AN EVALUATION OF THE HETEROGENEITY OF CYTOPLASMIC PARTICLES OBTAINED BY DIFFERENTIAL CENTRIFUGATION*

by

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Considerable interest has arisen in the biochemical properties of the various cell particles that have been isolated by means of differential centrifugation. Most studies have considered only nuclear, large granule (mitochondria), and small granule (microsome) fractions. While his studies have been open to criticism[1], CHANTRENNE[2] focused attention on the possibility that there is a continuous spectrum of particles in the cell varying in size and biochemical properties. Subsequently, investigations by others employing differential centrifugation[3–5] and density gradient[6] techniques under conditions which were not subject to the criticism of CHANTRENNE's experiments, have led to the conclusion that both the mitochondrial and microsomal fractions ordinarily prepared are heterogeneous mixtures. No attempt has been made to define a probable minimum number of biochemically distinct entities that exist within the cell.

The present study, which involves an extensive cytoplasmic fractionation of rat liver cells by differential centrifugation, does not attempt to define either a maximum or a minimum number of particles which may be isolated but rather has permitted a determination of the significance of the relative terms "heterogeneity" and "homogeneity", as applied to particulate fractions isolated by this technique, and as related to the number of fractions isolated.

EXPERIMENTAL

The cytoplasmic fractionation procedure used is shown in Fig. 1. Its unique features are the following: (1) During the separation of the mitochondria (*Mt*), the combined residues obtained after the nuclei had been removed were syringed[7] to dislodge the "poorly sedimentable" layer (*Ps*), and two fractions of the *Ps* layer were isolated. (2) In the treatment of the supernatant after the mitochondria had been sedimented, a short centrifugation (10 minutes) at $13{,}750 \times g$ was carried out two successive times. In each case two layers were obtained, a well-packed bottom one, and a diffuse "floating" layer (*F*). The well-packed pellets were treated further by syringing and centrifuging as in the case of the *Mt* fraction, to obtain a pellet and a poorly sedimenting layer. The supernatants obtained after the terminal centrifugation in each phase of the procedure were pooled. The entire procedure was carried out at 0–5° C, employing a type SS-1 Servall centrifuge.

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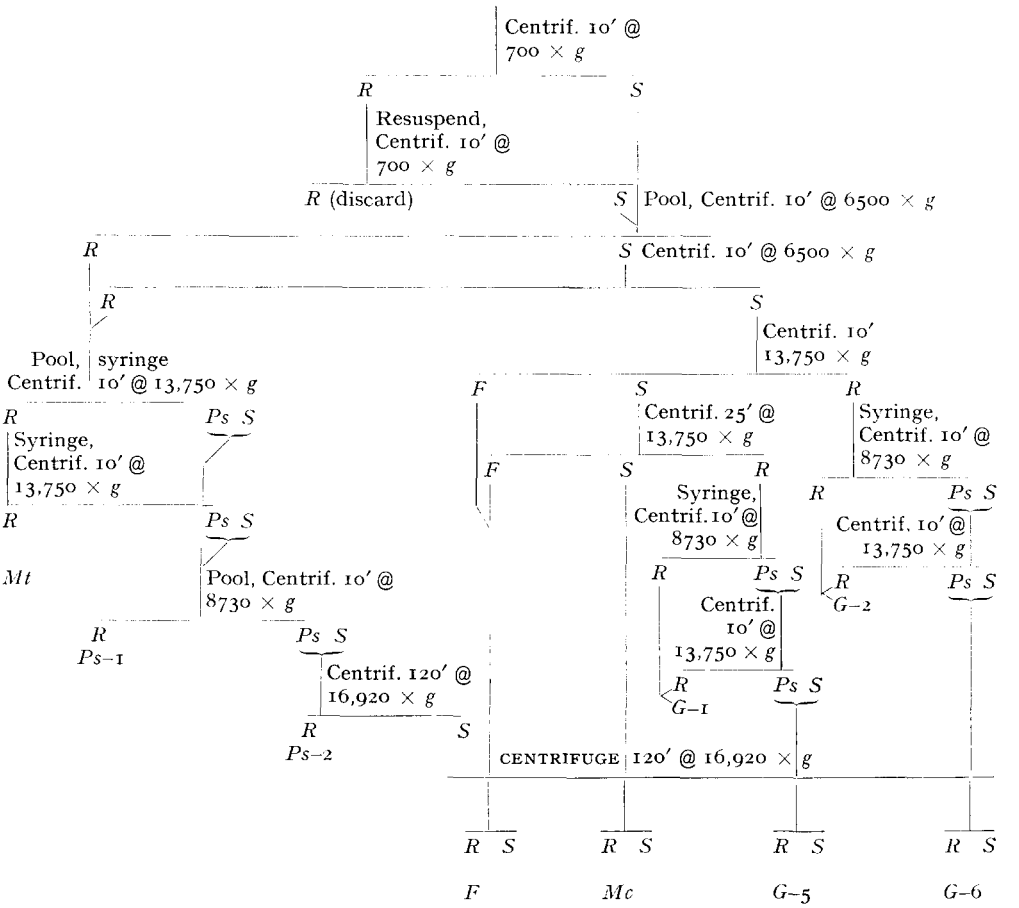
Fig. 1. 10 g liver, homogenized in 100 ml 8.5 % sucrose employing a Potter-Elvehjem type homogenizer with a rubber-covered steel pestle



