CELL POPULATIONS

Edited by
ERIC REID, PhD, DSc

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Editor's Preface

with comments on nomenclature

Cell isolation may seem an odd theme for a 'Subcellular Methodology Forum' (Guildford; July 1978) whilst clearly worthy of the desk-book form into which the material has now been hewn. Those concerned with isolating subcellular fractions are, however, increasingly aware of the problem of cell-type diversity that may imply biochemical diversity, and of the desirability of separating from their starting material a particular population of cells that are adequate in health, homogeneity and quantity for subcellular or other investigations.

Whilst this book does not obviate the need to consult literature, its content is largely non-specialist and appropriate for anyone venturing into an unfamiliar field entailing, for example, the study of particular types of blood cell, secretory cell or unicellular parasite. Cancer-cell studies get scant attention here, but will feature in the 7th Forum and in a resulting book (Vol. 11 of this series; 1981).

The evolution of this series.— In case readers or librarians find difficulty in discerning a 'pattern' (cf. the listing opposite the title page), an explanation may help. Like the Wolfson Bioanalytical Centre itself (an R & D laboratory), the meetings and the non-ephemeral books which they generate are distinctive in fostering good methodology, largely in two areas hitherto apt for the term 'biochemistry', albeit far apart. That one area concerns cells is evident from the present book and its companion, *Plant Organelles* (Vol. 9). On the other hand, Vols. 5 and 7 have dealt with the assay of biological fluids for drugs and hormones, i.e. small molecules. With intended extension of the latter area to environmental contaminants (Vol. 10), the term 'biochemistry' becomes inappropriate: the new designation for this sub-series will be *METHODOLOGICAL SURVEYS (A): Trace-Organic Analysis*. The present book inaugurates the sub-series *METHODOLOGICAL SURVEYS (B): Biochemistry*, and will still lean towards 'cellular' rather than 'molecular' aspects (cf. *Biochemical Journal* sections). Whilst volume nos. will remain consecutive, a librarian may prefer to specify only *A* or *B* when building up a run (retrospectively if desired). Editorial gratification is expressed concerning the evolving alliance with Ellis Horwood Limited, as initiated with Vol. 6.

Acknowledgements.– These are multiple and heart-felt, not least to Mrs. R Sarker for skilled sub-editing help as well as typing. The authors were mostly prompt, in spite of other pressures, and invariably good-natured. Dr. D.J. Morré was a notable ally in planning and running the Forum, along with other Honorary Advisers including Drs. G.B Cline, R. Coleman, H. Glaumann and G. Siebert. The Forum was co-sponsored by the European Cell Biology Organization, and received financial aid from company sources: Beckman-RIIC, Ciba Research, MSE, Nyegaard, and Pharmacia. Other publications have sometimes been drawn on, by courtesy of the Editor concerned, as is mentioned where applicable – particularly in certain Table headings and Fig. legends.

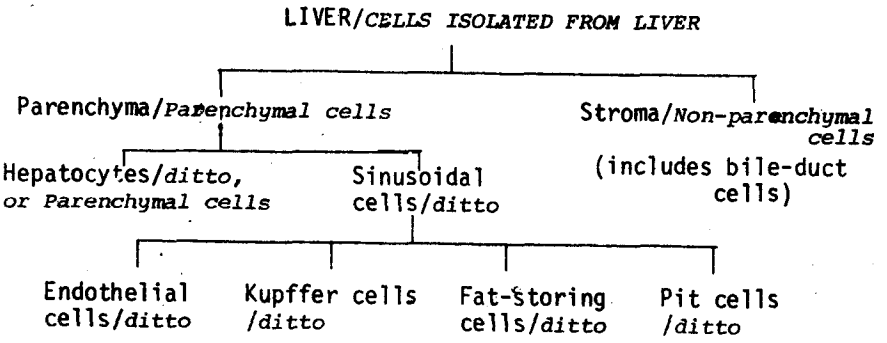
COMMENTS ON NOMENCLATURE

The terminology for cell types in liver, and for dispersions of cells that may or may not be homogeneous, shows inter-laboratory differences which are reflected in this book insofar as authors' preferences have been respected. However, as a contribution to international debate some thoughts are now set down.

