Separation of Cytoplasmic Particles by Centrifugation in a Density-Gradient

If cellular constituents from tissue homogenates are isolated by means of *differential centrifugation*[1], the separation of the various fractions depends upon differences in sedimentation rate, which is determined mainly by the mass and surface of the particles. This procedure yields particle fractions of relative purity only, so that, especially for small-sized particles, the possibility of an overlapping between the fractions always has to be taken into consideration[2].

Density-gradients were introduced by LINDERSTRØM-LANG[3] for density measurements; sucrose gradients have been used by PICKELS[4] and KAHLER and LLOYD[5] as a technical aid in counteracting the convection currents, which occur in angle-head centrifuges. The first to use sucrose gradients in the centrifuge as a means of separating particles of different density were E. N. HARVEY[6] and E. B. HARVEY[7] in their experiments on the stratification and separation of living egg cells in the centrifugal field, and BRAKKE[8] and HOLTER[9] proposed to use the same principle for the separation of cell constituents in homogenates. In the proposed method, the range of the density gradient is extended beyond the specific gravity of the heaviest component and centrifugation is prolonged until all particles have come to equilibrium with the medium at the corresponding level of density. Under such conditions, therefore, the *particles are separated according to their density*, since mass and surface affect only the sedimentation rate, but not the final position in the gradient.

Since all modern high-speed centrifuges are equipped with angle-heads, which are rather unsuitable for this type of work[10] the present work had to be postponed until a swinging bucket rotor had been made available as an accessory to the "Spinco" centrifuge.

BRAKKE[8] actually obtained a separation of plant virus particles by gradient centrifugation, but in his case, the particle separation was still due to sedimentation rate. Density equilibrium (although mentioned as a separation principle) was not attempted and was perhaps also unobtainable within the range of density employed (1·04 to 1·16). A similar technique was also used by BEHRENS and TAUBERT[11] mainly for the isolation of cell nuclei from acetone-fixed material in non-aqueous media.

The present note deals with a first set of experiments on previously isolated cytoplasmic particles of Xenopus-liver tissue. There is evidence that mitochondria and submicroscopic particles (microsomes) reach density equilibrium at different levels within the gradient. Moreover the density-range is wider for mitochondria-like particles than for microsomes, thus suggesting a qualitatively non-homogeneous mitochondria-population.

Preparation of the Gradients. A suitable gradient-medium must fulfill the following conditions: density ca. 1·30, chemical inertness, low viscosity, moderate osmotic effect and miscibility with water. As a matter of fact, the most serious problem consists in finding a solution corresponding to such a series of requirements.

By mixing sucrose-solutions with "Diodon" (Di-ethanol-amine-salt of 3,5-di-iodo-4-pyridone-N-acetic acid)[1] the following stock-solutions could be obtained:

Diodon	Sucrose and 0·03 m Versene				
	10%	20%	30%	40%	
23·33%	1·16*a*	1·20*b*	1·25*c*	1·30*d*	Density
11·66%	1·10*e*	1·15*f*	1·20*g*		

With a calibrated micro-pipette, 0·3 ml of each of the above stock-solutions (2°C) were carefully layered in plastic tubes of 1·5 ml capacity, to build up three-step gradients of 0·9 ml total volume. Three types of gradients with different density-ranges were used, namely:

Gradient 234 S (*b*, *c*, *d*)	density 1·20–1·30
Gradient 123 S (*a*, *b*, *c*)	density 1·15–1·25
Gradient 123 L (*e*, *f*, *g*)	density 1·10–1·20

To check the density-distribution in the gradients, the tubes were introduced into the optical system of an electrophoresis-apparatus (0°C). The resulting "Schlieren-Curves" indicated, that a continuous gradient in density from the top to the bottom of the tube was established after 10–20 h.

Isolation of the Cytoplasmic Particle Fractions. Liver homogenates of young Clawed Frogs (*Xenopus laevis* Daud.)[2], prepared in 0·3 m sucrose + 0·01 m "Versene" (Di-Na-salt of ethylene-diamine-tetra-acetic acid)[3], were separated into two fractions by differential centrifugation[4], after removing the cell-debris and nuclei. The large cytoplasmic particles (= *fraction A*) were sedimented at 5,000 g (30 min) and from the supernatant a second sediment was precipitated at 20,000 g (60 min) to which we refer as *fraction I + B*. Both sediments were washed twice in the sucrose-versene-medium.

