METRIZAMIDE, A NEW DENSITY-GRADIENT MEDIUM

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1. Introduction

Isopycnic centrifugation has proved to be an extremely versatile technique for the fractionation of biological materials. However, there are some restrictions on its use, mainly because of the physical and chemical characteristics of the compounds used to form the density gradients. Ionic strength, osmotic effects, viscosity, solubility and density all place limitations on the usefulness of each density-gradient medium. Therefore, the introduction of a new gradient medium such as metrizamide, the properties of which differ markedly from those of other density-gradient materials, represents a significant advance in the application of isopycnic centrifugation to the fractionation of macromolecules, subcellular components, viruses and cells. This review summarises the properties of metrizamide as a medium for the isopycnic centrifugation of biological particles, and compares the buoyant densities of particles in this medium with their buoyant densities in other commonly used media.

2. Physico-chemical properties

Metrizamide is a tri-iodinated benzamido derivative of glucose with the systematic name 2-(3-acetamido-5-*N*-methyl-acetamido-2,4,6-tri-iodobenzamido)-2-deoxy-D-glucose (mol. wt 789). It is an off-white powder which dissolves readily in water or dilute solutions of salts when added slowly to the solvent with constant stirring. Metrizamide has no ionizable groups and thus forms non-ionic solutions in water. Solutions of metrizamide can be stored indefinitely at $-20°C$ and they are stable at room temperature (in the absence of bacterial contamination) within the pH range 3 to 8.

Exposure of metrizamide solutions to light or excessive temperature leads to the release of iodine from the molecule, therefore all solutions should be kept in the dark and not heated above $55°C$. The iodinated aromatic nucleus of the metrizamide molecule gives rise to a large molar absorption in the ultraviolet region below 300 nm (λ_{max} 242 nm), therefore direct spectrophotometric analysis of nucleic acids or proteins is not possible. However, metrizamide is soluble in ethanolic and acid solutions, hence nucleic acids can be estimated following precipitation and washing. On the other hand, protein can be estimated directly in the presence of metrizamide using the method of Schaffner and Weissmann [1]. In addition, in the case of radioactively labelled material, metrizamide solutions do not cause unacceptable levels of quenching for liquid scintillation spectrometry.

The properties of metrizamide solutions in relation to density, viscosity and osmolality are compared with those of CsCl, sucrose and Ficoll solutions in fig.1. It can be seen that at all concentrations metrizamide forms denser, less viscous solutions than sucrose or Ficoll. In addition, in contrast to Ficoll, the osmolality increases approximately linearly with concentration. Solutions of metrizamide are less dense and more viscous than the corresponding CsCl solutions. However, unlike CsCl, metrizamide does not appear to be highly hydrated in solution so that the water activity of metrizamide solutions is close to unity [2,3]. In consequence, macromolecules and particles appear to be fully hydrated in metrizamide solutions and, therefore, the density of the solution required for isopycnic banding is in general much lower than that required in a solution of CsCl.

Gradients are formed when solutions of metrizamide are centrifuged at high speed in fixed-angle rotors [4,5],
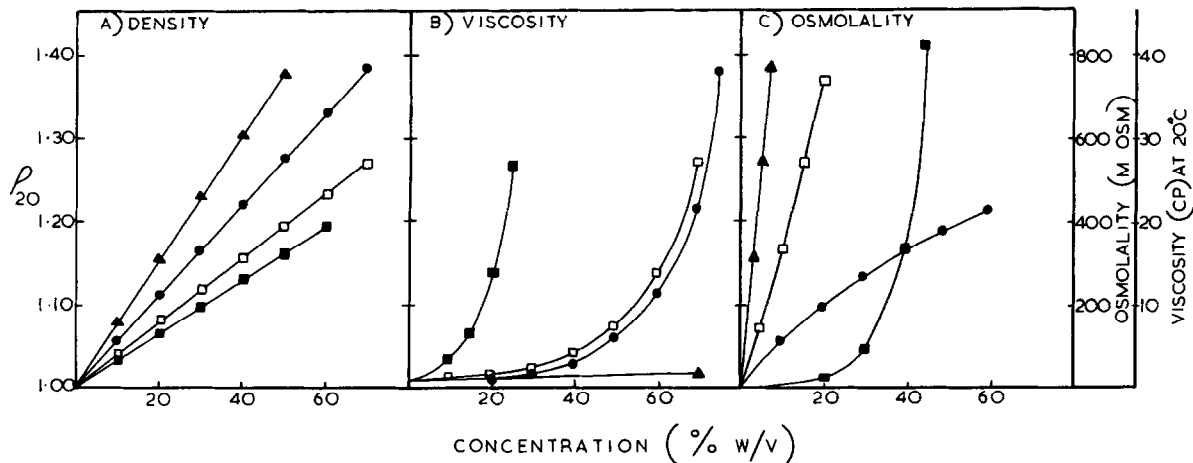
Fig. 1. Physico-chemical properties of CsCl, sucrose, Ficoll and metrizamide. The data is derived from references [32], [41], [42] and shows the effect of concentration on the density, viscosity and osmolality of solutions of CsCl (▲—▲—▲), sucrose (□—□—□), Ficoll (■—■—■) and metrizamide (●—●—●).

