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Tissue Fractionation Studies

18. RESOLUTION OF MITOCHONDRIAL FRACTIONS FROM RAT LIVER INTO THREE DISTINCT POPULATIONS OF CYTOPLASMIC PARTICLES BY MEANS OF DENSITY EQUILIBRATION IN VARIOUS GRADIENTS

BY H. BEAUFAY, P. JACQUES, P. BAUDHUIN, O. Z. SELLINGER,* J. BERTHET AND C. DE DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

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Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959) described experiments in which mitochondrial fractions from rat liver were partly resolved into their components by density equilibration in sucrose gradients, with either H_2O or D_2O as solvent. The results obtained in these investigations provided additional confirmation of the existence of lysosomes as a separate group of particles, distinct from mitochondria, and suggested further that urate oxidase, though almost

* Present address: Tulane University, Department of Biochemistry, New Orleans, La., U.S.A.

indistinguishable from the lysosomal enzymes on the basis of sedimentation rate, may belong to yet another population of cytoplasmic particles.

These experiments suffered from the fact that the particles had to be exposed to media of very high osmolarity and to fairly severe osmotic shocks. A search was therefore made for a high-molecular-weight solute of suitable density, solubility and biochemical innocuity to allow the formation of practically iso-osmotic density gradients of any desired osmotic pressure, in which particulate preparations could be subfractionated by density equilibration without osmotic complications. After various trials, glycogen was found to answer the purpose in a satisfactory manner.

The present paper describes experiments in which mitochondrial fractions from rat liver were centrifuged in glycogen gradients, with, as solvent for the polysaccharide, sucrose solutions of several well-defined concentrations. Experiments were also performed in gradients of sucrose in H_2O or D_2O, as in the work of Beaufay *et al.* (1959), but with some technical modifications.

One of the main outcomes of the present investigations has been the demonstration that urate oxidase is indeed associated with a separate group of particles, which appear to be also the bearers of catalase and of D-amino acid oxidase. As shown by Baudhuin *et al.* (1964), catalase and D-amino acid oxidase show distribution patterns very similar to that of urate oxidase in conventional fractionation experiments, but their association with the lysosomes could not be excluded on the sole basis of the results available.

These new experiments have also made it possible to study the influence of sucrose concentration on the density of cytoplasmic particles and to test some of the theoretical predictions formulated by de Duve, Berthet & Beaufay (1959). This analysis has provided further confirmation of the existence of three distinct groups of cytoplasmic particles in the mitochondrial fractions under study.

Some of the results described in the present paper have already been reported (de Duve *et al.* 1960; de Duve, Beaufay & Baudhuin, 1963) and their theoretical implications have been discussed (Beaufay & Berthet, 1963; de Duve, 1963*a*).

MATERIALS AND METHODS

All experiments were performed on washed mitochondrial fractions from rat liver, corresponding essentially to the sum of fractions M and L of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The procedures followed with different sucrose media are given in Table 1. When D_2O was used as solvent in the gradient, the particles were prepared in sucrose–H_2O, washed once in a sucrose solution of the same concentration in D_2O and resuspended in this medium. The final suspensions contained the particles from 0·5 to 1·0 g. fresh wt. of liver tissue/ml.

Subfractionation by density-gradient centrifuging was carried out according to the method of Beaufay *et al.* (1959), with the equipment and accessories described by de Duve *et al.* (1959). To facilitate the description of the results, the various gradients used have been labelled by an abbreviation indicating the nature of the main solute (G for glycogen, S for sucrose, F for ficoll), the solvent (h for H_2O, d for D_2O) and, in parentheses, the approximate limits of sucrose concentration, expressed as mole fraction $(N) \times 10^3$, the most convenient unit since it is a direct measure of the osmotic pressure of the medium and is independent both of the temperature and of the nature of the solvent water.

The gradients were prepared with a twin-syringe machine (de Duve *et al.* 1959) delivering two solutions through a miniature mixing chamber into the tube, in such a manner as to cause the relative proportion of the light solution to vary linearly from 1 to 0, and that of the heavy solution from 0 to 1, from the meniscus to the bottom of the tube. Thus the gradient obtained is a linear one between the limits set by the two solutions. Three different methods were used to add the particles to the gradient. With the polymer gradients and some of the sucrose gradients, the particles were incorporated in equal amounts (corresponding to 0·12 g. fresh wt. of liver/ml. of final suspension) to the two solutions used to prepare the gradients; they were thus distributed homogeneously throughout the gradient at the beginning of centrifuging. In some experiments with sucrose gradients, the particles were layered in a suitable sucrose solution above the gradient or, exceptionally, below it. The gradient itself had a volume of approx. 5 ml. in all cases and the total amount of particles subjected to subfractionation corresponded to 0·6 g. fresh wt. of liver tissue.

In Table 2 is given the composition of the solutions used to prepare the gradients. The solutions were prepared by weighing appropriate amounts of solutes and water and heating in hermetically stoppered flasks immersed in a boiling-water bath. After being cooled, the solutions were

Table 1. *Procedure for isolation of particles*

Homogenization and separation of nuclear fraction were combined, as described by de Duve *et al.* (1955), the three centrifugings being performed under the conditions indicated with head no. 253 of the International model PR–2 centrifuge. The mitochondrial fraction was separated from the cytoplasmic extract and washed once, in rotor no. 40 of the Spinco model L preparative ultracentrifuge. The conditions of centrifuging are given in the composite unit *g*-min. (de Duve & Berthet, 1953).

Concn. of sucrose in medium		$10^{-3} \times$ Amount of centrifuging (*g*-min.)	
(M)	$(10^3 N)$	Nuclear fraction	Mitochondrial fraction
0·25	4·7	10 and twice 6	Twice 250*
0·50	10·0	12 and twice 10	Twice 500
0·78	16·4	18 and twice 12	Twice 750
1·00	22·4	25 and twice 15	Twice 1000

* When D_2O was used, the particles were washed in sucrose–D_2O at $800 \times 10^3 g$-min.

either used as such for preparing the gradients, or mixed with the required volume of a particle preparation suspended in a sucrose solution of known concentration. In the latter case, provision was made, in calculating the necessary weights of solutes and solvent, for the amounts of sucrose and water introduced with the particles. In these calculations it was assumed that the particles from 1 g. of liver occupy a volume of 0·25 cm.³ in 0·25 M-sucrose (N $4·7 \times 10^{-3}$), and exchange water and sucrose with the medium in the manner foreseen by Beaufay & Berthet (1963) for their theoretical model of rat-liver mitochondria, when exposed to a different sucrose concentration. Only minor corrections were introduced by the latter considerations. The amounts given in Table 2 include those taken to be added with the particles and thus represent in all cases, as accurately as can be known, the actual composition of the medium in which the particles were subfractionated.

The glycogen used for making the gradients was extracted from mussels (*Mytilus edulis*) and purified according to a slight modification of the procedure of Somogyi (1934). Its apparent specific volume, as determined with a pycnometer on 10% (w/w) solutions prepared from samples subjected to prolonged drying over P_2O_5, was 0·638 cm.³/g. Its acid content amounted to 0·6 μmole/g. in one sample (A) and to 6·75 μmoles/g. in the other (B). All solutions were carefully neutralized before use for gradient work. Other substances included in some gradients were ficoll (Holter & Møller, 1958), obtained from A.B. Pharmacia (Uppsala, Sweden), and polyethylene glycols of average molecular weight 6000 and 20000 purchased from L. Light and Co. Ltd. (Colnbrook, Bucks.) (not mentioned in Table 2).

The density values given in Table 2 for the sucrose solution, excluding all polymers (ρ_m), and for the complete medium, apply to the temperature of the experiment and were calculated on the assumption that H_2O, D_2O and glycogen occupy under all conditions apparent specific volumes of 1·000, 0·9047 and 0·638 cm.³/g. respectively. The apparent specific volume of sucrose was computed for each concentration from the density data listed by de Duve *et al.* (1959). It was further assumed that sucrose occupies the same apparent specific volume at a given concentration, whether with H_2O or D_2O as solvent and whatever the concentration of glycogen.

The gradients were usually prepared immediately before the experiment, at a temperature close to 0° with H_2O, and to 4° with D_2O. Centrifuging was carried out for 2½ hr. at 39000 rev./min. ($19·2 \times 10^6 g$-min.; $W = 1·5 \times 10^{11}$ rad.²/sec.), with the precautions described by de Duve *et al.* (1959), in the swinging-bucket rotor SW39 of the Spinco model L preparative ultracentrifuge. In many experiments the centrifuge used was equipped with a diffusion pump (model L–HV) and with a low-temperature water-vapour trap, which allowed better vacuum and a more rigorous control of the temperature.

After centrifuging, the tubes were cut with the slicer described by de Duve *et al.* (1959); each subfraction was carefully homogenized above the knife, collected as completely as possible by means of a Pasteur pipette and added to a tared tube. The latter was weighed again, eventually after removal of a droplet (10 μl.) for measurement of the density, to determine the weight of the subfraction. The height of each cut through the tube was ob-

Table 2. *Composition of solutions used for preparation of gradients*

Details are given in the text.

Gradient	Solvent	Solution	Sucrose (g./100 g. of water)	Sucrose ($10^3 N$)	Sucrose (ρ_m)	Glycogen* (g./100 g. of water)	Density
Gh(2·3)	H_2O	Light	4·4	2·3	1·017	10·1	1·050
		Heavy	4·4	2·3	1·017	32·8	1·110
Gh(4·7)	H_2O	Light	9·0	4·7	1·034	—	1·034
		Heavy	9·0	4·7	1·034	28·9	1·113
Gh(10)	H_2O	Light	19·2	10·0	1·068	—	1·068
		Heavy	19·2	10·0	1·068	30·6	1·142
Gh(16–19)	H_2O	Light	31·6	16·4	1·104	6·7	1·120
		Heavy	36·4	18·8	1·117	38·6	1·193
Gh(22)	H_2O	Light	43·5	22·4	1·134	—	1·134
		Heavy	43·5	22·4	1·134	33·7	1·196
Sh(30–58)	H_2O	Light	59·7	30·4	1·170	—	1·170
		Heavy	117·0	58·0	1·260	—	1·260
Sh(36–54)	H_2O	Light	70·2	35·6	1·190	—	1·190
		Heavy	109·1	54·3	1·250	—	1·250
Sd(16–31)	D_2O	Light	27·8	16·0	1·190	—	1·190
		Heavy	55·5	31·4	1·250	—	1·250
Sd(21–41)	D_2O	Light	36·1	20·7	1·210	—	1·210
		Heavy	72·8	40·8	1·279	—	1·279
Sd(27–56)	D_2O	Light	47·7	27·1	1·235	—	1·235
		Heavy	101·0	55·7	1·316	—	1·316
Fh(10)	H_2O	Light	19·2	10·0	1·068	—	1·068
		Heavy	19·2	10·0	1·068	Ficoll: 74·0	1·200

* Neutralized by including, for each gram of glycogen added, 0·6 and 6·75 μmoles of NaOH respectively in series A and B.

tained directly from the tube slicer (the screw that serves to lift the tube displaces it by exactly 0·5 mm./turn and the number of turns is recorded on a counting device attached to the instrument) and was verified with vernier calipers. After being weighed, the subfractions were diluted with a known volume of an ice-cold solution of pH 7·6, containing sodium hydrogen carbonate (1 mM), EDTA (1 mM) and Triton X-100 (0·01%), and analysed for their content in protein and various enzymes.

Catalase was measured according to the method of Baudhuin *et al.* (1964), and D-amino acid oxidase as described by these authors but with 0·025% of sulphaguanidine as antiseptic in the incubation medium. It was found that under these conditions incubation could be prolonged up to 20 hr. without change in the enzymic activity, thus allowing the assay of very small amounts of enzyme. β-Acetamidodeoxyglucosidase was assayed by an adaptation to long incubation times of the method used by Sellinger, Beaufay, Jacques, Doyen & de Duve (1960), with 0·01% of sodium thiomersalate (Eli Lilly and Co., Indianapolis, Ind., U.S.A.) as antiseptic. All other enzymes and total proteins were measured as described by Beaufay *et al.* (1959).

