LYSOSOMES IN RAT THORACIC DUCT LYMPHOCYTES*

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In earlier studies on fractionation of rat spleen and other lymphoid tissues, evidence was obtained for the existence of two different populations of lysosomes in these tissues (1, 2). One, termed L_{19}, equilibrated around a density of 1.19 after isopycnic centrifugation in a sucrose gradient and contained all the acid hydrolases studied, most of them in amounts representing a large proportion of the total tissue content. The ability of these lysosomes to segregate materials injected intravenously suggested that they arose largely from macrophages, although their partial association with lymphocytes could not be excluded since they suffered a distinct reduction after cortisol treatment. A second population of lysosomes, designated L_{15}, was characterized by its distribution around a density of 1.15 and by its unusual enzyme composition. It was particularly rich in cathepsin D, an acid protease, contained some acid phosphatase, but appeared to lack, or to possess only low levels of, a number of other acid hydrolases, including cathepsins B and C. The disappearance of this population after treating rats with cortisol (2) led to the conclusion that it originates from lymphocytes. A similar conclusion was reached by Bouma and Gruber (3), who reported that 24 hr after a dose of 500 R, the spleen content of cathepsin D and acid phosphatase decreased by 65 and 15% respectively, while at the same time the levels of cathepsin B and C were unchanged.

In the present paper we report our findings on rat thoracic duct lymphocytes (TDL).[1] They indicate that cathepsin D is particularly abundant in these cells, where this enzyme seems to occupy a dual location: one in association with typical lysosomes not too dissimilar from the L_{19} population recognized in the solid lymphoid tissues, the other in a form that causes the enzyme to segregate with the soluble fraction upon fractionation, but which is believed to correspond to the L_{15} lysosomes. The latter particles apparently do not resist the procedure of homogenization in a hypotonic medium, which was necessary for adequate disruption of TDL.

Materials and Methods

Inbred HO (hooded) rats, obtained through the courtesy of Professor J. L. Gowans (Oxford University, Oxford, England) were bred and maintained in The Rockefeller University animal

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[1] *Abbreviations used in this paper*: E, postnuclear extract; HBSS, Hanks' balanced salt solution; N, nuclear fraction; P, high-speed pellet; S, high-speed supernatant; TDL, thoracic duct lymphocytes.

facilities. The thoracic duct was cannulated according to the method of Bollman et al. (4), and lymph was collected sterilely at room temperature for a 12–16-hr period in 5 ml Dulbecco and Vogt's solution (5) containing heparin (20 units/ml), streptomycin (100 μg/ml), and penicillin (100 units/ml). After filtration of lymph through sterile cotton, lymphocytes were centrifuged at 375 *g* for 10 min. The cells were then washed three times in Hanks' balanced salt solution (HBSS) (6). Viability of the cells, as judged by trypan blue exclusion, exceeded 99%. In some experiments, lymphocytes at a concentration of 7.5×10^6 cells/ml were incubated at 37°C in 4 ml of Dulbecco's modified Eagle's medium (7) containing 10% fetal calf serum; the Petri dishes (60 mm, No. 3002, Falcon Plastics, Oxnard, Calif.) were continuously gassed with a humidified atmosphere of 7% CO_2-air. Cell counts were made with a Coulter Counter, Model B (Coulter Electronics, Inc., Hialeah, Fla.).

For fractionation studies, cells were resuspended in 0.04 M KCl-0.01 ethylenediaminetetraacetate (EDTA), pH 7, for 4 min, after which they were vigorously homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) with 30 up-and-down strokes of the tight-fitting B pestle. Isotonicity was restored by mixing the homogenate with an equal volume of 0.24 M KCl-0.01 M EDTA, pH 7. The resulting preparation was then centrifuged at 650 *g* for 10 min, the supernatant removed, and the pellet rehomogenized according to the above procedure. After a second centrifugation at 650 *g* for 10 min, the supernatants were combined to form the postnuclear extract (E), and the remaining nuclear pellet (N) was taken up in 0.14 M KCl-0.01 M EDTA, pH 7. In some experiments, the postnuclear extract was fractionated further into high-speed pellet (P) and supernatant (S) by centrifugation at 100,000 *g* for 30 min. The pellet was resuspended in 0.14 M KCl-0.01 M EDTA, pH 7.

