Ecto-Enzymes of the Guinea Pig Polymorphonuclear Leukocyte

I. EVIDENCE FOR AN ECTO-ADENOSINE MONOPHOSPHATASE, -ADENOSINE TRIPHOSPHATASE, AND -P-NITROPHENYL PHOSPHATASE*

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SUMMARY

A suspension of intact guinea pig polymorphonuclear leukocytes hydrolyzed added ATP, AMP, and p-nitrophenyl phosphate under physiologically appropriate conditions. These enzymatic activities were not due to artifacts such as breakage of the cells during the incubation period. It thus seemed possible that the hydrolyses were being catalyzed by ecto-enzymes, i.e. enzymes on the plasma membrane with their active sites facing the external medium. Three types of experiment were designed to test this hypothesis.

First, the activities of intact cells were compared to those of homogenates, sonicates, and cells treated with detergent. Disruption of cells resulted in an approximately 2-fold increase in maximal ATPase and p-nitrophenyl phosphatase activities, suggesting that the plasma membrane was acting as a permeability barrier to the substrates involved. Disruption did not increase AMPase activity, leaving open the possibility that an ecto-enzyme is the only protein in polymorphonuclear leukocytes capable of hydrolyzing AMP.

Second, the products of ATPase, AMPase, and p-nitrophenyl phosphatase activities of intact cells were localized by using radioactively labeled substrates. The concentration of inorganic phosphate produced by these reactions was 18 to 100 times greater in the extracellular medium than in the intracellular milieu. This suggests that the substrates are cleaved outside the cells, or that they are cleaved inside and the products are transported out. The latter possibility was militated against by the following experiment. Cells were loaded with inorganic [*P]phosphate, then allowed to hydrolyze substrates labeled with *P. The distributions of the two isotopes were compared. Almost all of the inorganic [*P]phosphate was found outside of the cells, while 90% of the inorganic [*P]phosphate remained inside.

Third, the cells were treated with the diazonium salt of sulfanilic acid, a reagent known not to penetrate into intact erythrocytes. This treatment rapidly and dramatically inhibited the intact-cell ATPase, AMPase, and p-nitrophenyl phosphatase, while lactate dehydrogenase, a soluble cytoplasmic enzyme, was unaffected. Control experiments demonstrated that in sonicates lactate dehydrogenase was as susceptible to inhibition by the diazonium salt as were the other three activities.

The plasma membrane of cells may contain enzymes whose active sites face the external medium rather than the cytoplasm, and these enzymes are referred to as "ecto-enzymes." We have recently reviewed the ecto-enzymes of a number of cell types (3), and shall therefore make citations below only as necessary. As we have previously pointed out (3), the observed fact that particular intact cells act on an external substrate, even one that is commonly believed to be excluded from cells, does not provide sufficient evidence that the protein involved is an ecto-enzyme.

The experiments described below provide evidence along rigorous lines of argument to establish the presence of three ecto-enzymes on guinea pig polymorphonuclear leukocytes. An accompanying paper (4) discusses the properties of these ecto-enzymes and the possible uses to which they might be put.

MATERIALS AND METHODS

Chemicals

Unless otherwise stipulated, all chemicals used were purchased from commercial sources and were of reagent grade. For chemicals of particular importance, the source is given where the substance is mentioned for the first time.

Cells were prepared from guinea pigs of either sex (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 500 to 1000 g. The animals were fed Chow pellets (containing vitamin C) and water *ad libitum*. The cells were quantified either by suspending an aliquot in 3% acetic acid (to lyse the red blood cells present) and counting under the microscope in a Spencer Bright-Line hemocytometer or by protein determination (see below). Experiments with intact cells were always completed within 4 to 6 hours after harvesting.

Polymorphonuclear leukocytes were obtained by a slight modification of the method reported previously from this laboratory (5). The animals were injected intraperitoneally with 30 ml of a sterile solution of 12% (w/v) sodium caseinate (Difco Laboratories, Inc., Detroit, Mich.) in normal saline (0.9% NaCl) and 15 to 20 hours later they were killed by exposure to ether. The peritoneal cavity was opened, the cell suspension was removed with a pipette, and the cavity was rinsed out once or twice with normal saline. After filtration through nylon gauze to remove any hair or fat globules, the cells were spun down at <100 X g for 10 min, washed once in Krebs Ringer phosphate solution, pH

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Identification of peritoneal exudate cells

Polymorphonuclear leukocytes, monocytes, and lymphocytes were identified by doing a Wright stain; 300 to 1000 cells were counted for each differential. Eosinophils were identified with an eosin stain. Red blood cells were identified by light microscopy or by lysis and subsequent colorimetric determination of hemoglobin. Phase microscopy revealed no platelets in the preparations. Despite the use of sterile injection techniques, an occasional exudate was found to be contaminated with bacteria; such contaminated preparations were not used.

Table I

| Cell type                        | Relative numbers
|----------------------------------|--------------------|
| Polymorphonuclear leukocyte      | 94.9 (93.7-95.6)    |
| Monocyte                         | 2.1 (1.7-2.8)      |
| Lymphocyte                       | 2.1 (1.3-2.8)      |
| Eosinophils                      | 0.10 (0.04-0.17)   |
| Others*                          | 1.0 (0.2-1.4)      |
| Red blood cells                  | 1-8                |

* Mean and range (in parentheses) of the values from 8 to 11 determinations, except that eosinophils were counted in only four preparations. The total of all cells other than red blood cells is taken as 100.
* Plasma cells, macrophages, and unidentified cells.
* Although highly variable, red blood cell contamination was usually less than 3 or 4%. Highly contaminated exudates were not used.