but not in swing-out rotors [5,6]. The rate of formation of gradients is slower than in CsCl solutions; it depends on the viscosity of the original solution and the speed of centrifugation [4,5]. Because the gradients form only slowly, the shape of the gradients can be manipulated to optimise the resolution over any particular range of densities [4,5]. Gradients can also be preformed by any of the standard techniques; the shape of such gradients is not greatly changed by centrifugation in swing-out rotors [5,6]. After centrifugation, gradients can be fractionated by any of the usual methods. The density of each gradient fraction can be calculated from its refractive index (after correction for the salt present) using the relationship:

$$\rho_{5°C} = 3.453 \, \eta_{20°C} - 3.601$$

3. Buoyant densities of macromolecules

3.1. DNA

The media most commonly used for the isopycnic centrifugation of DNA are concentrated solutions of the salts of the alkali metals, eg. CsCl, Cs_2SO_4 and NaI. In such solutions DNA bands at high densities (table 1) which depend on the G + C composition.

Denatured DNA bands at densities higher than native DNA. Previously, most non-ionic media were not sufficiently dense to band nucleic acids; in contrast, DNA bands in metrizamide at an extremely low density (table 1), which corresponds to the expected density of fully hydrated DNA [2,3]. The buoyant density of native DNA appears to be independent of its base composition, though that of denatured DNA does seem to reflect the G + C content of the DNA (table 1). However, to prove this relationship rigorously other types of DNA, with a wider variation of base composition, have to be studied.

Since metrizamide solutions are non-ionic, another advantage of metrizamide gradients is that, for the first time, it is possible to study the effect of low concentrations of ions on the hydration of DNA under conditions of high ratios of salt to DNA. In this way we have shown that the hydration of DNA in solution, as reflected by its buoyant density, is affected by both the cations and the anions present in the solution [3]. Metrizamide itself does not interact with, or denature, free DNA [2].

3.2. RNA

For a number of reasons caesium salts are not entirely satisfactory for isopycnic sedimentation studies of RNA [7]. Some species of RNA precipitate in Cs_2SO_4, while even a saturated solution of CsCl is not dense

Table 1
Buoyant densities of macromolecules

	Buoyant density in metrizamide (g/cm^3)	Ref.	Buoyant density in other media (g/cm^3)	Ref.
Native DNA				
$E.\ coli$ (50% G + C)	1.120 (Na^+)	[2]	1.426 ($Cs_2 SO_4$)	[7]
			1.710 (CsCl)	[7]
Mouse (40% G + C)	1.118 (Na^+)	[2]	1.423 ($Cs_2 SO_4$)	[7]
	1.179 (Cs^+)	[3]	1.700 (CsCl)	[7]
			1.522 (NaI)	[8]
Denatured DNA				
$E.\ coli$ (50% G + C)	1.156 (Na^+)	[2]		
Mouse (40% G + C)	1.147 (Na^+)	[2]	1.715 (CsCl)	[7]
			1.574 (NaI)	[8]
RNA				
Ribosomal (60% G + C)	1.170 (Na^+)	[2]	1.61–1.65 (NaI)	[8]
Heterogeneous nuclear (40% G + C)	1.168 (Na^+)	[2]	1.61–1.65 (NaI)	[8]
Proteins				
Urease	1.28 (1.37–1.48)	[2]	1.30–1.32 (CsCl)	[35]
Catalase	1.27 (1.37–1.48)	[2]		
α-casein	1.24 (1.37–1.48)	[2]		
Cohn fraction V serum albumin	1.22 (1.37–1.48)	[10]		
Carbohydrates				
Blue Dextran	1.195	[2]		
Glycogen			1.67 (CsCl)	[39]
Lipids				
Mean value	1.0	[40]		

enough to permit RNA to be banded isopycnically. RNA does band in NaI, but in an extremely heterogeneous manner (range 1.61–1.65 g/cm^3), apparently irrespective of base composition [8]. In gradients of metrizamide, however, both nuclear and cytoplasmic RNAs form single, sharp bands (table 1). The buoyant density of RNA in metrizamide is apparently independent of base composition, though in view of the differences in the amount of secondary structure in these two types of RNA, which might affect hydration and hence buoyant densities, it is perhaps premature to make definite statements at this time.

