Analytical Technologies in Biotechnology

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Module - 05
Centrifugation techniques
Lecture - 04
Separation methods in preparative ultracentrifuges

In previous lectures concerning centrifugation, we have discussed about the basic principle of centrifugation techniques and also we have discussed about different types of centrifuges. In basic principle has if you could recall there are many factors on which the sedimentation rate depends. One is applied centrifugal field and it is expressed in terms of, most of the time relative centrifugal field, that is multiples of the gravitational field or g force, we call it. Also like we have seen there are other factors, which will determine the sedimentation rate, like the size of the particle, shape, density. In shape it is like whether a particle is spherical and non-hydrated or it is a spherical and hydrated.

Also the viscosity of the medium and the density of the medium will also play an important role in determining the rate of sedimentation. So, all these factors where you have angular velocity or it could be expressed in terms of revolutions per minute and it is being expressed in terms of relative centrifugal field. And sedimentation rate in terms of the acceleration due to the centrifugal force and the frictional force, which is generated due to the acceleration of the particle, in a medium under centrifugal field. All these factors play an important role and we have discussed about them.

Sedimentation rate or sedimentation velocity could be expressed in terms of sedimentation coefficient also, that is velocity per unit field. And it is expressed in terms of Svedberg units, that is one Svedberg unit is 10 is to power minus 13 seconds. So, higher the Svedberg unit then higher will be the sedimentation rate. Then we have discussed about different types of centrifuges. And if you could recall in last lecture, we have discussed from right from very small bench tops centrifuge to high capacity refrigerated centrifuge, high speed refrigerated centrifuge and the analytical ultracentrifuges. Also we have discussed about in detail about the analytical ultracentrifuges.

Now, in this lecture we are going to discuss about the separation methods which are based on preparative ultracentrifuges. And you can say these are preparative methods where you are aiming to get the material for further biochemical investigation. In analytical ultracentrifuge, in contrast it was for determining the sedimentation behavior and other aspects could be done. Because of an optical system, which is attached to the analytical ultracentrifugation technique instrument.

So, if you could recall analytical ultracentrifuge is mostly utilizes or you, samples which are taken are either purified or at least partially purified for characterization. So, let us start with certain or some separation methods, which are widely used in preparative ultracentrifuges. And also like, it is the, you can say high speed centrifuges also, we will start with, we are going to discuss in this section. One is differential centrifugation, which is very common method of separation particularly, sub cellular organelles or cell components. Then we are going to discuss about density gradient centrifugation where, we are going to discuss about two methods red zonal centrifugation and isopycnic or isodensity centrifugation method.

So, let us start with differential centrifugation method. Now, differential centrifugation method is the most common technique and it is called like, most common technique and is widely used for separation of particles from a liquid medium or to separate particles of different masses into separate fractions, into the pellet and supernatant. Now, separation is achieved primarily based on differences in the sedimentation rate of the biological particles, of different size and density.

Now here, the liquid medium is as simple particular density material so here mostly the separation will take place on the basis of mass of the particle or you can say, size and density in terms of how it sediments, what is the rate of sedimentation of that biological particle. So, various fractions or various components of, in a particular sample will be separated on the basis that, of their differential rate of sedimentation.

Now obviously, particles with large size will sediment much faster, as compared to small size particles. So, larger particles will be a pelleting first and will require lower centrifugal force and medium sized particle will then follow up with higher centrifugal force. And then finally, small size particles could be pelleted will require much higher centrifugal force. So for, if you consider for same mass particle, but having different

sizes, the centrifugation will mainly sediment higher density particles. So, if certain particle have same mass, but densities are differing then higher density particle will be sedimented first.

Particles having similar binding densities, at many times you have the sub cellular organelles, which have densities in the range of say, 1.1 to 1.3 gram per centimeter cube, in sucrose solution they could be separated, if there is a tenfold difference in their mass. So, there could be one way, could be that they differ, they might be of same density, but they differ in mass, they could be separated like, we will be discussing red zonal one or they differ in densities actually, so that they could be separated on the basis of their different density.

Now, commonly used like, in simple pelleting like, you obtain partially pure preparations of sub cellular organelles and macromolecules. We will be discussing this, that how when you start differential centrifugation. And your material is homogeneously distributed across the tube or the tube in which you are doing the sedimentation. You end up getting not completely pure, but partially pure preparations of different materials or components like organelles, macromolecules or sub cellular organelles. And for the study of sub cellular organelles or tissues or cells, they are, how do we go about when you have to say, you would like to study different sub cellular organelles from a tissue or cells. The first thing to be done is, that you have to disrupt the cells or you have to release the internal components into the solution.

And that is done by disruption so the crude is disrupted cell mixture, which is taken or it could be done by different methods, as we have discussed earlier. It could be like say, crude way of breaking the cells for example, freezing and thawing or it could be done by sonication or French press applications or through enzymatic like, enzymatic method could be utilized for releasing sub cellular organelles or breaking the cell wall.

Now once this is done, then crude disrupted cell mixture will be referred to as homogenate, which contains all these things in a solution in a homogeneously distributed here. So, during centrifugation of a cell homogenate, which you have taken after disruption of cell, you will take them in the tube and then sedimentation starts. Now in like, differential centrifugation like I said, it takes advantage of the different sedimentation rates of various components of the, homogenate. So, what is done is that,

the homogenate, the differential centrifugation is done in steps like, stepwise you keep on increasing the centrifugal force.

So that, different particles sediment at different centrifugal force or different revolutions per minute and so when you have one centrifuge for particular time, particular time at, particular centrifugal force then one component or one particular component will settle down, usually larger component. And then as you increase, as you increase the centrifugal force by increasing revolutions per minute. Then what you get is the smaller components will start pelleting in the order of their size or mass you can say. So, here larger particles like they sediment faster than a smaller ones and this is the basis for obtaining crude organelles fractions by differential centrifugation.