ATPase

Enzyme Assays

Cell Suspensions—The ATPase assay was based on the observation by Crane and Lipmann (12) that charcoal adsorbs adenosine phosphates but not inorganic phosphate. The standard assay mixture contained 1.2 to 1.8 X 10⁶ intact cells (or an appropriate amount of an homogenate or a sonicate), 5 mM p-nitrophenyl phosphate (Sigma phosphatase substrate 104) (see Ref. 4), and 1 mM 5′-AMP (Amersham-Searle) in KRP. The 15 mM inorganic phosphate in KRP did not inhibit this ATPase since similar results were obtained when this buffer was replaced with glycylglycine. The assay mixture was incubated at 37° for 20 or 30 min (shaking was not necessary) and then transferred to an ice bucket. Seventy-five seconds later 1 ml of a 10% (w/v) suspension of acid-washed Norit in 10% (w/v) trichloroacetic acid was added to the sample. After another 75 s in ice the charcoal was removed by filtration through Whatman No. 1 filter paper. 5′-AMP in an aliquot of the filtrate then was determined. A control without cells was routinely performed, and the assays were done at least in duplicate.

Under these conditions the hydrolysis of ATP by intact cells was linear for at least 30 min and proportional to cell concentration for at least the range 0.5 to 3 X 10⁶ cells per ml. The ATPase of homogenates and sonicates also was found to be directly proportional to time and concentration of protein. About 10% of the ATP present was normally hydrolyzed during the incubation period.

This assay could easily be scaled down 10-fold by incubating 1.0 to 1.5 X 10⁶ intact cells (or an appropriate amount of disrupted cells), 5 mM p-nitrophenyl phosphate, and 1 mM 5′-AMPATP having 5 times the usual specific activity in 0.2 ml of Krebs-Ringer phosphate solution at 37° for 30 min. The rest of the procedure was unchanged, except that 2 ml of 10% acid-washed Norit in 10% trichloroacetic acid were added to the sample instead of 1 ml.

An important aspect of this assay is that it is specific for the release of the γ-phosphate from ATP. ATPase assays based on the colorimetric determination of released inorganic phosphate must contend with possible contamination from the further breakdown of ADP to AMP and of AMP to adenosine.

Monolayers—This procedure was very similar to the assay in suspension, except that 1.5 to 1.8 X 10⁷ cells were first made into a monolayer as described below. The monolayer then was incubated for 20 to 30 min at 37° to the presence of labeled substrate and p-nitrophenyl phosphate. At the end of this period the supernatant fluid was removed and the monolayer was washed with 1 ml of Krebs-Ringer phosphate solution. After this wash less than 3% of the added radioactivity remains on the monolayer. Since only about 10% of the added ATP is hydrolyzed, even such a small residual amount of NP might be significant if the cells took up the product but not the substrate; however, as shown below, the product is localized outside the cells. The original supernatant fluid and the wash were mixed, 1 ml of this mixture was treated with Norit-trichloroacetic acid as detailed above for the determination of released radioactivity, and another aliquot was digested at room temperature overnight in 0.5 N NaOH for protein determination. Protein present on the monolayer and also was measured (see below). Total protein was used in the calculations. Duplicate or triplicate assays were routinely performed.

This ATPase assay was linear for at least 30 min and over the range of 50.0 to 300 μg of protein (0.6 to 3.5 X 10⁶ cells) per monolayer plate. ATPase values obtained by this monolayer method were about 95% of the values obtained by assay of the same cells in suspension, suggesting that adhesion to a surface renders few, if any, of the ATPase active sites inaccessible to substrate molecules.

AMPase

Since AMP also is adsorbed by charcoal (12), the hydrolysis of AMP was measured in suspension and on monolayers in a manner essentially identical to the assay of ATPase activity. The standard assay mixture consisted of 1 mM p-nitrophenyl phosphate (see below) and 1 mM 5′-AMP (Amersham-Searle) in KRP. The 15 mM inorganic phosphate in KRP did not inhibit the AMPase since similar results were obtained when this buffering species was replaced with Tris. Both with intact or disrupted cells in suspension and on monolayers this assay was found to be linear for at least 30 min and at least over the range of 1 to 8 X 10⁶ cells per assay vessel. Assays were performed in duplicate or triplicate and the whole procedure could easily be scaled down 10-fold.

p-Nitrophenyl Phosphatase

Two methods were employed to determine the hydrolysis of p-nitrophenyl phosphate. In both procedures 1.0 to 1.5 X 10⁶ intact cells (or an appropriate amount of a homogenate or sonicate) per ml of suspension or per monolayer plate were incubated with 1 mM substrate in KRP, pH 7.4, for 20 or 30 min at 37°. The 15 mM inorganic phosphate present in KRP apparently inhibited p-nitrophenyl phosphatase activity to some degree since the activity was 10 to 15% higher when this buffering species was replaced with Tris or glycylglycine. In the colorimetric assay the reaction was stopped by adding 3 to 5 ml of 0.1 N NaOH to the suspension or 1 ml of 0.1 N NaOH to the monolayer. The absorbance at 410 nm then was measured. Background absorbance at this wavelength due to cells alone and to nonenzymatic hydrolysis of the substrate was subtracted from the readings. In an assay procedure using radioactive substrate (Amersham-Searle) the reaction was stopped in the same manner as for the ATPase.
nitrophenyl phosphate also is adsorbed onto charcoal) and an aliquot of the filtrate subsequently was counted. The radioactive assay was used chiefly when the presence of a colored reagent would have interfered with the colorimetric determination. Direct comparison demonstrated that the assays gave identical results. Both assay procedures were found to be linear for at least 30 min and at least over the range of 0.2 to 3.0 × 10^6 intact or disrupted cells per assay vessel. Duplicates were routinely performed, and the assays could easily be scaled down 10-fold. The p-nitrophenyl phosphate measured on monolayers was about 98% of that measured with intact cells in suspension.

Acetylcholinesterase

Acetylcholinesterase was measured by a radioactive procedure similar to those reported by Potter (14) and Fonnum (15). Acetylcholine labeled with tritium in the methyl moiety of the acetyle group (New England Nuclear Corp.) was used. At the end of the incubation the reaction mixture was acidified and the free acetic acid was extracted into scintillation fluid and counted. That part of the esterase activity which could be inhibited by 10^{-4} M eserine was considered specific for acetylcholine.

