Semester – I

I7MBP112 BASIC PRACTICAL – II 4H – 2C

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9

Hours

1. Spontaneous Mutation – gradient plate technique
2. Induced Mutagenesis-chemical and physical - UV
3. Replica plating technique.
4. Transformation in Bacteria
5. Bacterial Conjugation
6. Induction of Lac operon
7. Measurement of growth-one step growth curve using a T even phage
8. Titration of phages (T4)
9. Nuclear staining for nucleic acid identification.
10. Spectrophotometric estimation of protein – BSA
11. Analysis of amino acid by Paper chromatography
12. Analysis of amino acid by Thin layer chromatography
13. Purification of proteins by column chromatography
14. Analysis of amino acid by HPLC – Demonstration

SUGGESTED READINGS

REFERENCES

1. Arora, B., and Arora, D.R., (2007). *Practical Microbiology*, (1st ed.). CBS Publishers and Distributors, Bangalore.
2. Benson, H.J. (1998). *Microbiological Application (Laboratory Manual in General Microbiology)*, (7th ed.). WCB.
3. Palanivelu, P. (2004). *Analytical Biochemistry and Separation Techniques*, (3rd ed.). Twenty First Century Publication, Madurai.
4. Chakraborthy, P., and Pal, N.K., (2008). *Manual of Practical Microbiology and Parasitology*, New Central Book Agency (P) Ltd, India.
5. Gaud, R.S., and Gupta, G.D., (1999). *Practical Microbiology*, 1st Ed.). Nirali Prakashan, Pune.

2017

List of practicals – lecture plan

S. No	Experiment name	Hours
1.	Spontaneous Mutation – gradient plate technique	1 week/4 hours
2.	Induced Mutagenesis-chemical and physical - UV	1 week/ 4hours
3.	Replica plating technique.	1 week/4 hours
4.	Transformation in Bacteria	1 week/4 hours
5.	Bacterial Conjugation	1 week/4 hours
6.	Induction of Lac operon	1 week/4 hours
7.	Measurement of growth-one step growth curve using a T even phage	1 week/2 hours
8.	Titration of phages (T4)	1 week/2 hours
9.	Nuclear staining for nucleic acid identification.	1 week/2 hours
10.	Spectrophotometric estimation of protein – BSA	1 week/2 hours
11.	Analysis of amino acid by Paper chromatography	1 week/2 hours
12.	Analysis of amino acid by Thin layer chromatography	1 week/2 hours
13.	Purification of proteins by column chromatography	1 week/2 hours
14.	Analysis of amino acid by HPLC – Demonstration	1 week/2 hours

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2017

Experiment no 1 – Spontaneous mutation

The following points highlight the top four techniques used for the isolation of microbial mutants. The techniques are: 1. Direct Observation2. Enrichment Technique 3. Replica-Plating Technique 4. The Ames Test.

1. Direct Observation Technique:

In some cases, a colony growing on an agar plate can easily be seen to be different from the normal parental type (wild-type). For example, if the parental strain is pigmented, the observation of non-pigmented colonies may indicate the presence of mutants. Indicators can also be incorporated into the medium to detect microorganisms with and without particular metabolic capabilities.

For instance, pH indicators can be used into the medium to detect the production of acidic products. The indication of acid production by one microbial strain and not by other of the same microorganisms growing under identical conditions would show the presence of a mutant.

2. Enrichment Technique:

ADVERTISEMENTS:

Enrichment technique is employed especially in isolating mutants resistant against phages, antibiotics, or toxic chemicals. Phage-resistant mutants can be isolated simply by plating the mutagenized, phenotypically expressed microbial population on plates containing phage particles.

Cells expressing the parental wild-type phenotype are killed; only phage-resistant mutants develop into colonies. Such colonies are isolated. Similarly, mutants resistant to an antibiotic or a toxic chemical can be isolated by plating the microbial population with the antibiotic or the chemical.

3. Replica-Plating Technique:

Replica-plating technique is often used to isolate nutritional mutants (auxotrophs) as well as various other type of mutants, e.g., antibiotic resistant mutants.

For convenience, if one wants to isolate nutritional mutants employing replica-plating techniques. The is required to follow the following steps:

(i) Bacterial cultures are diluted, and the cells are spread on the surface of semisolid nutrient agar medium in a Petri dish (called "master plate"). The medium in the master plate is a complete medium i.e., containing all the nutritional components required by the bacterial population. After a sufficient incubation period, each bacterium produces a visible colony on the surface of the agar in the master plate.

(ii) A piece of sterile velvet cloth is stretched over a cylindrical block of wood or metal that is slightly smaller in diameter than the Petri dishes used in the process.

(iii) The master plate is now inverted and gently pressed onto sterile velvet. Since the fibres of the velvet act as fine inoculating needle, some cells from each colony of the master plate stick to the velvet.

(iv) Other Petri dish (called "replica plate") is taken containing a minimal medium i.e., a medium deficient with specific nutritional component,

(v) The replica-plate is now inverted and gently pressed onto the velvet thus stamping the bacterial cells onto the surface of its minimal medium. The replica plate is identically oriented at the application on the velvet with respect to mark placed on its rim so that the colonies that appear on the replica plate after incubation occupy positions congruent with those of their siblings on the master plate.

(vi) After sufficient incubation, it is observed that a colony that develops on the complete medium of the master plate fails to develop on the minimal medium of the master plate that lacks a specific nutritional component. Such colony is marked on the master plate and is isolated; it represents mutant for that specific nutritional component not used in the minimal medium of the replica plate.

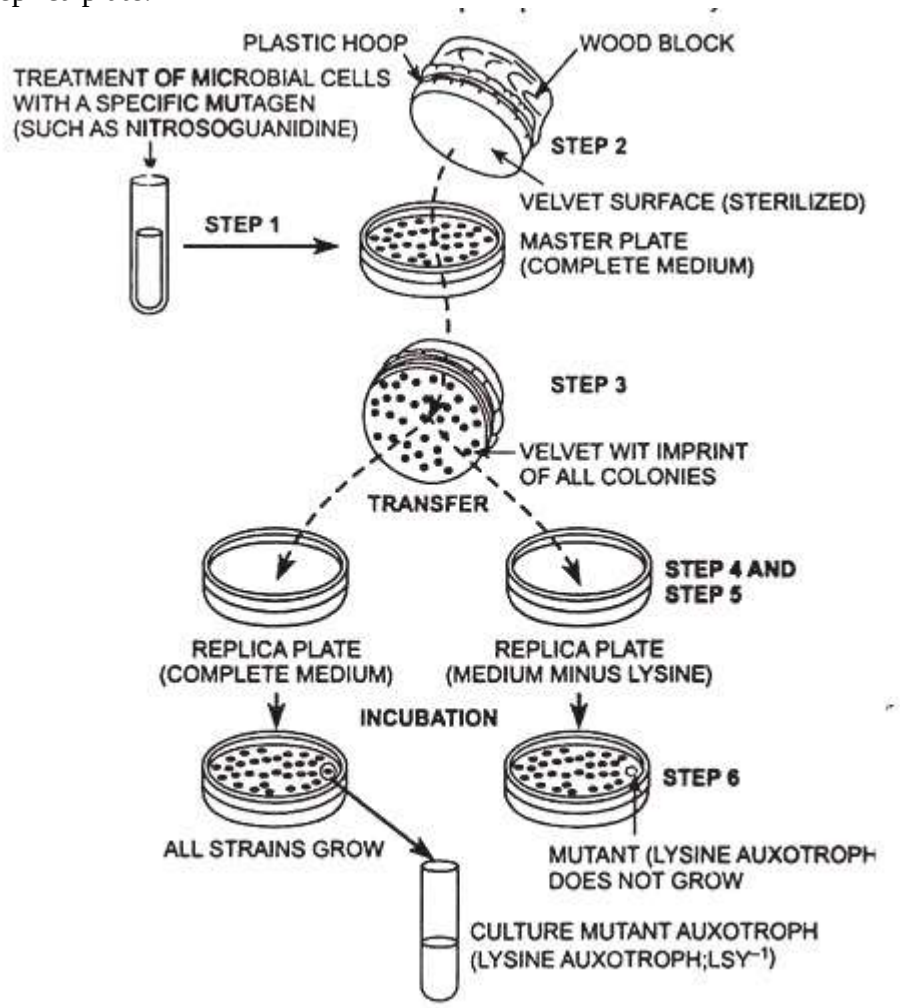

FIG. 29.13. Replica plating technique used for selection of nutritional mutants.

The replica-plating technique was developed by Joshua and Esther Lederberg in 1952 in order to provide direct evidence for the existence of pre-existing mutations originated spontaneously in microorganisms.

4. The Ames Test:

This test was developed by Ames and coworkers and is based on histidine-requiring (his^-) auxotrophic mutants of Salmonella typhimurium. Different his^- mutants carry different types of mutations, i.e., transitions, transversions and frame- shifts.

ADVERTISEMENTS:

In the Ames test, the frequency of reversion to his^+ (prototrophy) is scored in the especially constructed his^- mutants. This is done by placing a known number of mutant cells on medium lacking histidine and scoring the number of colonies formed. The frequency of cells forming colonies gives the frequency of reversion. The frequency of spontaneous reversion to his^+ is quite rare, i.e., 10^{-8}.

Ames test is routinely used to investigate the mutagenicity of various chemicals. Some of the chemicals may become mutagenic only when they are acted upon by liver enzymes.

For example, nitrates themselves are neither mutagenic nor carcinogenic. But in eukaryotic cells, nitrates are converted to introsamines, which are highly mutagenic and carcinogenic. In addition; some chemicals may be mutagenic only to replicating DNA.

The routine Ames test addresses to both these needs as follows:

1. The his^- cells are plated onto a medium that contains traces of histidine, which is enough to allow a few cell divisions, but inadequate for visible colony formation.
2. The test chemical is incubated with rat liver extract containing the liver enzymes, i.e., the microsomal fraction. This allows modification of the chemical in the same way as it would be in the liver of animals.

The procedure of Ames test is as follows. The bacterial cells are incubated with the liver extract, and then plated onto a medium containing traces of histidine; this serves as the control plate.

The test plates contain the same medium but the$^-$cells are not treated with liver extract. The test chemical is treated with the rat liver extract and a filter paper disc is soaked in this solution. The filter paper disc is placed onto the medium of test plate (Fig. 29.14).

The chemical present in the filter paper acts on the cells growing in the test plate. The frequency of colonies formed in the control plate and the test plate are compared. An increase in the frequency in the case of test plate will indicate the test chemical to be mutagenic. In order to increase the efficiency of the test, the his^- strains used in the test are defective in DNA repair, and have increased permeability to chemicals. It has been observed that more than 90% of the chemicals that are mutagenic are also carcinogenic.

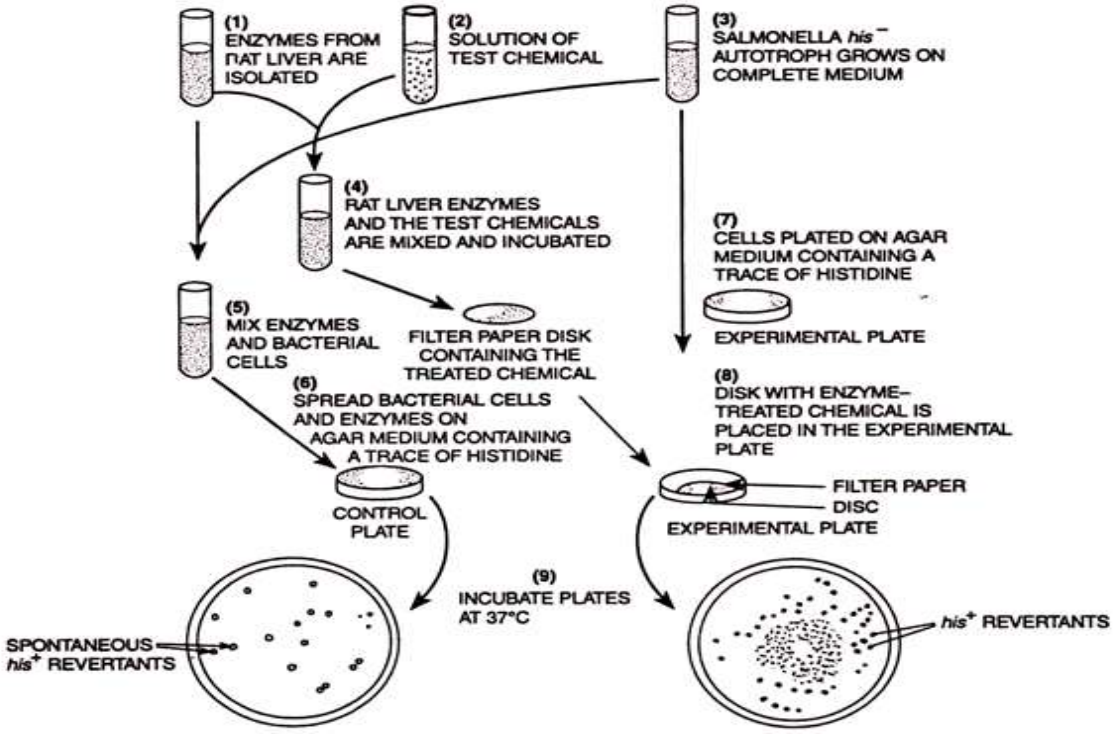

FIG. 29.14. The Ames test for mutagenicity. The medium in each Petri dish contains a trace of histidine and a known number of *his*$^-$ cells of a specific *Salmonella typhimurium* "tester strain". The control plate provides an estimate of the frequency of spontaneous reversion of the tester strain. The experimental plate shows the frequency of reversion induced by the test chemical.

