LYSOSOMES IN LYMPHOID TISSUE

III. Influence of Various Treatments of the Animals on the Distribution of Acid Hydrolases

WILLIAM E. BOWERS and CHRISTIAN DE DUVE

From The Rockefeller University, New York

ABSTRACT

The density-distribution patterns of various enzymes and of labeled materials have been determined by isopycnic centrifugation in a sucrose–0.2 M KCl gradient on homogenates of lymphoid tissues from rats injected with Triton WR-1339, ^{14}C-labeled dextran, ^{51}Cr-labeled erythrocytes, and cortisol. The results confirm and extend the conclusion, derived from previous investigations on normal animals, that the lysosomes of lymphoid tissues form two and possibly three, distinct populations. The evidence indicates that the L_{19} population belongs to macrophages and the L_{15} group to lymphocytes. The L_{30} population appears to be associated with a special type of phagocyte with a high capacity for dextran storage. All three populations seem to contribute to the activities found in soluble form in homogenates of normal lymphoid tissues.

INTRODUCTION

One of the main functions of lysosomes is concerned with the storage and digestion of exogenous materials taken up intracellularly by an endocytic process (for a review, see reference 12). In the present work, advantage has been taken of this phenomenon to characterize further the various lysosomal populations tentatively identified in rat lymphoid tissue as L_{15}, L_{19}, and L_{30} (4,5). The following treatments were selected for application to rats, from which the spleen was subsequently removed and fractionated by isopycnic centrifugation in a sucrose–0.2 M KCl gradient:

1. Injection of Triton WR-1339, a substance shown by Wattiaux and coworkers (39–41) to accumulate in liver and kidney lysosomes and to cause a marked decrease in the equilibrium density of these particles in a sucrose gradient.
2. Injection of ^{14}C-labeled dextran, a polysaccharide known from morphological observations to become concentrated in the lysosomes of liver (8,10) and of some spleen cells (9), and found to increase the equilibrium density of hepatic lysosomes in a sucrose gradient (2).
3. Injection of ^{51}Cr-labeled erythrocytes, as an easily detectable substrate for the well known erythrophagocytic function of spleen.

In a final series of experiments, the lympholytic effect of cortisol (13) was used to modify the cellular composition of spleen, thymus, and lymph nodes, and the changes incurred by the lysosomes of these tissues were investigated by density-gradient isopycnic centrifugation.

MATERIALS AND METHODS

Triton WR-1339 (Rohm & Haas Co., Philadelphia, Pa.), dissolved in saline, was injected at the dosage of 75 mg per 100 g rat into one of the lateral tail veins under light ether anesthesia.

Dextran was injected similarly at a dosage of 40–

60 mg per 100 g rat. The labeled material given consisted of a 5:3 mixture of cold dextran-500 (Pharmacia, Stockholm, Sweden) and of the same polysaccharide tagged peripherally by incubation in the presence of uniformly labeled sucrose-^{14}C and of dextran sucrase extracted from *Leuconostoc mesenteroides* (prepared by Dr. P. Jacques). This material was injected either as such, or with an equal amount of dextran-10.

Erythrocytes were obtained from rat or mouse blood collected into Strumia (38) mixture containing 0.1% of heparin. The rat blood was kept overnight at 2°C in this mixture, with addition of formaldehyde to a final concentration of 0.5%. It was then centrifuged at 600 *g* for 10 min and the sedimented cells were resuspended in Strumia mixture and incubated for 5 min at 37°C with 10 μcuries of Na-$^{51}CrO_4$ (Rachromate 51, Abbott Laboratories, Chicago, Ill.) (26). The cells were washed free from excess chromate by five to six washings with Strumia mixture and injected intravenously in an amount corresponding to 0.2–0.3 ml of blood per 100 g rat. The mouse blood was treated in the same way, except that the incubation with formaldehyde was omitted. It has been shown that in this labeling procedure ^{51}Cr binds to the beta chain of hemoglobin (31).

Cortisol (Sigma Chemical Co., St. Louis, Mo.) was injected subcutaneously twice daily at the dosage of 15 mg per 100 g rat.

The lymphoid tissues of the treated animals were fractionated by isopycnic centrifugation in a sucrose–0.2 M KCl gradient and analyzed, as described in the

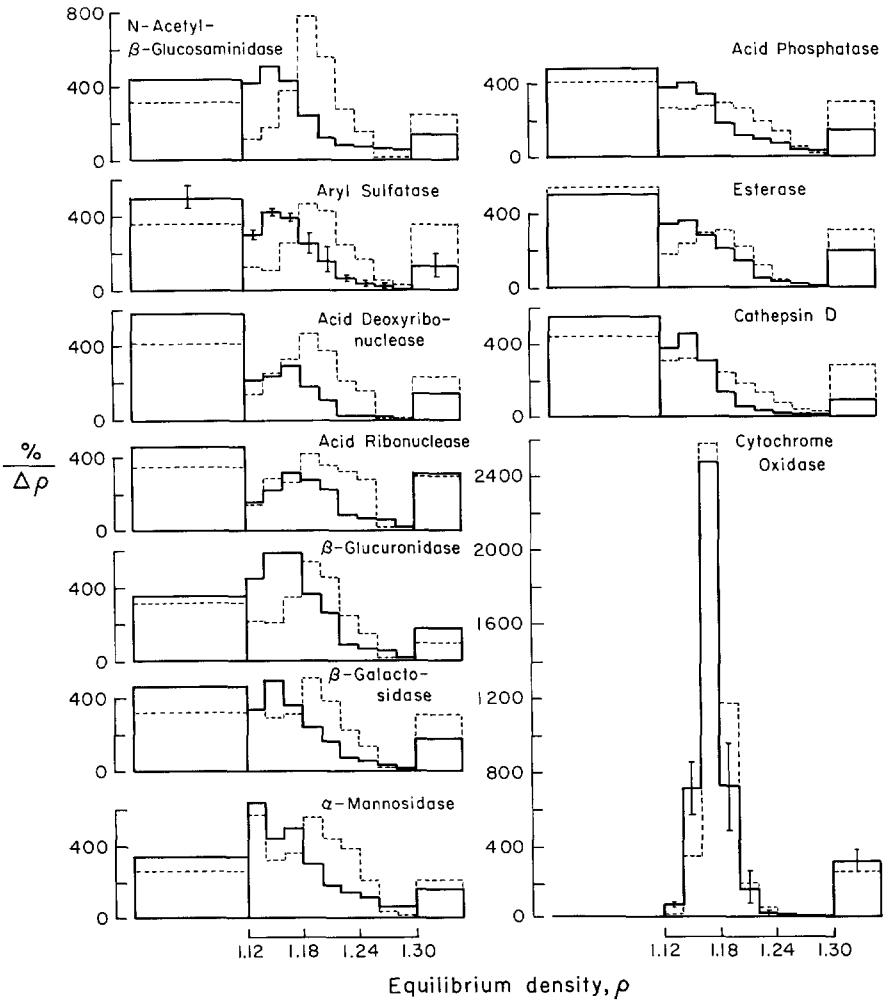

FIGURE 1 Influence of a single injection of Triton WR-1339 (75 mg/100 g body weight), given 2 days previously, on the density-distribution patterns of rat-spleen enzymes. Dotted lines show normal distributions (4).

