sedimentation and diffusion fluxes of the salt compensate each other. This equilibrium depends on the nature of the salt, the rotor speed and the temperature. If, initially, macromolecules have been added to the salt solution, and if their density in this solution is about equal to the solution density, during centrifugation they will form a more or less sharp band; they will occupy a position in the centrifugation medium where their apparent, or buoyant density, is equal to the local density of the salt solution. The position and the width of the band will also reach an equilibrium state.

III.2 THE DENSITY GRADIENT

Isopycnic equilibrium centrifugation is an equilibrium method, and hence could be studied very rigorously by the methods of equilibrium thermodynamics.

The value of the density gradient dp/dr, at each point of the centrifuge tube can thus be calculated, and is given by the general equation of equilibrium centrifugation (Svedberg and Pedersen, 1940; Meselsonetal.,1957: Vinograd and Hearst 1962), i.e.

III - 1
$$\frac{d\rho}{dr} = \frac{M\,(1 - \bar{\nu}\rho)}{RT} \times \frac{d\rho}{dLna} \times \omega^2 r$$

III - 2
$$\frac{d\rho}{dr} = \frac{\omega^2 r}{\beta}$$

The symbols of equation 111-1 have been defined in the preceding chapter. Let us specify that M, $\bar{\nu}$ and a are respectively the molecular weight, the partial specific volume and the thermodynamic activity coefficient of the gradient material. The term β takes together all the parameters which depend on the nature and concentration of the salt. Table A.3 gives the β-values as a function of density for a certain number of gradient materials; values relative to other salts are published by Hu et al. (1962), Ifft et al. (1970) and by Tsong (in Costello and Baldwin, 1972).

Actually, the density gradient given by equations 111-1 and III-2, or compositional density gradient, represents only a fraction, albeit the largest of the total, or effective density gradient/One should add two other terms to the equations, one being the macromolecular density gradient, the other being due to the difference of compressibility between the different components of the solution (Vinograd and Hearst, 1962). With the hypothesis of constant B (case of CsCI, around p = 1.7), one predicts that the compositional density gradient increases linearly with the distance to the rotor axis. But in the small swinging bucket rotors, and below 40,000 rpm this gradient is actually constant (Ifft et al., 1960: see also Figure 15). This constancy is probably an artifact, since the gradient material is redistributed during the

deceleration period of the rotor, especially at both ends of the gradient, and that it is only after the rotor has come to a rest that the measurements can be done.

One should especially remember that the density gradient dp/dr is proportional to the square of the rotor speed and that it depends on the nature of the gradient material.

In the example of Figure 15, the density of a few individual fractions has been plotted versus the fraction number. The slope of the resulting curve is necessarily equal to the compositional density gradient. Knowing that the distances of the meniscus and gradient bottom to the rotor axis are equal

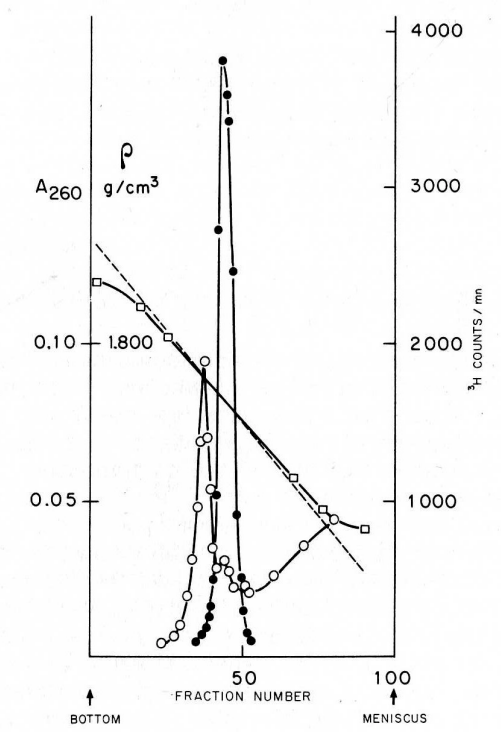

Figure 15. Equilibrium isopycnic centrifugation of a mixture of two DNA's

One of the DNA's, extracted from E. coli, was labeled with tritiated thymidine. The experiment was performed in the SW 39 rotor, at 32,000 rpm and 21°C. The polyallomer tubes contained 3 ml of CsCl solution, with an initial density of 1.76 g/cm , the pH was set to 12 with ammonia. The tubes have been filled up with paraffin oil. The gradients were fractionated from the bottom. • - •, radioactivity; O - O, absorption at 260 nm;-, experimental density variation, obtained from the refraction index of a few individual fractions; □—□ theoretical density variation (see text).

to r_m = 7.00 and rb = 9.75 cm respectively, one obtains an experimental density gradient equal to 7.15 $x10^{-1}$ g/cm^3. On the other hand, for a "mean" distance (see below the definition of the isopycnic point) of the gradient to the rotor axis of 8.41 cm, and for a mean p-value equal to $1.20x10^9$, one calculates according to equation III-2 that the compositional density gradient is equal to:

$$\frac{d\rho}{dr} = \frac{1.12 \times 10^7 \times 8.41}{1.20 \times 10^9} = 7.88 \times 10^{-2} \text{ gm/cm}^3$$

The small difference between the experimental and calculated values could be due to the redistribution of the CsCI during deceleration, or to a small error in the rotor speed.

For the centrifugation of cells, viruses, or subcellular fractions (section III.7), preformed gradients should often be prefered to equilibrium gradients. Special precautions have then to be taken in order to preserve the initial density gradient during centrifugation (section III.5.a).

III.3 MEASUREMENT AND SIGNIFICANCE OF THE BUOYANT DENSITY

One of the applications of isopycnic centrifugation is to measure the buoyant density of macromolecules or subcellular fractions which, in turn, yields some information about their composition (see below, and section III.7). However, the measurement of buoyant densities by preparative centrifugation is usually less accurate than by analytical centrifugation.

Let us consider the experiment of Figure 15. From the experimental density curve one observes that the fraction where the concentration of the radioactive DNA is maximum has a density of 1.765 g/cm^3; hence, this is the buoyant density of this DNA. It is known, however, that the density of E. coli DNA above pH 11.7 is equal to 1.766 (Vinograd et al., 1963). The agreement between both values seems particularly good, especially in view of the fact that after fractionating the gradient some evaporation of the water of the individual fractions may occur, which would lead to an overestimate of the densities. (An error of 0.003 g/cm^3 in the measurement of the buoyant density of DNA in neutral CsCI gradients, would introduce an error of 4 units in the estimation of the [G + C]/[A + T] percentage.)

Another method of measuring buoyant densities is to use a density marker. In the experiment of Figure 15 the radioactive DNA was actually used as a marker molecule. The figure yields a density difference of 1.778 - 1.765 = 0.013 g/cm^3 between both DNA's; knowing that the density of the marker is equal to 1.766 g/cm^3, one gets 1.766 + 0.013 = 1.779 g/cm^3 for the unknown DNA.

Both preceding methods make use of the density versus fraction number curve. It has already been noticed that in the first method the evaporation of water may give overestimates of the buoyant density. In the second method another source of error has to be considered: owing to the restricted diffusion at both ends of the liquid column, the density gradient decreases during the deceleration period of the rotor. Thus, the second method could lead to a certain underestimation of the measured buoyant density. In practice, however, the error is negligible if the two macromolecular species band in the central region of the gradient, which implies that the mean density of the gradient be adjusted correspondingly.

A third, very accurate method to measure a buoyant density, is to use two density markers, one on each side of the unknown band. One has to assume that the density between the three bands varies linearly with distance, which is usually the case (see Figure 15). This method has been used to measure the molecular weight of the DNA of phage K mutants which have either an insertion, or a deletion (Baldwin, 1971; Bellet et al., 1971). In case where the measurement is made via^the buoyant density of the whole phages, preparative centrifugation is suitable. The method has also been used to determine the DNA molecular weight of phages able to recombine with *K* (Baldwin et al., 1966). This method does not require the measurement of the density of individual fractions.

Still a fourth method can be used when no marker molecule is available or if, for some reason, the density of the individual fractions cannot be measured. In case where [3 can be considered as constant, the density difference p2- pi between two points whose respective distances to the rotor axis are r2 and n, is obtained upon integration of equation III-2, i.e.

