DISCUSSION

Compared with the procedures used previously to release bound hydrolases, the method described in this paper offers considerable advantages, in that it can be used with minimal quantities of material, requires no preliminary treatment of the granules and ensures complete activation without denaturation or inhibition of the enzymes. These advantages more than make up for the loss in accuracy which may affect some of the measurements, and justify the use of the method for routine determinations of total activity.

The results obtained on the graded release of the hydrolases may be added to the previous data, which indicated a parallel liberation of the five enzymes under the influence of a variety of agents (Gianetto & Duve, 1955; Duve *et al.* 1955). Such data form the main basis of the hypothesis that the enzymes may all belong to a single homogeneous population of granules. Since, however, the papers just mentioned contain evidence conflicting with this hypothesis, additional information would have been particularly welcome. It is unfortunate, therefore, that the conditions of the experiments did not allow a sufficient degree of accuracy to verify the significance of the finer differences which were occasionally observed. As mentioned in the Results section, these differences were not reproducible, and the fact that they occurred in the critical range of detergent concentration renders their significance doubtful. It must indeed be remembered that the granules were not in exactly the same environment during the assays, which, of necessity, had to be performed with different substrates. It is quite possible that these differences were sufficient to modify the degree of activation of such delicately balanced systems as must obtain in the neighbourhood of the critical detergent concentration. It may be significant in this respect that the two enzymes which were actually assayed together in the same mixture, namely acid phosphatase and ribonuclease, did show closely similar activities at all concentrations of detergent.

SUMMARY

1. The bound forms of acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin and β-glucuronidase were released quantitatively in preparations from rat liver exposed to 0·1 % (v/v) Triton X-100. This detergent had no inhibitory effect on the enzymes.

2. The above findings form the basis of a method whereby total activities of lysosomal enzymes can be assayed directly, without preliminary treatment of the liver preparations, simply by running the assays in the presence of 0·1 % (v/v) Triton X-100.

3. In mitochondrial preparations exposed to increasing concentrations of Triton X-100, the enzymes were released in a fairly abrupt fashion when the detergent concentration exceeded a certain critical level, which was about 0·035 % (v/v) for an amount of granules corresponding to 0·1 g. of original tissue/ml. All five enzymes were liberated in a roughly parallel manner, but the scattering of the results was such that finer differences could not be excluded.

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REFERENCES

Appelmans, F. & Duve, C. de (1955). *Biochem. J.* **59**, 426.
Appelmans, F., Wattiaux, R. & Duve, C. de (1955). *Biochem. J.* **59**, 438.
Duve, C. de, Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Gianetto, R. & Duve, C. de (1955). *Biochem. J.* **59**, 433.
Glassman, H. N. (1950). *Science*, **111**, 688.
Walker, P. G. (1952). *Biochem. J.* **51**, 223.
Walker, P. G. & Levvy, G. A. (1951). *Biochem. J.* **49**, 620.
Walker, P. G. & Levvy, G. A. (1953). *Biochem. J.* **54**, 56.

Tissue Fractionation Studies

8. CELLULAR LOCALIZATION OF BOUND ENZYMES

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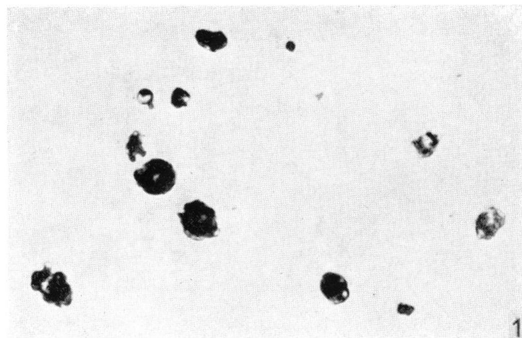
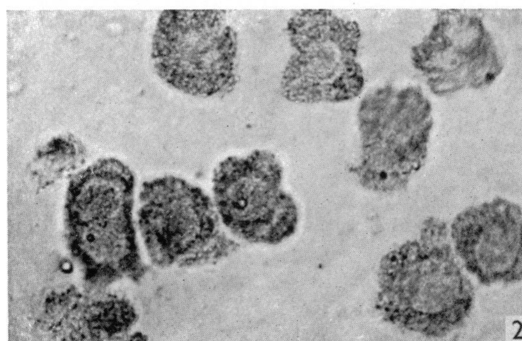
One of the difficulties in interpreting the results of tissue-fractionation experiments arises from the cellular heterogeneity of the starting material. In liver, accessory cell types represent only a small proportion of the total mass of the organ, but they could nevertheless be a source of complication if they should contain some specific systems. The recent identification of a special group of cytoplasmic granules (lysosomes), showing atypical distribution patterns and associated with only a small fraction of the total nitrogen (Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), has made





Microscopic appearance of isolated cells. Fresh unstained suspensions photographed in ordinary light (×400).
1, Reticulo-endothelial cell fraction; 2, parenchymatous cell fraction.