Fractionation of the postnuclear extract (E) by isopycnic density gradient centrifugation was performed with the automatic zonal rotor designed by Beaufay (8, 9). 10 ml of extract (E) were layered over 24 ml of a sucrose gradient extending linearly with respect to volume, in most cases between density limits of 1.10 and 1.20. The gradient rested on a 5 ml cushion formed by a sucrose solution with a density of 1.25. Loading and unloading were carried out at low speed; density equilibration of subcellular particles was achieved by centrifuging at 35,000 rpm for 37 min. Fractions of 2.5 ml were collected, and the weight and mean density of each fraction were determined. All procedures were performed as described by Leighton et al. (9).

Enzyme assays utilized fluorometric substrates (10) obtained from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England). The following 4-methylumbelliferone derivatives were used: 2-acetamido-2-deoxy-β-D-galactopyranoside, β-D-galactopyranoside, 2-acetamido-2-deoxy-β-D-glucopyranoside, β D-glucuronide, α-L-arabinopyranoside, β-D-cellobiopyranoside, β-L-fucopyranoside, α-D-mannopyranoside, and β-D-xylopyranoside. The substrates were diluted from 10 mM stock solutions immediately before use to 0.2 mM in 0.1 M acetate buffer (of appropriate pH: see below, Table II) containing 0.2% (w/v) Triton X-100. To start the reaction, 0.1 ml of the enzyme was added to 0.1 ml of the substrate mixture. Incubation was at 37°C. The reaction was stopped after an appropriate time by addition of 2 ml of a 50 mM glycine-5 mM EDTA solution adjusted to pH 10.4 with NaOH. Fluorescence was measured at 460 nm in a Perkin-Elmer Fluorescence Spectrophotometer 204 with an excitation wavelength of 365 nm (Perkin-Elmer Corp., Norwalk, Conn.). In the studies where enzyme activities of TDL were compared with those of spleen, the assay methods used chromogenic substrates (11). Cathepsin D was assayed as described previously (11), except that 10-min blanks were used and the trichloroacetic acid- (TCA)-soluble fragments were measured by the automated Lowry method described by Leighton et al. (9).

Under the conditions of the assays, all measured activities displayed linearity with respect to both incubation time and enzyme concentration. Enzyme activities are expressed in units, 1 unit being the amount of enzyme needed to hydrolyze 1 μmole of substrate/min under the assay conditions, except for cathepsin D activity, which is given as the chromogenic equivalence of 1 g of bovine serum albumin released/min.

The results of the fractionation experiments were calculated, plotted, and averaged with a

computer, using the procedures described by Beaufay et al. (12) and Leighton et al. (9). Recoveries, which are given in Table I, were somewhat variable, probably because of some clumping in the starting preparations and a resulting difficulty in pipetting reproducible samples. The distribution patterns themselves were very reproducible.

RESULTS

Total Activities and Optimal pH.—Table II records total activities and optimal pH values obtained for a number of hydrolases. All show maximal hydrolysis of their substrate at an acid pH, and total activities given were measured at the optimal pH value for each enzyme. Of the nine glycosidases assayed with fluorometric substrates, only the first four were studied further, since the low activity of the others rendered them unsuitable for routine assay. Cathepsin D,

TABLE I

Recovery Values

Component	No. of experiments	Average recovery ± standard deviation
β-Galactosidase	5	95.5 ± 18.5
β-Glucuronidase	13	85.5 ± 16.4
N-Acetyl-β-glucosaminidase	12	94.9 ± 19.9
N-Acetyl-β-galactosaminidase	5	81.6 ± 12.9
Cathepsin D	14	100.5 ± 18.7
Protein	8	104.9 ± 13.0

Recovery is defined as the percentage ratio of the sum of the values obtained on the fractions to the value obtained on the unfractionated homogenate or postnuclear extract.