Experiment no 2 – Induced mutagenesis – physical and chemical method

UV radiation has become a global concern due to the fact that it is a major environmental mutagen and carcinogen, leading to conditions such as skin cancer [8]. Coupled with the fact that ozone layer (the atmospheric layer that filters out solar UV radiation from the sun) is continuously depleted by pollution, there is pressure to better understand the mechanics in how mutations arise so that possible remedies in the future can be devised. UV radiation may be a hazard to the human population but it is also an environmental stress for other organisms such as bacteria. Such environmental stress caused by UV may in some way induce different evolutionary changes on bacteria that would have otherwise not been selected for. This area thus provides avenues of physiological, ecological, and genetic investigation because mutations play a key role in biological processes such as evolution, carcinogenesis, aging, and generation of somatic genetic diversity [8]. In this series of experiments, the K-12 strain of Escherichia coli were used because of its ease of cultivation, genetic characterization and because it is the most often used organism in UV mutagenesis experiments. The genetic markers chosen for mutational frequency analysis were rpoB (also known as rif) located at about 90 centisomes in the K-12 chromosome, ampA located at about 94 centisomes, and ompF located at about 21 centisomes [6]. Mutations at these sites confer resistance to rifampicin (an antibiotic that strongly inhibits RNA polymerase), ampicillin, and chloramphenicol respectively [9]. The lacZ locus, located at about 8 centisomes [6], was also selected as a genetic marker; lacZ− mutants are not able to use lactose. When deoxyribonucleic acid (DNA) is exposed to UV light (254nm), the most frequent DNA damage, or lesions, results at dimers of any two adjacent pyrimidine bases (T, thymine; C cytosine) causing T-T, C-T, and C-C dimers, but T-T dimers are the most common cyclobutane pyrimidine dimers [3, 10, 13, 18, 19]. Another type of DNA damage is the 6-4 pyrimidine-pyrimidone photoproducts. These occur at a lesser frequency than cyclobutane pyrimidine dimers but are less mutagenic because they're more efficiently repaired than cyclobutane pyrimidine dimers [3]. The remaining types of DNA lesions include pyrimidine hydrates, purine photoproducts, strand brakes, and DNA cross-links. These occur at a much lower frequency however [3]. Thus, UV radiation induces mutations such as frameshifts, base substitutions, deletions, recombination and other types of genetic rearrangements [13]. To cope with DNA damage, Escherichia coli has two main categories of repair, one is the "error-free" system and is independent of the SOS regulon or response (see below), while the other is the "error-prone" repair system and is part of the SOS regulon [15, 20]. There are three strategies employed by E. coli that are considered relatively errorfree, they are photoreactivation, excision repair and post-replication repair [21]. 32 Journal of Experimental Microbiology and Immunology (JEMI) Vol. 1:32-46 Copyright December 2001, M&I UBC However, not all DNA lesions can be repaired because it some situations, the cell may have sustained significant damage; the damage was poorly repaired; or the DNA damage occurred during the S phase of cell growth [17]. Under such stressful conditions, the cell must make a last, desperate effort to repair itself. Or else UV-induced lesions will block DNA polymerases from replicating its genetic material, ultimately leading to cell death. This push to repair itself is commonly referred to as the SOS response or SOS system [see reviews 1, 4, 7, 8, 16 for more detailed information]. Basically, the SOS system avoids replication blocks caused by UV induced lesions by activating a series of pathway that causes DNA polymerases to be highly tolerant of these bulky groups of photodimers. In order to achieve this tolerance to replicate pass these lesions, the proofreading ability and specificity of polymerases in faithfully replicating the template strand is greatly reduced. Consequently, the

wrong nucleotides are added during the elongation process of DNA replication and thus mutations are introduced to the genome. As a result, the SOS system is ultimately responsible for mutagenesis in a majority of the cases. When this project was initially designed, we planned on using the DH5α strain of Escherichia coli transformed with the plasmid pUC19 as our biological system to look at gene mutation frequency on lacZ located on pUC19 (see [12] for in-depth discussion of the lac system) because this system was used in one of the undergraduate biology courses at the University of British Columbia. Furthermore, their experiment involved naked pUC19 plasmid irradiation and subsequent transformation of irradiated pUC19 back into DH5α host which was useful for our purposes. We set out to repeat this experiment without realizing some of the constraints involved with the strains DH5α and INVα. Therefore, we expected to observe some kind of lac mutation frequency. The second part of the project evolved out of difficulties in our first set of experiments to include gene mutation frequency on the chromosome. With the second half of the project we expected the frequencies to show results similar to Miller's experiment [11]. The results from this project are discussed based on the SOS response and further avenues of investigation are provided. MATERIALS AND METHODS Chemicals and bacteria growth nutrients used in this project were standard grade reagents. Most equipment are standard scientific equipment. Bacterial Strains. Several strains of the K-12 line of Escherichia coli were used in this project (Table 1) The stock strains, provided by Dr. William Ramey (UBC Microbiology), were kept at -70°C in nutrient LB broth supplemented with 25% glycerol. LB broth agar plates were streaked with these freezer stocks to grow a working set of individual colonies. Individual colonies were picked for use of growing the overnights. Overnight cultures for the direct-plate irradiation were grown up in flasks containing 20ml of LB broth at 37°C in an orbit-shaking water bath (New Brunswick Scientific, model G76). Whereas overnight cultures for the liquid-plate irradiation were grown up in 16x125mm test tubes containing 5ml of LB broth placed on a rotary test tube rack (Departmental custom built) in the 37°C incubator room. All overnight cultures were grown to saturation. Strains of bacteria employed in this experiment. Direct-Plate Irradiation. For the series of direct-plate kill experiments, sub-cultures were grown by inoculating (1:50 dilution) LB broth (20ml final volume) with the overnights and grown to about 1 x 108 cells per ml, approximately 0.7 to 0.8 OD660nm. These sub-cultures were grown in flasks placed in a 37°C orbit-shaking water bath at power level 3.5. When the sub-culture reached the desired optical density, a series of dilutions of the sub-culture was prepared; these dilutions are 10-1, 10-2, 10-3, 10-4, and 10-5. These dilutions were then used to make final plates for the ultraviolet irradiation by spread-plating 0.1 ml of the diluted sub-cultures onto various nutrient plates. LB agar plates were used unless otherwise noted. Therefore, the final plated dilutions are 10-2, 10-3, 10-4, 10-5, and 10-6. For most experiments, the dilutions of 10-2, 10-3, and 10-4 were used for the treatment set while 10-5 and 10-6 dilutions were the control. After plating, the plates were kept in a black box (plastic cooler box) before and after irradiation unless otherwise noted. All UV irradiations were done in a custom-built UV chamber with a glass front. The UV lamp (Philips UVC lamp, 15W) could be adjusted vertically to a desired height of up to 1.2 metres. The majority of the experiments were done in the dark to avoid photoreactivation. The only time a light source was present was during the transfer of the plates into the UV chamber. Before each irradiation, the UV lamp was warmed up for at least 30 minutes. For each UV exposure time point one plate from each treatment set (10-2, 10-3, and 10-4 dilutions) were randomly placed in the centre of the chamber and irradiated for the set time; this was done by using a digital timer and by manually adjusting the power switch of the UV lamp. The lids of the treatment plates were removed before placing the plates

into the chamber to avoid shielding by the lids. After irradiation, the lids were replaced and the plates were immediately placed into the black box. All the plates were grown in the 37°C incubator for 24 to 36 hours before scoring the number of colonies. Kill curve variables. Escherichia coli strains DH5α(pUC19) and INVα(pUC19) were used in the initial tests to determine the appropriate parameters for UV irradiation involving the direct-plate irradiation protocol. Heights tested were 30 cm and 60 cm from floor of the chamber to the lamp. Various time sets were also tested, the first set involved exposures of 0, 10, 30 and 60 seconds. The second set involved exposures of 0, 5, 10 and 15 seconds. Lastly, the final set contained exposures of 0, 2, 4 and 6 seconds. Photoreactivation after UV mutagensis. The effects of natural light on UV irradiated cells were briefly investigated. This was done by carrying out the standard direct irradiation protocol with a new time set. This new time set had the same exposure length as that of the longest exposure to UV; the only difference is that the one of these sets were placed in natural light for approximately two hours before placing them into the incubator. Response of strains and plasmids to UV irradiation. Responses to UV irradiation in the different strains were compared by applying the direct-plate irradiation protocol. The effect of plasmids on UV response was looked at by comparing host strains with different plasmids, different hosts with the same plasmid, and hosts with or without the same plasmid. Negative screening of lac and Amp mutants. The direct-plate irradiation protocol was carried out with the α-complemented strains with the plasmid pUC19. After a day of incubation, UV survivors where randomly picked and transfer to a fresh LB agar plate to make a master stamp plate of 100 colonies per plate. The master plate was allowed to grow for 24 hours at 37°C before it was replicate-plated to negative-screen plates such as MacConkey agar and LB agar supplemented with 100ug/ml of ampicillin. These negative-screen plates were incubated for 24 to 48 33 Journal of Experimental Microbiology and Immunology (JEMI) Vol. 1:32-46 Copyright December 2001, M&I UBC hours in a 37°C incubator and then the number of negative mutants was counted. To quickly ensure that the mutants were not contaminants, they were Gram stained to check their microscopic properties. Further tests on the mutants were carried out by performing plasmid extraction using commercial mini-prep kits (Wizard), and then transforming α-complemented hosts to see whether the mutation was chromosomal related by screening the transformed hosts with the MacConkey and ampicillin supplemented LB agar plate

Experiment 3 – Replica plating

This procedure requires two specialty items: a replica block and sterile velvets. The replica block is a wooden or metal cylinder that fits snugly inside a petri plate (see Fig. 1.3.3). One method for constructing these has been described by Adams (1965). A metal ring is used to secure the velvets to the block. Squares of velvet should be cut so as to cover the base (a diameter of 14 cm is suggested). These velvets can be washed, autoclaved, and reused. If velvets are not available, pieces of sterile filter paper or disposable replica plates can be used ("Repli-Plate" Colony Transfer Pads, American Laboratories #59901). Replica plating also requires a master plate composed of well-separated colonies. The master plate can be a fresh plate onto which 50 to 100 colonies have been gridded (using toothpicks and the grid inFig. 1.3.4), or it can be a plate on which bacteria were spread that have now grown up into well-separated colonies.

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(vi) After sufficient incubation, it is observed that a colony that develops on the complete medium of the master plate fails to develop on the minimal medium of the master plate that lacks a specific nutritional component. Such colony is marked on the master plate and is isolated; it represents mutant for that specific nutritional component not used in the minimal medium of the replica plate.