3.3. Proteins

When protein is centrifuged to equilibrium in metrizamide, much of the protein bands at 1.27 g/cm^3 [9] which is somewhat lower than the buoyant density in CsCl, but which corresponds to fully hydrated protein [2,9] (table 1). However, a significant proportion of the protein bands at densities between 1.37 and 1.50 g/cm^3, that is, at densities greater than that of dehydrated protein [2]. This effect is seen with both monomeric and polymeric proteins, and simple and conjugated proteins, and we have shown that it is due to a transitory association of metrizamide with the

protein [9]. This interaction is extremely weak (K_{diss} > 10^{-2} M), and completely reversible [9]. The evidence available suggests that the interaction is between the iodinated aromatic nucleus of metrizamide and the protein [10]. It is important to note that this interaction is only observed when free proteins are banded; monodisperse nucleoprotein complexes always form single, symmetrical bands in gradients of metrizamide. Conjugated proteins in general band at lower densities than simple proteins. The buoyant densities of these proteins reflect the amount, and buoyant density of the conjugated material as is demonstrated by α-casein and Cohn Fraction V serum albumin (table 1).

The viscosity of the metrizamide gradients, and hence time of centrifugation, can be markedly reduced by using deuterium oxide (density, 1.105 g/cm^3) as the gradient medium [6]. Moreover, it has been shown that density-labelled proteins (containing deuterated amino acids) can be separated from non-labelled proteins on these gradients, and the resolution achieved is superior to that in CsCl or RbCl gradients [6].

3.4. *Other macromolecules*

Carbohydrate material for example Blue Dextran (Pharmacia Ltd.) bands at densities intermediate between those of nucleic acids and proteins (table 1). While extensive studies of the banding behaviour of carbohydrates have not yet been reported, it is likely that their buoyant densities will depend not only on the presence of charged groups, but also on the conformation of the molecules.

The buoyant densities of lipids have not been measured in metrizamide. However, for completeness, the mean density of lipids (kindly supplied by Dr R. Coleman) is included (table 1); it is probably similar in most kinds of non-interacting buoyant density-gradient media.

4. Studies of macromolecular interaction

Metrizamide gradients can be used to study the interaction of small molecules with macromolecules, as illustrated by the studies of the effect of ions on the buoyant density of DNA [3]. In addition, it is possible to study the interaction between macromolecules, in particular between nucleic acids and proteins, since DNA bands with a low density (1.12

g/cm^3), proteins band at higher densities (1.24–1.27 g/cm^3) and DNA–protein complexes band at intermediate densities, depending on the ratio of protein to DNA [2]. However, because of their low mol. wt, proteins are only banded when centrifuged for very long times at high speed, which in turn gives an extremely steep gradient [4,5], the resolution of which is extremely poor. However, when the samples are loaded into the bottom of the gradient the DNA rapidly bands near the top, while the proteins remain in the bottom half of the gradient. The unique advantage of this system over affinity column chromatographic methods is that the DNA–protein complexes formed can be isolated free of contaminating non-complexed DNA and protein. Thus, both the proteins of the complex and the DNA sequences to which they bind can be characterised. Using this system we have been able to study the specificity of the binding of chromatin non-histone proteins to DNA [11].

5. Buoyant densities of subcellular components

5.1. *Deoxyribonucleoproteins*

If nucleoproteins are banded in CsCl then the high ionic strength of the medium causes extensive dissociation of the complexes (see, for example [12]). Therefore, before banding in CsCl nucleoprotein particles must be 'fixed', usually by the formaldehyde method [13], which, however, precludes further analyses of the protein and nucleic acid components. Thus banding in CsCl can only give information on the ratio of protein to nucleic acid in the nucleoprotein complex. The other ionic medium which has been used for the isopycnic banding of chromatin is Renografin (a mixed salt of 3,5-diacetamido-2,4,6-triiodobenzoic acid), the structure of which is similar to the iodinated aromatic nucleus of metrizamide. In dilute solutions of Renografin, DNA bands at slightly higher densities than in metrizamide (1.14 g/cm^3), while chromatin bands at 1.20 g/cm^3 or 1.24 g/cm^3 depending on the method of preparation [14]. However, while dilute solutions of Renografin do not appear to cause extensive deproteinisation of the chromatin, the ionic nature of this compound restricts the possibilities of varying the ionic composition of the gradient.