Lactate Dehydrogenase

Lactate dehydrogenase was determined by following the disappearance of NADH at 340 nm upon conversion of pyruvate to lactate (16).

Miscellaneous Procedures

Monolayers—Monolayers were formed by the procedure reported previously from this laboratory (17). Briefly, 0.4 to 1.8 × 10^6 intact cells in 1.0 ml of Krebs-Ringer phosphate solution, pH 7.4, were added to a plastic tissue culture dish (Falcon Plastics, Oxnard, Calif.). These cells were incubated on the dish for 30 to 45 min at 37° with occasional swirling. At the end of this period the supernatant fluid was decanted and the cell monolayer was washed at least three times in Krebs-Ringer phosphate solution; this removed nonadherent cells, including almost all of the lymphocytes and red blood cells present. Subsequently, the monolayers were covered again with 1 ml of Krebs-Ringer phosphate containing various substrates and agents. Normally, 70 to 80% of the polymorphonuclear leukocytes applied stuck to the dishes, but there was enough variability to require that the amount of protein in the monolayers be assayed routinely. This was done by adding 2 ml of 0.5 N NaOH to the monolayers after removing the medium, allowing them to digest overnight at room temperature, and then removing an appropriate aliquot for protein determination.

Diazonium Salt of Sulfanilic Acid—This salt was synthesized by the indirect method (18). In short, 11.5 mg of NaN02 were dissolved in approximately 1 ml of cold water. This solution was added to 19.5 mg (100 pmoles) of sulfanilic acid and shaken until the diazonium salt precipitated out. After being allowed to stand for 15 min in an ice bucket, the precipitate was spun down and washed with a volume of cold water approximately equal to the volume of the pellet. If the pellet then was dissolved in 20 ml of Krebs-Ringer phosphate solution, thus giving a nominal concentration of 5 mM, the final pH of the solution was 7.25. The actual concentration of this final solution was determined several times by carrying out the reaction with [*4S]sulfanilic acid (New England Nuclear, Boston, Mass.) and determining the percentage of added radioactivity which was incorporated into the product. This procedure indicated approximately a 70% over-all yield of the diazonium salt.

Sonication—Sonication was carried out on a MSE 100-watt ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London, England) at a setting of 4 to 6 µm. The sonication vessel was immersed in an ice water bath and unless otherwise indicated, samples were sonicated for 90 s.

Radioactivity—Radioactivity was determined by counting in a toluene-ethanol-based scintillation fluid (10) using Omnifluor (New England Nuclear Corp.) as the scintillator. If insoluble tissue fragments were present in the sample to be counted, these were first dissolved in NCS solvent (Amersham-Searle) at 37° with occasional shaking.

Data Variability—Variability of the data is indicated by giving the range, the average deviation from the mean, or the standard deviation of the mean. Since most experiments were done only several times, the average deviation is most frequently used.

RESULTS

In considering possible ecto-enzymes of the polymorphonuclear leukocytes, acetylcholinesterase was the first to come to mind. This activity is present as an ecto-enzyme on red blood cells (20) and intact human leukocytes have also been reported to hydrolyze acetylcholine (21).

As illustrated in Table II, the acetylcholinesterase detectable in guinea pig polymorphonuclear leukocyte preparations was probably due to the few contaminating red blood cells. No special procedures, such as density gradient centrifugation, were employed to reduce this contamination further, because the present purpose was not to ascertain whether polymorphonuclear leukocytes had any ecto-acetylcholinesterase at all, but whether they had high enough levels of such an activity for it to be useful as a plasma membrane marker. It is clear from Table II that intact guinea pig polymorphonuclear leukocytes do hydrolyze acetylcholine, they do so at a rate not greater than, and probably substantially less than, 1 to 2% of the red blood cell activity. Contaminating red blood cells would thus seriously interfere with the use of any such weak acetylcholinesterase as a marker for the leukocyte plasma membrane (cf. Table I).

There seems to be a discrepancy between the present findings and those of Ross and Rosenbaum (21) who assayed acetylcholinesterase by allowing human white cells to hydrolyze acetylcholine and subsequently determined free sulfhydryl groups colorimetrically. They apparently did not inhibit the acetylcholinesterase with eserine in order to determine background levels of activity due to nonspecific esterases. A similar colorimetric acetylcholinesterase assay (22) was performed here on guinea pig polymorphonuclear leukocytes. The combined background due to cells without substrate and to eserine-insensitive esterase activity was very high, often 90% of the sample reading (cf. Ref. 24). The corresponding background in the radioactive assay used to do the measurements for Table II was less than 20%. When corrected for background, however, the colorimetric assay

| Preparation | Red blood cells present | Intact cell acetylcholinesterase* |
|-------------|------------------------|---------------------------------|
| Polymorphonuclear leukocytes        | 1.64 ± 0.65 (4)        | 93.8 ± 7.9 (3)                  |
| Erythrocytes                           | 1.64 ± 0.65 (4)        | 93.8 ± 7.9 (3)                  |

* Acetylcholinesterase activity is expressed as nanomoles of substrate hydrolyzed by 10^6 cells in a 15-min assay at 37°. The mean value is followed by the average deviation; the number of determinations is in parentheses.
gave a value for the guinea pig polymorphonuclear leukocyte that was 4% of the red blood cell activity, per cell; this compares favorably with the results in Table II.

Upon further investigation it was discovered that intact guinea pig polymorphonuclear leukocytes hydrolyze exogenous ATP, AMP, and p-nitrophenyl phosphatase.

Examination of Possibility that Enzymatic Activities of Intact Cells Arise from Artifacts or from Contamination—The most obvious artifact that could lead to the hydrolysis of substrates added to intact cells is the presence of broken cells or the breakage of cells under the assay conditions. The following observations rule out such artifacts in the present study.