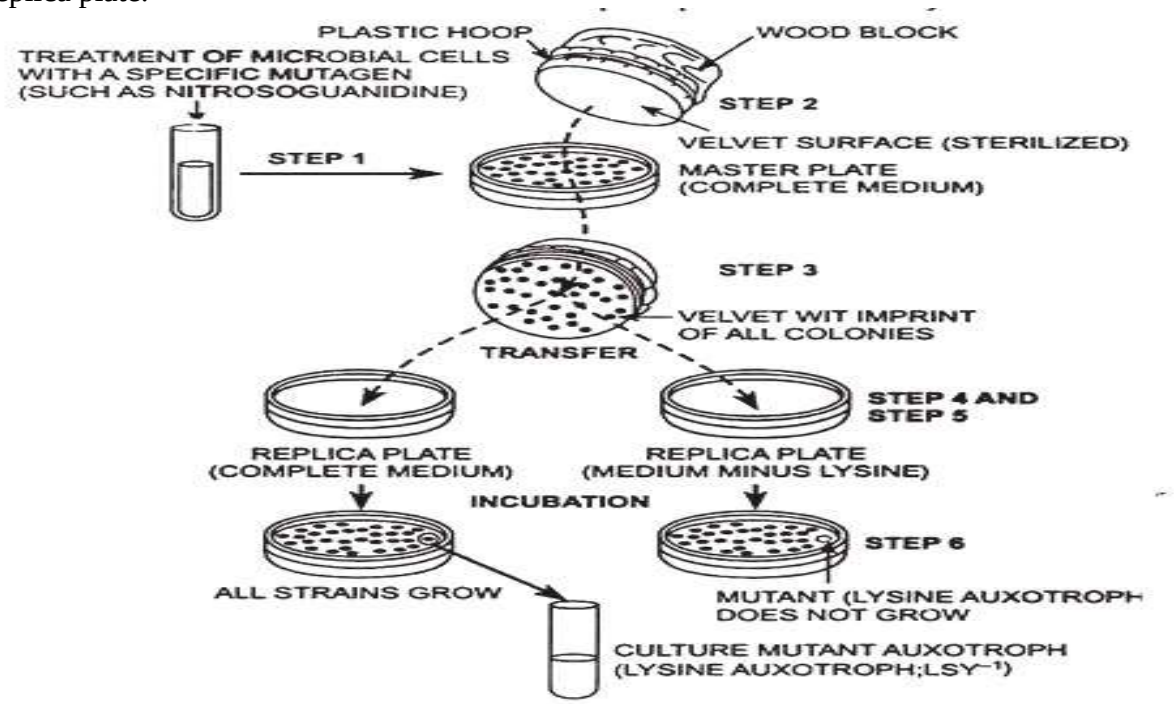

FIG. 29.13. Replica plating technique used for selection of nutritional mutants.

The replica-plating technique was developed by Joshua and Esther Lederberg in 1952 in order to provide direct evidence for the existence of pre-existing mutations originated spontaneously in microorganisms.

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Experiment 4 – Transformation in bacteria

Introduction

Transformation is the process by which foreign DNA is introduced into a cell. Transformation of bacteria with plasmids is important not only for studies in bacteria but also because bacteria are used as the means for both storing and replicating plasmids. Because of this, nearly all plasmids (even those designed for mammalian cell expression) carry both a bacterial origin of replication and an antibiotic resistance gene for use as a selectable marker in bacteria.

Scientists have made many genetic modifications to create bacterial strains that can be more easily transformed and that will help to maintain the plasmid without rearrangement of the plasmid DNA. Additionally, specific treatments have been discovered that increase the transformation efficiency of the bacteria and make them more susceptible to either chemical or electrical based transformation, generating what are commonly referred to as 'competent cells.'

Protocol: Transformation

Many companies sell competent cells, which come frozen and are prepared for optimal transformation efficiencies upon thawing. **For the highest transformation efficiency, we recommend that you follow the instructions that came with your competent cells.**

> ***Note:*** *Commercial competent cells range significantly in their transformation efficiency. The lowest efficiency cells (usually the least expensive) are fine for transforming plasmid DNA for the purposes of storage and amplification. The higher efficiency cells are more important if you will be transforming solutions with very small amounts of DNA or if you need nearly all of the DNA to be transformed from a single solution.*
>
> ***Note:*** *To save money, many labs also make their own competent cells. This is a relatively simple procedure and is useful for performing low efficiency transformations.*

Example Protocol: Standard heat-shock transformation of chemically competent bacteria

1. Take competent cells out of -80°C and thaw on ice (approximately 20-30 mins).
2. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature and then (optional) incubate in 37°C incubator.
3. Mix 1 - 5 µl of DNA (usually 10 pg - 100 ng) into 20-50 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.

   Note: *Transformation efficiencies will be approximately 10-fold lower for ligation of inserts to vectors than for an intact control plasmid.*
4. Incubate the competent cell/DNA mixture on ice for 20-30 mins.
5. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).
6. Put the tubes back on ice for 2 min.
7. Add 250-1,000 µl LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.

   Note: *This outgrowth step allows the bacteria time to generate the antibiotic resistance proteins encoded on the plasmid backbone so that they will be able to grow once plated on the antibiotic containing agar plate. This step is not critical for Ampicillin resistance but is much more important for other antibiotic resistances.*
8. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.

Note: *We recommend that you plate 50 µL on one plate and the rest on a second plate. This gives the best chance of getting single colonies, while allowing you to recover all transformants.*

Note: *If the culture volume is too big, gently collect the cells by centrifugation and resuspend in a smaller volume of LB so that there isn't too much liquid media on the agar plates. If the agar plate doesn't dry adequately before the cells begin dividing, the bacteria diffuse through the liquid and won't grow in colonies.*

9. Incubate plates at 37°C overnight.

How can I save time when carrying out transformations?

If you are not concerned with transformation efficiency (such as when you have a tube of plasmid DNA and just need to transform bacteria so that you can grow up more of the plasmid) you can save a lot of time by shortening or skipping many steps and will still get enough colonies for your next step. Remember that each of these shortcuts will reduce the efficiency of the transformation, so when higher efficiency is needed follow the complete protocol.

- Thaw the competent cells in your hand instead of on ice
- Reduce step 4 from 20 - 30 mins to 2 mins on ice before heat-shock
- Shorten or skip the outgrowth (for Ampicillin resistance it is ok to completely skip the outgrowth, for the other antibiotics it is a good idea to outgrow for at least 20-30 mins)

- **I get very few if any transformants when transforming large plasmids (>10 kb) or BACs, what can I do?**

  Chemically competent cells are fast and easy to use, but are less efficient at taking up larger plasmids. If you need to transform large plasmids, it is a good idea to use electro-competent cells. Instead of relying on the heat-shock to cause the cells to take up the DNA, an electro-magnetic field is applied to the cell/DNA mixture to induce membrane permeability. To do this you will need to have access to an electroporator and the appropriate cuvettes. Follow the manufacturer's instructions for each.

- **I got no transformants. What went wrong?**

  Check that you are plating on an LB Agar plate containing the correct antibiotic. The resistance gene on your plasmid must match the antibiotic on the plate. You should also add a positive control (many companies include a positive control plasmid with their competent cells) to ensure that your transformation procedure is working.

- **TIP: Sometimes less is more.**

  Although it may be counter-intuitive, you will often get higher transformation efficiencies with less DNA, especially when using highly competent cells. If you used 100-1000 ng of total DNA in a ligation you will often get more colonies if you use 1 µl of a 1:5 or 1:10 dilution rather than 1 µl directly.

Experiment 5 – Conjugation in bacteria

Aim:

To study the process of bacterial conjugation through transfer of genes coding for antibiotic resistance.

Introduction:

Bacteria possess several methods for gene transfer for transmission of genes between individual cells. These mechanisms not only generate new gene assortments, they also help to move genes throughout populations and from species to species.

The methods include transformation, transduction and conjugation. These methods occur by lateral gene transfer which is a potent evolutionary force that can create diversity within bacterial species. Conjugation is a recombination process where two live bacteria come together, and the donor cell transfers genetic material to the recipient cell. This process was first observed in 1946 by Joshua Lederberg and Edward Tatum in a series of experiments with E. coli. Principle: Conjugation is the mode of gene transfer in many species of bacteria. In 1950 William Hayes, Francis Jacob and Elie L. Wollman established that conjugating bacteria are of two mating types. Certain "male" types (designated as F+) donate their DNA and other "female types" (designated as F-) receive the DNA as shown in Figure 1. Fcells become F + when they acquire a small amount of DNA. Hence the F factor is called as the Fertility factor. In contemporary microbiology, the donor's F factors are known to be plasmids which are the extrachromosomal elements. The factors (plasmids) contain about 20-30 genes, most of which are associated with conjugation. These genes encode enzymes that replicate DNA during conjugation and structural proteins needed to synthesize special pili at the cell surface. Known as F pili or sex pili, these hairlike fibres contact the recipient bacteria, and then retract so that the surfaces of donor and recipient are very close or touching one another. At the area of contact, a channel or conjugation bridge is formed. Once contact via sex pili has been made, the F factor (plasmid) begins replicating by the rolling circle mechanism. A single strand of the factor then passes over or through the channel to the recipient. When it arrives, enzymes synthesize a complementary strand, and a double helix is formed. The double helix bends to a loop and reforms an F factor (plasmid), thereby completing the conversion of recipient from F- cell to F+ cell. Meanwhile, back in the donor cell a new strand of DNA forms, to complement the leftover strand of the F plasmid. The transfer of F factors involves no activity of the bacterial chromosome; therefore the recipient does not acquire new genes other than those on the F factor.

Experiment no 6 - Induction of Enzyme Activity in Bacteria: The Lac Operon

In E. coli, this enzyme is an important catalyst in the breakdown of the 12 carbon sugar lactose into two 6 carbon sugars, glucose and galactose. The breakdown of lactose provides an important source of energy for the bacteria. When E. coli are grown in a medium lacking lactose, $-galactosidase is either absent or present only in minute quantities. If lactose is added to the growth medium there is a gradual accumulation of $- galactosidase within the bacterium. This week we will investigate the mechanism by which E. coli regulates the intracellular appearance of $-galactosidase. The production of $-galactosidase is genetically controlled through the pathway shown below: (Actinomycin) (Chloramphenicol) DNA m-RNA Polypeptide chain Protein transcription translation on the ribosome assembly Many genetically controlled systems are energetically conservative. They remain in an "off" state unless the enzyme is required for a

cellular process. Systems of this type are used when a relative slow response is adequate. Other genetically controlled systems are capable of a more rapid response and are in an "on" state unless switched off. What is the advantage of keeping a system turned off most of the time? The production of $-galactosidase involves a number of steps. Each one of these can be probed to try to determine how the cell regulates production. In this laboratory we will try to distinguish between two hypotheses. Hypothesis 1: Lactose stimulates the synthesis of $-galactosidase by activating the DNA-RNA-protein pathway. This hypothesis suggests that the enzyme is not present, or is present in only very low concentration, in the bacterial cells until lactose is added to the medium. Therefore, the amount of $-galactosidase present in the cell will depend on how long the DNA-protein pathway is active (i.e., it is time dependant). Hypothesis 2: E. coli contains a form of the enzyme $-galactosidase which is present all the time but is inactive in its current configuration. The presence of lactose serves to activate the enzyme molecule. According to this hypothesis, lactose has no influence on the rate of $-galactosidase synthesis, but rather serves as an activator for the already present enzyme molecule. 90 II. Experimental Design: To differentiate between these two hypotheses we will stimulate the production/activation of $-galactosidase, use an antibiotic at successive time intervals to block protein synthesis, and assay for the amount of $-galactosidase present. A. Stimulation of the Bacteria: When bacteria are growing on lactose, the lactose in the medium is eventually used up and and the level of intracellular $-galactosidase drops. Our experimental design depends on having a constant level of lactose (the activator). Fortunately, there are several lactose analogs or "lookalikes" that also cause the production of $-galactosidae, but unlike lactose they are not broken down by the enzyme. One such molecule is isopropyl-$-d -thiogalactoside (IPTG). Its structure and that of lactose are shown below. Note the regions of similarity. B. Regulation of the DNA pathway: In this experiment we will use the antibiotic chloramphenicol to prevent translation of the message encoded in the m-RNA. Chloramphenicol prevents m-RNA from attaching to the ribosomes. Once chloramphenicol is added to the bacterial culture no additional $-galactosidase will be produced, but any already present will continue to function normally. Note ýý 1. If $-galactosidase is produced via the DNA - m-RNA - protein pathway then the amount of $-galactosidase in the E. coli culture will depend on how long the pathway is active before chloramphenicol is added (i.e., show a time dependency). ýý 2. If an inactive form of $-galactosidase is always present then the chloramphenicol should have no effect on the amount of $-galactosidase present. C. The Assay: As in last week's lab, the assay for the presence of functional $-galactosidase will involve the conversion of colorless ONPG (ortho-nitro-pheny-galactosidase) into the yellow compound ONP (ortho-nitrophenol). D. Experimental Cultures: All of the cultures you set up in your experiment will have the same basic ingredients, including a nutrient medium for growth of the bacteria (tryptone broth, TB), a stimulator (IPTG), a protein synthesis blocker (chloramphenicol) and an inoculum of cells (E. coli). Use care in setting up the cultures. The timing of chloramphenicol addition is critical to the success of the experiment. 91 Setting up cultures: Do not let the caps from culture bottles or tubes touch your hands, clothing or desk top. Do not leave any culture bottle or tube open for any longer than necessary while making a transfer. Wear gloves. The required proportions are given in Table 1 below. 1. Place the proper amount of TB in a large test tube then add the IPTG. 2. Add chloramphenicol only to the tubes that get it at T = 0. CAUTION: chloramphenicol is not added to tubes 6, 7 and 8 until they have been incubated for 15, 30, or 45 minutes respectively. 3. Start the cultures by adding 1.0 ml of E. coli to all the tubes except 2 and 5. 4. After mixing, incubate the cultures at 37oC for 60 minutes.

