UNIVERSITY OF NAIROBI

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF BIOCHEMISTRY

PRACTICAL MANUAL FOR BACHELOR OF SCIENCE IN BIOCHEMISTRY (BSC III)

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PRACTICAL ONE

FRACTIONATION OF LIVER HOMOGENATES BY DIFFERENTIAL CENTRIFUGATION

The following fractionation procedure in 0.25 M sucrose is essentially similar to that developed by de Duve et al. (1955) in connection with the characterization of lysosomes and peroxisomes. It is roughly equivalent to the classical procedure designed by Hogenboom and Schneider, except for the isolation of the additional mitochondrial fraction (L for light mitochondrial fraction) in which lysosomes and peroxisomes are better purified.

The liver homogenate is fractionated by successive sedimentation of the N, M, L and P fractions according to scheme 1: Note that each fraction is washed and fractionating is quantitative since washings are combined with supernatants. The volume of the resuspended fractions is carefully adjusted in such a way that the concentration of each fraction is known: These concentrations are expressed by the reciprocal of the volume in which is suspended the material from 1g of fresh liver. It is important to keep in mind that the results obtained with a given fractionation depend almost as much on the way supernatants are decanted as on the exact values of g min. It should be stressed again that adherence to this fractionation scheme does not guarantee that comparable results will be obtained with other tissues, or with pathological livers. The manner in which subcellular particles are distributed between the fractions depends on numerous factors, it is often best revealed by the distribution patterns of reference enzymes of known intracellular location.

The following selected marker molecules are used: DNA (nuclei), glutamate dehydrogenase (Mitochondrial matrix), cytochrome oxidase (Mitochondrial inner membrane), acid β-glycerophosphatase (lysosomes) urate oxidase (peroxisomes) and glucose-6-phosphatase (E.R membranes).

Experimental procedure

Preparation of the liver

Rats, fasted overnight are sacrificed by decapitation and allowed to bleed in the sink for one to two minutes. The liver is removed and immediately placed in a beaker of ice-cold 0.25 M sucrose solution. The weight of the liver is usually 7-10g per 200g rat. In addition, in all the

subsequent operations, great care must be taken to keep the tissue cold and to avoid contact of the tissue with tap water. In the cold room the liver is blotted on paper towels and adhering fat and fibrous tissue is removed. The liver is rinsed once with cold sucrose solution and blotted.

Homogenisation and isolation of the nuclear fraction (N) and of the cytoplasmic extract (E).

Ten grams of the liver are chopped up with scissors and homogenised (Teflon pestle) with about three volume 0.25 M sucrose. The Teflon pestle rotating at about 800 rpm, is pushed through only once: the crude homogenate (about 40 ml) is centrifuged at 2000 rpm (600 g) for 10 min. The supernatant is decanted by simple pouring. The pellet is re-homogenised with 30ml sucrose and centrifuged and washings are combined with the first supernatant. The final pellet, esuspended in sucrose, is the N fraction. It is brought to a concentration of 1/5. The combined supernatants and washings are brought to a volume of 10 ml per g of liver and thoroughly mixed; this is the 'cytoplasmic extract' E, concentration l/10

Isolation of the 'heavy' mitochondrial fraction (M).

60 ml of E (containing the equivalent of 6 g of liver) is distributed in two centrifuge tubes and spun at 5,500 rpm (3,500 xg) for 10 min. The supernatant is removed by means of a pipette with a bent tip, great care being taken to avoid touching the loosely packed material above the pellet.

The pellets are resuspended with a glass rod and diluted to a total volume of about 20ml in 1 tube and spun as before. The supernatant is combined with the first supernatant and mixed thoroughly; this total volume is recorded. The total pellets are resuspended in a small volume of fluid using a rotating Teflon pestle which fits loosely into the centrifuge tube: the suspension is brought to 12 ml to obtain a concentration of ½ and constitutes the M fraction.

Isolation of the 'light mitochondrial' fraction (L)

The combined supernatants of the previous centrifugations are centrifuged at 12,000 (8,000xg) for 15 min. The supernatant is decanted including as much as possible of the pink fluffy layer (consisting mostly of microsomes), which cover the pellet. It is difficult to remove the bulk of the fluffy layer at this stage.