The microscopical analysis of *fraction A* revealed the presence of typical mitochondria-like particles. The pellet corresponding to *fraction I + B*, was clearly composed of two parts, namely a thin reddish layer of small mitochondria-like spherules and a larger transparent and jelly-like pellet. The mitochondria-like substance could be easily washed away with sucrose-versene-medium, yielding a yellowish suspension, which we designate as "intermediate fraction" (= *fraction I*) on account of both the intermediate size[5] and the behaviour of these particles in the density-gradient. The remaining compact jelly-like sediment, after being stirred up in sucrose-versene-medium, gave a milky, bluish-opalescent suspension, containing submicroscopic particles of 0·1–0·2 μ diameter[5] (= *fraction B*).

The isolated particles were preserved in the "Diodon" –sucrose–solutions, and microscopically, there were no detectable morphological changes.

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High Speed Centrifugation of the Particle Suspension in Density-Gradients. Density-gradients, kept for 10–20 h at 2°C, with a thin top-layer of 0·05 or 0·1 ml suspension of the fractions *A*, *A* + *B*, *B*, and *I* respectively, were spun in the swing-out head of a refrigerated (*t* = *ca.* 5°C) *spinco-ultra-centrifuge* (model *E*) at 24,630 RPM (ca. 60,000 g for the bottom of the tubes). Every 30 min the run was interrupted, the rotor transferred to the coldroom (*t* = 2°C) and the tubes photographed in order to obtain an objective record on the sedimentation for the different particle fractions. To indicate the density levels, minute airfilled glass-beads of known density were introduced into the gradients, to serve as reference-bodies[1].

Samples for microscopic and electron-microscopical examination[2] were withdrawn from the various layers by means of a capillary pipette the tip of which was bent to an angle of 90° in order to minimize vertical disturbances due to the aspiration current.

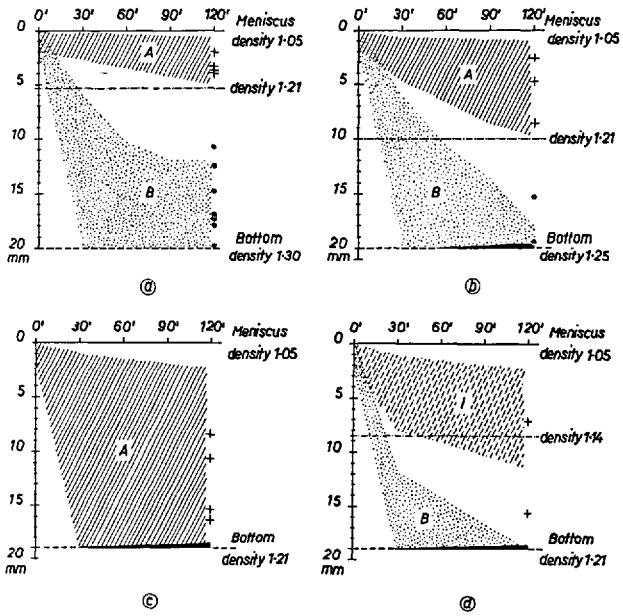

Fig. 1.—Approximate distribution of the particle fractions in different "Diodon"-sucrose-gradients after centrifugation
a = Gradient 234 S, *b* = Gradient 123 S, *c* and *d* = Gradient 123 L. The time of centrifugation is represented by the *x*-axis and the position of the particle fractions (*A*, *B*, *I*) is plotted on the *y*-axis as distance from the meniscus. The sedimentation of the different particle fractions is represented by the increasing thickness of the bottom line. The results of the microscopical analysis of the different samples are represented by: + sample rich in mitochondria-like particles, • sample without or with only few mitochondria (in the latter case probably due to contamination).

The data from different experiments involving the three types of "Diodon"-sucrose-gradients are assembled in Figure 1, *a–d*; the approximate positions of particle layers, after different times of centrifugation, are indicated schematically.