So tissue homogenate is divided centrifugally into number of fractions, by increasing the applied centrifugal field. Now, a cell homogenate can be centrifuged at a series of progressively higher g force and times to generate pellets of partially purified organelle. As you increase the g force and you start with certain g force, this could be, if you know about the components, that is they will pellet at particular g force. Then you can start with larger one which may be largest component, which will pellet at particular g force.

You can start with that g force and also time depending on what tubes or what rotors you are taking and if you know that, then times could be set and then you could start centrifugation. And so you will get pellet and supernatant now, again what you will do is pellet is taken and supernatant can again be taken for higher g force, again something pellets smaller molecule. And likewise, you can go on to sediment all the components of homogenate in a series, by increasing the centrifugal field.

Now, any type of particle present in homogenate may be found in pellet or supernatant or both fractions, depending upon the speed and time of centrifugation and size density, size and density of the particle. So, this will be, I will show you in a while now, initially all particles of the homogenate are homogeneously distributed throughout the tube. So, during centrifugation particles move down the centrifugal tube, at the sedimentation rates and start to form a pellet, on the bottom of the centrifuge. So, what happens here when a cell homogenate is centrifuged say, at a very low speed say, 1000.

So, remember in these preparative ultracentrifuges techniques, the ultracentrifugation of very high speeds, are used only for a certain components to be pelleted. So, when a cell

homogenate is centrifuged at a very low speed say, 1000 g force for say, 10 minutes then unbroken cells and heavy nuclei pellets to the bottom of the tube. So, what you get, you get a pellet and supernatant now, the supernatant can further centrifuged at 1000 g or any like you have to choose that for say, 20 minutes to pellet. Certain sub cellular organelles of intermediate velocities, such as mitochondria, lysosomes or other sub cellular fractions. Now some of these sedimenting organelles are obtained in partial purity and typically contaminated with other particles. So, why they are contaminated, let me show you on your screen this thing.

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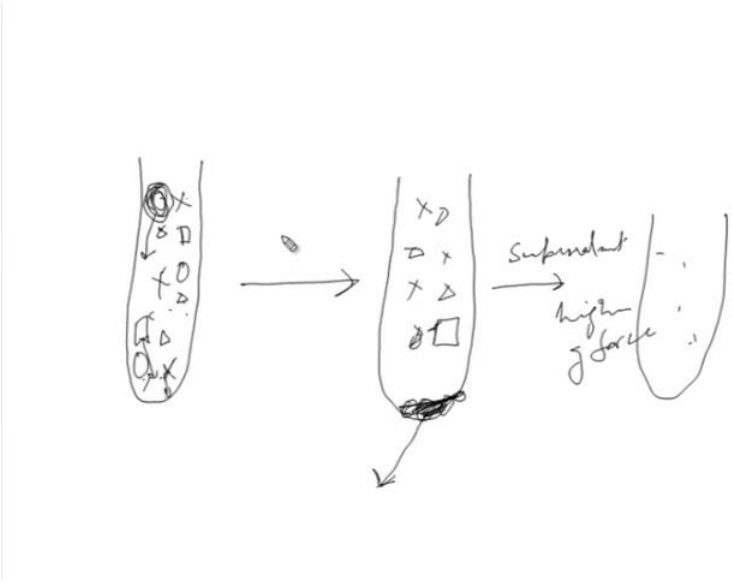

What is the homogenate, cell homogenate is taken in a tube and this is distributed all over. And this will contain different size fractions, this will be, there is one fraction here this is one another fraction, this could be another fraction, this could be another fraction and they are distributed all over actually. So ,what you have is, these are distributed all over now, when you apply a particular centrifugal field for example, say you apply a centrifugal field of say 1000 g. Then certain or larger component will settle like we said cell debris and other very heavy nuclei could be settling. So, what you will get is, you will get a pellet here depending on how, what kind of rotor you are using.

Pellet will form accordingly and you will get so pellet will contain what, pellet will contain say the biggest one molecule. Now, problem is here, why you get partial purity and you will also get these things which are again distributed all over actually. Now, why

you get partial purity when centrifugal field is applied then different molecules will sediment at different rates, but these molecules are homogeneously distributed all over the tube.

So, a molecule which is here will experience a particular force, a molecule which is here, these are molecules which are already present at the bottom, they will also experience certain force. So, what will happen is this molecule, which is larger molecule will travel down here, but these molecules which are at the bottom only, they will also travel down here. And so what you get is you get a pellet which might contain major fraction, portion of the larger molecule, but it will also contain, it will also contain other fractions, the other components. And this is because they are distributed across and the one which is very close to the bottom will also sediment in here.

So, unless you layer on top of here, this particular problem will be there. Now, what is happening so what you have to do to get pure purified, what is done is, you take the supernatant in next tube, you can decant it. And again the centrifugation, will be done at higher g force so you will do it at higher g force like say for, 1000 g force or whatever it is. Likewise, you can, you will take this pellet also and you will again homogenize in a particular suitable solvent solution or medium we can say.

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And this is again, this pellet is which is again, which will contain more of these, but it will also contain other smaller ones. Again it will be centrifuged and this process will be

done many times to, get the pure fraction. So, partial you can achieve reasonable purity by, you can achieve reasonable purity by going through these steps of repeating the centrifugation at that speed by, taking the pellet and then again doing it many times so that, you can get the pure fraction.

So what is done is that, centrifugation is continued long enough to pellet all the largest class of particle and the resulting supernatant is centrifuged at higher speed to separate medium sized particles. Like I said repeated washing of the pellet by re suspending it in isotonic solutions and repaletting may result in removal of contaminants, that are smaller in size. So, you could do it, you can purify to a large extent, a particular component by re suspending the pellet in a particular solvent and then again going through may cycles or at least few cycles of washing the pellet. And again sedimenting it and then finally, you get quite reasonable purified material.

