Novel Separation Processes
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Module No # 01
Lecture No # 40
Electrophoretic Separation Methods (Contd)

In the last class, we are discussing about the electrophoretic separation method, and this method becomes very important whenever we are talking about the separation of charged molecules are constant. Now, most of this has relevance in case of separation and purification and analysis of biomolecules, because most of the biomolecules are charged in nature. We have seen that these particular property or charged property of the biomolecules will be depending on its PH.

Most of the biomolecules will be having a particular PH, where the molecule is charge less or neutral. This particular PH is called Isoelectric PH. Now, various different biomolecules will be having different values of isoelectric PH. If you use the operating PH different from the isoelectric PH, the biomolecules will be charged according to your wish. Now, if you are set your operating PH less than the isoelectric point, the molecule will be positively charged. If you set your operating PH above the isoelectric point, the molecule will be negatively charged.

Now, in presence of an external electric field, if these molecules are placed in a solution, preferably in an electrolytic solution and apply an external electric filed then, these molecules will be moving to a particular electrode depending on its charge. So, therefore, if you set an operating PH, such that a mixture will be having a mixture of let us say four components. Let us say two of them will be having positively charge two of them will be negatively charge, at a particular PH then, if you apply an external electric field, the electrophoresis takes place and two molecules will be moving to one particular electrode and other two molecules will be moving to the other electrode with opposite polarity.

So, therefore, a mixture can be separated out, by using this particular method and the basic electrokinetic phenomena that is involved or responsible for this separation process is electrophoresis. So, electrophoretic separation method has become very important nowadays, in order to separate the charged molecules.

Now, this electrophoretic separation method can be utilized for fractionation purpose, for analysis purpose and things like that. Now, we, in the last class discussed some of the problems associated with the electrophoretic separation process. The major problem that will be arising out of it is the joule heating. It is because of the presence of electric external electric field; that means, the external electric field will be created if you apply a voltage difference between the two points of the cell then voltage difference divided by length will be electric field strength.

Now, because of the presence of electrolyte in the solution, there will be formation of electric double layer and the redistribution of the charges like co-ions and counter ions and formation of electric double layer. This double layer will result into free or excess charges and those will be moving under the influence of external electric filed producing a current. This current can be causing a joule type of heating like I square R. Now, this joule heating type of heating is very deleterious or harmful as far as the electrophoretic separation is concerned, because it causes the local temperature fluctuation that will lead to fluctuation in the density that will give rise to the buoyant force.

If the bond force is more than the viscous force then this will cause a natural circulation like natural convection sort of thing. So, the electrophoretic movement or the electrophoresis will be hampered. So, this is not desired. Secondly, at the higher temperature, the protein molecules may be denatured. So, under all circumstances, this thing has to be subdued or has to be prevented. In order to prevent joule heating and buoyant effect, generally a medium is placed into the solution that is either a gel or a membrane or paper. So, it is called Gel Electrophoresis or you know or Membrane Electrophoresis.

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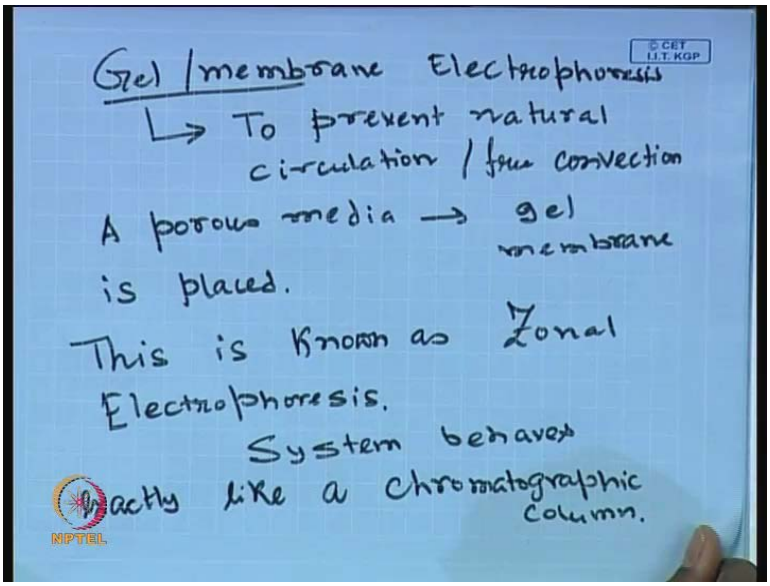

Now, in order to prevent, let us look into the various aspects of that. So, gel or membrane electrophoresis is done to prevent the natural convection or natural circulation in the system. Natural circulation like free convection and a porous media, either it is a gel or in the form of a gel or a membrane, is placed and this method is known as Zonal Electrophoresis. When you conduct the electrophoresis in presence of the gel or membrane, this is known as zonal electrophoresis. And the system behaves exactly like chromatographic column and therefore these stabilizing agent or the gel that you are going to use into the system, they must be having some desired properties.

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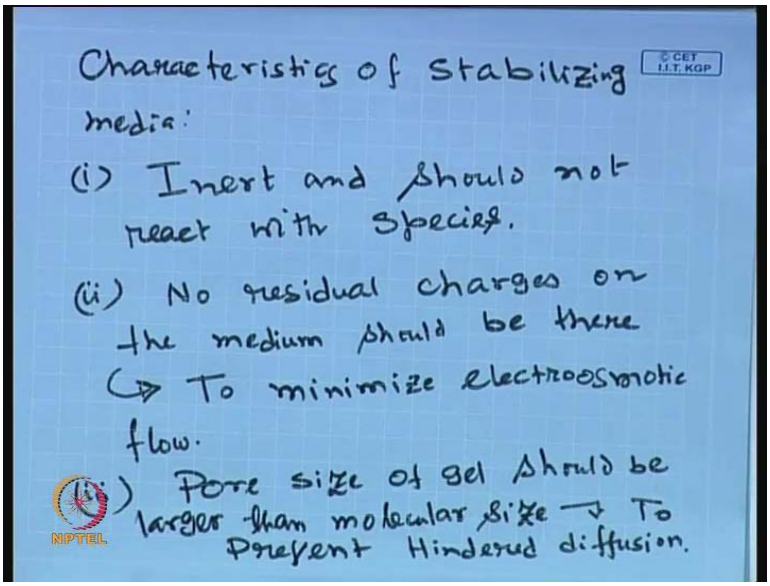

Now, let us tabulate these desired properties or the desired characteristics of the stabilizing media. Number one, it should be inert and should not react with the existing species those need to be separated. It should be inert and should not react with species. Secondly, residual charges on the media should not be there or no residual charges on the medium should be there. This basically occurs and this is basically another problem that crops up during electrophoresis. This is because of the electro osmosis flow. Now, if we know that if you have a solution of the electrolytes and then you have a charged wall.