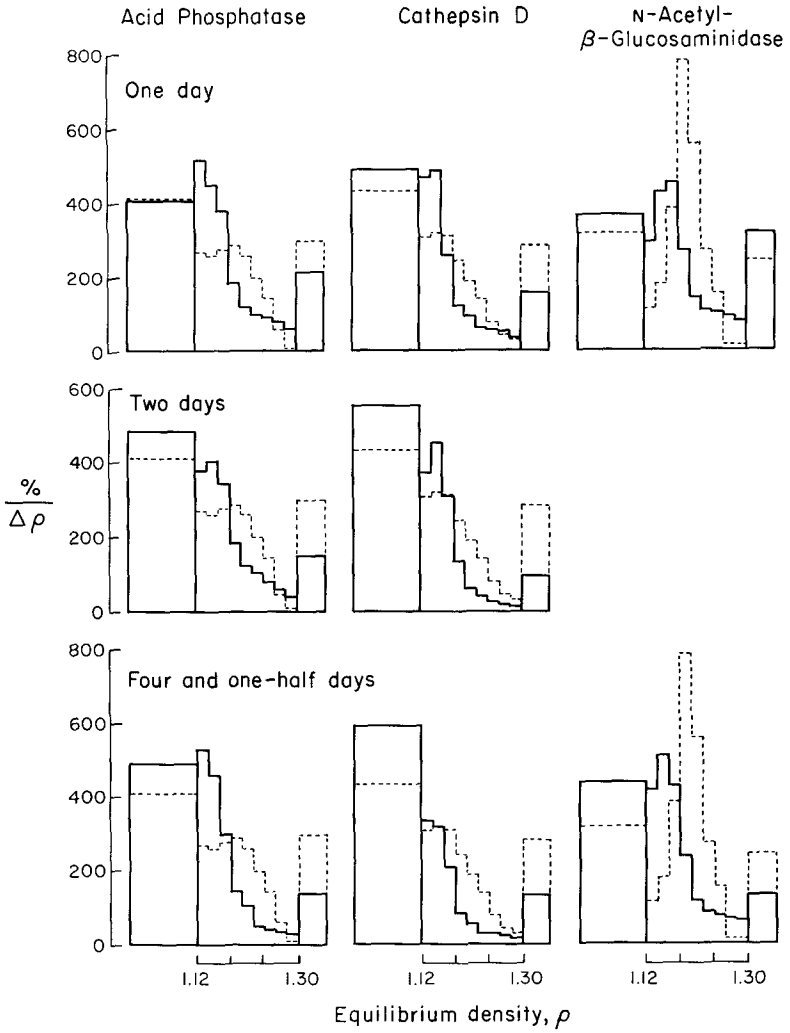

FIGURE 2 Influence of a single injection of Triton WR-1339 (75 mg/100 g body weight), given 1, 2, and 4½ days previously, on the density-distribution patterns of rat-spleen enzymes. Dotted lines show normal distributions (4).

preceding papers of this series (4, 5). ^{14}C was counted on 0.2–1.0 ml samples of the crude fractions by means of a Packard scintillation counter, in the scintillation mixture described by Bray (6). The degree of quenching was always determined by addition of a standard number of ^{14}C-formate counts. ^{51}Cr was counted on 0.5 ml samples of the fractions by means of a Nuclear-Chicago gamma counter.

RESULTS

Influence of Injection of Triton WR-1339

As shown in Fig. 1, a single injection of Triton WR-1339 causes a distinct shift towards lower densities of the density distribution curves of all the spleen hydrolases. The displacement appears most marked for enzymes such as *N*-acetyl-β-glucosaminidase and aryl sulfatase, which characterize what has been termed the L_{19} population; it is least for cathepsin D, which contributes most to the L_{15} population. Most enzymes also show a decrease in the dense sediment (L_{30} population), as well as an increase in the soluble fraction which probably reflects an enhanced fragility of the particles with respect to mechanical stresses. The distribution of cytochrome oxidase is essentially unaffected by the treatment. So apparently is the special 1.13 peak of α-mannosidase.

