Subcellular Distribution of Human Leukocytic Cathepsins

By M. A. STILES and J. FRAENKEL-CONRAT

IN STUDYING certain properties of peripheral leukocytic cathepsins in health and disease,1,2 we found that whole fresh cells contained two pH optima, 3.5 and 8.5, when denatured hemoglobin was used as a substrate. Mounter and Atiyeh,4 employing casein as a substrate for extracts of leukocytes, noted optima at pH 3.0, 5.5, and 8.0. Haschen and Krug,5,6 using denatured hemoglobin as substrate, reported the presence of proteases active at pH 3.5 and 7.5 as well as seven different peptidases, in normal and leukemic blood.

Since we considered it important to know the catheptic activity in the isolated leukocytes in order to compare leukemic cells with their normal counterparts, we separated the lymphocytes from the granulocytes7 and measured their respective catheptic activities at pH 3.5 and 8.5. We found that the lymphocytes displayed more catheptic activity per cell at pH 3.5 than the granulocytes, and the granulocytes more activity than the lymphocytes at pH 8.5. It then became apparent that at pH 3.5, lymphatic leukemia cells were considerably lower than normal lymphocytes and myelogenous leukemic and polycythemic cells much higher than normal granulocytes (polycythemic blood frequently contained as much as 85 per cent granulocytes). On the other hand, at pH 8.5, both types of leukemic cells were less active than their normal counterparts and polycythemic cells are approximately the same as normal granulocytes. These observations indicated that the differences noted in the leukemic cells were not due to a preponderance of one type of cell but rather to inherent deranged biochemical properties of the cells.2

Frei and his collaborators8 measured the catheptic activity of normal and leukemic leukocytes at pH 7.4, with casein as substrate. They found normal granulocytes to be almost four times as active as normal lymphocytes, cells of acute myelogenous leukemia less active, cells of chronic myelogenous leukemia more active than normal granulocytes and all lymphatic leukemia cells less active than normal lymphocytes.

This work was supported by Cancer Clinical Research Center, National Institute of Health Grant CA 08023-03.

First submitted July 17, 1967; accepted for publication November 27, 1967.

MARY ANN STILES, M.S.: Graduate student in Biochemistry, University of Missouri Medical Center and research assistant at Cancer Research Center. Some of the data presented in this paper was done in partial fulfillment of the requirements of Master of Science degree at University of Missouri. JANE FRAENKEL-CONRAT, Ph.D.: Director of Research Laboratories at the Cancer Research Center, Columbia, Missouri, and Assistant Professor of Oncology in the Department of Biochemistry, University of Missouri Medical Center, Columbia, Missouri. Present address: Department of Biology, University of Seattle, Pine Lake Campus, Issaquah, Washington.
Various laboratories have used rabbits as their source of leukocytes. Lapresle and his associates\textsuperscript{9,10} extracted and tested bone marrow, spleen, and leukocytes for cathepsins D and E, with pH optima 3.5 and 2.5, respectively. As yet no synthetic substrates have been found for these two proteases; hemoglobin or serum albumin is generally used as substrate. Bone marrow and polymorphonuclear leukocytes contained large amounts of cathepsins D and E; spleen and lymphocytes contained much D but exceedingly little E. Stefanovic\textsuperscript{11-13} has extended these investigations by isolating the enzymes from granulocytes, lymphocytes, spleen, and lymph nodes. Stefanovic, et al.\textsuperscript{10} have also ruptured granulocytes and studied the distribution of protease activity in nuclei, granular, and cytoplasmic fractions, finding most of the cathepsin D and half of cathepsin E in the nuclear fraction, and the rest of E in the granules and cytoplasm. Movat and his colleagues\textsuperscript{14} studied the granular fraction (lysosomes) and found not only cathepsins D and E but also some A. Cohn and Hirsch\textsuperscript{15} isolated and studied the specific cytoplasmic granules of the granulocytes and found the specific activity for nuclear acid cathepsins to be two-fifths that of the granular cathepsins and just a little higher than that of the supernatant cathepsins. They found no alkaline (pH 8.0) protease in any of the fractions.

