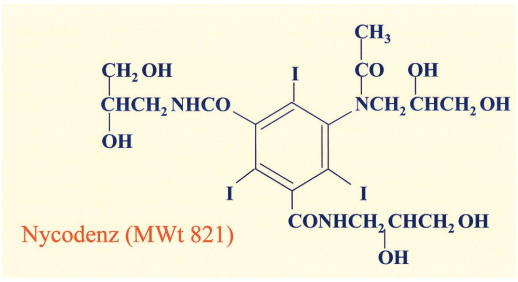
Iodinated density gradient media

Molecular structure

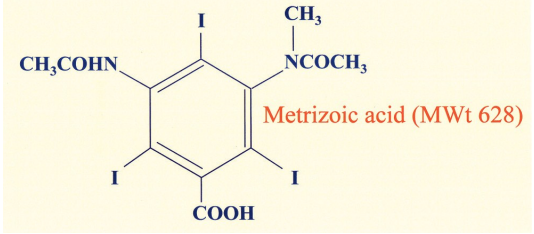
Iodinated media are derivatives of triiodobenzoic acid and capable of forming solutions that are uniquely capable of banding any biological particle, according to its buoyant density, often under isoosmotic conditions. In 1968 Bøyum published his famous density barrier method for the isolation of mononuclear cells from human blood that used sodium metrizoate (see figure) and a polysaccharide. This technique received universal approval and Nygaard & Co. in Norway was the first company to introduce a ready-made medium in 1973 based on his original formula. The product is called Lymphoprep™ and more recently metrizoate has been replaced by diatrizoate (see figure).

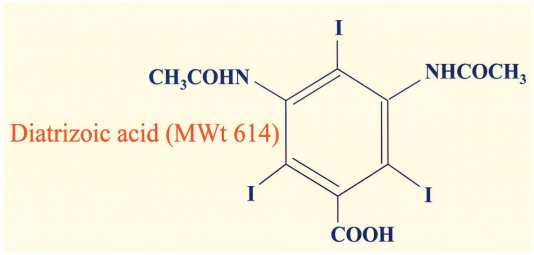
Both metrizoate and diatrizoate are ionic compounds which are able to interact with other charged groups on biological particles and also influence the distribution of ions across membranes, thus the development of non-ionic iodinated compounds was the next important step. Metrizamide was introduced in 1974 and Nycodenz® (iohexol) in 1982. Both of these non-ionic gradient media can form solutions of high density (>1 .30 g/ml at 60% w/v); in metrizamide the carboxyl group present in metrizoic acid is linked to glucosamine, while in iohexol the carboxyl group is linked to the amine group of 3-amino-1,2-propanediol. This difference gives iohexol some advantages over metrizamide, notably its lower toxicity towards cells and autoclavability. The systematic name of iohexol is 5-(*N*-2,3 dihydroxypropylacetamido)-2,4,6-triiodo-*N,N'*-bis(2,3-dihydroxypropyl)isophthalamide.

The molecular structure of Nycodenz® (iohexol) is given in the figure below. Its molecular mass is 821.



The latest addition to Axis-Shield's nonionic iodinated compounds is **iodixanol**. The systematic name of iodixanol is 5,5 -[(2-hydroxy-1,3-propanediyl)-bis (acetylimino)]bis-[*N,N* bis (2,3dihydroxypropyl)-2,4,6-triiodobenzenedicarboxamide] whose molecular structure is given in the figure below. Its molecular mass is 1550 and it is essentially a dimer of iohexol.

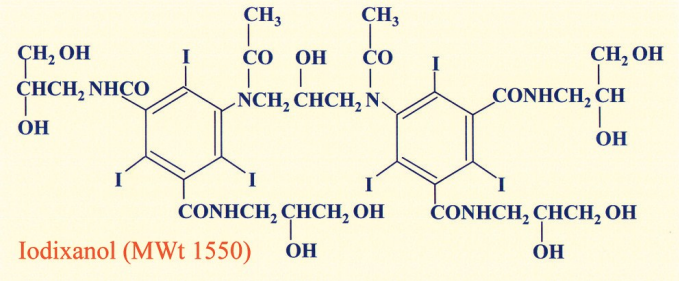




OptiPrep™ is the trademark name for a sterile 60% (w/v) solution of iodixanol in water.