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Experiment no – 7 One step growth curve

To construct the growth curve of a phage from laboratory data and determine its burst size. • To identify the phases of a phage growth curve. • To define the events involved in phage: bacterium interaction resulting in phage replication and release.

Viruses that attack bacteria are called bacteriophages or simply phages. Phages, like other viruses, cannot exist without a suitable host. In 1939, Ellis and Delbruck (J. Gen. Physiol. 22:365-385) proposed a technique to quantitate and monitor the growth of phage in a specific host. The bacteria are mixed with phage and incubated for a short period of time. The mixture is then diluted to drastically reduce the number of bacteria available for phage adsorption. Samples are removed at specified intervals and plated to quantitate the phage present in the culture. At the start of the experiment, the plaque count is relatively constant over a time period because each infected bacterium will yield only one plaque. A rise in plaque forming units (pfu) to a plateau level occurs as bacteria are lysed and the newly synthesized phage are released into the medium. These phage particles fail to meet susceptible bacteria (due to the dilution of the adsorption mixture) and thus remain free in the culture fluid. The average number of phage released per bacterium is called the burst size and this value may be calculated from the data. The burst size varies in accordance with the specific virus, and may range from 10 to 100 for the DNA transducing phages to approximately 20,000 pfu for the RNA viruses. To replicate, a virus should induce its host to synthesize components that are necessary for the assembly of new virus particles. The virus accomplishes this process by first attaching to the host (adsorption) and then injecting its nucleic acid into the cell (injection or penetration). The viral DNA can stay free in the cell and be replicated as such, or it can be incorporated into the host chromosome and be replicated simultaneously with it. Viral proteins are next synthesized with the host's machinery under the direction of viral DNA and the new virus particles are assembled mechanically. These particles can find their way out of the cell or lyse the cell and be released into the medium, ready to infect new cells. If the number of phage particles was monitored during growth, a growth curve could be drawn which would be similar to that of the bacterial growth curve except in the Microbiology. The phage growth curve starts with a latent or eclipse period (similar to the bacterial lag phase). During this phase, the infection, adsorption, injection and syntheses of new viral DNA and protein coat occur. The next phase is called the maturation or release stage (similar to the log phase in bacteria) when new phage particles are assembled and released. The cycle can then start over with the infection of new cells. In this manner, the shape of the curve would look step-wise and that is why the process is called "one-step phage growth curve". The single-step growth experiment of Ellis and Delbruck demonstrates the cyclic replication of the phage. These authors devised a method to demonstrate only a single step of the many steps of phage replication. Essentially they drastically diluted the mixture after attachment of phage to bacteria, so when the infected cells lysed, no new host cells could be found for a second round of infection. A number of modifications have been introduced since the original experiment was reported. For instance, instead of diluting the initial bacterium:phage mixture, antibodies specific for the phage attachment apparatus may be added to the mixture to 'neutralize' and thus render all of the unadsorbed phage unable to adsorb to any bacterium. In this laboratory experiment, we shall attempt to repeat the Ellis-Delbruck experiment. There are a number of steps and manipulations to perform and you are cautioned to be deliberate in your technique.

LABORATORY SUPPLIES Bacterial culture or host suspension 5 ml/group Phage lysate, 0.1 ml in small tt 1 tt/group Phage base plates 8/group Phage soft agar tubes 8/group Phage broth

(9.9 ml/tube) 2 tubes/group Sterile 1.0 ml pipettes as needed Water bath, 37°C 2/lab Water bath, 50°C 2/lab

PROCEDURE (The experiment will be performed by students at each table forming a group.) Note: First Session 1. The bacterial culture or the host suspension that we will be using is E. coli B and our phage is T4. You will find the host suspension (2 x 108 cfu/ml) and the phage lysate (4 x 106 pfu/ml) in the 37°C water bath in the lab. Keep them at this temperature all the time. To the "Adsorption Tube" which already contains 0.1 ml of the phage lysate, add 0.9 ml of host culture, mix well, record the exact time and return the tubes to the 37°C water bath. 2. Obtain 2 large test tubes containing 9.9 ml of phage broth and label them 10-2 and 10-4 and place them at 37°C. At precisely 10 min after starting the incubation of the Adsorption Tube, remove 0.1 ml of adsorption mixture and transfer to the tube marked 10-2 . Mix thoroughly by vortexing. 4. Use another sterile pipet to transfer 0.1 ml from tube 10-2 to tube 10-4 . Mix thoroughly by vortexing and place at 37°C. 5. At exactly 20 minutes into the experiment, add 0.1 ml from the 10-4 tube and 0.1 ml of the host culture to a soft agar tube and mix thoroughly by rolling the tube in the palms of your hands. Note: Soft agar (molten) tubes are kept at the 50°C water bath. Remove only one tube at a time to use immediately. The soft agar solidifies in the tubes in a few minutes, if the tube is placed at room temperature. So, once again: DO NOT REMOVE ANY SOFT AGAR TUBE FROM 50°C BATH UNTIL NEEDED! 6. Pour the soft agar onto a base plate labeled "20 min" and rotate the plate to evenly spread the soft agar across the surface of the entire plate. Allow the agar to gel, invert the plate, and incubate at 37°C overnight. 7. Repeat steps 5 and 6 at precisely 25, 30, 40, 50, 60, 70 and 80 minutes into the experiment. 8 Your plates will be moved to a refrigerator after 24 hours of incubation at 37°C.

Second Session 1. Calculate the multiplicity ratio in the Adsorption Tube. This is done by dividing the number of phage by the number of bacteria in the Adsorption Tube at the start of the experiment. This ratio provides an estimate of the number of phage available to infect each bacterium. 2. Obtain your plates. Count the number of plaques (pfu) per plate and complete the table in the "Results" section. Plates with a large number of plaques can be divided into 2 or 4 sections and only one section counted and then the result multiplied by 2 or 4 to get the total plaque number per plate. Microbiology BIOL 275 Dr. Eby Bassiri ebassiri@sas.upenn.edu 4 Figure: Plaques seen on a lawn of bacteria after 25 (picture on left) and 60 (picture on right) minutes into the experiment. Arrows show plaque forming units (pfu). Enlarge the pictures to see plaques better.

1. Calculate the multiplicity ratio in the Adsorption Tube (# of phage to # of bacteria): 2. How many bacteria are present for each phage in the Adsorption Tube? 3. Fill out the following table. Calculate the actual counts based for the number of plaques found on your plates multiplied by the dilution factor and the amount of inoculum used. Look at the actual counts and decide whether the very first or the average of the first few counts can be chosen as the base line (eclipse period). Divide all other counts by the base line to obtain the relative titers (maximum relative titer would be the burst size for the phage under study. What was the burst size of your phage in this experiment? 4. Graph the relative titers (y-axis) over time (x-axis).

Experiment no – 8 Titration of phages

Objectives:

1. To demonstrate the ability of bacteriophage to replicate inside a susceptible host cell.
2. To determine the concentration of phage particles in a suspension.

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Principle:

Bacteriophages (phage) are obligate intracellular parasites. They multiply inside a bacterium by making use of some or all of the host (ie., bacteria) biosynthetic machinery (viruses that infect bacteria are known as bacteriophage). They enter the bacterial cell by 'landing' on the cell wall and injecting their DNA into the bacterial cytoplasm. After entry, the phage DNA acts as a template for production of phage proteins. These proteins replicate the phage and subjugate the cell, eventually causing lysis and death of the host cell. A bacteriophage particle is even harder to see than a bacterium. Viruses are beyond the limits of resolution of the light microscope and can be seen only with electron microscopes. Fortunately, we can use a technique very similar to the colony-counting technique used to measure the number of bacteria to count phage particles, known as the plaque assay. Lytic phages are enumerated by this method.

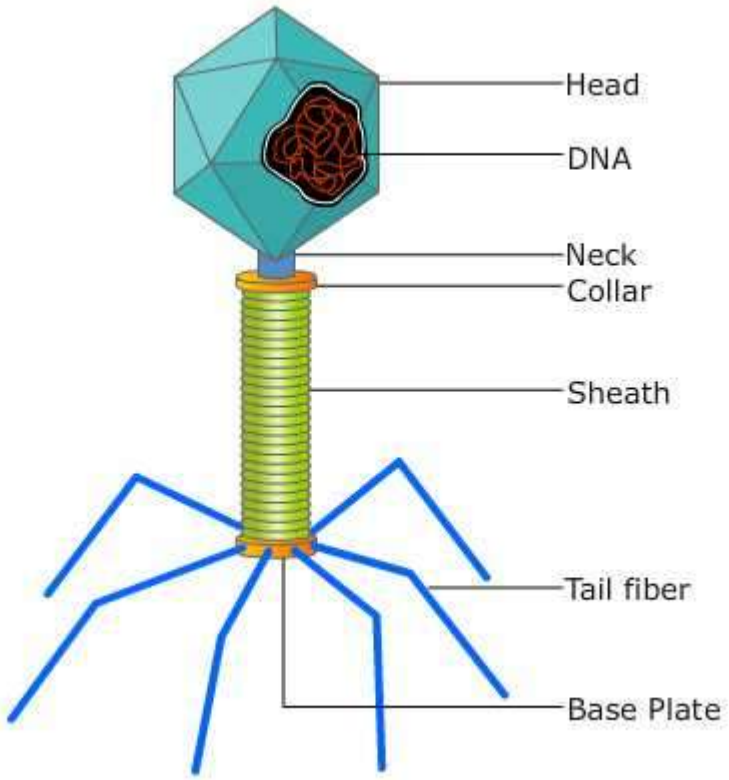


Fig:- A typical phage

The plaque assay is originally a virological assay employed to count and measure the infectivity level of the bacteriophages. But later, it was applied to measure and count the mammalian viruses as well. This assay is the most widely used technique for the isolation of virus and its purification, and to optimize the viral titers. The basis of plaque assay is to measure the ability of a single infectious virus to form a "plaque" on a concurrent monolayer culture cells. A plaque is developed as a part of infection of one cell by a single virus particle that is followed by the

replication of that virus, and finally, the death of the cell. The newly replicated virus particles will later infect and then kill surrounding cells.

For this technique, we will be provided with a virulent phage stock and a susceptible host cell culture. 10-fold dilutions of the phage stock are prepared. The procedure requires the use of a Double-Layer Agar (DLA) technique also known as double agar overlay method, in which the hard agar serves as a base layer (to form gel), and a mixture of few phage particles (diluted stock) and a very large number of host cells in a soft agar forms the upper overlay. When the plates are incubated, susceptible Escheria coli cells multiply rapidly and produce a lawn of confluent growth on the medium. When one phage particle adsorbs to a susceptible cell, penetrates the cell, replicates and release new phage particles which infect other bacteria in the vicinity of the initial host cell. The growth or spread of the new viruses is then restricted or limited to the neighbouring cells by the gel. This cycle is repeated until large numbers of bacteria have been destroyed. The destroyed cells produce single circular, non turbid areas called plaques in the bacterial lawn, where there is no growth of bacteria because the phage progeny originating from single virus particles have multiplied sufficiently to kill bacteria over an easily visible area. Eventually the plaque becomes too large to be visible to our naked eye. Each plaque represents the lysis of a phage-infected bacterial culture and can be designated as a plaque-forming unit (PFU) and is used to quantitate the number of infective phage particles in the culture. Dyes that stain the living cells are frequently used to enhance the contrast between the plaques and the living cells. Therefore the dead cells in the plaque will appear as unstained against the colored background. Only viruses that have the ability to cause visible damage of cells can be assayed using this way.