Now, if you apply an external electrical field then, the excess charges that is present they, will move under the external electric field in a particular direction giving rise to another electro kinetic phenomena and that is electro osmosis. So, electro-osmotic flow or the electro-osmotic velocity will be added to the electrophoretic velocity in that case and the separation will be mugged or the separation will be blocked. So, we would like to minimize two problems; one is the natural convection another is the electro-osmotic flow. So, no residual charges on the medium are desired in order to minimize the electro-osmotic flow.

Thirdly, the pore size should be greater than the molecular size so that no hindered transport occurs. Pore size of gel should be larger than molecular size of the species. Why this is required is to prevent hindered diffusion. So, diffusion should be spontaneous and it should go unhindered.

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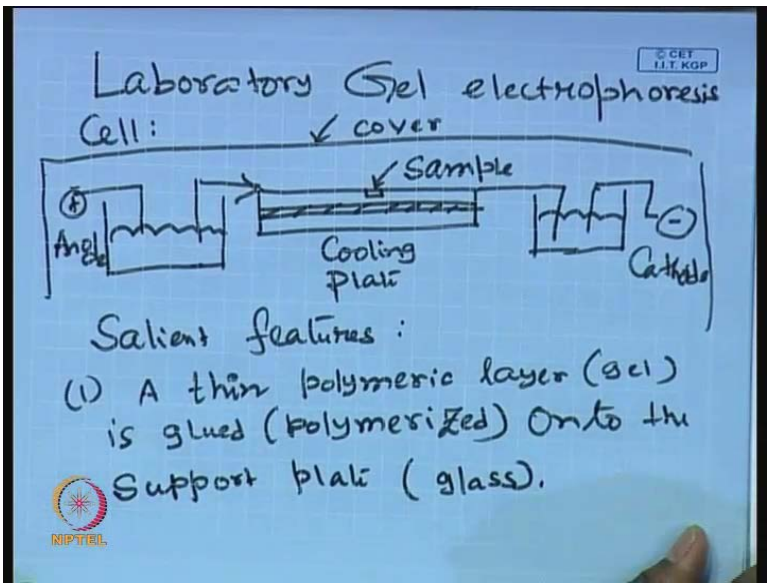

We will just draw a typical schematic of laboratory gel electrophoresis cell. This is a solution and this is the main cell and in between the gel is trapped. So, this is the gel and the sample is put here. At the bottom, there is a cooling plate in order to cool the system, to prevent the joule heating. You cannot avoid joule heating, you can minimize it. Then there is another bath and this will be given to the cathode and this will be going to the anode. The whole thing is kept under a cover.

Now, the salient features of this laboratory scale setup is that a thin layer of gel is polymerized onto the support plate, which is typically a glass plate, a thin polymeric layer, which is basically a gel and is glued means it is polymerized. It is polymerized on the base plate onto the support plate, which is typically a glass plate. Support plate is placed on a coolant plate so that the joule heating is prevented.

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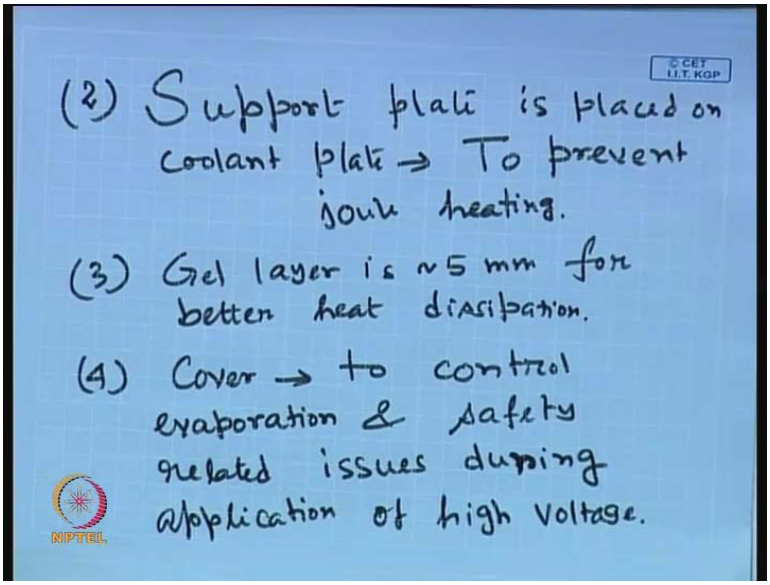

We have a coolant plate on which support plate is placed to prevent joule heating. Gel layer should be about 5 millimeter for better heat dissipation and that is a typical dimension which is about 5 mm for better heat dissipation. The cover must be there in order to control some amount of evaporation and safety related issues during application of high voltage.

So, generally the whole thing is kept under a cover. If there is some evaporation takes place at joule heating, the temperature will be more and to prevent the evaporation.

Suppose, we are using the high voltage and in order to have the safety in your whole apparatus, the whole apparatus is placed in a cover.

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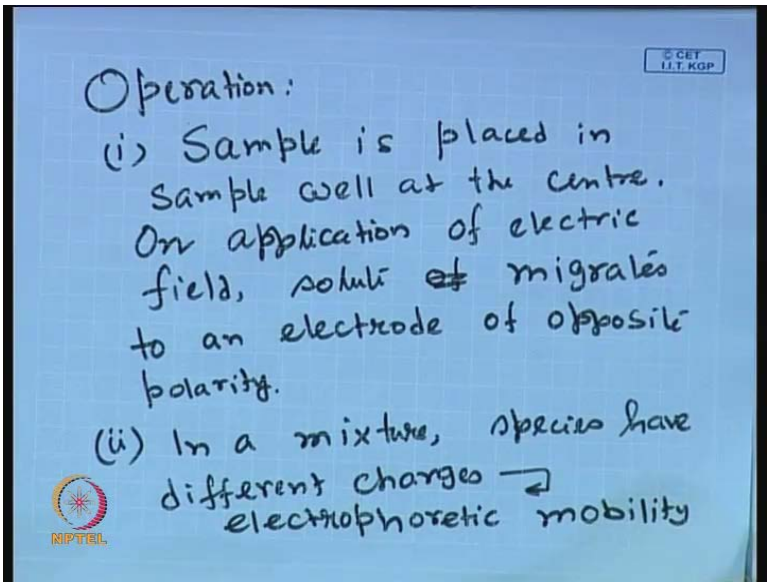

Now, what is the operation? Let us look into the salient features of the operation. The first one is that the sample is placed in the sample cell or the sample is placed in sample well and that is called sample well at the center of the glass slab. When current is on that means, when the voltage is applied, solute of appropriate polarity will go to or migrate to the electrode of opposite polarity. On application of electric field, solute migrates to an electrode of opposite polarity.