The effect of Triton WR-1339 is further illus-

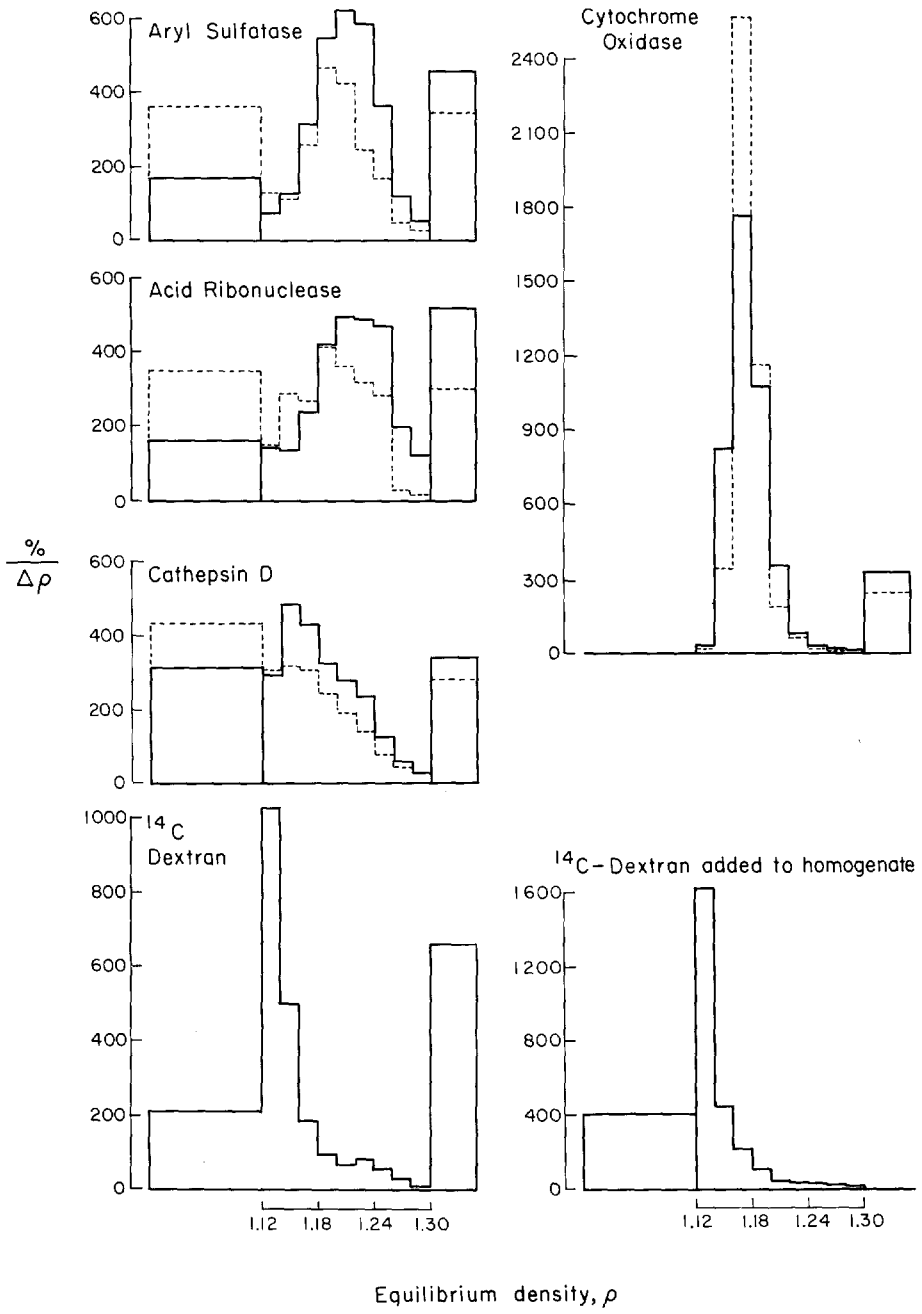

FIGURE 3 Influence of a single injection of dextran-^{14}C (40 mg of dextran-500/100 g body weight), given 1½ days previously, on the density-distribution patterns of rat-spleen enzymes. Dotted lines show normal distributions (4). Lower left-hand diagram represents distribution of ^{14}C in the same experiment. Lower right-hand diagram refers to a separate experiment performed identically on a normal rat-spleen homogenate to which dextran-^{14}C had been added.

trated in Fig. 2, which shows that the density shift of the particles, although perhaps not their mechanical fragility, already reaches a maximum 1 day after injection. This contrasts with the findings made on liver where about 4 days are required for maximum effect (39,40).

Influence of Injection of Dextran

In Fig. 3 are shown the results obtained on the spleen of rats killed 1½ days after receiving a single injection of dextran-^{14}C. Fig. 4 gives the results of a more complete experiment carried out on animals given four injections of dextran at daily intervals and killed 1 day after the last injection, which was made with dextran-^{14}C. These treatments do not significantly affect the distribution of cytochrome oxidase, but modify markedly the behavior of the hydrolases upon fractionation. Most prominent is a considerable decrease in the amount of soluble activity remaining on the top of the gradient, with a corresponding increase in particulate activity occurring both in the dense

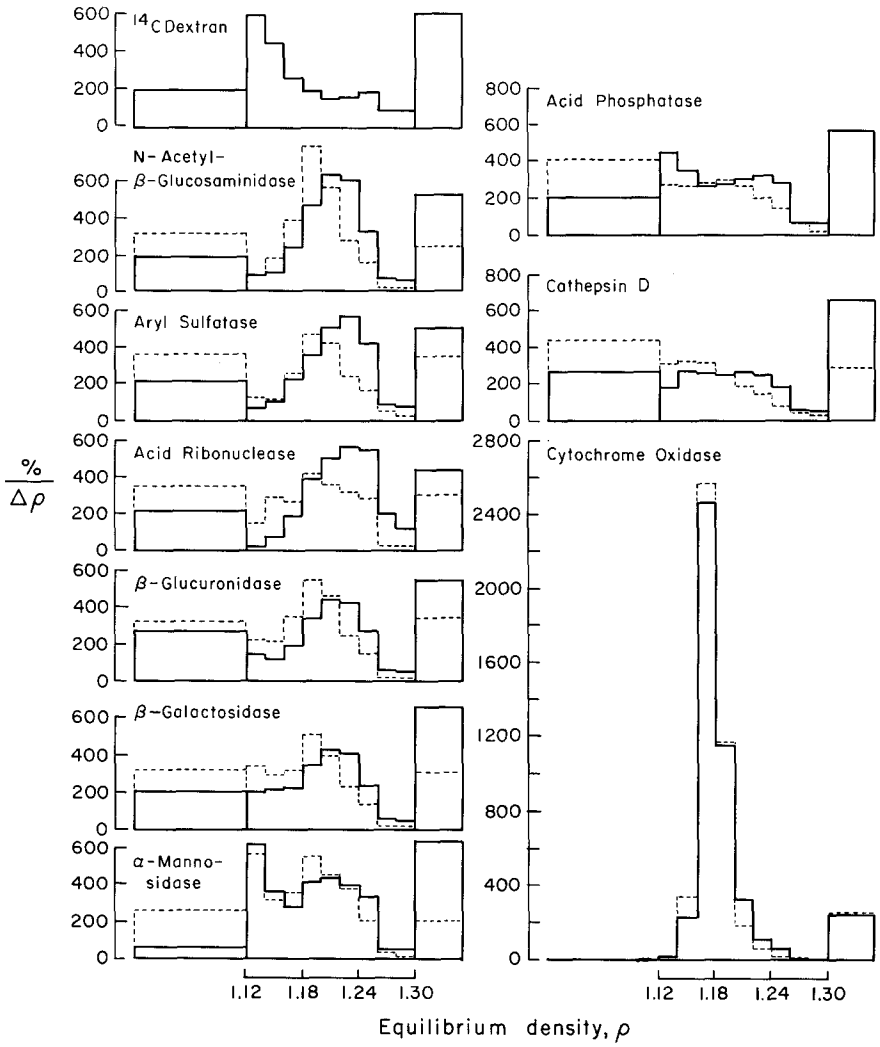

FIGURE 4 Influence of repeated dextran injections on the density-distribution patterns of rat-spleen enzymes. The animals were injected daily for 4 days with an equal mixture of dextran-10 and of dextran-500 (60 mg/100 g body weight) and were killed 1 day after the last injection which, alone, was made with dextran-^{14}C. Dotted lines show normal distributions (4). Upper left-hand diagram represents distribution of ^{14}C in the same experiment.

sediment (L_{30} population) and in the body of the gradient. In addition, all hydrolases exhibit a shift of their density distribution toward higher densities. This shift is more pronounced for enzymes characteristic of the L_{19} population, and least so for cathepsin D and acid phosphatase, which contribute most to the L_{15} population. There is little indication that repeated injections produce greater effects than a single injection, except in the case of cathepsin D, which exhibits an almost bimodal distribution after several dextran injections. Again, the 1.13 α-mannosidase peak appears unaffected by the treatment.