1. As will be discussed in more detail below, comparison of intact cell activities with the activities of homogenates or sonicates revealed that about 50% of the total cell ATPase, 55% of the total p-nitrophenyl phosphatase, and 100% of the total AMPase could be measured with intact cells. If these results are to be attributed to breakage artifacts, a very large percentage of the cells would have to be disrupted.

2. Ninety-nine per cent of the cells, however, excluded trypan blue before being used in an enzyme assay, and 95% of them still excluded this dye after the assay (6-8).

3. Catalase is a soluble cytoplasmic enzyme in guinea pig polymorphonuclear leukocytes (10, 25). If cells are disrupted under the assay conditions, collection of the cells by centrifugation after the assay should leave catalase behind in the supernatant. When this experiment was performed and the catalase of the supernatant fluid was determined (10), 5% or less of the total catalase was found.

4. The measured ATPase, AMPase, and p-nitrophenyl phosphatase activities were all linear with time, suggesting that cell breakage during the course of incubation was not adding appreciably to the activities.

5. The possibility that the intact cell activities studied here are actually due to lysosomal enzymes extruded during the assay period without actual cell breakage (20, 27) is excluded by Item 4 above. Furthermore, direct measurement revealed that, under the assay conditions for intact cell ATPase, AMPase, and p-nitrophenyl phosphatase, only 3.2% of the total acid phosphatase and 5.8% of the alkaline phosphatase of the leukocytes were released into the medium. The ATPase, AMPase, and p-nitrophenyl phosphatase activities were also shown not to be associated with the cell nucleus (28).

The exudate fluid itself was found to contain ATPase, AMPase, and p-nitrophenyl phosphatase activities. The levels of these activities and the dilution of the exudate fluid during harvesting, washing, and resuspension of the cells are such that less than 0.1% of the intact cell activities could be attributed to exudate fluid in the assay sample. However, adsorption to the cells might have occurred. The following considerations make it unlikely that the intact-cell enzymes are simply adsorbed soluble enzymes.

As depicted in Table III, washing the exudate cells 10 times with 50 to 100 volumes of either an ionic (KRP) or chiefly non-ionic (0.34 m sucrose containing 10 mM Tris, pH 7.4) buffer solution did not remove significant amounts of the three enzymes. In the case of the ATPase and the p-nitrophenyl phosphatase, the removal of adsorbed enzyme might be disguised by a growing leakiness of the cells caused by repeated centrifugation and resuspension; such leakiness might allow the expression of intracellular enzymes catalyzing the same hydrolyses. This was ruled out by comparing total cell ATPase and p-nitrophenyl phosphatase activities after disrupting the cells with a detergent (saponin, 1 mg per ml, was found to reveal all of these activities). Washing 10 times did not alter the total activities. As illustrated in Table IV, certain effectors could be used to distinguish between the cellular and exudate fluid enzymes. In addition, in exudates containing large numbers of cells, as much as 80 to 90% of the total ATPase, AMPase, and p-nitrophenyl phosphatase activities of the exudate were associated with the cells.

Evidence that Hydrolyses of ATP, AMP, and p-Nitrophenyl Phosphate by Intact Polymorphonuclear Leukocytes Are Catalyzed by Ecto-enzymes—It remains to be demonstrated that the substrates added to the assay media are actually cleaved by ecto-enzymes on the intact cells and do not simply enter the cells to be hydrolyzed inside. Three types of experiments have been performed.

1. As shown in Fig. 1, the 1 mM concentrations of ATP, AMP, or p-nitrophenyl phosphate routinely used in the assays represent virtually saturating levels of substrate for the three intact cell activities. Thus, if disrupted cells were shown to have higher ATPase, AMPase, and p-nitrophenyl phosphatase activities, this would be evidence for a permeability barrier in intact cells which the substrates could not cross by any nonsaturable process, e.g. diffusion. If the method of disrupting the cells fragmented the plasma membrane but did not seriously damage the membranes of intracellular organelles, this approach would suggest that the permeability barrier in question was the plasma membrane.

As shown in Table V, assay of the homogenates prepared by a method that does not seriously damage the permeability barriers of intracellular organelles (10, 28) revealed levels of ATPase and of p-nitrophenyl phosphatase that were about twice the corresponding activities of intact cells*. As also illustrated, these

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**Table III**

| Enzyme                        | Activity remaining after washing 10 times with 50 to 100 volumes of: |
|-------------------------------|---------------------------------------------------------------|
|                               | KRP    | Sucrose                      |
| ATPase                        | 103    | 97.5                         |
| AMPase                        | 102    | 91.3                         |
| p-nitrophenyl phosphatase     | 106    | 95.0                         |

* Sucrose, 0.34 M, containing 10 mM Tris, pH 7.4.

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**Table IV**

| Enzyme                        | Addition to assay mixture | Control activity |
|-------------------------------|---------------------------|-----------------|
|                               |                           | Intact cells    | Exudate fluid |
| AMPase                        | 0.5% (w/v) saponin        | 150%            | 84.3           |
| p-nitrophenyl phosphatase     | 10 mM EDTA                | 85%             | 18.8           |

* This increased AMPase is not simply due to lysis of the cells by the detergent because 100% of the total cell AMPase is detected when intact cells are assayed (see following paper, Ref. 4).

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This result does not provide evidence for the lysosomal hydrolysis of ATP and p-nitrophenyl phosphate by intact cells. The difference in the AMPase activity of intact cells and homogenates has been found to be a very effective energy poison for polymorphonuclear leukocytes (29), and levels of iodoacetate as high as 2 mM have been found to have no significant effect on the hydrolysis of ATPaae.

Active transport of ATP and p-nitrophenyl phosphate would require energy. Iodoacetate at a concentration of 3 × 10⁻³ M has been found to be a very effective energy poison for polymorphonuclear leukocytes (29), and levels of iodoacetate as high as 10⁻³ M have been found to have no significant effect on the hydrolysis of ATP and p-nitrophenyl phosphate by intact cells.

product formed by hydrolysis of ATP, AMP, and p-nitrophenyl phosphate by intact cells are as follows.