The pellets are resuspended with a glass rod, diluted to about 20 ml and the suspension is centrifuged as before. The pink fluffy layer is now clearly visible above the pellet and can easily be removed

with the supernatant. The pellet is resuspended with the tetlon pestle and the suspension is brought to 12 ml to obtain the L fraction. The supernatant and washings are combined.

Isolation of the microsomal fraction (P) and the particle-free supernatant (S)

Using the Beckman Ultracentrifuge the combined supernatants are centrifuged at 25,000 rpm (80,000g) for 30 min. The supernatant is decanted very carefully to avoid touching the loosely packed microsomes. The pellets is resuspended with the teflon pestle and the suspension is brought to 24 ml to obtain P fraction (concentration 1/4). The supernatant is the S fraction. The volume is recorded or adjusted and the concentration is computed.

Results for protein and marker enzymes.

For each marker,

1. First calculate the concentration in the fraction (units/ ml for enzymes 100µg/ ml for DNA mg/ml for protein); then calculate the amount equivalent of 1g of liver (units /g etc).

2. The sum H = E + N being taken as the activity in the unfractionated homogenate (i.e. 100%), and the sum N+M+L+P+S as the total recovered activity compute the recovery and express it in percentage.

3. Calculate for protein, for DNA and for each enzyme the amount recovered in each fraction as a percentage of the original total activity (E+N). Tabulate the results.

4. Calculate the Relative Specific Activity of each enzyme (and of DNA) in each fraction. To do this, divide the percentage of recovered marker by the percentage of recovered protein in the same fraction. Again, tabulate your results.
5. Draw distribution histograms for each marker, using blocks with height = RSA and base = % recovered proteins (de Duve diagrams)

Assay procedures

1. DNA estimation

The method for DNA estimation is based on colour reaction between deoxyribose and diphenylamine (Burton, K; Biochem. J, 62, 315, 1956)

Reagents

- Standard DNA (from calf thymus), 100 µg/ml
- Diphenylamine Reagent. Dissolve 1.5g recrystallize diphenylamine from petroleum. Ether in 100ml Analar acetic acid and add 1.5 ml conc. H2S04. Prepare fresh or store in a dark bottle. Before use, add 0.1ml acetaldehyde (16mg/ml) to every 20ml of reagent. Dilute acetaldehyde (1.5ml) before use in water
- 4% PCA

Procedure

1. Make aliquots of each fraction into 1ml with distilled, water and add 1 ml ice – cold 4% PCA. Stand in ice for 10 min then centrifuge and wash the precipitate three times with ice cold 2% PCA.
2. Disperse the precipitate in 1 ml 4% PCA.
3. Stand in a water bath at 70^0C for 25 min then cool in ice and centrifuge.
4. Transfer the supernatant carefully with a pasteur pipette into acid washed tubes.
5. Add 2 ml diphenylamine-acetaldehyde reagent.
6. Stand the tubes for 16 h (Overnight) at 28°C in the dark.
7. Read the colour developed at 600 nm and by reference to a standard
8. Calculate the concentration of DNA in each fraction.

2. Lactate dehydrogenase

Reagents

- Phosphate buffer, pH 7.4, 0. 1 M
- NADH, 2.5mg /ml
- Pyruvate, 2.5 mg/ml

Procedure

- To 2.7 ml phosphate buffer in a spectronic tube add 0.1 ml NADH and a suitable aliquot of each fraction.
- Mix well, wait 2 min and check initial O.D at 340 nm.
- Add 0.2 ml pyruvate and take further readings at 30-sec. intervals for 5 min.
- Calculate the initial change in O.D per minute, and hence the enzyme activity as units/ml (i.e. µmoles/min/ml).

