

# LYSOSOMES IN RAT THORACIC DUCT LYMPHOCYTES FRACTIONATED BY ZONAL CENTRIFUGATION

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## ABSTRACT

A method of zonal centrifugation was developed which separates rat thoracic duct lymphocytes (TDL) mainly according to size. The validity of the fractionation method was supported by light microscope observations, Coulter Counter sizing, and *in vivo* and *in vitro* labeling of lymphocytes. The distributions of lysosomal acid hydrolases in TDL fractionated by zonal centrifugation are similar to the distribution obtained for the cells. This result indicates that the large lymphocyte is not the sole bearer of either lysosomes or the large amount of soluble cathepsin D found in homogenates of TDL. Both reside mainly in small lymphocytes. This point was clearly established by fractionating homogenates of purified small lymphocytes by means of differential centrifugation and isopycnic density gradient centrifugation.

## INTRODUCTION

In a previous paper (1), it was shown that rat thoracic duct lymphocytes (TDL)<sup>1</sup> have a remarkably high content in cathepsin D, and that this enzyme occurs largely in soluble form in homogenates of osmotically shocked cells. In contrast, four other TDL acid hydrolases were found to be associated, together with a small fraction of the total cathepsin D, with typical lysosomal particles having a modal equilibrium density of about 1.18 in aqueous sucrose gradients. In this paper these particles will be designated as lysosomes. For simplicity's sake, the excess cathepsin D found in soluble form in TDL homogenates will be referred to as "nonlysosomal," even though we favor the possibility that in intact cells the enzyme is contained within special lysosomes which are particularly fragile to me-

chanical or osmotic disruption and which are related to the cathepsin D-rich particles of low density previously identified in lymphoid tissues (2, 3).

An important problem raised by these findings concerns the identity of the lymphocytes that contain the "nonlysosomal" cathepsin D. Are they identical to the cells that have the lysosomes, or do they represent a separate cell type? To try and answer this question, we have developed a method of zonal centrifugation that separates rat TDL mainly according to their size. The results indicate that, although large lymphocytes have higher enzyme activities per cell than have small lymphocytes, they account for only a small fraction of the total TDL content of any enzyme, including cathepsin D, and cannot possibly be the sole bearers of either the lysosomes or the "non-lysosomal" cathepsin D. The small lymphocytes must therefore contain both, and data are presented to support this conclusion.

<sup>1</sup> *Abbreviations used in this paper are:* TDL, thoracic duct lymphocytes; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum; N, nuclear fraction; P, high-speed pellet; S, high-speed supernatant.

## MATERIALS AND METHODS

### *Animals*

Young adult male and female rats of the inbred HO (hooded) strain were used.

### *Operative Procedures*

Thoracic duct cannulation was performed according to the method of Bollman et al. (4).

### *Handling of Thoracic Duct Lymphocytes*

Before use in experiments TDL were washed three times in Hanks' balanced salt solution (HBSS), as described previously (1). Cell counts were made with a Coulter Counter, model B (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.).

### *Labeling of TDL*

In vivo labeling was accomplished by infusing into the lateral tail vein of restrained 200-g rats at a flow rate of 2 ml/h a solution of [ $^3\text{H}$ ]thymidine (9.1 Ci/mmol, Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) containing 5  $\mu\text{Ci/ml}$  in phosphate-buffered saline (5). The duration of infusion was 36 h, including the 12-h collection period. In vitro labeling was achieved by incubating for 1 h at 37°C three times-washed TDL at a concentration of  $10 \times 10^6$  TDL/ml of Dulbecco's modified Eagle's medium (6) containing 1  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]thymidine.