So, the separation here achieved may be, improved by this particular procedure and re centrifugation and paletting and re centrifugation, this could be. So, you can obtain partially purified organelles by, differential centrifugation and it serves as a preliminary step for further purification, using other types of centrifugal separation technique. Like, we will be discussing density gradient separation so though you get partially impure fraction here, but this is the very useful technique, a very much used technique for various application.

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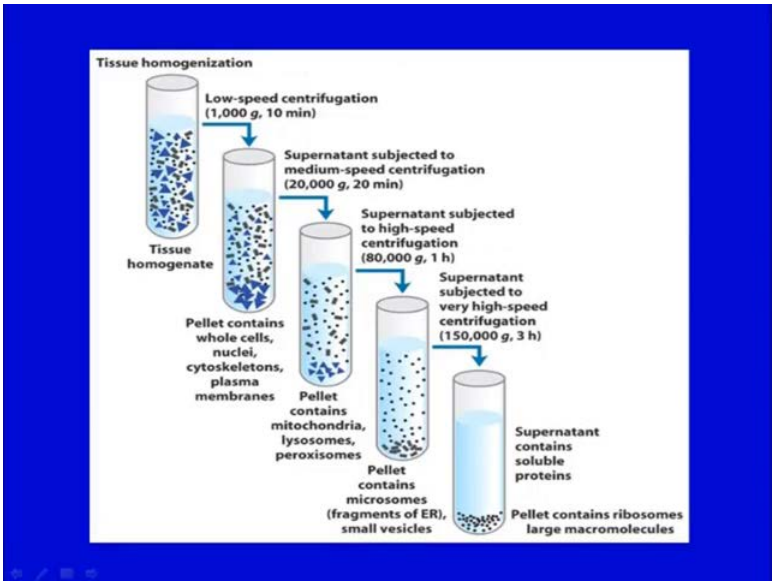

Let us pay attention, whatever we have said, let us see how this is done in here. So, if you see this figure on your screen, what you have is, you have, you have a tissue homogenization. And remember you have to disrupt the cell by any technique and then components are released into the solution and they are called homogenate. So, tissue homogenate is obtained or cell homogenate is obtained here and you can see, there are lot of different components of different sizes, they are equally distributed or homogeneously distributed all over the tube.

Now, what you do first thing is, you start with very low speed or g force and you centrifuge at a particular time say 10 minutes, what will happen once you centrifuge this for 10 minutes the largest one these triangles settle down here. So, the pellet contains whole cells, nuclei, cytoskeletons, plasma membrane and other things are there, which pellets in here. Now, again like I said this pellet is not completely pure or does not constitute only the largest one, there will be smaller ones also.

So, the re suspending and again re centrifugation couple of times is required. Now, what is done, pellet and supernatants are removed here and then this supernatant is taken in a fresh tube and then again centrifugation is performed. Now, at this time you will go for higher centrifugation, like here it is 20,000 g force for 20 minutes or it could be like, you can go little smaller steps like, you can start with like 10,000 g also, this is just to understand the whole concept.

So, what will happen it will pellet, certain other sub cellular organelles like say mitochondria or lysosomes or peroxisomes, these will be sedimented. So, next bigger like, this will be the largest one and the medium sized particles are being pelleted here. So, again you will get a pellet, this pellet will be partially pure or not completely pure so again it has go through re suspension and re centrifugation and to get the reasonable purified sample.

Now, supernatant here would be again taken in a separate tube and then again centrifuged at much higher like say 80,000 g here for 1 hour. Now, you can see that for larger molecules you require low g force as well as less time, but as you go through these steps, the smaller molecules require higher g force as well as you require more time for centrifugation. So, again you get a pellet which contains microsomes, fragments of ER, small vesicles etcetera and again, this could be purified.

The supernatant again will be taken and again done, again it will be recentrifuged, supernatant will be centrifuged at much higher say 1,00,000 g or force. So, what you will get pellet will contain ribosomes, macromolecules and other things. Supernatant is still might contain certain small soluble proteins, which might not pellet as such. So, this is how differential centrifugation is done in steps and lot of things could be separated on the basis of their rate of sedimentation, which is dependent on the centrifugal applied centrifugal field and their mass.

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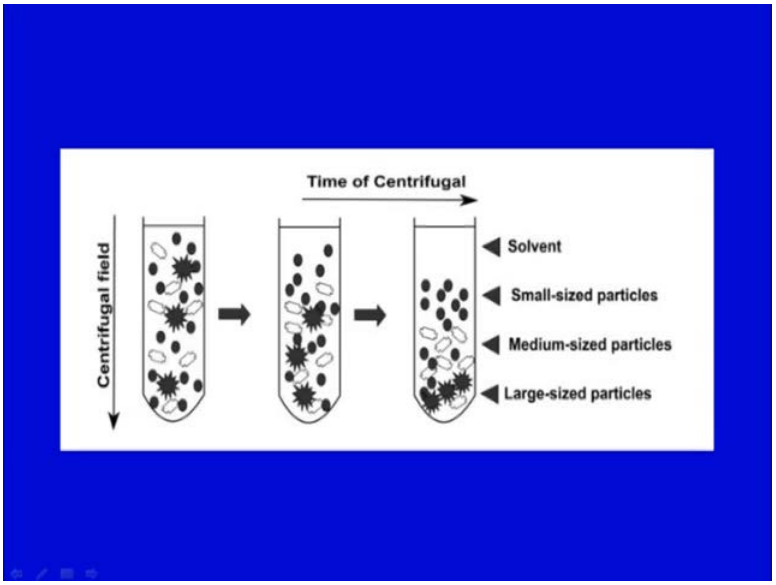

So, this is how centrifugation is done in differential centrifugation. Now, this is very simple way of showing this, that you have a centrifugal field in this direction. And as you go along, you get different sized fragments and separated and could be purified as we go along in here.