The number of phage particles contained in the original stock phage culture is determined by counting the number of plaques formed on the seeded agar plate and multiplying this by the dilution factor. For a valid phage count, the number of plaques per plate should not exceed 300 nor be less than 30. Plates showing greater than 300 PFUs are too numerous to count (TNTC); plates showing fewer than 30 PFUs are too few to count (TFTC).

The main difference between Plaque Assay and Colony-Counting is that, to count bacteria, we spread about 100 - 400 bacteria over the surface of the agar and incubate the plate. To count phage, we spread 100 - 400 phage particles mixed with a very large number of host bacterial cells over the surface of the agar and incubate the plate. In colony-counting, we directly observe the accumulation of large numbers of bacteria, each pile being a colony. In plaque formation, we directly observe the absence of bacteria against a background of dense bacterial growth, each zone of destruction being a phage plaque (hole) in the lawn of bacteria. Each hole represents one phage in the sample applied to the plate.

2017

Experiment no 9 – Nuclear staining

Nuclear Staining by Giemsa's Method and its Details

Aim

To stain bacteria nuclear material by Giemsa's staining technique.

Approach

DNA is a principle genetic material of all micro-organisms except some viruses.Bacterial cell lacks well organised nucleus.It is very essential structure of a living cell.Nucleus performs important functions like growth,metabolic activities,multiplication and transfer of heridatory characters.Nuclear material of bacteria is also called as nucleochromatin body.Bacteria cell have haploid nuclear content and they posses single copy of double stranded DNA material.Besides DNA some bacteria may posses extra chromosomal material called as plasmid. The nuclear material of bacteria is stained by using Giemsa's method where as other method's that can be used are Feulgen's method and Robinow's method.

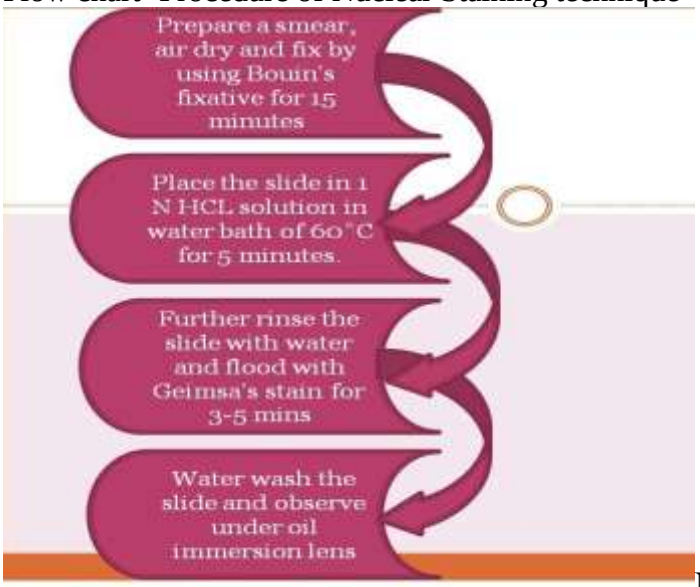
Requirements

1. Bouin's fixative
2. 1 N HCL
3. Water bath
4. Giemsa's stain
5. Cell suspension

Procedure

1. A clean grease free slide it taken and a thick smear is prepared by using a sterile wireloop.
2. The slide is allowed to air dry and then the smear is fixed by using Bouin's fixative for about 15 minutes here we avoid heat fixation step.
3. Further after chemical fixation the slide is treated with 1 N HCL solution in water bath at 60°C for about 5 minutes.
4. After 5 mins the slide is rinse with water and flooded with Giemsa's stain for about 3 to 5 minutes after that slide is observe under oil immersion lens.

Flow chart- Procedure of Nuclear Staining technique



Nuclear Staining by Giemsa's method

Mechanism

Giemsa's stain is prepared by mixture of two stains that are methylene blue (basic dye) and eosin(acidic dye) so the resulting stain has properties of both dye.The chromatide of a cell is highly acidic in nature so when it reacts with Giemsa's stain it gives a reddish purple color to DNA.The RNA of a cell is removed by acid hydrolysis step in this step the smear is treated with 1 N HCL solution in a water bath of about 60°C temperature for 5 minutes during this step due to heat and acid the double bonds between the base pairs of RNA molecule get week and break.Where as DNA material has triple bonds in between some base pairs so DNA molecule doesn't get hydrolyzed and hence only DNA molecule get stain and cytoplasm remains colourless.

Observation

The cytoplasm appears colourless where as nuclear material appears purple in colour.

Experiment no 10 – Estimation of protein by lowry's method

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

AIM:

To estimate the amount of Protein present in given unknown solution.

PRINCIPLE:

Alkaline CuSo4 catalyses the oxidation of aromatic amino acids with subsequent reduction of sodium potassium molybdate tungstate of Folin's reagent giving a purple colour complex the intensity of the colour is directly proposition to the concentration of the aromatic amino acid in the given sample solution.

REAGENTS REQUIRED:

1. Stock Solution: Bovine Serum albumin of 100mg is weighed accurately and dissolved in 100ml of distilled water in a standard flask (concentration 1 µg /ml). 2. Working Standard: The Stock Solution of 10 ml is distilled to 100ml with distilled water in a standard flask (concentration 100 mg/ml). 3. Folin's Phenol Reagent: Folin's Phenol Reagent is mixed with distilled water in the ratio 1:2. 4. Alkaline copper reagent: Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide. Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate. Solution A, B, C is mixed in the proportion of 50:1:0.5. Unknown Preparation: The unknown protein is made upto 100 ml with distilled water.

PROCEDURE:

Working standard of 0.2 -1ml is pipette out into clean test tube and labeled as S1-S5. Test solution of 0.2ml is taken into test tube and labeled as T1. The volume is made upto 1ml of distilled water. Distill water of 1ml serve as blank. To all the test tube 4.5ml of alkaline CUSO4 reagent is added and incubated at room temperature for 10 minutes. All the test tube 0.5ml of folin's phenol reagent is added. The contents are mixed well and the blue colour developed is read at 640 rpm after 15 minutes. From the standard graph the amount of protein in the given unknown solution is calculated.

RESULT:

The amount of protein present in the given unknown solution is mg (µg of protein).

2017

Experiment no 11– Analysis of amino acid by paper chromatography

Chromatography is a common technique for separating chemical substances. The prefix "chroma," which suggests "color," comes from the fact that some of the earliest applications of chromatography were to separate components of the green pigment, chlorophyll. You may have already used this method to separate the colored components in ink. In this experiment you will use chromatography to separate and identify amino acids, the building blocks of proteins. The proteins of all living things are composed of 20 different amino acids, some of which are described below.

Chromatography is partially characterized by the medium on which the separation occurs. This medium is commonly identified as the "stationary phase". Stationary phases that are typically used include paper (as in this experiment), thin plates coated with silica gel or alumina, or columns packed with the same substances. The "mobile phase" is the medium that accompanies the analyzed substance as it moves through the stationary phase. Both liquids and gases can be used as mobile phases depending on the type of separation desired. To refer to gas or liquid chromatography, chemists often use the abbreviations GC or LC, respectively. These abbreviations explicitly identify the phase of matter of the mobile phase. The term "paper chromatography" used in this experiment's title identifies the composition of the stationary phase. The compositions of the stationary and mobile phases define a specific chromatographic method. Indeed, many different combinations are possible. However, all of the methods are based on the rate at which the analyzed substances migrate while in simultaneous contact with the stationary and mobile phases. The relative affinity of a substance for each phase depends on properties such as molecular weight, structure and shape of the molecule, and the polarity of the molecule.

In this experiment, very small volumes of solutions containing amino acids will be applied (this process is sometimes called "spotting") at the bottom of a rectangular piece of filter paper. For ready comparison of each trial, it is vital that each solution be applied on the same starting line. After the solutions have been applied, the paper will be rolled into a cylinder and placed in a beaker that contains a few milliliters of the liquid mobile phase. For this separation, a solution containing n-propanol, water and ammonia is the optimum mobile phase. As soon as the paper is placed in the mobile phase, the solution (sometimes called the eluting solvent) will begin to rise up the paper. This phenomenon is called capillary action, a concept that is described in Chapter 12 of your text. As the mobile phase rises on the paper it will eventually encounter the "spots" of amino acids. The fate of each amino acid in the mixture now depends on the affinity of each substance for the mobile and stationary phases. If an amino acid has a higher affinity for the mobile phase than the stationary phase, it will tend to travel with the solvent front and be relatively unimpeded by the filter paper. In contrast, if the amino acid has a higher affinity for the paper than the solvent, it will tend to "stick" to the paper and travel more slowly than the solvent front. It is these differences in the amino acid affinities that lead to their separation on the paper. The affinities of these amino acids for the mobile phase can be correlated to the solubility of the different amino acids in the solvent (i.e., an amino acid that is highly soluble in the eluting solvent will have a higher affinity for the mobile phase than an amino acid that is less soluble in the solvent.). When the solvent front comes near the top of the filter paper, the paper is removed from the beaker and allowed to dry. At this point, the various amino acids are invisible.

The acids can be visualized by spraying the paper with a compound called ninhydrin. Ninhydrin reacts with amino acids to form a blue-violet compound. Therefore, the sprayed filter paper should show a number of spots, each one corresponding to an amino acid. The further the spot

from the starting line, the higher the affinity of the amino acid for the mobile phase and the faster its migration. The relative extent to which solute molecules move in a chromatography experiment is indicated by Rf values.

The Rf value for a component is defined as the ratio of the distance moved by that particular component divided by the distance moved by the solvent. Figure 1 represents the migration of two components. Measurements are made from the line on which the original samples were applied to the center of the migrated spot. In the figure, dA is the distance traveled by component A, dB is the distance traveled by component B, and dsolv is the distance traveled by the eluting solution. In all three cases, the travel time is the same. dA dsolv dB solvent front solvent front pencil line with spot of mixture solvent level in beaker A B

. Thus the Rf values for components A and B are Rf(A) = dA/dsolv Rf(B) = dB/dsolv Note that Rf values can range from 0 to 1. In this example, Rf(A) is obviously larger than Rf(B). Although Rf values are not exactly reproducible, they are reasonably good guides for identifying the various amino acids.

Paper chromatography is most effective for the identification of unknown substances when known samples are run on the same paper chromatograph with unknowns. To best understand why different amino acids have unique Rf values, it is important to understand the structural features of these molecules. As the name suggests, each amino acid contains an amino group, -NH2, and a carboxylic acid group, -COOH. The molecular structure of a generic amino acid is provided below: C H R H2N COOH Hydrogen Amine group Acid group Side chain The 20 different amino acids that make up our proteins, and those of most other living things, differ in the identity of the side chain R. In glycine, the simplest amino acid, R is a hydrogen atom. Eight amino acids have R groups that consist of carbon atoms with attached hydrogen atoms. Two examples are valine for which R is –CH(CH3)CH3, and phenylalanine, which contains a benzene ring with R equal to –CH2(C6H5). These nonpolar hydrocarbon side chains are hydrophobic or "water-hating."

