that this dose of antimycin A reduced

Table 1. Decrease in extracellular uric acid after incubation with alveolar macrophages. Cells were first incubated for 1 hour with uricase, then washed 10 times. Control cells were not incubated with uricase. Results are given as the decrease in uric acid (micrograms per milligram of protein per hour). The difference between control and experimental cells is significant ($P < .01$).

Control	Incubated with uricase
0.03	0.6
0.08	1.0
0.06	1.5
0.09	3.1
0.18	0.4
0.04	2.4
0.08 ± 0.05 (Mean $\pm$ S.D.)	1.50 ± 1.06 (Mean $\pm$ S.D.)

the formation of pinocytotic vesicles to less than 10 percent of control values. In the present studies, the reduction in uricase activity in cells exposed to antimycin A suggests that energy derived from oxidative phosphorylation is required for the acquisition of uricase activity.

Red cells treated with uricase showed a slight, but statistically significant increase in uricase activity ($P < .05$, $n = 6$). The decrease of uric acid in the medium (micrograms per milligram of protein per hour) was 0.04 ± 0.01 in control cells and 0.06 ± 0.01 in uricase-exposed cells. Thus, a small fraction of uricase appeared to be irreversibly bound to the cell surface, while retaining some degree of biologic activity.

The possibility that uptake of uricase by macrophages did not involve endocytosis seems unlikely from the differences in uricase activity found in alveolar macrophages and red cells. Uricase activity was approximately 20 times greater in uricase-treated macrophages than in untreated macrophages or treated red cells. It could be argued the differences in uricase activity found in the two cells were due to differences in surface area. Red cells are smaller than macrophages, and the surface area of macrophages further increases in solution because pseudopod-like projections are formed. As a result, greater nonspecific binding to macrophages might be expected. However, the results with antimycin A in macrophages favor intracellular uptake rather than nonspecific binding as the mechanism.

The rate of oxygen consumption ($\dot{Q}O_2$) was increased in alveolar macrophages exposed to uricase as compared to unexposed cells. Cell suspensions (4 to 5 mg of cell material in 4 ml of Ringer-tris solution, *p*H 7.40), with or without uricase (2 units), were tested at 37°C with a polarographic oxygen electrode system (*11*). The $\dot{Q}O_2$ was continuously measured and recorded on a Varicord 43 recorder. The reaction chambers of the oxygen monitor were filled with the cell suspensions, and 5 minutes were allowed for equilibration of the system. The $\dot{Q}O_2$ remained constant after the initial equilibration period, and measurements were made for the next 30 minutes. The $\dot{Q}O_2$ increased 31 percent in cells exposed to uricase as compared to unexposed cells ($P < .01$, $n = 6$). The $\dot{Q}O_2$ (microliters of oxygen per milligram of protein per hour) was 23.9 ± 4.1 in exposed cells and 18.2 ± 4.7 in control cells. The increase in $\dot{Q}O_2$ with no net change in lactate production in uricase-exposed cells is similar to the results of Oren *et al.* (*12*) for alveolar macrophages during phagocytosis. It seems reasonable to conclude that the increased $\dot{Q}O_2$ was related to the active incorporation of uricase into the cell (*13*).

It appears improbable that the increased $\dot{Q}O_2$ during uricase exposure or the reduced uricase activity following treatment with antimycin A could result from nonspecific binding of uricase to the external cell surface. These results support the premise that the incorporation of uricase into alveolar macrophages is an active process requiring energy derived from aerobic metabolic pathways.

It is possible that intracellular uptake of uricase was nonspecific, that is, a result of bulk endocytosis. This would not modify the overall conclusion, that a living cell has acquired a new metabolic function by exposure to a foreign enzyme.

A similar mechanism has previously been demonstrated in the ciliate *Didinium nasutum* by Doyle and Patterson (*14*). When paramecia were ingested by this organism, the enzyme dipeptidase, which originated in the paramecia, could be recovered quantitatively from the intracellular phase of the ciliate. If intracellular localization can be proved with other biologically active macromolecules, this approach may have broad application for testing regulatory biochemical mechanisms. For example, uptake of a specific

Table 2. Decrease in extracellular uric acid after incubation with alveolar macrophages previously exposed to antimycin A ($10^{-6}M$) and uricase. Cells were incubated for 1 hour with enzyme and drug (or enzyme alone, for the control), then washed free of uricase in the extracellular fluid before incubation with uric acid. Decrease in uric acid is given as micrograms per milligram of protein per hour. The difference between control and drug-treated cells is significant ($P < .05$).

Uricase only	Uricase and antimycin A
4.7	2.2
5.0	2.7
5.6	4.6
1.7	0.8
1.0	0.8
3.6 ± 2.1 (Mean $\pm$ S.D.)	2.2 ± 1.6 (Mean $\pm$ S.D.)

messenger RNA might result in specific and predictable alteration of protein synthesis. Also, heritable disorders in which regulatory macromolecules are absent might be treated by this method. Exposure of macrophages to the missing macromolecule might result in sufficient cellular uptake to reverse the disorder. This approach has been tried in patients with inborn errors of metabolism. Enzyme replacement was attempted by direct infusion of enzyme in patients with types 2 and 4 glycogenosis (*15*), metachromatic leukodystrophy (*16*), or Fabry's disease (*17*).

Finally, these studies suggest an important mechanism by which environmental agents produce genetic alteration. Cell uptake of regulatory macromolecules may lead to alterations of the DNA system in dividing cells and could produce transmissible alterations of cell function.