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it particularly desirable to obtain some information concerning the cellular location of bound enzymes and of their particulate support.

This problem has recently become accessible to experimental investigation, thanks to the work of St George, Friedman & Byers (1954), who have succeeded in separating reticulo-endothelial from parenchymatous cells, by combining the procedure described by Anderson (1953) for the preparation of whole-cell suspensions with the magnetic method originally used by Rous & Beard (1934) to isolate reticulo-endothelial cells loaded with iron from liver perfusates. Their method has been applied in the present work to ascertain the cellular origin of the hydrolytic enzymes belonging to the lysosome group, and of uricase, as well as of cytochrome oxidase and glucose 6-phosphatase, the representative enzymes for mitochondria and microsomes.

METHODS

The technique used for separating the cells consisted essentially of an adaptation of the procedure of St George *et al.* (1954). Rats were injected intravenously for 3 days consecutively with 1 ml. of a 15% (w/v) suspension of carbonyl iron (grade F, average diameter 3 μ.) in isotonic saline containing 5% (w/v) starch. The carbonyl iron was a gift from the Mond Nickel Co., London. The animals were killed 1 or more days after the last injection and the liver was perfused, first through the dorsal aorta and then backward through the hepatic vein, with an ice-cold solution of 0·25 M sucrose containing 0·01 M ethylenediaminetetraacetic acid neutralized to pH 7·4. As shown by Anderson (1953), the presence of a fairly concentrated calcium-binding agent in the perfusion fluid causes a softening of the intercellular cement and is essential for the isolation of intact cells. The subsequent fractionation was performed in 0·25 M sucrose containing 0·001 M ethylenediaminetetraacetic acid.

Tissue freed from blood, and greyish-black in colour, was forced through 0·7 mm. holes by means of a small hand-press (garlic press), and the resulting coarse brei was further dispersed by gentle stirring with two or three volumes of fluid. In two experiments the preparation was ground manually in a loose glass homogenizer, as recommended by Anderson (1953), but excessive cell breakage was observed and this procedure was abandoned. The suspension was then filtered under gentle pressure through silk cloth to remove undispersed tissue fragments, poured into a beaker and held above a powerful electromagnet placed vertically. The field developed by the magnet was regulated by means of a rheostat to be just sufficient to cause the cells containing iron to sediment. The supernatant was sucked off while the magnetic field was held on. The cells were resuspended and washed twice by means of the same separation method. The supernatants were combined, subjected to the maximal field developed by the magnet to remove traces of iron, and centrifuged at 700 *g*-min. The sediment of parenchymatous cells was washed twice under the same conditions. After microscopical examination, the cell suspensions were further ground and eventually fractionated in the manner described previously for whole liver (Duve *et al.* 1955). Iron was removed from the disrupted cell fractions by means of the magnet.

The microscopic appearance of the two cell fractions is shown in Plate 1. The reticulo-endothelial cells appeared as small bodies, filled with iron granules which obscured all details of internal structure. Some showed evidence of damage and could be seen shedding their iron content. The proportion of injured cells and of isolated iron granules appeared to depend on the size of the magnetic field used for the separation. The cells showed a great tendency to cluster together, and extensive agglutination occurred in the preparation as soon as washing was completed. The parenchymatous fraction formed a creamy suspension, consisting mostly of fairly well-preserved whole cells together with some nuclei and cell debris.

The methods used for the enzyme assays were essentially those of Duve *et al.* (1955), except that total activities of lysosomal enzymes were determined in the presence of 0·1% (v/v) Triton X-100, as described by Wattiaux & Duve (1956). This detergent was purchased from the Rohm and Haas Co., Philadelphia.