Now, if you have more than one species this solute migration velocity will be there and that means, the electrophoretic velocity will be different, because each of them will be carrying different charges. So, in a mixture, species have different charges, may be of opposite signs also. Even in the same sign, they will be having different values; because of this the electrophoretic mobility will be different for this species. Mobility differs for the species and on the application of external electric field, since the electrophoretic mobility is defined as the electrophoretic velocity per unit field strength, so on application of electric potential difference then this electrophoretic velocity of various species will be different and they will migrate at different velocities after sometime. So, at the end of the residence time, at different regions different species will appear.

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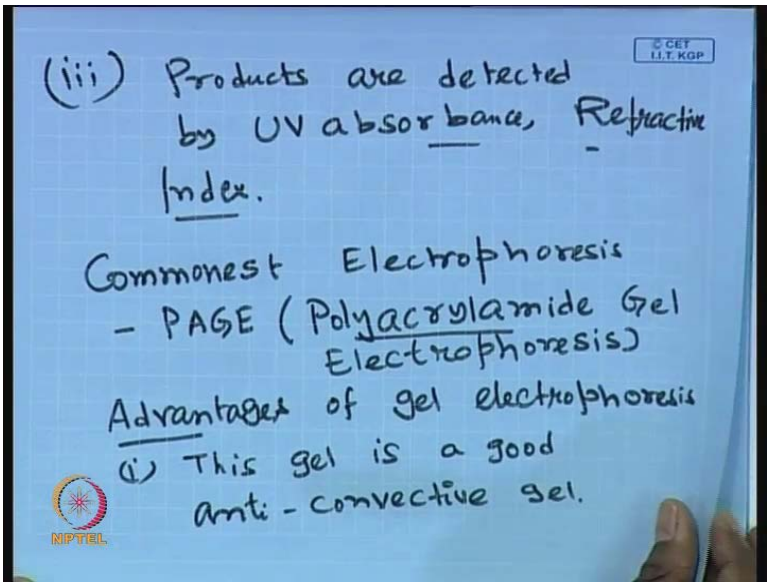

The products are therefore, products are detected by ultraviolent absorbance or refractive index. By using these, the products are detected and they are connected to the concentration of various species exactly like the chromatographic separation process. The most common electrophoresis is Polyacrylamide Gel Electrophoresis or PAGE. Commonest electrophoresis is PAGE and that is polyacrylamide gel electrophoresis. And then let us look into the advantages of gel electrophoresis. Let us list down the advantages of gel electrophoresis. This polyacrylamide gel is an excellent anti-convective gel. I am talking about this polyacrylamide gel. This gel is a good anti-convective gel; that means, that gel structure is such that the convection is not allowed. So, it is basically subdued.

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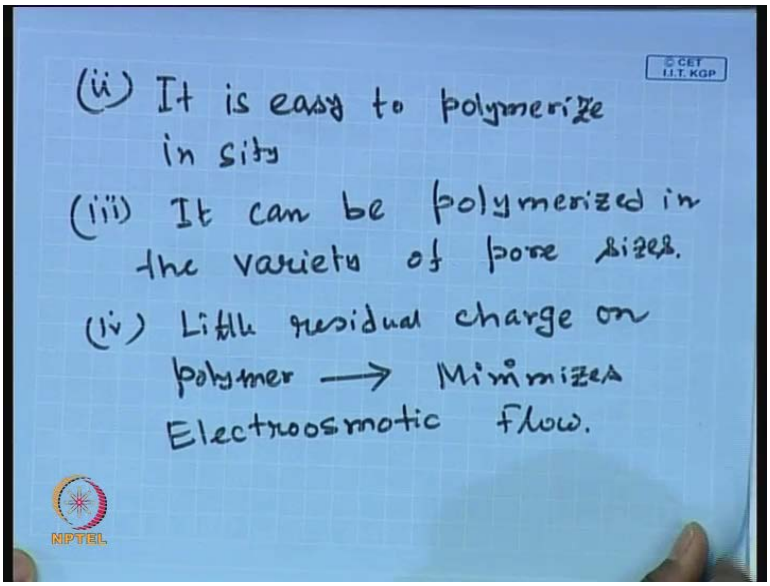

Secondly, it is easy to polymerize in place; polymerize in situ, in the same place. Third one is that it can be polymerized in the variety of pore sizes; it can be polymerized. The fourth one is there is little residual charge is present on the polymer. Charge on polymer, this simply allows the minimization of electro-osmotic flow. This minimizes electro-osmotic flow. So, these are the various advantages of page or polyacrylamide gel electrophoresis.

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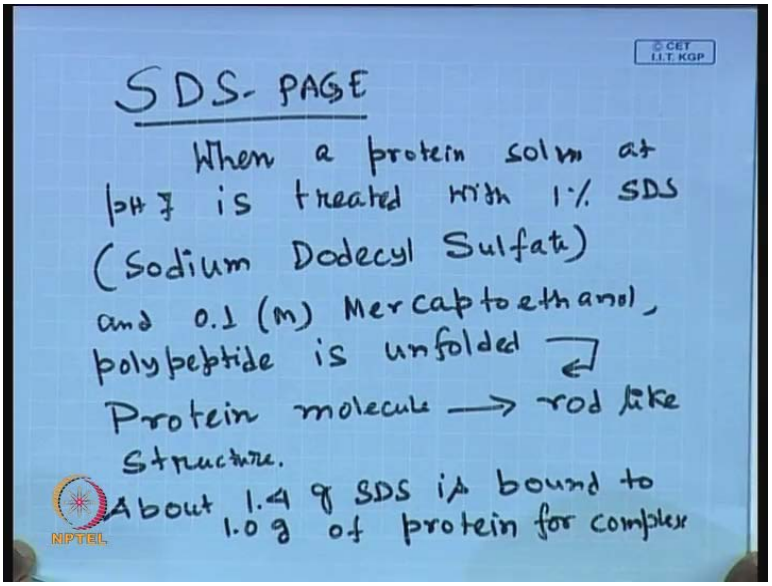

There is another page that is quiet common in use and that is called SDS-PAGE. So, in this case, a protein, when a protein solution at PH seven is treated with one percent SDS that is sodium dodecyl sulfate, this is a surfactant, this is an anionic surfactant. Sodium dodecyl sulfate and 0.1 molar mercaptoethanol, the polypeptide chain is unfolded. The polypeptide chain present in the protein is unfolded and what it results is this. This result into the protein molecule gets converted to a rod like structure; protein molecule becomes a rod like structure. About 1.4 gram SDS is bound generally to one gram of protein to make a complex. For complex formation and for each protein the attachment occurs in the same ratio.