The chemical properties and stability of Nycodenz® and iodixanol are related to their structure. Their high density derives from the presence of the substituted triiodobenzene rings which are linked to a number of hydrophilic groups.

Solubility, stability and spectroscopic properties



The highly hydrophilic side chains of **iohexol** and **iodixanol** make these media readily soluble in water. At pHs below 9 solutions are stable almost indefinitely. Above pH 9 there is a very slow generation of inorganic iodine, but even after 12 months this represents a degradation of only 0.01% of the compound. Concentrations in water up to 80% (w/v), equivalent to a density of approx 1.426 g/ml can be prepared. Iohexol and iodixanol are also soluble in formamide, dimethylformamide and in ethanolic solutions; concentrations up to 50% (w/v) are possible. Non-aqueous media may be of particular use for minimizing loss of water soluble molecules from some biological particles, particularly nuclei and mitochondria.

Prolonged exposure of Nycodenz® and iodixanol to sunlight over a period of months, and to a lesser extent, to artificial light, may lead to the release of iodine from these compounds, but the extent of the reaction is generally insignificant when working with solutions on a day to day basis.

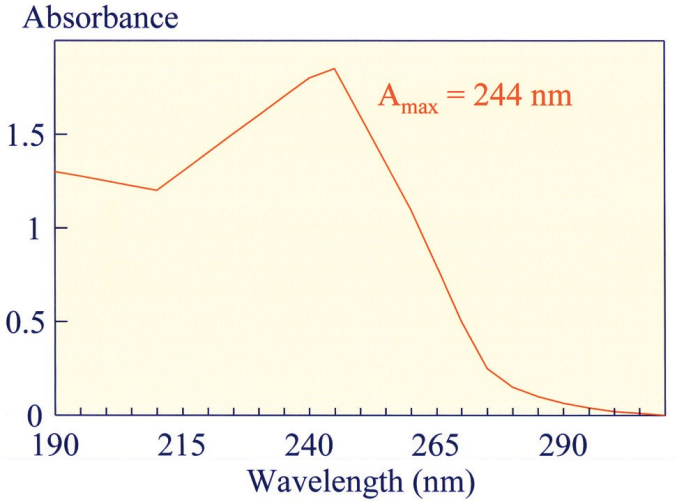
The iodinated aromatic nucleus absorbs strongly in the ultraviolet region of the spectrum (see figure below) with an absorbance maximum of approx 244 nm; this can be used as a direct measurement of the concentration of the medium in gradient fractions (see “Analysing density gradients”).

Iodixanol and Nycodenz® in solution are stable to heat and may be autoclaved; stability to autoclaving is enhanced by the presence of millimolar concentrations of an organic buffer such as Tris and also Ca/Na-EDTA.

Density, osmolality and viscosity

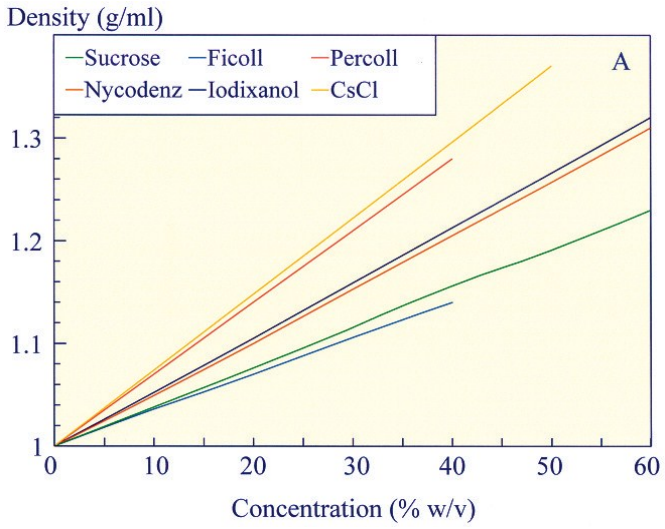
These important physicochemical properties of Nycodenz® and iodixanol solutions have been fully investigated and some of the data is summarized in the figure on the next page, which shows the relationships between density, osmolality, viscosity and concentration of aqueous solutions, measured at 20°C. Comparative data for some other media in common use for the fractionation of biological material is also included.

