# 5′-NUCLEOTIDASE ACTIVITY OF MOUSE PERITONEAL MACROPHAGES

## I. Synthesis and Degradation in Resident and Inflammatory Populations*

By PAUL J. EDELSON‡ and ZANVIL A. COHN

*(From The Rockefeller University, New York 10021)*

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase EC 3.1.3.5) is a widely distributed enzyme which hydrolyzes monophosphate 5′-ribonucleotides to their corresponding nucleosides with the release of inorganic phosphate. In the resident mouse peritoneal macrophage, Werb and Cohn (20) have shown that this enzyme can be detected in association with the plasma membrane. When the cells are allowed to ingest latex particles, the enzyme is internalized in association with the membranes enclosing the phagosomes and is subsequently inactivated when the phagosomes fuse with lysosomes.

Previous reports have characterized certain differences between the resident peritoneal macrophage population and cells obtained from an inflammatory focus. Inflammatory cells show an ability to ingest complement-coated sheep red blood cells in the absence of IgG antibody (5), display elevated rates of synthesis and secretion of a variety of neutral proteases (19), and maintain elevated rates of pinocytosis (8). In this work, we report on the 5′-nucleotidase activity of resident and inflammatory macrophages and on the rates of synthesis and degradation of the enzymes in these cells. We also examine the effect of serum deprivation on the metabolism of the enzyme in resident macrophage cultures.

## Materials and Methods

*Macrophages.* Cells were obtained and cultivated as previously described (7) from NCS mice of either sex weighing 22–30 g. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated fetal calf serum (DMEM-20% FCS)[1] in a humidified incubator in a 95% air-5% $CO_2$ atmosphere at 37°C. For serum-free cultivation, cells were plated and maintained in DMEM supplemented with 0.2% lactalbumin hydrolysate (LH). Culture medium, serum, and LH were purchased from Grand Island Biological Co., Grand Island, N. Y. Endotoxin or thioglycollate-stimulated cells were obtained as described previously (8). Cells were generally maintained in culture for 24 h before use.

*Assays.* 5′-nucleotidase was assayed by the method of Avruch and Wallach (1), using [$^3$H]adenosine monophosphate (adenosine-[2-$^3$H]-5′-monophosphate, ammonium salt, Amersham-Searle Corp., Arlington Heights, Ill.) as substrate in a 54 mM Tris buffer, pH 9.0, containing 12

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[1] *Abbreviations used in this paper:* AMP, adenosine monophosphate; DMEM-20% FCS, Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated fetal calf serum; LH, lactalbumin hydrolysate.

mM $MgCl_2$. Cells to be assayed for activity were rinsed twice in serum-free DMEM and then scraped into a small volume of freshly prepared 0.05% Triton X-100. For macrophage monolayers prepared on 35-mm plastic culture dishes, the usual working vol was 1 ml. Lysates were assayed immediately for enzyme activity, or else immediately frozen, and thawed directly before assay. In general, 0.1 ml of cell lysate was assayed for activity in 0.5 ml substrate. Under these conditions substrate was in considerable excess during the entire incubation period. After incubation at 37°C, the unhydrolyzed substrate was precipitated with zinc sulfate and barium hydroxide, and separated from the supernate by centrifugation. A 0.5-ml aliquot of the supernate was mixed with 10 ml of Bray's solution (New England Nuclear, Boston, Mass.) and counted in a Nuclear-Chicago scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Enzyme blanks were included in each assay. Over 97% of the substrate radioactivity was precipitable with barium sulfate. Protein was assayed by the method of Lowry and colleagues (15) using egg white lysozyme as standard.

*Pinocytic Rate Assay.* Pinocytic rates were measured by the technique of Steinman and Cohn (17), as previously described (8), based on the spectrophotometric assay of the uptake of horseradish peroxidase, using *o*-dianisidine as the substrate.

*Estimation of Rates of Enzyme Synthesis and Degradation.* Berlin and Schimke (4) have presented an analysis of the estimation of rates of enzyme synthesis and degradation in cells under the assumptions that degradation proceeds as a first-order process with regard to enzyme concentration, while synthesis is independent of enzyme concentration. If these assumptions are applied to a system in which an equilibrium enzyme level has been perturbed, the rate of approach to a new equilibrium state will be determined solely by the rate of enzyme degradation characteristic of the new state. The rate constant for degradation can then be calculated from the half-time for approach to equilibrium from the relation: $K_d = 0.693/t^1/_2$, where the symbols are self-explanatory. The new equilibrium level reached will, of course, depend both on the rate of synthesis and the rate of degradation, thus allowing calculation of the synthetic rate from the relation $K_s = E/K_d$.

*Reagents.* Trypsin was purchased from Worthington Biochemical Corp., Freehold, N. J. Cycloheximide, adenosine monophosphate (AMP), and Triton X-100 (octylphenoxy polyethoxyethanol, Rohm and Haas, Philadelphia, Pa.) were purchased from Sigma Chemical Co., St. Louis, Mo. Bovine plasma albumin, crystallized, was purchased from Armour Pharmaceutical Corp., Chicago, Ill. Pronase was purchased from Calbiochem, San Diego, Calif. Proteolytic activity was standardized with hide powder substrate (Azocoll, general proteolytic substrate, Calbiochem). Plasminogen, prepared by chromatography on lysine-Sepharose, and urokinase were generously provided by Dr. Saimon Gordon and Ms. Rene Zweibel, The Rockefeller University.