### *Scintillation Counting*

Cell samples, to which 0.5 mg of bovine serum albumin was added, were precipitated with 5 ml of 5% trichloroacetic acid and left for 1 h in ice. The precipitate was sedimented at 1500 g for 10 min at 2°C and, after removal of the supernate, dissolved in 0.5 ml of 0.1 N NaOH at 37°C. The samples were precipitated and dissolved three more times. The final 0.5-ml sample was neutralized and transferred to 10 ml of Bray's solution (7) for counting in a Packard 3375 scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Counts were corrected for quenching.

### *Autoradiography*

Smears were fixed in methyl alcohol and dried. They were dipped in Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) and exposed for 1-2 wk. After development, the slides were washed thoroughly with tap water, stained with Giemsa, and washed further with distilled water.

### *Zonal Centrifugation*

TDL were resuspended in 20 ml of HBSS-2% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.) and fractionated by zonal differential sedimentation through a continuous Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gradient in a B-XIV rotor<sup>2</sup> (8) operated by a Spinco model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The gradient rested on 50 ml of an 8% (wt/vol) Ficoll solution and extended from 2% to 3.1% (wt/vol) Ficoll in a volume of 560 ml. The gradient was generated by adding 4% Ficoll-2% FCS to the gradient-forming device, described by Anderson and Rutenberg (9), containing 355 ml of 2% Ficoll-2% FCS. The 20-ml sample of cells was introduced on top of the gradient and was overlaid with 20 ml of HBSS-2% FCS. After spinning for 13 min at 1,000 rpm, the contents of the rotor were pumped out with 26% (wt/vol) sucrose at a rate of 80 ml/min. 40-ml fractions were collected. The speed of the rotor was checked with a stroboscopic flash (Strobotac type 1531-A, General Radio Co., Concord, Mass.). All operations were carried out at 2°C. The viability of the cells, as judged by trypan blue exclusion, exceeded 96%.

The 2%, 4%, and 8% Ficoll solutions used for zonal centrifugation of cells were prepared from a concentrated, dialyzed Ficoll solution which was diluted to isotonic conditions in Dulbecco's phosphate-buffered saline lacking the calcium and magnesium chloride salts (5). The solutions were then autoclaved, and streptomycin (100  $\mu\text{g/ml}$ ) and penicillin (100 U/ml) were added. Just before use, the Ficoll solutions were adjusted to 2% with FCS and to 1 U/ml with heparin.

### *Presentation of Results*

Two different methods of presenting the results were employed. (a) Owing to the geometry of the zonal rotor, successive fractions of equal volume are not situated at equal intervals from the axis of rotation. Histograms have therefore been constructed in which the ordinate expresses the percent of total activity found for each fraction divided by its thickness in centimeters, and the abscissa gives the distance in centimeters from the axis of rotation. The area occupied by the histogram equals 100%. (b) The second type of graphic representation records along the ordinate the percent of total activity for

<sup>2</sup> The B-XIV rotor was put at our disposal by Dr. N. G. Anderson, Oak Ridge National Laboratory, under subcontract no. 3081, under W-7405-Eng 26, between Union Carbide Corporation and The Rockefeller University.

each fraction divided by the percent of total cells which it contains, and the abscissa denotes the distance in centimeters from the axis of rotation. Any value greater than one indicates an enrichment in enzyme activity or radioactivity per cell in that fraction.

### Differential Centrifugation

Homogenates of rat TDL were fractionated by the method of differential centrifugation described previously (1).

### Isopycnic Density Gradient Centrifugation

Postnuclear extracts were centrifuged and histograms constructed according to the methods described previously (1).

### Enzyme Assays

The assays for various acid hydrolases were described in a previous publication (1). Recoveries for the experiments are given in Table I.

## RESULTS

### Fractionation of Rat TDL by Zonal Centrifugation

Rat TDL are comprised mainly of small lymphocytes which make up 90–95% of the total population. The remainder are cells of increasing size, which in part constitute a separate population termed large lymphocytes. A method of zonal differential sedimentation was developed

in order to take advantage of the difference in size between the small and large lymphocytes.