As shown in Figs. 3 and 4, part of the injected ^{14}C counts was found in the layer above the gradient; the remainder formed two distinct peaks, one in the upper fractions and the other in the dense sediment. In order to ascertain the significance of this distribution, dextran-^{14}C was added to a normal rat-spleen homogenate and the mixture was subjected to isopycnic centrifugation in the usual manner. The resulting distribution of labeled material is illustrated in Fig. 3. Sedimentation of the layered macromolecules causes them to enter the upper part of the gradient, where they pile up into a sharp peak as a result of the steep rise in medium density and viscosity existing at the interface. It is evident that the same phenomenon takes place in the spleen homogenates from the animals injected with dextran. At least 50% of the label must belong to free dextran molecules, which are recovered partly in the upper layer and partly in the first peak, as in the control experiment. On the other hand, the second dense peak which is found only when the polysaccharide has been injected and which comprises some 30–35% of the total counts recovered in the homogenate, is not duplicated in the control experiment and must belong to material which either has become sufficiently aggregated to sediment right through the gradient, or has become incorporated within particles equilibrating in the bottom region. In any case, some in vivo process must be responsible for the appearance of this second peak.

Consideration of the dextran graphs of Figs. 3 and 4 suggests that more labeled material accumulates below the first peak within the body of the gradient when the polysaccharide has been injected to the animal than when it has been added to the homogenate. The difference is particularly marked in the experiment of Fig. 4 where the labeled dextran was injected after three preceding injections of unlabeled material. This indicates that some radioactivity is also associated with particles equilibrating within the gradient, especially if the animal has been preloaded with unlabeled dextran.

A few additional experiments were carried out in order to explore further the significance of the relatively specific association of ^{14}C counts with the dense fraction. Table I shows the results of an abbreviated fractionation by differential centrifugation performed on the spleen of a rat subjected to a single dextran-^{14}C injection 1 day previously. The nuclear fraction contains about the same proportion of ^{14}C counts and of aryl sulfatase as the dense fraction isolated by isopycnic centrifugation. So does the combined cytoplasmic particulate fraction M + L + P, which actually contains more counts than appeared to be associated with particles equilibrating within the body of the gradient in the experiment of Fig. 3. In Fig. 5 are depicted the results of an isopycnic fractionation experiment performed on a cytoplasmic extract freed from the nuclear fraction by a preliminary centrifugation. The dense fraction is practically devoid both of ^{14}C counts and of aryl sulfatase, indicating a clear relationship between this fraction and the nuclear fraction. A small ^{14}C peak coinciding with the aryl sulfatase peak in the body of the gradient reflects the association of a small amount of radioactive material with the L_{19} particles (which have been displaced to a modal density of 1.21 as a result of the injection of dextran). In this experiment, a duplicate of the first tube was centrifuged for 6 hr instead of 2½ hr. This resulted in no change in the aryl sulfatase distribution, demonstrating that true equilibrium was achieved in 2½ hr for the L_{19} particles. On the

TABLE I

Distribution of ^{14}C, Aryl Sulfatase, and DNA in Spleen Fractions from Rat Injected with Dextran-^{14}C

Fraction	Percentage of total recovered content		
	^{14}C counts	Aryl sulfatase	DNA
N	33.8	28.7	89.3
M + L + P	30.3	30.2	1.5
S	35.9	41.1	9.2

Abbreviated fractionation (4) of a spleen homogenate from a rat injected 1 day previously with 40 mg of dextran-^{14}C per 100 g body weight.

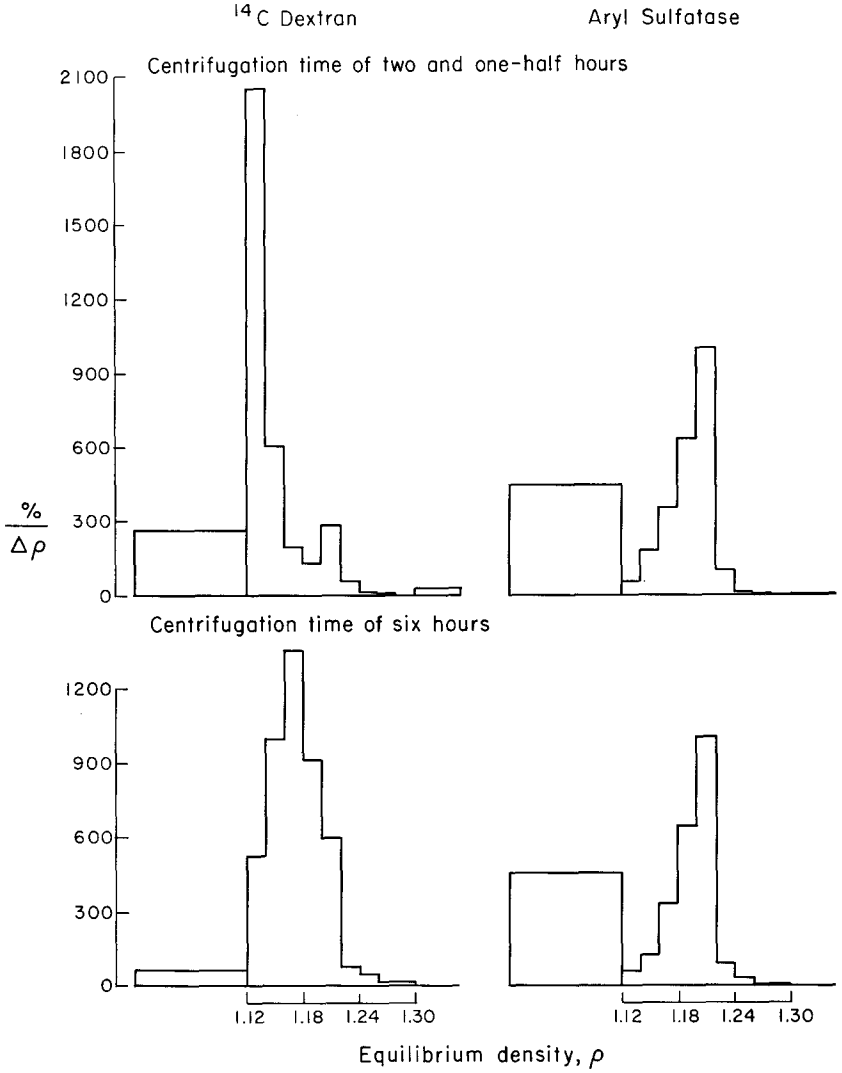

FIGURE 5 Distribution of ^{14}C and of aryl sulfatase, as recorded after centrifuging, for 2½ and for 6 hr, a cytoplasmic extract from the spleen of a rat injected 1 day previously with dextran-^{14}C (40 mg of dextran–500/100 g body weight), layered above a sucrose–0.2 M KCl gradient. Note that this experiment, unlike all the others, was carried out on a homogenate freed of the nuclear fraction by differential centrifugation (4).

other hand, the dextran peak has moved further down the tube, confirming our interpretation of this peak as a zone of sedimenting dextran molecules.

