Chapter-3

(Materials and Methods)

3.1 Preparation of phosphate buffer solution

Biological systems are extremely sensitive to pH. Human beings can only operate in the pH range of 7.35 to 7.45. How do we maintain such a stringent pH? Fortunately our blood is buffered with the carbonic acid / bicarbonate buffer. In fact, most biologically active systems require a buffered environment. Enzyme reactivity is extremely sensitive to solution pH and requires buffered solutions to function. A pH meter is the best way to determine the pH of a solution. To get a measure of a solution pH is a universal indicator. A universal indicator has a color associated with each pH unit over a wide pH range useful over the pH 4 to 10. The color pH code is:

- pH 12: purple
- pH 9: teal
- pH 7: yellow (greenish)
- pH 5: orange (yellow)
- pH 3 and lower red
- pH 10: blue
- pH 8: green
- pH 6: yellow
- pH 4: red (orange)

The solution has a large buffer capacity", but there are also quantitative descriptions of buffer capacity. There is not universal agreement on a quantitative expression of buffer capacity, one description is:

\[
\text{Buffer Capacity} = \frac{\text{(Moles of OH}^{-}\text{ or H}^{+}\text{ added)}}{\Delta pH \times \text{(Volume of buffer in Lt)}}
\]
We will use this quantitative description of buffer capacity to describe a phosphate buffer. The phosphate buffer solution ($\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$) is one of the most common biological buffers, because it buffers so near to pH 7.3. The simplest way to prepare a phosphate buffer solution is to use PBS buffer tablets. They are formulated to give a ready to use PBS solution upon dissolution in a specified quantity of distilled water. They are available in the standard volumes: 100, 200, 500 and 1000 ml. Sterilization may not be necessary depending on its use. PBS can be stored at room temperature, but may warrant refrigeration to prevent bacterial growth if solution is not sterile and is kept for long periods of time. However, concentrated stock solutions may precipitate when cooled and should be kept at room temperature until precipitate has completely dissolved before use.

A phosphate buffer solution is a handy buffer to have around, especially for biological applications. Because phosphoric acid has multiple dissociation constants, you can prepare phosphate buffers near any of the three pH, which are at 2.15, 6.86 and 12.32. The buffer is most commonly prepared at pH 7 using monosodium phosphate and its conjugate base, disodium phosphate.

Phosphate Buffer Materials

- monosodium phosphate
- disodium phosphate
- DM water
- Phosphoric acid to make the pH more acidic or sodium hydroxide to make the pH more alkaline.
- pH meter
- glassware
- hot plate with stirring bar
**Prepare the Phosphate Buffer**

1. Decide on the concentration of the buffer. Most buffers are used at a concentration between 0.1 M and 10 M. If you make up a concentrated buffer solution, you can dilute it as needed.

2. Decide on the pH for your buffer. This pH should be within one pH unit from the pKa of the acid/conjugate base. So, you can prepare a buffer at pH 2 or pH 7, for example, but pH 9 would be pushing it.

3. Use the Henderson-Hasselbach equation to calculate how much acid and base you need. You can simplify the calculation if you make 1 liter of buffer. Select the pKa value that is closest to the pH of your buffer. For example, if you want the pH of your buffer to be 7, then use the pKa of 6.9:

   \[
   \text{pH} = \text{pKa} + \log \left( \frac{[\text{Base}]}{[\text{Acid}]} \right)
   \]

   \[
   \text{Ratio of } \frac{[\text{Base}]}{[\text{Acid}]} = 1.096
   \]

   The molarity of the buffer is the sum of the molarities of the acid and conjugate base or the sum of [Acid] + [Base]. For a 1 M buffer (selected to make the calculation easy), \([\text{Acid}] + [\text{Base}] = 1\)

   \[
   [\text{Base}] = 1 - [\text{Acid}]
   \]

   Substitute this into the ratio and solve:

   \[
   [\text{Base}] = 0.523 \text{ moles/L}
   \]

   Now solve for [Acid]. \([\text{Base}] = 1 - [\text{Acid}]\) so \([\text{Acid}] = 0.477 \text{ moles/L}\)

4. Prepare the solution by mixing 0.477 moles of monosodium phosphate and 0.523 moles of disodium phosphate in a little less than a liter of water.

5. Check the pH using a pH meter and adjust the pH as necessary using phosphoric acid or sodium hydroxide.
6. Once you have reached the desired pH, add water to bring the total volume of phosphoric acid buffer to 1 L.
7. If you prepared this buffer as a stock solution, you can dilute it to make up buffers at other concentrations, such as 0.5 M or 0.1 M.
8. To calculate the buffer capacity of your phosphate buffer you will measure the moles of acid required to decrease the buffer pH by a known amount. With the other solution the pH will be raised by a known amount through the addition of a strong base.

3.2 Human serum albumin solution Preparation & storage.

Albumin is a protein of about 66,000 Daltons present in blood at a very high concentration. In addition to maintaining blood osmotic pressure, it is also known to play a role in transporting fatty acids and a variety of other substances by forming complexes with them. Serves as an indicator of liver function. Essentially fatty acid free human serum albumin standard solution (33.5 mg/ml) was prepared in a phosphate buffer (pH 7.4) the same protein solution was used in all experiments. This HSA solution was checked at regular intervals. The serum albumin Standard Solution is stable for up to 1 week after preparation when stored at 4°C, or for up to 1 month at -20°C.

All chemicals were of analytical grade. The solutions were prepared with demineralized and purified water. Most nicotinates used in this study were synthesized in our laboratory according to known methods. The remaining nicotinic acid esters were supplied by pharmaceutical companies or obtained from commercial sources.
Esterase-Like Activity of HSA as a Function of pH and Temperature.