The upper graph in Fig. 1 presents the results of eight experiments in which rat TDL were subjected to zonal centrifugation. The nearly symmetrical distribution is skewed slightly toward those fractions farthest from the axis of rotation, presumably by the presence of a minor population of large cells sedimenting more rapidly than the major population of smaller cells.

A number of criteria established the validity of the separation method. Examination of the fractions in the light microscope revealed that

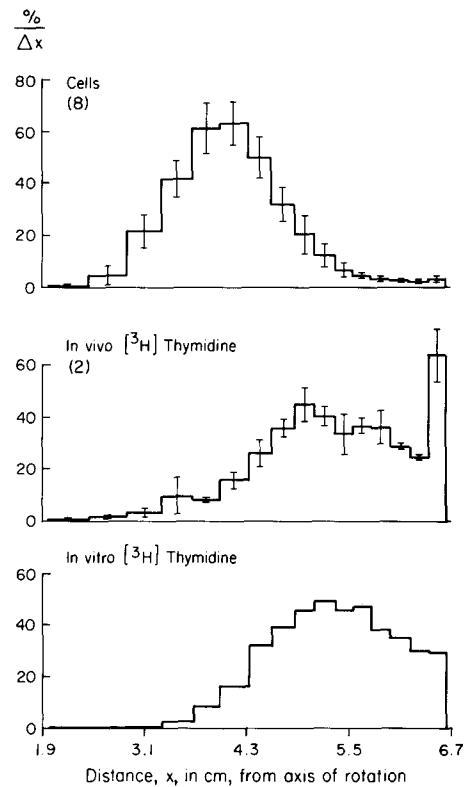


FIGURE 1 Distribution after zonal centrifugation of rat TDL and of  $^3\text{H}$ thymidine incorporated in vivo and in vitro by TDL. Histograms were constructed according to Materials and Methods. Standard deviations are given for each distribution, and the number of experiments appears in parenthesis. The top histogram presents the distribution of TDL. The middle histogram shows the distribution of  $^3\text{H}$  counts after zonal centrifugation of TDL which were labeled in vivo for 36 h. The bottom histogram gives the distribution of  $^3\text{H}$  counts for TDL, which were labeled in vitro for 1 h. and subjected to zonal centrifugation.

TABLE I  
Recovery Values

Component	No. of experiments	Average recovery $\pm$ standard deviation
Cells	10	95.7 $\pm$ 9.1
$^3\text{H}$ counts (in vivo labeling)	2	83.5 $\pm$ 0.5
$^3\text{H}$ counts (in vitro labeling)	1	92.1
$\beta$ -Galactosidase	3	104.3 $\pm$ 4.8
$\beta$ -Glucuronidase	4	106.4 $\pm$ 17.6
<i>N</i> -Acetyl- $\beta$ -glucosaminidase	5	101.6 $\pm$ 9.5
Cathepsin D	6	95.5 $\pm$ 7.0

Recovery is defined as the percentage ratio of the sum of the values obtained on the fractions to the value obtained on the unfractionated preparation.

those situated nearer the axis of rotation were almost devoid of large lymphocytes and consisted of nearly pure preparations of small lymphocytes. The frequency of large lymphocytes increased progressively with increasing radial distance, but even the most distal fractions contained some small lymphocytes. Some cells accumulated in the final fraction because they encountered an 8% Ficoll cushion which retarded further sedimentation.

A second means of assessing the separation method was to size each fraction by means of a Coulter Counter. These results agreed with the observations made in the light microscope and showed that the more distant the fraction was situated from the axis of rotation, the more it was enriched in large cells.

#### *In Vivo Labeling of Lymphocytes*

A different way of assessing the validity of the separation method was afforded by the fact that most of the large lymphocytes appearing in thoracic duct lymph can be labeled by an intravenous infusion of [<sup>3</sup>H]thymidine for a short time (24 h) before the collection of TDL.