Following the announcement of de Duve and his colleagues\textsuperscript{16} a decade ago that cells contained discrete granules, lysosomes, a little lighter than mitochondria and rather heavier than microsomes, much work has been carried out by both cytologists and biochemists to ascertain the importance of these granules in the economy of the cell and of the organism as a whole. The lysosomal concept has changed from “suicide sacs,” membrane-encased acid hydrolases which could cause the death and disintegration of their host cells, to a much more comprehensive concept as admirably reviewed by de Duve.\textsuperscript{17,18} Bowers and de Duve\textsuperscript{19} have recently investigated the intracellular distribution of acid hydrolases in lymphoid tissue, finding them largely associated with cytoplasmic particles of lysosomal nature.

It is interesting to note that, already in the prelysosome era, Mayer and Greco\textsuperscript{20} investigated the intracellular distribution of cathepsin (pH 3.5, hemoglobin substrate), benzoylarginine amidase, and leucine amidase activities in normal rat tissues and primary rat hepatoma. They found cathepsin in the crude mitochondrial fraction. More recently, several investigators\textsuperscript{21,22} have studied the role of lysosomes in tumor formation and chemotherapy.

Since we were interested to know the localization of the leukocytic cathepsins and to learn how this information might fit into the lysosomal concept in health and disease, we investigated the subcellular distribution of human leukocytic cathepsins.

It has been known for a long time that leukocytes contain phosphatases, both acid and alkaline in granulocytes and acid in lymphocytes, and that these become altered in various blood dyscrasias.\textsuperscript{23-25} A number of laboratories\textsuperscript{15,26-28} have investigated the distribution of these enzymes in the subcellular fractions of leukocytes, finding much of the activity in the granular fractions. Since acid phosphatase has always been associated with lysosomes, even though not ex-
CLUSIVELY, WE FOLLOWED THE POPULAR TREND OF DETERMINING THE DEGREE OF OUR PRELIMINARY SEPARATIONS OF THE SUBCELLULAR FRACTIONS BY USING ACID PHOSPHATASE AS A MARKER. ONCE A SATISFACTORY SEPARATION OF ACTIVITY WAS ACHIEVED, WE EXTENDED THE INVESTIGATIONS TO ACID AND ALKALINE CATHEPSINS AND ALKALINE PHOSPHATASE.

MATERIALS AND METHODS

Isolation of Leukocytes

Blood was obtained from donors, 500 to 600 ml. from normal subjects, 100 ml. from leukemic patients, and white cells harvested or separated into granulocyte- and lymphocyte-rich fractions as described previously, consisting of up to 98 per cent of the respective cell type. These fractions contained an average of 2 per cent monocytes and 4 per cent eosinophils in the granulocyte-rich fraction, and 3 per cent monocytes and 1 per cent eosinophils in the lymphocyte-rich fraction. To free the cell suspension of contaminating erythrocytes, the cells were suspended briefly in iced distilled water which was immediately brought to isotonicity by the addition of one-ninth the volume of 10 per cent saline, and centrifuged.

Disintegration of Cells

The cells were suspended in 0.25M sucrose, pH 6.7-7.0, at a concentration of around 200,000 to 300,000 per cu. mm., sonicated for 1 min. at maximum frequency of a Branson Sonifier with a microtip and Rosette cooling cell. This sonication produced virtually 100 per cent lysis, as measured by direct hemocytometer counting technique, while the granules remained intact as seen by phase-contrast microscopy and stained smears.

Preliminary experiments on disruption of leukocytes and differential centrifugation were carried out on fresh hog blood. Only after the method was satisfactorily worked out was human blood employed.

Differential Centrifugation

Figure 1 shows a simplified flow diagram of the following procedure. The sonicated material was diluted two- to fourfold with 0.25M sucrose and subjected to differential centrifugation in a Sorvall RC-2 refrigerated centrifuge, Rotor SS 34. The first centrifugation was at 250 g. for 30 min. The pellet was resuspended in 0.25M sucrose and the suspension again centrifuged at 250 g. for 30 min. This 7,500 g. minute fraction (I) was composed primarily of nuclei and debris, an occasional cell, and some trapped particles, as seen under phase-contrast microscopy.

The supernatant of the first 250 g. centrifugation was centrifuged at 3,000 g. for 30 min.; the pellet washed with 0.25M sucrose and the recentrifuged at 3,000 g. for 30 min. This 90,000 g. minute fraction (II) was composed primarily of large, highly refractile granules as observed under phase-contrast microscopy.