3. Glutamate Dehydrogenase

Reagents

- Phosphate buffer pH 7.4 0.lM
- NADH 2 .5 mg/ml
- ADP 0.05M (neutralize)
- Ammonium chloride 0.5 M
- α-Ketoglutarate 0.2 M (neutralize)
- 2% (w/v) Tritron X-100

Procedure

1. To 2.6 ml phosphate buffer in spectronic tube add 0.1 ml NADH, 0.05 ml ADP 0.2 ml ammomium chloride and a suitable aliquot of each fraction.
2. Add 0.1 ml Triton, mix well wait 2 min and Check initial O.D at 340 nm.
3. Add 0.l ml α-ketoglutarate and take readings at 30 sec interval for a further five minutes.
4. Calculate the initial change in O.D per min and hence the enzyme activity as units/ml.

4. Assay of cytochrome oxidase

Cytochrome oxidase can be determined by several procedures. The polarographic procedure described below is rapid and convenient.

The consumption of oxygen is measured with a clark electrode at room temperature (about 23°C) in an assay system containing in a final volume of 2 ml, 0.033 M phosphate buffer, pH 7.4, 0.07 mM cytochrome C, 0.0114 M Na-ascorbate and 0.1 mM TMRD. A blank with ascorbate alone must run since ascorbate may undergo some auto-oxidation. Cytochrome oxidase activity will be derived graphically from the trace obtained on any strip chart recorder and will be expressed in atoms of oxygen consumed per mg of protein per min. The solution of ascorbate must be prepared immediately before use by adding 1 ml of 0.1N NaOH to 20 mg, of ascorbic acid.

5. Acid phosphatase

Reagents

- 0.5 M Sodium β-glycerophosphate adjusted to pH 5 with dilute HCL

- 1M sodium acetate-acetic acid buffer pH 5
- 1M Sucrose
- 8% (w/v) TCA
- 2% (w/v) Triton
- Reagents for phosphate determination

Procedure

1. The 2 ml assay system comprise 0.2 ml β-glycerophosphate, 0.1 ml acetate buffer 0.25 ml sucrose solution, 0.35 ml water, 0.1 ml Triton and 1.0ml enzyme.
2. The assay is started by adding the enzyme to the test tube containing the substrates and previously equilibrated at 37°C.
3. The tubes are incubated for 10min at 37°C after which the reaction is stopped by addition of 10ml TCA.
4. After centrifugation, inorganic phosphate is determined in a 1 ml supernatant sample (see below).

Blanks are run by incubating the enzyme with all components of the assay mixture except β-glycerophosphate which is added after the TCA. One unit of enzyme catalyses the hydrolysis of 1μmole of substrate per min.

6. Glucose 6-phosphatase

Reagents

- Citrate buffer 0.1 M pH 6.5
- EDTA 0.01 pH 6.5
- Glucose -6- phosphate 0.08M pH 6.5
- TCA 8% w/v
- Reagents for determination

Procedure

1. The total volume in the assay is 1ml made of 0.25ml of glucose -6- phosphate solution, 0.05 ml of EDTA solution, 0.6 ml of citrate buffer and 0.1ml of the enzyme preparation. [undiluted]
2. After a 30 minutes incubation at 37°C the reaction is stopped by adding 5 ml of TCA.
3. The denatured proteins are removed by centrifugation and inorganic phosphate is determined

on a l ml sample of the filtrate.

4. Blanks are run by incubating the enzyme with all the components of the assay mixture except glucose-6-phosphate. One unit of glucose -6- phosphatase is the amount of enzyme which catalyses the hydrolysis of 1 μmole of substrate per minute.

7. Urate oxidase:

The rate of the reaction is measured by following the disappearance of urate spectrophotometrically at 292 nm.

Reagents

- 0.125 mM sodium urate, prepared by diluting l00-fold a fresh solution and dissolving 210mg of uric acid in 100ml.
- Enzyme diluent containing: 1.25 mM EDTA, 1.25 mg/ml Triton X-100, 6.5mM sodium phosphate buffer pH 7.4

Procedure

1. The two reagents described above are kept in a water-bath at 37°C, 1 ml of the enzyme preparation is mixed with 4ml of the enzyme diluent, 2 ml of this mixture are pipetted in each of two 1 cm spectrophotometric cuvettes (quartz). 1 ml of urate solution is added to the first (test), 1ml of water is added to the other blank.
2. The disappearance of urate is followed during the next 12 minutes by recording the O.D at 292nm of the rest cuvette, taking the blank as zero OD
3. The molar extinction coefficient of urate at 292 nm is 1.22 x 10 cm^{-1} M.
4. Calculate the activity of urate oxidase, in each fraction. One unit of urate oxidase is defined as the amount of enzyme which catalyses the oxidation of 1 μmole of urate per minute.