## Results

*5′-Nucleotidase Activity in Resident and Inflammatory Macrophages.* As Werb and Cohn previously observed (20), 5′-nucleotidase activity is readily detected in resident mouse peritoneal macrophages cultivated overnight in DMEM-20% FCS to remove contaminating lymphocytes. The activity measured, using an adaptation of the assay described by Avruch and Wallach (1), is directly proportional to the quantity of cell lysate included in the assay mixture (Fig. 1*a*) and to the length of time the substrate and enzyme and the incubation conditions routinely employed in this study.

Enzyme activity detected is higher when the cells are lysed in Triton X-100 (52.3–58.0 U/mg protein) than when they are lysed in distilled water (39.9 U/mg), but there is no systematic change in the enzyme activity with increasing concentrations of the detergent, between concentrations of 0.05 and 0.5%. Cells were therefore regularly lysed in 0.05% Triton X-100.

The phosphatase activity detected in this assay is not attributable to a nonspecific alkaline phosphatase, as 94% of the control activity can be detected in the presence of β-glycerophosphate (5 mM).

Resident mouse peritoneal macrophages cultivated for 24 h in DMEM-20% FCS have a 5′-nucleotidase activity of 58.3 nm AMP hydrolyzed/min per mg cell

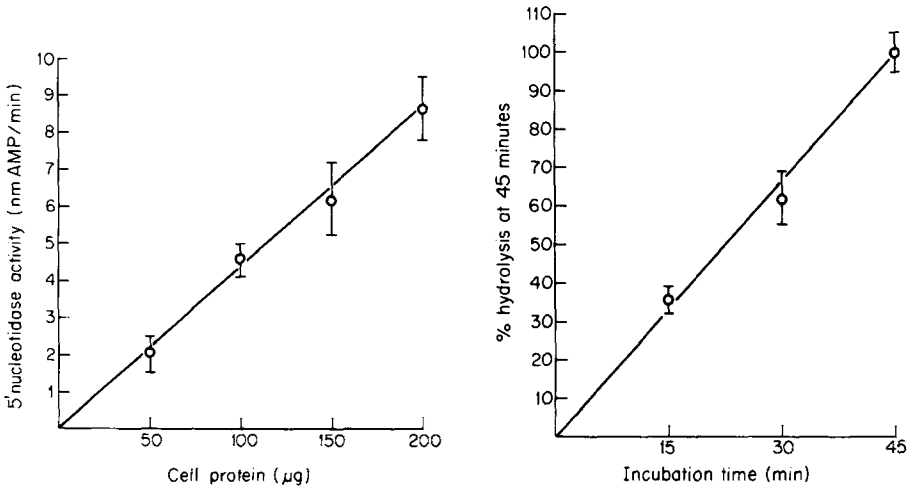


FIG. 1. Hydrolysis of AMP by macrophage cell lysates. (*a*) Rate of hydrolysis as a function of the cell protein added. Reactions were allowed to proceed for 30 min at 37°C. (*b*) Time-course of hydrolysis. Substrate was incubated with 100 μg of cell protein.

TABLE I
*5'-Nucleotidase Activity of Unstimulated and Activated Mouse Peritoneal Macrophages*

| Stimulant | 5'-nucleotidase activity* |
|---|---|
| | *nm AMP/min per mg protein* |
| None (14)‡ | 58.3 ± 8.0 |
| Endotoxin (30 μg) (6) | 20.9 ± 0.4 |
| Thioglycollate medium (4) | 0.7 |

* All cells were cultivated for 24 h in DMEM-20% FCS before assaying for enzyme activity.
‡ Number of experiments performed.

protein (Table I). When peritoneal macrophages are obtained from mice that have been injected 4 days earlier with endotoxin (30 μg), the 5'-nucleotidase activity measured is reduced to 20.9 nm AMP/min per mg protein. In cells obtained from animals previously injected with thioglycollate medium, 5'-nucleotidase activity is essentially undetectable.

*Effects of Cultivation on 5'-Nucleotidase Activity.* There is a progressive increase in enzyme activity in unstimulated cells maintained in culture in DMEM-20% FCS for 48–72 h (Table II). The mean enzyme activity after 48 h is approximately 1.5 times the activity of cells maintained in culture for 24 h, while 72-h cultures have about 2.5 times more activity than the 24-h monolayers. Cells obtained after thioglycollate injection may show the development of low levels of 5'-nucleotidase activity upon cultivation for several days. This increased activity is abolished after exposure of the monolayers to trypsin (500 μg/ml) for 30 min at 37°C. Similar treatment has no effect on the activity of unstimulated cells. As such treatment can remove certain contaminating cell

TABLE II

*5′-Nucleotidase Activity of Unstimulated and Thioglycollate-Stimulated Macrophages Cultivated in DMEM-20% FCS*

| Cell type | Time in culture | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| | *nm AMP/min per mg protein* | | |
| Unstimulated* | 64.4 | 107.1 ± 30.7 | 163.2 ± 43 |
| Thioglycollate stimulated | 0.7‡ | 5.3 | 9.1 |
| Thioglycollate stimulated (trypsinized)§ | 0.7 | – | 0.4 |

* Activity of unstimulated cells cultivated for 4 h in DMEM-20% FCS is 62.0.