The supernatant of the original 3,000 g. fraction was centrifuged at 5,000 g. for 30 min.; the pellet washed with 0.25M sucrose and recentrifuged at 5,000 g. for 30 min. This 150,000 g. minute fraction (III) contained a heterogeneous population of different-sized granules. If this 150,000 g. fraction were not removed, the 750,000 g. fraction would then be contaminated with larger granules.

The supernatant of the first 5,000 g. fraction was centrifuged at 25,000 g. for 30 min.; the pellet washed with 0.25M sucrose and recentrifuged at 25,000 g. for 30 min. This 750,000 g. minute fraction (IV) was composed of very small, nonrefractile granules as observed under phase-contrast microscopy. The supernatant of the original 750,000 g. fraction was saved and constituted the soluble fraction (V).

All fractions were then dialyzed either (1) against distilled water in a multiple dialyzer at 5 C. overnight or (2) against running tap water for one hour and then distilled water in a multiple dialyzer for another hour before being assayed for enzymic activity and analyzed for protein content. All operations were carried out at 5 C., using plastic containers.
Table 1.—Effect of Sonication on Cell Count* of Sucrose Suspensions of Leukocytes

<table>
<thead>
<tr>
<th>Before Sonication</th>
<th>After Sonication</th>
<th>Before Sonication</th>
<th>After Sonication</th>
</tr>
</thead>
<tbody>
<tr>
<td>31,450</td>
<td>250</td>
<td>43,500</td>
<td>200</td>
</tr>
<tr>
<td>70,000</td>
<td>750</td>
<td>56,000</td>
<td>230</td>
</tr>
<tr>
<td>35,000</td>
<td>250</td>
<td>49,500</td>
<td>200</td>
</tr>
</tbody>
</table>

*Cell count by hemocytometer, expressed as number of cells per c. mm. suspension.

Fig. 1.—Simplified flow diagram of distribution of intracellular particles upon differential centrifugation.

Assay of Acid Phosphatase

Acid phosphatase activity was determined by the release of inorganic phosphate from sodium beta-glycerophosphate at pH 5.0 in barbital-acetate buffer incubated at 37°C for one hour. The reaction was stopped by the addition of trichloracetic acid. The control tubes were treated in the same manner as the experimentals, except that the unknown was added to the solution after the trichloracetic acid. Specific activity is expressed as optical density per milligram protein nitrogen per hour.

Assay of Catheptic Activity

Catheptic activity was assayed by a modification of Anson’s method as applied to leukocytes, using acid denatured hemoglobin at pH 3.5 and alkali-urea denatured hemoglobin at pH 8.5. In earlier work, we used the Folin-Ciocalteu reagent to determine the tyrosine in the protein-free filtrates but have now changed exclusively to reading the absorbance at 275 μm after we substituted Whatman #42 filter paper for the Whatman #2, from which the trichloracetic acid erratically extracted substances absorbing in the ultraviolet. Specific activities are expressed as gamma tyrosine released per milligram protein nitrogen.
Fig. 2.—Sucrose suspension of leukocytes before sonication. Phase-contrast microscopy 1000 ×.

**Nitrogen Determination**

Total nitrogen of the various fractions assayed for enzymic activity was determined by the microkjeldahl method, using selenium pellets as catalysts.

**Assay of Alkaline Phosphatase**

Alkaline phosphatase was assayed fluorometrically at pH 9.7, using disodium betanaphthol acid phosphate as the substrate, as described by McCoy. Reaction mixture without substrate was incubated 2 min. at 37 C. The substrate was then added and the rate of release of beta-naphthol was measured by the change in fluorescence at 350 mu and 420 mu. Specific activity is expressed as μM beta-naphthol liberated per milligram protein nitrogen per hour.

**Use of Synthetic Peptides as Substrates**

Fractions were assayed to determine hydrolysis of the synthetic substrates for cathepsins A, B, and C; carboxbenzoyl-L-glutamyl-L-tyrosine; benzoyl-L-argininamide; and glycyl-L-phenylalanine. The substrates were made 0.1M in a 0.1M citrate buffer at pH 5.0. Equal portions (0.5 ml.) of the substrate and unknown were incubated at 37 C. Aliquots of 0.2 ml. were withdrawn at 0, 30, and 60 min. The rate of hydrolysis was determined by the liberation of carboxyl groups which were titrated in acetone with standard HCl, using benzzenazo-alpha-naphthyl-amine as the indicator.