Prior to the introduction of metrizamide some

attempts were made to band nucleoprotein complexes, particularly chromatin, in non-ionic media. Sucrose combined with glucose [15] has been used, but the high concentrations of sucrose required results in partial dehydration of the chromatin, thus increasing its density. Moreover, these solutions are so extremely viscous, even at room temperature, that only unsheared material can be banded isopycnically [15]. In addition, these viscous gradients are extremely difficult to fractionate. The other medium used to band chromatin is chloral hydrate [16]. However, these gradients are difficult to handle and, in addition, chloral hydrate denatures free DNA [16], and irreversibly inhibits RNA polymerase [17].

When single homogeneous nucleoprotein complexes (e.g. chromatin or histone—DNA complexes) are banded in metrizamide the complex gives a single symmetrical peak, the buoyant density of which is related to the ratio of protein to DNA [2]. However, in all cases the complex bands at a density higher than that estimated from the relative amounts of nucleic acid and protein. The difference between the calculated and observed densities is directly related to the ratio of protein to DNA (fig.2), and may reflect changes in the conformation of DNA and/or protein which result in changes in the hydration of the complex. Ribonucleoprotein particles also band at a higher density than expected, but the proportion of water lost from the complex differs from that of DNA—protein complexes.

In gradients of metrizamide, lightly sheared chromatin forms a single band with a density of 1.200 g/cm^3 (table 2) [18]. When this lightly sheared chromatin is banded at 2°C in metrizamide at low ionic strength, the ribonucleoprotein particles, including the nascent RNA, are released from the chromatin and, because of their greater density, are separated from the chromatin [18]. Thus, for the first time, it is possible to isolate chromatin almost completely free of contaminating ribonucleoprotein material. When chromatin which has been sheared to small fragments is centrifuged to equilibrium in metrizamide, it is found that chromatin consists of interspersed protein-rich and protein-poor regions, which are separable by virtue of the differences in the ratio of protein to DNA [2,4,18]. Metaphase chromosomes can also be banded in metrizamide gradients. However, in order to retain the morphology of the chromosomes, they must be centrifuged in the presence of a stabilising agent such
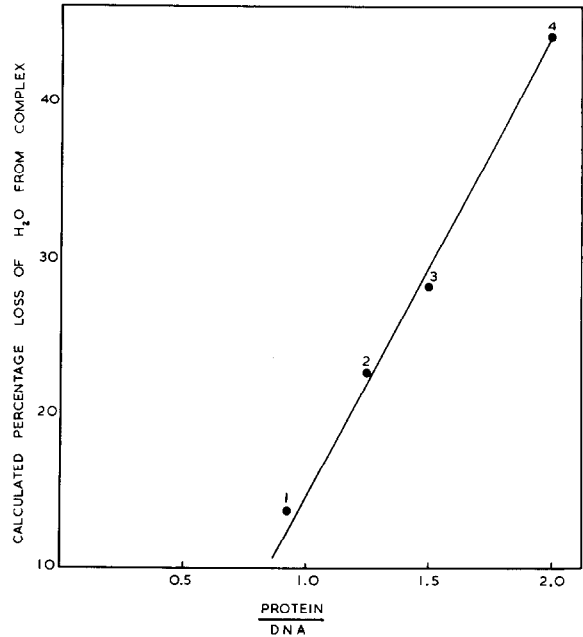

Fig. 2. Effect of composition on the loss of water from DNA—protein complexes. The loss of water has been calculated for: 1) histone—DNA complex; 2) light fraction of sonicated chromatin; 3) unfractionated chromatin, and 4) dense fraction of sonicated chromatin.

as the hexylene glycol buffer described by Wray and Stubblefield [19], or low concentrations of divalent cations [20]. Metaphase chromosomes centrifuged through gradients of metrizamide in the presence of hexylene glycol buffer band at 1.24 g/cm^3 [21], that is at a slightly higher density than interphase chromatin.