TABLE II

Acid Hydrolases of Thoracic Duct Lymphocytes

Component	No. of determinations	milliunits/10^9 cells	Optimal pH
β-Glucuronidase	19	33.0 ± 17.7	4.0
β-Galactosidase	16	21.5 ± 9.2	4.5
N-Acetyl-β-glucosaminidase	15	11.6 ± 3.2	5.0
N-Acetyl-β-galactosaminidase	14	6.4 ± 2.6	5.0
α-Mannosidase	2	1.2 ± 0.2	5.5
α-Arabinosidase	1	0.5	5.5
β-Xylosidase	1	0.4	5.5
β-Cellobiosidase	1	0.03	5.5
β-Fucosidase	1	0.03	4.5
Cathepsin D	18	1.78 ± 0.53	3.5
Protein (*mg/10^9 cells*)	13	18.0 ± 5.9	

With the exception of cathepsin D, all enzymes were measured fluorometrically. Values given are means ± standard deviation.

which is expressed in different units, displayed a high activity and was systematically included in subsequent studies.

Because earlier studies provided information on acid hydrolases in rat spleen, some measurements were performed with chromogenic substrates (11) in order to compare enzyme activities for TDL with those obtained on spleen. The results are presented in Table III, and they show that TDL possess lower activities than spleen for all acid hydrolases except cathepsin D. This enzyme is at least as abundant in TDL as it is in spleen.

Comparison of Tables II and III reveals that both the β-glucuronidase and β-galactosidase of TDL showed distinctly higher specific activities on the 4-methylumbelliferone glycosides than on the chromogenic substrates, even though the latter are used at much higher concentration (12.5-fold for β-glu-

TABLE III

Acid Hydrolase Activities in Thoracic Duct Lymphocytes and Spleen

Enzyme	Thoracic duct lymphocytes		Spleen	
	No. of experiments	units/g protein	No. of experiments	units/g protein
Arylsulfatase	3	2.34 ± 0.20	8	9.83 ± 3.22
β-Glucuronidase	7	1.03 ± 0.49	6	6.94 ± 1.61
β-Galactosidase	1	0.44	3	3.75 ± 0.92
N-Acetyl-β-glucosaminidase	5	5.55 ± 1.94	3	13.5 ± 3.13
α-Mannosidase	2	1.24 ± 0.24	4	4.36 ± 2.64
Cathepsin D	11	0.088 ± 0.027	2	0.074 ± 0.012

All assays were performed with chromogenic substrates according to Bowers et al. (11), except for cathepsin D which was measured by the modified method referred to in this paper. Values for the spleen enzymes, except cathepsin D, are from Bowers et al. (11).

curonidase and 50-fold for β-galactosidase). In contrast, the other two glycosidases assayed were much less active on the fluorometric than on the chromogenic substrates. The reasons for these differences were not explored further.

Latent Activity.—Homogenates were assayed for an acid hydrolase in substrate mixtures containing or lacking 0.1% Triton X-100, a nonionic detergent. Total activity is measured in the presence of Triton X-100 and free activity in its absence; the difference between these two activities gives a measure of the latent activity, which can be expressed as a per cent of the total activity. Three separate homogenates of rat TDL gave latent activities for *N*-acetyl-β-glucosaminidase of 55.4, 52.3, and 56.4%.

Fractionation by Differential Centrifugation.—Homogenates of rat TDL fractionated by means of differential centrifugation into nuclear (N), high-speed pellet (P), and supernatant (S) fractions yielded the results recorded in Table IV. All three enzymes contribute a similar per cent of total activity to the nuclear

fraction, but cathepsin D differs markedly from β-glucuronidase and *N*-acetyl-β-glucosaminidase in its relative contribution to the high-speed pellet and supernatant. Three times as much cathepsin D activity appears in the supernatant as compared with the two other enzymes.