Cells within or from liver

At the risk of being in a minority, the Editor and his colleague Dr. R.H. Hinton are inclined to favour stretching the term *parenchyma* to include sinusoidal cells, as in Scheme 1. This scheme

Scheme 1. Nomenclature preferred by the Editor and Dr. R.H. Hinton for cell types observed in liver *or (italics) isolated from liver*, in accord respectively with A.W Ham [1] and E.R. Weibel and co-authors [2]. Note that in some laboratories, especially biochemical, the term *parenchymal (or parenchymatous)* is restricted to hepatocytes, and sinusoidal cells are termed *non-parenchymal*.



gives terms encountered in histology and *(italicized)* in some studies with isolated cells. Elsewhere in this book, however, there is implicit adherence to a simpler scheme and, moreover, designation of sinusoidal cells as *non-parenchymal*, as is noted editorially. "Nonparenchymal cells in the liver of a young adult rat consist of sinusoidal cells, haemopoietic cells, bile duct cells, connective tissue cells and blood vessel wall cells" [3]. With able initiative from D.L. Knook, debate continues in the columns of *Bull. Kupffer Cell Foundation*.

1. Ham, A.W. (1965) *Histology* (5th edn), Lippincott, Philadelphia.
2. Blouin, A., *et al.* (1977) *J. Cell Biol. 72,* 441-455.
3. Knook, E.L. & Sleyster, E.Ch. (1977) *—Ref. 1 in #B-2, this vol.*

Designation of cell preparations in relation to homogeneity

Guidance on nomenclature was sought by the Editor from Honorary Advisers before the Forum, and from Forum participants in a debate chaired and ably summarized by D.R. Headon. Amongst those who contributed helpful views were R.J. Hay, F. Leighton, G. Siebert, J.F. Tait, and notably C.N.A. Trotman — who advocates the term *Isolated cells*, with no cell-type specified, for suspensions of single cells derived from a tissue. Whilst he is in good company, the risk of confusion with purified cells inclines this Editor to favour the nomenclature in Scheme 2, where the first category has alternatives that are acceptable to Trotman (as is *Cell suspension*). Either *Purified* or *Enriched hepatocytes* could, however, be ranked as *Isolated hepatocytes* if desired.

Near-equivalent terms; starting material	*Suspended cells* - as already present in suspension cultures or fluid 'tissues'. *(Dispersed cells)* esp. from *Dissociated cells* a tissue.
From a secondary step (→ fractions)	*Separated cells* - from a fractionation acc. to (e.g.) size or density; individual cell type *not* implied.
Selectivity perhaps by mere digestion; maybe no secondary step	*Purified cells* - attainment of a homogeneous cell population for the desired aim; e.g. *purified hepatocytes*. An *Enriched cell preparation* (e.g. of *hepatocytes*) does not imply assured purity.

Scheme 2. Nomenclature which does not entail use of the term *isolated*. Note that *separated* is an operational term, and that .....*cytes* (e.g. *astrocytes*) implies proved/probable near-homogeneity. The starting material *(cell suspension)* may be sub-categorized as *dissociated*.

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9 April, 1979

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#A Features of Isolated Liver Cells

#A-1
CRITERIA OF METABOLIC COMPETENCE OF ISOLATED HEPATOCYTES

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No single test supplies full information about the metabolic competence of isolated hepatocytes. Exclusion of trypan blue does not always parallel metabolic competence. The trypan blue exclusion test is useful only in a limited way in that if a high percentage of the cells do not exclude the dye the suspension is unsatisfactory. Additional tests are the assay of the total adenine nucleotides, the measurement of the rate of O_2 uptake, of gluconeogenesis from lactate and of the synthesis of urea. The response to glucagon provides information on the integrity of the outer membrane. When a metabolic process is used as a criterion, it must be measured under optimum conditions.

We reiterate that there is no simple single criterion of metabolic competence. We stress this point because many authors offer in their publications the comment that their hepatocytes appeared normal under the light microscope and that over 80% or 90% excluded trypan blue. One gains the impression that this satisfies them that their cells are competent (or as some people like to call it, viable). The trypan blue exclusion test is useful but only in a very limited way. If a high proportion of cells do not exclude trypan blue the suspension is unsatisfactory. Thus the test is a quick and simple indicator of inadequate cells. On the other hand dye exclusion is not a guarantee of metabolic competence. The following examples illustrate this statement.