RESULTS

In Table 1 are summarized the main biochemical properties of the whole-cell suspensions before their fractionation by the magnetic method. The data obtained by Duve *et al.* (1955) on dispersions of unperfused whole liver are given for comparison.

About one-third of the total nitrogen was lost as the result of the removal of blood, connective tissue and unbroken liver fragments retained on the press and on the filtering cloth. The specific enzymic activities were usually somewhat higher in the cell suspensions than in the whole-liver dispersions, with the exception of deoxyribonuclease and β-glucuronidase. These were less abundant in the cell suspensions, though not significantly so.

The values observed for the distribution of nitrogen between the two cell fractions are shown in Table 2. Except in Expt. 3, where a distinct contamination by parenchymatous cells was observed microscopically, the isolated reticulo-endothelial cells accounted for 1·8–2·0% of the nitrogen present in the original suspension, irrespective of the method used for dispersing the tissue. In contrast, the recovery of parenchymatous cells was very low in the two cases when a loose glass homogenizer was used, and amounted to about 20% in the others.

In Table 3 are shown the detailed results of Expt. 5, which was the most reliable on every count. The distribution of all investigated enzymes paralleled roughly that of nitrogen, with the difference that the specific activities were decreased in the final supernatant and correspondingly increased in at least one of the cell fractions. The highest specific activity was found in the reticulo-endothelial cells for ribonuclease, deoxyribonuclease and cathepsin, in the parenchymatous cells for the other enzymes.

Table 1. *Biochemical properties of unfractionated cell suspensions*

Results refer to the properties of the filtered whole-cell suspensions obtained from the perfused livers of animals treated with iron, as analysed prior to their fractionation by the magnetic method.

Enzyme	Enzymic activities, units*/g. of N (±S.E.M.)	
	Cell suspensions (means of 5 experiments)	Dispersions of unperfused whole liver†
Cytochrome oxidase	1210±78	948±74 (17)
Acid phosphatase	195±21	187±13 (19)
Ribonuclease	129±35	83·6±5·6 (8)
Deoxyribonuclease	36·8±6·3	40·5±4·6 (6)
Cathepsin	60·3±5·4	45·2±4·1 (9)
β-Glucuronidase‡	17·4±3·7	24·2±1·7 (4)
Uricase	12·5±2·2	9·0±0·83 (5)
Glucose 6-phosphatase	591±98	565±38 (9)
mg. of N/g. of liver	21·2±0·7	32·3±0·25 (19)

* For definition of units, see Duve *et al.* (1955).
† Data from Duve *et al.* (1955); figures in parentheses refer to number of experiments.
‡ Uncorrected for inhibition by sucrose (approximately 28%).

Table 2. *Nitrogen content of isolated cell fractions*

Figures give the proportion of nitrogen of the original cell suspensions which was recovered in the two cell fractions. RE, Reticulo-endothelial cells; PA, parenchymatous cells.

Expt. no.	Percentage of N of original cell suspension		Remarks
	RE	PA	
1	1·8	18·7	—
2	2·0	5·5	Excessive cell breakage owing to use of glass homogenizer
3	4·8	6·8	Excessive cell breakage owing to use of glass homogenizer. RE cells contaminated by some PA cells
4	1·9	21·6	—
5	1·8	23·2	—

Table 3. *Distribution of enzymes amongst isolated cell fractions*

Results of Expt. 5. The starting material was a filtered cell suspension prepared from the perfused liver of a rat treated with iron. Reticulo-endothelial cells (RE) were separated and washed by means of the magnetic method. Parenchymatous cells (PA) were isolated from the residue by low-speed centrifuging; this left a supernatant containing broken cells. The three fractions and the original suspension were analysed for nitrogen and enzymic activities.

Enzyme	Percentage of activity of original cell suspension				Relative specific activity*			
	RE	PA	Supernatant	Recovery	RE	PA	Supernatant	Recovery
Nitrogen content	1·8	23·2	73·0	98·0	1·00	1·00	1·00	1·00
Cytochrome oxidase	1·2	32·6	56·0	89·8	0·67	1·41	0·77	0·92
Acid phosphatase	1·8	37·8	61·5	101·1	1·00	1·63	0·84	1·03
Ribonuclease	4·4	23·5	65·5	93·4	2·44	1·01	0·90	0·95
Deoxyribonuclease	3·6	23·5	70·5	97·6	2·00	1·01	0·97	1·00
Cathepsin	2·9	26·1	59·0	88·0	1·61	1·13	0·81	0·90
β-Glucuronidase	2·1	39·2	56·0	97·3	1·17	1·69	0·77	0·99
Uricase	1·8	45·5	47·3	94·6	1·00	1·96	0·65	0·97
Glucose 6-phosphatase	1·0	38·6	65·4	105·0	0·56	1·66	0·90	1·07

* Ratio: $\frac{\text{Percentage of activity}}{\text{Percentage of nitrogen}}$.