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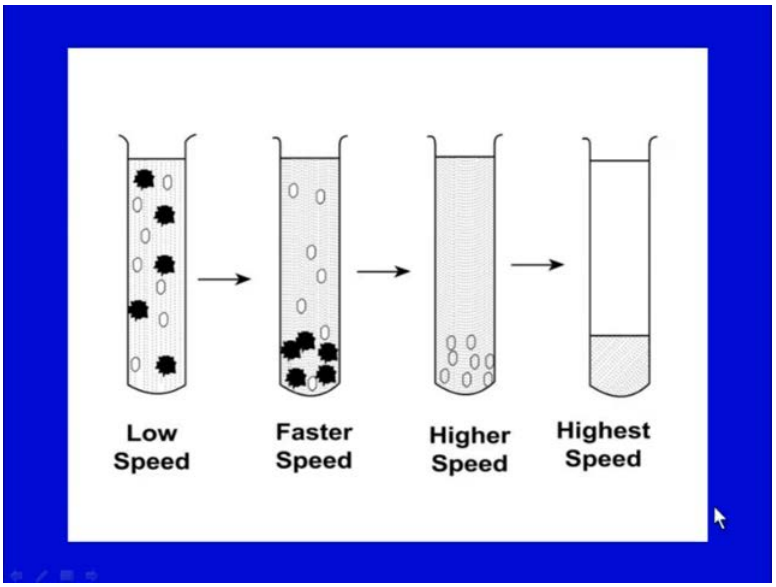

So, first is low speed and faster speed, higher speed and highest speed tester, a sequence of events which occur in the centrifugation method, this centrifugation method.

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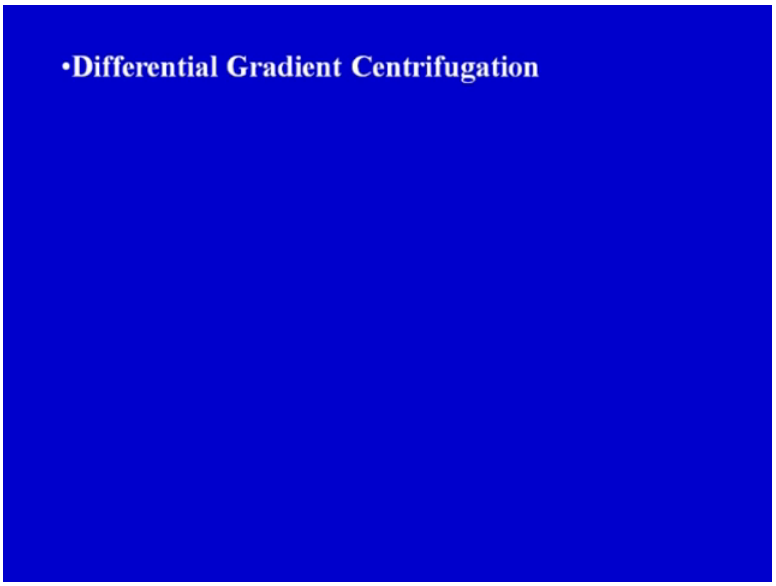

So, to summarize differential gradient centrifugation, takes advantage of the differential sedimentation rate or the differences in applied centrifugal field for sedimentation in a particular time. Different components larger, medium sized and the smaller components will be separated by gradually or stepwise increasing the centrifugal, applied centrifugal field and for different times. And you will separate large molecules first, which will

sediment first then medium sized and then finally, a smaller size molecules will be sedimenting, at much higher applied centrifugal field.

So, we will move onto the next method that is density gradient centrifugation Now, density gradient centrifugation is the preferred method, to purify sub cellular organelles and macromolecules. It is pretty good and gives, can purify lot of different types of sub cellular organelles and macromolecules like, proteins. Now, density gradients can be generated by placing layer after layer of gradient media.

Such as sucrose in a tube, with the heaviest layer at the bottom and the lightest at the top, in either, this could be a discontinuous like stepwise gradient or it could be a continuous gradient. The cell fraction to be separated or the particular molecule or material to be separated is placed on top of the layer and centrifuged. In density gradient centrifugation what is done is that, you can very well separate sub cellular organelles and what is done is…

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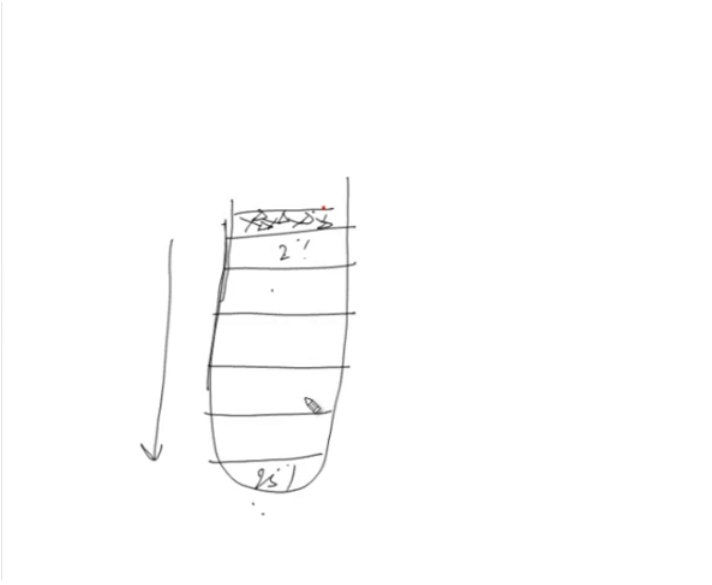

Like I said, there needs to be formation of a gradient so gradient can be formed, the gradient is in, it is a increasing like concentration, it is a increasing concentration of the gradient material in here. So, as you go along there will be one concentration then you can like say in terms of percentage, if I say 2 percent to say 25 percent or so it could be different.