Two separate experiments were performed, each involving one rat. Smears were prepared for autoradiography, and the percent of labeled large lymphocytes was determined. Table II shows that virtually all large lymphocytes were labeled. These results are in agreement with the observations of Howard (10).

TDL collected from these two rats during the last 12 h of the 36-h infusion period were centrifuged by the zonal method and the label in each fraction was determined. The results are presented in the middle graph of Fig. 1. They show that the distribution of counts differs considerably from the distribution of cells, and that the most distal

fractions make a substantial contribution to the total counts.

This point is demonstrated more clearly on the upper graph of Fig. 2, where the percent of total tritium counts in each fraction has been divided by the percent of total cells found for that fraction. Only those fractions in the distal half of the gradient show an enrichment in radioactivity per cell, which rises sharply to a maximum value in the last fraction.

#### *In Vitro Labeling of Lymphocytes*

As seen in Table II, the infusion of [<sup>3</sup>H]-thymidine leads to the labeling of a small percentage of small lymphocytes, in addition to the nearly complete labeling of all large lymphocytes. Because approximately 90% of the cells in thoracic duct lymph are small lymphocytes,

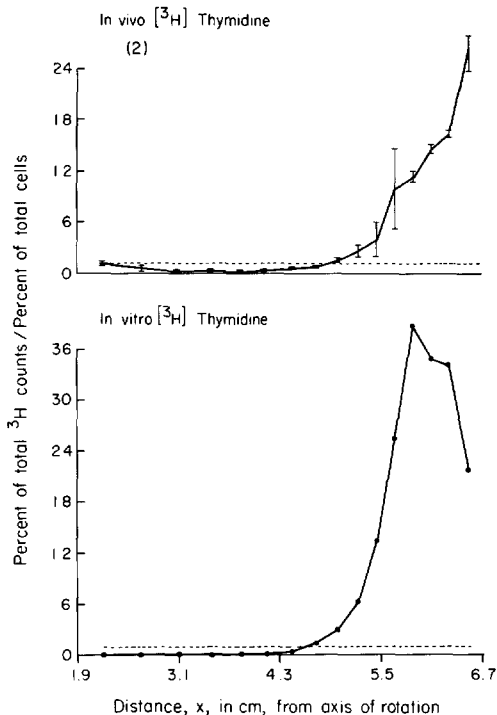


FIGURE 2 Distribution of <sup>3</sup>H counts per cell for [<sup>3</sup>H]-thymidine incorporated in vivo and in vitro by rat TDL. Same experiments as shown in Fig. 1. Values  $\pm$  standard deviation are plotted along the axis of rotation at the position locating the midpoint for each successive fraction. Any value above one, denoted by the dotted line, indicates an enrichment in radioactivity for that fraction. The number of experiments appears in parenthesis.

TABLE II  
[<sup>3</sup>H]Thymidine Labeling of Rat TDL

[ <sup>3</sup> H]Thymidine	% of labeled lymphocytes		% of total lymphocytes labeled
	Small	Large	
In vivo*	1.8	90.6	4.3
	1.5	78.4	4.3
In vitro	0.05	60.9	3.0

\* Smears were made from TDL collected during the last 12 h of the 36-h infusion period.

even a low labeling frequency means that these cells contribute a significant percent of the total tritium counts. In the experiments described above, it is not known precisely where the labeled small lymphocytes localize in the Ficoll gradient. For this reason, and in order to overcome the difficulty of categorizing arbitrarily some labeled cells as either small or large lymphocytes, TDL were labeled *in vitro* with [<sup>3</sup>H]thymidine. This procedure labels exclusively large lymphocytes, although as seen in Table II, only 60.9% are labeled.

When TDL labeled *in vitro* were centrifuged in the zonal rotor, the results presented in the lower graph of Fig. 1 were obtained. The distribution of tritium counts is similar to that found for TDL labeled *in vivo*, except that fewer total counts associate with the most distal fractions. As shown in the lower graph in Fig. 2, the radioactivity per cell increases with radial distance, as in the *in vivo* labeling situation, except that it tends to drop again in the very last fractions. It is possible that the larger cells have completed their DNA synthesis and do not incorporate any more thymidine.