ASSESSMENT OF METABOLIC COMPETENCE

Hepatocytes from 48 h starved rats were prepared by our modification [1] of the method of Berry & Friend [2]. A small sample (0.2 ml) of cell suspension was mixed with 0.1 ml of trypan blue (0.2% in 0.9% NaCl). At least 200 cells were counted in a haemacytometer. Table 1 shows that aerobic or anaerobic storage of cell suspensions for 3 h at 22° had relatively little effect on

trypan blue exclusion compared with the effect, especially of anaerobic storage, on the subsequent capacity of the cells to synthesize glucose from lactate. Ketogenesis from butyrate, being a mitochondrial process, was resistant to the damaging effects of storage.

Striking differences between changes in dye exclusion and changes in gluconeogenic rate were found on addition of surface-active agents. As illustrated in Table 2, 0.025% Triton X-100 inhibited gluconeogenesis completely but increased the proportion of cells taken up the dye by only 16%. In the case of Cetavlon there was a rough parallelism between dye exclusion and biosynthetic capacity.

Butanol and *n*-pentanol (amyl alcohol), which are also surface-active agents, at certain low concentrations markedly inhibited gluconeogenesis without affecting dye exclusion (Table 3). On the other hand toluene decreased dye exclusion near-parallel with the rate of gluconeogenesis. Again ketogenesis from butyrate was less affected, which confirms that mitochondrial processes are less sensitive to surface-active agents than cytosolic processes. These agents are chemically relatively inert, and they are taken to interfere with cell activity on account of either their lipid solubility or their adsorption at interfaces [3]. In the present context the precise mechanism of their action is unimportant as it is the object of the exercise to demonstrate the limited value of dye exclusion tests. An alternative to the trypan blue test (which stains nuclei) is the addition of succinate together with iodonitrotetrazolium salt (INT) which stains mitochondria within a few minutes. INT is reduced within the mitochondria to a red formazan by the flavoprotein of succinate dehydrogenase. This is essentially a test for the exclusion of succinate which normally does not enter hepatocytes. To 0.2 ml cell suspension are added 0.04 ml of a solution containing 13 mM INT, 60 mM succinate and 80 mM NaCl. In general the two methods give the same results except when the suspensions are treated with both dyes simultaneously. Under these conditions about 5% stain with trypan blue only, possibly because the mitochondria have leaked out of the cell through major ruptures of the plasma membrane (p.m.).

DISCUSSION

The upshot is that the results of dye exclusion tests do not necessarily run parallel with metabolic impairment. Mammalian cells are very complex systems with numerous components, some of which are more sensitive to damaging circumstances than others. Hence there is no short-cut, by way of a simple dye test, to the assessment of metabolic competence.

Table 1. Effect of storage of hepatocytes on trypan blue exclusion and on subsequent capacity to form glucose and ketone bodies. Substrates and initial concns.: 10 mM lactate, 10 mM NH_4Cl, 2 mM ornithine, and 1 mM oleate (for glucose synthesis) or 10 mM butyrate (for ketone body synthesis). Hepatocyte suspensions were stored without albumin. Albumin (25 mg/ml) was present during incubation with substrates for 60 min. The values are the means of 4 experiments, with % change given in parentheses ().

Cell material	Trypan blue exclusion,%	Gluconeogenesis, µmol/min/g	Ketogenesis, µmol/min/g
Fresh	86	1.49	3.07
Stored 3 h; 22°; 5% CO_2 in O_2	71 (-17%)	0.83 (-44%)	2.89 (-6%)
Stored 3 h; 22°; 5% CO_2 in N_2	68 (-21%)	0.50 (-66%)	2.38 (-22%)

Table 2. Effects of surface-active agents (detergents) on trypan blue exclusion and gluconeogenesis from lactate. Substrates and initial concns.: 10 mM lactate, 1 mM pyruvate, 2 mM lysine, 1 mM oleate and 0.1 mM dibutyryl cyclic AMP. Incubation for 30 min at 37°. Dye exclusion was determined after incubation. The % change is given in parentheses.