2. The second series of experiments was directed at localizing the products of ATP, AMP, and p-nitrophenyl phosphate hydrolysis by intact cells. The possible distributions of the products formed by hydrolysis of ATP, AMP, and p-nitrophenyl phosphate by intact cells are as follows.

The results are presented.

Another possibility, that a product is bound to the cell membrane, is suggested by findings that the γ-phosphate of ATP added to intact Ehrlich ascites carcinoma cells can be transferred to serine or to threonine (30, 31). Product bound to the plasma membrane is equivalent in the following experiments to product inside the cells. A final possibility is that the substrate is cleaved while crossing the plasma membrane.

The experimental design was as follows. A thick cell suspension in KRP was incubated in the presence of 0.5 mM ATP, AMP, or p-nitrophenyl phosphate at 37 °C for long enough to hydrolyze all of the substrate. A concentration of 0.5 mM is still nearly saturating for all three enzymes (see Fig. 1); but because it is one-half the normal concentration, the incubation time required for total hydrolysis was substantially reduced. There was less risk of movement of products into or out of cells. The substrates were labeled with ³²P (as usual, only the γ-phosphate of ATP was radioactive). In addition, several experiments were done with ATP and AMP labeled with tritium in the adenosine moiety. The usual concentrations of p-nitrophenyl phosphate were used to prevent hydrolysis of ATP and AMP by the p-nitrophenyl phosphatase. [³⁵S]Inulin also was present.

After an incubation period, an aliquot was removed for the usual determination of the amount of substrate hydrolyzed. The remaining cells were pipetted into a finely calibrated hematocrit tube and centrifuged for 1 min. The total volume of the hematocrit tube contents was noted. Subsequently, the supernatant was removed and the volume of the pellet was noted. Aliquots of the supernatant and of the pellet (resuspended in a known volume of KRP) were digested in NCS (see "Materials and Methods." Table II) and the radioactivity was determined.

Comparison of ATPase, AMPase, and p-nitrophenyl phosphatase activities of intact and disrupted cells

| Enzyme                  | Activity in° | Homogenate | Sonicat* | Saponin-treated cells* |
|-------------------------|--------------|------------|----------|------------------------|
| ATPase                  | 100          | 210 ± 18   | 213 ± 20 | 254 ± 25               |
| AMPase                  | 100          | 102 ± 10   | 107 ± 6  | 159 ± 9                |
| p-nitrophenyl phosphatase | 100          | 170 ± 8   | 178 ± 9  | 186 ± 16               |

° The figures represent means of the number of determinations indicated in parentheses. Standard deviations are given for five or more determinations; for fewer values, the average deviation is presented.
* Prepared as described under "Materials and Methods."
* Saponin, 0.5% (w/v), was found to give maximum activities of AMPase and p-nitrophenyl phosphatase, while 1.0% was optimal for the ATPase.
The protein content of a known number of cells in order to determine the volume of the extracellular fluid (inulin space) from the total volume of the extracellular space present in the pellet (32). The appropriateness of this method was checked as follows. The cell was divided by their total number. Using this result and the formula for the volume of a sphere, the diameter of a single cell was approximated as 9.97 ± 0.46 μm (mean and standard deviation of nine determinations). This calculated diameter was obtained from the figures in the third and fourth columns as follows: (100% outside cells/100% volume outside cells)/( inside cells/100% volume inside cells).

In an attempt to eliminate the latter possibility the following experiment was performed. Cells in KRP were allowed to take up inorganic 32P-labeled products of ATP, AMP, and p-nitrophenyl phosphate which did not mix. For instance, the inorganic 32P-labeled phosphate might have been incorporated into some other compound. However, the loading was performed in the cold specifically to prevent such metabolism. Furthermore, investigations by Aleyassine and Frei (35) indicate that incorporation of inorganic phosphate into other compounds by guinea pig exudate polymorphonuclear leukocytes is relatively slow. It seems reasonable to conclude that the dissimilar distributions of inorganic 32P-labeled phosphate and inorganic 33P-labeled phosphate depicted in Table VII arise because the 32P-labeled products of ATP, AMP, and p-nitrophenyl phosphate hydrolysis have never been inside the cells and so have not mixed with the intracellular pool of inorganic phosphate.

Localization of inorganic [32P]phosphate formed by hydrolysis of ATP, AMP, and p-nitrophenyl phosphate by intact cells

See the text for details of this experiment. The second, third, and fourth columns of figures represent the means of the determinations ± the average deviation.

| Enzyme                  | Length of incubation | Substrate hydrolyzed | Volume of incubation mixture which was inside cells | Product 32P outside cells/32P inside cells | 32P outside cells/32P inside cells | 33P outside cells/33P inside cells | 33P outside cells/33P inside cells |
|-------------------------|----------------------|----------------------|------------------------------------------------------|------------------------------------------|---------------------------------|-------------------------------|-------------------------------|
| ATP                     | min                  | %                    | %                                                   | %                                        | 2.29                            | 30.7                          | 0.71                          |
| AMP                     | 1.5-2.0              | 100                  | 42.0                                                | 0.71                                     | 101                             |                                |                               |
| p-nitrophenyl phosphatase | 1.5-2.0              | 99                   | 43.0                                                | 4.02                                     | 18.0                            |                                |                               |

| Substrate               | Length of incubation | Volume of incubation mixture which was inside cells | 33P inside cells | Product 33P inside cells |
|-------------------------|----------------------|------------------------------------------------------|-----------------|-------------------------|
| None                    | 7.0                  | 50.2                                                 | 89.9            | 0                       |
| ATP                     | 7.0                  | 48.7                                                 | 65.6            | 0                       |
| AMP                     | 2.0                  | 55.1                                                 | 93.6            | 1.2                     |
| p-nitrophenyl phosphate | 2.0                  | 53.1                                                 | 94.8            | 0.8                     |

* This value was calculated from the figures in the third and fourth columns as follows: (% 33P outside cells/100% volume outside cells)/( % 33P inside cells/100% volume inside cells).

and Methods "); 3H, 14C, and 32P were determined in these aliquots by differential scintillation counting.