Fractionation by Isopycnic Centrifugation.—Postnuclear extracts of TDL were fractionated by means of isopycnic centrifugation in sucrose density gradients. The averaged results of three experiments are presented in Fig. 1. They show, in confirmation of the data of Table IV, that considerably more cathepsin D than β-glucuronidase or *N*-acetyl-β-glucosaminidase activity remains in the starting layer in unsedimentable form. They reveal further that two other enzymes, β-

TABLE IV

Fractionation of Homogenates of Thoracic Duct Lymphocytes by Differential Centrifugation

Enzyme	No. of experiments	Fraction	Per cent of total activity
β-Glucuronidase	8	N	13.5 ± 4.3
		P	64.5 ± 6.8
		S	22.0 ± 5.5
N-Acetyl-β-glucosaminidase	8	N	14.2 ± 6.6
		P	64.6 ± 7.6
		S	21.2 ± 8.4
Cathepsin D	9	N	14.6 ± 7.0
		P	20.0 ± 7.1
		S	65.4 ± 8.0

For preparation of the fractions, see Materials and Methods. Values are means ± standard deviations.

galactosidase and *N*-acetyl-β-galactosaminidase behave essentially like their sister glycosidases; and that the sedimentable portion of all five enzymes equilibrates almost identically in the gradient, around a modal density of 1.175.

Most of the protein associated with the postnuclear supernatant is soluble and unsedimentable. The remainder makes no significant contribution to any one fraction, but is dispersed rather uniformly throughout the gradient. In the case of the acid glycosidases, an enrichment of five- to six-fold over the starting postnuclear extract is achieved for the peak fraction.

Fractionation by Isopycnic Centrifugation after Short-Term Culture of TDL.—When TDL are cultured it is frequently observed that a few cells adhere to the plastic surface of the Petri dish. Many of the adherent cells can be identified as macrophages, which are known to be rich in lysosomal enzymes (13). Sufficient numbers of such cells could influence significantly the total enzyme activity attributed to lymphocytes or modify the gradient distributions of the enzymes.

After 2 hr in culture the nonadherent cells were removed by gentle pipetting and the dishes were rinsed twice. The cells were then washed three times with HBSS, and assayed as described in Materials and Methods. Three separate experiments were performed, and the total enzyme activities are presented in

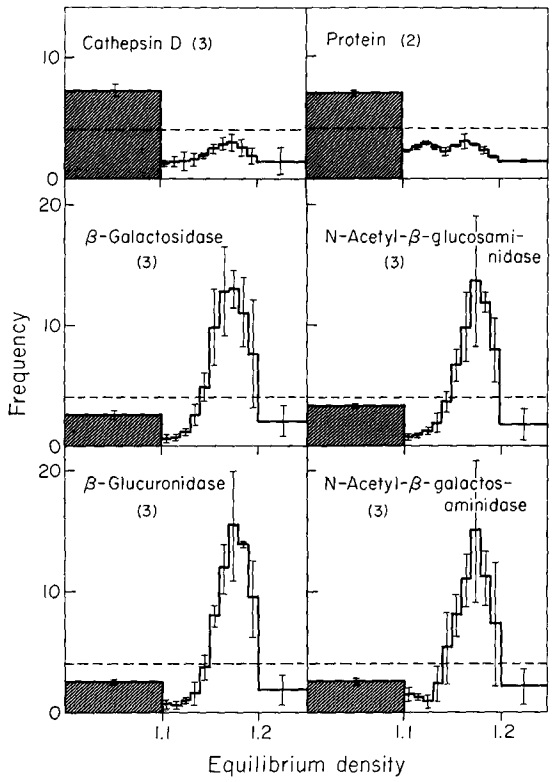

FIG. 1. Distribution of acid hydrolases and protein after isopycnic centrifugation in sucrose density gradients of postnuclear extracts of TDL. Shaded block with density below 1.10 has an arbitrary density interval and represents the position of the sample layer. The arbitrary density span above 1.20 denotes the position of the "cushion" of sucrose, $\rho = 1.25$. The diagram shows the average of results with standard deviation, and the number of experiments between parentheses.