**Lysis Treatment of Fractions**

Subcellular fractions, obtained as described above, were subjected to triple rapid freezing in an acetone dry-ice bath and thawing in warm water, in order to ascertain whether addi-
Fig. 3.—Sonicated sucrose suspension after centrifugation at 250 g. Phase-contrast microscopy 1000 ×.

Table 2.—Distribution of Acid Phosphatase Activity*

<table>
<thead>
<tr>
<th>Centrifugal Fraction</th>
<th>Optical Density†</th>
<th>Protein‡</th>
<th>Specific Activity§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>I</td>
<td>.096</td>
<td>(.03–23)</td>
<td>.0769</td>
</tr>
<tr>
<td>II</td>
<td>.35</td>
<td>(.30–37)</td>
<td>.1306</td>
</tr>
<tr>
<td>III</td>
<td>.18</td>
<td>(.15–21)</td>
<td>.1293</td>
</tr>
<tr>
<td>IV</td>
<td>.152</td>
<td>(.14–17)</td>
<td>.1009</td>
</tr>
<tr>
<td>V</td>
<td>.13</td>
<td>(.04–14)</td>
<td>.3158</td>
</tr>
</tbody>
</table>

*Average of 4 experiments, following sonication of mixed population of leukocytes.
†Optical density measured at 660 nm.
‡mg protein N/ml. fraction measured by microkjeldahl technique.
§Optical density/mg. protein N/hr. × 1000.

Additional lysis would effect an increased release of enzymes. Treated samples and untreated controls were then assayed for catheptic activity in the usual manner.

RESULTS

The results are summarized in Tables 1 through 8 and in Figures 1 through 3. After the leukocytes had been harvested, either as a mixed population or as isolated granulocytes or lymphocytes, they were sonicated in 0.25M sucrose. Cell counts were taken on the suspension of harvested cells before and immediately after sonication; lysis was essentially complete, as can be seen in
Table 3.—Distribution of Catheptic Activity of Normal Leukocyte Fractions

<table>
<thead>
<tr>
<th>pH</th>
<th>3.5*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Same Day†</td>
</tr>
<tr>
<td>Centrifugal Fraction</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,044 (181)</td>
</tr>
<tr>
<td>II</td>
<td>1,175 (448)</td>
</tr>
<tr>
<td>III</td>
<td>1,202 (254)</td>
</tr>
<tr>
<td>IV</td>
<td>782 (236)</td>
</tr>
<tr>
<td>V</td>
<td>392 (170)</td>
</tr>
<tr>
<td>Intact Cells</td>
<td></td>
</tr>
<tr>
<td>γ Tyr./mg. prot. N</td>
<td>989 (609)</td>
</tr>
<tr>
<td>mg. Tyr./10¹⁰ WBC</td>
<td>173 (92)</td>
</tr>
</tbody>
</table>

*γ tyrosine liberated per mg. protein-N, mean values; numbers in parentheses are standard deviations (σ) of the mean.
†Five experiments.
‡Ten experiments.

Table 1 and Figures 2 and 3. Furthermore, we feel that a minimal number of granules have been disrupted since a great multitude are visible under phase-contrast microscopy (see Fig. 3), and the supernatant following differential centrifugation contains very little enzymic activity (see Tables 2 and 3).

Our primary interest was to localize the catheptic activity of the leukocytes. However, the preliminary centrifugal separations of the granules were checked by following the distribution of acid phosphatase, since cathepsins in other tissues have also been associated with acid phosphatase, which is more readily assayed than cathepsin. Table 2 shows the distribution of acid phosphatase among the various fractions.

The results of the experiments on the distribution of cathepsins in the subcellular fractions are summarized in Tables 3 through 7. Tables 3 and 4 summarize the catheptic activities obtained subsequent to differential centrifugation of sonicated normal leukocyte suspensions. At pH 3.5, the enzyme seems to be fairly evenly distributed among the particulate fractions. At pH 8.5, much more activity is found in Fractions I (nuclear) and II (large granules) than in IV (small granules). In both cases, essentially no activity is found in Fraction V, indicating that all the activity is particulate bound.

