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ISOLATION OF TRYPANOSOMES IN REFERENCE TO THE PROGRESS IN MICROBIOLOGICAL PURIFICATION TECHNIQUES

by

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Summary — The most relevant literature data illustrating the progress of separation techniques of cells and viruses are sumarized. The possibilities of centrifugation, filtration, gel-filtration, (liquid-interface) counter-current distribution and chromatography on ion exchangers for the fractionation of microorganisms are reviewed. For each technique mentioned, the applicability to the isolation of trypanosomes is discussed and completed by personal observations. The effect of these techniques on the more fundamental research is illustrated with a few examples.

KEYWORDS : Microbiology; Cells; Viruses; Trypanosoma; Centrifugation; Filtration; Gel filtration; Adsorption; Countercurrent distribution; Chromatography; Ion exchange; Electrophoresis.

Introduction

The separaton and purification of intact cells and viruses is one of the first problems encountered before making a cell culture or an antigen and starting a microbiological production process. The behaviour of a cell under harsh conditions in temperature, pH and pressure makes chemical purification and extraction procedures as so impossible, they provoke unwanted lysis or damage of the cells. Since 1964 (Rabinowitz) no review on this topic has been published. The last decade however modern cell separation techniques were developed, the most important of them will be the subjects of this paper. Trypanosome isolation will receive special attention.

Highly specific isolation techniques, based on temperature optima, resistance to extreme temperatures and culture on selective media, were not treated e.g. the purification of unicellular Blue-green algae by temperature selection, early transfer of colonies and phototactical isolation of the motile strains (Stanier *et al.*, 1971). Such techniques are used for the isolation of many viruses and lymphocytes (Knight *et al.*, 1972; Eiko Suzuki *et al.*, 1972; Kimura and Dulbecco, 1972).

1. *Centrifugation and Sedimentation followed by Decantation*

General Applications

The most commonly used cell fractionation technique is centrifugation or density gradient centrifugation, which separates according to differences in size and density. Fractionation of complex mixtures is possible using suitable sucrose or CsCl gradient conditions. Sometimes two different sucrose gradients are applied to the rotor thereby allowing rate zonal and isopycnic centrifugation simultaneously in the same run (Chucholowius and Rott, 1972). Sucrose and CsCl solutions of the required density however may have high osmotic pressures (Pretlow *et al.*, 1969-1970; Wiebe *et al.*, 1972), therefore solutions of high molecular weight material, e.g. Ficoll, Renografin (E. R. Squibb and Sons) and Dextran derivatives (Holter and Møller, 1959, Stewart *et al.*, 1972) have been alternatively proposed.

Bovine Serum Albumin-gradients in equilibrium centrifugation is applied to separate several differentiation stages of a given cell line (Williams *et al.*, 1972).

Density gradient contrifugation is a well developed tool for the fractionation of different types of erythrocytes (Boyd *et al.*, 1967; Williams and Shortman, 1972), avian erythroid cells (Williams, 1972), leucocytes (Wildy and Ridley, 1958; Böyum, 1968 a and b; Harris and Ukaejiofo, 1970; Shortman *et al.*, 1972) macrophages (Zembala and Asherson, 1970), spermatozoa O'Donnell, 1968), rat brain glial cells (Flangas and Bowman, 1968), hepatocytes (Castagna and Chauveau, 1969 a and b), spleen cells (Mage, 1968 and Plotz, 1967), viruses and rickettsia (Canonico *et al.*, 1972; Dubois *et al.*, 1972; Toplin and Sottong, 1972).

Trypanosome Isolation

The separation of trypanosomes from blood by centrifugation is a well known technique for diagnosis and antigen preparation. To facilitate the laborious centrifugation of trypanosomes many modifications have been proposed e.g. the centrifugation in sucrose gradient (Williamson and Cover, 1966) and the use of hemagglutinine (Simmons *et al.*, 1964).

Limitations and Special Applications

In the case of very heterogeneous starting mixtures however, time spilling centrifugation procedures alone cannot solve all problems, so many workers claim for more efficient isolation techniques, based on other physical and chemical properties, which may be used alone or in combination with centrifugation to complete or to complement purification.

In the industries working with microbiological procedures, engineering problems require more sophisticated and better controlable purification procedures than the classical sedimentation alone. A helpful technique in combination with sedimentation is a flocculation-deflocculation procedure. The flocculation in question is the formation of a loose aggregate of individual cells provoked by adding a small amount of metallic ions such as Al^{+++} or Ca^{++}. Deflocculation can be subsequently obtained by increasing the rotation speed of the impeller. It is now possible to control the whole flocculation-deflocculation mechanism as shown by the experimental and mathematical approach of Shuici Aiba and Masaharu Nagatami (1971).