III - 3
$$\rho_2 - \rho_1 = \frac{\omega^2}{2\beta}(r_2^2 - r_1^2) = \frac{\omega^2}{\beta} = (r_2^2 - r_1^2)\frac{r_2 + r_1}{2}$$

In particular, this equation is applicable when $r_2 = r_o$ is the distance to the rotor axis of the macromolecular band center and when $r_1 = r_i$ is the corresponding distance of the point where the density is equal to the mean density, or initial density pi of the gradient. The buoyant density θ of the macromolecules is then given by:

III - 4
$$\theta = \rho_i + \frac{\omega^2}{2\beta}(r_o^2 - r_1^2)$$

The major drawback of this method is that it requires an accurate knowledge of ρ_i, r_i, which is called the isopycnic point, and r_o.

ρ_i can be obtained, either from the knowledge of the initial gradient material concentration, or from its direct measurement on an aliquot before centrifugation (section 111.8).

According to Ifftetal. (1961), the isopycnic point is calculated from the distances to the rotor axis of the meniscus (r_m) and gradient bottom (r_b). For cylindrical tubes it is given by:

III - 5 $$r_i^2 = \frac{r_m^2 + r_m r_b + r^2}{3}$$

whereas for sector-shaped cells and, hence, for zonal rotors, one has:

III – 5bis $$r_i^2 = \frac{r_m^2 + r_b^2}{2}$$

Equations III-5, hold only if β is constant, which is the case in CsCI gradients for densities comprised between 1.60 and 1.75 g/cm^3, i.e. for DNA centrifugation. If 5 ml cellulose nitrate tubes contain 3 ml of gradient solution, one has $r_b - r_m = 2.75$ cm; the r_b values of the SW39, SW50, SW50.1 and SW65 rotors are given in Table A.1. With r_b and r_m, one than calculates r_i. Knowing the total fraction number and the fraction where the concentration of a given macromolecular band is maximum, the knowledge of r_b and r_m allows the calculation of r_o. In the experiment of Figure 15 one finds $r_i = 8.41$ cm, and, for the unlabeled DNA, $r_o = 8.65$ cm. Applying equation III-4, it follows that $\theta = 1.781$. Owing to the relative inacurracy of the different distances, the agreement with the buoyant density determined by the second method, appears to be exceptionally good.

Equation III-3 has an important practical conclusion, namely that the distance between two bands is almost inversely proportional to the square of the rotor speed. We shall come back to this point during the discussion of the resolving power.

Buoyant densities as determined by the four preceding methods, can sometimes be completely wrong, especially with DNA (Sinclair, 1974). With large concentrations, the viscosity of the DNA bands becomes very important and the flow of the gradient through the fractionation hole is no longer lamellar. It follows that DNA will appear in fractions which precede those in which it should be normally contained. In order to avoid this artefact, the DNA concentration should be as small as possible. Sinclair (1974) also suggests to reduce its molecular weight (if allowed by the purpose of the experiment) by several passages through a syringe needle.

Finally, a fifth method, similar to those used in analytical centrifugation, can be used to measure the buoyant density of DNA. If the DNA is labeled with ethydium bromide (Watson et al., 1971; see also section lll.7.a), the tubes are photographed, and the photographs are read with a microphotodensitometer. With unlabeled DNA, the centrifugation is performed in quartz tubes which are scanned at 260 nm with an appropriate apparatus (Olpin and Burger, 1972). In both cases, it is necessary to use a density marker which can be a known DNA, special fluorinated macromolecules, or plastic beads (Olpin and Burger, 1972), and equation III-3 is applied to both bands. With

the Watson et al. (1971) method, and replacing the cesium chloride by sodium iodide, it should be particularly easy to measure buoyant density differences between two DNA's; in addition, the method requires only 0.05 jig of DNA.

What is the significance of buoyant densities measured by isopycnic centrifugation? Williams et al. (1958), Baldwin (1959) and Hearst and Vinograd (1961, a) have shown that if 0 designates the buoyant density of a given macromol.ecule, then

III – 6 $$\frac{1}{\theta} = \frac{\bar{v}_m + \Gamma'\bar{v}_2}{1 + \Gamma'}$$

where $\bar{v}_m$ and $\bar{v}_2$ are the partial specific volumes of the anhydrous macromolecule and of the solvation water, respectively, Γ' is the solvation of the macromolecules in grams of water per gram of macromolecules. Since the thermodynamic activity of water varies very much from one salt solution to another, the solvation of DNA strongly depends on the nature of the salt used as a gradient material (Hearst and Vinograd, 1961, a; Vinograd and Hearst, 1962).

This is the reason why the buoyant density of a given duplex DNA is about 1.1 g/cm^3 in metrizamide, 1.4 g/cm^3 in Cs_2S0_4,1.7 g/cm^3 in CsCI, and 1.9 g/cm^3 in cesium acetate. The precise values depend on the base composition of the individual DNA's (see, for example, Schildkraut et al., 1962). It is for the same reason that the buoyant density of single stranded DNA in CsCI at pH 7 is 0.015 g/cm^3 larger than the density of the corresponding duplex DNA; in Cs_2S0_4 the density increase is equal to 0.024 g/cm^3 (Erik-son and Szybalski, 1964). Above pH 11.5 instead, the density increase of single - stranded DNA (0.062 g/cm^3 beyond the density of the homologous double-stranded DNA) is almost entirely due to the titration of the G and T residues (Vinograd et al., 1963). Thus, it is always necessary to specify the exact experimental conditions used for a particular isopycnic centrifugation.

Plotting the buoyant density of T4 DNA measured in various cesium salt solutions (it is necessary to use the same cation), versus the activity of water and extrapolating to zero activity, Hearst and Vinograd (1961, a) found for this DNA, $\bar{v}_m = 0.473$ cm^3/g^1. They also determined that in CsCI the cesium salt of T4 DNA has a degree of hydration equal to 30% by weight. For bacteriophage λ, these figures are equal to 0.57 cm^3/g^1 and 30%, respectively (Costello and Baldwin, 1972).

According to preliminary results of Birnieetal. (1973), the hydration of any type of macromolecules, as well as its perturbation by salts, can easily be measured in metrizamide gradients.

The experiments made in sodium iodide gradients suggest that the buoyant density of DNA also depends on other factors than simply its secondary

structure and preferential solvation (Anetand Strayer, 1969, b; De Kloetand Andrea, 1971; Birnie, 1972; Lohman et al., 1973). More particularly, it seems that certain anions, like iodide, directly interact with the bases (Robinson and Grant, 1966).

Subcellular fractions, like ribosomes, and viruses are made up of different types of macromolecules, each of them having its own buoyant density. The density of such a particle will then be a certain mean of the density of its components, and its measurement should allow the determination of the proportion of each of them. While Weiglé et al. (1959) were the first to measure deletions, or insertions, in λ phage DNA, from the measurement of the buoyant density of the whole phages, Costello and Baldwin (1972) reconsidered this problem in detail. These authors have shown that the density of the whole phage not only depends on the proportions of DNA and protein, and on the hydration of each of these components, but that the actual hydration of the DNA, i.e. the density of the phage, depends on the free volume available in each particle. After calibration of the method, the proportions of DNA and protein and the size of the DNA molecule can be measured accurately (Belief etal., 1971).

Similarly, the proportions of RNA and protein in ribosomal subunits can be determined from the buoyant density of the whole subunits. The smaller the density, the greater the proportion of proteins. Unlike the case of *X* phage, a completely empirical relation has to be used, in cesium chloride and cesium sulfate (Hamilton, 1971) as well as in sodium perchlorate gradients (Liautard and Kohler, 1972). The rule of the additivity of the hydration of each macromolecular component (Costello and Baldwin, 1972) is much less applicable to such complex structures like ribosomes, than to bacteriophages.

III.4 THE SHAPE OF THE MACROMOLECULAR BAND

Meselson et al. (1957), and Hearst and Vinograd (1961, a) have shown that at sedimentation equilibrium, the curve of the concentration of the macromolecules (homogeneous with respect to molecular weight and buoyant density) versus distance has a gaussian shape. If the distances are measured from the center of the band, then the concentration c at the point of abcissa 6, is given by:

$$c = c_o \exp(-\delta^2/2\sigma^2) \qquad \text{III} - 7$$

c_o being the concentration at the center of the band and σ the standard deviation of the gaussian curve. With preparative centrifuges the fractionation procedures of the gradients usually cause some distortion of the bands, and their gaussian character disappears (Figure 15).

The preceding authors have also shown that the standard deviation is given by:

$$\sigma^2 = \frac{\theta}{M_{app}} \frac{RT}{\left(\frac{d\rho}{dr}\right)_{eff} \omega^2 r_o} \qquad \text{III} - 8$$

where θ is the buoyant density of the macromolecules, $(d\rho/dr)_{eff}$ the effective density gradient, and r_o the distance from the band center to the rotor axis. Mapp is an apparent molecular weight of the solvated macromolecules (Baldwin, 1959; Hearst and Vinograd, 1961, a) whose actual value increases for decreasing concentrations (Schmid and Hearst, 1969).