Influence of Injection of Erythrocytes

In Fig. 6 are reproduced the results of two experiments performed on the spleens of rats injected 1½ days previously with ^{51}Cr-labeled erythrocytes. The first experiment was done with autologous erythrocytes kept overnight in the presence of 0.5% of formaldehyde, the second one with freshly drawn mouse erythrocytes. Identical results were obtained in both cases. Like the administration of dextran, the injection of erythrocytes causes a decrease in the amount of hydrolases occurring in soluble form, but this decrease, far from being reflected in a corresponding increase in the activity of the dense fraction, is actually associated with a partial depletion of the fraction, at least as regards

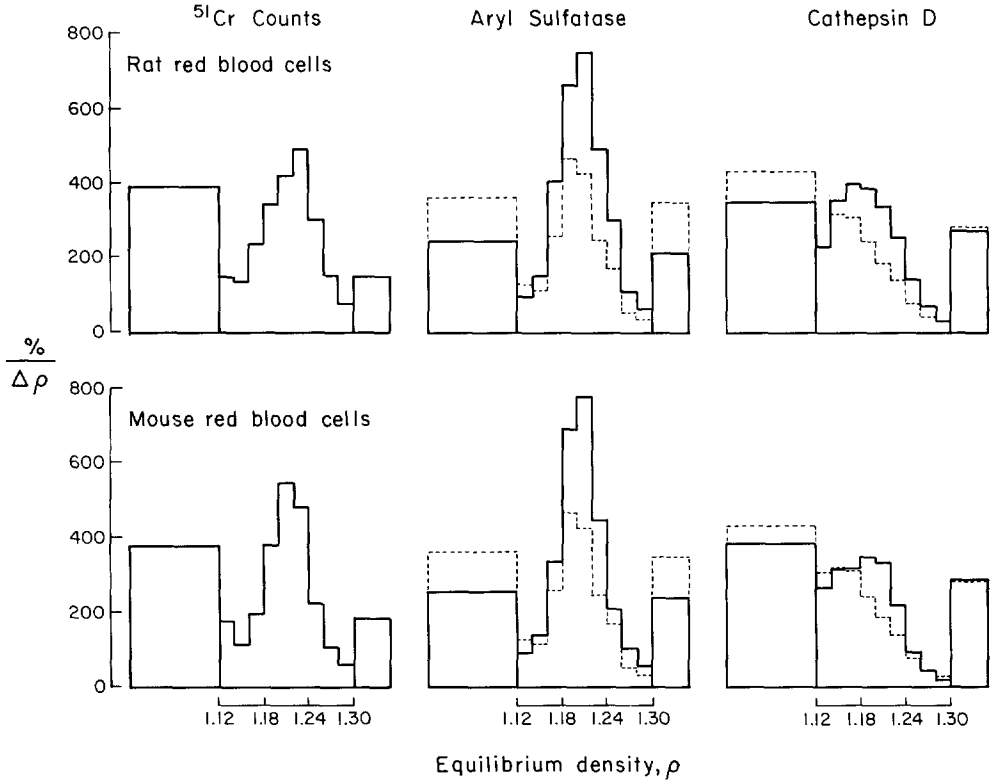

FIGURE 6 Influence of a single injection of ^{51}Cr-labeled erythrocytes, given 1½ days previously, on the density-distribution patterns of rat-spleen enzymes. Dotted lines show normal distributions (4). Distribution of ^{51}Cr is shown at left.

aryl sulfatase, and with a considerable increase in the amount of enzyme equilibrating in the body of the gradient. As after dextran, the density distribution of the hydrolases is displaced towards higher densities.

Of the ^{51}Cr counts, about 50% occurred in the soluble fraction; except for a small percentage which was associated with the dense fraction, the remainder equilibrated within the gradient, forming a distribution pattern closely similar to that of aryl sulfatase.

Influence of Injection of Cortisol

It has been reported by Bouma and Gruber (3) that whole-body X-irradiation of rats causes a selective loss of cathepsin D from the spleen, amounting to about 65% of the organ content. In contrast, cathepsins B and C were hardly affected by irradiation and their concentrations in the tissue rose proportionately to the reduction of its wet weight. Acid phosphatase behaved in an intermediate fashion, exhibiting a 15% loss and a moderate rise in concentration. These results were interpreted by the authors as indicating that a large fraction of the cathepsin D content of the spleen and possibly a smaller fraction of its acid phosphatase, but little or no cathepsin B or C activity, are associated specifically with radiosensitive cells (lymphocytes?). Our own observations having led to an almost identical conclusion with respect to the enzyme complement of L_{15} lysosomes, the results of Bouma and Gruber (3) suggested that the cathepsin D–rich L_{15} population may actually belong to cells of lymphocyte lineage. In order to test this hypothesis, we decided to use cortisol to cause a depletion of lymphocytes in lymphoid tissues.

As shown by the results gathered in Tables II and III, the effects of cortisol injections on the enzyme content of the tissues did not entirely duplicate the effects of X-irradiation. In spleen, a considerable loss of cathepsin D, associated with a

TABLE II

Influence of Cortisol Injections on Enzyme Content of Rat Spleen

Values given are means ± standard error. Figures in parentheses indicate number of experiments.

No. of injections*	Acid phosphatase			Aryl sulfatase			Cathepsin D			Spleen weight mg/100 g rat
	U/g wet wt.	U/g protein	U/100 g rat	U/g wet wt.	U/g protein	U/100 g rat	U/g wet wt.	U/g protein	U/100 g rat	
—	5.25 ± 0.15 (61)	33.0 ± 4.16 (6)	1.40 ± 0.18 (4)	1.64 ± 0.14 (20)	9.83 ± 1.14 (8)	0.45 ± 0.10 (11)	2.52 ± 0.24 (18)	18.3 ± 0.21 (8)	0.66 ± 0.02 (10)	240 ± 20 (14)
2	7.75 ± 0.98 (3)	42.8 ± 3.75 (3)	1.10 ± 0.08 (2)	2.02 ± 0.08 (3)	11.2 ± 1.20 (3)	0.30 ± 0.05 (4)	3.41 ± 0.11 (4)	18.2 ± 1.20 (4)	0.50 ± 0.04 (4)	150 ± 13 (4)
4	8.00 (1)	44.8 (1)	1.31 (1)	1.80 (1)	10.1 (1)	0.30 (1)	3.30 (1)	18.5 (1)	0.54 (1)	160 (1)
6	10.7 (1)	69.7 (1)	1.17 (1)	2.30 (1)	14.9 (1)	0.22 (1)	2.65 (1)	17.2 (1)	0.25 (1)	90 (1)
12	17.5 ± 2.21 (2)	112 ± 28.2 (2)	1.38 ± 0.39 (2)	2.88 ± 0.56 (2)	17.7 ± 1.23 (2)	0.24 ± 0.02 (2)	2.36 ± 0.21 (2)	14.7 ± 0.63 (2)	0.18 ± 0.01 (2)	80 ± 10 (2)

* Twice daily, 15 mg of cortisol/100 g body weight.

small decrease in specific activity, did eventually occur; but acid phosphatase was retained quantitatively, showing a more than 3-fold increase in specific activity, whereas aryl sulfatase, which might have been expected to be conserved like cathepsins B and C, suffered a partial loss. A somewhat similar situation was observed on thymus, where cortisol induced a greater loss of cathepsin

TABLE III

Influence of Cortisol Injections on Enzyme Content of Thymus and Lymph Nodes

Each line refers to a single experiment performed on the pooled organs from several rats.