In many experiments, the density of each subfraction and of the solutions used to prepare the gradient was measured by a technique similar to that of Hvidt, Johansen, Lindestrøm-Lang & Vaslow (1954). A small droplet of the fraction was allowed to settle in a cold thermostatically controlled gradient of *o*-dichlorobenzene and light petroleum (b.p. 80–100°) and its position was read with a cathetometer. The gradient was calibrated with KBr solutions of known density.

CALCULATION AND PRESENTATION OF RESULTS

Composition of subfractions. The actual experimental values are: the inner cross-sectional area, A (cm.²), of the tube, the height, h (cm.), of each section through the tube, the weight, w (g.), of each subfraction, the volume, v (ml.), of diluent added to the subfraction, the average density, ρ, of the subfraction (either measured directly or estimated from the known shape of the gradient) and the various analytical results, C', expressed in mg. of protein or in units of enzyme activity/ml. of diluted subfraction. For the top subfraction, the height, h (cm.), is estimated by multiplying the volume, w/ρ, of the subfraction by the average ratio of height to volume for the three following subfractions. The height of the bottom subfraction, which includes the remainder of the fluid and the sediment, is corrected for the sphericity of the tube by subtracting one-third of the tube radius.

The final concentration, C, of the measured component in the undiluted subfraction is obtained from

$$C = C' \frac{w+\rho v}{w} \quad (1)$$

The amount, Q, of the measured component is calculated from

$$Q = ChA \quad (2)$$

The initial concentration, C_i, is given by

$$C_i = \frac{\Sigma Q}{AH} \quad (3)$$

in which H represents the total height, Σh, occupied in the tube by the gradient itself. When the material has been incorporated initially within the gradient, C_i has a direct physical meaning and corresponds to the initial concentration of the assayed component. When a layering technique has been used, C_i represents the concentration which the material would have if it were distributed homogeneously within the whole gradient, none being present in the superimposed layer.

This method of calculation is essentially that followed by Beaufay *et al.* (1959). It introduces only two implicit corrections; one is for the inevitable sampling losses and is brought about by using the theoretical volume, hA, rather than the measured volume, w/ρ, in eqn. (2). This correction rarely exceeds 10%. The other is for the analytical errors and for the changes in activity that the material may have suffered as a result of the experimental procedures; it is introduced in eqn. (3) by assuming that the sum of the activities of the subfractions is equal to the total activity of the material subjected to subfractionation. An estimate of the magnitude of this correction is provided by the recovery values, which are calculated as the percentage ratio of C_i to the concentration measured on a mixture containing equal volumes of the two particle suspensions which have served to make the gradient, or on a suitably diluted sample of the material layered, depending on the technique used.

Average recovery values for all the experiments mentioned in the present paper are listed in Table 3, together with their statistical dispersion. They make it clear that the

Table 3. *Average recoveries of measured constituents*

Experimental details are given in the text.

Constituent	No. of values	Percentage recovery		
		Mean	S.D.	S.E.M.
Protein	26	101·9	7·4	1·5
Cytochrome oxidase	18	98·7	16·5	3·9
Urate oxidase	19	96·7	15·1	3·5
Catalase	22	108·9	12·2	2·6
D-Amino acid oxidase	12	93·9	7·4	2·1
Acid phosphatase	25	103·6	8·7	1·7
Cathepsin	6	96·5	12·1	4·9
Acid ribonuclease	5	95·2	13·7	5·6
Acid deoxyribonuclease	23	102·8	10·4	2·2
β-Glucuronidase	6	101·7	14·4	5·9
β-Acetamidodeoxyglucosidase	3	104·5	3·8	2·2

calculations are not affected by any gross systematic error. Recoveries do not differ significantly from 100%, except for catalase and D-amino acid oxidase, the activities of which appear to be influenced slightly by subfractionation. However, the deviation is small and unlikely to affect the final significance of the results. In view of these findings it has not been deemed necessary to list individual recovery values.

Distribution patterns. The observed distributions are represented graphically by a succession of rectangles of base h and of height C/C_t, aligned along the abscissa in the order of the position of the subfractions in the tube from the meniscus (left) to the bottom (right). For the sake of uniformity, h is expressed as a percentage of H. The surface area of each rectangle is thus equal to $Q \times 100/\Sigma Q$, the percentage of total recovered activity present in the subfraction. By definition, the total area of each distribution pattern is equal to 100. In such diagrams the top and bottom fractions include material the density of which may lie beyond the limits of the gradient. To underline this fact the corresponding rectangles have been filled in the graphs. Whenever the average densities of the subfractions have also been measured these are plotted in a similar discontinuous manner.

This method of presentation, which differs somewhat from that adopted by Beaufay *et al.* (1959), has been chosen as providing the most factual and objective image of the experimental results. However, especially with greatly distorted gradients, it fails to provide one of the most useful pieces of information that can be derived from such experiments, namely the density-distribution curve of the assayed components. To obtain this it is necessary first to reconstruct the final shape of the gradient from the average density values. This is done graphically by fitting the smoothest possible continuous curve to the experimental points (for an example see Fig. 3). This curve is used to determine the limits of density between which each subfraction was isolated, as well as their difference, $\Delta\rho$. The average frequency of the component over the density range spanned is then obtained for each subfraction, by dividing the fractional amount present, $Q/\Sigma Q$, by $\Delta\rho$. A plot of the frequency against density gives the equilibrium-density distribution of the component.

In view of the irregularities of many of the distributions and of the relatively small number of cuts, these plots have also been made in histogram form. All the histograms constructed in this manner have a total area equal to unity (or less when subfractions falling beyond the density limits of the gradient have been left out), and the relative areas for each subfraction are the same in the two types of representation adopted. The only additional uncertainty introduced in the density distribution diagram is the value of $\Delta\rho$, which determines the two linear dimensions of the corresponding rectangles. This uncertainty is negligible as long as the gradient deviates but little from linearity, but may become relatively large in those regions where the gradient shows large changes in slope.

In some experiments with sucrose gradients in which density measurements were not performed, approximate density distributions were constructed from the values of Table 2 by assuming perfect linearity of the gradient.

Quantitative and comparative data. To answer the need for quantitative characterization, the median position of each component was evaluated as the abscissa value, expressed as a percentage of H, intersected by a vertical line dividing its distribution diagram into two halves of equal area. The median equilibrium density was similarly computed from the density-distribution histograms. In exceptional cases where distinctly bimodal distributions were observed, the modal position and modal equilibrium density were also estimated graphically.

Differences between two distributions recorded in the same experiment are expressed quantitatively by the differences in median position (or in median density) and by the area displacement, defined as the percentage of the total area not overlapped by the other distribution.

RESULTS

Glycogen gradients

Two main groups of experiments were carried out. They differ in the nature of the glycogen used and in a number of experimental details explained below. Series A, which forms the basis of the calculations reported by Beaufay & Berthet (1963), is the most recent one and served principally to localize catalase and D-amino acid oxidase. Series B represents an earlier group of experiments in which a larger number of acid hydrolases were studied. In addition to these two groups, several other experiments were carried out, especially with the aim of investigating the possible use of ficoll as a substitute for glycogen.

Series A. In this series, the particles were all isolated in 0·25M-sucrose, irrespective of the concentration of sucrose established in the gradient. The experimental results obtained are shown in Fig. 1. A significant distortion of the gradient takes place during centrifuging, owing to the sedimentation of glycogen. As would be expected, this phenomenon depends both on the viscosity of the medium and on the concentration of the glycogen. Consequently, it is greatest at lower sucrose concentrations and in the upper part of the tube, especially when the glycogen concentration on top of the gradient is zero. It has been estimated from the position of the boundary in gradient Gh(4·7) that the sample of glycogen used had an average molecular weight of about 900000.

With regard to the enzyme distributions, the most striking finding disclosed by the use of glycogen gradients is represented by the fairly high degree of dissociation that obtains between urate oxidase, catalase and D-amino acid oxidase on one hand, and acid phosphatase and acid deoxyribonuclease on the other. Whereas the former enzymes tend to accumulate in the upper regions of the gradients, the acid hydrolases are concentrated in lower regions and in the sediment. Cytochrome oxidase and the bulk of the proteins occupy an intermediary position, moving closer to that of the other oxidases as the sucrose concentration increases.

The graph of Fig. 1 also indicates that the various particles with which the assayed enzymes are associated increase in density as the sucrose concentration in the medium increases. Their density-distribution histograms are shown in Fig. 9; the corresponding median equilibrium density values are listed in Table 4.

Series B. In this series, the gradients were identical in composition with those of series A, but another sample of glycogen was used and the particles were prepared each time in a medium of the same sucrose concentration as was used in the gradient (Table 1); density measurements were not carried out. The main results are shown in Fig. 2.

The results obtained in this series differ to a relatively large extent from those recorded in series A. In the first three gradients of series B, most enzymes occupy a lower position than in the corresponding experiments of series A, but the situation is reversed in gradient Gh(22). This can

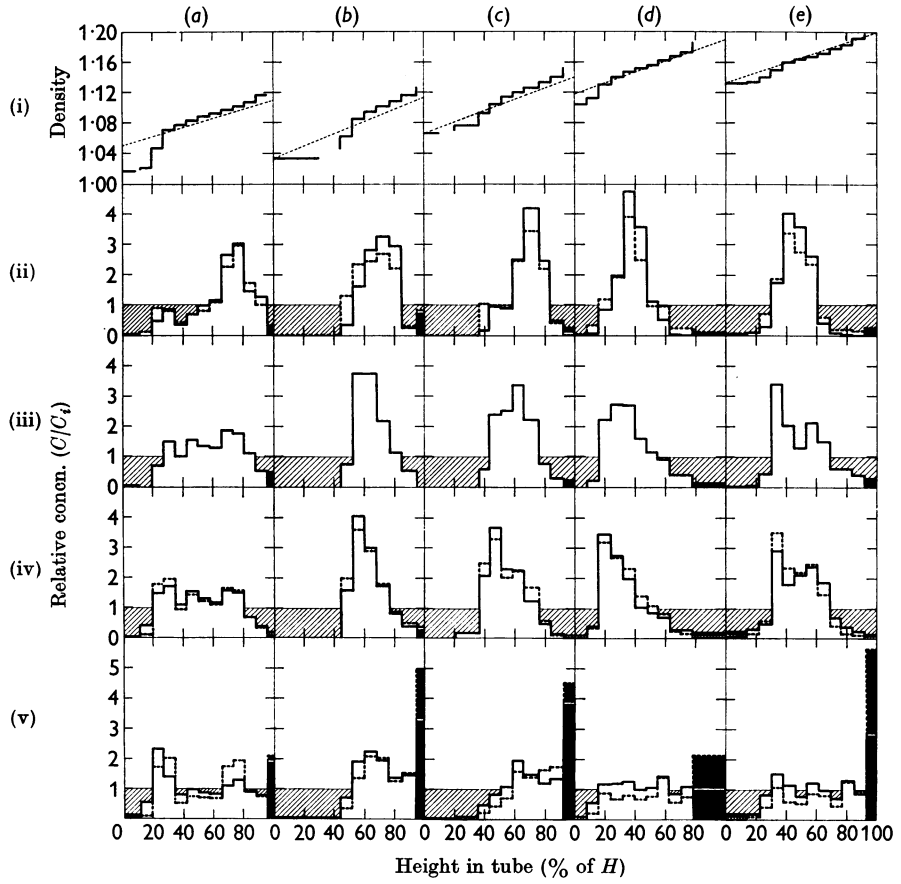

Fig. 1. Distribution patterns of enzymes after centrifuging in glycogen gradients (series A). Subfractions are represented on the abscissa scale in the order of their position in the tube, from meniscus (left) to bottom (right). (i) Measured average density of the subfractions (——) and the starting gradient (- - - - - -) as calculated from the data of Table 2. The other graphs show the relative concentration (C/C_i) of proteins and enzymes in each subfraction: (ii) cytochrome oxidase (——) and protein (- - - - -); (iii) urate oxidase; (iv) catalase (——) and D-amino acid oxidase (- - - - -); (v) acid phosphatase (——) and acid deoxyribonuclease (- - - - -). The gradients were: (*a*) Gh(2·3); (*b*) Gh(4·7); (*c*) Gh(10); (*d*) Gh(16–19); (*e*) Gh(22). Filled blocks (■) are used for the top and bottom subfractions to indicate that they include material falling beyond the limits of the gradient and cannot therefore serve for the estimation of density distributions. The horizontal line through ordinate 1 indicates the initial position of enzymes; free areas below this line have been shaded (▨) to allow better visual appreciation of the manner in which enzyme distributions have become reorganized during centrifuging. For further explanations, see the section on Calculation and Presentation of Results. Median equilibrium densities are given in Table 4, and median positions in Table 5.