8. Protein determination bv the Falin procedure (Lowry, et al., Biochem L 193. 265. 951

Reagents

- 1% $CuSO_4$ 5 H 2 0
- 2% Na-K tartrate
- 2% Na_2CO_3 in 0. 1 M NaOH
- 0.5 ml A+ 0.5 ml B + 49 ml C (prepared before use)

- Folin - Ciocalteu reagent diluted 1.1 ml with water
- Standard protein (BSA, 1 mg/ml)

Procedure

1. Make a suitable aliquot of each sample up to 0.1ml with water, add 3.0 ml Reagent D. Mix, wait for 10 minutes. Add 0.3 ml reagent E mix, wait at least 30 min. read at OD at 750 nm
2. Treat a blank (0.1 ml water) and two standards (0.05 ml standard protein+ 0.05 ml water) in the same way as the unknowns.

9. Inorganic phosphate determination *(for* acid phosphatase and Glucose-6-phosphatase assavs)

To 1ml of each TCA supernatant, to 1ml 0.5mM, standard phosphate add to 1 ml water (the blank) Add in succession, 1 ml acid molybdate, 1 ml 20% Na_2S0_3 and 0.5% hydroquinone. Mix the tube contents immediately after each addition and read the O.D in the spectronic at 660 nm after 30-60 minutes against the blank. Try read all tubes (exp. and standard) at approximately the same time after addition of Hydroquinone.

PRACTICAL TWO

LIPIDS - ISOLATION OF A PHOSPHOLIPID

Lipids are an important class of biochemical compounds which fulfil several functions, including energy storage (e.g. triglyceride) and membrane structure (e.g. cholesterol and phosphatidyl choline, also called lecithin). The basic structure of the major class of phospholipids, glycerides, is a glycerol molecule to which are attached fatty acids at carbon 1 and 2. The hydroxyl group on carbon 3 may be free (e.g. diacylglyceride) or esterified with fatty acids (e.g. triacylglycerides) or another organic group (e.g. phosphorylcholine group of lecithin). Depending on the nature of the substituent at carbon 3, a lipid may either be polar (e.g. lecithin) or non-polar (e.g. triacylglycerides). This difference is exploited in the resolution of lipid mixtures by thin layer chromatography (TLC). Spots of lipids are revealed on the TLC plate by incubation with iodine vapour. The iodine molecules bind reversibly to double bonds of unsaturated fatty acids. Such fatty acids are often substituted at carbon 2.

You will extract lecithin from egg yolk using an organic solvent and purify it by precipitation with acetone. Egg yolk also contains triacylgyceride, sphingomyelin and cholesterol; however, sphingomyelin is insoluble in ether and cholesterol is not precipitated by ether and acetone. The degree of purity of the lecithin can be estimated by measuring its phosphate content, since one mole of phosphate would be derive from one mole pure lecithin. Alternatively, contaminating lipids may be resolved and identified by TLC. In the following practical, you will use your purified lecithin to study the action of the enzyme phospholipase A.

Reagents and materials

- Egg yolk (previously weighed)
- Ether (15 ml)
- Ethanol – ether mixture (2:1, v/v) (100 ml)
- Acetone (30 ml)
- 5 M sulphuric acid
- Ammonium molybdate reagent
- Standard phosphate solution (1.0 micromol/ml) – NaH_2PO_4
- 0.5 M trichloroacetic acid (TCA)
- 10% Na_2SO_3
- 0.5% hydroquinone