According to equation III-8, the standard deviation depends on the effective density gradient. It follows that the band width can be modified in two ways: by the choice of the density gradient material, and by changing the rotor speed (section III.2). Since for equilibrium gradients, dρ/dr is itself proportional to ω^2 (equation III-2), it follows from equation III-8 that the band width is finally inversely proportional to the square of the rotor speed. This property, too, will be reconsidered with more detail during the discussion of the resolving power.

The width of a macromolecular band in equilibrium in a density gradient is the smaller, the greater the molecular weight. But the bandwith is independent of the diffusion coefficient of the macromolecules. More particularly, this means that two density gradients with different viscosities will yield bands of equal width, provided £ is constant.

Equation III-8 suggests that the measurement of the standard deviation should allow the determination of the molecular weight of the macromolecules. With preparation centrifuges this is almost impossible, owing to the deformation of the bands during the fractionation of the gradients. If the gradients are collected from the bottom of the centrifuge tubes, the deformation will be minimal if the bands are in the lower third of the gradient (Meselson and Weigle, 1961). Another difficulty arises from the fact that the molecular weight can only be obtained through an extrapolation of Mapp to zero concentration (Schmid and Hearst, 1969). In this respect, the use of preparative centrifuges represents a considerable advantage, since extremely small amounts of macromolecules can be used. But even under such ideal conditions, Zipser (1963) found 3.0×10^5 daltons for the molecular weight of p-galactosidase, whereas the true value is equal to 5.4×10^5 daltons (Craven etal.,1965).

If the macromolecules are heterogeneous with respect to molecular weight and/or buoyant density, their bands suffer an additional broadening. Baldwin and Shooter (1963) have shown how to measure both of these heterogeneities. Since their method has, apparently, only been applied to their particular material, and since its description would go beyond the limits of the present monograph, the interested reader should refer to the original article. Nevertheless, it is noteworthy that these heterogeneity measurements can only be performed in preparative centrifuges, because the density

gradients have to be formed prior to the centrifugation. The SW40, and SW41 rotors have particularly long tubes, and forthis reason, are particularly well suited for this kind of experiments.

Bands which are symmetric, or even gaussian, are no proof that the macromolecules are homogeneous with respect to mass and density. If, for example, the density distribution is of gaussian shape, the macromolecular band will retain a gaussian shape too (Baldwin, 1959).

III.5 THE DURATION OFTHE CENTRIFUGE RUN

Equilibrium isopycnic centrifugation implies that two equilibria be established: the equilibrium of the density gradient material, and the equilibrium of the macromolecules. The present state of the theory implies that in order to study the latter, the former has already to be established. But this disadvantage is compensated by the fact that the behavior of macromolecules in preformed gradients can easily be studied. With equilibrium gradients it is (most often) necessary that both equilibria be reached which implies relatively long centrifugation times. With preformed gradients, instead, the centrifugation time should be short enough to maintain the initial shape of the density gradient, at least at the center of the tubes.

a) The equilibrium of the gradient material

As has already been mentioned in section 111.1, centrifugation of a salt solution, for example CsCI, yields a continuous increase of its concentration between the meniscus and the bottom of the liquid column. The concentration first changes rapidly at both ends, whereas it remains relatively stable, for some time, at the center. However, after a suitable centrifugation time, depending on the particular experimental conditions, the concentration change throughout the gradient becomes stable and tends towards an equilibrium state. According to Van Holde and Baldwin (1958), the time (in hours) necessary to reach the equilibrium of a CsCI gradient within less than 1% is given by the following, practical equation:

III – 9 $$t'_{es} = 5.6\,(r_b - r_m)^2$$

For this equation use was made of a diffusion coefficient of CsCI equal to 220 Ficks (Trautmanetal.,1962: Baldwin and Shooter, 1963). $r_m$and r_b have been defined above. For a different gradient material, one should notice that t'_{es} is inversely proportional to the diffusion coefficient.

It follows from equation III-9 that the equilibration time of the density gradient is practically independent of the rotor speed. Instead, it is proportional to the square of the total length of the gradient. With the small swinging

bucket rotors and with the usual 3 ml gradients, the time to reach equilibrium is about 40 hours. This time can be considerably reduced if three layers of 1 ml with the appropriate density are superimposed. These individual, initial densities depend on the expected overall density range of the gradient, i.e. on the gradient material and on the rotor speed (equation III-3). In the particular case of a 3 ml CsCI gradient, with a mean density of 1.50 g/cm^3, to be spun at 35,000 rpm, one predicts an overall density variation of 0.2 g/cm^3, and thus, three initial layers with the following mean densities: 1.43,1.50 and 1.57 g/cm^3. The equilibrium will be reached in $40/(3)^2 < 5$ hours.

For the same reason that a certain time of centrifugation is required for a homogeneous solution to reach its sedimentation equilibrium, a preformed gradient will maintain its initial shape for a given length of time, especially at its center. Baldwin and Shooter (1963) have shown that a preformed CsCI gradient will be maintained at the center of the liquid column during a time (in hours) given by:

III – 10 $$t'_i = 0.3(r_b - r_m)^2$$

With the SW41 rotor, whose tubes are completely filled with a CsCI gradient, this time is equal to 20 hours. During about one half of this time the initial gradient will be maintained in the central third of the tube.

(In zone centrifugation one usually assumes that the preformed sucrose gradients are not modified upon centrifugation. In rigorous terms, this is not true. But the length of time during which the initial gradient is maintained is inversely proportional to the diffusion coefficient of the gradient material [Van Holde and Baldwin, 1958]. Now, the diffusion coefficient of sucrose is about five times smaller than the one of CsCI; furthermore, the diffusion coefficient of sucrose decreases rapidly for increasing concentrations and decreasing temperature. It thus follows that the preceding hypothesis is practically valid if the centrifugation time does not exceed 24 hours at 5°C.)

b) The equilibrium of the macromolecular band

In order to determine the length of time necessary to achieve the sedimentation equilibrium of the macromolecules, an estimate of their sedimentation coefficient is necessary, and one has to assume that the density gradient (preformed, or at equilibrium) is stable.

- Equilibrium gradients

In this first case, the centrifugation time is estimated from an equation of Meselson et al. (1957), modified by Baldwin (personal communication) i.e.

III – 11 $$t_{eM} = \frac{\beta(\theta - \rho)}{\omega^4 r_o^2 s}\left[1.26 + \mathrm{Ln}\frac{r_b - r_m}{\sigma}\right]$$

with $(r_b - r_m) >> \sigma$, which is always satisfied for molecular weights larger than10^6daltons. θ is the buoyant density of the macromolecules, and s their

sedimentation coefficient in a sedimentation medium (with the same gradient material) whose density is equal to p. r_0 is the equilibrium position of the band. Since teM varies only very little with the gradient length, we shall assume that $(r_b-r_m)/\sigma = 50$. The following, practical equation is then obtained:

III – 11bis $$t'_{eM} = \frac{120\beta\,(\Theta-\rho)}{N_o^4 r_o^2 S}$$

where N_o is the rotor speed in thousands of rpm, S the sedimentation coefficient of the macromolecules in Svedberg units, t'_{eM} being given in hours.

From equation III-11, the following important conclusions are drawn: with equilibrium gradients, the centrifugation time is inversely proportional to the fourth power of the rotor speed, and it depends on the nature of the gradient material.

As far as the latter parameter is concerned, the centrifugation time not only depends on p, but also on the viscosity. In equation 111-11 bis, S is the sedimentation coefficient which would be measured in a solution of the gradient material which is used for the isopycnic centrifugation. Since S is inversely proportional to the viscosity of this solution, (equation II-9), it turns out that the time to reach equilibrium is proportional to the viscosity of the density gradient.

For a duplex DNA of $30x10^6$ daltons, to be centrifuged to equilibrium in a CsCI gradient at 25,000, 35,000 and 50,000 rpm, the equilibrium will be attained after 110, 25 and 6.5 hours, respectively. Since the two latter values are smaller than the time needed to equilibrate the density gradient (section III.B.a), the centrifugation time will have to be extended to 40 hours. If the resolving power will be such that the run can be performed at 50,000 rpm, it will be advantageous to use a partially preformed gradient, which will considerably reduce the centrifugation time.

As a second example, let's consider the purification of phage A whose sedimentation coefficient is equal to 290 S in a CsCI solution with p = 1.2 (Costello and Baldwin, 1972). According to equation 111-11 bis, the equilibrium will be attained within 5 hours at 25,000 rpm. This shows that with large particles, it is always advantageous to use preformed gradients.