‡ Same experiment as reported in Table I.

§ Cultures exposed to trypsin (500 μg/ml) for 30 min at 37°C.

types from macrophage monolayers, including fibroblasts, it seems likely that this modest activity can be attributed to a minor contaminating cell population and does not represent 5′-nucleotidase associated with the stimulated macrophages themselves.

One reason that thioglycollate cells might fail to express 5′-nucleotidase activity would be the presence of an enzyme inhibitor or inactivator in these cells. However, when unstimulated cells were cocultivated for 24 h with thioglycollate-stimulated macrophages the 5′-nucleotidase activity recovered was directly proportional to the number of unstimulated cells originally plated (Fig. 2). Similar results were obtained when lysates of separately cultivated unstimulated and thioglycollate-stimulated macrophages were mixed and assayed, or when unstimulated cells were exposed overnight to medium previously conditioned by thioglycollate-stimulated cells. In addition, treatment of thioglycollate-stimulated cells with trypsin (1 mg/ml for 15 min at 37°C) failed to disclose any latent enzyme activity. These experiments indicate that the absence of 5′-nucleotidase activity in thioglycollate-stimulated cells is not due to an inhibitor produced by these cells.

*Effects of Proteolytic Enzymes on 5′-Nucleotidase Activity of Unstimulated Macrophages.* Certain macrophage plasma membrane functions, such as the receptor for the third component of complement, are sensitive to proteolytic enzymes (14). The absence of enzyme activity in thioglycollate-stimulated cells, which secrete large amounts of several neutral proteases (19), along with its presumptive location on the plasma membrane, raised the possibility that this enzyme, too, was sensitive to proteases in situ. Although mixing experiments described above rule out a role for the secreted proteases in reducing the enzyme activity of stimulated cells, it seemed important nevertheless to examine the effects of several proteolytic enzymes on 5′-nucleotidase activity of intact cells (Table III).

Trypsin, in concentrations from 10 μg/ml to 500 μg/ml had no effect on the 5′-nucleotidase activity of unstimulated cells. Pronase was also ineffective at reducing the normal levels of enzyme activity, but may have slightly increased the activity at 100 μg/ml or higher concentrations. Treatment of the cells with

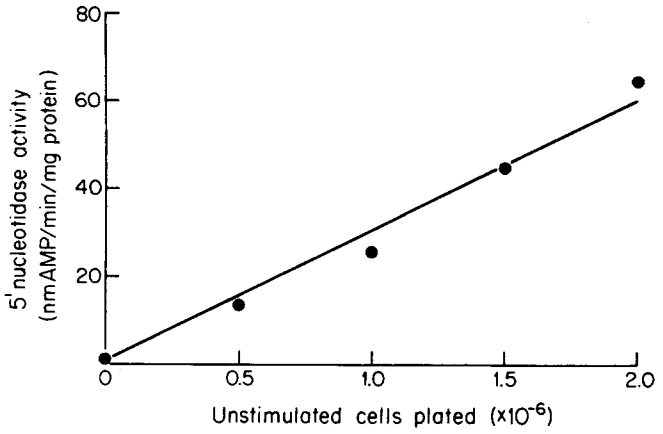


FIG. 2. 5'-nucleotidase activity of normal cells cocultivated with thioglycollate-stimulated cells. A total of 2 × $10^6$ cells were plated and maintained in culture for 24 h before being assayed for enzyme activity.

TABLE III
*Effects of Proteolytic Enzymes on 5'-Nucleotidase Activity of Unstimulated Macrophages*

| Enzyme* | 5'-nucleotidase activity |
|---|---|
| | *nm AMP/min per mg protein* |
| Trypsin 0 μg/ml | 65.1 |
| 10–500 | 66.3–74.5 |
| Pronase 0 μg/ml | 77.8 |
| 10–500 | 71.2–92.2 |
| Urokinase 0 Plough units | 75.6 |
| 1 Plough unit | 102.9 |
| 1 Plough unit + plasminogen 5–25 μg | 68.8–105.0 |

* Cell monolayers were exposed to enzyme for 30 min at 37°C in HBSS.

urokinase or urokinase and plasminogen also appeared to increase enzyme activity. However, none of these enzymes was effective in significantly diminishing the 5'-nucleotidase activity of unstimulated cells.