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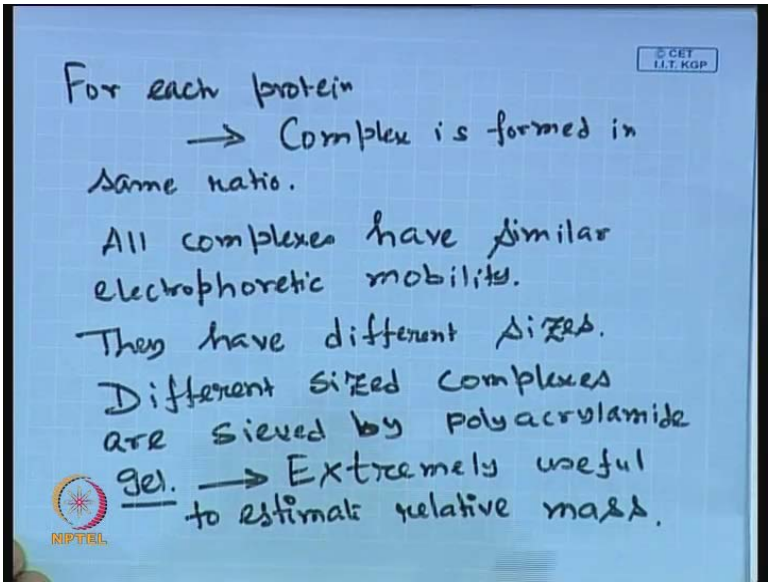

Surprisingly, for each protein, the complex is formed in the same ratio. So, therefore, the protein SDS complex has the same charge to mass ratio. Therefore, all the complexes have similar electrophoretic mobility in free solution but, they have different sizes and these different sized complex then can be sieved or separated by the polyacrylamide gel. Different sized complexes are sieved or filtered or they can be separated using by the polyacrylamide gel.

So, this is the basic principle and since SDS page, the value of electrophoretic does not play an important role. Only the molecular rate and the size of the species play a very important role. This method is very useful, extremely useful in determining the relative

mass of the protein; extremely help useful to estimate relative mass of the protein particles.

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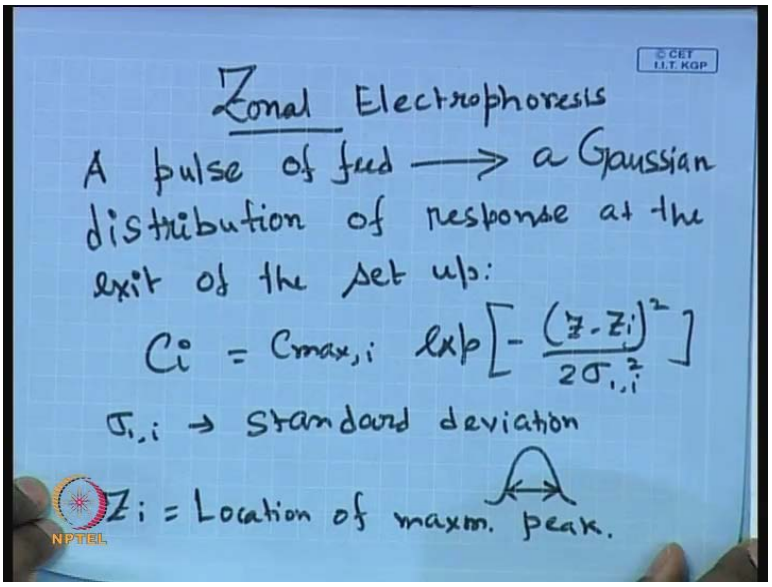

Let us talk about the zonal electrophoresis and how to quantify them? It is just exactly like the chromatographic column. A pulse of the feed results a Gaussian distribution response at the exit of the setup. A pulse of the feed; it results a Gaussian distribution of response at the exit of the setup. What is the distribution? The distribution is just like Gaussian expression. It will be something like this. C i is C max of the component i, exponential of minus Z minus Z i square of that divided by 2 sigma 1 i square. Sigma one i is nothing but the standard deviation of the response. If this is the Gaussian distribution at the exit, then the width is basically the standard deviation and Z i is the location of maximum peak location.

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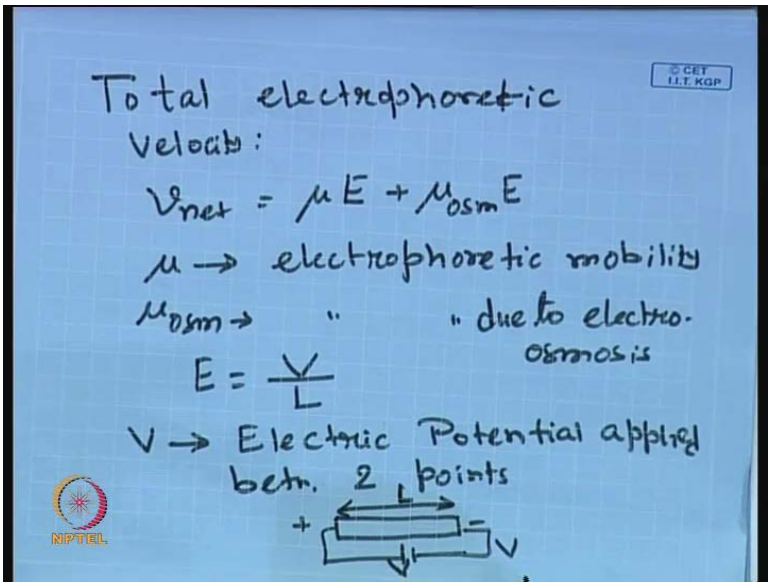

Now, we can quantify the various quantities those are involved in the calculation. The total electrophoretic velocity is nothing but a sum of electrophoretic velocity plus electro-osmotic velocity; that is a net electrophoretic velocity. Velocity is given as V net and this will be nothing but mu times E plus mu osm times E. What is mu? Mu is the electrophoretic mobility. So, electrophoretic mobility is the velocity per unit field strength. So, electrophoretic mobility multiplied by the electric field strength that will give you the electrophoretic velocity.

Similarly, mu osm is the electrophoretic mobility due to electro osmosis and what is E? E is the field strength and it is the potential difference that is applied between the two points by L; L is the length of the set up, V is the electric potential applied between the two points. That means, if I am having a cell, something like this and let us say this is positive charged and this is negatively charged and you just apply an external electric volt of voltage V between the two points and one is the cathode, another is the anode and this length is L.