A simple and safe method to estimate the centrifugation time is to determine it experimentally in an analytical centrifuge, and then, considering that it is inversely proportional to N$rg, to calculate the time for the preparative centrifuge.

In numerous cases, the maximum resolving power of equilibrium isopycnic centrifugation can only be fully exploited at relatively moderate rotor speeds, around 25,000 rpm (section III.6.a). With DNA's of a few million daltons this could lead to exceedingly long centrifugation times, 200 to 300 hours. But this can be shortened by the use of one, out of two "tricks".

The first one (Anet and Strayer, 1969, a) is now largely in use, and consists in beginning the run at a relatively high rotor speed during a short time, and then reducing the speed to the initially determined value. During the

first period of time, the macromolecular bands will build up rapidly, and during the second period they will move to their final equilibrium position. This method allows a two to four fold shortening of the centrifugation time. An example is given in Figure 19.

In the second "trick" (Brunk and Leick, 1969), one superimposes two solutions of the gradient material, and only one of them contains the macromolecules. If the buoyant density of the macromolecules is very different from the density of the solution in which they are dissolved, they will very rapidly sediment to their equilibrium position. Here too, the gain of time is two to four-fold.

Since the centrifugation time is proportional to p, it follows that density gradient materials with a good resolving power (see below, equation 111-14) require longer run times.

- Preformed gradients

Preformed gradients are especially useful for the centrifugation of particles whose sedimentation coefficient is larger than SOS: the run time can be shortened, the resolving power can be increased (section III.6), and the density heterogeneity of the particles can be determined (Baldwin and Shooter, 1963). These advantages of preformed gradients can only be exploited if the particles reach their equilibrium within a lapse of time shorter than the time during which the initial density gradient is conserved (equation 111.10).

With completely preformed gradients, onto which a small sample solution is initially layered, Baldwin and Shooter (1963) have shown that the particles reach simultaneously their equilibrium width and position. They are within 1% of equilibrium at a time given by:

III – 12 $$t_{eP} = \frac{(\Theta - \rho)\,\mathrm{Log}\left[\frac{r_o - r_m}{r_m} - \mathrm{Log}\,10^{-2}\right]}{\omega^2 r_o s \frac{d\rho}{dr}}$$

With the rotor speed in thousands of rpm, the sedimentation coefficient (measured in a solution of density p) in Svedberg units, and the time in hours, the following, practical equation is obtained:

III – 12bis $$t_{eP} = \frac{2.5 \times 10^5\,(\Theta - \rho)\left[\mathrm{Log}\frac{r_o - r_m}{r_m} + 4.61\right]}{N_o^2 r_o S \frac{d\rho}{dr}}$$

Unlike the preceding case, the centrifugation time is now inversely proportional to the square of the rotor speed, instead of its fourth power. As for equilibrium gradients, the run time depends only slightly on the total length

of the gradient. Similarly, it is proportional to the viscosity of the gradient material solutions.

With the SW41 rotor at 36,000 rpm (maximum allowable speed for p = 1.5) and a 12 ml CsCl gradient whose extreme densities are equal to 1.45, and 1.55 g/cm^3, respectively, phage A will come to equilibrium within less than 6 hours. One should notice that this is, indeed, shorter than the time during which the initial shape of the density gradient is maintained in the central third of the centrifuge tube (section III.5.a).

All the centrifugation times which have been calculated above have been tested experimentally in the author's laboratory on many occasions. They show that the very common, extremely long run times can often be substantially reduced.

III.6 RESOLVING POWER AND ROTOR SPEED

a) Equilibrium gradients

Ifft et al. (1961) defined the resolving power of equilibrium isopycnic centrifugation, as

III – 13 $$\Lambda = \frac{\Delta r}{\sigma_1 + \sigma_2}$$

where Δr is the distance which separates two macromolecular bands, and σ_1 and σ_2 are their standard deviations. Using equations III-2 and III-8, they have shown that:

III – 14 $$\Lambda = \frac{\Delta\Theta}{2}\left[\frac{\overline{M}\,\beta}{\overline{\Theta}\,RT}\right]^{1/2}$$

where $\Delta\Theta$ is the buoyant density difference between the two macromolecular species, M their mean molecular weight, and $\overline{\Theta}$ their mean buoyant density.

According to equation lli-14, and unlike the situation of zone centrifugation, the resolving power of equilibrium isopycnic centrifugation is independent of the rotor speed. This property follows implicitely from equation III-3, where both Δr and $(\sigma_1 + \sigma_2)$ are inversely proportional to ω^2. Another difference with zone centrifugation, is that Λ is independent of the length of the density gradient. Here, the length should be such that, given the rotor speed, and the gradient material, the density range covered by the gradient, includes the buoyant density of the macromolecules to be centrifuged simultaneously. Most often gradient lengths of 1 to 3 cm are satisfactory. Since β is proportional to RT (equation III-1 and III-2), the resolving power is almost independent of the temperature.

Finally, the resolving power depends essentially on β, i.e. the gradient

material. The following cesium salts have increasing resolving powers: sulfate, bromide and oxalate, chloride, formate, iodide, acetate. The increased resolving power of Nal, relative to CsCI has been clearly established (section lll.7.a). Owing to its large buoyant density, RNA cannot be banded in CsCI gradients, whereas it can be done in cesium sulfate and oxalate (Zolotor and Engler, 1967), and in sodium iodide (section lll.7.a), the latter salt giving the best resolution. In his study on the subunits of (B-galactosidase (Zipser, 1963) used RbCI gradients whose resolving power is about 30% larger than the resolving power of CsCI.

Although the resolving power of equilibrium isopycnic centrifugation is independent of the rotor speed, and despite of the shorter centrifugation time the greater the speed, equilibrium runs have very often to be performed at relatively moderate speeds. Increasing the rotor speed decreases the distance between bands. But the closer the bands are to each other, the more difficult it is to keep them separated during the fractionation of the gradient. The limits of the fractionation procedure, set the upper limit of the rotor speed.

It is impossible to give particular recommendations for the choice of the rotor speed. In each particular case, one should consider the properties of the gradient material, the molecular weight of the macromolecules, and their buoyant density difference. Owing to the availability of rotors with a high limiting speed (SW65) or of rotors with long tubes (SW41), the use of high resolution salts (large β values) is now greatly facilitated. In practice, two bands can be well separated, if their width (measured in number of fractions) is two times smaller than the distance which separates the maximum of their respective concentrations (see also section II.6.b).

As for zone centrifugation, certain analytical applications of isopycnic centrifugation do not necessarily require a good resolving power (Figure 16).

With equilibrium isopycnic centrifugation - and only with this method - the use of fixed angle rotors increases the resolving power (Hershey et al., 1965; Flamm et al., 1966). During centrifugation the density difference between the meniscus and bottom of the gradient depends on the horizontal distance between these two points while the tube is tilted. But, at the end of the run, when the tube is brought to the vertical position for fractionation, this same density difference is spread over a larger distance, namely the distance between meniscus and bottom as measured along the axis of the tube.

Finally, the rotor speed still depends on the following factors: first, the high concentration of the gradient material at the bottom of the gradient should always be below its limiting solubility; second, the maximum speed of the rotors should be decreased for densities larger than 1.2 g/cm^3, as recommended by the manufacturers.

b) Preformed gradients

With a constant, preformed gradient, equal to dp/dr, the distance which separates two bands is equal to Ar = A0/(dp/dr). Knowing, on the other

hand, that their band width is given by equation 111-8, the following equation of the resolving power is obtained:

III – 15

$$\Lambda = \frac{\Delta\Theta}{2}\left[\frac{M\omega^2 r}{\frac{d\rho}{dr}\,\overline{\Theta}RT}\right]$$

r is the mean distance of the bands to the rotor axis; the other symbols are defined as for equation III-14.