Tables 5 and 6 summarize the results obtained under the same conditions as those reported in Tables 3 and 4, except that the lymphocytes had been separated from the granulocytes before sonication and centrifugation. The distribution of enzymic activity at pH 3.5 among the various fractions is similar to that noted for fractions isolated from mixed populations of leukocytes (see Table 3), although the lymphocytic Fraction IV seemed somewhat
Table 4.—Distribution of Catheptic Activity of Normal Leukocyte Fractions

<table>
<thead>
<tr>
<th>pH 8.5*</th>
<th>Time</th>
<th>Same Day†</th>
<th>Next Day†</th>
<th>Ratio</th>
<th>Same Day/Next Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugal Fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>21,703</td>
<td>568</td>
<td>(5090)</td>
<td>(233)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>17,978</td>
<td>5,004</td>
<td>(8630)</td>
<td>(1064)</td>
<td>3.6</td>
</tr>
<tr>
<td>III</td>
<td>22,186</td>
<td>4,242</td>
<td>(9860)</td>
<td>(857)</td>
<td>5.2</td>
</tr>
<tr>
<td>IV</td>
<td>7,024</td>
<td>2,779</td>
<td>(2120)</td>
<td>(557)</td>
<td>5.2</td>
</tr>
<tr>
<td>V</td>
<td>1,081</td>
<td>321</td>
<td>(344)</td>
<td>(138)</td>
<td></td>
</tr>
<tr>
<td>Intact Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ Tyr./mg. prot. N</td>
<td>9,867</td>
<td>(3600)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg. Tyr./10^10 WBC</td>
<td>1,593</td>
<td>(566)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*γ tyrosine liberated per mg. protein-N, mean values; numbers in parentheses are standard deviations (σ) of the mean.
†Four experiments.
†Ten experiments.

Table 5.—Distribution of Catheptic Activity in Cellular Fractions of Normal Isolated Leukocytes

<table>
<thead>
<tr>
<th>pH 3.5*</th>
<th>Time</th>
<th>Same Day†</th>
<th>Next Day†</th>
<th>Ratio</th>
<th>L/S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td>Lymphs</td>
<td>Segs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugal Fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,887</td>
<td>1,860</td>
<td>0</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>3,569</td>
<td>1,167</td>
<td>3.1</td>
<td>85</td>
<td>231</td>
</tr>
<tr>
<td>III</td>
<td>3,642</td>
<td>1,478</td>
<td>2.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV</td>
<td>4,666</td>
<td>1,458</td>
<td>3.1</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>424</td>
<td>146</td>
<td>2.9 (Av.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ Tyr./mg. prot. N</td>
<td>1.012</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg. Tyr./10^10 WBC</td>
<td>154</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*γ tyrosine liberated per mg. protein-N, mean values.
†Three experiments.
†One experiment.

more active than Fractions II or III. It should be noted that the lymphocytic granular fractions are about three times as active as the corresponding granulocytic fractions, this is in keeping with earlier experiments on whole cells in which the acid catheptic activity of lymphocytes was found to be higher than that of the granulocytes.2

At pH 8.5, the results summarized on Table 6 are somewhat less clear-cut.
Table 6.—Distribution of Catheptic Activity in Cellular Fractions of Normal Isolated Leukocytes

<table>
<thead>
<tr>
<th>pH</th>
<th>Time</th>
<th>Same Day†</th>
<th>Next Day‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>Lymph</td>
<td>Segs</td>
<td>Ratio S/L</td>
</tr>
<tr>
<td>Centrifugal Fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4,226</td>
<td>41,075</td>
<td>202</td>
</tr>
<tr>
<td>II</td>
<td>44,394</td>
<td>41,284</td>
<td>0.9</td>
</tr>
<tr>
<td>III</td>
<td>30,761</td>
<td>72,160</td>
<td>2.3</td>
</tr>
<tr>
<td>IV</td>
<td>9,085</td>
<td>22,617</td>
<td>2.5</td>
</tr>
<tr>
<td>V</td>
<td>136</td>
<td>1,803</td>
<td>1.9 (Av.)</td>
</tr>
</tbody>
</table>

Intact Cells
γ Tyr./mg. prot. N | 8,738 |
mg. Tyr./10⁶ WBC | 1,560 |

*γ tyrosine liberated per mg. protein–N, mean values.
†Three experiments.
‡One experiment.