5.2. Ribonucleoproteins

These particles also can be banded isopycnically in metrizamide. In the absence of divalent cations the banding densities of ribonucleoproteins reflect the true ratio of protein to RNA in the particles; thus the 50S ribosomal subunit from EDTA-treated polysomes bands at a slightly lighter density than the 30S subunit (table 2) [22]. However, in contrast to deoxyribonucleoproteins, the buoyant density of ribonucleoproteins is dependent on the ionic composition of the gradient. In the presence of Mg^{2+} (3 mM) the densities of the ribosomal subunits, particularly of the larger one, are markedly increased, while that of the messenger ribo-

Table 2
Buoyant densities of subcellular components

	Buoyant density in metrizamide (g/cm^3)	Ref.	Buoyant density in other media (g/cm^3)	Ref.
50S ribosomal subunit	1.214 (Na^+)	[22]	1.60 (CsCl)	[36]
30S ribosomal subunit	1.223 (Na^+)	[22]	1.59 (CsCl)	[36]
60S ribosomal subunit	1.315 (Mg^{2+})	[23]	1.57 (CsCl)	[37]
40S ribosomal subunit	1.230 (Mg^{2+})	[23]	1.49 (CsCl)	[37]
80S ribosome	1.305 (Mg^{2+})	[23]	1.55 (CsCl)	[37]
Myosin mRNP	1.205 (Mg^{2+})	[23]	1.39 (CsCl)	[23]
Chromatin (high mol. wt)	1.200	[18]	1.39 (CsCl)	[13]
			1.20, 1.24 (Renografin)	[14]
			1.357 (sucrose-glucose)	[15]
			1.40—1.60 (chloral hydrate)	[16]
Metaphase chromosomes	1.24	[21]		
Microsomal membranes	1.14—1.26	[24]	1.14—1.26 (sucrose)	[24]
Brain nuclei				
Neuronal	1.275—1.283	[25]	1.287 (sucrose)	[25]
Oligodendroglial	1.262—1.268	[25]	1.348 (sucrose)	[25]
Lysosomes	1.15—1.20	[24]	1.20—1.25 (sucrose)	[24]
Mitochondria	1.20—1.25	[24]	1.20—1.25 (sucrose)	[24]

nucleoprotein particles is essentially unchanged (table 2) [23]. Thus, buoyant density centrifugation permits the isolation of messenger ribonucleoprotein particles irrespective of size and thus allows even large messenger ribonucleoprotein particles to be prepared free of contamination with ribosomal subunits. Using this technique it has been possible to isolate the messenger ribonucleoprotein particle containing the 26S myosin messenger RNA and study both the RNA and the proteins present in the complex [23]. One problem, however, is that of RNA degradation during fraction-ation, since, in the absence of Mg^{2+}, RNA-depleted ribosomal subunits may band at densities greater than expected [22]. Centrifugation times should therefore be kept to a minimum, and it may be preferable to use preformed gradients for these separations. Also, in the case of gradients run in the presence of Mg^{2+} it may be expedient to use D_2O as the gradient solvent in order to minimise the viscosity of the gradient [6].

5.3. Membranes

Few studies of the buoyant densities of membranes in metrizamide have yet been done. However, Aas [24]

has shown that microsomal membranes, as detected by marker enzymes, band over a broad range from 1.14—1.26 g/cm^3, that is at densities similar to those in sucrose. The relatively wide range of banding densities may reflect the heterogeneity of these membranes, particularly with respect to the ribonucleoprotein content of the membranes.

5.4. Nuclei

Whereas in the case of chromatin the banding density is related only to the ratio of protein to DNA, nuclei are more complex in that they may contain a considerable amount of ribonucleoprotein material; in addition, they are enclosed in a double membrane. Moreover, in order to maintain the integrity of the nucleus, it is necessary to add divalent cations to the gradient medium. The actual composition of nuclei is related to their trans-criptional capacity. Thus, nuclei which are actively synthesizing RNA tend to have a high ratio of protein to DNA and, moreover, they contain high amounts of RNA as compared to nuclei from inactive cells (e.g. avian erythrocytes). Therefore it is possible to frac-tionate nuclei by isopycnic sedimentation, as was

Table 3
Buoyant densities of cells and viruses

	Buoyant density in metrizamide (g/cm^3)	Ref.	Buoyant density in other media (g/cm^3)	Ref.
Cells				
Rat liver parenchymal	1.12	[32]	1.09−1.14 (Ficoll)	[31]
Rat liver non-parenchymal	1.08	[32]	1.09−1.14 (Ficoll)	[31]
Viruses				
Murine sarcoma virus (Harvey)	1.11−1.14	[38]	1.16 (sucrose)	[38]
Vesicular stomatitis virus (VSV)	1.11	[33]		
VSV Core	1.13	[33]		

shown by the results of Mathias and Wynter [25] who fractionated brain nuclei from different cell types according to their transcriptional activity. Thus neuronal nuclei, which are actively synthesizing RNA, band at a higher density than the more inactive oligodendroglial nuclei (table 2).