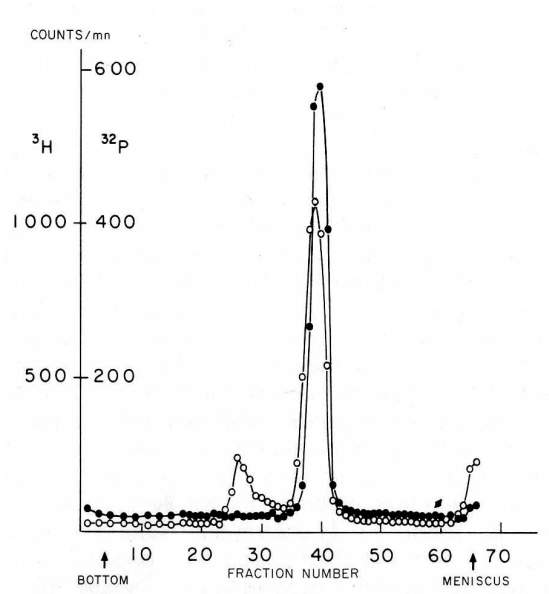

Figure 16. Isopycnic centrifugation of λ bacteriophages

This experiment was aimed to show that the progeny phages obtained after infection of bacteria incorporate part of the DNA of the infecting phages. The density of the infecting phages was increased through labeling with ^{15}N, and 13C; in addition, their DNA was labeled with ^{3}H-thymidine. The multiplicity of infection was equal to 1. The progeny phages have been purified, and then mixed with "light" ^{32}P labeled phages. The mixture was centrifuged to equilibrium in a 3 ml CsCl gradient whose mean density was equal to 1.5 g/cm^3. The SW39 rotor was spun at 26,000 rpm during 36 hours. The gradient was fractionated dropwise in a series of liquid scintillation vials. The radioactivity (o, 3H; • ^{32}P) has been plotted versus fraction number. The density increases from the right to the left.

The result shows that part of the heavy phages retained their initial density (they did not infect bacteria), but that the majority of the tritium has been incorporated into phages with a much smaller buoyant density, but slightly denser than the "light" phages. Since their band is as narrow as the light band, one concludes that they incorporated a well defined fraction of the parental phage genome. From their position relative to the "light", and "heavy" phage bands, it follows that this fraction is equal to 10% to 15% of the parental DNA.

It appears from equation 111-15, that unlike equilibrium gradients, the resolving power of preformed gradients is proportional to the rotor speed. But this property has only a limited interest, since increasing the rotor speed narrows the band width, which is essentially necessary for low molecular weight macromoles. But for such molecules, the duration of the centrifuge run would be such that the initial density gradient would no longer be maintained. Thus it appears once more that preformed gradients are especially useful for large particles, like viruses or subcellular fractions.

Instead, it is a remarkable property of preformed gradients that their resolving power can be changed by a single change of dp/dr. With weak preformed gradients it is possible to achieve better resolving powers than with equilibrium gradients. With such weak gradients, and in order to cover the proper density range, it might be necessary to use rotors with long tubes; these rotors have the additional advantage that the initial density gradient is maintained for a greater length of time.

In their study concerning the isopycnic centrifugation of ribonucleoproteic particles in preformed sodium perchlorate gradients, Liautard and Kohler (1972) report that the relatively large viscosity of this gradient material gives a better resolution than CsCl gradients. Since the equilibrium width of the bands is independent of the diffusion coefficient of the macromolecules, the viscosity should only be considered as long as the macromolecules are not in equilibrium in the gradient. The increased resolving power is then due to the sharpening effect which has been discussed in connection with zone centrifugation (section II.6.b). And, indeed, the mentioned authors stop the centrifuge before the bands come to their equilibrium position.

III.7 DENSITY GRADIENT MATERIALS, AND THEIR APPLICATIONS

The properties of the ideal gradient material to be used for equilibrium gradients have been enumerated very often: low molecular weight (in order to obtain short equilibrium times) and absence of ionization of the gradient material; low viscosity large density, transparency to UV light, and a good resolving power of its solutions; good solubility and stability of the macromolecules which are dissolved in the solutions. Since equilibrium isopycnic centrifugation is essentially, if not exclusively, used for the study of macromolecules, the gradients which are the closest to the ideal one, are salt gradients.

Whole cells, subcellular particles, or viruses instead, come very rapidly to equilibrium, and preformed gradients should thus be favored. For such particles, one should then look for gradient materials of high molecular weight. These will have the two-fold advantage that the preformed gradient will be maintained for a longer time, and that the osmotic pressure can be

kept closer to its physiological level. Several substances can be used to this end.

Thus, we shall try to review shortly the most common gradient materials in view of their applications.

a) Nucleic acids

It is certainly useless to analyze, even shortly, the wealth of applications of the cesium gradients in the study of the properties and the replication mechanism of DNA. We shall only recall three particular applications.

- The separation of complementary DNA strands

Vinograd et al. (1963) have shown that at relatively high pH (pH > 11.5), the hydrogen bonds of duplex DNA are disrupted, and that the buoyant density of the strands is increased by about 0.06 g/cm^3. This density increment is essentially due to the titration of the G and T residues, and its precise value depends on the exact proportion of those residues in each strand. Thus, if the proportions of G and T are different enough in two complementary strands, they should be separable on their density difference basis. Using this property, Doerfler and Hogness (1968) were able to separate the complementary strands of *X* phage DNA by repeated equilibrium isopycnic centrifugations in alkaline CsCI. Only one such centrifugation is necessary for the separation of the strands of the satellite DNA's of mouse and guinea pig (Corneoetal., 1968,1970).

In many cases, the separation of the complementary strands is largely enhanced if the mixture of the strands is hybridized with synthetic polynucleotides rich in guanine, and centrifuged in neutral CsCI gradients (Szybalski and Szybalski, 1971). The polymer which gives the best separation appears to be poly (U, G). An example of its use is given in Figure 17. After centrifugation, several methods can be used to remove the polymer.

- Centrifugation in the presence of silver or mercury ions

Isopycnic centrifugation of linear duplex DNA in CS2S04 gradients in the presence of mercury (Nandi etal.,1965: Skalka, 1971), or silver ions (Jensen and Davidson, 1966), yields an increase of the buoyant density of the order of 0.1 g/cm^3. For a given DNA, the precise density increment depends on the concentration of Hg (II), or Ag^+. According to the preceding authors, at neutral pH mercury binds preferentially to A, T rich DNA, and silver to G, C rich DNA. Centrifugation in the presence of Hg (II) allowed Wang et al. (1965), and Skalka etal. (1968) to amplify the natural density difference which exists between the two halfs of A DNA and to purify each of them.

In cesium chloride, the density difference between double-stranded and single-stranded DNA is equal to about 0.015 g/cm^3, whereas in the presence of Hg (II) or Ag^+ it is equal to, or larger than 0.1 g/cm^3 (same group of authors). Although the resolving power of Cs2S04 is about 1.3 times smaller than the resolving power of CsCI (equation 111-14), the use of mercury or

silver renders the separation of both DNA's extremely easy, even on a large, preparative scale.

Accumulating evidence indicates that the buoyant density increase of a given DNA in the presence of Ag^+ not only depends on the average base composition of that DNA, but also on its precise nucleotide sequence (Daune et al., 1966; Filipski et al., 1973). Both specificities of silver binding to DNA must be considered to explain the purification of satellite DNA's from mouse and guinea pig (Corneo et al., 1968, 1970), the fractionation of the entire bovine genome into more than seven DNA species (Filipski et al., 1973), or the fractionation of the DNA from bacteriophage *k* after treatment by Eco RI endonuclease (Figure 18).

The precise experimental conditions have to be adjusted to each particular kind of experiment. Most often, the best separations are obtained at pH 9.2 (5x10-^{3}1VI Na2B40y), with 0.3 to 0.4 moles of Hg (II), or Ag^+ per mole of DNA phosphate. It is obvious that any traces of substances (EDTA, citrate, Tris, etc.) which could complex the metal ions, have to be avoided.

After centrifugation, the metal ions are removed through extensive dialysis against 2 M NaCI (suitably buffered), upon which the DNA recovers its native structure and biological properties.

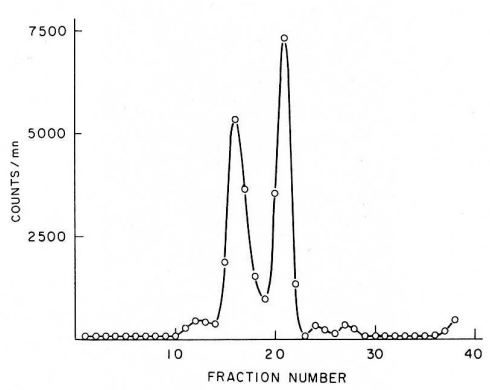

Figure 17. Separation of the complementary strands of \ bacteriophage DNA

0.1 ml of a ^{14}C thymidine labeled phage suspension, containing about 100 µg of DNA, was mixed with 0.05 ml of a poly (U, G) solution at 2mg ml^{-1}. After dilution with water, to reduce the DNA concentration to 175 ng ml, an equal volume of 0.02 M EDTA, 0.02% sarkosyl, pH 7, was added. The phage was lysed and the DNA denatured by heating the mixture at 100°C for 2 mn. The DNA/poly (U, G) hybridization occurs upon addition of 1/20 volume of 4 M NaCI and letting the mixture stand at room temperature for 15 mn.