### Distribution of Acid Hydrolases

Fig. 3 presents the distributions of four acid hydrolases in TDL fractionated by zonal centrifugation. Fig. 4 gives the enzyme activities per cell. These are remarkably constant for all four enzymes in the middle of the gradient where the bulk of the small lymphocytes are located. As shown by the tail end of the distribution patterns, it appears that the large lymphocytes also contain the four enzymes assayed, but in amounts differing somewhat from the quantities found in the small lymphocytes. Apparently the large cells have more *N*-acetyl- $\beta$ -glucosaminidase and  $\beta$ -glucuronidase, and less  $\beta$ -galactosidase than the small cells. The two cells have about the same cathepsin D content.

### Subcellular Fractionation

Small lymphocytes were separated from TDL by zonal centrifugation. Fractions 3-7 (those lying between 2.9 and 4.6 cm from the axis of rotation) were pooled. These nearly pure small lymphocytes comprised 78.3% of the total starting

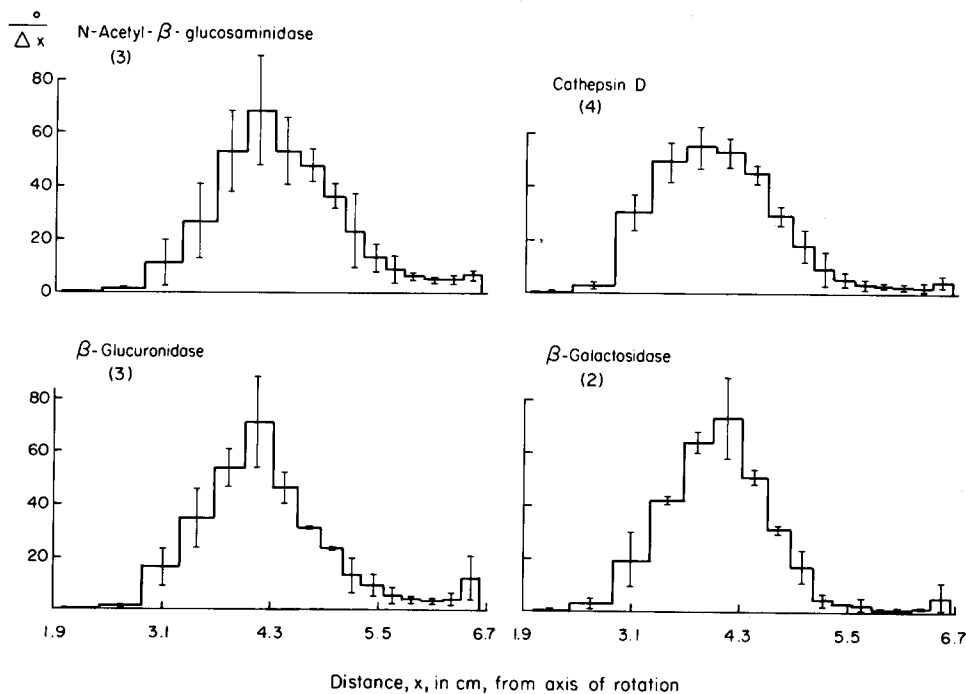


FIGURE 3 Distribution of acid hydrolases after fractionation of rat TDL by zonal centrifugation. Histograms plotted according to Materials and Methods. Values  $\pm$  standard deviation are given, and the number in parenthesis indicates the number of experiments.