Agent added	Final concentration,%	Trypan blue exclusion,%	Gluconeogenesis, µmol/min/g
None	-	85	2.08
Triton X-100	0.00625	78 (-8%)	0.90 (-57%)
	0.0125	80 (-6%)	0.39 (-81%)
	0.025	72 (-16%)	0.05 (-98%)
	0.05	2 (-98%)	0.04 (-98%)
Cetavlon	0.00625	85 (0%)	1.68 (-19%)
	0.0125	89 (0%)	1.51 (-27%)
	0.025	28 (-67%)	0.93 (-55%)
	0.05	0 (-100%)	0.20 (-91%)

The intactness of the cell depends above all on the permeability characteristics of the p.m. and of internal membranes, especially the inner mitochondrial membrane, because these membranes are responsible for maintaining the normal and specific intracellular and intraorganelle environment necessary for the physiological operation of the enzymes. If the membranes do not

Table 3. Effects of surface active agents (higher alcohols and toluene) on trypan blue exclusion and on gluconeogenesis and ketogenesis.
Substrates and initial concns.: 10 mM lactate, 2 mM lysine, 1 mM oleate, and 0.1 mM dibutyryl cyclic AMP (for glucose synthesis) or 10 mM butyrate (for ketone body synthesis). Incubation for 60 min at 37°. Dye exclusion was determined after incubation. The values are expressed as % of control.

Agent added	Final concentration, %	Dye exclusion	Gluconeogenesis	Dye exclusion	Ketogenesis
n-Butanol	0.25	98	83	100	96
	0.5	87	47	100	91
	1.0	64	13		
n-Pentanol	0.25	80	2	84	41
	0.5	44	1	47	5
Toluene	0.25	86	88	100	94
	0.5	58	50	69	85
	1.0	2	2		

function normally, especially when they become leaky, the cells discharge low molecular constituents and even larger molecules such as enzymes; this upsets the internal environment.

The implication is that a series of criteria such as those listed below must be used for a reliable assessment of the functional integrity of the cells.

Appropriate criteria

(1) Loss of refraction, as revealed by phase contrast microscopy, indicates unduly swollen and presumably damaged cells.

(2) Staining with trypan blue or other dyes, as already mentioned, is a rough test in as much as major staining is definitely indicative of major damage.

(3) The assay of the total adenine nucleotides provides information on whether the key agents in energy transformations are maintained. Normal values imply that cell respiration and oxidative phosphorylation function normally.

(4) The rate of oxygen uptake is a useful quantitative measurement of the degree of integrity, especially when the time course is followed manometrically.

(5) Rates of gluconeogenesis from lactate or urea synthesis from ammonium chloride under optimal conditions are criteria of the adequacy of energy supply and energy utilization. Urea synthe-

sis is somewhat less exacting than glucose synthesis because it is less dependent on physiological adenine nucleotide concentrations [4].

(6) A further test directed especially towards the check of the integrity of the outer membrane is the response to glucagon. The peptide hormones are taken to exert their effects through receptor sites on the p.m. It would not be surprising if receptors were damaged by the crude collagenase at the perfusion stage, but experience indicates that the receptors still function though it is not certain how much of their original capacity is retained. Tests with glucagon on the stability of glycogen show that very low glucagon concentrations down to 10^{-9} M accelerate glycogenolysis. The responsiveness of gluconeogenesis to glucagon is an alternative check of the integrity of the p.m.

When testing for metabolic competence it is essential to carry out the tests under optimum conditions where rates are at their maximum. It is not good enough to demonstrate merely that gluconeogenesis from lactate occurs. Unless the capacity is stretched to the maximum, partial losses of activity would not be detected. In the case of gluconeogenesis from lactate maximum rates occur in the presence of 10 mM lactate, 1 mM pyruvate, 2 mM lysine (which proved the best agent for restoring and maintaining the amino acids needed for the aspartate shuttle [5]), 1 mM oleate (source of energy) and 0.1 mM dibutyryl cyclic AMP.

For testing the maximum rate of urea synthesis it is necessary to add not only NH_4Cl as a precursor, but also 1 mM ornithine as a catalyst, 1 mM oleate as a source of energy and 2 mM lactate as a precursor of intracellular aspartate required for the formation of argininosuccinate.

There are many other properties one could assess, e.g. the ability to maintain normal intra/extracellular K^+ and Na^+ gradients. However gluconeogenesis appears to be rather insensitive to alterations of the gradients: ouabain markedly decreases the ion gradients and the intracellular concentration of alanine without affecting the rate of gluconeogenesis from alanine [6].