*Estimation of Rates of Enzyme Synthesis and Degradation by Inhibiting Protein Synthesis.* As analysed by Berlin and Schimke (4), and reviewed above, measurement of the rate at which an enzyme activity approaches a new equilibrium state, following perturbation of an initial state of equilibrium, allows a direct calculation of the rate of enzyme degradation characteristic of the new state. This, and the value of the new equilibrium enzyme activity, then define the rate of enzyme synthesis.

A convenient way to perturb the normal equilibrium state of any cell protein is to inhibit protein synthesis. The rate at which the enzyme activity decays can then be used to estimate the rate of enzyme degradation in these cells. Such an approach was therefore taken by exposing unstimulated, or endotoxin-stimulated macrophages, to cycloheximide.

Monolayers were placed in serum-supplemented medium containing cycloheximide (10 μg/ml) for periods of up to 8 h. As shown in Fig. 3, 5′-nucleotidase activity decays exponentially under these conditions, with the rate of decay considerably faster in endotoxin-stimulated macrophages than in unstimulated cells. As this concentration of cycloheximide is sufficient in macrophages to completely inhibit the incorporation of [$^3$H]leucine into trichloroacetic acid precipitable material within 1 h of exposure (P. J. Edelson, unpublished observations), one may assume that the cycloheximide-treated cells are approaching an extrapolated equilibrium state for which E = 0. Decay rates and rates of degradation and synthesis were therefore calculated (Table IV). The rates of synthesis in the two cell populations are about the same. However, the rate of degradation of enzyme is about twice as rapid in the endotoxin-stimulated cells as it is in the unstimulated population. Thus, the decrease in enzyme activity in endotoxin-stimulated cells may be almost entirely accounted for by its more rapid decay in these cells.

To interpret the data from these experiments validly, it is necessary to be certain that cycloheximide has not had any effect on the rate of degradation of the enzyme in the treated cells. To examine this point, we made similar measurements on cells that were exposed for 6 h to cycloheximide, rinsed, and then reincubated in fresh medium without cycloheximide (Fig. 3, insert). The calculated rates of degradation and synthesis are quite similar to those obtained in cells in the presence of the inhibitor (Table IV), indicating that there is no significant degradative artefact introduced by cycloheximide in this system.

*Effects of Serum-Free Conditions on the Metabolism of 5′-Nucleotidase.* When unstimulated macrophages are maintained in medium supplemented with 0.2% lactalbumin hydrolyzate and 1% or less fetal calf serum, they exhibit lower levels of 5′-nucleotidase activity than do comparable cells maintained in 5% or higher concentrations of serum and 0.2% lactalbumin hydrolyzate (Table V). This reduction in enzyme activity is not permanent and may readily be reversed by increasing the serum content of the culture medium (Table VI).

Unstimulated cells placed in serum-free culture conditions show an exponential loss of enzyme activity until a new equilibrium state is reached about 9 h later (Fig. 4). Using a similar analysis to the one applied to the study of enzyme metabolism in cycloheximide-treated cells, it is possible to estimate the rates of synthesis and degradation of 5′-nucleotidase in serum-free culture conditions (Table VII). The rate of degradation of enzyme under these conditions is nearly twice that of similar cells maintained in 20% FCS-supplemented medium, while the synthetic rate is probably unchanged. This increase in the rate of degradation of 5′-nucleotidase can be completely prevented by the addition of bovine serum albumin (50 μg/ml) to the culture medium, although under these conditions the enzyme synthetic rate may be somewhat decreased.

Because serum deprivation leads to a new equilibrium state of enzyme activity, it is possible, by measuring the rate of recovery of enzyme activity of these cells after serum supplementation, to obtain another estimate of the rates of synthesis and degradation of the enzyme in unstimulated cells in complete culture medium (Fig. 4, insert). The rates calculated in this way are in good