Then the electric field strength given by V by L and V is as you know, you must be having some external electric power supply, either a battery or some voltage external voltage supplier. So, V by L is given by the electric fields strength. Therefore, the expression of z i or the location.

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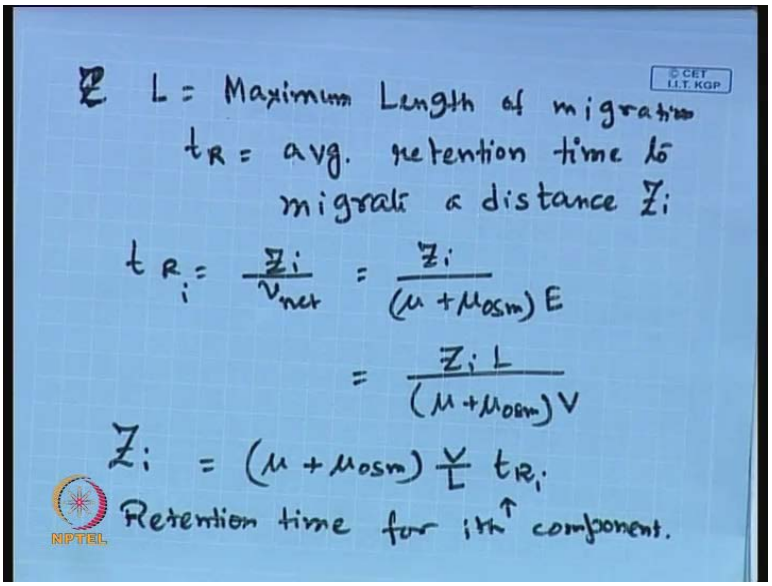

What is the L? L is the maximum length of migration. If t R is the average retention time to migrate a distance Z i; t R is average retention time to migrate a distance Z i, then the expression of the retention time becomes Z i divided by V net; the distance divided by the velocity. So, if we put the expression then this becomes Z i and by V net. V net is written as mu plus mu o s m times E, and instead of E, I am going to substitute by V by L, because E is not the operating variable; direct operating variable is voltage and length is known to you.

So, it will be Z i times L divided by mu plus mu o s m times V. So, E is replaced by V by L. So, expression of Z i can be obtained as mu plus mu o s m times V by L times t R i. So, let us say t R i is the retention time corresponding to i th component. So, t R i is the retention time for i th component. Now, for say since that chromatographic run is for a fix time we have basically this retention time is nothing but the experimental time and we know what this value is.

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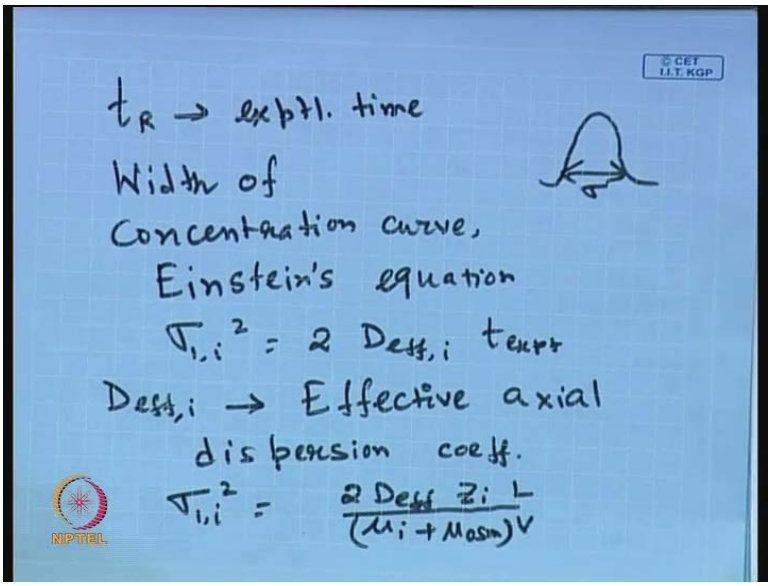

This retention time is nothing but the experimental time; that means, the SDS is SDS page is run for, electrophoresis run for, let us say for two hours, then two hours will be the retention time. So, different particles will travel different distances, because of the difference in electrophoretic mobilities. The width of the concentration curve, so basically the concentration curve that will be getting at the exit is given by the width of the sigma i.

So, width of concentration curve is given by Einstein's equation. What is that equation? Sigma 1 i square is two times D effective i into t experimental; t experimental is same as the retention time and it will be it will be basically an operating condition. You are going to run the system for 2 hour or half hour or 5 hours something like that.

What is D effective i? It is nothing but the effective axial dispersion coefficient in the electrophoresis system coefficient. So, one can have the sigma 1 i square as 2 D effective Z i times L divided by mu i plus mu osmotic multiplied by V voltage. So, that is basically the width of the concentration curve and one can have, if there are two components or two species present, one can have the degree of resolution on how good they are separated.

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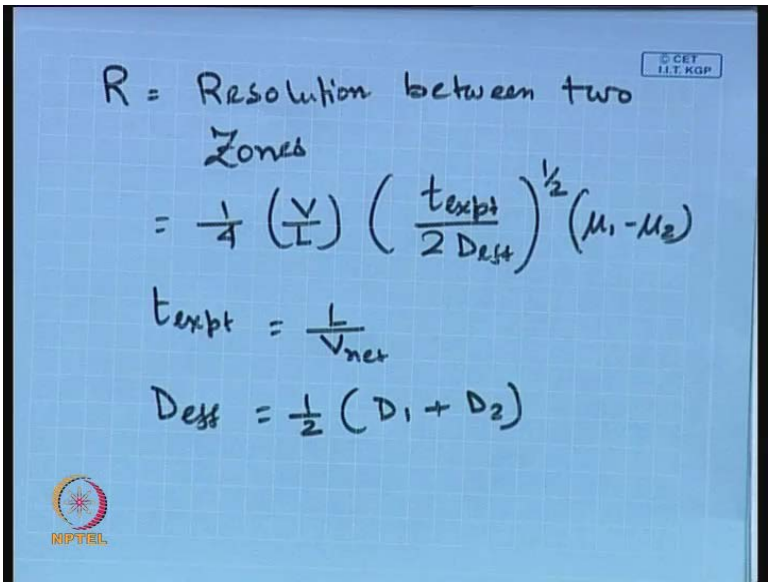

So, resolution or resolution between the two zones can be defined and this is define as 1 by 4 V by L t ex experimental divided by 2 D effective raise to the power half into mu 1 minus mu 2. So, degree of resolution becomes very important and your t experimental you already obtained as earlier; t experimental is L by V net. What is D effective? D effective, in a typical system is an arithmetic mean of the diffusivity of the two species those are present in the system that is half of D 1 plus D 2. Now, based on this, you can determine what the degree of resolution for this system is; if close up to be one then that means, it is there and the degree of resolution is very good. Now, with this theoretical background, I will be just solving couple of problems, just to I have an idea how this system work and how to get down into the some numerical fillings of the system.