A 3 ml CsCI solution at 1.72 g/cm^3 was prepared with the hybrid solution, the proper amount of CsCI cristals, and 0.01 M Tris, 0.001 M EDTA, pH 7.5. Polyallomer tubes were used .(they adsorb less DNA than cellulose nitrate tubes). The SW50.1 rotor was spun at 30,000 rpm, 15°C, during 48 hours. The gradient was fractionated in 38 equal volume fractions; 5 (il of each fraction was used to measure the radioactivity. Within experimental error, each DNA/poly (U, G) band contains the same amount of 1^{4}C.

- Purification of superhelical DNA

The superhelical structure is encountered for many DNA's: DNA from mitochondria and animal viruses, cellular and bacterial plasmids, or intracellular forms of phage DNA's. In order to study their physico-chemical properties, and their replication mechanism, these DNA's have to be purified. This can be done by several methods (Freifelder, 1971) but we shall only describe the method which is now the most commonly used. Following preliminary work of Le Pecq (1965), Vinograd and his collaborators (Radloff etal., 1967; Bauer and Vinograd, 1968,1970, a, 1970, b) designed and studied with much detail the method which uses equilibrium isopycnic centrifugation in CsCI, in the presence of ethydium bromide (ETB).

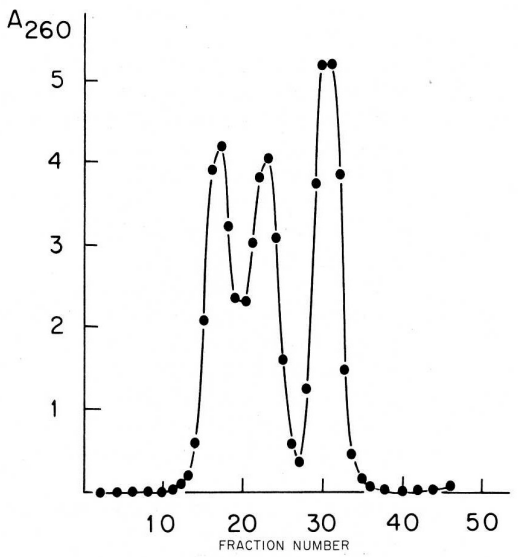

Figure 18. Isopycnic centrifugation of Eco Rl A. DNA fragments in the presence of silver

DNA from bacteriophage λ imm434 ts S7 has been hydrolysed with the Eco RI restriction enzyme (Yoshimuri, 1971). 7 fragments are obtained whose molecular weights are comprised between 1.1 x10^6, and 14x10^6daltons (Tiollaisand Rambach, 1974). After phenol treatment the DNA was extensively dialysed against 0.005 M Na2B407, pH 9.2 where upon 510 fig of DNA were diluted to 100 jig mH with borate buffer, and 0.001 M AgNOs was added to a final concentration of 0.3 Ag+ ions per DNA base. Finally, Cs2S04 crystals and borate buffer were added to obtain 9 ml of solution at the density of 1.50 g/cm^3. The fixed angle rotor 65 was used. Filling of the polyallomer tubes was completed with light mineral oil. The temperature was 20°C. The rotor was first spun during 24 hrs at 35,000 rpm; then 72 hrs at 29,000 rpm. After centrifugation, the centrifuge tube was punctured at the bottom, and 46 fractions of 8 drops each were collected. The absorption at 260 nm has been plotted versus the fraction number. The density increases from the right to the left. (From Fritsch and Tiollais, 1975.)

The principle as well as the use of the method, are relatively simple. At ETB concentrations of about 100 fig/ml, the molecules of the dye are intercalated between the bases of duplex DNA. For linear, or relaxed circular DNA molecules, this leads to a buoyant density decrease of about 0.13 g/cm^3, whereas for superhelical circles, the density increment is of the order of 0.09 g/cm^3 (Bauer and Vinograd, 1968). The buoyant density difference of 0.04 g/cm^3 between both conformations is large enough for the purification of superhelical DNA from bacterial extracts (Figure 19), or from cellular extracts which contain a small proportion of superhelical DNA (Radloff etal., 1967).

At an ETB concentration of 300 fig/ml, the precise density difference between a superhelical DNA and the homologous, relaxed, or linear DNA molecule, depends on the initial density of superhelical turns per base pair

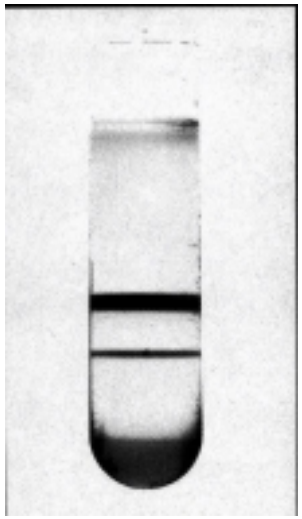

Figure 19. Purification of superhelical, bacterial plasmid DNA

A strain of Streptococcus faecalis (group D) harbors two plasmids responsible of its resistance to tetracyclin, and erythromycin, respectively (Courvalin et al., 1974), In order to purify the plasmids, 100 ml of an exponential culture at 10^9 bacterial/ml were washed, resuspended in 9 ml of 0.05 M Tris, 0.001 M EDTA (pH 8.0), and treated during 1 hour with 400 μg ml^{-1} of lysozyme, at 37°C. The resulting protoplasts were then lysed with sarkosyl (0.5% final concentration). The lysate was centrifuged at 25,000 g, 4°C during 30 mn, in order to pellet the majority of the chromosomal DNA. 8 grams of supernatant were then mixed with 8 grams of CsCI, to obtain a density of 1.51 g/cm^3, and ETB was added to a final concentration of 400 (ig m l . The mixture was centrifuged at 44,000 rpm, 20°C, during 36 hrs in the 50Ti rotor. After centrifugation, the tube was illuminated with 350 nm UV light, and photographed. The most dense band contains the mixture of both superhelical plasmid DNA's, whereas the less dense band contains residual chromosomal DNA and relaxed circular plasmid DNA. (The experimental results have been kindly provided by P. Courvalin.) [Negative Image]

(Bauer and Vinograd, 1970, b). This property is used for the measurement of the number of superhelical turns. One single preparative centrifuge run is sufficient and the DNA's do not need to be completely purified (Gray et al., 1971).

After centrifugation, bands with about 5 fig of DNA are visible by the naked eye due to their red colour. Bands which contain about 0.5 jig of DNA can be made visible as well, through the excitation of the fluorescence of the dye with a UV lamp (ETB is excited at 365 nm). 0.05 fig of DNA can be detected by photography (Watson et al., 1971). If the fractions of the density gradient are collected on a sheat of parafilm, those which contain the DNA are detected through illumination of the sheat with UV light (Cuzin, personal communication).

After analysis of the gradient, the ETB can be removed, either by passage on a Dowex 50 column (Radloff et al., 1967), or by extraction with isopropanol (Cuzin etal., 1970).

In view of their large buoyant density, RNA's cannot be banded in cesium chloride gradients. But this can be achieved in cesium sulfate, cesium formate (Davern and Meselson, 1960), or in cesium oxalate (Zolotor and Engler, 1967), the most widely used salt being cesium sulfate. Among the recent applications, we shall mention the characterization of the double-stranded RNA from bacteriophage 06 (Semancik et al., 1973) and the detection of RNA in the replication intermediates of polyoma virus DNA (Magnusson et al., 1973). The methods used to avoid the precipitation of RNA have been summarized by De Kloetetal. (1971).

For a number of applications, cesium salts tend to be replaced by sodium iodide (Ayet and Strayer, 1969, b; De Kloet and Andraa, 1971; Birnie, 1972; Lohman et al., 1973). According to this group of authors, the advantages of Nal relative to CsCI can be summarized as follows: its resolving power is well above the resolving power of CsCI; the buoyant density of DNA is not changed by the presence of ETB, so that the fluorescence of the latter can be used for the measurement of the buoyant density (section III.3), i.e. the base composition, of very small amounts of any DNA (De Kloet and Andraa, 1971); the buoyant density difference between double-stranded and single-stranded DNA's is much larger than in CsCI and sufficient for their preparative separation; in the absence of trace amounts of heavy metals, no precipitation of RNA occurs; one can band DNA, DNA/RNA hybrids and RNA in one single density gradient. A remarkable property is that the buoyant density of DNA in Nal gradients, not only depends on its primary structure, but also seems to be related to its secondary structure, whose study could be probably undertaken by this method.