Inulin was included in the incubation mixture to serve as a measure of the extracellular space present in the pellet (32). The appropriateness of this method was checked as follows. The volume of an individual cell was calculated by subtracting the volume of the extracellular fluid (inulin space) from the total volume of the pellet to give the volume of the cells present. The protein in an aliquot of the resuspended pellet was compared to the protein content of a known number of cells in order to determine the number of cells in the pellet. The total volume of the cells was divided by their total number. Using this result and the formula for the volume of a sphere, the diameter of a single cell was approximated as 9.97 ± 0.46 μm (mean and standard deviation of nine determinations). This calculated diameter agrees well with values arrived at through morphological studies (33, 34); since it depended upon inulin space regarded as extracellular volume, it would appear that inulin is in fact excluded.

From calculations using the percentage of the total incubation mixture and the percentage of the total 32P which was inside cells, Table VI was constructed. It is clear from the table that the substrates are either hydrolyzed outside the cell and the products remain there, or they are hydrolyzed inside the cell and the products are actively transported out.

In an attempt to eliminate the latter possibility the following experiment was performed. Cells in KRP were allowed to take up inorganic phosphate labeled with 32P (New England Nuclear Corp.) for 30 to 45 minutes at 0°. 32P can easily be distinguished from 33P and 34P by differential scintillation counting. The loaded cells then were washed free of extracellular inorganic 32P phosphate and used to repeat the experiment described above. One change in the process was the use of inulin labeled with 3H instead of 14C so that this compound could be distinguished from both 32P and 33P. The ATP and AMP used in these experiments were labeled only with 32P. If the inorganic 32P phosphate produced by hydrolysis of ATP, AMP, and p-nitrophenyl phosphate is at any time inside the cells, it would mix with the pool of inorganic 32P phosphate. Such mixing would result in an identical distribution of 3P and 32P in the extra- and intracellular fluids. Table VII shows that the distribution of these isotopes was far from identical.

Although it seems unlikely, these dissimilar distributions might have resulted from the existence of different intracellular pools of inorganic 32P phosphate and inorganic 33P phosphate which did not mix. For instance, the inorganic 32P phosphate might have been incorporated into some other compound. However, the loading was performed in the cold specifically to prevent such metabolism. Furthermore, investigations by Aleyassine and Frei (35) indicate that incorporation of inorganic phosphate into other compounds by guinea pig exudate polymorphonuclear leukocytes is relatively slow. It seems reasonable to conclude that the dissimilar distributions of inorganic 32P-labeled phosphate and inorganic 33P-labeled phosphate depicted in Table VII arise because the 32P-labeled products of ATP, AMP, and p-nitrophenyl phosphate hydrolysis have never been inside the cells and so have not mixed with the intracellular pool of inorganic phosphate.

Thus, localization of the inorganic phosphate produced by hydrolysis of ATP, AMP, and p-nitrophenyl phosphate by intact cells strongly suggests that these reactions are catalyzed by ectoenzymes. The results were less definitive when the localization of the other products was examined. Since p-nitrophenol has a pK of about 7.15, a substantial amount of this product would be present in the unionized form in KRP (pH 7.4) and would thus be expected to enter cells readily; indeed, most of the released p-nitrophenol was found inside the cells. The use of tritiated ATP and AMP in the above experiments generally gave values for the ratio of the concentration of 3H outside the cells to the concentration inside of 3 or less. The easiest explanation for this finding is that the ADP produced is subsequently hydrolyzed to AMP (the intact cells do exhibit an ADPase activity; see below) which then is hydrolyzed to adenosine, and adenosine is taken up relatively rapidly by the cells (36). The enzymatic identification and "trapping" of the products of the intact cell ATPase and AMPase (discussed in the next paper, Ref. 4) provide better evidence that the adenosine-containing products of these enzymes are originally localized outside the cells.

3. The third set of experiments designed to show that the intact cell ATPase, AMPase, and p-nitrophenyl phosphatase are ecto-enzymes was based on the reasoning that if a substance which does not penetrate into the intact cells can inhibit these enzymes, they are probably located on the plasma membrane. An initial attempt was made to inactivate the intact cell activities by adding a protease (trypsin, pronase, or papain) to the medium. This approach has provided evidence that erythrocyte acetylcholinesterase is an ecto-enzyme (37). If the polymorphonuclear leukocytes were in suspension, they formed large clumps shortly after the protease was added; so the experiment was performed with cell monolayers, a procedure which effectively eliminated clumping. Table VIII shows that none of the proteases had an impressive effect on the intact cell hydrolases. These results are
TABLE VIII

Effect of proteases on intact cell enzymes

Cell monolayers were formed and then treated with trypsin (1 mg per ml), pronase (1 mg per ml), or papain (2 mg per ml) for 30 min at 37°. The incubations were carried out in KRT plus 10 mM CaCl₂ for trypsin and pronase and in KRP minus divalent cations for papain. After removal of the proteases by washing, enzyme assays were performed as described under "Materials and Methods."

| Enzyme                           | Activity remaining after treatment with |
|----------------------------------|----------------------------------------|
|                                  | Trypsin | Pronase | Papain |
| ATPase                           | 106     | 70.6    | 77.2   |
| AMPase                           | 111     | 111     | 85.0   |
| p-nitrophenyl phosphatase        | 108     | 112     | 104    |

Fig. 2. Time course of the effect of the diazonium salt of sulfanilic acid on enzymes of intact cells. Monolayers were incubated at 37° with the diazonium salt of sulfanilic acid at a concentration of 3.5 mM for the time periods indicated on the abscissa. The reaction was terminated by washing as described in the text. The key to the figure is as follows: O-O, lactate dehydrogenase; O-O, ATPase of intact cells; X-X, AMPase of intact cells; O-O, p-nitrophenyl phosphatase of intact cells.