2. *Filtration*

General applications

Filtration, ultrafiltration and sieving, although one of the oldest purification techniques known, may serve in modern applications to loose problems. Using membranes of mixed cellulose-esters (Millipore, Schleicher-Schüll, Diaflo) water, beer (Millipore Application Guide) wine, urine and other biological liquids may be sterilized (Millipore ADM-20 and Millipore ADM-60). These membranes serve also to concentrate viruses (Rhim *et al.*, 1968; Guskey and Wolff, 1972) and cells (Millipore ADM-50; Wang *et al.*, 1968, Werby and Monley, 1964). Using filter discs with varying pore diameter, ranging from ten microns to a few millimicrons, microorganisms may be isolated according to their size.

Trypanosome Isolation and Special Application

Special membranes are available to combine centrifugation with ultrafiltration (Centriflo®).

Membrane filtration of trypanosomes is also possible and will be discussed in the next paper by Van Meirvenne *et al.* (1972).

Haller (1965) was able to isolate T. M. V., T. R. S. and SBM viruses with special glass filters. These filters which are commercially available, have pore sizes ranging from 20 millimicrons to 250 millimicrons and are therefore not suitable for the filtration of trypanosomes.

3. *Gelfiltration*

General applications

Gelfiltration can be conveniently applied to isolation and purification processes on a micro and on a large scale as well.

Gelfiltration on Sephadex® G-25 completely frees various types of viruses from salts and phenol (Matheka, 1961). Since 1963 many authors have described purification of viruses by gelfiltration (Stegeman and Loeshcke, 1963; Beis and Marx, 1962). Steere (1963) fractionated T. M. V. viruses on agarose according to their length.

On sepharose® Bengtsson and Philipson (1964) isolated Polio virus type 1, Influenza virus type A and Echo virus type 7. Polyoma virus was purified by Inglot *et al.* (1964), Polio virus by Bell and Engler (1964). Fridborg *et al.* (1965) isolated S. V. and T. N. V. viruses, Steere and Ackers (1962) separated T. M. V. and Southern bean mosaic virus, Hollinshead *et al.* (1967) searched for the relationship between the subunits of Adeno virus type 12. Lyons and Moore (1965) isolated the mouse mammary tumor virus. Sugar Cane mosaic virus was purified too by gelfiltration (Baudin *et al.*, 1968). Joustra (1970) separated adeno virus type 5 and polio virus type 1 on Sepharose.

Trypanosome Isolation and Limitations

Gelfiltration on Sephadex and Sepharose, being an excellent technique for the purification of viruses cannot be used to isolate trypanosomes from blood cells, therefore gels with a lower cross-linkage would be needed, which are not available yet. From a cell — and particle — free medium or suspension trypanosomes however can be isolated be gelfiltration (Moors and Van Meirvenne, 1971), gelfiltration on Sepharose 2B offers the opportunity not only to isolate the trypanosomes but also to obtain, with the same run, a good insight in the molecular composition of the fractionated (cell — and particle free) medium; e.g. trypanosomes can be separated from serum or from a buffered solution of macromolecules (with molecular weight below 25,000,000) while the present molecules are fractionated according to their molecular weight which facilitates the research of antibodies, exoantigens and or metabolites. The ideal tool to change completely the buffer of a trypanosome suspension without further fractionation is a gelfiltration on Sephadex G-25.

The viscosity of the samples often limites the cell concentration, that in classic gelfiltrations with columns can be used. To obtain trypanosomes and other protozoa in a suitable buffer solution on a preparative scale (for example for antigen preparation), the basket centrifuge technique with Sephadex is very promising (Gelotte and Emnéus, 1966). It permits the treatment of viscous solutions and gives no sample dilution.

4. *Adsorption*

General applications

By fractionating microorganisms and cells with chemical adsorbentia (aluminium hydroxide and calcium phosphate gel), the greatest part of the living material is irrecoverable. Using such materials a few purification procedures for viruses have been tried (Woods and Robbins, 1961; Pederson *et al.*, 1972) none of them give entire satisfaction.

A more specific adsorption procedure is that of Mosier (1967) using plastic dishes to collect the adhering spleen cells. Cosenza *et al.* (1971) modified the technique of Mosier to obtain a more regular and complete cell separation. The best known techniques however to differentiate lymphocytes is the active adherence method for Shortman *et al.* (1971) which fractionates mouse spleen cells to produce a lymphocyte preparation extensively depleted of phagocytic cells adhering on glass by passage through different columns of glass beads. Feldmann and Palmer (1971) also used this technique in their study of the secondary immune response and Shaipanik and Ciaciola (1972) used this technique in the rapid and large scale separation of macrophages.