Table 4. *Relative enzymic activities of isolated cell fractions*

Mean results of Expts. 1–5. For experimental details and meaning of symbols, see Tables 2 and 3. The supernatant containing cell debris was analysed only in the last three experiments. All results are expressed in terms of the values obtained on the original unfractionated cell suspensions; *n* refers to the number of experiments.

	Relative specific activity* (±S.E.M.)				
Enzyme	RE $n=5$	PA $n=5$	Supernatant $n=3$	Recovery $n=3$	RE/PA $n=5$
Cytochrome oxidase	0·82±0·09	1·25±0·15	0·86±0·15	0·89±0·03	0·68±0·09
Acid phosphatase	0·96±0·16	1·48±0·11	0·90±0·05	0·97±0·07	0·64±0·07
Ribonuclease	2·50±0·64	0·96±0·13	0·98±0·04	0·97±0·06	2·62±0·60
Deoxyribonuclease†	1·74±0·25	0·79±0·08	0·93±0·04	0·94±0·09	2·19±0·33
Cathepsin	1·95±0·18	0·93±0·07	0·84±0·08	0·85±0·04	2·21±0·25
β-Glucuronidase	1·16±0·11	1·51±0·16	0·77±0·12	0·87±0·08	0·80±0·12
Uricase	0·95±0·23	1·47±0·26	0·73±0·15	0·93±0·09	0·63±0·10
Glucose 6-phosphatase‡	0·63±0·05	1·58±0·39	0·96±0·05	0·89±0·10	0·49±0·18

* Ratio: $\frac{\text{Percentage of activity}}{\text{Percentage of nitrogen}}$.

† Expt. 3 omitted, owing to very low recovery.

‡ Expt. 2 and 4 omitted, owing to excessive recoveries; see text.

Table 5. *Fractionation of purified parenchymatous cells*

Means of two experiments. Parenchymatous cells were isolated as described under Methods and further fractionated according to Duve *et al.* (1955). N, nuclear fraction; M, Heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

	Percentage of cytoplasmic extract + nuclear fraction					
	N	M	L	P	S	Recovery
Nitrogen	6·9	30·5	11·8	30·3	26·3	105·8
Cytochrome oxidase	0·6	55·5	14·8	2·1	0·3	73·3
Acid phosphatase	2·4	25·1	48·5	20·3	7·2	103·5
Ribonuclease	4·6	20·3	28·7	18·8	15·2	87·6
Deoxyribonuclease	4·8	26·1	40·7	9·2	9·2	90·0
Cathepsin	4·8	18·1	41·4	9·8	9·8	83·9
β-Glucuronidase	2·8	13·6	27·5	42·0	7·2	93·1
Uricase	2·7	26·9	48·0	11·7	5·7	95·0
Glucose 6-phosphatase	3·2	6·2	14·9	76·3	1·5	102·1

The other experiments, including those in which the yield of parenchymatous cells was low, furnished essentially comparable results, as is shown by the summarized data in Table 4. In two of them, however, the parenchymatous cells had abnormally high glucose 6-phosphatase activities, resulting in relative specific activities of 7·2 (Expt. 2) and 4·5 (Expt. 4), and associated with excessive overall recoveries. This phenomenon could be due to the removal of an endogenous inhibitor (Beaufay, Hers, Berthet & Duve, 1954), but it is puzzling that it did not occur systematically. The glucose 6-phosphatase values obtained in these two experiments were excluded from the statistical evaluations in Table 4, as were those recorded in Expt. 3 for deoxyribonuclease, which showed a very deficient recovery (55 %).

In two other experiments, purified parenchymatous cells were isolated and further fractionated according to the procedure of Duve *et al.* (1955). The observed distributions, which are given in Table 5, were comparable with those established previously on unperfused whole liver. Recoveries were deficient in some cases, especially for cytochrome oxidase, presumably as a result of the additional time and manipulations required.