Hence, they tend to lower the water solubility of the corresponding amino acids. Six amino acids have polar but neutral R groups that tend to promote water solubility. For example, for serine R is –CH2OH. In two amino acids, glutamic acid and aspartic acid, the side chains carry carboxylic acid groups. For example, in glutamic acid, R is –CH2CH2COOH. Finally, three amino acids have basic R groups. One of these is lysine, for which R is – CH2CH2CH2CH2NH2. Both acidic and basic R groups tend to promote water solubility, though the solubility will be pH dependent. In fact, the water solubility of all amino acids varies with the acidity of the solution, i.e. the H+ ion concentration that is commonly communicated via pH values. This is because all amino acids, even those with neutral side chains, contain an acidic –COOH group and a basic -NH2 group. T

he most prevalent ionic form of an amino acid in solution therefore depends on the pH of the solution. As the equation below suggests, in solutions of low pH (high H+ concentration), the amino and acid groups are both protonated and this contributes a net plus charge. Near the neutral pH of 7, an H+ has dissociated from the carboxylic acid group and the positive and negative charges balance each other. In solutions of still higher pH (low H+ concentration), the amino group is in the –NH2 form and the net charge is negative because of the –COO- . This means that the rate of migration of an amino acid will depend on the pH of the mobile phase, and that the details of this dependence will vary from amino acid to amino acid. The presence of an acidic or basic R group further complicates this pH dependence. C H R H3N COOH High pH + C H R H3N COO + _ pH ~ 7 C H R H2N COO _ Low pH + H+ + 2 H+ The acidic group of one

amino acid can react with the basic group of another to form what is called a peptide bond, with the elimination of a water molecule. This process can be repeated to form polypeptides or proteins. Many proteins contain well above 100 amino acids. When a protein is heated in the presence of acid or base, it is hydrolyzed, the peptide bonds are broken, and the constituent amino acids are released. One of the samples for study in this experiment is such a hydrolyzed mixture.

Experimental Procedure Obtain a sheet of filter paper, and draw a faint pencil line about 1 to 2 cm from one of the long edges and parallel to that edge. This will be the bottom of the chromatogram. Mark off seven equally spaced points along this line. (They should be separated by about 2 cm). Your samples will be applied to these spots. The laboratory contains solutions of four identified amino acids and a sample of a hydrolyzed protein. In addition, you will be given a numbered unknown that will contain one or more of the known amino acids. The samples can be applied to the paper by using a narrow capillary tube. The procedure is pretty simple, but it is a good idea to practice making sample spots on a separate sheet of filter paper before you start on your chromatographic paper. Dip the open end of a clean capillary into the solution to draw up a small volume of the solution into the tube. Lightly and briefly touch the tube to the paper and allow the sample to transfer. The spot should be about 2-3 mm in diameter. Once you have mastered the technique, place one spot of each of the four known amino acids on the separate points that you previously marked on the filter paper. In addition, apply samples of your unknown to two of the points, and the hydrolyzed protein to another. Be careful not to contaminate either the solutions or the spots. Label each spot (with pencil and below the starting line) to indicate its identity. Finally, it's a good idea to avoid getting fingerprints on the chromatographic paper. When you have finished spotting your paper, allow it to dry by waving it in the air or using a heat lamp or hair dryer. (Don't get it too hot.)

Meanwhile, in the hood, pour about 15-20 mL of the eluting solution (n-propanol and concentrated aqueous ammonia) into a clean, dry 600 mL beaker and cover the beaker with a watch glass or plastic wrap. When the sample spots have dried, roll the paper into a cylinder, with the short sides almost touching. Use a bit of "Scotch" tape along the top of the paper to hold the cylinder together. Evenly lower the paper cylinder, sample side down, into the beaker. The solvent will wet the paper, but the sample spots should not be immersed. In addition, the paper should not touch the walls of the beaker. At this point, cover the beaker with a watch glass or plastic wrap and place the beaker in the hood. When the solvent front gets within about 1 or 2 cm of the top of the paper (in perhaps about 2 hrs), remove the paper, use a pencil to mark the solvent front at several points, unroll the cylinder, and let the chromatography paper dry in the hood. When the paper is dry, spray it with ninhydrin reagent. Allow the paper to dry, perhaps using the hair dryer, heat lamp, or an oven at about 100o C, but don't overcook it! When the chromatographic paper has fully dried, outline the spots, mark the centers of each of the spots, and note their colors. (Not all amino acids give the same color with ninhydrin). Measure and record the distances the solvent and each of the amino acids traveled from the origin. Use these distances to calculate Rf values for each sample. Comparison of the spots should enable you to identify the amino acid(s) present in your unknown sample. Also note whether the known amino acids are present in the hydrolyzed protein.

Report Your report should include the following experimental data and conclusions:

1. Your marked chromatograph
2. Rf calculations and results

3. The identification of the amino acid(s) in your group's unknown and support for your conclusions
4. Conclusions about the amino acids present in the hydrolyzed protein In addition, your report should include the following information or answers:
1. The formulas for the four amino acids you chromatographed
2. The most prevalent ionic form (structure) of these amino acids in the very basic eluting solution
3. Explanations for the observed differences in Rf values for these four amino acids
4. An explanation for the difference in appearance of the chromatograph for the hydrolysed protein and for the pure amino acids
5. Why are you advised to mark the paper with pencil and warned not to get fingerprints on it?

Experiment no 12– Analysis of amino acid by thin layer chromatography

Introduction

Method basics Planar chromatograpy belongs to the family of chromatographic methods used for separation and determination of substances. It allows to carry out qualitative and sometimes also quantitative analysis of chemical components in complex mixtures. Substances to be determined are transferred onto the layer of sorbent as a spot or band using a capillary, microsyringe or special aplicator. Sorbent may comprise of a layer (0,05...0,2 mm thick) of fine-grain material (silicagel, cellulose, aluminium oxide, ion exchange resin) on a solid support (glass, plastic, metal). Instead of sorbent, porous materials (paper, plastic etc) can be used as well. Spots of samples are applied on the plate in a straight line called starting line. The mobile phase starts moving upwards due to capillary forces when the plate edge is inserted into the eluent in chromatographic chamber. While the eluent is moving the components of sample separate from each other because of the interactions between the molecules of eluent, sorbent and substances to be separated. The elution process is stopped when the eluent has traveled up the plate until 5-10 mm from the upper edge of the chromatographic plate. This eluent front is marked and this line is called stop line. The elution distance, h0, is the distance between the starting line and the stop line. After the chromatographic separation, all the components of the analysed mixture are located somewhere between the starting line and stop line. In order to visualize the separated substances (if they are not colored) different techniques are used: chemical reactions, adding fluorescence indicators to the sorbent layer during the process of preparation of the plates or spraying the plates with fluorescent solutions and then observing under ultraviolet lamp. As the distances travelled by the different substances differ, their mobilities can be used for qualitative analysis. Therefor, the distance between the starting line and the center of the spot of substance hx (mm) characterizes each substance. Retention factor, RF, provides better way to indentify substances. RF is calculated from the following formula: 0 h h R x F = Provided that exactly the same amount of sample is applied, the intensity of the spot can be used for quantitative determination: the bigger the amount of substance in the mixture, the more intensive is the spot. Also the size of the spot can give quantitative information – the bigger the spot, the bigger the content of this compound in the mixture. Intensity of the spots is evaluated by comparing with the intensities of analyte spots with known amounts visually or using densitometer (device for measuring optical density).

Amino acids Proteins consist of amino acids interconnected by peptide bonds. depicts the general structure of α-amino acids (carboxylic acid and amino groups attached to the same carbon), which are found in living organisms.

Instruments, chemicals and glassware
1. Eluent1 . [Mix n-butanol, acetic acid (purity 98 – 100 %) and distilled water in volume ratio 5:1:5. Stir for 10 minutes, then let the layers separate. Use upper layer as eluent.]
2. Solution on ninhydrin. [Dissolve 0.3 g of ninhydrin in 100 ml n-butanol. Add 3 ml of glacial acetic acid.]
3. 0.02 M solutions of amino acids (leucine, methionine, alanine and serine). [Dissolve: 0.026 g leucine, 0.030 g methionine, 0.018 g alanine and 0.021 g serine in distilled water and bring the volume to 10 ml.] 4
Chromatographic paper with dimensions 85 x 50 mm
5. Elution chamber with approximate internal dimensions: 7 cm high and with 5.5 cm diameter. Upper edge of the chamber is grinded and the chamber is covered with two lids, the first lid has a slot for chromatographic paper.
6. Glass capillaries for spotting the samples. 7
. Graduated test-tube.
8. Graphite pencil.
9. Drying oven at ~ 60° C.
10. Filter paper 10 x 10 mm or 12 cm in diameter.
11. Solution of ninhydrin in spraying bottle. 1
2. Ruler.
13. Scissors.
14. Rubber gloves.
Analytical procedure
Rubber gloves must be used during this work to avoid contamination of chromatographic paper with amino acids from skin, and for protecting skin from solvents and ninhydrin while working with the sprayer or sprayed paper. While the paper is being prepared for chromatographic analysis it should be kept on a piece of filter paper. Mark the starting line on the paper approximately 1 cm from the edge with graphite pencil (very slight line!). Also mark the locations where the samples will be spotted. The neighboring spots should be about 8 mm apart from eachother and at least 5 mm away from the paper's edge. Usually the spot of unknown substance is applied to the center of the starting line. Before applying samples to the paper and filling the elution chamber fit the length of chromatographic paper with the height of elution chamber. Cover the chamber with the first lid and insert the chromatographic paper through its slot. With pencil mark the place where the paper extends from the slot. Remove the paper from chamber and fold it back 1 mm below the mark so that eventually the paper would hang down from the lid. The spots of individual amino acids and sample solutions are applied to the chromatographic paper. Use separate clean and dry glass capillary for each solution. Dip the capillary into solution – some solution is drawn into the capillary. With the filled capillary touch the prepared location on chromatographic paper. 1 Instructions in square brackets are for information only. Students don't prepare those solutions. Determination of amino acids using thin layer chromatography. 08.09.15 Meditsiiniline keemia/Medical chemistry LOKT.00.009 http://tera.chem.ut.ee/~koit/arstpr/ah_en.pdf 4 The spot on the paper should not be bigger than 2-3 mm. It is advised you can exercise spotting on a sheet of filter paper with distilled water. After application of samples let the spots dry.To start the analysis, insert the chromatographic paper through the slot in the first lid and cover it with the second lid. Check if the paper reaches the eluent surface. Elution is stopped when the solvent front has traveled up the plate until 7-10 mm

from the lid. Remove the paper from elution chamber and place it on a sheet of filter paper. After 2-3 minutes mark the eluent front with pencil and dry the paper in oven. When the paper is dry, take it into the fume hood and spray it with solution of ninhydrin until the paper is slightly damp. Chromatographic paper and the paper supporting it should lie at 45° angle while spraying. The chromatographic paper is again put in the drying oven for 15 min to speed up the reactions. Use the time to wash the gloves (let the gloves be on until washed). Use distilled water to rinse the capillaries and put into oven for drying. Pour the eluent from the elution chamber into residues bottle and let the chamber dry. Remove chromatographic paper from drying in the oven, draw the contours and centers of the chromatographic bands.
Calculate RF values by the method described above.
Compare retention of standard substances and components in sample and determine which amino acids were present in the sample.
Results Names of amino acids found in the mixture

Experiment no 13- protein purification by column chromatography

The exercise is divided into two parts. In the **first part** you will receive a sample containing three different biomolecules - two are proteins and the third is a small cofactor. The aim of this practical is to **design a purification protocol** by which you are able to separate the molecules from each other. To do this you will have to take such properties as molecular weight (=size) and charge (isoelectric point/pH) into account.
In the **second part** of the practical you will study the ability of the serine protease trypsin to bind to a benzamidine column. This is an example of affinity chromatography but the experiment will also serve as an introduction to the enzymes part of the course.
The methods you will apply are different types of column chromatography that are used in the purification of proteins. For analysis of the molecules you will use simple enzyme assays and/or colour observations. Absorbance measurments may also be used.

Introduction to protein separation

In order to study proteins it is crucial for the biochemist to yield a sample wich contains only the molecule he is interested in. Also in the production of proteins for commercial purposes the demand for purity is high.

A purification scheme might consist of a combination of the following steps:

Crude cell extract

↓

Ammonium sulfate precipitation

↓

Affinity chromatography

↓

Ion exchange chromatography

This particular sequence of steps is of course not applicable in all cases which often means that a unique purification protocol has to be developed for each new substance you wish to isolate. Most important is that the steps complement each other and that the degree of purity increases in each step. The number of steps included the protocol depends on the state of the starting fraction and on how pure you want your substance.
To get a good recovery of the substance i.e. minimizing losses it is desirable that each step is as specific as possible. To check purity and yield you may use absorbance measurements, various types of electrophoresis and preferably also some kind of activity measurement. The column chromatography part may be performed in different ways more or less manually. In the course practical we will use manual ways. For more routine purifications this system can be built out to monitor absorbance etc. and quite often one uses a FPLC which is a programmable system with more powerful pumps.