What is done is, then so you have you can make it a step gradient, that is you can layer first 25 percent and then the next one, the next one and the lightest one at the top or it could be by a gradient maker. As we have discussed in earlier sections by, you can use a gradient maker to make a continuous gradient, where the lowest, at the bottom of the tube contains highest gradient material. And the upper one or the starting of the tube has, lighter or you can say lower concentration. The sample will be placed in here, of a particular material or a particular say components which could be macromolecules or sub cellular organelles and then centrifugation is performed.

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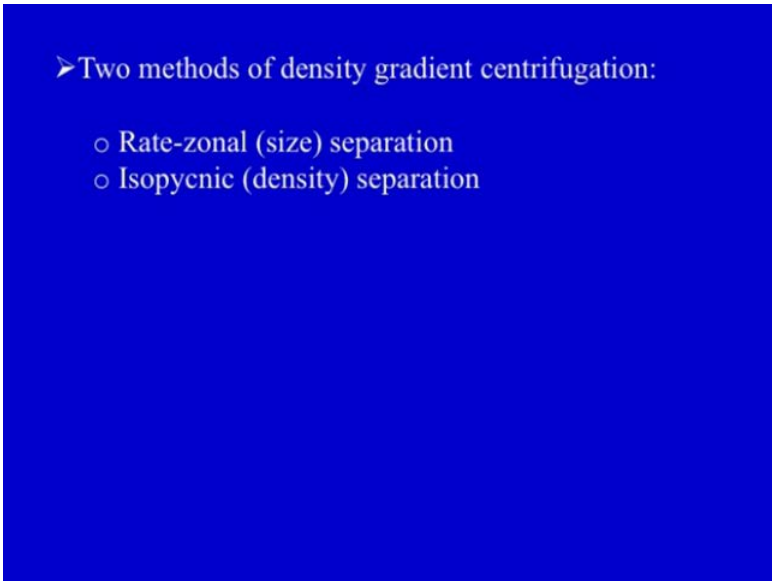

So, what are the two methods actually, which we were discussing about in density gradient centrifugation. One is called rate zonal separation, this is based on size of a particular analyte and second method is isopycnic or isodensity separation. Now, both of these methods are used for, quantitative separation of components and determination of bion densities. And also for estimation of sedimentation coefficients that could be utilized.

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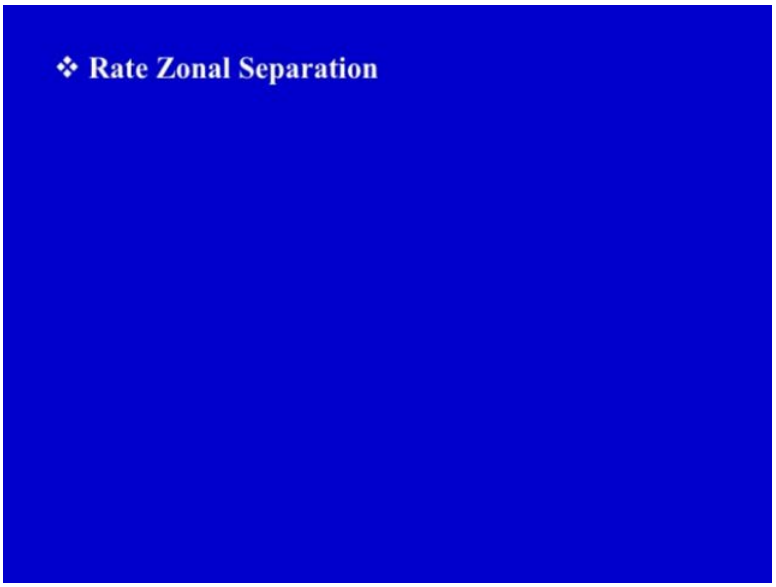

So, let us start with first one rate zonal separation now, these are based on differences in size, shape, density of the particle, density and viscosity of the medium and applied centrifugal field. Now, rate zonal separation takes advantage of a particle size actually, and mass instead of particle density. So, what is done here is for sedimentation of similar type of biological particles, which have almost like same density, but they have differences in their mass or size.

Now, when you are preparing the density gradient in a tube then in this particular method maximum density of the gradient is chosen, not to exceed that of the densest particle to be separated. So, this has to be understood that, the maximum density of the gradient you have chosen in and you are keeping, you are putting it in the tube, is such that it will not exceed the density of the, densest particles in your sample. So, what does that means that, your sample largest particle can be pelleted in here because the largest, it is, the largest density of the gradient is smaller than the densest particle of the sample.

Now, examples of common applications here includes separation of cellular organelles, particularly which has different sizes then separation of proteins because proteins mostly have similar densities in a particular range. And but they have different sizes actually so if you have 3 to 4 fold differences in the mass then they could be separated on rate zonal separation method. Now, sample is applied in a thin zone at the top of the centrifuged tube on a density gradient solution.

So, what is done first you have formed the density gradient in the tube and once that is formed, it could be continuous or a stepwise gradient, continuous is better always then sample is will be applied on top of this density gradient. Then under centrifugal force what will happen, the particles will begin sedimenting through the gradient in separation zones, that is spaced according to the relative velocities of the particle.

And they will be moving based on their size, shape and density now here function of gradient is mostly to stabilize the liquid column in the tube and to improve the resolution of the gradient. So, what is done is, what is happening is here they are mostly moving on the basis of size and obviously, the density like certain components might have densities which are in the range of the density gradient. So, they will not move, but these are separating on the basis of size and density like, many particles will move through because densest part of the density gradient is not is smaller than the component materials here.

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Now, what is the criteria for successful separation here so what we have said density of the sample solution must be less than that of the, that of the lowest density portion of the gradient. Now, density of the sample particle which we have taken, which are, which will be in separated must be greater than that of the highest density portion of the gradient.