5.5. Lysosomes and mitochondria

Both of these types of organelles are also enclosed in a membrane but they are more sensitive to changes in the osmotic strength of the medium than are nuclei. One of the major problems in working with either of these organelles is cross-contamination with the other. They are of similar size and, when sedimented to equilibrium in sucrose, both band over the same range of densities. However, in metrizamide, while the mito-chondria band at the same density as in sucrose, the lysosomes band over a lower range of densities, perhaps reflecting the differential swelling of these organelles in the slightly hypotonic medium. Thus, by using metriz-amide gradients, it is possible to obtain a complete separation of these organelles by isopycnic sedimen-tation [24].

6. Buoyant densities of cells and viruses

6.1. Cells

Cells, like lysosomes and mitochondria, are very sensitive to changes in the osmotic strength of the medium in which they are suspended. Therefore, sucrose is unsuitable for fractionating cells by isopycnic

sedimentation, since the osmolality is extremely high at the densities required. Other compounds which have been used for this purpose include colloidal silica [26] and metrizoate, either by itself [27] or in combination with Ficoll [28]. However, colloidal silica can bind to membranes and alter enzymic activities [29], while metrizoate binds extremely tightly to proteins [10,30]. Solutions of Ficoll alone have also been used to fractionate cells [31]. However, isotonic solutions of Ficoll only have a density of 1.13 g/cm^3, while isotonic metrizamide solutions have a density of 1.21 g/cm^3 [32]. Moreover, the viscosity of Ficoll solutions is much higher than that of metriz-amide solutions (fig.1). The fractionation of rat-liver cells in gradients of metrizamide has been reported [32], and the separation appears to be superior to that obtained with Ficoll gradients. Another advantage in using metrizamide gradients is that cells killed or damaged during the manipulations prior to fractiona-tion on the gradient band at extremely high densities (>1.16 g/cm^3), and they can thus be completely sepa-rated from the viable cells [32]. Metrizamide does not appear to affect the integrity of the cells or alter irre-versibly their enzymic activities.

6.2. Viruses

Viruses are frequently purified by isopycnic centri-fugation in dense solutions of sucrose. However, this method is hampered by the viscosity of the medium and such virus preparations may be contaminated by co-sedimenting cellular debris (for example ribosomal material). Metrizamide gradients offer some advantages,

since they are much less viscous, and the ribosomal particles are generally much denser than the virus particles. This method has now been used for murine sarcoma virus (Harvey), vesicular stomatitis virus and herpes simplex virus. In the case of vesicular stomatitis virus it has proved possible to separate virion cores from intact virions on metrizamide gradients [33]. The infectivity of herpes simplex virus recovered after centrifugation in metrizamide is dependent on the pH of the gradient, the minimum amount of inactivation (approx. 30%) occurring when the gradients are run at pH 6.5 in phosphate buffered saline containing 0.2% calf serum [34]. Prolonged centrifugation (longer than 12 hr) of the virus does lead to the production of non-infective virus, which, however, bands at a different density from the intact virus [34].

7. Conclusions

The usefulness of any density gradient medium is extremely dependent on the reactivity of the compound towards biological materials. Therefore the weak interaction of metrizamide with protein is one distinct disadvantage of this new compound. However, this binding appears to be significant only when free proteins are banded, and there is, as yet, no evidence that metrizamide interacts with other biological macromolecules. The large molar absorption of metrizamide in the ultraviolet region is also a disadvantage in that it hampers optical analysis of the gradients, but frequently alternative methods of analysis are readily available. On the other hand, metrizamide does have many advantages over other buoyant density-gradient media. Its non-ionic nature, chemical inertness and low degree of hydration in solution, coupled with the relatively low viscosity of its aqueous solutions and the wide range in densities over which these solutions can be used, should make metrizamide extremely useful for the separation of biological particles and macromolecules by buoyant density-gradient centrifugation.

Acknowledgements

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