Table V. It can be seen by comparison with the data of Table II that the elimination of adherent cells from preparations of TDL did not significantly alter the total enzyme activities.

Postnuclear extracts of nonadherent, cultured TDL, fractionated by means of isopycnic centrifugation, yielded the distributions shown in Fig. 2. The results are similar to, but not identical with, those obtained on uncultured TDL (Fig.

TABLE V

Total Activities for Nonadherent Thoracic Duct Lymphocytes after 2 hr in Culture

Component	No. of experiments	milliunits/10^8 cells
β-Glucuronidase	3	58.4 ± 22.5
β-Galactosidase	2	27.3 ± 10.3
N-Acetyl-β-glucosaminidase	2	9.6 ± 1.3
N-Acetyl-β-galactosaminidase	2	4.3 ± 1.2
Cathepsin D	3	1.90 ± 0.50
Protein (*mg/10^8 cells*)	3	16.3 ± 4.5

With the exception of cathepsin D, all enzymes were measured fluorometrically. Values given are means ± standard deviation.

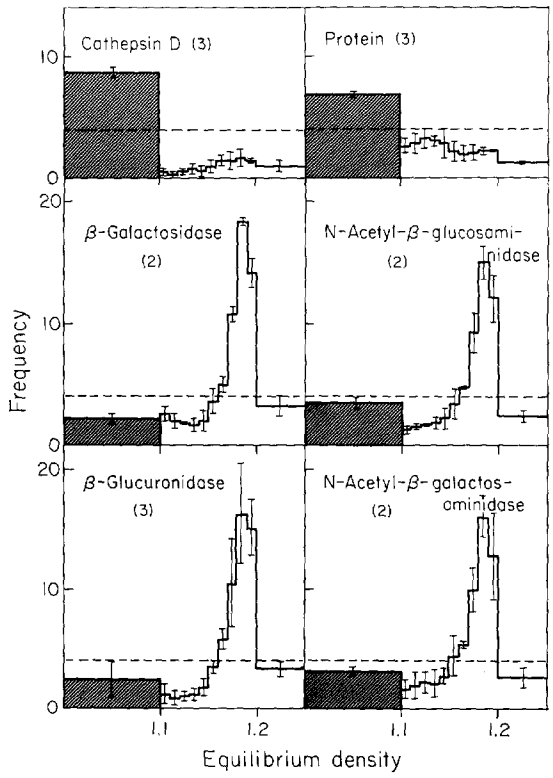

FIG. 2. Distribution of acid hydrolases and protein after isopycnic centrifugation, in sucrose density gradients, of postnuclear extracts prepared from nonadherent TDL cultured for 2 hr. Diagrams are constructed as in Fig. 1.

1). After culturing, the distributions of the particulate enzymes are shifted slightly towards a higher density, from a modal value of 1.175 to one of 1.185. Also a larger proportion of the cathepsin D (87% as against 72%), but not of the acid glycosidases, was recovered in unsedimentable form.

DISCUSSION

The biochemical evidence reported in this paper shows that 10 hydrolases known to be lysosomal in other cells occur in rat TDL. All 10 enzymes display optimal activity at an acid pH, and the behavior of some, which were studied in more detail, fulfills two of the biochemical criteria for assuming a lysosomal location: sedimentability and latency. In fractionation experiments, four TDL acid glycosidases were found to be largely sedimentable and to equilibrate in sucrose gradients around a density of about 1.18. Part of the cathepsin D activity showed a similar behavior. Furthermore, one of the glycosidases, *N*-acetyl-β-glucosaminidase, was found to display latency, although to an extent that did not entirely match its degree of sedimentability. Rupture of some particles under the assay conditions was probably responsible for this discrepancy, and it may be safely concluded, by analogy with what is known for a large number of cell types, that the sedimentable acid hydrolase activities of TDL are associated with a population of lysosomes.