Table 7.—Catheptic Activity of Leukocyte Fractions

<table>
<thead>
<tr>
<th>Cells</th>
<th>Normal Isolated Lymphocytes*</th>
<th>Chronic Lymphatic Leukemia Cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Centrifugal Fraction‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1,887</td>
<td>4,226</td>
</tr>
<tr>
<td>II</td>
<td>3,569</td>
<td>44,394</td>
</tr>
<tr>
<td>III</td>
<td>3,642</td>
<td>30,761</td>
</tr>
<tr>
<td>IV</td>
<td>4,662</td>
<td>9,085</td>
</tr>
<tr>
<td>V</td>
<td>424</td>
<td>136</td>
</tr>
</tbody>
</table>

Intact Cells, Mixed Population
γ Tyr. mg. prot. N | 1,012 | 8,738 | 479 | 931 |
mg. Tyr./10⁶ WBC | 154 | 1,560 | 44 | 86 |

*Three experiments.
†One experiment.
§Numbers in parentheses represent values calculated on duplicate determinations of tyrosine but only single determinations of protein, due to paucity of samples; all other values reported represent duplicate determinations of both tyrosine and protein per experiment.

There is more proteolytic activity in Fractions II and III than in IV, as was the case with mixed population experiments (see Table 4). In most fractions, the granulocytes show more activity than the lymphocytes but not as much as would have been expected from studies with whole cells.²,⁸

Table 7 shows the catheptic activity of subcellular fractions of chronic lymphatic leukemic cells compared to their normal counterparts. Except for Fraction I, the leukemic subcellular fractions show considerably less catheptic activity than those of normal isolated lymphocytes at both pH 3.5 and 8.5. This substantiates the findings on unfractionated lymphatic leukemic cells.
Table 8.—Distribution of Alkaline Phosphatase

<table>
<thead>
<tr>
<th>Centrifugal Fraction</th>
<th>Alkaline Phosphatase†</th>
<th>Protein‡</th>
<th>Specific Activity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.42</td>
<td>2.25</td>
<td>0.63</td>
</tr>
<tr>
<td>II</td>
<td>0.95</td>
<td>3.18</td>
<td>0.30</td>
</tr>
<tr>
<td>III</td>
<td>1.56</td>
<td>1.55</td>
<td>1.01</td>
</tr>
<tr>
<td>IV</td>
<td>28.40</td>
<td>3.06</td>
<td>9.28</td>
</tr>
<tr>
<td>V</td>
<td>0</td>
<td>1.05</td>
<td>0</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>4.61</td>
<td>8.84</td>
<td>5.24</td>
</tr>
</tbody>
</table>

*One experiment, duplicate determinations.
†μM β-naphthol released/50X sample/hour.
‡Mg. protein nitrogen/ml. sample measured.
§μM β-naphthol/mg. protein N/hour.

compared to normal lymphocytes as reported earlier, referring activity to 10^10 white cells rather than per unit protein.

Table 8 shows results of subsequent experimentation on the distribution of alkaline phosphatase in the subcellular fractions. As can be seen, the activity is highest in Fraction IV while being remarkably lower in Fractions II and III. This enzyme is also obviously particulate bound but concentrated in different fractions than either the cathepsins or acid phosphatase.

**DISCUSSION**

Our results compare favorably with those of Cohn and Hirsch on rabbits regarding the distribution of leukocytic acid cathepsin as well as acid and alkaline phosphatase, also with others in the distribution of acid and alkaline phosphatases, most of which are particle-bound. Our experiments indicate that there is a pattern of acid phosphatase and acid cathepsin activity being rather widely distributed among the particulate matter in the cell, compared with that for alkaline phosphatase and alkaline cathepsin being more specifically granule-bound, albeit alkaline cathepsin is more active in the large granules and the alkaline phosphatase in the small granules. That our results do not substantiate the report of Cohn and Hirsch, that there was no granulocytic alkaline cathepsin might be explained on the basis of species difference, since Barnes found some time ago that rabbit polymorphonuclear cells lack neutral and alkaline protease. Furthermore, Antonioli has shown that granulocytes can have different enzyme activity if taken from the blood or peritoneal exudate, although morphologically they seem identical.