Fig. 3. Concentration dependence of the effect of the diazonium salt of sulfanilic acid on enzymes of intact cells. Monolayers were incubated at 37° for 30 min with the diazonium salt of sulfanilic acid at the concentration indicated on the abscissa. (Note that the concentrations are given on a log scale.) The reaction was terminated by washing as described in the text. The key to the figure is as follows: O-O, lactate dehydrogenase; O-O, ATPase of intact cells; X-X, AMPase of intact cells; O-O, p-nitrophenyl phosphatase of intact cells.

Experiments were performed with monolayers of polymorphonuclear leukocytes because of the extreme speed and ease with which reagents can be removed from these preparations (17). After incubation with the diazonium salt at 37°, the supernatant was decanted from the monolayer plates and they were washed nine times with KRP; the whole procedure took 30 s or less (17). Plates which received the reagent and were then immediately washed had the same ATPase, AMPase, and p-nitrophenyl phosphatase activities as untreated plates; thus, the washing was effective. In addition to being rapid and convenient, this washing procedure almost certainly harms the cells less than repeated centrifugation and resuspension.

Figs. 2 and 3 illustrate the effects of incubating the cell monolayers with 3.5 mM diazonium salt for various lengths of time and with different concentrations of diazonium salt for 30 min. Inhibition of the ATPase, AMPase, and p-nitrophenyl phosphatase of intact cells was rapid and dramatic. After a 5-min incubation with 3.5 mM reagent, the ATPase and AMPase were inhibited more than 70%, but inhibition of the p-nitrophenyl phosphatase was not as severe at this early time point. Using a 30-min incubation the concentration of diazonium salt that brought about...
a 50% inhibition was about 0.01 mM for the ATPase and the AMPase and about 0.1 mM for the p-nitrophenyl phosphatase. Incubation for 30 or 40 min with 3.5 or 0.75 mM diazonium salt turned the cells orange, not an unexpected effect (41), but even these most severe treatments caused only 10 to 20% of the cells to come off during the washing procedure following the incubation. The precursors of the diazonium salt, sulfanilic acid and NaNO₂, had no effect on the enzymes of the intact cells.

Fig. 4 represents data concerning penetration of the diazonium salt into the cells. During the first 15 min of incubation the reagent was found to react much more rapidly with a sonicate than with a cell monolayer. Between 30 and 40 min of incubation the rate of reaction with intact cells approached that with the sonicate. The easiest explanation of these results is that the plasma membrane of intact cells acts as a permeability barrier to the reagent molecules; thus, most of the groups capable of reacting with the diazonium salt are initially inaccessible. After a time changes in the membrane brought about by the attachment of reagent molecules cause this permeability barrier to break down. Berg (18) used a similar analysis to demonstrate that human erythrocytes remain impermeable to the diazonium salt of sulfanilic acid for about 60 min at 37°.

However, it is apparent from Fig. 4 that even though the diazonium salt reacts more slowly with intact cells, the extent of this reaction is substantial even at early incubation times. Furthermore, when the cells were scraped from the monolayer plates, sonicated, and centrifuged at 100,000 × g for 1 hour, about 50% of the total reagent molecules bound to the cells were found in the supernatant fluid. This was true even at the earliest incubation times, which suggests that some diazonium salt molecules were reacting with soluble cytoplasmic proteins from the very beginning of the incubation and therefore must have entered the cells.

It therefore must be shown that the molecules that penetrate into the cells are not the ones responsible for the observed inhibitions of the ATPase, AMPase, and p-nitrophenyl phosphatase exhibited by intact cells.

An intracellular enzyme could be used as an indicator for the extent of reaction between the diazonium reagent and proteins inside intact cells. Lactate dehydrogenase, which is generally a cytoplasmic enzyme, was examined. When guinea pig polymorphonuclear leukocytes were homogenized so as to cause minimal damage to intracellular organelles (40) and the homogenate centrifuged at 100,000 × g for 1 hour, all of the lactate dehydrogenase remained in the supernatant. Thus, this enzyme is a soluble cytoplasmic protein in these cells.

Figs. 5 and 6 indicate that in sonicates lactate dehydrogenase, ATPase, AMPase, and p-nitrophenyl phosphatase were all about equally susceptible to inhibition by the diazonium salt. There may be some problem in interpreting the results for ATPase and p-nitrophenyl phosphatase since only about 50% of these total

![Graph](image-url)
sonicate activities are actually due to the corresponding activities exhibited by intact cells (see Table V). However, there seems to be little difference between the inhibition profiles of the various enzymes when sonicates are used.

On the other hand, Figs. 2 and 3 demonstrate that in intact cells the soluble cytoplasmic enzyme was much less susceptible to inhibition by the diazonium salt than were the three potential ecto-enzymes. Thus, after as much as 30-min incubation of cell monolayers with 3.5 mM diazonium salt, lactate dehydrogenase was only slightly (6.8%) inhibited. This treatment should have inhibited the ATPase, AMPase, and p-nitrophenol phosphatase of intact cells to about the same extent if these activities were due to intracellular proteins; however, it inhibited them more than 80%. Similarly, treatment of cell monolayers with 0.35 mM diazonium salt inhibited the phosphatase activities shown by intact cells 60 to 90%, but did not affect lactate dehydrogenase at all. The simplest explanation of these results is that the permeability barrier of the plasma membrane of intact cells restricts the entry of diazonium salt molecules and thereby severely limits the rate of inhibition of intracellular enzymes by this reagent. The proteins involved in ATPase, AMPase, and p-nitrophenol phosphatase activities of intact cells must have functional groups located outside the permeability barrier of the plasma membrane.