Tissue	No. of injections*	Aryl sulfatase			Cathepsin D			Weight
		U/g wet wt.	U/g protein	U/100 g rat	U/g wet wt.	U/g protein	U/100 g rat	mg/100 g rat
Thymus	—	0.56	7.00	0.18	2.02	25.25	0.63	314
	2	0.96	9.14	0.09	3.10	29.52	0.29	94
	6	1.56	25.57	0.055	2.36	38.69	0.084	35
Lymph nodes	—	0.89	14.1	0.14	1.72	27.3	0.28	161
	9	1.23	53.5	0.02	2.62	113.9	0.043	17

* Twice daily, 15 mg of cortisol/100 g body weight.

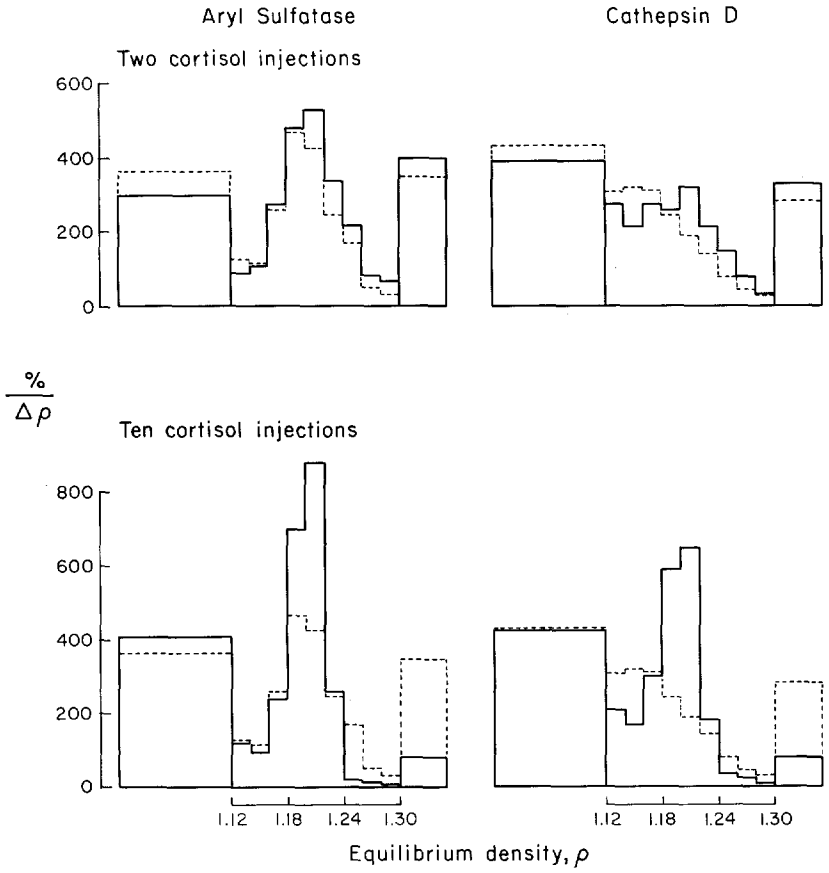

FIGURE 7 Influence of cortisol injections on density-distribution patterns of rat-spleen enzymes. Cortisol was given twice daily at dosage of 15 mg/100 g body weight. Dotted lines show normal distributions (4).

D than of aryl sulfatase, but an even greater reduction in organ weight and protein content, so that the specific activity of both enzymes was increased. In lymph nodes, the two enzymes appeared to show the same loss and the same increase in specific activity.

It seems likely that the differences between the effects of the two treatments are due to the fact that

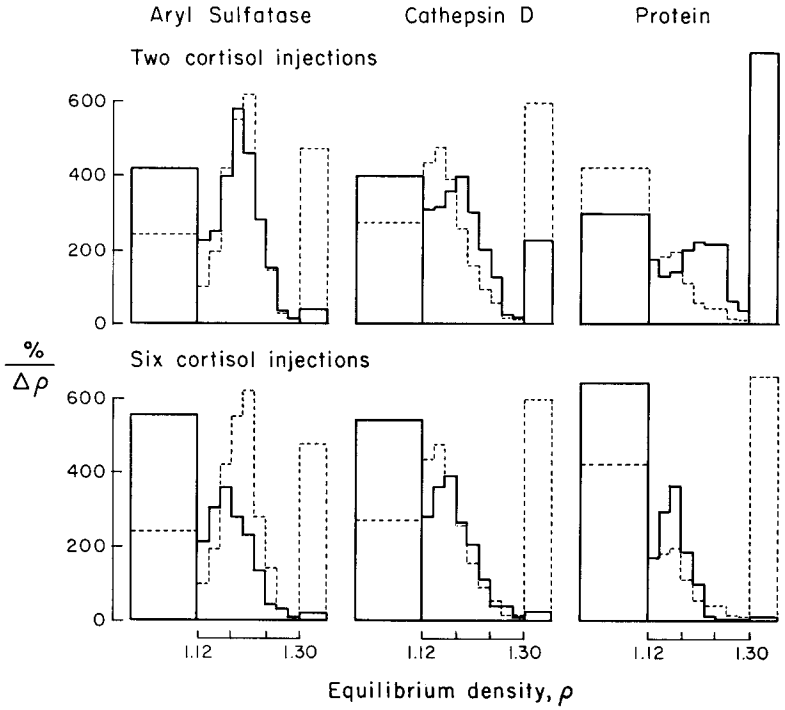

FIGURE 8 Influence of cortisol injections on density-distribution patterns of rat-thymus enzymes and protein. Cortisol was given twice daily at dosage of 15 mg/100 g body weight. Dotted lines show normal distributions (4).