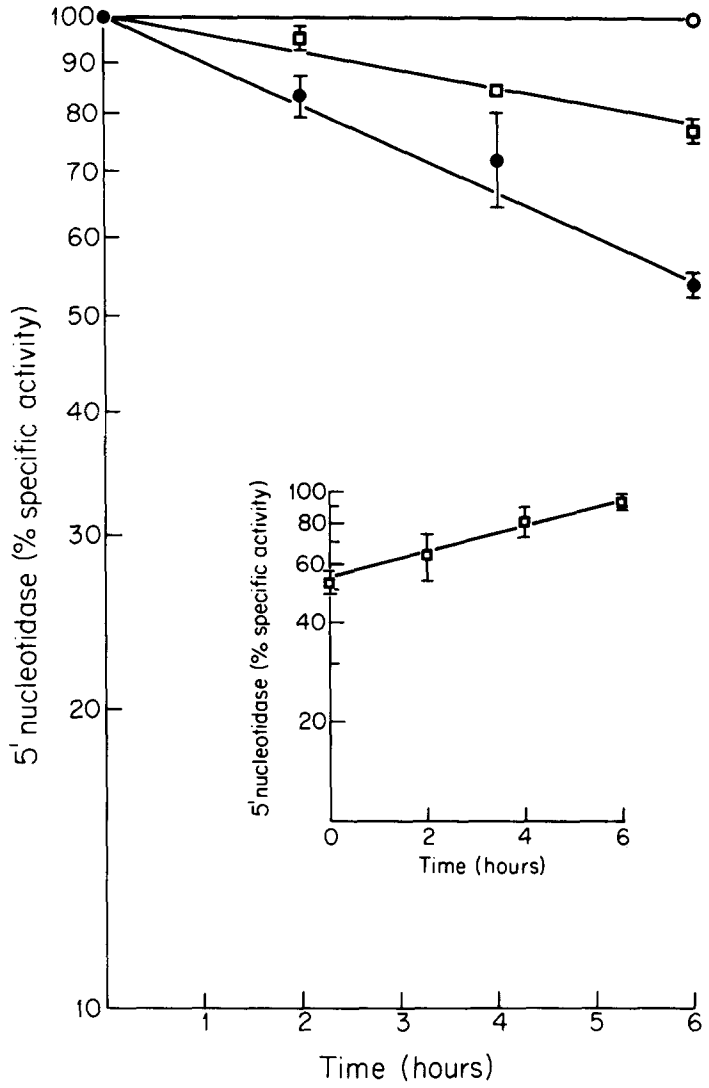


FIG. 3. Loss of 5'-nucleotidase activity by cells exposed to cycloheximide (10 μg/ml). 24-h cultures were placed in cycloheximide at time t = 0 and maintained in the drug until assay. Curves were fit to the data by the method of least-squares. (□) unstimulated macrophages; (●) endotoxin-stimulated macrophages; (○) unstimulated cells not exposed to drug. (inset) Recovery of enzyme activity by endotoxin-stimulated cells after 6 h incubation in cycloheximide (10 μg/ml) and further incubation in fresh drug-free medium.

agreement with the estimates obtained using cycloheximide (Table VII).

*Pinocytic Rate of LH Cells.* Endotoxin cells, which have a somewhat higher pinocytic rate than unstimulated cells, were found to degrade 5'-nucleotidase more rapidly. We therefore examine the pinocytic rate of unstimulated cells maintained for 24 h in serum-free medium to see whether this was related to their increased rate of enzyme degradation. As shown in Table VIII, the pinocytic rate of these cells is not different from control cells maintained in serum.

TABLE IV
*Rates of Synthesis and Degradation of 5′-Nucleotidase in Unstimulated and Endotoxin-Stimulated Macrophages**

| Cell type | $t_{\frac{1}{2}}$ | $K_d$ | $K_s$ |
|---|---|---|---|
| | *h* | $h^{-1}$ | *U/h* |
| Unstimulated (4)‡ | 13.9 ± 2.9 | 0.052 ± 0.01 | 3.03 ± 1.1 |
| Endotoxin stimulated (2) | 6.8 ± 1.1 | 0.105 ± 0.02 | 2.2 ± 0.4 |
| Endotoxin stimulated (1) (recovery from cycloheximide) | 5.8 | 0.121 | 2.4 |

* Macrophages were cultivated in DMEM-20% FCS for 24 h before exposure to cycloheximide (10 μg/ml).
‡ Number of experiments.

TABLE V
*5′-Nucleotidase Activity of Unstimulated Macrophages Cultivated in Various Concentrations of Fetal Calf Serum**

| Culture supplement | | 5′-nucleotidase |
|---|---|---|
| LH | FCS | |
| % | | *nm AMP/min per mg protein* |
| 0.2 | 0 | 52.8 |
| 0.2 | 1 | 58.9 |
| 0.2 | 5 | 77.2 |
| 0.2 | 10 | 65.6 |
| 0.2 | 20 | 81.1 |
| 0.2 | 40 | 74.9 |
| 0 | 20 | 68.3 |

* Unstimulated macrophages were cultivated for 24 h in DMEM-20% FCS, rinsed, and then transferred to DMEM supplemented with increasing concentrations of fetal calf serum as indicated. 8 h later, the cells were lysed and the lysates assayed for enzyme activity.

TABLE VI
*Recovery of 5′-Nucleotidase in Cells Maintained Sequentially in Serum-Free and Serum-Supplemented Media*

| Culture conditions* | | 5′-nucleotidase | |
|---|---|---|---|
| 0–24 h | 25–48 h | 24 h | 48 h |
| | | *nm AMP/min per mg protein* | |
| FCS | FCS | 58.0 | 59.2 |
| LH | FCS | 26.1 | 54.5 |
| LH | LH | 26.1 | 27.9 |

* Unstimulated macrophages were cultivated for 1 h in DMEM-20% FCS, rinsed, and then maintained in either DMEM-20% FCS or DMEM-0.2% LH as indicated.