DISCUSSION

As shown by the results of Table 1, neither the preliminary treatment of the animals with iron nor the removal of blood and connective tissue had any marked effect on the enzymic activities of the liver suspensions; nor had the further elimination of reticulo-endothelial cells, which obviously cannot have been the exclusive source of any of the investigated enzymes, for one would then have to assume that their recovery never exceeded 5 % and that they were contaminated by many times their weight of parenchymatous material (to account for their low specific activities). The microscopical examinations render these possibilities extremely unlikely. Since, in addition, the isolated parenchymatous cells retained considerable amounts of every measured enzyme and furnished typical distribution patterns upon further fractionation, it

appears safe to conclude that all eight enzymes, and consequently also the lysosomes, are true components of the parenchymatous cells of the liver.

The observed results do, however, bring to light a systematic difference between cytochrome oxidase, acid phosphatase, β-glucuronidase, uricase and glucose 6-phosphatase on one hand, and the two nucleases and cathepsin on the other. The former enzymes are regularly more concentrated in the parenchymatous cells than in the original suspension, probably owing to contamination by granules and soluble enzymes originating from damaged cells, possibly also as the result of leakage of inactive (soluble?) material from the parenchymatous cells. In contrast, there is, if anything, a decrease in the relative specific activity of the nucleases and cathepsin in the parenchymatous cells, and the probabilities are, in view of the observations made on the other enzymes, that this decrease would be even more marked if there had been no complicating artifact. These results suggest that parenchymatous cells are not the exclusive source of the hepatic nucleases and cathepsin, a conclusion which is further supported by the fact that these three enzymes are the only ones to be significantly concentrated in the reticulo-endothelial cells. Whether any of the other enzymes are actually present in these cells cannot be decided with certainty, for even a relatively small degree of contamination by parenchymatous elements could account for their observed activities.

In an earlier paper, attention has been called to the fact that the five lysosomal enzymes do not show identical distribution patterns (Duve *et al.* 1955). As pointed out in that paper, the observed discrepancies may be due to a variety of causes and do not necessarily indicate that lysosomes are enzymically heterogeneous or consist of several species. The present findings bring to light an additional complication, showing that the nucleases and cathepsin may have a dual cellular origin. The distribution patterns of these enzymes appear to follow that of acid phosphatase somewhat more closely in purified parenchymatous cells than in whole liver (Table 5). However, the final recoveries are not good enough to warrant a definite conclusion.

SUMMARY

1. Whole-cell suspensions were prepared from the perfused livers of rats injected with iron particles, and further fractionated into reticulo-endothelial cells containing iron, which were separated by means of a magnet, and parenchymatous cells, which were isolated by centrifuging. In two experiments, purified parenchymatous cells were further fractionated into one nuclear and four cytoplasmic fractions, according to the scheme of Duve *et al.* (1955). All fractions were analysed for nitrogen, cytochrome oxidase, acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin, β-glucuronidase, uricase and glucose 6-phosphatase.

2. All investigated enzymes were present in significant amounts in the parenchymatous cells and showed the same distribution amongst the fractions isolated from these cells as was observed previously on unperfused whole liver.

3. The reticulo-endothelial cell fraction also contained all the activities, but in this the possibility that some of the enzymes were derived from contaminating components of parenchymatous origin could not be ruled out with certainty. Least open to this objection were the results obtained on ribonuclease, deoxyribonuclease and cathepsin, the specific activity of which was increased in the reticulo-endothelial cell fraction.

4. Applying these results to the problem of the cellular origin of lysosomes, it was concluded that these particles belong essentially to the parenchymatous cells, but that some of their characteristic enzymes may also be present in the reticulo-endothelial cells, a possibility which could account, at least partly, for the apparent heterogeneity of lysosomes commented upon in an earlier paper.

REFERENCES

Anderson, N. G. (1953). *Science*, **117**, 627.

Beaufay, H., Hers, H. G., Berthet, J. & Duve, C. de (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1539.

Duve, C. de, Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.

Rous, P. & Beard, J. W. (1934). *J. exp. Med.* **59**, 577.

St George, S. S., Friedman, M. & Byers, S. O. (1954). *Science*, **120**, 463.

Wattiaux, R. & Duve, C. de (1956). *Biochem. J.* **63**, 606.