Table 4. *Median equilibrium densities in glycogen gradients of series A*

Values were computed from the data of Fig. 1.

Gradient	Median equilibrium density						
	Protein	Cytochrome oxidase	Urate oxidase	Catalase	D-Amino acid oxidase	Acid phosphatase	Acid deoxyribonuclease
Gh(2·3)	1·098	1·098	1·092	1·087	1·087	1·087	1·094
Gh(4·7)	1·099	1·101	1·094	1·092	1·092	1·103	1·107
Gh(10)*	1·125	1·127	1·120	1·112	1·113	1·131	1·138
	(1·124)	(1·126)	(1·119)	(1·111)	(1·110)	(1·131)	(1·136)
Gh(16–19)	1·149	1·149	1·147	1·143	1·144	1·158	1·173
Gh(22)	1·162	1·162	1·163	1·163	1·161	1·171	1·189

* Values given in parentheses are averages of two or three experiments (Table 6).

already be seen by comparing Fig. 2 with Fig. 1, and is made obvious by the median positions listed in Table 5.

It is possible that the final gradients were different in the two series owing to differences in the rate of sedimentation of the two glycogens. Density values are not available in series B to test this point, but in one experiment of this series, Gh(4·7), the total reducing sugars released by acid hydrolysis were measured on the subfractions by the method of Nelson (1944), which gives identical values for glucose and fructose. The amounts of sucrose, glycogen and water present in the medium for each subfraction were evaluated from these results by a careful calculation that took into account the volume occupied by the particles (estimated from the assays of cytochrome oxidase), the weight ratio of the saccharides to their hydrolysis products and the fact that the mole fraction of sucrose was $4{\cdot}7 \times 10^{-3}$ throughout the gradient. Average densities were computed from these data and served to reconstruct the approximate shape of the final gradient. This is shown in Fig. 3, together with the shape of the final gradient in the corresponding experiment of series A, as reconstructed from the measured average densities. According to these estimates, one should indeed expect a certain shift towards the bottom of the tube of the activities collected between 47 and 75 % of the fluid column in gradient B, as compared with gradient A; but the shift should occur in the other direction between 75 and 100 %. Therefore a difference between the gradients cannot apparently account for the higher activities found in the bottom subfractions in the three first experiments of series B, and it must be concluded that the particles themselves had different density distributions in the two series. The factors responsible for this difference are not known. There are indications that the glycogen used in series B was of poorer quality than that used in series A. It appears to have been more polydisperse (Fig. 3) and its content in acidic groups was more than 10 times as high. As a result of the latter difference, the gradient of ionic strength ranged from a value near zero to about 2×10^{-3} in series B, as opposed to $1{\cdot}7 \times 10^{-4}$ in series A. The method of preparation of the particles introduces another variable, which, however, cannot be invoked to explain the differences observed in gradient Gh(4·7).

Whatever the reasons for the quantitative differences between the two series, the second group of experiments does confirm in a qualitative manner the main findings brought to light by series A and shows in addition that cathepsin, acid ribonuclease, β-glucuronidase and β-acetamidodeoxyglucosidase exhibit properties very similar to those of acid phosphatase and acid deoxyribonuclease. The dissociation in three groups containing, respectively, urate oxidase and catalase, cytochrome oxidase and the bulk of the proteins, and the acid hydrolases, shows up in a particularly clear-cut fashion in gradients Gh(10) and Gh(16–19).

Effect of ficoll and polyethylene glycols. The substance ficoll, introduced by Holter & Møller (1958), was tried in two experiments with gradient Fh(10). The gradient was essentially undisturbed by centrifuging and there was no evidence of a separation of the tube contents into two phases. Nevertheless, all enzymes and the bulk of the proteins were found to be concentrated about eightfold in a narrow band centred around a density value of 1·177 (Table 6).

To investigate this effect of ficoll further, the substance was included in Gh(10) gradients, at a roughly constant concentration of 5 % (5 g. added to 100 ml. of each of the two complete particle suspensions prepared as described in Table 2). As shown in Table 6, which also illustrates the degree of reproducibility that can be achieved with the same sample of glycogen, a slight but significant increase in equilibrium density was observed under those conditions for cytochrome oxidase, total protein and especially catalase, but not for acid phosphatase. Replacement of ficoll with polyethylene glycol of average molecular weight 6000

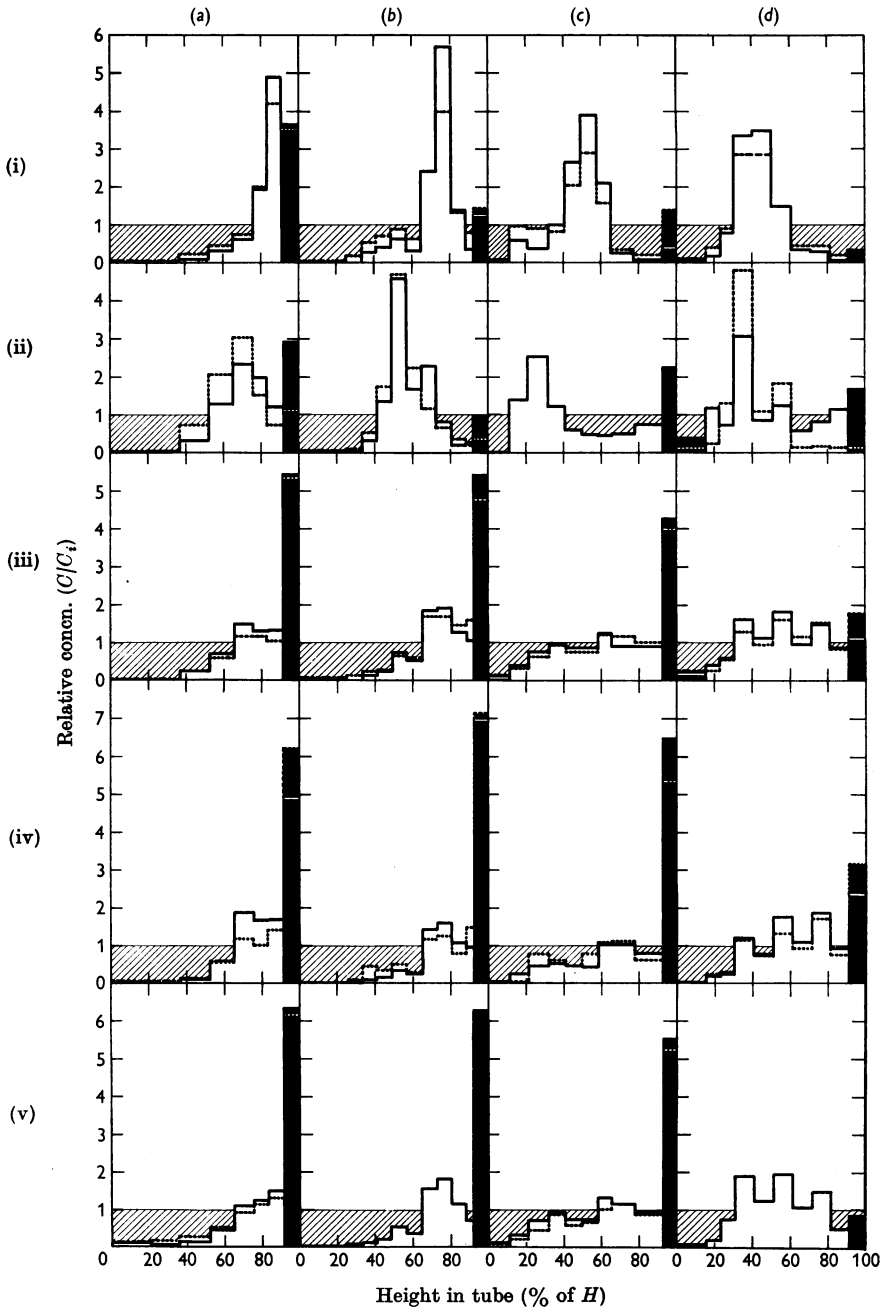

Fig. 2. Distribution patterns of enzymes after centrifuging in glycogen gradients (series B): (i) cytochrome oxidase (——) and protein (- - - - - -); (ii) urate oxidase (——) and catalase (- - - - - -); (iii) acid phosphatase (——) and cathepsin (- - - - - -); (iv) acid ribonuclease (——) and acid deoxyribonuclease (- - - - - -); (v) β-glucuronidase (——) and β-acetamidodeoxyglucosidase (- - - - - -). The gradients were: (*a*) Gh(4·7); (*b*) Gh(10); (*c*) Gh(16–19); (*d*) Gh(22). For explanation of the graphs see Fig. 1. Median positions are given in Table 5.

Table 5. *Median positions in glycogen gradients of series A and B*

Values were computed from the data of Figs. 1 and 2.

	Median position (% of *H*)											
	Protein		Cytochrome oxidase		Urate oxidase		Catalase		Acid phosphatase		Acid deoxyribonuclease	
Series ...	A	B	A	B	A	B	A	B	A	B	A	B
Gradient												
Gh(4·7)	67·7	86·7	71·4	87·6	63·7	76·3	60·9	68·4	73·3	90·9	78·4	87·8
Gh(10)	68·1	73·8	69·7	75·5	60·7	56·8	52·1	54·9	76·3	85·7	83·6	93·0
Gh(16–19)	37·6	51·0	37·8	51·3	34·9	38·3	30·0	—	54·1	72·4	74·5	90·2
Gh(22)	46·8	43·8	47·1	43·1	48·7	53·4	49·6	38·0	64·8	57·3	87·3	69·7

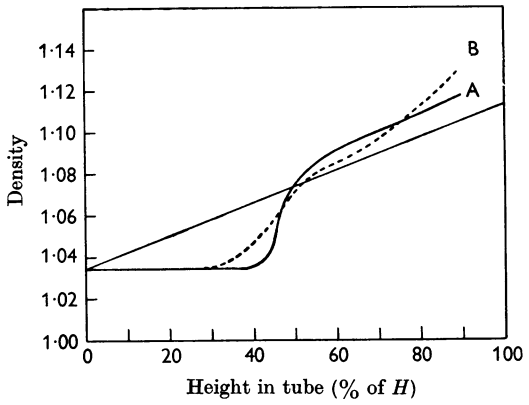

Fig. 3. Shape of final density gradient in iso-osmotic sucrose, Gh(4·7). ——, Series A, as reconstructed from average densities; - - - - - -, series B, as estimated from reducing sugars present after acid hydrolysis; the straight line indicates the initial gradient in both series.

caused a much smaller increase in median density (Table 6), indicating that, if degradation of the polymer was responsible for the observed changes, it must have led to a considerable decrease in average molecular weight. In a similar experiment performed with polyethylene glycol of average molecular weight 20000, the system broke into two phases of average density 1·08 and 1·17 and the particles were all concentrated at the interface.

Sucrose gradients

Sucrose–D_2O gradients. In view of the observation by Beaufay *et al.* (1959) that a particularly clear-cut dissociation between cytochrome oxidase and urate oxidase can be obtained in sucrose–D_2O gradients, their experiments were repeated with the object of determining the behaviour of catalase and of D-amino acid oxidase under the same conditions. Fig. 4 shows the results recorded in two such experiments.