Gel filtration (Size exclusion chromatography)

Gel filtration is used to separate proteins of different sizes. You may also determine the native molecular weight of a protein by this method since there is a linear correlation between the elution volume of proteins and the logarithms of their molecular weights (MW)
The system contains two phases, one stationary and one mobile. The stationary phase usually consists of a cross-linked polysaccharide which forms porous beads. The mobile phase normally consists of a buffer. The separation depends on the ability of molecules to enter the pores. Smaller molecules can diffuse into the beads and move more slowly down the column. Molecules are therefore eluted in order of decreasing molecular size. By varying the degree of cross-linking the gels are optimized for different molecular weight ranges.

Elution profiles
The result from a gel filtration experiment is often plotted as the variation of substances eluted as a function of the **elution volume, V_e** (see figure below). V_e is however not the only parameter needed to describe the behaviour of a substance since this also is determined by the total volume of the column and from how it was packed.
By analogy with other types of partition chromatography the elution of a solute may be characterized by a distribution coefficient (K_d). K_d is calculated for a given molecular type and represents the fraction of the stationary phase that is available for the substance. In practice K_d is difficult to determine and it is usually replaced by K_{av} since there is a constant relationship between K_{av}:K_d. K_{av} is obtained from
$$Kav = (V_e-V_0)/(V_t-V_0)$$
The total volume of the column (V_t) is simply calculated from $p \times r^2 \times h$ and the void volume (V0) is determined by passing a large substance that does not interact with the beads (like blue dextran) through the column.

Ion exchange chromatography

The ability to reversibly bind molecules to immobilised charged groups is used in ion exchange chromatography (IEC). Which type of charged group one chooses - positive or negative - depends on the net charge of the protein which in its turn depends on the pH. IEC is maybe the most commonly used technique today for the separation of macromolecules and is almost always

included as one of the steps in the purification protocol. The experiment may be divided into four different parts. See also figure below.

1. Equilibration of the ion exchanger in a buffer in such a way that the molecule(s) of interest will bind in a desirable way.
2. a) Application of the sample. Solute molecules carrying the appropriate charge are bound reversibly to the gel.

b) Unbound substances are washed out with the starting buffer.

3. Elution with a gradient of e.g. NaCl. This gradually increases the ionic strength and the molecules are eluted. The solute molecules are released from the column in the order of the strengths of their binding i.e. the weakly bound molecules elute first.
4. Substances that are very tightly bound are washed out with a concentrated salt solution and the column is regenerated to the starting conditions.

This is a type of adsorbtion chromatography in which the component to be purified is specifically and reversibly bound to a ligand that has been immobilized on a matrix. Any component may be used as ligand as long as it can be covalently attached to the chromatographic bed material. Examples of this type of chromatography is antigen-antibody, enzyme-substrate analogue etc.

SDS-Polyacrylamide gel electrophoresis (not performed in this course).
Sodium Dodecyl Sulfate-PolyacrylAmide Gel Electrophoresis (SDS-PAGE) is an excellent and commonly used method to analyze purity and homogeneity of protein fractions. It may furthermore be used to estimate the molecular weight of protein subunits.
In general, fractionation by gel electrophoresis is based on differences in size, shape and net charge of macromolecules. Systems where you separate proteins under native conditions cannot distinguish between these effects and therefore proteins of different sizes may have the same mobility in native gels. In SDS-PAGE this problem is overcome by the introduction of an anionic detergent SDS which binds strongly to most proteins. When hot SDS is added to a protein all non-covalent bonds are disrupted and the proteins acquire a negative net charge. A concurrent treatment with a disulfide reducing agent such as □-mercaptoethanol or DTT (dithiothreitol) further breaks down the macromolecules into their subunits. The electrophoretic mobility of the molecules is now considered to be a function of their sizes i.e. the migration of the SDS-treated proteins towards the anode is inversely proportional to the logarithms of their molecular weights, or more simply expressed: Small proteins migrate faster through the gel. Compare this with the situation in gel filtration.
The polyacrylamide gel is formed by co-polymerization of acrylamide and a cross-linking monomer N,N'- methylene. To polymerize the gel a system consisting of ammonium persulfate (initiator) and tetramethylene ethylene diamin (TEMED) is added. The concentration of the monomers may be varied to give gels of different density, usually gels with 10-20% acrylamide are used.

Part 1. Separation of three biological molecules.

The aim is to **design a purification protocol** or rather a combination of methods/protocols by which you are able to separate the molecules from each other. At the end of this experiment you

should present three fractions/pools containing each molecule. To do this you will have to take such properties as molecular weight (=size) and charge (= isoelectric point and pH) into account.

You will receive a sample containing a mixture of:
Catalase (10 mg/ml), cytochrome c (10 mg/ml) and riboflavin (0.01 mg/ml).

Some properties of the biological molecules in your sample are summarized in the table below.

Molecule	Molecular weight (Dalton or units)	Isoelectric point (pI)	Other properties
catalase	240.000	5.4	Enzyme that produces water and oxygen from hydrogen peroxide (H_2O_2). Weakly brownish.
cytochrome c	12.000	10.0	Transports electrons across cell membranes. Contains a heme group. Strong red colour.
riboflavin	376	-	Also called vitamin B2. Used in the synthesis of the coenzymes FAD and FMN. Yellow colour.

Available materials and solutions:

- For gel filtration:

Sephadex G25 (PD10 column).
Molecules larger than 10-20 000 in molecular weight will migrate with the void volume on this column. Smaller molecules will be more or less retarded. (In practice this means that most proteins will migrate with the void volume in this gel.)

- For ion exchange chromatography:

CM-Sepharose, negatively charged ion exchange material.

- 50 mM Tris HCl pH 8.0
- 50 mM Tris HCl pH 8.0, 0.5 M NaCl
- Pasteur pipettes
- Glass wool.
- Small test tubes, 3-5 ml
- Small waste beakers
- Pipettes, tips
- 10 % Hydrogen peroxide H_2O_2 - for the catalase enzyme activity assay

Methods:
Gel filtration - Size exclusion chromatography
How to perform the gel filtration experiment:
The type of column you will use can be purchased already packed with a Sephadex G25 gel filtration substance (for more information see above).
The total volume of the column is 9 ml.
You will also need ~50 ml of 50 mM Tris HCl pH 8.0

1. Mount the Sephadex G25 (PD10) column vertically in a stand. Take off the lid and remove the liquid on top of the filter. Also remove the seal at the bottom of the column with a pair of scissors. Equilibrate the column with 20-25 ml of 50 mM Tris HCl pH 8.0 i.e. 2-3 column volumes.
2. Put 15 small test tubes in a rack and put the column above the first tube. Apply 1 ml of your sample.
3. Elute stepwise with 1.0 ml of the buffer and collect at the same time 1 ml fractions. Note at which volumes the proteins and riboflavin elute. The proteins are red/brown coloured and the riboflavin yellow. Continue until you have collected 15 fractions.

Hint: The void volume usually is ~1/3 of the total volume of a gel filtration column.

4. To identify the molecules in your fractions you should check colour(s) and perform the catalase test (see below).

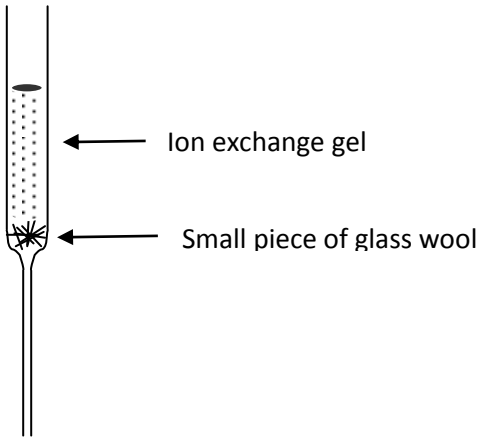
Ion exchange chromatography (IEC).

In this experiment you will pack a simple ion exchange column with the aid of a Pasteur pipette some glass wool and ion exchanger. You will also need the following buffers

- Buffer A: ~10 ml 50 mM Tris HCl pH 8.0
- Buffer B: 3 ml 50 mM Tris HCl pH 8.0, 0.5 M NaCl.

Procedure:

1. Put a small piece of glass wool in the Pasteur pipette and mount it in a stand. The glass wool will stop the gel from being rinsed out of the column (See figure). Put a waste beaker under the column outlet.



2. Add carefully approximately ~1 ml of ion exchange material to the pipette. You avoid air bubbles if you apply the solution along the wall of the pipette. Equilibrate the column with ~3 column volumes of buffer A.
3. Place the column over a rack with 7 tubes and apply 1-2 ml of your sample. Collect the flow through in the first tube. Move to the next tube.
4. Continue to elute stepwise 3 x 1.0 ml with buffer A (tubes 2-4). Then elute with 3 x 1.0 ml of buffer B (tubes 5-7).

The fractions should be analyzed for colour and catalase activity as in the previous experiment.

Finally:

Look at your results and pool fractions from the two chromatographic steps in such a way that you obtain 3 tubes/fractions each containing ~2 ml. One fraction should contain riboflavin the

other catalase and the third cytochrome c. At this point you may also check the absorbance of these three fractions. Try to figure out the appropriate wave lengths.
Did you succeed? Present the result to your lab teacher.

Catalase (H_2O_2) test:
Catalase catalyses the formation of water H_2O and oxygen O_2 from hydrogen peroxide H_2O_2.
See Stryer (6^{th} ed) p. 518. This reaction can be monitored with a simple enzyme assay in the laboratory. Withdraw ~20 ul from each fraction (use e.g. an automatic pipette) and place the drops on a numbered glass or plastic plate. Then add 1 drop of 10 % H_2O_2 to all drops you want to test. Observe gas formation (bubbles) which is an indication of O_2 production.

Results and discussion points that should be included in your lab report (as part of the text):

1. Elution data from both columns. In what order did the molecules elute. Which substance(s) bound or did not bind to the ion exchange resin?

Tip: make a table for each type of column with information about the content in the fractions. Remember to note such things as enzyme activity and colour observations.

2. Is the result from the ion exchange column consistent with the information about the isoelectric points given for each protein?
3. Imagine that you had used a buffer at pH 5.0 when you did the ion exchange experiment. How would that influence the elution pattern of the proteins?
4. Can you think of a more advanced gel filtration experiment where you would separate these three molecules with just one column?
5. Could these molecules have been separated by some other methods?
6. Proteins are often detected by UV measurements. There is a general wavelength that we often use to monitor proteins. Which one and why is this wavelength used?

Affinity chromatography - Binding of trypsin to the inhibitor benzamidine

Trypsin is an enzyme belonging to the serine proteinase family. Several trypsin inhibitors have been characterised and one of them is benzamidine (Figure 1). In the following experiment benzamidine has been covalently linked to Sepharose beads and this column material has been packed into small pre-packed columns. If you apply a solution containing trypsin to this column material the protein should reversibly bind to the ligand benzamidine and later be recovered by either a pH change or addition of excess free ligand to elute the protein. In our case we will decrease the pH to elute the protein.

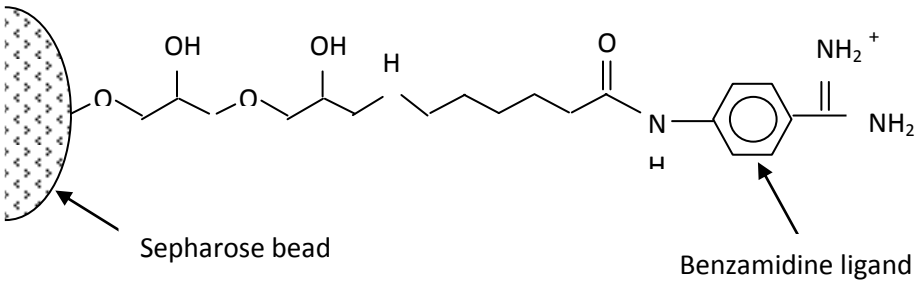


Figure 1. Partial structure of Benzamidine Sepharose.

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To detect trypsin we will use an artificial substrate p-nitrophenyl-p'-guanidinobenzoate (NPGB). When this substrate is cleaved p-nitrophenol a strongly yellow coloured compound is formed (Figure 2).