Trypanosome Isolation and Special Applications

An other example of the use of adsorbentia is the separation of trypanosomes from blood cells by filtration through glass-gauze.

Recently ultra-specific cell separation and adsorption methods have been developed. Cells were separated by attachement of highly charged haptens, by the specific adsorption of antibody-antigen complexes or by passage through antibody coated poly-urethane foam (Rhie and Sehon, 1972; Basten *et al.*, 1972; Evans *et al.*, 1969; Wofsy *et al.*, 1971). The application of this ultra-specific technique on Trypanosome isolation gives theoretically no problems it is however not published yet.

5. Partition Chromatography

General Applications

The classical partition chromatography (Craig and Graig, 1956) with nocuous organic solvents cannot be used to purify cells and viruses. Recently (Albertsson, 1960; Philipson *et al.*, 1960; Baird *et al.*, 1961; Walter *et al.*, 1968 a and Albertsson, 1970) special two phase systems have been composed for biological material, in which for example two immiscible aqueous mixtures of two different high molecular weight polymers are involved.

Liquid-interface counter-current distribution has been applied on a number of cells. Different strains of *Escherischia coli* (Albertsson, 1961; Albertsson and Baird, 1962) and of *Chlorella pyrenoidosa* (Walter *et al.*, 1970 a) have been separated. The separation of blood cells by this method has been extensively studied (Walter and Selby,1966; Walter *et al.*, 1967; Walter *et al.*, 1968 b; Walter, 1969; Walter *et al.*, 1969). Using this method spleen cells have been equally fractionated in colony forming cell types, granulocytes and antibody producing cells (Brunette *et al.*, 1968).

Different strains of polio virus also have been fractionated (Frick, 1961; Bengtsson and Philipson, 1962-1963). With this technique mutant strains of some viruses could be separated from the wild type; also mutants of the phage $\Phi \times 174$ have been isolated (Walter *et al.*, 1970).

Trypanosome Isolation and Limitations

Theoretically the isolation of trypanosomes from blood and the separation of trypanosome types would be possible with this technique although this was never tried.

Partition chromatography is based on the surface properties of the cell and therefore can complement centrifugation. Two disadvantages have to be mentioned. The counter-current distribution apparatus designed for work with aqueous polymer biphasic systems is more expensive than an ordinary chromatography installation, and secondly the quantities normally handled by the apparatus are relatively small (1-100 mg).

6. Chromatography on ion exchangers

General application

The ion exchangers known before 1940, based on natural or synthetic aluminiumsilicates and polystyrene resins, are not useful for the fractionation of living material, because they all provoke adsorption and denaturation.

Ionic group and Form (counter ions)	Name of the group	Matrix	Trade-mark *
$-O-C_2H_4-NH-C(N^+H_2)=N^+H_2$ Cl^-	guanidoethyl	cellulose	cellex GE
$-O-C_2H_4-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}-C_2H_5$ Cl^-	triethanolamine	cellulose	cellex T
$-O-C_2H_4-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}-CH_2-CH(OH)CH_3$ Cl^-	diethyl 2, hydroxy-propyl aminoethyl	sephadex	QAE sephadex
$-CONH-CH_2-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}H$ Cl^-	diethylamino amidomethyl	polyakryl	biogel DM
$-O-C_2H_4-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}H$ * * Cl^-	diethylaminoethyl	sephadex cellulose polyakryl	DEAE sephadex DEAE cellulose cellex D biogel DE
$-O-R-NH_2$	ecteola (amino-alkyl)	cellulose	cellex E
$-O-C_2H_4-NH_2$	aminoethyl	cellulose	cellex AE
$\vdash(CH_2-CH_2-NH)$ $\vdash CH_2-CH_2-NH_2$	polyethylenimin	cellulose	cellex PEI
$-O-CH_2-C_6H_4-NH_2$	paraäminobenzyl	cellulose	cellex PAB
$-O-CH_2-COO^-$ Na^+	carboxymethyl	sephadex cellulose polyakryl	CM-sephadex CM-cellulose cellex CM biogel CM
$-O-P{<}^{O^-}_{O^-}(O)$ Na^+ Na^+	fosforic acid	cellulose	biorex 70 cellex P
$-O-C_2H_4-SO_3^-$ Na^+	sulfomethyl	cellulose	cellex SE
$-O-C_3H_6-SO_3^-$ Na^+	sulfopropyl	sephadex	SP-sephadex

Ion exchangers available to fractionate cells and viruses.