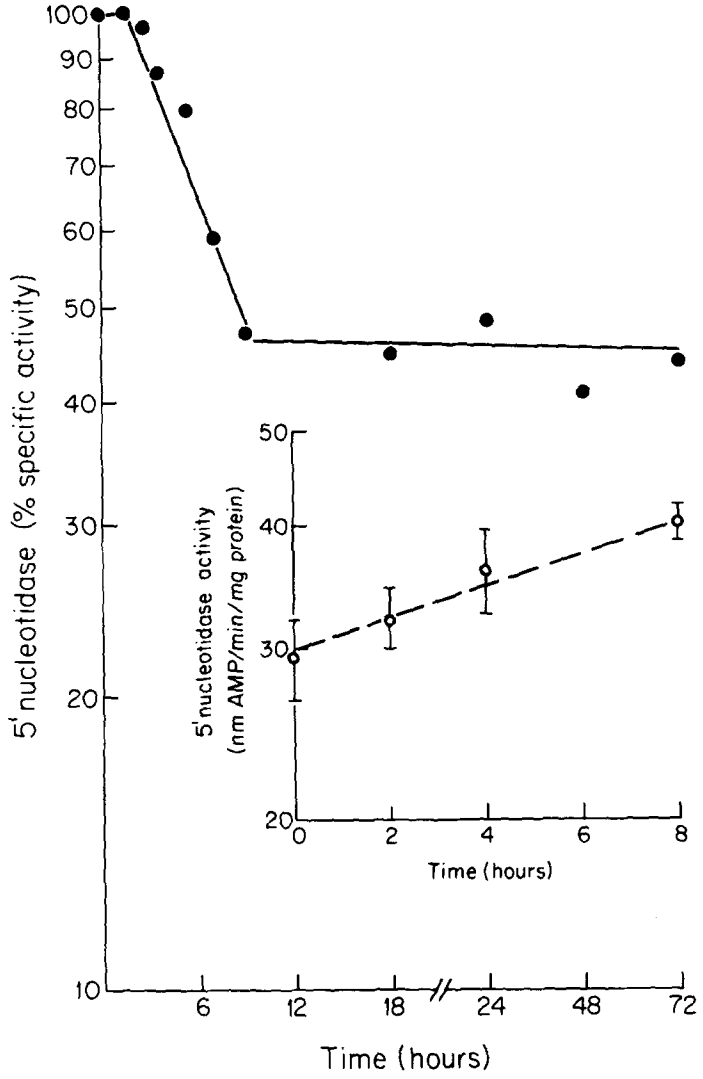


FIG. 4. Loss of 5′-nucleotidase activity by unstimulated macrophages cultivated for 24 h in DMEM-20% FCS and then transferred at t = 0 to DMEM-LH. (inset) Recovery of enzyme activity by unstimulated cells maintained in DMEM-LH for 24 h and then transferred to DMEM-20% FCS.

## Discussion

Specific 5′-nucleotidase activity is readily detected in unstimulated mouse peritoneal macrophages cultivated in serum-supplemented medium for 24 h. Using [$^3$H]AMP as substrate, these cells hydrolyze an average of 58 nm AMP/min per mg protein, which is quite comparable to the levels of activity previously detected in these cells by assays that measured the inorganic phosphate released from AMP, or which coupled the reaction to adenosine deaminase (20). The assay is convenient to use, avoids the use of $^{32}$P, and with minor modifica-

TABLE VII
*Rates of Synthesis and Degradation of 5'-Nucleotidase in Unstimulated Cells Cultivated in Serum-Free Conditions*

| Culture conditions | 5'-nucleotidase activity | $t_{\frac{1}{2}}$ | $K_d$ | $K_s$ |
|---|---|---|---|---|
| | *nm AMP/min per mg protein* | *h* | $h^{-1}$ | *U/h* |
| DMEM + 20% FCS* | 58.3 | 13.9 | 0.052 | 3.03 |
| DMEM + 0.2% lactalbumin hydrolyzate | 26.6 | 6.9 | 0.100 | 2.66 |
| DMEM + 0.2% lactalbumin hydrolyzate + BSA (50 μg/ml) | 40.8 | 13.8 | 0.051 | 2.10 |
| DMEM + 20% FCS (after 8-h incubation in 0.2% LH) | – | 15.6 | 0.044 | 2.93 |

* From Table II.

TABLE VIII
*Pinocytic Rates of Unstimulated and Inflammatory Macrophages*

| Cell | $t_{\frac{1}{2}}$ | Pinocytic rate |
|---|---|---|
| | *h* | *nl/h per 100 μg cell protein* |
| Unstimulated | 13.9* | 56.0‡ |
| Endotoxin stimulated | 6.8* | 85.0‡ |
| Unstimulated: serum-free conditions | 6.9§ | 48.7 |

* From experiments reported in Table V.
‡ From reference 8.
§ From experiments reported in Table VIII.

tions is capable of reliably detecting levels of enzyme activity which are 1% of normal macrophage values. Detergent solubilization seems necessary for optimum results, but the consistency of activity over a range of detergent concentrations suggests that Triton X-100 is not artefactually stimulating enzyme activity.

As previously noted by Werb and Cohn (20), there appears to be no nonspecific alkaline phosphatase activity detectable in peritoneal macrophage lysates. β-glycerophosphate, which effectively competes with AMP as substrate for nonspecific phosphatases, but not for 5'-nucleotidase (3), had essentially no effect on the amount of [$^3$H]AMP hydrolysed.