Since it is rather difficult to compare our results with the others mentioned due to use of different starting materials and reports of results in different terms, we chose to use Cohn and Hirsch's system of expressing enzyme activity per unit amount of protein. We did not aim for total recovery, but rather for maximum separation of particulate fractions.

We have found that there are nuclear alkaline cathepsins as well as acid cathepsins. Our results indicate that in mixed populations of human leukocytes, both acid and alkaline enzyme concentrations are higher in Fractions II and III than in IV. This is also true for those of isolated granulocytes. In the case of isolated lymphocytes, the acid cathepsin appears to have more activity in Frac-
SUBCELLULAR DISTRIBUTION

129

In Fractions IV than in Fractions II and III, while the alkaline enzyme has a similar
distribution to that of the granulocytes. The higher percentage of granulocytes
than lymphocytes in normal blood most probably accounts for the distribution
of enzyme activity in whole blood fractions mirroring that of the polymorpho-
nuclear cells.

Experiments in which the granules had been rapidly frozen and thawed
time displayed no increase in available cathespin in the assay. This was
not too surprising since we had found1 that homogenization of the leukocytes
prior to cathespin assay did not lead to an increase in activity at pH 3.5. We
assumed that the extremely hypotonic assay solution led to rupture of the cells
and released their contents into the surrounding fluid containing the substrates.
Also, Shibko and his colleagues25 observed that 95 per cent of the kidney
lysosomal acid phosphatase was released merely by incubating the lysosomes
at 37 C. for two hours.

If one tries to incorporate these results into the lysosome concept, several
questions arise. De Duve17 was not hesitant to state that not all acid hydro-
lases belong to lysosomes. However, now, by definition, lysosomes contain acid
hydrolases. We have found much of our acid cathespins in the granular frac-
tions of leukocyte homogenates; yet we have found more alkaline cathespins in
every fraction. Could these be in the same sacs as the acid hydrolases, since
all the lysosomal enzymes are in a latent state? Or could these be “alkaline
lysosomes”? De Duve has only considered this possibility in passing but has
not yet developed the idea completely.

In various tissues, e.g., spleen and liver, the major portion of the lysosomes
sediment at a higher centrifugal force than the mitochondria. However, in
phagocytes,15,26 as well as in other tissues,18,19,37-42 it has been amply demon-
strated that lysosomes represent a heterogenous array of cytoplasmic organelles
which cannot be readily separated from mitochondria and microsomes by
classic technics of differential centrifugation. Several investigators18,36,37,41-44
have proposed the existence of two types of lysosomes: primary, storage sacs
containing only native acid hydrolases; and secondary, those which have at
some time been involved in digestive activity and thus contain extraneous ma-
terial. If the secondary lysosomes are heavier than the primary ones, our results
would indicate that all of the leukocytes in the peripheral blood contain both
types of lysosomes, mostly secondary except perhaps in the case of the isolated
lymphocyte. But this cell, as well as the granulocytes, contain more alkaline
protease activity in the heavier granules.

Most investigators working on the subcellar particles of leukocytes have
concentrated on the granulocytes because of their exciting phagocytic action
and role in inflammation which can be followed microscopically and chemi-
cally.15,45-50 We feel, however, that the lymphocytes, although more modest
in the display of their activities, contribute a very vital element to the blood
and to the body. There are six to seven times as many lymphocytes outside
the circulation as granulocytes.34 The small lymphocyte has the ability to
become transformed into a metabolically very active cell with increased mitosis
and an increased number of lysosomes.51-53 This can occur not only in vitro
under the influence of phytohemagglutinin or other mitogenic substance but also in vivo to give rise to plasma cells which are capable of antibody formation. Wherever active protein synthesis, as in growth and regeneration, is proceeding, there are always increased lysosomes.\textsuperscript{54-58}