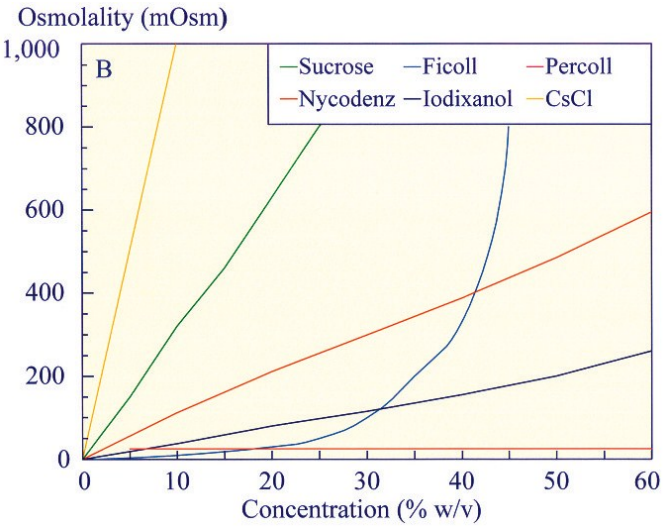
Expressed in terms of % (w/v), for any given concentration, solutions of Nycodenz® are denser than those of

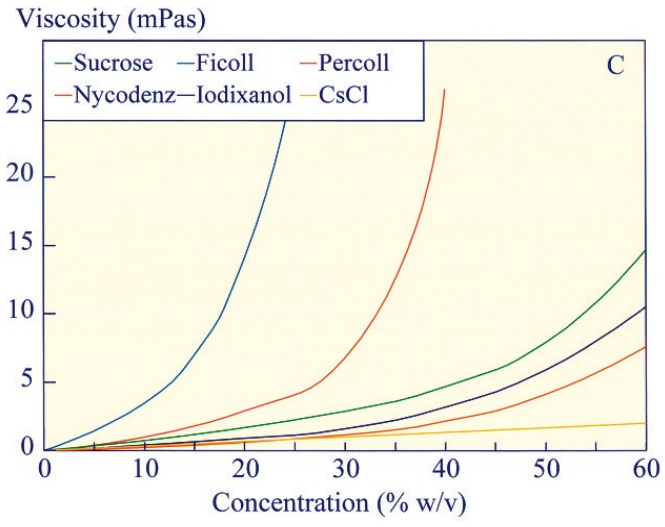


sucrose or Ficoll®, less dense those of CsCl or Percoll® and almost identical to those of iodixanol.

The osmotic behaviour of solutions is often critical when choosing a gradient medium. When separating cells (lacking a rigid cell wall) or subcellular organelles it is most important to maintain a constant osmolality throughout the gradient. Exposing cells, organelles and membrane vesicles to changing osmolality will alter not only their volume and hence their density, but their viability and function might also be impaired. The resolution of these biological particles depends on differences in density, making osmotic conditions in the gradient an important factor. Less obviously, the density of many viruses, nucleic acids and proteins, and consequently







their banding and resolution in gradients, may also be significantly affected by the osmolality of the medium. Moreover changes in the hydration of nucleic acids and proteins which occur in media of high osmolality may affect their molecular stability.