The disadvantages of Nal are two-fold: this salt cannot be used for the purification of superhelical DNA's or for centrifugation in the presence of silver ions, and it is not preceded by the marvellous history of CsCI.

The use of metrizamide as a density gradient material deserves special mention. This substance has the particular property to be non-ionized; hence, nucleic acids are fully hydrated and their buoyant density is equal to 1.12 g/cnrr^3,

which is below the density of proteins. It can be used for the preparative separation of single-stranded and double-stranded DNA's. Unfortunately, metrizamide absorbs UV-light very strongly (Birnie etal., 1973).

b) Proteins

Isopycnic centrifugation of proteins is difficult for two reasons: at high ionic strength proteins are often insoluble and enzymes are inactivated; on the other hand, because of the low molecular weight of proteins (relative to most DNA's), long centrifugation times are needed and the resolving power is relatively poor.

Despite these disadvantages, fi-galactosidase could be banded (Hu etal., 1962) and the number of subunits could be determined (Zipser, 1963) in RbCI equilibrium gradients. Serum-albumin (Ifft and Vinograd, 1962), collagene (Fessler and Hodge, 1963) and a-amylase (Filner and Varner, 1967) have been studied in CsCI gradients. Whereas threonine-deaminase precipitates in CsCI gradients, it remains soluble and active in potassium citrate gradients (Changeux and Fritsch, unpublished). The regulation of the biosynthesis of certain plant enzymes has been studied through D20 labelling and isopycnic centrifugation in CsCI (Attridge et al., 1974; Attridge and Smith, 1974).

Since the buoyant density of glycoproteins in CsCI (1.45 g/cm^3) is much larger than the density of proteins (1.3 g/cm^3) and smaller than the density of sugars (1.6 g/cm^3), they can be purified through isopycnic centrifugation in CsCI gradients (Creeth and Denborough, 1970).

In a very elegant work, Wilcox et al., (1969) purified the lipoproteins of human plasma in preformed NaBr gradients, whose extreme densities were equal to 1.00 and 1.40 g/cm^3, respectively. Through the use of zonal rotors, 30 ml of plasma could be treated at a time. The plasma was introduced at the bottom of the gradient, so that at the end of the centrifugation, the lipoproteins were contaminated neither with proteins, nor any other high density material. In order to separate the very light and light lipoproteins from the heavy ones, they proceeded in two steps. An excellent separation of the three classes of lipoproteins has been obtained, although their sedimentation equilibrium was not completely achieved.

Anderson and Breilatt (1971) propose a very sensitive and rapid method for the detection and the measurement of extremely small amounts of antigen and antibody. The antibody is adsorbed on latex beeds whose diameter is 0.109 (im and their density 1.050 g/cm^3. The density of the complex is larger than the density of the naked beeds. The addition of a specific antigen leads to an additional density increase of the latex-antibody complex. Densities are measured through 1 hour centrifugation in preformed sucrose gradients at 27,000 rpm. The method allows the detection of about 2x10^9 viral particles, or 2x10" antibody molecules. In addition, the method allows the measurement of masses in the range of 10 ~^{12} to 10~^{14} grams.

Marx etal.. (1974) purified the Sendai virus nucleocapsid in heavy water-

sucrose gradients and showed that it contained the activity of the RNA dependent reverse transcriptase.

Since metrizamide is non-ionized, it is particularly well suited for isopycnic centrifugation of proteins. Whereas enzymes are not inactivated in such gradients, they nevertheless give rise to two bands with different buoyant densities; the reason of this phenomenon still remains unknown (Birnie etal.,1973).

c) Subcellularfractions

Isopycnic centrifugation is more and more used as a purification method of cellular components, and very often it is combined with zone centrifugation. Once more, we do not aim to provide an exhaustive analysis of all the applications, but rather to point out the main features of each type of application.

Membrane fractions from E. coli have a buoyant density of the order of 1.2 g/cm^3, which is below the density of any other bacterial component and allows such fractions to be purified in preformed sucrose gradients (Kaback and Stadtman, 1966; Miura and Mizushima, 1968; Quigley and Cohen, 1969). In order to study the biogenesis of bacterial membranes, Foxetal. (1970) used bromostearate as a density label and isopycnic centrifugation in sucrose. Such gradients have also been used for the investigation of the attachment of bacterial DNA to membrane fragments (Sueoka and Quinn, 1968; Worcel and Burgi, 1974). Huff et al. (1964) instead, use CsCl gradients for banding bacterial cell walls.

In an article devoted to the various purification methods of plasma membranes, Wallach and Lin (1973) discuss the use of various gradient materials with regard to the osmotic pressure of their solutions and their penetration into membrane vesicles. Cytoplasmic membranes and membranes from mitochondria and microsomes (Wells and Sheeler, 1973; Amar-Costesce, 1973; Headon and Duggan, 1973; El Aaser, 1973) as well as cholinergic-receptor rich membranes (Cohen et al., 1972) or nerve endings (Bretz and Baggiolini, 1973), are purified in sucrose gradients.

Instead, the purification of nuclei (Loir and Wyrobek, 1972) and intact lysosomes (Wolf and Pertoft, 1972) by isopycnic centrifugation, requires the use of gradient materials with low osmolality. If colloidal silica gels are much used, it might well be that they will be rapidly replaced by metrizamide (Mathias and Wynter, 1973; Aas, 1973). Nevertheless, sucrose gradients have been used to characterize lysosome rich fractions (Stauber and Bird, 1974). The derepression of yeast mitochondria in their respiratory function has been studied in sorbitol gradients (Neal et al., 1971). Chloroplasts have been purified in isoosmotic gradients of sucrose and sorbitol, or Ficoll (Vasconcelos et al., 1971; Nobel, 1969; Price, 1973, b), or colloidal silica (Lyttleton, 1970).

The isopycnic banding of nucleoproteic particles (ribosomes, polysomes, informosomes) in concentrated salt gradients requires some special precautions. The presence of magnesium (Brenner et al., 1961; Cohen and Nisman, 1961) only partly avoids (Meselson et al., 1964) their dissociation into RNA

and protein. This can be completely avoided if, prior to banding, the particles are "fixed" with either formaldehyde (Spirin et al., 1964) or glutaraldehyde (Baltimore and Huang, 1968). It becomes then possible to use CsCl, CS2S04 (Hamilton, 1971) or sodium perchlorate gradients (Liautard and Kohler, 1972; Morin, 1972) to measure the RNA/protein ratio. Figure 20 shows the result of an isopycnic centrifugation of formaldehyde fixed ribonucleoproteic particles. This experiment and the one of Figure 13 show that certain particles can be characterized by the combined use of zone, and isopycnic centrifugation, whereas only one of the two methods would be insufficient (Tiollaisetal. 1971).

Unfortunately, the "fixation" of the particles is irreversible. Here again, metrizamide appears to become an excellent substitute of salt gradients, since no fixation is required (Mullock and Hinton, 1973).

The separation of nuclear fractions (chromatin, nucleol, nuclear membrane) in preformed sucrose and glucose gradients does not seem to raise any particular problem (Raynaud and Ohlenbusch, 1972; Tata and Baker, 1974). But the centrifugation of chromatin in CsCl requires fixation with formaldehyde. Even so, it has been shown that the buoyant density of chromatin (comprised between 1.3 and 1.5 g/cm^3) depends on its biosynthetic activity and on the nature of the basic proteins which are attached to it (Moav et al., 1974). In metrizamide gradients, chromatin does not need to be fixed. (Rickwood et al., 1973).

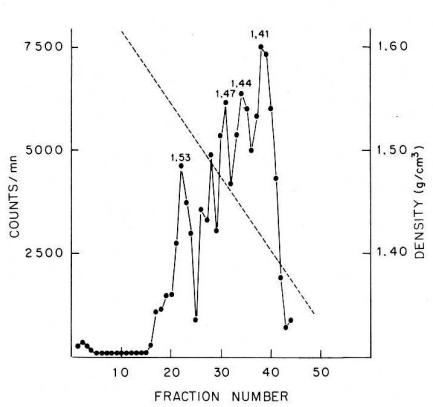

Figure 20. Isopycnic centrifugation of nucleoproteic particles

L5178 Y exponentially growing cells were labeled during 40 mn with ^{3}H uridine. The polysomes were then centrifuged as in Figure 13, except that the centrifugation time was raised to 18 hrs, which yields a better separation of the ribosomal subunits. The ^{3}H labeled particles of the 45 S zone were then dialysed against a buffer containing 4% HCHO. Finally, this solution was diluted in the same buffer and used to prepare a 8 ml CsCl solution, which was centrifuged in the SW41 rotor at 36,000 rpm, 20°C, during 20 hrs. The gradient was fractionated, and the radioactivity (•-•), and CsCl solution density (---) were plotted versus the fraction number. The bands whose buoyant densitities are equal to 1.53 g/cm^3, and 1.41 g/cm^3, correspond to the small ribosomal subunit and to informosomes, respectively. The intermediate bands correspond presumably to precursors of the subunit. (From Tiollais et al., 1971.)

d) Viruses

One particularly fecund application of isopycnic centrifugation is the purification of viruses. Here again, we consider that it is not necessary to describe the extremely wide use of CsCI gradients for the purification, or characterization of E. coli or B. Subtilis bacteriophages. An example is given in Figure 16.