So, the path length of the gradient must be sufficient for the separation to occur remember if you, so time is very important in here. So, because like if you perform the very long runs, that is if you perform a run beyond particular time then the particles which is densest particles will certainly pellet at the bottom of the tube. And that will not be like, they will not remain in the density gradient solution and that will be a problem. So, what is done is, that it is the time limited run has to be performed, that is you have to determine for how much time this run has to go so that, to ensure that the particles do not pellet at the bottom of the tube

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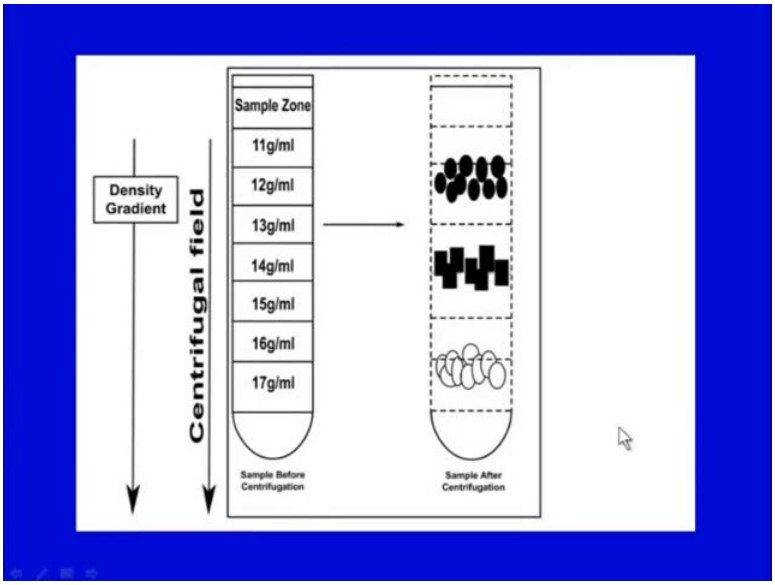

So, here it is shown very clearly that you have a density gradient and you have this density gradient in here, you have a sample zone which is layered on top of the density gradient. And then centrifugation is performed for a particular period of time now, remember this material should not be sedimenting or pelleting here. Rather, they should be separated in the column liquid column only so in a particular time when it is switched off these particles are separated on the basis, mostly on the basis of their size actually.

Like I said these particles are mostly of a same density kind so they will be separating on the basis of their size here. So, this is how rate zonal separation is achieved. So, what are the key features of rate zonal separation, in rate zonal separation one is that the, it is like, it is done for a particular period of time, it cannot be unlimited, it cannot be run for unlimited time.

Then, the density gradient which is formed, the densest part of the density gradient should be lesser than, less than the densest component of the sample so that, pelleting does not take place. And as you do centrifugation for limited time, these molecules will be separated on the basis of their size, if they are of the same density, but they are differing in 3 to 4 fold in their masses then they could be separated.

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Let us move on to the next one that is, isopycnic or isodensity centrifugation now, isodensity or isopycnic centrifugation is depends upon the bion density of the particle. And it is not dependent on its size and shape here so it is a density which is the most important, it is exclusively depending on the density. Now, in this type of separation a particle of, a particular density will sink or move during centrifugation until a position is reached, where the density of the surrounding solution is exactly same as the density of the particle.

So, once that is reached the particle will be settled in that density or equal density. So, once this quasi equilibrium is reached then the length of centrifugation does not really influence on the migration of the particle because particle will not move further, as it is floating on the cushion of a higher density material. So, these are very useful for separation of, this particular method is very useful for separation of sub cellular organelles, which are almost of the same size, but they differ in density actually.

Like for example, you have like, certain sub cellular organelles like have 1.1 gram per centimeter cube mitochondria, peroxisomes they have 1.19 and 1.23 gram per centimeter cube, density in sucrose solution. These could be, all these sub cellular organelles which have a particular density and they might be roughly equal in size. This method could be used for the separation of these sub cellular organelles.

A common example for this method is, the separation of nucleic acid in a caesium chloride gradient. Now, remember so this is like, one is you have layered the sample on the top of the density gradient. So, then when you do centrifugation these travels, the components travel and they settle down at their isodensity. And you can say, they are floating on a cushion of higher density because they cannot move further, you can run it for anytime or at any centrifugal field, they will not move further because the density of the gradient will be higher than their own density. So, they will just settle down where there is an equal density.

And there could be another method where, rather than overlaying the sample on the top of the density gradient, the sample could be mixed in a particular density gradient material. And as the centrifugation goes, the density gradient material forms the gradient and the components of the sample, will automatically reach their isodensity. So, a very common method is, which is very well known is the separation of nucleic acid in caesium chloride gradient.

Now, these methods are combination of sedimentation and floatation, at involves a layering sample not on the top of density gradient, but it is mixed in here. You can layer it also, on top of that after making the gradient, like you have to run it for a longtime to make caesium chloride gradient. This is common these methods are commonly used in analytical centrifugation to determine bion density of the particle, base composition of double extended DNA and to separate linear forms of DNA.

So, they could be, these methods could be utilized, this method could be utilized for determining bion density of the particle and lot of other like I said, the isopycnic density gradients separation could be utilized for a various application. Like for separation of sub cellular organelles and for determining bion density or for separation of DNA and other materials could be done on isodensity or isopycnic separation method. So, we have done

in here, we have discussed about two methods in in density gradient centrifugation that is, rate zonal and isopycnic both are different and they cater to particular needs.

Now, let us move on to the few more important points like for example, what are the criteria for successful isopycnic or isodensity separation. Now, in this particular method density of the sample particle must fall within the limits of the gradient densities. Like whatever gradient material you are utilizing and the range of gradient you are making or forming the sample density of the sample or the components of the sample, should also be falling within those limits. Then here in isopycnic any gradient length is acceptable, as it is isodensity or floatation method, there is no problem about the gradient length as long as the sample density falls within this range.