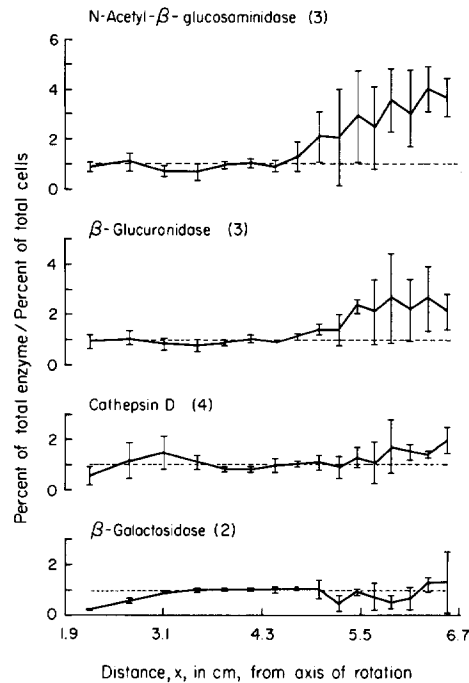


FIGURE 4 Distribution of acid hydrolase activity per cell for rat TDL fractionated by zonal centrifugation. Same experiments as shown in Fig. 3. Values  $\pm$  standard deviation are plotted as described for Fig. 2. The number of experiments appears in parenthesis.

TDL preparation. When they were homogenized and fractionated by differential centrifugation, the results given in Table III were obtained. As found before with complete TDL (1) cathepsin D was largely soluble and *N*-acetyl- $\beta$ -glucosaminidase was largely particulate.

In another experiment, small lymphocytes separated in the same fashion and representing 64.3% of the starting TDL preparation, were subjected to isopycnic density gradient centrifugation. The results of this experiment are shown in Fig. 5. Cathepsin D, in contrast to the other enzymes, is recovered mostly as soluble, un-sedimentable activity, whereas the other three enzymes,  $\beta$ -glucuronidase, *N*-acetyl- $\beta$ -glucosaminidase, and  $\beta$ -galactosidase, show a sedimentable activity distributed around a modal density of 1.165. This is essentially what we found previously for complete TDL, except that the modal density of the lysosomes was higher (1.18) and the proportion of soluble enzyme activities was lower (1). Possibly the denser lysosomes were

TABLE III  
Fractionation by Differential Centrifugation of Small Lymphocytes Obtained by Zonal Centrifugation

Enzyme	Fraction	% of total activity
Cathepsin D	N	14.6
	P	12.8
	S	72.6
<i>N</i> -Acetyl- $\beta$ -glucosaminidase	N	13.5
	P	50.3
	S	36.2

Differential centrifugation leading to nuclear (N), high-speed pellet (P), and high-speed supernatant (S) fractions was performed as described by Bowers (1). Fractions containing purified small lymphocytes represented 69% of the total cathepsin D and 55% of the total *N*-acetyl- $\beta$ -glucosaminidase activity found for unfractionated TDL.

preferentially ruptured upon homogenization of the small lymphocytes.

#### DISCUSSION

Two parameters determine sedimentation rate: size and density. It was not certain a priori that the method of zonal centrifugation we developed would successfully fractionate TDL according to size, since in the case of lymphocytes larger size probably goes together with lower density, and the relative importance of the two factors could not easily be predicted. In practice, the results make it clear that a fairly good resolution of TDL in different size classes has been achieved. Visual examination, Coulter Counter sizing, as well as both *in vivo* and *in vitro* labeling with [ $^3$ H]-thymidine, all converge to support this contention. However, the resolution is not perfect. Our technique successfully separates a large proportion (60–70%) of the small lymphocytes from the large ones, but it does not allow the isolation of large lymphocytes free of small ones. Whether some small cells sediment as fast as much larger ones by virtue of their higher density or owing to some agglutination artifact could not be determined. We saw no evidence of agglutination microscopically and included heparin in all solutions specifically to avoid this complication. In any case, even if it did occur, it was on a minor scale, of little importance with respect to our primary aim.