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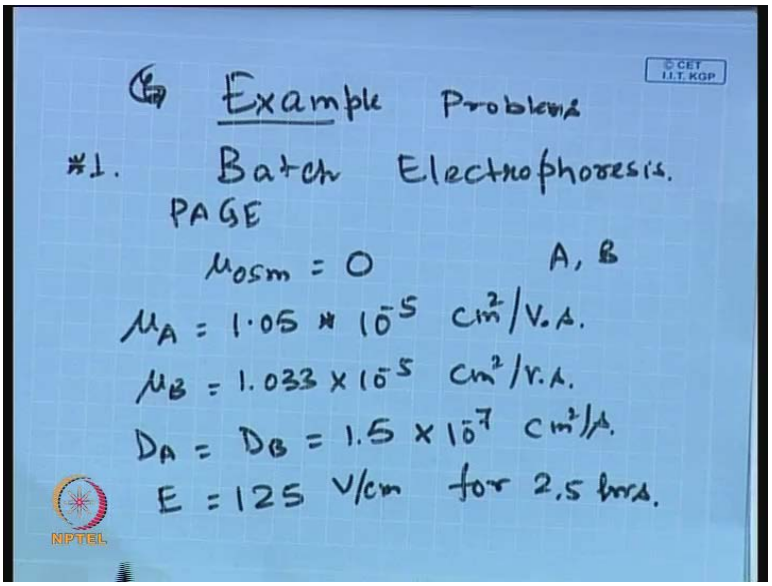

So, some example problems and let us consider the first example talked about talks about a batch electrophoresis system. For this is done with PAGE. The system is so efficient that the electrophoretic mobility due to electro osmosis is 0 and mu of a there are two species present A and B. The electrophoretic mobility for species one is given as 1.05 into 10 to the power minus 5 centimeter square volt per second, and mu of b is 1.033 in to 10 to the power minus 5 centimeter square per volt second.

Diffusivity of the two components are very close and they will be equal for this particular example and it is 1.5 into 10 to the power minus 7 centimeter square per second. So, if you look into the order of magnitude of diffusivity they will correspond 10 to the minus 11 meter square per second. They are typically diffusivity of the protein solution and the electric fields strength that we are going to apply here is 125 volt per centimeter and we are applying it for 2.5 hours. So, we are applying this much of electric field strength of 125 volt per centimeter for 2.5 hours and what we have to find out.

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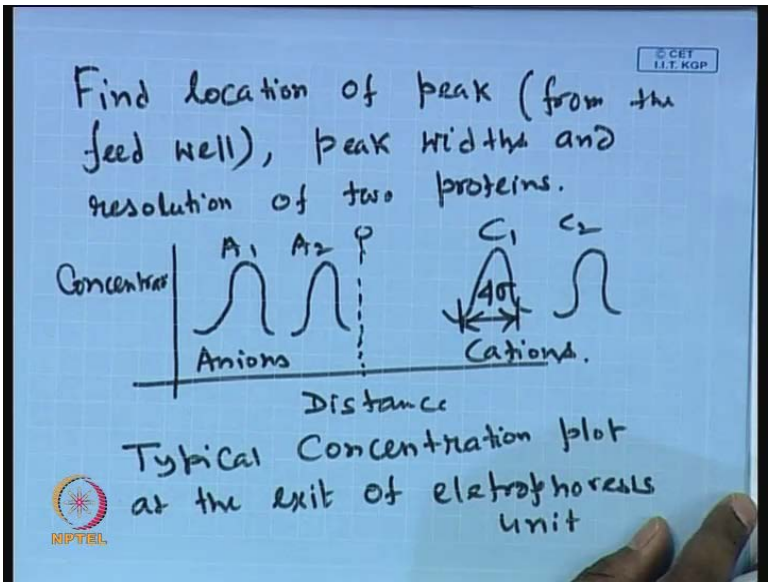

We have to find out the location of the peak, basically from the feed well; the location where we have injected the feed from the particular position, the peak widths and resolution. These quantities we have to find out.

Now, just before going into do this or looking into this thing, now let us, just like to see a typical concentration plot at the exit of electrophoresis unit. This distance is basically is the location where the feed is being injected. So, this concentration and this is the location or distance. This is zero location. Now, suppose you will be having the anions and cations, both are present; that means, you will be setting your operating PAGE such that some of the species will be positively charged and some of them are negatively charged.

So, they will be attracted to the opposite polarity. So, let us say these are A 1 and A 2 and A 1, A 2 are the anions. These are C 1, C 2 and they are the cations. They move in the opposite direction and this distance between the widths of the concentration peak is given by 4 times sigma and this is the location where the 0 charge is present.

So, this is the isoelectric point. Suppose, you are setting an operating PH 4 and if you have some protein which is having a isoelectric point at 4 that will be staying here and if for the other isoelectric points, for the other proteins which will be having the other isoelectric points depending on their polarity, they will move to the different electrodes and this is the width of the concentration curve that we are talking about.

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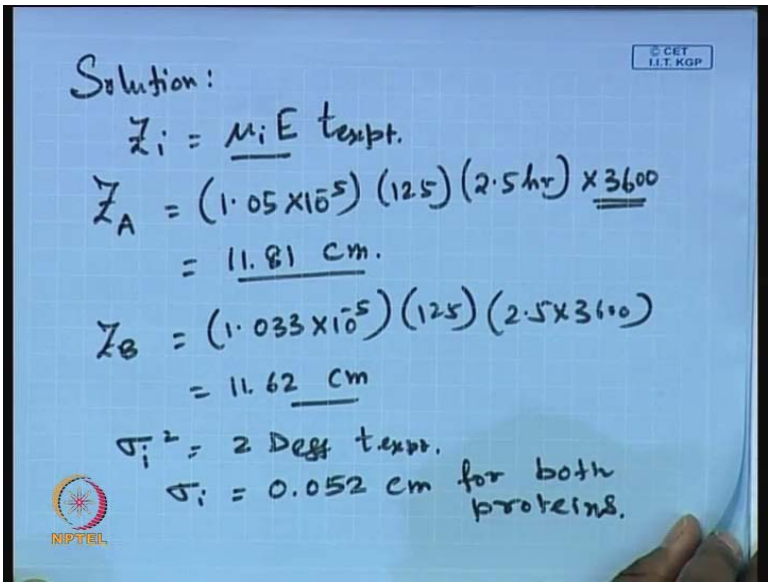

Now, let us you know get down to the calculations. Z i is mu i E t E x experimental, mu i E is the electrophoretic velocity. So, for the protein A the distance Z A is 1.05. You just put the values corresponding to protein A into 125 volts per centimeter into 2.5 hrs, since it has been operated for 2.5 hours, into 3600; this conversion factor is given for to convert hour into second, because the electrophoretic mobility is given in second. This Z A turns out to be 11.81 centimeter and Z B, correspondingly you can calculate 1.033 into 10 to the power minus 5 into 125 into 2.5 into 3600 and this turns out to be 11.62 centimeter. So, these are the regions where the maximum peak of A and maximum peak of B will be occurring.