We should therefore, like to suggest that the higher catheptic activity at pH 3.5 in the lymphocytes, compared to that of the granulocytes, and especially in the light granule Fraction IV, may indeed be indicative of their great potential to transform and work outside the circulation. With an increase in lysosome and acid cathepsins, there would be an increase in the digestion of albumin and other proteinaceous material taken up by the cell, and perhaps even an increase in subsequent protein synthesis! In some unpublished experiments a number of years ago, we found that the incubation of benzoyl derivatives and amides of glutamic acid and of leucine with normal leukocytes resulted in the production of traces of polymers. Although leukocytes do not contain cathepsins B and C, which are known to participate in transamidation,\textsuperscript{57,58} perhaps their capthepsins are similar to others in various tissues which can lead to polymerization of amino acid derivatives.\textsuperscript{59,60}

**Summary**

1. Methods are presented for the disruption of human peripheral leukocytes by sonication and the separation of the released particulate matter by differential centrifugation.

2. The distribution of catheptic activity in the various fractions was investigated both on material obtained from mixed populations of leukocytes and on that from isolated lymphocytes and granulocytes.

3. All of the leukocytic fractions tested contained cathepsins active at pH 3.5 and pH 8.5.

4. At pH 3.5, the catheptic activity was rather evenly distributed among nuclear (I), and light (IV) and heavy (II) granular fractions, with practically no activity in the soluble fraction (V).

5. At pH 8.5, there was always more catheptic activity in the heavier (II) than the lighter (IV) granules, variable amounts in the nuclear fraction (I) and very little in the supernatant (V).

6. At pH 3.5, the lymphocytic granules showed considerably higher proteolytic specific activity than those from granulocytes.

7. At pH 8.5, all fractions except the heavy granules (II) had higher proteolytic specific activities in granulocytes than in lymphocytes.

8. At pH 3.5, and to a lesser extent at pH 8.5, the light (IV) and heavy (II) granules from chronic lymphatic leukemia cells contained much lower catheptic activity than those from normal lymphocytes.

9. Cathepsins in each subcellular fraction are very labile, losing as much as 80 per cent of their activity upon storage at 4 C. overnight in distilled water.

**SUMMARIO IN INTERLINGUA**

1. Es presentate methodos pro le disruption de human leucocytos peripheric per medio de sonicisation e separation del liberate materia particulate per centrifugation differential.

2. Le distribution de activitate catheptic in le varie fractiones esseva investigate tanto
in material obtenite ab populationes mixte de leucocytos e in material ab isolate lymphocytos e granulocytos.

3. Omne le fractiones leucocytic testate contineva cathepsinas active a pH 3.5 e a pH 8.5.

4. A pH 3.5, le activitate catheptic esseva distribuite satis uniformemente inter le fractiones nucleari (I), granulo-leve (IV), e granulo-pesante (II), con practicamente nulle activitate in le fraction solubile (V).

5. A pH 8.5, un facto invariabilemente notate esseva le plus marcate activitate catheptic in le granulos plus pesante (II) in comparation con le granulos plus leve (IV), le variabile quantitate de ille activitate in le fraction nucleari (I), e le sparstate de illo in le supernatante (V).

6. A pH 3.5, le granulos lymphocytic monstrava considerabilemente plus forte activitate proteolytic specific que le granulos ab granulocytos.

7. A pH 8.5, omne le fractiones-excepte le pesante granulos (II)habeva plus alte grados de activitate proteolytic specific in granulocytos que in lymphocytos.

8. A pH 3.5 e-minus marcatemente-a pH 8.5 le leve granulos (IV) e le pesante granulos (II) ab cellulas a chronic leucemic lymphocytic contineva minus activitate cathethic que illos ab lymphocytos normal.

9. Cathepsinas in cata-un del fractiones subcellular es labilissime. Ils perde usque a 80 pro cento de lor activitate in le curso de lor thesaurisation transnocturne a 4 C in aqua distillate.

ACKNOWLEDGMENTS

The authors would like to take this opportunity to express their deep gratitude to Dr. Reginald Pugh for his helpful criticism; Mr. James Vardiman, Mrs. Teresa Stoy, and Mrs. Juanita Cunningham for technical help; and Miss Bobbie Fox and Mrs. Cunningham for clerical assistance. They also want to thank Dr. Milton Bailey for continued interest and for the preparation of photomicrographs.

REFERENCES


14. Wasi, S., Murray, R. K., Macmoriane,


Subcellular Distribution of Human Leukocytic Cathepsins

MARY ANN STILES and JANE FRAENKEL-CONRAT