The experiment in Fig. 4(*a*) is identical with that shown in Fig. 8 of Beaufay *et al.* (1959) and gives distributions for cytochrome oxidase, protein, urate oxidase and the acid hydrolases essentially similar to those seen previously. Catalase and D-amino acid oxidase resemble urate oxidase in being largely concentrated in the denser subfractions, though with a shift of their peak towards the less dense regions which is greater for D-amino acid oxidase than for catalase. To characterize the tail end of the distribution curves more completely, the second experiment was carried out over a range of higher density values (Fig. 4*b*). Though confirming the relative positions observed for the various enzymes in the preceding one, this experiment disclosed a general shift of the distribution curves towards the denser regions. Since this shift could possibly have been due to differences between the two particle preparations, a third experiment was performed with the same particle suspension, samples of which were equilibrated simultaneously in two different gradients. As clearly shown by the protein distributions depicted in Fig. 5, the main factor responsible for the observed shift is represented by the density range covered by the gradient.

To investigate this phenomenon further, samples of the same particle preparation were centrifuged simultaneously in identical gradients Sd(21–41), but starting from different positions. In one case, shown in Fig. 6(*a*), the particles were layered above the gradient in a sucrose–D_2O solution of mole fraction $4{\cdot}7 \times 10^{-3}$ and all moved centrifugally towards their equilibrium position; in another (Fig. 6*c*) they were layered below the gradient in a sucrose–D_2O solution of mole fraction $57{\cdot}8 \times 10^{-3}$ and moved centripetally; in the third (Fig. 6*b*) they were distributed homogeneously within the gradient and moved partly in both directions. When moving from an upper layer, the proteins formed an almost unimodal population, with a median equilibrium density of 1·229, and a modal density estimated at 1·227. After lower layering, their distribution was again almost unimodal, though somewhat more flattened than after upper layering, with a median equilibrium density of

Table 6. *Influence of polymer on median equilibrium densities*

Particles were isolated in 0·5 M-sucrose in Expt. 1, and in 0·25 M-sucrose in Expts. 2–4. All glycogen gradients were made with glycogen A. Additions were made to two solutions used for preparing gradient, in the ratio of 5 g. to 100 ml. of final particle suspension. Details are given in the text. Experiments bearing the same number were run simultaneously on samples of the same preparation of particles.

Expt. no.	Gradient	Addition	Median equilibrium density: Protein	Cytochrome oxidase	Urate oxidase	Catalase	D-Amino acid oxidase	Acid phosphatase	Acid deoxyribonuclease
1	Gh(10)	—	1·124	1·125	1·118	1·106	1·106	1·135	1·134
2*	Gh(10)	—	1·125	1·127	1·120	1·112	1·113	1·131	1·138
3	Gh(10)	—	1·123	—	—	1·114	—	1·128	—
3	Gh(10)	Ficoll	1·131	—	—	1·126	—	1·132	—
4	Gh(10)	Ficoll	1·136	1·136	—	1·128	—	1·135	—
4	Gh(10)	Polyethylene glycol†	—	1·128	—	1·118	—	1·132	—
3	Fh(10)	—	1·178	—	—	1·177	—	1·177	—

* Expt. of Fig. 1 (Table 4). † Average mol.wt. 6000.

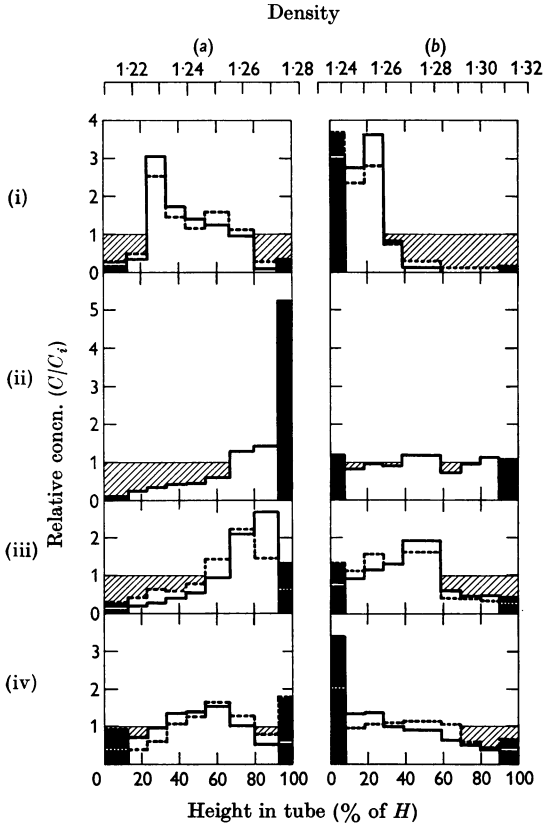

Fig. 4. Distribution patterns of enzymes after centrifuging in sucrose–D_2O gradients: (i) cytochrome oxidase (——) and protein (- - - - -); (ii) urate oxidase; (iii) catalase (——) and D-amino acid oxidase (- - - - -); (iv) acid phosphatase (——) and acid deoxyribonuclease (- - - - -). (*a*) Gradient Sd(21–41) (Expt. 1 of Table 7); (*b*) gradient Sd(27–56) (Expt. 2 of Table 7). For explanation of the graphs see Fig. 1. The density scale is based on calculated values (Table 2).

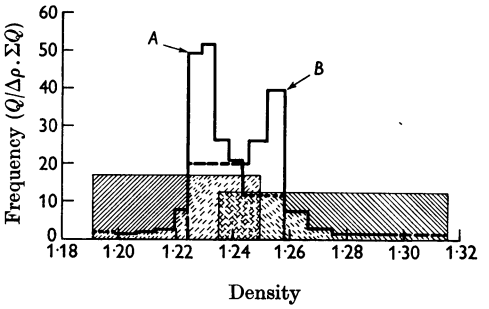

Fig. 5. Density-distribution histograms of proteins (Expt. 3 of Table 7), with samples of the same preparation of particles equilibrated in: *A*, gradient Sd(16–31); *B*, gradient Sd(27–56). Distributions are represented on a single density scale computed from the data of Table 2 on the assumption of perfect linearity of the gradients. The thin lines limiting shaded areas (▨, ▨) represent initial distribution of particles (ΣQ/total $\Delta\rho$). Histograms are completed on both sides by means of broken lines covering density limits ($\Delta\rho$) selected arbitrarily for ease of comparison. Rectangular areas delimited by these broken lines represent to scale the relative amounts of protein recovered in the top or bottom subfractions of each gradient. For further explanations see the section on Calculation and Presentation of Results.

1·248, very close to the modal density. The two modes are clearly represented when the particles have been homogeneously distributed within the gradient; the median equilibrium density falls between the two modal values, at 1·237.

It appears from these various results that the main component of the preparation, obviously represented by the mitochondria, has two distinct isopycnic points in sucrose–D_2O solutions, one at a density value of about 1·227, corresponding to a mole fraction of 25×10^{-3}, and the other at a density value of about 1·248, corresponding to a

mole fraction of 31×10^{-3}. As further illustrated by the median equilibrium density values listed in Table 7, the proportion of particles that eventually come to rest at each position depends essentially on the density range spanned by the gradient and on the initial position of the particles.

The existence of two modes has not been clearly observed for any of the non-mitochondrial enzymes assayed. As shown by the results of Table 7, they all tend to show a shift in median equilibrium density comparable with that of the mitochondria, but it must be remembered that, with the exception of urate oxidase, for which too few values are available, they are all liable to be released partly in soluble form. As pointed out below, this fact suffices to explain largely the observed displacements of the median equilibrium density.

Sucrose–H_2O gradients. In the investigations of Beaufay *et al.* (1959), very poor resolution was achieved by centrifuging particles initially distributed in a homogeneous fashion in a sucrose–H_2O gradient covering a density range from 1·19 to 1·25 (from mole fraction 36×10^{-3} to 54×10^{-3}). In view of the results described above, the possibility had to be considered that the conditions of this experiment favoured the equilibration of mitochondria at a second equilibrium position of higher density and that better resolution might be obtained by layering the particles above the gradient. That such is indeed the case is clearly shown by the results given in Fig. 7 and in Table 8. As in sucrose–D_2O gradients, the mitochondria, whether estimated by total protein or by cytochrome oxidase, appear to have two isopycnic points in sucrose–H_2O solu-

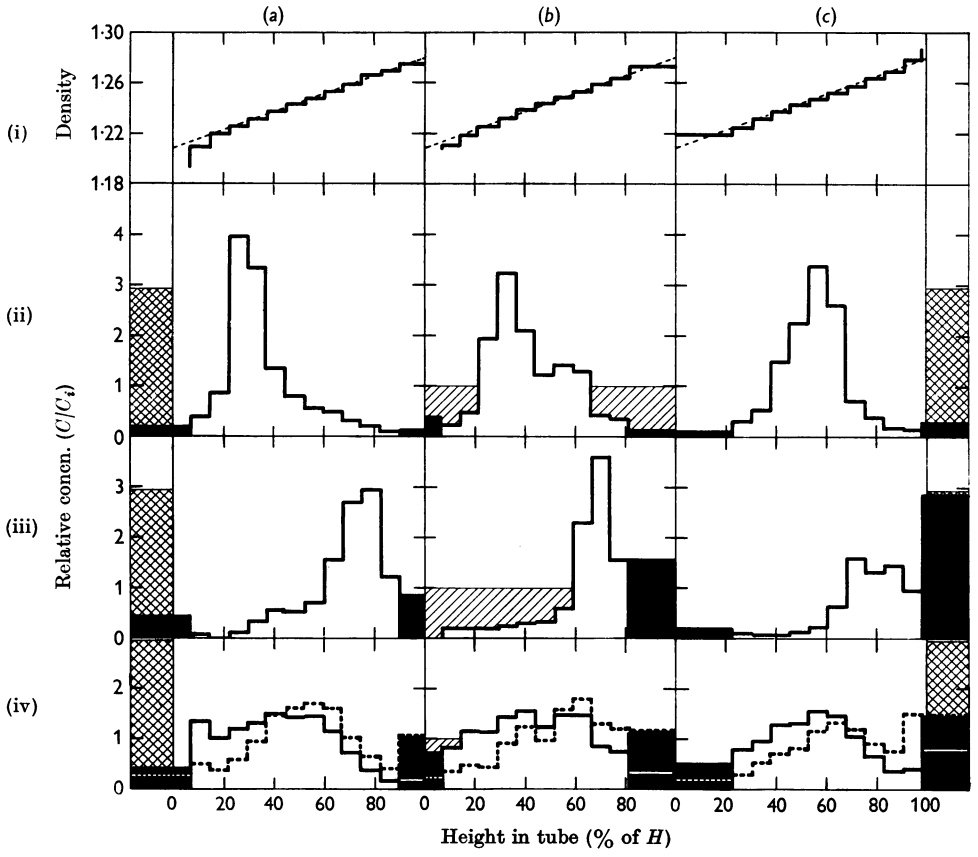

Fig. 6. Influence of initial position of particles on distribution patterns in sucrose–D_2O gradient: (i) measured average density of the subfractions (——) and the starting gradient (- - - - -); (ii) protein; (iii) catalase; (iv) acid phosphatase (——) and acid deoxyribonuclease (- - - - -). The gradient was Sd(21–41) (Expt. 5 of Table 7). Samples of the same preparation of particles were: (*a*) layered above the gradient in sucrose–D_2O (N $4{\cdot}7 \times 10^{-3}$); (*b*) incorporated homogeneously in the gradient; (*c*) layered below the gradient in sucrose–D_2O (N $57{\cdot}8 \times 10^{-3}$). In (*a*) and (*c*), the line above the cross-hatched area (▨) gives the initial concentration of particles at half-scale. The width of the layer is shown to scale. For further explanations see Fig. 1.

Table 7. *Median equilibrium densities in sucrose–D_2O gradients*

Experimental details are given in the text. Experiments bearing the same number were run simultaneously on samples of the same preparation of particles.