Scheme showing the fractionation of rat liver cells. The following observations were made regarding the appearance of the fractions: *Mt*, tan, opaque, red center; *Ps*-1, turbid red, white halo; *Ps*-2, similar to *Ps*-1; *F*, red, opalescent; *G*-1, clear bluish; *G*-2, opalescent, bluish with slight red cast; *G*-5, opalescent, with reddish tinge; *G*-6, similar to *G*-5; *Mc*, clear, cherry red color; *S*, faint turbidity, pink tinge.

In a typical experiment a male Fisher strain rat weighing 300–350 g received a single injection of radioactive phosphorus*, as $Na_2H^{32}PO_4$, containing 0.45 microcuries/g of body weight. Four hours later the animal was sacrificed and the liver fractionated as described above. The individual cell fractions were separated into acid-soluble inorganic and organic phosphorus (P), phospholipide, phosphoprotein, desoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA) fractions. This procedure as well as total P and radioactivity measurements have been previously described[8,9].

The results shown (Table I) are the averages of five fractionations. The data were analyzed statistically, and those differences were considered significant in which the probability factor was equal to or less than 0.05[10]. With this criterion of difference, the number of times a given P fraction showed a significant difference from each of the other nine cell fractions was calculated. The total possible number of possible differences is 54 (9 fractions and 6 phosphorus groups). Thus a criterion of "homogeneity" was established for each fraction in terms of the number of differences found,

* The ^{32}P used in these experiments was furnished by the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

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TABLE I

UPTAKE OF RADIOACTIVE P BY VARIOUS P-CONTAINING FRACTIONS OF CYTOPLASMIC FRACTIONS*

Fraction	*A-S Inorganic*	*A-S Organic*	*P-protein*	*PNA*	*P-Lipid*	*PN*
Mt	3720 ± 320	2110 ± 770	1110 ± 140	220 ± 38	585 ± 18	70 ± 1
Ps-1	1640 ± 710	1530 ± 211	550 ± 110	50 ± 11	810 ± 50	120 ± 9
Ps-2	210 ± 26	965 ± 194	1170 ± 166	80 ± 16	903 ± 98	172 ± 1
F	880 ± 280	1260 ± 118	1430 ± 167	70 ± 10	919 ± 34	200 ± 2
Mc	1670 ± 690	1450 ± 160	1540 ± 222	90 ± 12	891 ± 23	160 ± 18
G-1	230 ± 50	300 ± 54	320 ± 34	40**	600 ± 345	340 ± 107
G-2	130 ± 10	600 ± 86	310 ± 65	25**	790 ± 207	170 ± 42
G-5	400 ± 170	1350 ± 210	1050 ± 210	60 ± 10	920 ± 41	160 ± 9
G-6	590 ± 190	1110 ± 100	640 ± 94	60 ± 11	930 ± 64	170 ± 8
S	3330 ± 300	2268 ± 422	2380 ± 325	150 ± 18	785 ± 78	80 ± 5

* These values are concentration coefficients calculated as (counts per minute per microgram of phosphorus found)/(counts per minute injected per microgram of body weight). They represent the averages and standard errors of 5 animals.