Most of the time, you can use the continuous or stepwise gradient, but it is preferable to use a continuous gradient for best results. The run time for isopycnic separation must be sufficient for the particle to bend at their isodensity point or isopycnic point. Excessive runtimes have no adverse effect, but if you run for a shorter period of time then may be, the bending might not occur at the isodensity point. So, runtime must be sufficient and that has to be determined.

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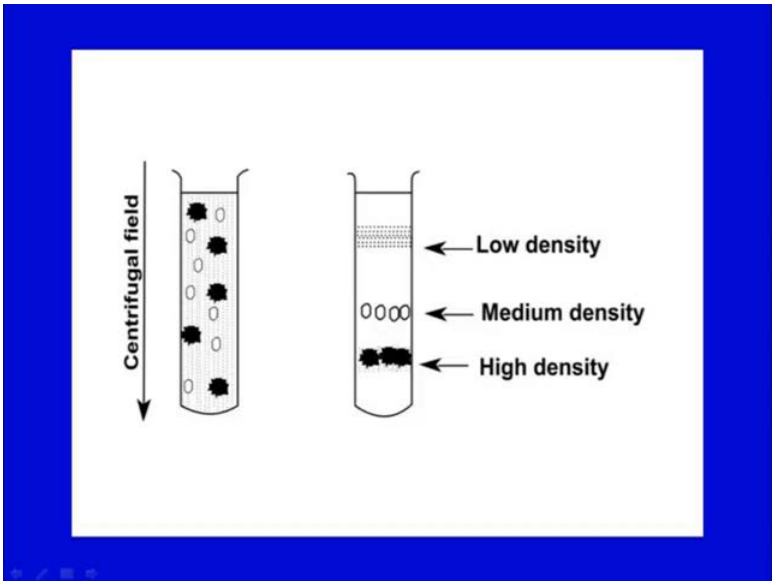

Now these are so if you want to like, here it is explained in this figure on your screen. So, your material could be distributed or it could be layered on the top as you centrifuge and as the gradient is formed. Then different materials will be distributed according to

their density. So, low density material will be at the top and very high density material will be at the bottom and in between will be medium density samples. So, according to their density they will settle down at their isopycnic or isodensity layer and then they could be separated easily or recovered easily.

Like I said as an alternative to layering, these particle mixtures could be mixed with the gradient solution and so there are two ways you can do it, sample application. One is layering the particle mixture on the top of the preformed gradient and another is mixing the material with gradient medium. And finally, the gradient, self-forming gradient materials are used like caesium chloride and they will be separating.

And this is called equilibrium isodensity method, as the material, they will be forming the density gradient automatically, as the centrifugation is performed. And also, the samples to be purified will layer on their isodensity, many salts of heavy metals like caesium or rubidium and other materials are utilized for forming the gradient, for certain applications.

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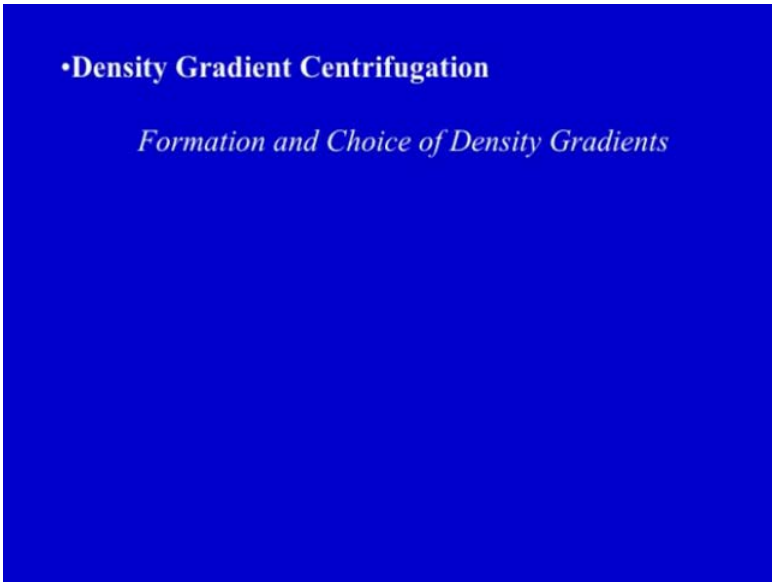

Now, let us discuss some more points about, the density gradient separations. One is formation and choice of density gradients now, all density gradient method involve a supporting column of liquid, whose density increases towards the bottom of the centrifuged tube. And the function of density gradient here is, one is to stabilize the

column of liquid in the centrifuged tube, to prevent mixing of the separated part particle due to convection currents.

To improve resolution of the separated components by eliminating factors, such as mechanical vibrations and thermal gradients and also density gradients. So, these are certain points which are very important, which you can say these are points or the function which determine the function of these density gradient in these methods.

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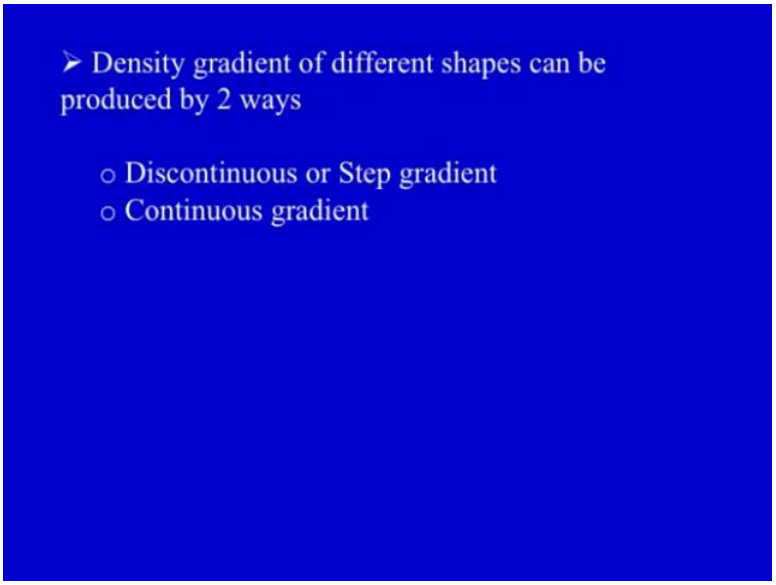

Density gradient of different shapes can be produced and like we were discussing, it could be discontinuous or step gradient or it could be continuous gradient. Now, discontinuous or step gradient, it is done by over layering method. So, what is done is known volumes of solution, of decreasing densities are allowed to run slowly down the side of the centrifuged tube, to form layers over each other. It could be done by over layering method or under layering method, that is a layer of lightest density is introduced into the centrifuged tube, with series of layers of increasing density placed over the first layer, by pipetting.