Expt. no.	Figure no.	Gradient	Initial position of particles*	Median equilibrium density: Protein	Cytochrome oxidase	Urate oxidase	Catalase	D-Amino acid oxidase	Acid phosphatase	Acid deoxyribonuclease
4	—	Sd(21–41)	Upper (4·7)	1·227	—	—	1·261	—	1·240	1·244
5	6(*a*)	Sd(21–41)	Upper (4·7)	1·229	—	—	1·259	—	1·236	1·245
3†	5(*a*)	Sd(16–31)	Homogeneous	1·232	—	—	—	—	—	—
1†	4(*a*)	Sd(21–41)	Homogeneous	1·241	1·238	1·270	1·263	1·257	1·244	1·252
4	—	Sd(21–41)	Homogeneous	1·239	—	—	1·253	—	1·241	1·247
5	6(*b*)	Sd(21–41)	Homogeneous	1·237	—	—	1·260	—	1·242	1·253
2†	4(*b*)	Sd(27–56)	Homogeneous	1·249	1·249	1·276	1·270	1·265	1·256	1·268
3†	5(*b*)	Sd(27–56)	Homogeneous	1·248	—	—	—	—	—	—
5	6(*c*)	Sd(21–41)	Lower (57·8)	1·248	—	—	1·279	—	1·248	1·262

* Values in parentheses indicate mole fractions ($\times 10^3$) of sucrose in layer containing the particles.
† Densities estimated on assumption of perfect linearity of gradient.

tions, at density values of about 1·19 and 1·22, corresponding to mole fractions of 36×10^{-3} and 45×10^{-3} respectively. Accordingly, their final distribution depends on the density range spanned by the gradient and on the initial position occupied by the particles.

The results of Table 8 provide no clear indications that urate oxidase may suffer a similar type of displacement. Considerable variability was observed for the other enzymes, especially for

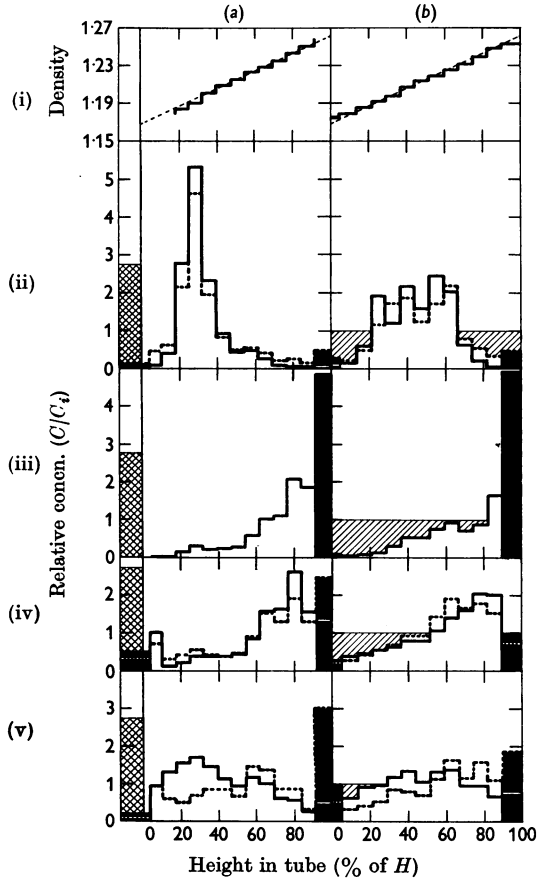

Fig. 7. Influence of initial position of particles on distribution patterns in sucrose–H_2O gradient: (i) measured average density of the subfractions (——) and the starting gradient (- - - - - -); (ii) cytochrome oxidase (——) and protein (- - - - - -); (iii) urate oxidase; (iv) catalase (——) and D-amino acid oxidase (- - - - - -); (v) acid phosphatase (——) and acid deoxyribonuclease (- - - - - -). The gradient was Sh(30–58) (Expt. 2 of Table 8). Samples of the same preparation of particles were: (*a*) layered above the gradient in sucrose–H_2O (N $4{\cdot}7 \times 10^{-3}$); (*b*) incorporated homogeneously in the gradient. In (*a*), the line above cross-hatched area (▨) gives the initial concentration of particles at one-third the scale. The width of the layer is shown to scale. For further explanations see Fig. 1.

Table 8. *Median equilibrium densities in sucrose–H_2O gradients*

Experimental details are given in the text. Experiments bearing the same number were run simultaneously on samples of the same preparation of particles.

Expt. no.	Figure no.	Gradient	Initial position of particles*	Median equilibrium density						
				Protein	Cytochrome oxidase	Urate oxidase	Catalase	D-Amino acid oxidase	Acid phosphatase	Acid deoxyribonuclease
2	7(*a*), 8(*b*)	Sh(30–58)	Upper (4·7)	1·192	1·192	1·250	1·234	1·234	1·203	1·227
3	8(*c*)	Sh(30–58)	Upper (25·8)	1·189	—	1·245	1·220	1·196	1·196	1·206
4	—	Sh(30–58)	Upper (25·8)	1·195	—	—	—	—	—	—
1	8(*a*)	Sh(36–54)	Upper (4·7)	—	<1·197	1·238	1·227	1·225	1·208	1·215
2	7(*b*)	Sh(30–58)	Homogeneous	1·215	1·212	>1·249	1·230	1·227	1·216	1·229

* Values in parentheses indicate mole fractions ($\times 10^3$) of sucrose in layer containing the particles.

catalase and D-amino acid oxidase. The main reason for this variability is revealed by the histograms of Fig. 8, which illustrate three different layering experiments. In all three experiments, the top subfraction, which includes the layer where the particles were originally deposited and the interface between this layer and the gradient, contains practically no urate oxidase, but retains considerable amounts of catalase and D-amino acid oxidase. This difference is largely made up by a corresponding excess of urate oxidase over the other two enzymes in the bottom subfraction. In between, all three enzymes show almost identical distributions. Also, the amounts of catalase and D-amino acid oxidase are much greater in the top subfraction and correspondingly smaller in the bottom subfraction when the mole fraction of sucrose in the upper layer is $25{\cdot}8 \times 10^{-3}$ than when it is $4{\cdot}7 \times 10^{-3}$ (Fig. 8*c*), whereas the distribution of urate oxidase is hardly affected by the sucrose concentration of the upper layer. It is clear from the additional results shown in Table 9 that the presence of variable amounts of catalase and D-amino acid oxidase in the upper layer may be ascribed to a release of these enzymes in soluble form which is

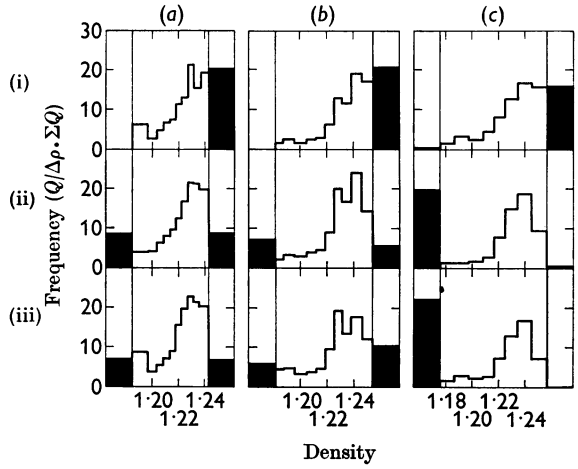

Fig. 8. Density-distribution histograms of enzymes in sucrose–H_2O gradients, with particles initially layered above gradient: (i) urate oxidase; (ii) catalase; (iii) D-amino acid oxidase. Filled blocks (■) represent to scale amounts present in top (including upper layer and interface) and bottom subfractions respectively over arbitrary abscissa values chosen equal in all cases to facilitate comparison. (*a*) Gradient Sh(36–54), mole fraction (N) of sucrose in upper layer = $4{\cdot}7 \times 10^{-3}$ (Expt. 1 of Table 8); (*b*) gradient Sh(30–58), mole fraction (N) of sucrose in upper layer = $4{\cdot}7 \times 10^{-3}$ [Expt. 2 of Table 8, pattern (*a*) of Fig. 7]; (*c*) gradient Sh(30–58), mole fraction (N) of sucrose in upper layer = $25{\cdot}8 \times 10^{-3}$ (Expt. 3 of Table 8). For further explanations see the section on Calculation and Presentation of Results.

Table 9. *Solubilization of catalase and* D-*amino acid oxidase in molar sucrose*

Samples of the same preparation of particles representing 0·8 g. fresh wt. of liver were resuspended in 5 ml. of 0·25 M-sucrose (N $4·7 \times 10^{-3}$) and of 1·0 M-sucrose (N $22·4 \times 10^{-3}$). Both tubes were centrifuged at $19·2 \times 10^6 g$-min. (as in the gradient experiments). Supernatant and sediment were separated and analysed.

$10^3 \times$ Concn. of sucrose (N)	Percentage of total amount recovered in supernatant			
	Protein	Urate oxidase	Catalase	D-Amino acid oxidase
4·7	2·0	0	10·9	3·9
22·4	4·0	0·2	24·5	16·8

enhanced by exposure of the particles to a high concentration of sucrose.

General view

In order to facilitate the discussion, the main results described above have been summarized in Fig. 9 and Table 10. Fig. 9 illustrates, with a number of representative experiments, the influence of the concentration of sucrose on the density distribution of the main enzymes studied, together with the correlations and dissociations that have been brought to light. In Table 10, the latter relationships have been expressed quantitatively in terms of area displacement and of displacement of median position. In view of the variety of conditions investigated and of the unequal number of experiments in each group, the average values listed in this Table are meaningful only if contained within sufficiently narrow limits. However, they have been given systematically, since they may in some cases help to assess the significance of an extremum.

DISCUSSION

Localization of enzymes

The present investigations represent an attempt to apply in the field of tissue fractionation a familiar approach of chemical fractionation in general, which consists in varying systematically the properties of the medium in which a given type of physical separation is attempted. The rationale behind this approach is that, though two unlinked components may behave identically under a given set of conditions, they are less likely to do so consistently when one or more of the parameters conditioning their behaviour are varied systematically. Logically, the most meaningful results in experiments of this type are those that indicate separability, provided that artifacts can be ruled out, and also independence of behaviour of two components, as revealed for instance by a quantitative analysis of their response to changes in medium composition. Identity of behaviour is never entirely conclusive, but becomes increasingly significant the more numerous and varied are the conditions tried.

As elaborated by de Duve (1964), the application of these principles to tissue fractionation meets with greater difficulties than in ordinary chemical fractionation, owing to the greater risk of artifacts and, especially, to the lack of completely unambiguous criteria of identification for subcellular components. In the present investigations, as in previous work from this Laboratory, it is assumed that cytoplasmic particles are sufficiently homogeneous biochemically to allow meaningful extrapolation from enzymes to host particles. The limits of validity of this assumption, as also the possible complications introduced by artifacts, are examined in each case.

Cytochrome oxidase and protein. Little need be said about the correlation between these two constituents, which is only to be expected in a preparation of which the major particulate component is known to be the cytochrome oxidase-bearing mitochondria. In general, though their peaks coincide closely in all cases, the distribution of protein tends to be flatter than that of cytochrome oxidase, thus accounting for their observed area displacement (Table 10). In our opinion, the latter difference should not be interpreted as an indication of biochemical heterogeneity of the mitochondria, but rather as evidence that the preparations were contaminated fairly heavily with extraneous elements, mainly soluble proteins and microsomal material. From the ratio of cytochrome oxidase to protein in the purest subfractions, this contamination may be estimated at 15–30 % of the total protein content. It is somewhat higher than was encountered by Beaufay *et al.* (1959) and is explained by the fact that the particles were washed only once and that care was taken to avoid decantation losses leading to a biased sampling of the particle populations under study.

In view of these considerations, there seems to be no need to alter the opinion expressed by Beaufay *et al.* (1959) that the distribution of cytochrome oxidase reflects faithfully that of mitochondrial mass or protein and may serve as a valid reference standard against which to compare other enzymic distributions.