** 1 observation.

TABLE II

DIFFERENCES BETWEEN CELL FRACTIONS AS DETERMINED BY PHOSPHORUS UPTAKE IN SIX P-CONTAINING COMPONENTS

Fraction:	*Mt*	*Ps-1*	*Ps-2*	*F*	*Mc*	*G-1*	*G-2*	*G-5*	*G-6*	*S*	*"Homogeneity"*
Mt	—	5	4	4	4	5	4	4	5	2	37/54
Ps-1	5	—	3	2	2	4	2	3	2	4	27/54
Ps-2	4	3	—	1	0	3	2	0	1	5	19/54
F	4	2	1	—	0	1	3	1	2	5	19/54
Mc	4	2	0	0	—	4	4	0	1	2	17/54
G-1	5	4	3	1	4	—	1	2	2	5	27/54
G-2	4	2	2	3	4	1	—	2	3	4	25/54
G-5	4	3	0	1	0	2	2	—	0	4	16/54
G-6	5	2	1	2	1	2	3	0	—	5	21/54
S	2	4	5	5	5	5	4	4	5	—	39/54

The figures given are the number of differences found between that fraction and the other nine cell fractions at a significance level of $P < 0.05$. See text for explanation.

divided by the total possible differences. A completely homogeneous and totally distinct fraction would therefore have the value 54/54. The tabulation of these values is shown in Table II.

The *Mt*, *Ps-1*, *G-1*, *G-2*, and *S* fractions each differed significantly in one or more respects from all other fractions, while neither the *Ps-2*, *F*, *Mc*, *G-5*, nor the *G-6* fraction differed significantly in any way from each other or from the remaining fractions. Moreover, certain fractions which appeared to be identical biochemically to the same fraction were not identical to each other. For example both the *Ps-2* and *F* fractions are identical to the *Mc*, but they differ from each other, and the *Mc* and *G-6* fractions are both identical to the *G-5*, but they differ from each other (Table II). It should be noted at this point that the statistical significance level selected ($P = 0.05$) probably accounts for this, and furthermore contributes to the number of individual fractions discernable. At a level of significance of 0.1, all fractions can be shown to be unique; at a level of $P = 0.05$, as pointed out, 5 of the 10 fractions are not significantly different; at a level of $P = 0.01$ only two fractions (*Mt* and *S*) may be shown to be different, the remaining 8 being identical to each other. This observation emphasizes the fact that any study attempting to describe a degree of heterogeneity in the cytoplasmic particles of a cell must be considered in the light of a statistical analysis. Unfortunately, this has not been done as frequently as might be desired in the past.

None of the fractions isolated were homogeneous, and differences in degree existed between them. Thus there was a range from 39/54 (*S* fraction) to 16/54 (*G-5* fraction). With the exception of the supernatant liquid, the degree of "heterogeneity" increased as the fractionation proceeded. For example the particulate fractions, listed in order of purity, are (with brackets surrounding equivalents levels), *Mt*, (*Ps-1*, *G-1*), *G-2*, *G-6*, (*Ps-2*, *F*), *Mc*, *G-5*, while the order in which they were obtained in the separation procedure was *Mt*, *Ps-1*, (*G-1*, *G-2*), (*Ps-2*, *F*, *Mc*, *G-5*, *G-6*).

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DISCUSSION

In subdividing a heterogeneous material one assumes that there exists a finite number of entities separable one from another, and that the identity of any one of them will not undergo gross change during the fractionation procedure. Fundamental to this process is a means of characterizing a fraction as unique; that is, of recognizing its individuality among all the other fractions produced. Recently investigators have turned to the use of biochemical properties to distinguish cytoplasmic fractions from one another, as a supplement to the earlier use of microscopical properties.

The number of unique fractions that can be isolated by differential centrifugation from cytoplasm may be large, but finite. The results of the fractionation described herein indicate that the degree of uniqueness of the fractions prepared will decrease as the fractionation procedure is continued. It would be useful, therefore, to employ some means that would enable one to recognize when further fractionation is no longer profitable. This recognition is linked closely to the number of tests required to distinguish successive fractions, or alternatively, to the number of comparisons between fractions which are significantly different. Further fractionation yielded no "new" fractions in the present experiments when about 50% of the comparisons failed to distinguish one fraction from another.