A reason why gluconeogenesis from lactate is a particularly exacting criterion is the fact that it is a complex process which depends on the intactness of the energy supply, the intactness of the intracellular environment (especially the concentrations of the amino acids needed for the aspartate shuttle, the intactness of ATP formation and of the intracellular ATP concentration, the intactness of regulatory mechanisms, the intactness of the p.m. and the mitochondrial membranes). Thus *one* measurement, that of the rate of gluconeogenesis under maximum conditions, tests several key properties. It might be thought that an alternative to gluconeogenesis is urea synthesis because both

involve the intactness of the p.m. and the mitochondrial membrane, but measurement of these rates in slices shows that gluconeogenesis is more easily damaged than urea synthesis. We sometimes do both tests as these are rather simple. In addition, we often measure O_2 uptake because this gives the time course if carried out manometrically. The rate of O_2 uptake should be almost constant during the period of the experiment. The suggestion is that anyone who proposes to work on isolated hepatocytes should satisfy himself that he can reproduce the maximum rates reported in the literature [see 7] before studying new aspects. Merely looking at cells – at their shape and at dye exclusion – is not good enough. Nor is reproducibility of data, because this does not reveal systematic errors. Thus a second or third sample is not a reliable reference standard. An acceptable reference standard for a new test is the isolated perfused intact liver.

Concluding comments

What has been said so far applies to rat hepatocytes. Chicken hepatocytes may require different criteria. We find that chicken liver cells are more resistant to unphysiological conditions. While rat hepatocytes usually lose about 10% of their metabolic capacity per hour, such losses are less in chicken hepatocytes. Furthermore in rat liver the rate of gluconeogenesis from lactate, as already stated, depends on the presence of oleate, cyclic AMP and lysine. In chicken liver these substances do not increase gluconeogenesis from lactate, which always gives high rates (about 6 μmol/min/g wet wt). This is more than twice the maximum rates observed in rat liver.

Finally, we would make a plea for uniform expression of metabolic rates. Some authors use μmol/min per g dry wt. or μmol/min per g wet wt. which are both acceptable. Others use as a reference the number of cells. This makes comparison of the rates of different authors difficult. It also complicates the comparison of different species. Avian hepatocytes are about 10 times smaller than mammalian hepatocytes.

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#A-2

PROPERTIES OF THE CELL SURFACE OF ISOLATED HEPATOCYTES

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The effects on the hepatocyte plasma membrane (p.m.) of enzymic dissociation of liver tissue is evaluated from the viewpoint of damage to the intercellular junctions and to p.m. ectoenzymes. The consequences to the hepatocyte's functional polarity of removal from an organized epithelium are discussed.

The process of dissociating tissues and organs into the constituent cells is somewhat traumatic. In liver, a range of methods for tissue disaggregation have been explored to obtain primary monolayer cultures [1], and current methods now rely almost exclusively on enzymic dissociation using collagenase [2, 3; *see also* P.O. Seglen, *this volume*]. In the present account, the consequences to the p.m. of liver tissue dissociation are discussed from three aspects. First, the effects of the dissociation regime on the intercellular junctions are discussed in the context of current knowledge of junctional morphology and their molecular architecture. Second, damage to p.m. ectoenzymes is assessed by examining the recovery from isolated cells of p.m. 5'-nucleotidase and nucleotide pyrophosphatase. Finally, the effects of removal of hepatocytes, in rendering the cells free from an organized epithelium that separates blood and bile compartments, is examined from the viewpoint of whether biochemically characterized p.m. subfractions are still obtainable.

EXPERIMENTAL

Tissue dissociation and cell separation

Rat livers were dissociated *in situ* by perfusion through the portal vein with a Ca^{2+}-free Hank's medium pH 7.4 at 37°, equilibrated in O_2/CO_2 (19 : 1 by vol.) using a Miller-type re-circulating perfusion apparatus [4]. After flushing out blood from the organ with 50 ml of Hank's medium, perfusion was continued at 10-25 ml/min with 100 ml Hank's medium containing 50 mg collagenase (Sigma Type 1 prepared from *Clostridium histolyticum*) and