In the heaviest gradient (234 S) the particles of all fractions (*A*, *B*, *I*) are kept in suspension. According to the photographic record, equilibrium is established after approximately 2 h at 60,000 g. The *fractions A* and *I* respectively form a thin layer just below the meniscus, whereas the particles of *fraction B* appear as a dense bluish cloud above the bottom of the tube (Fig. 1*a*). If the density of the gradient is decreased, the layers due to *fractions A* + *I* spread out towards the bottom (1*b*). Thus the particles of *fraction A* have almost completely sedimented if the bottom-density is 1·20. On the other hand, *fraction I* at the same conditions still forms a concentrated top-layer (Fig. 1*d*) of light particles, which probably represents the smaller mitochondria (= "intermediate particles"). The submicroscopic particles of *fraction B* (microsomes) sediment more readily than mitochondria, since at a bottom-density of 1·25 they are partially and at 1·20 they are completely sedimented after a run of two hours at 60,000 g.

Our experiments thus indicate the possibility of separating at least mitochondria from microsomes, if centrifugation is performed in a "Diodon"-sucrose-gradient with a suitable density-range. Judging from the equilibrium positions of the particle layers in the different gradients and from the conditions for sedimentation to the bottom, we may assume a density-range of 1·10–1·20 for mitochondria-like particles (*fraction A* + *I*) and 1·25–1·30 for microsomes (*fraction B*).

Assuming that this separation method is only determined by the density of the cell-constituents and in no way affected by the mass and the surface of the particles, it would follow that the positions of the various fractions reflect in some respect their qualitative composition. Thus the narrow density-range found for microsomes (*fraction B*), would indicate a qualitatively rather uniform population of cytoplasmic particles. On the other hand, the wider range, estimated for mitochondria-like particles (*fractions A* and *I*), might be considered as an indication for a heterogeneous nature of these cell constituents.

Unfortunately there is little information on the density of cell particles. Some values for plant-virus particles were calculated to 1·25[1] and 1·27[2] respectively and may to a certain extent serve as comparison with our density-range for microsomes (1·25–1·30).

On this occasion we should not forget that the density of cytoplasmic particles, as they were used in our experiments, are dependent on both the homogenate- and the gradient-medium. Furthermore it must be stressed, that we have no information about the possible influence of the medium on the density, the chemical composition and the enzymatic activity of these particles. So far we can only state that the equilibrium position of the layers was stable, and that there was no detectable morphological alteration of the particles by the gradient-medium. Further experiments are necessary in order to elucidate these questions, to determine the yield of the various particles and to compare particle fractions isolated by the present method and by differential centrifugation.

H. Holter, M. Ottesen, and R. Weber[3]

Carlsberg Laboratorium, Copenhagen, May 31, 1953.

Note added in proof: While the present communication was in print, there appeared a note by Schneider, Dalpon, Kuss, and Felix, Nature *172*, 161 (1953), reporting the successful isolation of Golgi substance from Rats epididymis by similar technique.

[1] Such beads have been used for years at the Carlsberg Laboratory for the characterization of gradients. Although cumbersome to make they are better suited for the purpose than any substitute so far tried, on account of their stability.

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Zusammenfassung

Erste Versuche zur Ultrazentrifugierung isolierter Zytoplasmapartikelfraktionen aus Krallenfroschleber (*Xenopus laevis* Daud.) in «Diodon»-Saccharose-Gradienten mit verschiedenen Dichtebereichen zeigen, dass mitochondrien- und mikrosomenartige Zellpartikel in verschiedenen Zonen der Gradienten ins Dichtegleichgewicht gebracht werden können. Die Lage der Partikelzonen in den Gradienten ist abhängig von den gewählten Dichteextremen, woraus abzuleiten ist, dass die Trennung der Fraktionen im wesentlichen durch die Unterschiede in der Partikeldichte bedingt ist. Für die isolierten Mitochondrien wurde so eine Dichte von 1,10 bis 1,20, für die Mikrosomen hingegen eine solche von 1,25 bis 1,30 gefunden.

Hydrogen Peroxide in Tumours

Its Possible Significance in Carcinogenesis

During the last twenty years, we have studied the action of hydrogen peroxide on proteins and high split-products of proteins consisting in an aggregation of molecules with an increase of N precipitable by trichloroacetic acid[1] and of turbidity[2]. This phenomenon was further investigated and called[3] "aggregation effect" of H_2O_2. While formerly we considered such effect as chiefly due to an interaction with an enzymatic system, and therefore corresponding to a true polymerisation, we were lately inclined to admit an oxidation of hydrophilic groups of proteins and peptides molecules and subsequent process of physical aggregation. The formation of S-S-bridges between molecules through oxidation of SH-groups was also taken into consideration.