The likelihood that one can determine either the actual numbers of particles present in the cytoplasm of a cell, or their homogeneity, by this type of technique, seems remote. The cutting and subdividing of fractions, as well as the repeated changing of sedimentation conditions which are inherent in this method appear to lend themselves more readily to a dispersion of properties associated with a range of particles than to a collection and centralization. Indeed, as pointed out above, the data obtained in this study, when viewed at a high level of significance ($P = 0.01$), indicate that only two unique fractions had been prepared. On the other hand, the "concentration gradient" method introduced by HARVEY[11], and later employed by other investigators[6,12,13] in which centrifugation is carried out until the various particles come to equilibrium with the medium at the corresponding level of density, would seem to circumvent many of these difficulties. Here again, however, one must consider the number of tests that are necessary to establish individuality of the fractions.

SUMMARY

1. As a cell fractionation procedure of the differential fractionation type is continued in order to create new fractions, the degree of heterogeneity of each fraction will increase. Sufficient criteria must be employed to differentiate between fractions. However, on increasing the number of criteria, one tends also to increase the similarities between fractions. In the present study, when about 50% of the comparisons did not distinguish one fraction from another, further fractionation yielded no "new" fractions.

2. It is unlikely that one can determine either the actual number of particles present in the cytoplasm of a cell, or their homogeneity, by the technique of differential centrifugation employing a single medium. The "concentration gradient" method may be more desirable from this standpoint.

3. It is necessary that data describing the biochemical properties of subcellular particles be subjected to statistical analysis, in order that their value in determining the uniqueness of a fraction be properly assessed.

RÉSUMÉ

1. Au fur et à mesure qu'un procédé de fractionnement des contenus cellulaires du type différentiel est poursuivi, le degré d'hétérogénéité de chaque fraction augmente. Des critères suffisants doivent être utilisés pour différencier les fractions. Cependant si l'on augmente le nombre de ces

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critères, on tend également à accroître les similitudes entre les fractions. Dans le présent travail, lorsque 50% des comparaisons ne distinguent pas une fraction d'une autre, un fractionnement ultérieur ne donne pas de "nouvelles" fractions.

2. Il est peu vraisemblable que l'on puisse déterminer soit le nombre réel des particules présentes dans le cytoplasme d'une cellule, soit leur homogénéité, par la technique de centrifugation différentielle en employant un seul milieu. La méthode du "gradient de concentration" peut être préférable de ce point de vue.

3. Il est nécessaire de soumettre les résultats décrivant les propriétés biochimiques des particules subcellulaires à l'analyse statistique, de façon à établir convenablement leur valeur dans la détermination de l'homogénéité d'une fraction.

ZUSAMMENFASSUNG

1. Während ein Zellenfraktionierungsverfahren, vom Typus Differentialfraktionierung, verfolgt wird, um neue Fraktionen zu schaffen, wird der Heterogenitätsgrad der einzelnen Fraktionen anwachsen. Es müssen Kriterien in genügender Anzahl angewandt werden, um zwischen den Fraktionen unterscheiden zu können. Indem man jedoch die Anzahl der Kriterien erhöht, neigt man dazu, auch die Ähnlichkeit zwischen den Fraktionen zu erhöhen. Wenn bei unseren Forschungen ungefähr 50% der Vergleiche die Fraktionen nicht voneinander unterscheiden konnten, so ergab eine weitere Fraktionierung keine "neuen" Fraktionen.

2. Es ist unwahrscheinlich, dass man entweder die tatsächliche Anzahl der im Zellencytoplasma gegenwärtigen Partikel, oder deren Homogenität an Hand der differentialen Zentrifugationstechnik, unter Anwendung eines einzigen Mediums, feststellen könnte. Die Methode des "Konzentrationsgradienten" dürfte in dieser Hinsicht vorteilhafter sein.

3. Es ist notwendig, die Angaben, welche die biochemischen Eigenschaften von subzellularen Partikeln betreffen, der statistischen Analyse zu unterwerfen, um ihren Wert für die Bestimmung der Einzigartigkeit einer Fraktion abzuschätzen.

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