Now, let us look into the width of the concentration curve. Sigma i square is given as two D effective times t experimental and if you put the value of sigma i, then it turns out to be 0.052 centimeter for both proteins.

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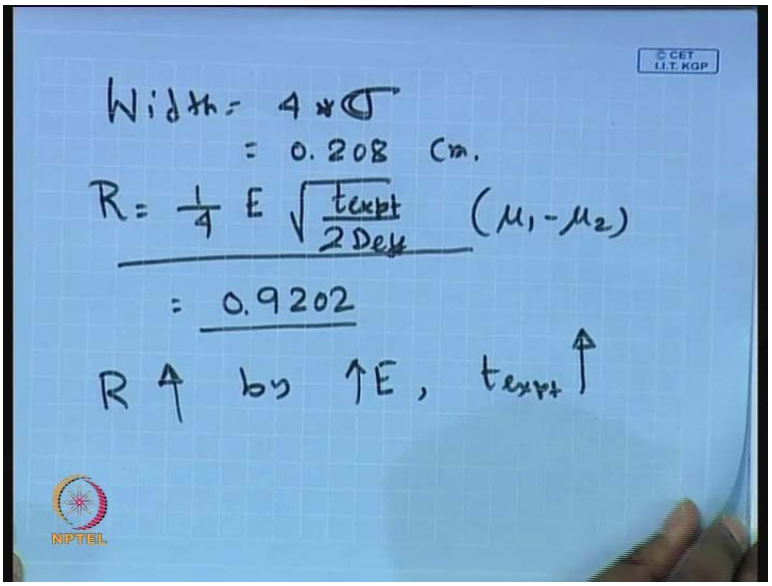

And the width is nothing but the four times of sigma. This is a peak width of four times sigma and this will be 0.208 centimeter. You can look into the expression of resolution, the expression resolution becomes 1 by 4 times E under root t for experimental duration divided by 2 D effective multiplied by mu 1 minus mu 2. If you put the various values, this turns out to be 0.9202. Now, if you look into this and that is the resolution we are talking about and if you look into the expression of resolution, you can check see that R increases, the more be the resolution it will be better. R increases by increasing E, if you increase E then R increases and if you increase t experimental it will increase then that means, if you conduct the experiment for longer duration the resolution is suppose to increase.

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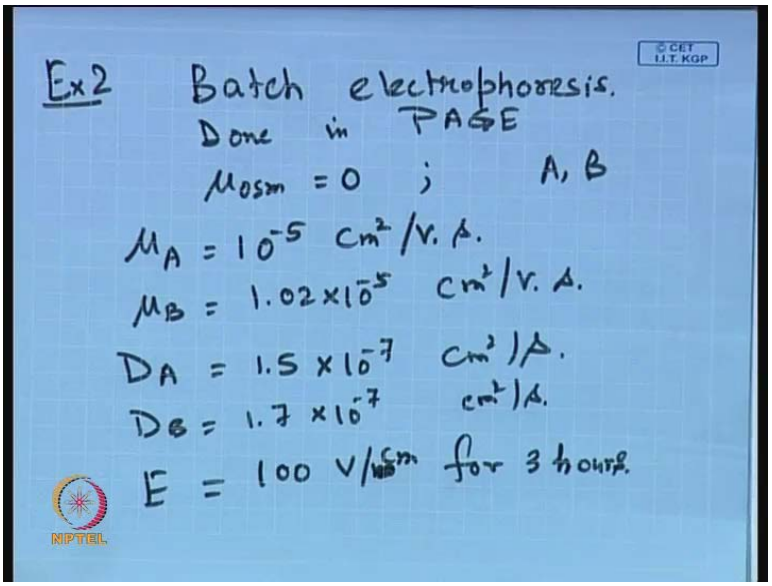

Then we will look into one more example. Here, we will be having different values of diffusivities. Now, again we are talking about a batch electrophoresis and is done in PAGE with osmotic mobility. Electroosmotic mobility is 0 and the mobility of the two proteins are given and again we are having two proteins A and B. Mobility of protein A is 10 to the power minus 5 centimeter square per volt per unit time and that is for B is 1.02 into 10 to the power minus 5 centimeter square per unit volt per unit time. The diffusivities are D A is given as 1.5 into 10 to the power minus 7 centimeter square per second and D B is 1.7 into 10 to the power minus 7 centimeter square per second. The experiment is conducted at the operating electric field is 100 volt per meter and this for 3 hours. This is 100 volt per centimeter, I guess. So, now we have to again find out the location of the peaks, peak width and the resolution of the two proteins as we have solving the earlier problem.

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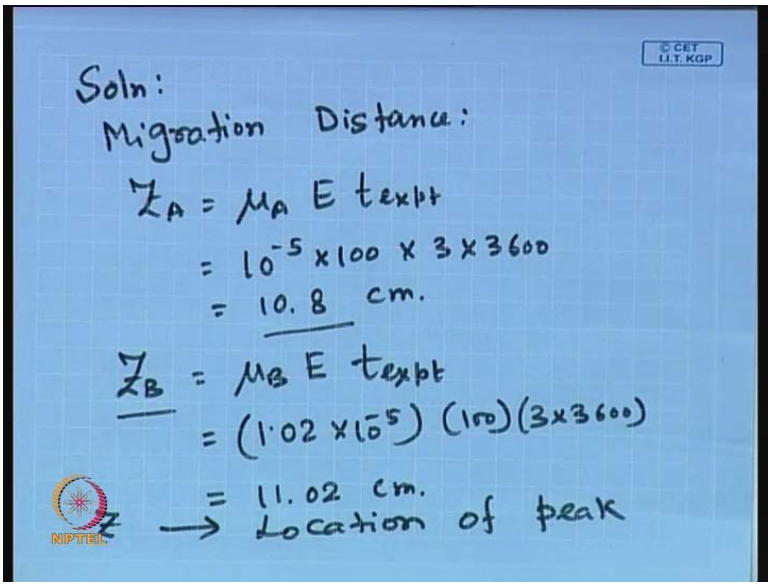

Now, the solution is almost same thing like the earlier one. The migration distance can be obtained by putting the values there. z A is mu A E t E x experimental. So, mu A is given as 10 to the power minus 5 into 100 into 3 into 3600, for 3 hours. So, this distance is 10.8 centimeter. On the other hand, you can find out the migration distance for the protein B as mu B times E time for experimentation that is three hours. So, just put the values 1.02 into 10 to the power minus 5 times 100 into 3 into 3600. So, it turns out to be 11.02 centimeter. So, what does this mean? This means that location of peak for component A will be at 10.8 centimeter and the component and the location of the peak for component B will be at 11.02 centimeter. So, Z values they represent the location of the peak at the exit of the system.