Acid hydrolases. The results obtained in glycogen gradients provide additional evidence of the

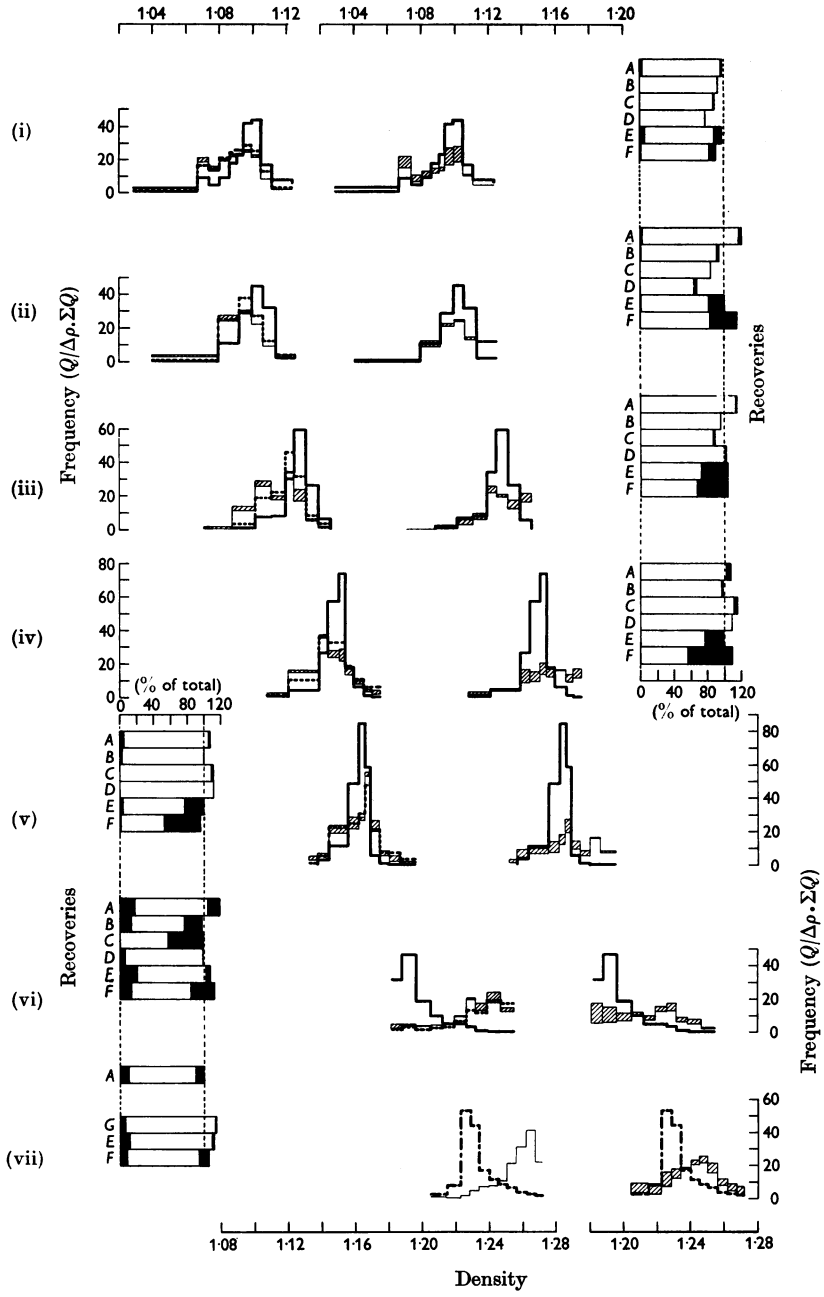

Fig. 9. Density-distribution histograms in different media. All histograms are constructed as explained in the section on Calculation and Presentation of Results. Block graphs at right or left of histograms represent percentage recoveries (total height) and, by means of filled-in areas (■), amounts found in top (left) and bottom (right) subfractions: *A*, catalase; *B*, D-amino acid oxidase; *C*, urate oxidase; *D*, cytochrome oxidase; *E*, acid phosphatase; *F*, acid deoxyribonuclease; *G*, protein. The results of each experiment have been represented in two separate graphs constructed side by side. Overlapping density scales for each diagonal row are drawn above and below the Figure. In (i)–(vi) the left-hand graphs show the distributions of urate oxidase (- - - - - -) and, without distinction, of catalase and D-amino acid oxidase (▨). In (i)–(vii) the right-hand graphs show, without distinction, the distributions of acid phosphatase and of acid deoxyribonuclease (▨). In (i)–(vi) the distribution of cytochrome oxidase (——) is represented in both graphs of each pair to facilitate comparison. In (vii) the distributions of protein (– - – -) and, in the left-hand graph, of catalase (——) are shown. (i) Gradient Gh(2·3); (ii) gradient Gh(4·7); (iii) gradient Gh(10); (iv) gradient Gh(16–19); (v) gradient Gh(22); (vi) gradient Sh(30–58); (vii) gradient Sd(21–41). The experiments represented are those of: (i)–(v) series A glycogen gradients; (vi) Expt. 2 of Table 8 (Figs. 7*a* and 8*b*); (vii) Expt. 5 of Table 7 (Fig. 6*a*).

Table 10. *Comparative survey of enzyme distributions*

Area displacements are calculated as explained in the section on Calculation and Presentation of Results. Displacements of median position, expressed as percentages of H, are given positively when enzyme in the left-hand column has a higher median equilibrium density than enzyme in the right-hand column, and negatively in the reverse case. Enzyme pairs are listed in each group in order of increasing average area displacement. Correlations believed to be significant are shown in **bold** print (see the Discussion section). Catal. = catalase; DAA.Ox. = D-amino acid oxidase; Prot. = protein; A.Pase = acid phosphatase; A.DNase = acid deoxyribonuclease; Ur.Ox. = urate oxidase; Cyt.Ox. = cytochrome oxidase; Cath. = cathepsin; β-Gase = β-glucuronidase; A.RNase = acid ribonuclease; β-N-Gase = β-acetamidodeoxyglucosidase.

10^3 × Range of sucrose concn. scanned (N)	Enzymes		No of comparisons	Percentage displacement: Area Minimum	Area Average	Area Maximum	Median position: Lower extreme	Median position: Average	Median position: Upper extreme
2·3–58·0	**Catal.**	**DAA.Ox.**	**12**	**5·1**	**9·7**	**20·9**	**− 1·3**	**+ 4·0**	**+23·5**
	Prot.	**Cyt.Ox.**	**17**	**8·1**	**14·0**	**20·4**	**− 3·7**	**− 0·2**	**+ 4·4**
	A.Pase	**A.DNase**	**22**	**8·0**	**18·2**	**30·6**	**−25·5**	**−12·3**	**+ 3·1**
	Ur.Ox.	**DAA.Ox.**	**12**	**9·6**	**24·8**	**45·0**	**+ 2·5**	**+15·1**	**+46·7**
	Ur.Ox.	**Catal.**	**15**	**11·0**	**25·0**	**43·0**	**− 0·9**	**+10·6**	**+23·2**
	Prot.	A.Pase	25	17·2	28·8	44·2	−22·7	− 7·2	+18·8
	Prot.	DAA.Ox.	11	21·5	33·5	58·4	−42·5	− 2·8	+19·6
	DAA.Ox.	A.DNase	12	15·2	34·0	59·1	−43·2	−12·9	+17·8
	DAA.Ox.	A.Pase	12	20·9	35·2	50·2	−30·5	+ 0·1	+35·4
	Catal.	A.DNase	20	15·3	37·5	70·1	−44·5	− 5·8	+24·1
	Ur.Ox.	A.DNase	17	15·3	38·8	63·2	−51·9	− 7·3	+41·2
	Prot.	A.DNase	21	20·8	39·3	62·9	−40·5	−20·8	+ 5·5
	Cyt.Ox.	A.Pase	21	26·0	40·0	57·0	−22·1	− 8·9	+19·0
	Prot.	Ur.Ox.	16	19·1	40·6	74·7	−56·9	− 9·7	+17·0
	Ur.Ox.	A.Pase	18	22·9	41·1	64·0	−34·1	+ 5·3	+52·4
	Catal.	A.Pase	25	20·7	41·3	64·5	−30·8	+ 2·1	+41·7
	Prot.	Catal.	23	21·9	43·1	71·3	−44·7	− 7·5	+19·4
	Cyt.Ox.	DAA.Ox.	11	32·0	45·4	73·1	−46·9	− 7·2	+19·8
	Cyt.Ox.	Ur.Ox.	17	24·5	47·8	83·8	−70·3	−11·2	+18·7
	Cyt.Ox.	Catal.	17	27·6	49·5	77·2	−49·6	− 1·1	+20·6
	Cyt.Ox.	A.DNase	16	29·2	49·9	69·4	−40·2	−21·3	+ 5·7
4·7–22·4	**A.Pase**	**Cath.**	**5**	**4·0**	**6·5**	**9·5**	**− 4·8**	**− 0·5**	**+ 1·6**
	A.Pase	**β-Gase**	**5**	**8·1**	**8·5**	**9·2**	**− 8·2**	**− 2·0**	**+ 6·8**
	A.RNase	**A.DNase**	**4**	**8·5**	**11·1**	**14·7**	**−13·8**	**− 1·0**	**+ 6·7**
	A.RNase	**β-Gase**	**4**	**3·5**	**11·5**	**25·6**	**− 4·2**	**+ 3·4**	**+17·6**
	Cath.	**β-Gase**	**5**	**10·0**	**12·0**	**14·0**	**− 7·8**	**+ 1·0**	**+ 7·7**
	A.DNase	**β-Gase**	**5**	**6·7**	**13·9**	**20·3**	**− 7·5**	**+ 4·4**	**+14·2**
	Cath.	**A.RNase**	**4**	**9·7**	**14·0**	**19·3**	**−11·0**	**− 7·0**	**− 3·6**
	Cath.	**A.DNase**	**5**	**12·6**	**14·7**	**18·8**	**−17·4**	**− 8·6**	**− 2·2**
	A.Pase	**A.RNase**	**4**	**9·0**	**14·8**	**20·5**	**−15·8**	**− 7·6**	**− 3·6**
	Ur.Ox.	Cath.	5	22·5	34·3	53·8	−34·5	−22·1	− 8·7
	Prot.	β-Gase	5	29·1	36·8	45·8	−29·6	−16·8	− 8·6
	Ur.Ox.	β-Gase	5	25·5	37·1	59·0	−42·3	−23·7	− 2·1
	Prot.	Cath.	5	32·4	38·9	42·0	−22·4	−15·2	− 3·3
	Ur.Ox.	A.RNase	4	27·7	39·5	60·5	−38·1	−28·0	−18·2
	Prot.	A.RNase	4	39·2	44·6	47·9	−29·3	−20·4	− 7·8
	Cyt.Ox.	β-Gase	5	38·1	45·5	59·2	−29·3	−16·2	− 7·7
	Catal.	Cath.	3	42·6	47·6	53·8	−29·2	−25·0	−21·6
	Cyt.Ox.	Cath.	5	38·9	48·6	54·7	−21·8	−14·7	− 2·4
	Catal.	β-Gase	3	35·2	49·1	59·0	−36·9	−27·1	−17·5
	Cyt.Ox.	A.RNase	4	46·4	52·3	55·7	−30·0	−19·8	− 6·9
	Catal.	A.RNase	3	52·4	57·5	64·0	−37·9	−33·0	−26·1
4·7–18·8	**β-Gase**	**β-N-Gase**	**2**	**5·5**	**6·0**	**6·4**	**+ 0·5**	**+ 0·7**	**+ 0·8**
	A.RNase	**β-N-Gase**	**2**	**5·9**	**6·7**	**7·5**	**− 3·7**	**− 1·9**	**0**
	Cath.	**β-N-Gase**	**2**	**9·3**	**9·6**	**9·9**	**− 7·3**	**− 5·9**	**− 4·5**
	A.Pase	**β-N-Gase**	**3**	**9·2**	**11·0**	**14·2**	**−18·4**	**− 9·9**	**− 3·6**
	A.DNase	**β-N-Gase**	**2**	**9·3**	**13·7**	**18·0**	**− 6·7**	**+ 1·7**	**+10·1**
	Ur.Ox.	β-N-Gase	2	32·1	34·4	36·6	−41·8	−30·0	−18·2
	Prot.	β-N-Gase	3	38·6	42·3	48·7	−29·1	−20·7	− 7·8
	Cyt.Ox.	β-N-Gase	3	47·0	51·4	59·4	−28·8	−19·4	− 6·9
4·7–10·0	Catal.	**β-N-Gase**	2	**52·3**	58·3	64·3	−40·6	−33·2	−26·1

association of these enzymes with a special group of particles distinct from the mitochondria, the lysosomes. This is made clear by the results given in Table 10, which show close correlations between all six hydrolases, as well as relatively important displacements with respect to cytochrome oxidase and protein.