Influence of pH on the catalytic activity of HSA was investigated. The explorable pH range was limited by the constraints of the method, such that the hydrolysis of substrate produces stoichiometric amounts of protons only at pH values higher than the liberated acid, the initial rate constants of hydrolysis catalyzed by HSA were markedly pH-dependent and increased about 5-fold from pH 6.0 to 8.2. The rates of chemical hydrolysis were obviously also affected by P^H, a 10-acceleration being observed from pH 6.0 to 8.2.

Temperature also had a marked influence on rates of HSA-catalyzed hydrolysis. The highest activity was observed between 37° and 42°C, whereas 59 ± 3% and 17± 2% of maximal activity were found at 25° and 51°C, respectively.

Influence of Organic Co-solvents and Chemical Structure of Buffers on the Esterase-Like Activity of HSA.

A number of nicotinates in this study were not sufficiently water-soluble to be examined without using a co-solvent. To assess the influence of co-solvents on enzymatic activity, three organic solvents (MeCN, MeOH, and DMSO) were tested at three concentrations 1%, 2%, and 4%. The general observations DMSO Was used as a co-solvent owing to its better solubilizing properties, and its concentration was fixed at 1% (v/v) in all assays.
3.3 **Used organic and inorganic chemicals.**

<table>
<thead>
<tr>
<th>Organic reagents</th>
<th>Inorganic chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid $(C_6H_5NO_2)$</td>
<td>Sodium bi carbonate NaHCO$_3$</td>
</tr>
<tr>
<td>6-Bromonicotinic acid $(C_6H_4BrNO_2)$</td>
<td>Sodium sulphate (Na$_2$SO$_4$)</td>
</tr>
<tr>
<td>Tri-methylboroxin $(C_7H_{11}BO)$</td>
<td>Sodium chloride (NaCl)</td>
</tr>
<tr>
<td>Thionyl chloride $(SOCl_2)$</td>
<td>Potassium carbonate (K$_2$CO$_3$)</td>
</tr>
<tr>
<td>Bromine $(Br_2)$</td>
<td>Sodium metal (Na)</td>
</tr>
<tr>
<td>Iso-nicotinonitrile $(C_6H_4N_2)$</td>
<td>Sodium hydroxide (NaOH)</td>
</tr>
<tr>
<td>2-Bromoisonicotinic acid $(C_6H_4BrNO_2)$</td>
<td>Ferric chloride (FeCl$_3$)</td>
</tr>
<tr>
<td>Oxalyl chloride $(COCl)_2$</td>
<td>Sodium hydride (NaH)</td>
</tr>
<tr>
<td>Pyridine $(C_5H_5N)$</td>
<td>Sodium carbonate (Na$_2$CO$_3$)</td>
</tr>
<tr>
<td>3-Bromopyridine-2-carbonitrile $(C_6H_3BrN_2)$</td>
<td>Monosodium phosphate (NaH$_2$PO$_4$)</td>
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<tr>
<td>2-Aminonicotinic acid $(C_6H_6N_2O_2)$</td>
<td>Phosphoric acid (H$_3$PO$_4$)</td>
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<tr>
<td>Ethyl iodide $(C_2H_5I)$</td>
<td>Silica gel (SiO$_2$)</td>
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<tr>
<td>Diethyl1,3-Acetonedicarboxylate</td>
<td>Disodium phosphate (Na$_2$HPO$_4$)</td>
</tr>
<tr>
<td>Triethylorthoformate</td>
<td></td>
</tr>
<tr>
<td>Acetic anhydride $(CH_3CO)_2O$</td>
<td><strong>Solvents</strong></td>
</tr>
<tr>
<td>Phosphorusoxichloride $(POCl_3)$</td>
<td>Ethyl acetate (EtOAc)</td>
</tr>
<tr>
<td>Ammonia solution Aq. $(NH_3 30%)$</td>
<td>Ethyl acetate (EtOAc)</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>Methanol (CH$_3$OH)</td>
</tr>
<tr>
<td>Methyl alcohol $(CH_3OH)$</td>
<td>Diethyl ether (C$_2$H$_5$)O</td>
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<tr>
<td>Tetrakis(Triphenylphosphine) palladium(0)</td>
<td>n-Pentane</td>
</tr>
<tr>
<td>Tetrakis(Triphenylphosphine) palladium(0)</td>
<td>n-Hexane</td>
</tr>
<tr>
<td></td>
<td>1,4-Dioxane</td>
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<tr>
<td></td>
<td>Dichloromethane (DCM)</td>
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<tr>
<td></td>
<td>Dimethyl formamide (DMF)</td>
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<tr>
<td></td>
<td>HCl solution (12N)</td>
</tr>
</tbody>
</table>
Common lab instruments and materials.

- Round bottom flask of different sizes (100 ml, 250 ml, 500 ml, 1 lt, single neck, two necks, three necks etc.)
- Chilled water glass condenser with supply pipes.
- Magnetic stirrer with oil bath and thermotech.
- Digital pH meter.
- Glass column of different sizes (500 mg, 1 gm, 5 gm, 10 gm etc.)
- Column stand and glass conical.
- Water bath, balloons and capillaries box.
- Separating funnel, needles & syringes.
- Measuring cylinder, magnetic beads and spatula.
- Vacuum pump, TLC (Thin layer Chromatography) plate.
- Preparative TLC plate and TLC chamber.
- UV (ultra violet) light chamber.
- Face mask and hand gloves.
- Ball pen, HB pencil and lab note book etc.