So, it could be both methods could be utilized, discontinuous gradients can be used directly by layering of sample to be separated as a narrow zone, on the top of the layer or if allowed to stand, layers slowly merge by diffusion to provide a continuous linear gradient. Many times what happens is, the step gradient or discontinuous gradient, if

allowed to merge they will also merge and form a not a typical continuous, but sort of continuous linear gradient.

They can be used for isopycnic centrifugation and these are suitable for separation of whole cells or sub cellular organelles from plants or animal tissue homogenates and for purification of say, viruses or other components. So, there was discontinuous gradient then continuous gradient for continuous gradient making, you require a spatial apparatus called density gradient maker. Our density gradient maker is capable of generating linear concave or convex gradients and these gradient makers consists of two chamber device, for making continuous gradients.

So, what you have is two identical chambers a and b connected by particular tap channel, contains equal volumes of the high density and low density material solutions respectively. And they, the magnetic stirrer is there which keeps on mixing actually a particular chamber. So, the chamber b which sits on a magnetic stirrer bears a delivery tube, which reaches to the bottom of the centrifuged tube by a low pulsation peristaltic pump.

So, when the labels of a liquid in the two chambers fall, synchronously dense solution from a mixes continuously with the higher solution in b. So, that the pump delivers the solution of ever increasing density, to the bottom of the centrifuged tube. So, the two stirring bars ensure that, the label of the liquid in a and b is the same. So, if the two solutions have very different densities and if the string is not sufficiently vigorous, the dense solution may be, may flow under the lighter solution. So, this is very important that, mixing should be proper here.

Now, if the placement of the two solutions is reversed then the tendency of the less dense solution to float up through the denser solution, in b will improve mixing considerably. So, in this format the tip of the delivery tube needs to be placed against the wall of the tube, near its top actually so that, a solution of decreasing density flows to the bottom. And now, this can be used for rate zonal or isopycnic centrifugation and different types of gradients can be made in here.

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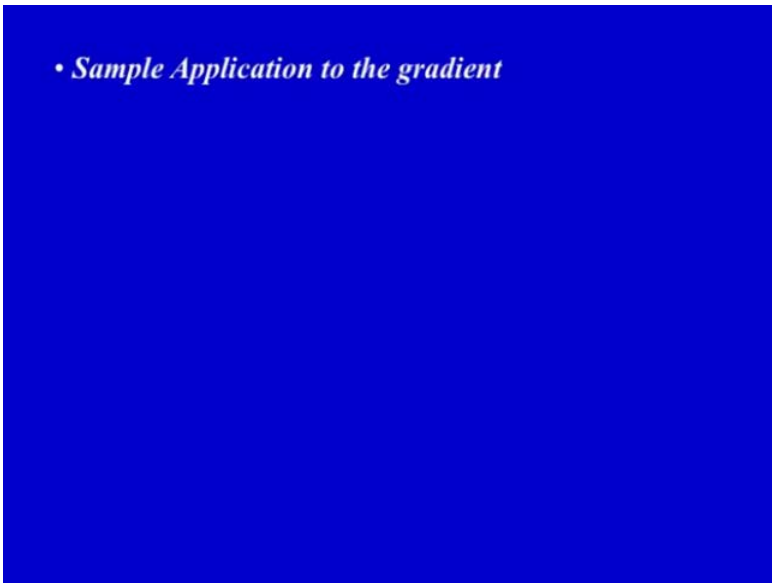

Once the gradient is formed then sample applications to the gradient has to be performed as we have seen. Now, optimum volume of the sample here and the concentration should be determined, as each gradient has a defined sample capacity. The volume of the sample that can be applied is, a function of the cross sectional area of the gradient exposed to the sample. So, sample volumes in the range of say 0.2 to 0.5 ml may be added to the say, 1 to 1.6 centimeter diameter and sample volumes up to 1 ml or 1 centimeter cube to tubes with diameter of 2.5 centimeter could be added.

Effective separation could not be achieved with larger sample volumes remember, this is also very much important that how much sample you are applying. For proteins and nucleic acids, sample concentration of say 1 microgram per ml to 1 milligram are recommended in a sucrose gradient. For rate zonal separations, samples should be applied with a narrow bored piped slowly and gently to reduce mixing with the gradient.

So, we will so in this lecture, we will continue our discussion in the next lecture about, the formation and different aspect of the density gradient. So, in this lecture we have discussed about two methods, one is differential centrifugation and another is gradient centrifugation methods. In differential centrifugation method, it is the different sedimentation rate, that is at different applied centrifugal field, particular components of the sample sediment. And that is taken, that criteria is taken and in steps, the increasing centrifugal field is applied to sediment components at different centrifugal fields.

In density gradient centrifugation, there are two methods we have discussed, rate zonal and the isopycnic separations. In rate zonal separation, mostly on the basis of size like for example, many proteins with same density, but different sizes can be separated. And isodensity or isopycnic separations, it is based on the density. So, many sub cellular organelles having roughly same size, but different densities could be separated in here. In the next lecture, we will continue with the discussion of gradient formation and different points like, recovery and monitoring of gradients. And also we are going to start with the, with the rotors, different types of rotors which are used. And we will continue our discussion in the next lecture.

Thank you.