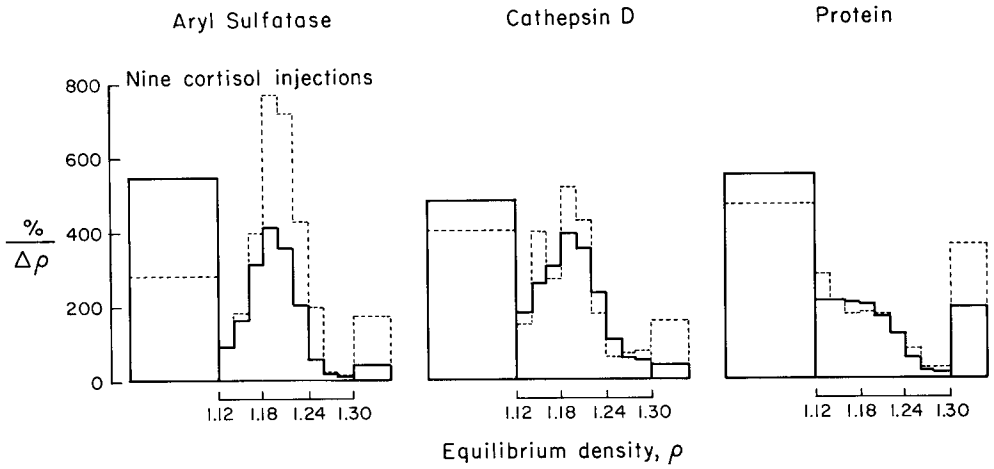

FIGURE 9 Influence of cortisol injections on density-distribution patterns of rat lymph-node enzymes and protein. Cortisol was given twice daily at dosage of 15 mg/100 g body weight. Dotted lines show normal distributions (4).

cortisol, in addition to being lympholytic, causes other cellular transformations that are not induced by X-rays. Nevertheless, there appeared little doubt, in view of the considerable reduction in weight suffered by the lymphoid tissues of the cortisol-treated rats, that the hormone did produce a widespread destruction of lymphocytes, and it seemed therefore of interest to examine the qualitative changes, if any, undergone by the lysosomes in these tissues. The results obtained are summarized in Figs. 7 to 9.

The main finding brought to light by these experiments is that, in all three lymphoid tissues, cortisol alters the distribution of cathepsin D, causing it to resemble that of aryl sulfatase. After 3 or more days of treatment with the steroid, the distribution patterns of the two enzymes are practically identical. It must be noted that the change affects mostly cathepsin D. Only in thymus, after repeated cortisol treatment, is a slight shift of the aryl sulfatase peak towards lower densities observed. Cathepsin D shares this displacement.

A second effect of cortisol is to empty almost completely the dense fraction of hydrolases. This phenomenon appears to take place more readily in thymus than in spleen. In thymus and lymph nodes, but not in spleen, cortisol caused a relative increase in the proportion of hydrolases occurring in soluble form.

DISCUSSION

The differential effects recorded in the present investigation support the existence of two and possibly three distinct populations of lysosomes in lymphoid tissues; they provide suggestive indications concerning the cellular location and functional properties of each of them. Table IV, which presents a qualified summary of our results, will serve as a guide to the following discussion.

The L_{19} Population

The particles in this group have all the properties of highly functional lysosomes. They contain a full complement of hydrolases and they accumulate considerable amounts of chromium-labeled erythrocytes (or hemoglobin), as shown by direct counts of ^{51}Cr, and of Triton WR-1339, as indicated by the marked decrease in their density following injection of this substance. Strangely enough, they contain relatively little ^{14}C-labeled material in the spleen of animals treated with dextran-^{14}C, especially after a single injection of the labeled polysaccharide. In considering these results, it is necessary to remember that the dextran

TABLE IV

Summary of Effects Observed on Spleen

Treatment	L_{15}	L_{19}	L_{30}	Soluble	α-Mannosidase (1.13 peak)
Triton WR-1339	Little affected	Somewhat decreased, density decreased	Decreased	Increased	Unaffected
Dextran-^{14}C	Little affected	Density increased, slightly labeled	Increased, heavily labeled	Decreased, heavily labeled	Unaffected
^{51}Cr erythrocytes	Little affected	Increased, density increased, heavily labeled	Unaffected or decreased, moderately labeled	Decreased, heavily labeled	—
Cortisol (repeated injections)	Decreased (also in thymus and lymph nodes)	Proportionately increased (decreased in thymus and lymph nodes)	Decreased (also in thymus and lymph nodes)	Increased or unaffected (greatly increased in thymus and lymph nodes)	—

used was labeled at the periphery of the molecule. If the particles should contain an active exodextranase, an enzyme which has been detected in rat intestine (11) and in hepatic lysosomes (P. Jacques, personal communication), this hydrolase could remove the outer glucosyl units which are the only ones to contain ^{14}C, leaving a residue which, though still quite bulky, would show little radioactivity. There are some indications that this might have taken place. Preliminary experiments have shown that spleen does contain an acid exodextranase and that part of the enzyme equilibrates in the L_{19} region. After injection of dextran, the L_{19} particles show a distinct increase in density, which could be due, as in liver, to storage of the dense polysaccharide. However, this argument is not conclusive, since injection of erythrocytes causes a similar density shift, the mechanism of which is not clear. Finally, the L_{19} particles seem to retain more ^{14}C when unlabeled polysaccharide has been injected several times previously, as would be expected if dextranase were responsible for their low content in ^{14}C.

Our results also indicate that the L_{19} particles represent the richest group of lysosomes in lymphoid tissues and that they form the main population present after repeated cortisol injections. The latter fact suggests that L_{19} lysosomes are associated with cells that are not destroyed by the steroid treatment. This view is consonant with numerous reports in the literature indicating that many acid hydrolases, with the exception of cathepsin D (3, 27), are retained in an essentially quantitative manner in the regressing lymphoid tissues of animals treated with adrenal glucocorticoids (37,42) or with X-rays (3, 14–16, 18, 22, 28, 32–36, 42). Our own results do not entirely bear out this point, since we have found a distinct loss of aryl sulfatase, a typical L_{19} enzyme, from all three lymphoid tissues after cortisol treatment. However, the loss in cathepsin D, which is only partly associated with the L_{19} population, was considerably greater, at least in spleen and thymus, and the change in the density-distribution pattern of this enzyme after cortisol treatment suggests strongly that the remainder is associated exclusively with L_{19} lysosomes.

Thus it appears, on the basis of these various arguments, that the L_{19} lysosomes belong to active phagocytes, resistant to cortisol and X-rays, in other words, to macrophages or related cells. This conclusion is supported by the cytochemical findings of Daems and Persijn (9) who have observed "erythrocyte fragments in different stages of digestion" together with ferritin-like granules in acid phosphatase–positive inclusion bodies present in mouse-spleen macrophages, and who further state that the same bodies store dextran or polyvinylpyrrolidone after injection of these substances. These observations are in keeping with our own results with ^{51}Cr-labeled erythrocytes and with our interpretation of dextran effects. Rahman (34) has taken advantage of the selective lympholytic effect of X-rays to separate a lysosome-rich fraction from rat thymus; the particles in this fraction, which are likely to be L_{19} lysosomes, have the structure of dense bodies loaded with ferritin-like material. However, in the final interpretation of our results, account must also be taken of the observations of Meijer and Willighagen (25), who report that dextran injected to mice was stored in spleen "in the cytoplasm of the reticulum cells surrounding the follicles, while only a few of the reticulum cells of the reaction centres of the follicles had stored." This point will be considered in relation to the significance of the L_{30} lysosomes.