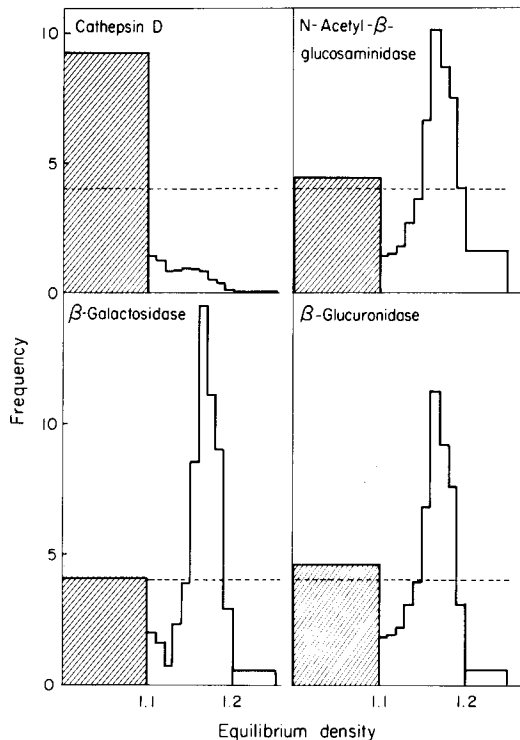


FIGURE 5 Distribution of acid hydrolases after isopycnic centrifugation in a sucrose density gradient of a postnuclear extract of small lymphocytes. These cells were purified by collecting fractions 3-7 after zonal centrifugation of rat TDL. For details, see text. Shaded block with density below 1.10 has an arbitrary density interval and represents the position of the sample layer. The arbitrary density span above 1.20 denotes the position of the "cushion" of sucrose,  $\rho = 1.25$ . The dotted line indicates the histogram expected if enzyme activity were homogeneously distributed.

According to our previous results, at least 50% of the total cathepsin D of TDL does not belong to lysosomes. Were this "nonlysosomal" cathepsin D associated specifically with the large lymphocytes, a clearly bimodal distribution would have been observed for this enzyme. Conversely, if the lysosomes were restricted to the large lymphocytes, the distribution of the glycosidases would have paralleled that of the tritium counts. Neither one nor the other result was observed, and we may therefore conclude that the small lymphocytes definitely contain both lysosomes and "nonlysosomal" cathepsin D. This conclusion is unequivocally confirmed by the fractionations performed on the small lymphocytes separated by zonal centrifugation.

There is little doubt that the large lymphocytes also possess lysosomes. These particles seem to contain all four enzymes found in the lysosomes of the small lymphocytes, but in somewhat different relative quantities. The results do not allow one to decide whether or not the large lymphocytes also have "nonlysosomal" cathepsin D, not associated with the lysosomes, since either the presence of "nonlysosomal" enzyme or a sufficient increase in lysosomal cathepsin D, comparable for instance to that of *N*-acetyl- $\beta$ -glucosaminidase, could account for the fact that the cathepsin D activity per cell does not decrease in the distal part of the gradient.

The cells used in all experiments were collected from the thoracic duct only during the first day after the operation. This preparation consists almost exclusively of lymphocytes. Previous results (1) have shown that macrophages are present in exceedingly low numbers in our preparations and, as such, do not have a detectable effect on the results. Except for the occasional presence of a few erythrocytes, no other contaminating cells have been observed, and thus we feel justified in interpreting our results as due solely to lymphocytes.

The finding that both lysosomes and "nonlysosomal" cathepsin D reside in small lymphocytes raises the question of whether both are contained in each small lymphocyte or whether they are distributed among different subclasses of small lymphocytes. Both thymus-derived (T) and bone marrow-derived (B) lymphocytes are known to be present in rat thoracic duct lymph (11), and thus it is conceivable that lysosomes and "nonlysosomal" cathepsin D are segregated into these two functionally distinct classes of lymphocyte. However, other explanations are possible as well. No available evidence can be brought to bear on this point, and further studies are required.

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