JAMES THEODORE
JULIO C. ACEVEDO
EUGENE D. ROBIN
Departments of Medicine and
Physiology, Stanford University
School of Medicine,
Stanford, California 94305

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Growth Hormone Responses to L-Dopa in Depressed Patients

Abstract. *Plasma human growth hormone responses to oral administration of 500 milligrams of L-dopa were analyzed in three groups of subjects: normals, age 20 to 32; normals, age 48 to 68; and unipolar depressed patients, age 45 to 68. While only 7 percent of the young normals had deficient human growth hormone responses to this stimulus, 36 percent of the older normals and 77 percent of the depressed patients failed to have adequate responses, suggesting an effect of age and a further effect of depressive illness. Because the release of human growth hormone appears to be closely related to brain catecholamine metabolism, the deficient responses in the depressed patients may provide further support to the concept of a neurochemical defect in depressive illness.*

The hypothalamic regulation of growth hormone (GH) release from the anterior pituitary appears to be closely related to brain catecholamine metabolism (*1*). Intraventricular infusions of norepinephrine provoke GH release in animals (*2*). Norepinephrine depletors, such as reserpine and α-methyl-*p*-tyrosine inhibit the normal GH response to insulin-induced hypoglycemia, whereas prior treatment with a monoamine oxidase inhibitor reverses the reserpine blockade (*3*). Intravenous infusion of phentolamine, an alpha adrenergic blocking agent, inhibits the GH response to hypoglycemia (*4*). Single oral doses of 500 mg of L-dihydroxyphenylalanine (L-dopa), a precursor of dopamine and norepinephrine, stimulate human growth hormone (HGH) release in patients with Parkinson's disease (*5*) and in normal young adults (*6*). Intravenous infusion of phentolamine inhibits the HGH response to L-dopa (*6*).

Since there is increasing evidence for a functional depletion of brain norepinephrine in at least certain depressive illnesses (*7*), we may hypothesize that such patients would manifest an impairment of HGH release to the usual stimuli. Sachar *et al.* have indicated that a substantial subgroup of depressed patients failed to release HGH in adequate amounts in response to insulin-induced hypoglycemia (*8*). Powell *et al.* have also reported that children with the syndrome of maternal deprivation and failure to grow also failed to release HGH in response to insulin-induced hypoglycemia; after a period of emotionally supportive hospital care, the HGH response returned and growth was resumed (*9*). We have now measured the plasma HGH response to a single oral dose of 500 mg of L-dopa in 16 normal subjects, age 20 to 32; 14 normal subjects, age 48 to 68; and 13 unipolar depressed patients, age 45 to 68.

All subjects were medically healthy and were receiving no medication. None of the subjects had received regimens of drugs that affect HGH secretion within 2 months of the test. None of the normal subjects had a history of significant psychiatric disturbance. All 43 subjects but one were within 25 percent of ideal body weight, and none were cachectic. All the depressed patients were unipolar by history (that is, no history of manic episodes), and none had a history of schizophrenic episodes. The depressive syndromes were all characterized by marked depressive mood, fatigue, sleep disturbance, pessimism, loss of interest, decreased responsiveness to the environment, and anhedonia; most also manifested agitation or retardation, loss of appetite, and guilty and worthless feelings.

On the experimental day, a sterile cannula was inserted in a forearm vein of the subject, who had fasted for at least 6 hours. While the subject reclined in bed, blood samples were drawn (and heparinized) every 15 minutes for 1 hour before and 2¼ hours after administration of 500 mg of L-dopa orally. The plasma was separated by centrifugation; samples were frozen and subsequently analyzed in duplicate for HGH by radioimmunoassay, with the use of dextran-coated charcoal to separate bound from free hormone (*10*), so that we were able to make accurate measurements at concentrations as low as 0.5 ng/ml. Subjects whose HGH concentration exceeded 4 ng/ml just prior to L-dopa administration were eliminated from the study. This premature HGH release, presumably due to anxiety induced by the procedure (*11*) required elimination of eight young normals, but none of the older normals or depressives. The minimum plasma HGH response to L-dopa considered adequate was above 5 ng/ml, a conservative figure accepted as the clinical standard (*12*).

The maximum HGH responses occurred 60 to 120 minutes after L-dopa ingestion, consistent with reports by

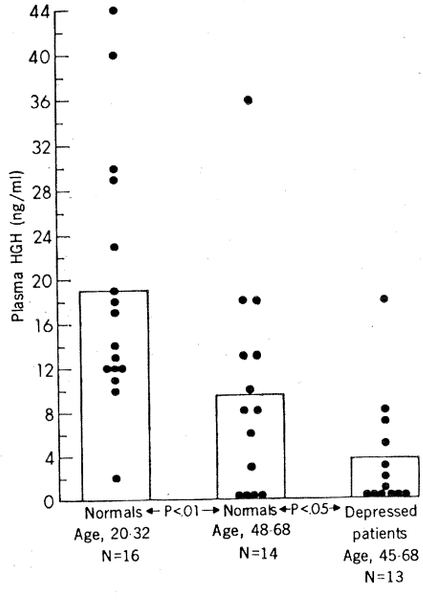

Fig. 1. Maximum HGH responses to L-dopa in normals and depressed patients. Circles represent individual HGH responses. Bars represent mean HGH response for each group.