The enzyme activity of unstimulated macrophages of 58.3 nm AMP/min per mg protein is quite consistent with the earlier measurements of Werb and Cohn (20). Enzyme activity increases over several days of culture in serum-supplemented medium. 1 ml of fetal calf serum hydrolyzes about 1 nm AMP/min, with about one-half of this activity due to a specific 5'-nucleotidase and the remainder due to nonspecific alkaline phosphatase activity (P. J. Edelson, unpublished

observations). Heat-inactivated fetal calf serum has somewhat lower activities. Thus, the serum supplement, in DMEM-20% FCS, could contribute only 0.2 U of enzyme activity to a culture containing more than 100 $\mu$g of cell protein. This is far too little to account for the increase in cell enzyme activity seen which extended cultivation, and indicates that serum is not itself the source of the increased activity. Consistent with this conclusion is the observation that thioglycollate-stimulated macrophages, when exposed to serum, fail to increase their levels of 5′-nucleotidase activity. Serum components may, however, provide either general nutritional support or specific supplementation either capable of stimulating enzyme production or necessary for its expression. For example, cholesterol has been shown to be required for the recovery of 5′-nucleotidase activity after extensive interiorization of latex by macrophages (20). The ability of bovine serum albumin to substitute for serum may indicate a role for fatty acids, hormones, or other ligands which are normally associated with albumin in the regulation of enzyme levels in cultivated cells.

Cells in culture increase in size and in apparent surface area, and the increase in specific 5′-nucleotidase activity may reflect a relatively greater increase in plasma membrane as compared with cell volume. The behavior of 5′-nucleotidase in culture is qualitatively similar to that of several lysosomal hydrolases, particularly acid phosphatase, whose specific activities also increase in culture. While the stimulus for the increases in the lysosomal enzymes may be the continued ingestion of degradable material (2), regulators of cell 5′-nucleotidase activity are at present unknown.

As others have previously noted (A. L. Hubbard, personal communication, 13), 5′-nucleotidase activity is strikingly reduced in inflammatory macrophages. In cells obtained after endotoxin stimulation, average activity was reduced to about 21 nm AMP hydrolysed/min per mg protein, while in thioglycollate-stimulated cells, no 5′-nucleotidase activity was detectable even after prolonged cultivation away from the presence of the thioglycollate broth. In mixing experiments, or in experiments where thioglycollate-stimulated and resident macrophages were cocultivated, no evidence was obtained for an inhibitor or inactivator of the enzyme which would account for this lack of activity. Although thioglycollate cells secrete considerable amounts of several neutral proteases (19), these enzymes, in fetal calf serum-containing medium, had no effect on the 5′-nucleotidase activity of unstimulated cells. Although serum protease inhibitors may have diminished the effectiveness of these secreted enzymes, in serum-free conditions neither trypsin, pronase, urokinase, nor plasmin could effectively diminish 5′-nucleotidase activity of intact cells. Although one possible explanation for this lack of sensitivity to these proteases would be that 5′-nucleotidase is simply not exposed to the cell exterior, experiments with a relatively impermeable reagent, the diazonium salt of sulfanilic acid, which are reported in the following paper, indicate that about 80% of the total enzyme is accessible from the cell exterior. There is precedent for a plasma membrane component which is externally disposed, but is insensitive to various proteases, in the macrophage Fc receptor (5).

Although there was some increase in 5′-nucleotidase activity in cultures of thioglycollate-stimulated cells, this probably was associated with a small population of fibroblasts which occasionally contaminate these cultures. The activity

could be removed with trypsin, a maneuver that detaches the fibroblasts, but not the macrophages, from the culture dish.

Efforts to uncover latent 5′-nucleotidase activity in thioglycollate-stimulated macrophages by treating the cells with trypsin, or cultivating them in serum-free conditions, were unsuccessful. If thioglycollate-stimulated cells synthesize 5′-nucleotidase at the rate of unstimulated cells, their rate of enzyme degradation would have to be about 50 times greater than that of resident cells to account for the absence of detectable enzyme. While this elevated rate of degradation is possible, it is also conceivable that these cells fail to synthesize an active 5′-nucleotidase, either due to failure to synthesize a gene product altogether, synthesis of an inactive molecule, or absence of some cofactor necessary for enzyme function.

Endotoxin-stimulated cells synthesize 5′-nucleotidase at rates similar to those of resident peritoneal macrophages, about 2–3 enzyme U/mg protein per h, where 1 U of enzyme activity can hydrolyze 1 nm AMP/min. Endotoxin-stimulated cells, though, degrade 5′-nucleotidase at about 11% per h, or about twice the rate of degradation in unstimulated cells. Therefore, the endotoxin-stimulated cells have an equilibrium enzyme activity about half that of unstimulated cells. Rigorous application of the analysis of Berlin and Schimke requires that cells under study be examined between two equilibrium states. As the 5′-nucleotidase specific activity does increase somewhat while the cells are in culture, this state is by definition not one of strict equilibrium. However, the change is small enough over the time the experiments are carried out (about 10–15% over 8 h) that we have chosen to treat the undisturbed culture state as if it were one of equilibrium for the purposes of the analysis.