In all systems investigated, the distributions of the lysosomal enzymes are extremely flat and irregular, and, in addition, vary somewhat from one hydrolase to the other. These facts, which illustrate again the heterogeneity of lysosomes, have become much more easily understandable in the light of our increasing knowledge of the biological function of these particles (for reviews see de Duve, 1963*b*, *c*). Special reference should be made in this connexion to the observations of Wattiaux, Wibo & Baudhuin (1963*a*, *b*), which indicate that the equilibrium density of lysosomes in sucrose–H_2O gradients can be modified considerably by the injection of suitable substances that accumulate in these particles.

From a practical point of view, the enzymic heterogeneity of lysosomes raises the problem of a suitable reference enzyme. In most experiments, the widest differences were observed between acid phosphatase, which appears to be more concentrated in the less dense particles, and acid deoxyribonuclease, which tends to be particularly abundant in the denser ones. For this reason, it has become customary, whenever measurements of numerous acid hydrolases proved impracticable, to rely on these two enzymes for an estimate of the distribution of their host particles. It is assumed, though, of course, not demonstrated, that the distribution of the particles themselves lies somewhere in between those of the two reference enzymes.

Urate oxidase, catalase and D*-amino acid oxidase.* The previously expressed suspicion that urate oxidase may belong to a third type of particle (de Duve *et al.* 1955; Beaufay *et al.* 1959) has been amply confirmed by the present results. Its dissociation from the mitochondria, which has already been demonstrated before both by differential centrifuging and by density equilibration (de Duve *et al.* 1955; Beaufay *et al.* 1959), is particularly clear-cut in the presence of high concentrations of sucrose, whether with H_2O or with D_2O as solvent, whereas the possibility, introduced by the use of glycogen as solute, of achieving density equilibration in the presence of low concentrations of sucrose has now made it obvious that urate oxidase is not a constituent of lysosomes either. This point has been further confirmed by Wattiaux *et al.* (1963*a*, *b*), who have succeeded in separating the two groups of particles completely, by taking advantage of the finding, referred to above, that the density of lysosomes can be modified selectively by appropriate treatment of the animals.

Consideration of Fig. 9 and Table 10 shows that the same conclusions are valid for catalase and D-amino acid oxidase. Baudhuin *et al.* (1964) found that both enzymes were concentrated largely in the light-mitochondrial fraction, together with urate oxidase and the lysosomal hydrolases, and it was deduced from these results that at least a considerable proportion of them must be associated with non-mitochondrial particles. The new observations described in the present paper make it very probable that both enzymes are entirely absent from the mitochondria. They have been dissociated more than 70 % from cytochrome oxidase in sucrose gradients, and the shape of the observed distributions suggests strongly that overlapping and not true association is responsible for the lack of complete resolution. On the other hand, the results obtained in glycogen gradients indicate that catalase and D-amino acid oxidase resemble urate oxidase much more than they do the lysosomal hydrolases, from which they can also be largely separated by density equilibration in media of low sucrose concentration. The relationship between the three enzymes raises a more delicate problem.

That a close association must exist between catalase and D-amino acid oxidase seems hardly to be denied. In eight out of 12 experiments performed under a variety of conditions, their distribution patterns overlapped more than 90 %, and in only one case (Fig. 4*a*) did their area displacement exceed 13 %. In addition, both enzymes share the peculiar property of being partly released in soluble form on exposure of their host particles to a high concentration of sucrose (see Fig. 8 and Table 9). It is also clear that they have many properties in common with urate oxidase, in particular the remarkable dependence between equilibrium density and sucrose concentration that causes all three enzymes to equilibrate at a lower density than the mitochondria and the lysosomes in media of low sucrose concentration, and at a higher density than these particles in highly concentrated sucrose. As is recalled below, mathematical analysis of this dependence has led Beaufay & Berthet (1963) to assign very similar properties of a unique nature to the particles bearing the three enzymes.

To these correlations, which come out statistically in the results given in Table 10, must be opposed a systematic difference in equilibrium density that causes urate oxidase to equilibrate at somewhat higher density values than the other two enzymes. This difference is particularly great in sucrose gradients; it may lead to area displacements of up to 45 % (Table 10) and to the separation of sediments containing considerable amounts

of highly purified urate oxidase and much poorer in catalase and D-amino acid oxidase, sometimes even practically devoid of these enzymes (Fig. 8). In previous papers in which this discrepancy was briefly discussed (de Duve *et al.* 1960; Beaufay & Berthet, 1963) it was taken to reflect either an intrinsic heterogeneity of a single population of particles or the existence of more than one group of particles with analogous properties, or, possibly also, to be the result of an artifact. Closer analysis of the experimental findings and especially examination of the results shown in Fig. 8 and in Table 9 causes us to lean strongly in favour of the third interpretation.

As can be shown in a variety of ways, urate oxidase is firmly bound to the insoluble framework of its host particles, whereas catalase and D-amino acid oxidase are fairly easily released in soluble form. This phenomenon can be accomplished mechanically and may account for the presence of a significant proportion of the latter enzymes in the final supernatant when complete fractionation of liver tissue is performed (Baudhuin *et al.* 1964). It also takes place when the particles are exposed to high concentrations of sucrose. As shown in a particularly clear-cut fashion by the results of Fig. 8, it appears that the denser particles are especially sensitive to this kind of damage, with the result that losses of catalase and D-amino acid oxidase, instead of affecting all subfractions uniformly, are reflected selectively in the sediment. Whether the particles suffer a further increase in density as a result of this loss is not known, but a change of this sort does not have to be postulated in order to interpret the results. Obviously, this phenomenon can be clearly recognized only in layering experiments of the type shown in Fig. 8. When the particles are distributed homogeneously within the gradient, it will lead to a more diffuse displacement of the distributions and, as seen systematically, to the isolation of sediments containing more urate oxidase than either catalase or D-amino acid oxidase.

In conclusion, present indications are that urate oxidase, catalase and D-amino acid oxidase are associated together and, possibly, in an essentially homogeneous fashion (see the three distributions of Fig. 8) with a single group of particles, and that the differences in distribution that are observed between them can be explained entirely on the basis of a selective loss of the soluble catalase and D-amino acid oxidase from the denser particles. Such losses may, exceptionally, affect one of these enzymes more than the other, thus accounting for the differences that have occasionally been observed between them.

As already briefly reported by Baudhuin & Beaufay (1963), these new particles are probably identical with the so-called 'microbodies' described by various authors. Their biological function raises intriguing problems on which our knowledge of their enzymic complement has so far thrown little light.

Obviously, the particles cannot qualify as metabolic units in the usual sense, since neither urate oxidase nor D-amino acid oxidase appears to be accompanied in them by any of their possible ancillary enzymes. Most other enzymes involved in purine catabolism are located in the soluble phase, whereas those that might participate in the further metabolism of D-amino acids (in themselves rather unusual metabolites) are distributed between the mitochondria and the soluble phase (for a review see de Duve, Wattiaux & Baudhuin, 1962).

At present, the only link between the three enzymes so far identified as constituents of the new particles appears to be hydrogen peroxide, which is formed by the oxidases and destroyed by catalase. Considered from a teleological point of view, the observed association could be advantageous either as a protection against the accumulation of hydrogen peroxide formed by the oxidases, or, alternatively, as ensuring a proper supply of hydrogen peroxide for a specific peroxidatic function of catalase.

Many other experimental results will have to be reconsidered in the light of the present findings, especially those relating to the concentration and turnover of hepatic catalase in normal and tumour-bearing animals. Special reference should be made, in this respect, to the investigations of Price, Sterling, Tarantola, Hartley & Rechcigl (1962), Roodyn, Suttie & Work (1962), Higashi & Peters (1962) and Peters & Higashi (1963), suggesting that catalase is renewed rapidly by a biosynthetic mechanism located in microsomal vesicles and is subsequently transferred to the 'mitochondria'. It is not yet possible to say whether these results apply to the renewal of the new particles as a whole or only to their catalase content.

Physical properties of particles

Beaufay & Berthet (1963) have attempted to fit some of the results described in the present paper to the theoretical model analysed by de Duve *et al.* (1959). These publications must be consulted for details and only the most salient facts bearing on this problem are examined in the present paper.

The model considered was originally suggested by the observations of Werkheiser & Bartley (1957) on mitochondria. It is that of a particle surrounded by a membrane and made up of a hydrated matrix enclosing two formally distinct spaces, one accessible only to water and behaving as a perfect osmometer (osmotic space) and the other accessible to water and to solutes of low molecular weight,

including sucrose, but not to macromolecular substances (sucrose space). The following equations, restated in terms of mole fraction, describe the relationships between the density of such a particle and the properties of the medium (concentration of sucrose and nature of the solvent water):

$$\rho_p = \rho_w \frac{\rho_d \alpha M_w + (\rho_d - \rho_d \alpha M_w \times 10^{-3} + \rho_m \beta)\, N \times 10^3}{\rho_d \alpha M_w + (\rho_w - \rho_d \alpha M_w \times 10^{-3} + \rho_w \beta)\, N \times 10^3} \quad (4)$$

$$\rho_d = (1-\psi)\, \rho_a + \psi \rho_w \quad (5)$$

in which ρ_p, ρ_w, ρ_d, ρ_m and ρ_a are the densities respectively of the particle, of the solvent water, of the hydrated matrix, of the external sucrose solution (assumed also to fill the sucrose space) and of the dry matrix; α is a measure of the relative amount of osmotically active solutes present in the osmotic space, expressed in m-osmoles/g. of hydrated matrix; β is the relative volume of the sucrose space, expressed in ml./ml. of hydrated matrix; M_w is the molecular weight of the water; N is the mole fraction of sucrose in the outside medium (and in the sucrose space); and ψ is the relative volume of hydration water, expressed in ml./ml. of hydrated matrix.

Thus, if the volume of the hydrated matrix is represented by ϕ_d, the particle is assumed to contain $\rho_d \alpha \phi_d$ m-osmoles of osmotically active solutes, and to include $\psi \phi_d$ ml. of hydration water and $\beta \phi_d$ ml. of a sucrose solution of mole fraction N and density ρ_m.

In their calculations, Beaufay & Berthet (1963) have used the values of median equilibrium density observed in the glycogen gradients of series A [except those from gradient Gh(2·3) obviously vitiated by exposure of the particles to a medium of low osmolarity] and some of the median (or modal in the case of the mitochondria) equilibrium density values determined in sucrose gradients. They have further assumed that these values, which are referred to as typical, apply in all cases to the same representative particles, that the concentration of glycogen is without influence on the physical properties of the particles, that the hydration water is rapidly and quantitatively exchangeable with D_2O, and finally that all the parameters in eqns. (4) and (5) are independent of the mole fraction of sucrose (except for the mitochondrial ψ, for which a transitional change must be postulated at high sucrose concentration). They have arrived at the values listed in Table 11. The fit between the functions calculated by using these values and the experimental points mentioned in the present paper is illustrated in Fig. 10. Only the points obtained in experiments in which the particles were initially incorporated homogeneously within the gradient are shown on this graph, since they are all affected in about the same way by errors due to the presence of soluble enzyme activities.

As clearly stated by the authors, their deductions require so many assumptions and involve so many parameters that too much significance cannot be attached to the underlying theory, simply on the basis of a mathematical fit. However, it is remarkable that the predicted properties of mitochondria agree closely with those measured experimentally by a number of authors, in particular with respect to the existence and dimensions of two distinct water spaces in these particles (for a review of the literature and a complete discussion of this point, see Beaufay & Berthet, 1963). It is also striking that the calculations made on urate oxidase, catalase and D-amino acid oxidase lead, for all three enzymes, to the assignment to their host particles of a very large sucrose space and of a negligible osmotic space, features that are borne out by studies of the release of catalase (P. Baudhuin, unpublished work). On the other hand, the values computed for the lysosomal enzymes have so far received no experimental confirmation and must be considered with great caution, in view of the great irregularity of the distributions on which they are based.