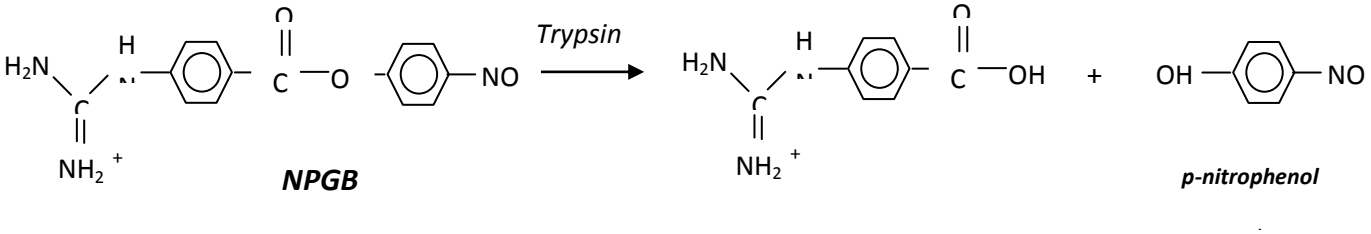

Figure 2. Cleavage of the substrate p-nitrophenyl-p'-guanidinobenzoate (NPGB) by trypsin.

Materials:

- Column: HiTrap Benzamidine Sepharose 4 Fast Flow (1 ml) with Luer lock adapter
- 1 ml syringe
- 12 small test tubes
- Buffer A = Binding buffer: 15-20 ml 50 mM Tris-HCl pH 8.0 containing 0.5 M NaCl
- Buffer B = Elution buffer: 10 ml 50 mM Glycine-HCl pH 3.0.
- Sample: 0.5 ml trypsin (10 mg/ml)
- 1 ml NPGB (1.0 mg/ml, freshly made!)
- 1 ml 1 M TrisHCl pH 8.0
- Small beaker with distilled water
- Waste beaker

Procedure:

To avoid contact with buffers and other solutions it is advisable that you wear gloves during this experiment

1. Put 12 test tubes in a rack and number them 1-12. Add 100 ul (100 **micro**liter) 1 M Tris-HCl pH 8.0 to numbers 7-11 You will later elute the protein by decreasing the pH to 3.0 into these tubes and the extra concentrated pH8-buffer will help increasing the pH back in the fractions to a more physiological level. Add 1ml of buffer A to number 12. This will be used as a control tube.

2. The HiTrap system consists of convenient pre-packed columns that you may run either connected to a pump (e.g. FPLC/ÄKTA) or manually with the aid of a syringe. The column is stored in ethanol and you should start by washing it with distilled water. Replace the top lid of the column with a Luer lock connection and remove the bottom nut. Fill a syringe with distilled water, connect it to the top of the column and flush it SLOWLY at a speed of 1 ml/min. A recommended wash volume is 3 ml. All the subsequent solutions including your sample are applied like this with the syringe.

3. Continue to wash the column with 5 ml of buffer A (binding buffer). Now the column is ready to use.

4. Apply 0.5 ml trypsin to the column (use the syringe speed of ~1 ml/min). Start collecting the eluted fluid in the first tube. Then leave the column with the added protein for 1-2 min to allow the trypsin to bind to its ligand.

5. Apply 5 x 1 ml of buffer A (binding buffer). Collect in tubes 2-6.

6. Apply 5 x 1ml of buffer B (elution buffer). Collect in tubes 7-11.

7. Add 50 ul NPGB to all tubes 1-12. Mix and observe any colour change. Leave the tubes for 5 minutes and observe them again.

8. Wash the column with 5 ml of water and put back the lids.

Results and discussion points that should be included in your lab report:

1. Does trypsin have an affinity for benzamidine?
2. What happens when you add the low pH buffer? Can you explain this at a molecular level?
3. Could you have used the same type of column for any other proteins?

Experiment no 14- Analysis of amino acid by HPLC – Demonstration

Introduction High performance liquid chromatography, commonly known as HPLC, has a variety of applications in the chemical biology research laboratory. This protocol provides some basic background theory, some tips for getting ready to use the HPLC for your particular purification, and guidelines for doing an HPLC purification using our (fill in brand name) instrument. Click here to refer to the HPLC glossary for definitions of many of the terms used in the text. Chromatography is a general analytical technique used to separate a mixture into its individual components. You should already be familiar with thin layer chromatography (tlc), which is used in organic chemistry to separate molecules based on structural differences. The individual components, or analytes, can then be analyzed free of interference from the other components. In chemical biology, individual analytes, such as peptides, are often chromatographically purified for use as a functional tool (e.g., binding to another molecule, enzymatic activity). High performance liquid chromatography (HPLC) is a method used to analyze and separate liquid samples. The separation apparatus is coupled to a UV detector to characterize the analytes as they are separated. In chemical biology laboratories, HPLC is considered indispensable for the purification of peptides (synthesized manually or automated with a synthesizer) and other small to medium-sized organic molecules. Fundamentally, HPLC consists of passing a liquid sample (mixture of components, e.g. a crude peptide synthesis which will typically contain contaminants from the synthesis reagents, various truncated forms of the peptide, etc.) through a column under high pressure. This mobile phase passes through the material in the column, which is called the stationary phase. The analytes passing through the

column interact at different rates between the mobile and stationary phases, primarily due to different polarities of the analytes. The analytes that interact least with the stationary phase or interact most with the mobile phase will exit the column faster. Propagated along the length of the column, these repeated interactions result in a separation of the analytes.

Mixtures of various analytes can be analyzed by changing the polarities of the stationary phase and the mobile phase. There are many types of HPLC columns developed for specific applications. The right choice of column is critical for obtaining good HPLC results. Column choice is governed by characteristics of components in the mixture we wish to separate. For example, we can separate components based on size, charge, hydrophobicity, aromatic character, even chirality. Variable factors include the polarity of the stationary phase, column dimensions, and pore sizes (which can be varied to allow certain sized analytes to pass through at different rates). Another variable that impacts the efficiency of the HPLC separation is the polarity of the mobile phase. Multisolvent delivery systems change the polarity of the mobile phase over the course of an HPLC run, at a rate that defines the "gradient" (e.g., 20% Buffer B to 100% Buffer B over 60 minutes). The use of a gradient improves the separation of analyte mixtures of varying polarities. Typically, the stationary phase in an HPLC column is prepared by reacting an organochlorosilane with the reactive hydroxyl groups on silica.

The organic functional is often a straight chain octyl (C-8) or octyldecyl (C-18) hydrocarbon. When the stationary phase is polar (silica or alumina) and the mobile phase relatively less polar (nhexane, ethyl ether, chloroform), this is referred to as ‘normal-phase chromatography.’ An example of normal phase chromatography is a silica gel “flash” column, often used in organic chemistry to separate relatively non-polar water-insoluble organic compounds. Flash columns are used for the purification of synthetic b-amino acids. When the mobile phase is more polar than the stationary phase (as is the case with a C- 8 or C-18 bonded phase), this type of chromatography is called ‘reversed-phase chromatography.’

Reversed-phase chromatography separations are carried out using a polar aqueous-based mobile phase mixture that contains an organic polar solvent such as methanol or acetonitrile. Because of its versatility, reversed-phased chromatography is the most frequently used HPLC method. Applications include non-ionic compounds, polar compounds (such as peptides), and in certain cases ionic compounds. Analytes exiting the column can be detected by refractive index, electrochemical, or ultraviolet-absorbance changes in the mobile phase.

The detector measures a signal peak as each analyte leaves the column. The signal intensity corresponds to the amount of analyte leaving the column, and can yield quantitative data when compared to a known amount of that particular analyte. The time it takes for the peak to show up, known as the retention time, is characteristic of a particular compound and thus enables identification of the peak of interest. Our HPLCs use a photodiode array detector (PDA) to continuously scan various wavelengths of the UV spectrum. As an analyte peak is detected, the UV spectrum is recorded. Over time, this compiled output yields a time-based plot called a chromatogram The mechanics of the HPLC system are controlled by Windows-based software on a PC. This software controls the gradient of the mobile phase, the solvent flow rate, mobile phase pressure, and measures the signals produced by the detector. A specific HPLC protocol is stored as a method, the parameters of which can be adjusted as necessary. Finally, the results of your sample run can then be interpreted and printed in a variety of report formats.

Very Important Note Everything that goes into the HPLC must be filtered first, through a 0.45 mm or 0.2 mm filter and special glassware to remove particles that can get caught up on the

column and interfere with absorption and separation. This includes your buffers and your sample. Omission of this step can result in damage to the instrument.
B. Sample preparation The crude peptide, prepared by manual or automated synthesis, will be supplied as a lyophilized (dried by freezing in a high vacuum) substance. For b-peptides, the sample is dissolved in 50% H20/50% CH3CN (or a range of others; solvent selection depends on solubility of the sample). Filter your sample. C. Buffer Preparation Buffer A and Buffer B are prepared according to the following recipes: Schepartz Lab Protocols Buffer A Buffer B 80 mL CH3CN 3200 mL CH3CN 3920 mL H20 800 mL H20 2.4 mL TFA* 2.0 mL TFA*

Safety precaution: Trifluoroacetic acid (TFA) is highly corrosive and causes severe burns when inhaled or upon contact with skin. This chemical should only be handled in the fume hood while wearing safety goggles, gloves, and protective clothing. Filter your buffers, using the designated glassware and following the specific instructions provided by your TA. This can be done prior to use and buffers stored at room temperature until you are ready to use the HPLC.
D. HPLC Operation Your TA will provide specific instructions pertaining to the use of the HPLC. Typically, a run starts by attaching your buffers and washing the column (100% Buffer B for 5-10 minutes). Next allow the column to re-equilibrate to conditions that will start your run. For a run with a gradient of 20% Buffer B to 100% Buffer B, this means allowing about 5-10 minutes for the starting conditions for injection to be achieved (that is, to get the entire column in 20% Buffer A). Once a specific separation method is specified, you may review the parameters such as pump flow gradient, run time, and the PDA setup (acquisition).
On some instruments, you will need to specify the lamp used for detection. Your TA will supply the details for the instrument you are using. When making an injection, choose the amount based on the type of column you are using and the approximate amount of your sample.
For a- and b-peptides, the following general guidelines apply: Column scale Amount peptide per injection Analytical Up to 0.01 mg Semi-preparative Up to 0.05 - 0.1 mg Preparative Up to 0.1 – 0.5 mg Use either a glass syringe or a disposable plastic syringe fitted with a luer lock needle (only use flat-tipped needles). Before drawing up your sample, wash out the syringe several times with Buffer B. Draw your sample into the syringe, then carefully remove ALL bubbles from the sample by inverting the syringe, tapping gently, and expelling air until liquid just appears at the needle tip. Load your sample as instructed by your TA. You will want to adjust the view on the PC screen for convenient monitoring of the run, which means selecting the appropriate wavelength(s).
For a- and b-peptides, 214 nm (the absorption frequency of peptide bonds) and 280 nm (the absorption of tyrosine and tryptophan) are recommended. Notice the retention times listed (in minutes) at the bottom of the graphs as well as in the status bar at the top of the screen (this may vary depending on the software used; your TA will clarify this). You will need to record the retention times as you collect peaks so you can correlate your fractions with peaks on the chromatogram. For the first injection of a peptide you've never purified before, you will need to carefully analyze the output. To do this, label a set of 15-20 tubes (15-ml conical vials usually work; you may want to do this ahead of time and loosen the caps so they are Schepartz Lab Protocols ready for collecting peaks as they come off the column. Once you have collected all the relevant peaks from the first injection, you will analyze them by mass spectrometry and determine which fraction or fractions contain your peptide by looking for its molecular weight (calculated in advance). Matching these fractions to their corresponding peaks will give you the retention time for your molecule. At this point, further injections will be simplified as you can

accurately predict the retention time of your sample, and you'll know where to expect the peak containing your molecule. The first peaks that come off the column (after 3-4 minutes dead time for the semi-prep column, 5 minutes dead time for a prep column) represent a variety of leftovers from the synthesis (usually incomplete removal of reagents during wash steps). Once you get beyond this point you should collect every peak as it comes off the column, noting the retention time (for example: 11.23-11.5) for each numbered tube. Try to separate shoulders from main peaks, and isolate peaks that appear within multiple peaks. Keep in mind that the method you choose will impact the appearance of the chromatogram, and hence your ability to collect a pure, isolated fraction. For example, a longer method will give better resolution, but broader peaks. You will get better at this technique with practice, and bear in mind that it usually takes at least two passes through a column to purify a crude peptide synthesis.