For the purification of animal viruses, potassium tartrate is a common gradient material (McCrea etal., 1961; Pfau and McCrea, 1963; Norcross etal., 1963); in addition to its good resolving power, it does not inactivate hemagglutinins. Silica sols (Ludox AM; Ludox HS; Ludox SM) have the following advantages: very short centrifugation times; physiological osmotic pressure and ionic strength (Juhos, 1966; Pertoft et al., 1967). Trautman et al. (1962) devised an elegant method for the purification of the foot and mouth disease virus. In a 5 ml tube of a small swinging bucket rotor, they superimpose the three following phases: 0.9 ml of a CsCI solution with a density of 1.42 g/cm^3 (which is the buoyant density of the virus); 0.9 ml of a dense, organic phase; finally, 3.3 ml of a suspension of non-purified virus. Upon centrifugation, only the virus sediments into the CsCI phase, whereas all cellular debris are retained in the organic phase.

The use of zonal rotors for the industrial purification of viruses, deserves special mention. A large number of references, and half-a-dozen particular applications are mentioned or analyzed by Chervenka and Elrod (1972). Other examples have been the subject of communications at the "European Symposium of Zonal Centrifugation" of 1973 (Editions Cit§ Nouvelle, Paris). Since the majority of viruses have buoyant densities below 1.3 g/cm^3, the most commonly used gradient material is sucrose. In many cases, continuous flow rotors are used; the virus suspension flows along a preformed sucrose gradient into which the viruses sediment; in the gradient they build up a narrow band at a position where the density is equal to their own buoyant density. The rotor speed and the flow rate of the virus suspension have to be mutually adjusted, so that during their passage in the rotor, the viruses will have enough time to penetrate into the gradient (for details, see Instruction Manuals).

e) Cells

Whereas the viability of viruses is usually maintained in highly concentrated salt or sucrose solutions, this is no longertruefor bacteria (Pollard and Grady, 1967; Dubert, personal communication), or for cells of higher organisms. Banding of entire cells in density gradients requires low osmotic pressure sedimentation media, i.e. high molecular weight gradient materials.

Silica sol (Ludox AM) gradients have been used by Juhos (1966) to separate bacteria from non-specific phages. Tamirand Gilvarg (1966) used urografin gradients for the separation of vegetative cells and spores from B. megaterium (their buoyant densities are equal to 1.135 and 1.305 g/cm^3, respectively).

In their review about the purification of cells and membranes, Wallach and Lin (1973) emphasize that isopycnic centrifugation is the most commonly

used method, owing, especially, to the large amount of material which can be treated at a time. Since the work of Leif and Vinograd (1964) on the buoyant density of red blood cells as a function of their age, the most used gradient material is serum albumin. The density gradients have necessarily to be formed prior to centrifugation. Leif etal., (1972) show how to use them in swinging bucket rotors. In his work on the density heterogeneity of mesoblastic cells from chick embryo wings (it seems to depend on the degree of differentiation), Flower (1972) shows that the serum albumin gradients have to be adapted to each particular application, especially with regard to the resolving power, the number and the viability of the cells. Moavet al. (1974) used the method for the fractionation of cells, from testis and here again, found that their buoyant density depended on their age.

Instead of serum albumin, Pertoft etal. (1968) separated the various blood cells in gradients made of silical sols and polyvinyl-pyrrolidone.

More recently, Munthe-Kaas and Seglen (1974) have shown that, owing to the low osmolality of metrizamide, its gradients are particularly suited for the separation of cells. More particularly, they have shown that the buoyant density of parenchymal cells from rat liver is well above the density of the other cells, and that they recover their whole metabolic activity after removal of the metrizamide. Munthe-Kaas and Seglen also designed special methods to preform the gradient, and to introduce the cells in the gradient (in order to preserve their viability).

III.8 PRACTICAL ASPECTS OF ISOPYCNIC CENTRIFUGATION

The choice of the gradient material, the time and speed of centrifugation have been discussed in preceding sections. Preformed gradients are established by the same methods and require the same precautions as for zone centrifugation (section II.2.c and II.2.d); similarly, the same gradient fractionating devices are used (section ll.2.f). The use of photographic and spectrophotometric methods to locate macromolecular bands in the gradients have also been mentioned (section III.3).

Isopycnic centrifugation needs the preparation of solutions with precise densities. In equilibrium centrifugation, the density difference between two points depends only on the rotor speed (equation III-3), but the absolute value of the densities at those points depends on the density of the initial solution. With preformed gradients, the two extreme densities have to be set correctly. Table A.2 (appendix) gives the correlation between salt concentration and solution density for CsCI, Cs2S04, NaCI and sucrose. The concentrations are expressed in weight fraction of the gradient material and are designated by F

In order to prepare v ml of solution, with a density equal to p, the mass of gradient material to be used is given by:

III – 16 $$m_o = v\rho F$$

and the mass of buffer, and/or water, and/or macromolecular solution is given by:

III – 17 $$m = v\rho - m_o$$

Forthe experiment of Figure 15, a CsCl solution with v = 3 ml, and p = 1.760 g / cm^3 had to be prepared. For this density, one has F = 0.589. Hence, m_0 = 3x1.76 x 0.589 = 3.110 g of CsCl were weighed and dissolved in m = 3 x 1.760 - 3.110 = 2.170 g of buffer containing the DNA's to be centrifuged. With the reasonable assumption that the density of this solution was equal to 1.00 g/cm^3, the salt was finally dissolved in 2.17 ml of buffer.

It is also possible to dilute a concentrated gradient material solution in the right proportions. To obtain v ml of solution with a density equal to p (the corresponding concentration is F), from a more concentrated solution of density p_0 (concentration F_0), the volume of the latter is given by:

III – 18 $$v_o = v\,(F/F_o)(\rho/\rho_o)$$

and the volume of buffer (whose density is supposed to be equal to 1.00) to be added is:

III – 20 $$\Delta v = \rho v - r_o v_o$$

Once a gradient material solution has been prepared, it is often safe to check its density, either by pycnometry or refractometry. In the first case, pycnometers with a volume of 0.1 to 0.3 ml are particularly convenient. In the second case, one uses an Abbe refractometer, which requires only 0.05 ml of solution, and the relation between refraction index and density of the respective solutions (Table A.4). In cases where the final solution should contain substantial concentrations of materials (buffer) other than the macromolecules and the gradient material, only the pycnometric method will give the density within less than 0.002 g/cm^3: but in preparative centrifugation such an accuracy is only rarely needed.

In equilibrium isopycnic centrifugation, the macromolecules are usually uniformly mixed with the initial gradient material solution. One exception (Brunk and Leick, 1969), aimed to reduce the centrifugation time, has been mentioned in section lll.5.b. Gradients of 2 to 3 cm length are most often sufficient; in the small swinging bucket rotors, this corresponds to a volume of 3 ml. To avoid the collapse of the tubes, their filling has to be completed with light mineral oil (for example, parafin oil).

Preformed gradients, instead, should usually be as long as possible (section lll.6.b), and the sample solution is layered on top of the gradient as for zone centrifugation (section II.2.d).

The total amount of macromolecules which a given gradient can support, is still completely empirical. As a first approximation, one can consider that equilibrium CsCI gradients, in swinging bucket rotors, can contain up to 20 fig of DNA per cm^2, without any major loss of resolution. In the experiment of Figure 18, where a CS2S04 gradient and a fixed angle rotor were used, each band contains about 160 fig of DNA; if the vertical elliptical section of the tilted tube is considered, this corresponds approximately to 30 fig/cnv^2. The resolving power of this experiment is only slightly below the resolution of the equivalent analytical centrifugation, where 20 times less DNA per cm^2 was used. It is a remarkable property of isopycnic centrifugation that the macromolecular load is about 10 times greater than for zone centrifugation.