The use of polysaccharides such as Ficoll® is limited not only by their density range but also by the non-linear relationship between concentration and osmolality. Sucrose, Nycodenz® and iodixanol exhibit a near linear relationship between concentration and osmolality. The osmolality of Nycodenz® solutions is considerably lower than that of sucrose (and CsCl) but above a density of approx 1.16 g/ml (approx 30% w/v) Nycodenz® solutions are hyperosmotic, although even at 50% (w/v), which is equivalent to a density of 1.265 g/ml (greater than that of the great majority of biological particles) the osmolality is only 485 mOsm. At the densities which are commonly used to isolate subcellular organelles, sucrose (and inorganic salt) solutions have osmolalities in excess of 1500 mOsm. Percoll® solutions in water do not exhibit any significant osmolality since Percoll®, is a colloid rather than a true solute.

Because of the higher molecular mass of iodixanol, the osmolality of its solutions (calculated from freezing point depression measurements) is less than half that of Nycodenz® solutions of the same density. It is technically difficult to measure the osmolality of concentrations of iodixanol above 50% (w/v). Vapour pressure measurements for example give a lower value (170 mOsm) than freezing point measurements (approx 260 mOsm) at 60% (w/v). The reason for this is not clear but may be related to the tendency of iodixanol to form oligomers at these higher concentrations and thus reduce the effective number of particles in solution. This has no effect on the density but it affects colligative properties such as osmolality. However when OptiPrep™ is diluted with an isoosmotic medium to provide a solution containing 40-50% (w/v) iodixanol, the osmolality is easily measured and reproducible and in the range 285-305 mOsm.

In this manner working solutions can be produced which are iso-osmotic with the biological particles being fractionated. See “Preparation of gradient solutions” for more details.

Solutions of Nycodenz®, are less viscous (see viscosity figure) than any other gradient medium, except CsCl, thus particles sediment very rapidly in this medium. When very dense gradients are required, the viscosity can be reduced even further by using deuterium oxide

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(D_2O) as the gradient solvent (ρ = 1.105 g/ml). However, partial substitution of D_2O for H_2O in the hydration shell of particles is likely to lead to an increase in their effective buoyant densities.

Although the viscosity of iodixanol solutions is slightly higher than those of Nycodenz®, it is lower than those of sucrose. Since however many particles exhibit densities in iodixanol that are much lower than those in sucrose (and often lower than those in Nycodenz®), the density range of gradients of iodixanol required to band the particles is also lower. Consequently these gradients usually have a very low viscosity.

Biological properties

A number of lines of evidence point to the lower toxicity of Nycodenz® and iodixanol compared to all other gradient media. Both Nycodenz® and iodixanol, as widely used X-ray contrast media (Omnipaque™ and Visipaque™ respectively), have undergone extensive clinical testing. Comparative clinical studies with other X-ray contrast media have shown the non-toxic nature of Nycodenz®. In clinical trials iodixanol has been shown to have extremely low acute toxicity in rodents and the LD_{50} was higher than for any other X-ray contrast medium tested. Upon injection it is rapidly excreted by the kidneys in an unchanged form and in clinical trials iodixanol showed a lower frequency of adverse effects, compared to other media.

Studies have shown that iodixanol does not bind to proteins in human plasma and neither iodixanol or Nycodenz® have significant effects on cell morphology or on cell growth, nor are they metabolized by cells. MOLT-4 T cells have been grown as monolayers in a standard RPMI medium supplemented with 5% (w/v) iodixanol. After 1-72 h the increase in viable cell number, as judged by the MTT Test was identical to control cells grown in the absence of the medium. Confluent monolayers of human embryo lung fibroblasts can be exposed to 30% (w/v) iodixanol in culture medium for up to 3 days without any change in cell viability or subsequent plating efficiency. Nycodenz® solutions have also been shown to be very resistant to bacterial degradation.

References are available on request. More information can be found at: www.axis-shield-density-gradient-media.com

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