There are strong grounds for the belief that this lysosomal population arises from true lymphocytes. Cannulation of the thoracic duct of rats yields preparations of leukocytes that all have the morphological appearance of lymphocytes. Although no granulocytes have ever been seen, cells such as monocytes or macrophages could contaminate our preparations without being easily detectable. However, restricting the lymph collection to only the 1st day after cannulation minimizes the number of monocytes or macrophages that could appear in the lymph. In addition, such cells, if they were present, would tend to adhere to the glass surface of the collecting flask during the lengthy collection period, and thus their number would be reduced even further. And finally, in those experiments specifically designed to eliminate the few adherent cells remaining in our preparation (never exceeding more than 4 or 5 adherent cells/million nonadherent cells), there was no indication that the 1.18 lysosomes had been removed, even in part. The occurrence of such lysosomes in lymphocytes explains our earlier finding that the L_{19} population is partly depleted after cortisol treatment (*2*).

The intracellular localization of the nonsedimentable acid hydrolase activities poses a more difficult problem. For the acid glycosidases, the proportion of total activity occurring in soluble form in the TDL homogenates amounts to no more than about 20%. This is less than is found for many other cell types and it can be safely attributed to lysosome breakage. The proportion is, however, much higher for cathepsin D. Three possibilities could account for this finding: (*a*) part of the enzyme, amounting to as much as 80% of the total, is truly soluble and occurs in the cell sap; or (*b*) the enzyme is associated predominantly with fragile members of the 1.18 lysosome population; or (*c*) a large fraction of the enzyme belongs to a separate population of lysosomes, more fragile than the 1.18 lysosomes.

There is at present no way of distinguishing between these three interpretations, but the third is the most likely one, because so far no evidence has been

obtained for the occurrence of free cathepsin D in other cell types, and especially because cathepsin D happens to be most abundant in the special population of lysosomes identified in solid lymphoid tissues as belonging to the lymphocytes and distinguished in these tissues by their low equilibrium density (1.15) in sucrose gradients (1, 2). If this view is correct, one may ask why the L_{15} lysosomes should rupture more easily upon homogenization of TDL than of solid tissues. It is important to point out in this respect that the homogenization conditions are not the same. The lymphoid tissues were disrupted in an isotonic medium whereas the TDL were subjected to an osmotic shock, and also to greater mechanical stress. It is possible that the L_{15} lysosomes are more sensitive to osmotic and/or mechanical disruption than are those of higher density.

Upon differential sedimentation, about 14% of the three acid hydrolases assayed were recovered with the nuclear fraction. Most likely, this value represents largely intact cells, since if cosedimentation of lysosomes with the nuclei had occurred, one would expect to find only one-third as much cathepsin D as either β-glucuronidase or *N*-acetyl-β-glucosaminidase in the nuclear fraction.

Because the thoracic duct lymph of rats contains several different types of lymphocytes, it is not possible from our data to know the origin of these two populations of lysosomes. This problem forms the subject of a future paper.

SUMMARY

10 acid hydrolases known to be lysosomal in other tissues were detected in rat thoracic duct lymphocytes (TDL). Except for cathepsin D, all these enzymes occurred in lower levels in TDL than in rat spleen.

Fractionation by means of differential centrifugation revealed two types of distribution patterns. Most of the total cathepsin D activity appeared in a high-speed supernatant (S), whereas the other acid hydrolases associated mainly with a high-speed pellet (P). Further studies by isopycnic centrifugation in a sucrose density gradient showed that all the enzymes, including a small portion of cathepsin D, sedimented around a modal density of 1.18. In contrast, most cathepsin D activity was recovered in a soluble, unsedimentable form. These results suggest the presence of two populations of lysosomes. Because the possibility could be excluded that other cell types contaminated the lymphocyte preparations, it is concluded that both lysosomal populations arise from TDL.

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