It is possible that the diazonium salt of sulfanilic acid inhibits the hydrolysis of ATP, AMP, and p-nitrophenol phosphate by intact polymorphonuclear leukocytes simply by preventing the entry of these substrates into the cells. The functional groups of the outer cell surface that are attacked by the reagent might be involved in determining the permeability of the plasma membrane or in transporting the three substrates in. If this is the case, the prediction would be that sonicates prepared from cells that had been incubated with the diazonium salt would have the same levels of ATPase, AMPase, and p-nitrophenol phosphatase activities as sonicates of untreated cells. Table IX shows that this prediction was not fulfilled.

The data with nonpenetrating reagent do not provide absolute evidence that the active sites of the three enzymes studied are outside the permeability barrier of the plasma membrane. It seems entirely possible that a plasma membrane enzyme with its active site facing the cytoplasm and some other part of the molecule exposed to the external medium could be inhibited by attack on the externally exposed groups. In fact, this seems to have occurred in a study on human erythrocytes performed by Ohta and his co-workers (42; for review see Ref. 3). The best evidence that the active sites of the ATPase, AMPase, and p-nitrophenol phosphatase of intact polymorphonuclear leukocytes are outside the permeability barrier of the plasma membrane comes from the localization of the inorganic phosphate produced, but in fact the evidence addressed in each of the contents discussed should be taken together.

**DISCUSSION**

It is clear from the experiments reported here that guinea pig polymorphonuclear leukocytes have an ecto-ATPase, an ecto-AMPase, and an ecto-p-nitrophenyl phosphatase. It is also clear, from the evidence presented, that virtually all the hydrolysis of exogenous ATP, AMP, and p-nitrophenol phosphate by intact polymorphonuclear leukocytes is catalyzed by these ecto-enzymes.

Before applying the criteria we have used to define ecto-enzymes it had to be demonstrated that the intact cell activities were not simply due to soluble contaminants or to the leakage of intracellular enzymes. The importance of eliminating the possibility of such artifacts is well recognized. In their investigation of ATP hydrolysis by intact Ehrlich ascites carcinoma cells, Wallach and Ulrey (43) showed that there was no leakage of ATPase from the cells in the medium during incubation. Similar evidence was provided by Mustafa and his co-workers (44) in their investigation of ATP hydrolysis by intact alveolar macrophages and by Ronquist (45) in his study of the enzymatic activities of intact erythrocytes. The first indication of the presence of ecto-enzymes on guinea pig polymorphonuclear leukocytes was the detection of AMPase, ATPase, and p-nitrophenol phosphatase activities with intact cells, and increased activities with the latter two enzymes upon disruption of the cells. Of course, as seen with the ecto-AMPase, no such increase will occur if there is no intracellular enzyme catalyzing the same reaction. Several other groups of investigators have also used this approach. Studying Ehrlich ascites carcinoma cells, Wallach and Ulrey (43) found that homogenates had 2.5 times the ATPase activity of intact cells. Sonication of these cells, and increased activities with the latter two enzymes upon disruption of the cells. Of course, as seen with the ecto-AMPase, no such increase will occur if there is no intracellular enzyme catalyzing the same reaction. Several other groups of investigators have also used this approach. Studying Ehrlich ascites carcinoma cells, Wallach and Ulrey (43) found that homogenates had 2.5 times the ATPase activity of intact cells. Sonication of alveolar macrophages resulted in a 60% increase in ATPase activity over that of the intact cells (44). The simplest explanation of such findings, and the first criterion for an ecto-enzyme, is that some cellular structure presents a permeability barrier to substrate molecules and that active sites capable of acting on substrate added to the medium of intact cells lie outside of this barrier. This first criterion was also made use of by Patton and Trams (46) in their investigation of bovine milk fat globules. These globules are surrounded by the plasma membrane of mammary gland cells and were found to exhibit 5'-nucleotidase activity. There was no increase in this activity if the globules were disrupted. This suggests that bovine mammary gland cells also have an ecto-5'-nucleotidase that is the only enzyme catalyzing this reaction in these cells as appears true of guinea pig granulocytes. Care was taken in the present study to disrupt the cells in a manner that caused relatively little damage to membranes

| Enzyme                   | Activity in: |
|-------------------------|--------------|
|                          | Before treat-| After reagent |
|                          | nt with reagent | Un-            |
|                          |            | treated       |
|                          |            | cells          |
|                          |            | Cells treated |
|                          |            | with reagent   |
| ATPase                  | 100         | 16.3          | 228            | 125              |
| AMPase                  | 100         | 11.6          | 105            | 17.7             |
| p-nitrophenol phosphatase | 100        | 18.9          | 190            | 92.2             |

* All activities are expressed as a percentage of the activity of untreated cells (Column 1).
other than the plasma membrane (28), and this would strengthen the conclusion that it is the plasma membrane that acts as a permeability barrier to the substrates.

The second criterion involves localization of the products of intact cell enzymatic activity, an approach that also has been used by other investigators (3). ATP added to the medium of Ehrlich ascites carcinoma cells was hydrolyzed but did not affect intracellular levels of inorganic phosphate or acid-labile phosphate (43), suggesting that neither substrate nor product was entering the cells. Ninety-five to ninety-six per cent of the 5'-monophosphate (43), suggesting that neither substrate nor product was entering the cells. Ninety-five to ninety-six per cent of the intact Ehrlich ascites carcinoma cells was hydrolyzed but did not affect products of intact erythrocyte enzymatic activities were found intact cell enzymatic activity, an approach that also has been used by other investigators (3) and to Dr. Howard Berg for advice regarding the nonpenetrating reagent.

The third criterion utilized here was the inhibition of intact cell enzymes by a “nonpenetrating” reagent. In a similar transport by erythrocytes with a nonpenetrating sulfhydryl reagent inhibited the ecto-acetylcholinesterase, as well as the facilitated diffusion of glucose 1-phosphate by rat intestinal loops was recovered in the medium. Furthermore, it was demonstrated that the intra- and extracellular pools of inorganic phosphate had not mixed. A similar demonstration that the inorganic phosphate produced by ecto-enzyme activity was never inside the cells is provided by the present studies.

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