The L_{15} Population

These are incomplete lysosomes, largely characterized by cathepsin D and by smaller relative amounts of acid phosphatase, β-galactosidase, and esterase, but either very poor in, or possibly devoid of, numerous other hydrolases, including cathepsins B and C, aryl sulfatase, *N*-acetyl-β-glucosaminidase, and the acid nucleases. Our density-gradient results indicate that this population either disappears or becomes undistinguishable from the L_{19} group after repeated cortisol injections. Supporting the former interpretation are the results of Bouma and Gruber (3) who have reported a selective loss of cathepsin D from the spleen following X-irradiation, in contrast with the numerous observations mentioned above, including their own, indicating that a number of other lysosomal enzymes are retained after this treatment.

Although less striking than those of Bouma and Gruber (3), our own data tend to confirm the greater sensitivity of cathepsin D to cortisol treatment, at least in spleen and thymus. A moderate loss of total cathepsin D, associated as in our results with a greater reduction in organ weight and therefore with an increase in specific activity, has been noted by Nagel and Willig (27) on the thymus of rats injected with prednisolone.

In view of these various results, it seems likely that the L_{15} lysosomes belong to lymphocytes. Their functional significance raises very interesting problems. According to our observations, they do not seem to participate significantly in the storage of injected foreign materials and are obviously ill suited for a role in intracellular digestion, since they can only carry out a very incomplete breakdown of proteins and other substances. Perhaps they are essentially dormant and functionally inactive, until a transforming stimulus triggers off the synthesis of a more complete complement of enzymes, as suggested by several recent reports (1, 19–21, 30). On the other hand, it is tempting to attribute some significance to the presence in them of an enzyme capable of causing a partial breakdown of proteins, apparently unaccompanied by the ancillary proteases required to pursue this breakdown further. It has been postulated that antibody formation may be preceded by a partial fragmentation of antigen molecules (23), and Franzl (17) has found that injected antigens persist longest in particles associated predominantly with the L fraction, in which L_{15} lysosomes also tend to be concentrated.

The L_{30} Population

The first question raised by the observations made on the dense fraction relates to the lysosomal nature of the hydrolase-containing bodies recovered in this fraction. We have been unable to separate these bodies from the nuclei and the most obvious interpretation of this failure is that the enzymes actually belong to the nuclei themselves. Arguing against this interpretation are the facts that injected materials are also found in the dense fraction and that its enzyme content is significantly altered by the injection of foreign substances. Such observations apply typically to lysosomes and there exists no evidence, either biochemical or morphological, that storage and digestion of foreign materials can occur in nuclei.

Granting that the L_{30} particles are true lysosomes, the question then arises whether they form a distinct population or are made up of L_{19} lysosomes which agglutinate with the nuclei. It is true that the L_{30} particles resemble the L_{19} lysosomes in many ways, both enzymatically and functionally. Also their high density suggests an artifact since lysosomes of such high density have never been encountered in another tissue. The L_{30} particles do differ from the L_{19} lysosomes in their apparent sensitivity to cortisol, but this does not necessarily disprove the artifact theory since cortisol depletes the lymphoid tissues of nuclei, the cause of the assumed artifact. Also arguing in favor of an agglutination artifact is the particular richness of the thymus in L_{30} lysosomes; the thymus has the largest proportion of nuclear material on a weight basis. However, there remain the preferential storage of dextran-^{14}C and the enrichment of the L_{30} population by dextran injection, as definite indications of a difference between the two groups.

If the L_{30} lysosomes belong to the L_{19} population, then it must be admitted that those L_{19} particles which store the largest amount of dextran or are least able to digest the polysaccharide either become dense enough to equilibrate below the limit of the gradient or are selectively carried down with the nuclei. The former possibility is not easily reconciled with the virtual absence of intermediary forms of density 1.26–1.30, even after repeated dextran injections (Fig. 4), but the latter cannot be excluded.

An alternative interpretation is that the L_{30} lysosomes belong to a special type of phagocytic cell, particularly active in storing dextran or relatively deficient in dextranase. As mentioned above, Meijer and Willighagen (25) have claimed that dextran is selectively stored in the reticulum cells surrounding the spleen follicles. Cells of a related type could be present in thymus and account for the relatively high content in L_{30} lysosomes of this organ. Unfortunately, no data, either morphological or biochemical, are available on dextran storage in thymus. If this theory is correct, the high-density position of the L_{30} lysosomes and their depletion following cortisol treatment still remain to be explained. An agglutination artifact could account for both facts and would be more easily understandable in this case, since it would affect the whole population rather than certain of its members. On the other hand, the L_{30} lysosomes could have an unusually high density, perhaps as a result of loading with some dense material such as hemosiderin, and their disappearance after repeated cortisol injections could be due either to a true cortisol sensitivity of their host cells, or to an increase in their mechanical fragility causing their enzymes to appear in the soluble fraction.

The Soluble Fraction

The enzyme levels in the soluble fraction were decreased after the injection of dextran and after that of chromium-tagged erythrocytes. The corresponding increase in particulate activity occurred

mostly in the L_{30} group in the former case, and in the L_{19} group in the latter. Thus it would appear that both populations contribute to the soluble fraction under normal conditions. Furthermore, in view of the high proportion of soluble cathepsin D, a contribution from the L_{15} particles must also be assumed.

Triton WR-1339 caused an increase in the amount of soluble activities, presumably due, as in liver, to the increased fragility of the swollen lysosomes filled with detergent. The increase in soluble activity found after cortisol treatment confirms earlier observations of Sachs et al. (37) on thymus and recalls similar findings on X-irradiated animals (29, 33, 35, 36). Its significance is unclear. It may reflect a true intracellular release of enzymes associated with autolysis, or the enhanced phagocytic activity of the macrophages, in particular, as mentioned above, of the cells containing the L_{30} lysosomes. Studies by Cohn and Wiener (7) have shown that the initiation of phagocytosis in isolated macrophages causes a release of lysosomal enzymes in soluble form. Strangely enough, the uptake of dextran and of erythrocytes (or hemoglobin) caused a decrease in the amount of soluble activities and seemed therefore to stabilize the particles against mechanical damage. This effect is not easily explained, especially when compared with the large proportion (about 50%) of both ^{14}C- and ^{51}Cr-labeled material found in the soluble fraction.

The 1.13 α-Mannosidase Peak

Neither the injection of Triton WR-1339 nor that of dextran appeared to have any influence on the 1.13 α-mannosidase peak. The significance of this peak and its possible relation to the low levels of soluble α-mannosidase observed by us (4) and especially by Levvy and Conchie (24) remain to be explored. It is hoped that the combination of morphological examination with the biochemical approaches opened by this work will help to solve this as well as the other unanswered questions that have been raised.

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