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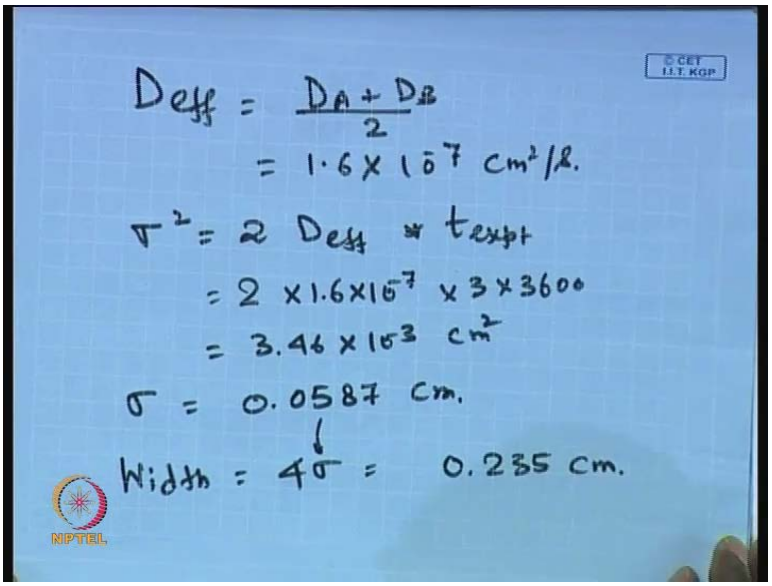

Now, what you have to do is you have to calculate the effective diffusivity, the effective diffusivity will be D A plus D B divided by 2. If you substitute the values, it turns out to be 1.6 into 10 to the power minus 7 centimeter square per second. So, you can find out the sigma square and sigma square is nothing but two D effective multiplied by t experimental. Now, if you put the values, this turns out to be 2 into 1.6 into 10 to the power minus 7 into 3 into 3600, and this is 3.46 into 10 to the power minus 3 centimeter square and sigma turns out to be 0.0587 centimeter. The width of the peak becomes four times sigma and this is if you just put the value, this value here it turns out to be 0.235 centimeter.

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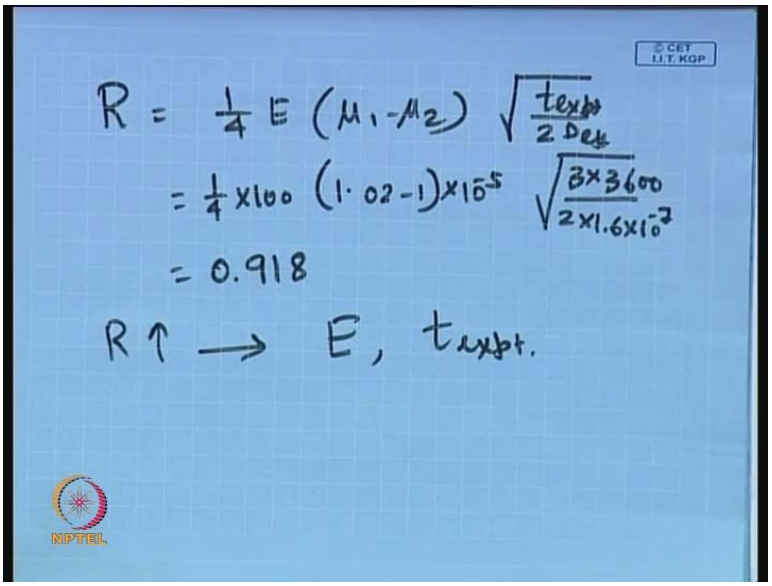

So, once you know the different parameters you will be in a position to calculate the resolution of the peaks as well. So, resolution is 1 by 4 times E into mu 1 minus mu 2 times under root t experimental divided by two times D effective. Now, you just put different values, 1 by 4 into 100 into 1.02 minus 1 into 10 to the power minus 5 under root 3 into 3600 divided by 2 into 1.6 into 10 to the power of minus 7. This turns out to be point around 0.92; the resolution is around 0.92.

R can be increased, as we have talked earlier, decided earlier that by tuning the operating conditions. What are the operating conditions? The operating condition is electric field strength; that means, if you apply more voltage then electric field strength will be higher, if you apply, if you conduct experiment for a larger duration, the duration of the experiments will be higher and one can get and one can increase the resolution and one can get a higher separation.

So, this basically gives a demonstration of how electrophoretic separation process takes place and if you summarize that this is very important as far as the separation analysis of the biomolecules are concerned because of the charged properties of the biomolecules, and you must be having some PH. So, every biomolecules will be having their characteristic p i or isoelectric point, where the where the charge of the particular biomolecule will be neutral.

Now, based on the operating PH, you can really set the or you can tailor make the charge of the of the biomolecules and now if you apply an external electric field, then these biomolecules will proceed to various electrodes depending on their polarity. Now, this process, the electrophoresis, will be giving rise to several complications. Number one is that because of the presence of the electrolytes in the solution, it will produce a current and that current will give rise to joule type of heating, which may degenerate the protein or it may give rise to natural circulation, like natural convection, which will hamper the electrophoretic movement.

So, one has to go for a gel type of medium or a porous medium, so that the effect of natural convection will be minimized number one. Number two is that the porous of porous medium must be large enough so that the diffusion of the species through them should not be hindered.

Number three characteristic is that there should not be any electro-osmotic velocity and that has to be minimized, otherwise the whole thing will be mugged and there will be no resolution. You will be getting and therefore you must be having a gel, which will be polymerized typically, so that charge will be residual surface charge will be minimal. So, that the zeta potential of that will be extremely small and you will be having almost no electro-osmotic velocity. So, that is more or less completes the electrophoretic separation method. So, in the next class, I will stop here, in the next class, we will move on to the next separation method that will be the supercritical fluid extraction.

Thank you.