Estimates of rates of degradation of enzyme, using cycloheximide, may not necessarily reflect the rate of degradation in untreated cells. Schimke (16) has noted several examples in which inhibition of protein synthesis also inhibited enzyme degradation or inactivation. Our approach to this issue was to compare the rate of degradation measured in the presence of cycloheximide with the rate in cells recovering from cycloheximide. These rates were quite similar, indicating that the cycloheximide is not artefactually affecting enzyme degradation.

In addition, we used cells recovering from serum deprivation as an alternate way of estimating synthesis and degradation under normal culture conditions, and again confirmed our estimates of synthetic and degradatory rates using cycloheximide.

Conditions of serum deprivation cause a decline in 5′-nucleotidase activity to levels about 50% those of control cells. This decline is due to an increase in the rate of degradation of enzyme under these conditions, the synthetic rate remaining essentially unchanged. This increased enzyme degradation does not occur in the presence of bovine serum albumin (50 $\mu$g/ml). As discussed earlier, it is not clear whether this effect of albumin is simply due to nutritional supplementation, either with protein or with albumin-associated free fatty acids, or is related to other molecules normally occurring in association with circulating albumin. Additionally, we have not examined the issue of metabolic specificity, that is, whether the rate of degradation of 5′-nucleotidase is specifically increased in serum-free conditions, or whether this is part of a general increase in the rates of protein turnover in these cells.

Several workers have estimated the turnover rates of either particular plasma membrane components, usually proteins, or of the plasma membrane as a whole. Gurd and Evans, studying mouse liver cells (10), and Kaplan and Moscowitz, studying rhesus monkey kidney cell lines (12), concluded that the rates of degradation of various internally or externally labeled membrane components were quite heterogeneous.

Tweto and Doyle (18) and Hubbard and Cohn (11) have reported more homogeneous rates of degradation of plasma membrane components. In these studies, enzymatic iodination of the cells was used to specifically distinguish plasma membrane species. Tweto and Doyle reported that hepatoma tissue culture (HTC) cell plasma membrane components are degraded with a half-time of 100 h. Hubbard and Cohn found that L-cell plasma membrane species capable of being labeled in intact cells with $^{125}I$ and lactoperoxidase divide into two major classes. One class decays with a half-time of about 2 h, while a second, major class, exhibits a half-time of 25–33 h. These turnover rates are similar to the turnover rate estimated by Devreotes and Fambrough (6) for the acetylcholine receptor in chick or rat myotube plasma membrane of 22–24 h. In comparing these rates to our estimate of 14 h for the half-life of 5′-nucleotidase activity, it is important to bear in mind that our studies only measured the rate of enzyme inactivation, while the previous studies depended upon the complete degradation of the labeled proteins to amino acids, and the subsequent release of monoiodotyrosine into the medium.

Results that indicate a relatively homogenous rate of degradation of plasma membrane constituents are consistent with a mechanism of membrane renewal which involves bulk replacement of relatively heterogeneous portions of membrane. Heterogenous turnover rates would permit the differential segregation, turnover, and replacement of rather restricted membrane components, including single molecules. It is interesting, in relation to this issue, that the rate of degradation of 5′-nucleotidase is elevated in the more rapidly endocytizing endotoxin-stimulated cells. This would be the expected result if replacement of membrane proceeded in bulk, and particularly if pinocytic interiorization of plasma membrane were an important step in this process. Pinocytic rate is not, however, the sole determinant of the rate of degradation of 5′-nucleotidase, as cells in serum-free medium degrade the enzyme more rapidly than do normally cultivated cells, but have the same pinocytic rate as cells maintained in serum.

## Summary

Mouse resident peritoneal macrophages display sufficient 5′-nucleotidase activity to hydrolyze 58 nm AMP/min per mg cell protein. This activity increases approximately 163 nm AMP/min per mg after 72 h in culture. The enzyme is renewed in unstimulated cells with a half-time of 13.9 h. The activity is not reduced by treatment of intact cells with a variety of proteolytic enzymes, including trypsin, pronase, urokinase, and plasmin. Cells obtained from an inflammatory exudate have diminished or absent levels of enzyme activity. Endotoxin-elicited cells display enzyme activity of 20.9 nm AMP/min per mg, while thioglycollate-stimulated macrophages have no detectable activity. The reduced level of activity in endotoxin-stimulated cells is due to their elevated rate of enzyme degradation, with a half-time of 6.9 h. Their rate of enzyme

synthesis is essentially normal. No evidence for latent enzyme activity could be obtained in thioglycollate-stimulated cells, nor do these cells produce any inhibition of normal cell enzyme activity.

Serum deprivation reduces the enzyme activity of resident cells to about 45% of control activity. These conditions do not significantly affect the rate of enzyme synthesis, but again are explainable by an increase in the rate of enzyme degradation.

Pinocytic rate is elevated in endotoxin-stimulated cells which show a more rapid rate of enzyme degradation than unstimulated cells do. However, in serum-free conditions, the rate of enzyme degradation is doubled with no change in the pinocytic rate of the cells.

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