* Each type ion exchanger is delivered by more manufactories and in many porosity types, only one (trade mark) is given.

* * Double DEAE-groups (pK=9,5 and pK=5,7) are responsible for the second shoulder in the titration curve according to the reaction :

$$\text{Matrix}\ O-CH_2-CH_2-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}-CH_2-CH_2-\overset{C_2H_5}{\underset{C_2H_5}{N}} + H^+ \rightarrow \text{Matrix}\ O-CH_2-CH_2-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}-CH_2-CH_2-\overset{C_2H_5}{\underset{C_2H_5}{N^+}}H$$

Only ion exchangers with cellulose (Peterson and Sober, 1956), Sephadex (Porath and Lindner, 1961) and polyacryl matrices allow effective separations of biological material. The most common types of these ion exchangers with their functional ionic group are represented in table 1.

Since 1964, viruses have been purified on these ion exchangers (Nicol *et al.,* 1964; Giron and Hellman, 1964; Czakó, 1966).

Dextran sulphate and DEAE-Dextran in solution behave as minute ion exchange particles. A simple method for recovering rickettsiae from whole egg homogenates using dextran sulphate has been described (Davies and Patrick, 1965). The purification of viruses on insoluble polyelectrolytes is also possible (Wallis *et al.,* 1972).

Trypanosome Isolation

Separation of trypanosomes from host blood by anion exchangers is also possible (Lanham, 1968, Lanham and Godfrey, 1970); the literature on this subjects as well as a technique for diagnostic purposes will be given by Van Meirvenne *et al.* (1972) in the next paper.

Limitations

The use of ion exchangers for cell fractionation is limited by the following factors. Cells have to be handled in suitable conditions of pH (stability region) and osmolarity (isotonicity). For these reasons sometimes only one or two types of ion exchangers give satisfaction.

An other restriction is that all the mentioned ion exchangers have charged groups in their beads, these groups will not influence the elution of the cells, so the ion exchanger works at a capacity which is lower than the theoretical total one. To surmount partly these inconveniences, it is essential that the flow rate of the buffer is regulated so that it favours the attainment of equilibrium. This generally necessitates low flow rates, the use of a pump is therefore recommended.

7. *Electrophoresis*

General applications

Block and column electrophoresis (Bloemendal, 1963; Maurer, 1971) and electrochromatography (Nerenberg and Pogojeff, 1969) are suitable tools for the isolation of cells. Isolation of viruses and bacteria by isoelectric focusing or by immunoelectroosmophoresis has recently been proposed by Golubev and Gruber (1972), Jeansson (1972), Rice and Horst (1972) and Zhilinskayor *et al.* (1972).

Trypanosome Isolation

The literature about cell separation by electrophoresis is very restricted. The electrophoretic behaviour of trypanosomes has been studied by Hollinshead *et al.* (1963) and by Broom *et al.* (1936).

Samenvatting — **Trypanosoomisolatie in het licht van de vooruitgang op het gebied der microbiologische scheidingstechnieken.**

De literatuur, die de vooruitgang op het gebied van de scheidingstechnieken voor cellen en virussen beschrijft, wordt gegeven. De mogelijkheden geboden door filtratie, gelfiltratie, centrifugatie, tegenstroomverdeling, ionenuitwisselingschromatografie en elektroforese bij de fraktionatie van microörganismen worden aangetoond. Bij iedere behandelde techniek krijgt de trypanosoomisolatie speciale aandacht.

Enkele beperkingen, die bij celfraktionatie nog altijd blijven voortbestaan, worden besproken. De weerslag, die deze technieken zullen hebben op het meer fundamentele onderzoek, wordt verduidelijkt met enkele voorbeelden.

Résumé — **L'isolement des trypanosomes à la lumière des progrès récents dans les techniques de séparation en microbiologie.**

Les auteurs décrivent les progrès récents dans les techniques de séparation de cellules et de virus. Les possibilités offertes par la centrifugation, la filtration classique et sur gel, la séparation à contre courant, la chromatographie sur échangeur d'ions et l'électrophorèse sont indiquées. Une attention particulière est donnée à l'application de ces méthodes à l'isolement des trypanosomes. Certaines restrictions aux techniques de fractionnement cellulaire sont discutées. La répercussion future de ces techniques sur la recherche plus fondamentale est illustrée par quelques exemples.

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