A relation of this effect of H_2O_2 to similar processes and their importance for the protein synthesis were largely discussed[4].

YAMAFUJI and coworkers[5] paid great attention to the action of H_2O_2 on proteins, considering it to be a true polymerisation[5]. They think that poisoning catalase in the living organism (e.g. by hydroxylamine), permitting the H_2O_2 formed in the oxidative metabolism of cells to escape destruction by this enzyme, may give rise to polymerisation of some protein constituents and bring about the formation of virus particles. These authors therefore admit an endogenous origin of some virus by means of abnormal H_2O_2 concentration in tissues.

One of the most important features of enzymatic pattern in tumours is represented by a strong decrease of catalase activity. This was first shown by GREENSTEIN and coworkers[6], who studied the activity of some enzymes in rats' hepatomas, in normal and regenerating livers. The relationship between catalase activity in hepatoma and in normal adult liver was 1:1000; whereas the differences concerning other enzymes were much smaller. Regenerating liver has nearly the catalase activity of normal liver. A decrease of catalase activity in livers of tumour-bearing rats was also demonstrated by GREENSTEIN and coworkers, and later throughly investigated by other authors[1]: thus CUDKOWICZ[2] found a strong decrease of catalase in livers of benzpyrene-treated rats as soon as 60 days after the injection of the hydrocarbon, i.e. before the appearance of a sarcoma at the injection site.

A working hypothesis was advanced by RONDONI[3], who said: "The strong diminution of catalase activity in a tissue may induce an accumulation of H_2O_2 having an aggregation or polymerisation effect on some proteins and high split-products of proteins. Such aggregation effect may be the starting point for that proteins rearrangement, perhaps ultrastructural in nature, which brings about the malignant change in cell" (p. 272).

According to such hypothesis the carcinogenic agents might be considered as catalase poisons: really CUDKOWICZ found that liver catalase can be partially inhibited *in vitro* by two powerful carcinogenic hydrocarbons (3,4-benzpyrene and methylcholanthrene) dissolved with caffein, and not by some non-carcinogenic hydrocarbons. However, water suspensions of hydrocarbons were in every case devoid of inhibiting activity.

It may be mentioned that according to recent investigations the mode of action of ionising and exciting radiations on cells can be at least partially explained by the formation of hydroxylic radicals from water, i.e. a chemical mechanism involving oxidation reactions and very likely some kind of protein denaturation. Besides this, a formation of H_2O_2 by irradiation of water or aqueous solutions has been found in some experiments[4].

A direct proof of the role of H_2O_2 in carcinogenesis has neither been given nor proposed, so far as we know. Such a demonstration may be very difficult, because the peroxide after formation in sufficient amount to induce the cellular change can be very quickly destroyed in the metabolic pool. However, we have attempted to estimate the contents of H_2O_2 in normal and tumour tissues (rats and mice) by the following method, permitting an approximative and comparative evaluation.

Method.—The animals were killed by bleeding. The tissues were immediately homogenised in an aq. 6·5%-solution of KCN in order to destroy every enzymatic activity. Estimation of dry weight. A 20%-solution of trichloroacetic acid is added (1:1) to the homogenate. After a few minutes filtration through paper, a clear solution is obtained, to a known amount of which some milliliters of a solution of titanium sulphate are added. Such a solution is made by dissolving the titanium salt in warm n H_2SO_4. A yellow colour is developed, which is photometrically (FISCHER electrophotometer) checked against a standard (homogenate + trichloroacetic acid + n H_2SO_4 without titanium salt). As the colour changes to some extent, an empirical curve ought to be established for every homogenate (using a part of homogenate where H_2O_2 has been destroyed by silver powder and another with known amount of H_2O_2), in order to evaluate the H_2O_2 contents from the photometric figures.

We must remark that photometric values do not always give a regular straight line, and the colour derived from the reaction $H_2O_2 + Ti_2(SO_4)_3$, which is quite stable in simple water solution, fades away at a different speed in the homogenates.

We may summarize our relative results in the following Tables.

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