Table 11. *Typical properties of cytoplasmic particles*

The data are from Beaufay & Berthet (1963).

Parameter	Mitochondria	Lysosomes		Other particles		
Enzyme ...	Cytochrome oxidase*	Acid phosphatase	Acid deoxyribonuclease	Urate oxidase	Catalase	D-Amino acid oxidase
α	0·110	0·102	0·276	0	0	0
β	0·76	1·05	0·34	2·58	2·55	2·40
ρ_d	1·200 (1·233)	1·226	1·257	1·248	1·231	1·224
ρ_a	1·315 (1·313)	1·300	1·331	1·322	1·319	1·315†
ψ	0·36 (0·26)	0·25	0·22	0·22	0·28	0·28†

* Values in parentheses correspond to the 'dense state' of mitochondria exposed to high sucrose concentrations.
† Values calculated from new results reported in the present paper.

One difficulty that is not discussed by Beaufay & Berthet (1963) is raised by the differences in equilibrium density observed in the presence of different polymers (Tables 5 and 6). It is possible that these differences are related to factors such as colloidal osmotic pressure, presence of impurities of low molecular weight influencing the total osmotic pressure or specific interactions (with ficoll, for instance), which were not operative in the glycogen gradients of series A, but their existence should be remembered in any evaluation of the results. They undoubtedly indicate that the density of subcellular particles may be influenced by other factors besides those which are taken into account in eqns. (4) and (5), a point that may be of both theoretical and practical importance. It has indeed been found by Bartley (1961) that changes in both the sucrose space and the osmotic space of mitochondria can be induced, for instance by the addition of small amounts of salts to the suspension medium.

A property that appears to be peculiar to mitochondria is represented by the transition to a denser form which takes place at high sucrose concentration. As deduced from density measurements in H_2O and D_2O, it appears to be due essentially to a partial loss of hydration water (Beaufay & Berthet, 1963). It occurs in sucrose–H_2O when the mitochondria are exposed to a sucrose mole fraction slightly higher than 36×10^{-3}, which corresponds to the isopycnic point of normally hydrated mitochondria. The partially dehydrated particles have another isopycnic point at

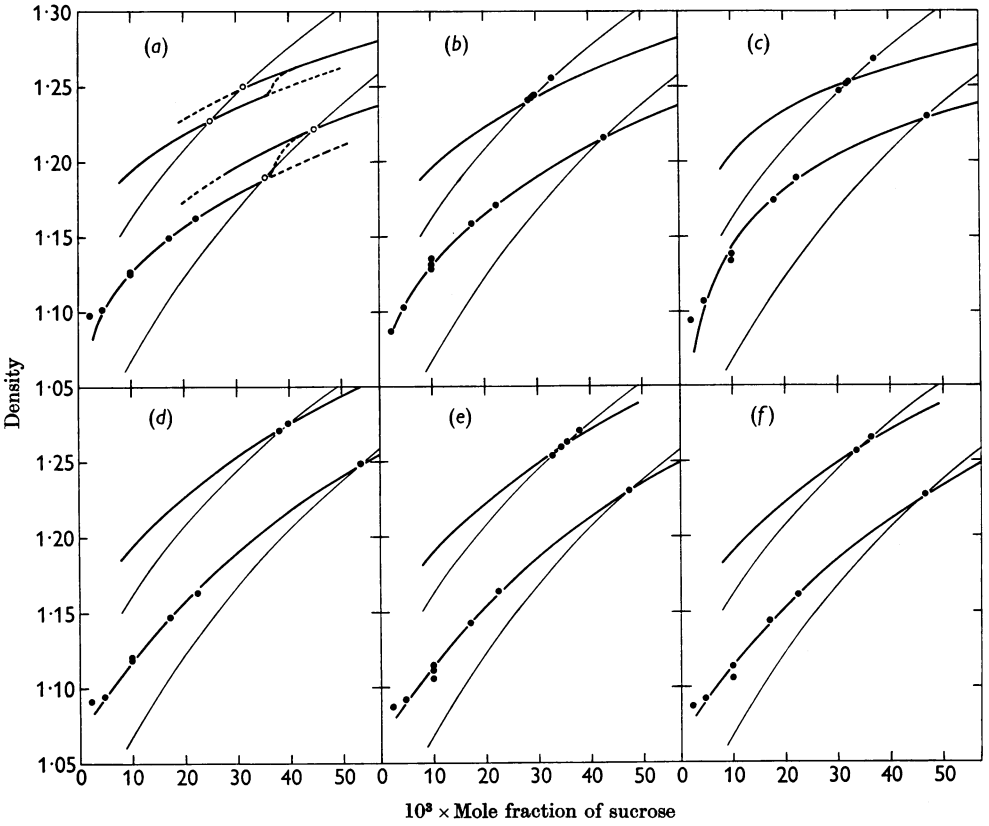

Fig. 10. Influence of sucrose concentration on the density of cytoplasmic particles: (*a*) cytochrome oxidase; (*b*) acid phosphatase; (*c*) acid deoxyribonuclease; (*d*) urate oxidase; (*e*) catalase; (*f*) D-amino acid oxidase. The thin curves repeated on each graph show density of medium (ρ_m) as a function of the mole fraction of sucrose, in H_2O (lower curve) and D_2O (upper curve). Thick curves are representations of eqns. (4) and (5), calculated for each enzyme with the parameters listed in Table 11, in H_2O (lower curve) and D_2O (upper curve). ●, Median equilibrium densities observed in the experiments in which the particles were initially incorporated homogeneously within the gradient; ○, modal equilibrium densities, estimated graphically. For cytochrome oxidase, the curves corresponding to the 'normal' and to the 'dense' state are shown, the transition from one to the other being indicated as occurring at N 37×10^{-3} in both solvents.

mole fraction 45×10^{-3}. Thus the transition occurs between these two isopycnic points. As shown in Fig. 10, a third metastable isopycnic point may be present between the two stable ones, provided that the particles can exist in an intermediate state. Trimodal distributions of the type shown in Fig. 7(*b*) could possibly be explained on this basis.

In D_2O, the two corresponding stable isopycnic points are at mole fractions 25×10^{-3} and 31×10^{-3}. Examination of the distributions observed in various gradients [Figs. 4(*a*), 4(*b*), 5 (*A* and *B*) and 6(*b*); and also Fig. 8 of Beaufay *et al.* (1959)] indicates strongly that the transition does not take place between these two points, but rather, as in H_2O, at a mole fraction of about 37×10^{-3}. In other words, only those mitochondria that have been exposed to a sucrose mole fraction of 37×10^{-3} or more equilibrate at the upper isopycnic point of 31×10^{-3}. From this it may be concluded that the sucrose concentration is the main factor responsible for the partial dehydration of the mitochondrial matrix, H_2O and D_2O being bound about equally strongly, and that this phenomenon is essentially irreversible. It has been depicted as such in Fig. 10.

The actual mechanism responsible for the transition is itself entirely unknown. It should be pointed out that the calculated hydration ratio is higher for the mitochondria than for the other particles and falls down to the value estimated for the latter in the 'dense state'. Apparently, therefore, the mitochondria possess additional and relatively weak binding sites for water that do not exist in the other particles, and this peculiarity explains why they alone are liable to suffer the observed transition. Whether this property is related to the existence of a double-membrane system in the mitochondria is a matter for further investigation.

SUMMARY

1. Mitochondrial fractions from rat liver have been subfractionated by density equilibration in gradients of glycogen at several different concentrations of sucrose and in gradients of sucrose with H_2O or D_2O as solvent. Measurements of total protein and of a number of enzymes in the various subfractions have led to the recognition of three distinct populations of cytoplasmic particles in the preparations under study. Some physical characteristics of these particles have been deduced from the manner in which their density is affected by the sucrose concentration and by the replacement of H_2O by D_2O.

2. The behaviour of the mitochondria, as ascertained from cytochrome-oxidase and, accessorily, protein assays, is consistent with the assumption, supported by other experimental results, that they contain both a sucrose space and an osmotic space. When they are exposed to a sucrose concentration higher than 37×10^{-3} (mole fraction), they suffer a relatively sudden increase in density which is due to a partial dehydration of their matrix. This phenomenon appears to be irreversible.

3. Further evidence of the existence of lysosomes as a separate group of particles has been obtained from determinations of up to six different acid hydrolases. The results of these experiments suggest that the lysosomes may also contain a sucrose space and an osmotic space. However, this conclusion should be considered with great caution in view of the highly heterogeneous character of their physical and biochemical properties.

4. Urate oxidase belongs to a third group of particles, which appear to include catalase and D-amino acid oxidase also. These particles are characterized by a very large sucrose space and the lack of an osmotic space. When exposed to a high concentration of sucrose, they lose part of their catalase and D-amino acid-oxidase activities (apparently released in a selective fashion from the denser particles) without releasing urate oxidase. This phenomenon may cause relatively large shifts in the distribution of catalase and D-amino acid oxidase with respect to that of urate oxidase.

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The Reaction of 1-Fluoro-2,4-Dinitrobenzene with Bone

STUDIES ON THE RELATIONSHIP BETWEEN BONE COLLAGEN AND APATITE

BY R. E. WUTHIER, P. GRØN AND J. T. IRVING
Forsyth Dental Center, Harvard School of Dental Medicine, Boston, Mass., U.S.A.

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A number of investigators have now utilized FDNB* (Sanger, 1945) to study the reactivity of the ϵ-amino groups of collagen lysine and hydroxylysine in either intact fully mineralized bone or in bone in various stages of demineralization. The reasoning has been that a logical association between the mineral and collagen would be an interaction between the positively charged ϵ-amino groups and the negatively charged phosphate ions of the hydroxylapatite.

Solomons & Irving (1955) first described experiments in which the number of reactive ϵ-amino groups in collagen of hard tissues were estimated with FDNB during decalcification *in vitro*. They reported a linear relationship between the release of mineral and the reactivity of the ϵ-amino groups and suggested that this was evidence for a bonding between these groups and apatite. These results were amplified (Solomons & Irving, 1956, 1958) by comparison of the reactivity of collagens from hard and soft tissues. Under conditions of simultaneous demineralization and treatment with FDNB nearly all the ϵ-amino groups of hard tissues reacted, compared with only two-thirds in soft tissues.

Glimcher (1960, 1961), presenting data on bone of various densities, suggested that the extent of the reaction of the ϵ-amino groups of mineralized bone with FDNB was limited by diffusion (i.e. governed by the degree of occlusion of the collagen by apatite crystals). Demineralization would thus remove the diffusion barrier and permit FDNB to react with the ϵ-amino groups of collagen.

Cartier & Lanzetta (1961), however, reported that 53% of the total ϵ-amino groups of collagen in finely ground ox bone reacted with FDNB independently of the degree of demineralization until 97% removal of the mineral. Beyond this stage an additional 22% reacted, giving a total of 75% of the lysine and hydroxylysine residues in bone collagen. The lack of effect of demineralization on the reaction with FDNB, except at almost complete removal of bone salt, contra-indicated a diffusion phenomenon.

The present studies were undertaken to clarify these apparently contradictory results and to elucidate the nature of the relationship between the mineral phase and the ϵ-amino groups of bone collagen. Our objectives were: (1) to test the reactivity of the ϵ-amino groups of bone collagen with FDNB in fully demineralized bone and at various stages of demineralization; (2) to evaluate the effect of diffusion on the rate and extent of the reaction of the ϵ-amino groups of bone collagen with FDNB and different particle sizes of bone. The result of these first experiments showed that it was necessary to test the reactivity of the ϵ-amino groups of bone collagen with FDNB in intact fully mineralized bone in an anhydrous reaction system in which hydrolysis of FDNB was kept at a minimum.

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.