Procedure

1. Crush the egg yolk with a glass rod and mix with ethanol-ether (75ml). Allow to stand for about 10 min, stirring occasionally.
2. Filter through a fluted filter paper moistened with ethanol-ether. Remove the residue from the paper, mix well with another portion of ethanol-ether (20ml) and filter. Combine the two filtrates and discard the residue.
3. Take a sample (1 ml) of combine filtrates and save it for analysis in the next practical. Evaporate the remainder to dryness on a hot plate.
4. While waiting, **prepare in duplicate** a range of phosphate concentrations at suitable intervals between 0 and 1.0 ml of standard phosphate solution (0 - 1.0 μM). Make each volume up to 1.0 ml with water. To each tube add 0.5 M TCA (2.0 ml).
5. Dissolve the residue from step 3 in ether (10 ml). With continual stirring, slowly pour the solution into acetone (30 ml) and continue stirring until the particles of precipitated lecithin stick together.
6. Collect the precipitate by filtration and dry it between filter paper. Weigh the lecithin in a tared paper and record the yield. Weigh the filter paper.
7. Weigh 10 mg lecithin into a sample bottle. Add 5 M sulphuric acid (0.5 ml) and incubate the bottle in a boiling water bath to digest the lecithin.
8. Save the remaining lecithin for the next experiment (**Do not discard the remaining lecithin**).
9. During this time finish doing the standard assay procedure as follow. To the tubes containing phosphate solution and acid, add ammonium molybdate reagent (1.0 mL) to each and after mixing well, incubate in the dark for 20 – 30 mins. Read the absorbance at 600 nm.
10. After the digestion of lecithin is complete, allow the bottle to cool before adding slowly 3 mL water (CARE). Heat this diluted solution for about 10 min in a boiling water bath to hydrolyse any pyrophosphate formed during the digestion. Filter the solution through a moist filter paper. Make the volume up to 25 mL with water.
11. Determine the total phosphate content of the solution by taking a sample (1.0 mL) and applying the same procedure as for the phosphate standard assay method.
12. Draw the standard curve for phosphate, plotting micromole of phosphate on the X-axis. Calculate the % content of lecithin in the egg yolk and the % purity of your lecithin sample> keep your lecithin preparation and the egg yolk solution for the next practical.

Discussion:

Comment on your results and the purity of your lecithin sample. Incorporate the results of the next practical in your discussion.

PRACTICAL THREE

LIPIDS – CHARACTERISATION OF PHOSPHOLIPIDS

Higher animals breakdown ingested lipids in the small intestines by the action of hydrolytic enzymes (lipases) present in the secretion of the pancreas. One such enzyme, phospholipase A, catalyses the conversion of lecithin (phosphatidyl choline) and other phospholipids (phosphatides) to fatty acid residue from carbon 2 position. Fatty acids from this position are generally saturated. The source of the phospholipase in this experiment is an extract of pancreas (pancreatin).

Since lecithin is almost insoluble in water and therefore unavailable to the enzyme, its effective concentration is increased by preparation of a lecithin emulsion using an inorganic solvent. The emulsion also contains calcium ions (to activate the enzyme and precipitate the fatty acid as it is formed) and a buffer at pH 8.0 (to maintain optimal activity of phospholipase). The products of the enzymatic reaction are analysed by thin layer chromatography (TLC) in two different solvents: Solvent A separates only polar lipids whereas Solvent B separates only non-polar lipids.

Reagents and materials

- Lecithin (from the previous practical). Lipid sample should be made fresh before application
- Egg yolk solution (from previous practical)
- Lysolecithin (marker lipid, 10 mg.ml in methanol)
- Oleic acid (marker lipid, 5 mg/ml in chloform)
- Chloroform-methanol solution (3:1, v/v)
- Pancreas solution (50 mg/ml in 0.2 M borate buffer, pH 8.0 containing 20mM $CaCl_2$
- Sodium tetraboraye adjust the pH, in Boric acid or HCl
- Capillary tubing
- Sample bottles
- Tank with iodine vapour
- Solvent A
    - Chloroform: methanol: acetic acid: water (65:25:8:4)
- Solvent B
    - Hexane: diethyl ether: glacial acetic acid (80:20:1 by volume)

- TLC plates coated with silica gel G.

Procedure

1. Weigh out 30 mg of the lecithin preparation from the previous experiment into a sample.
2. Dissolve the lecithin in chloroform-methanol solution (1.0 mL).
3. Using the capillary tubing apply two or three drops of the lecithin solution to each of the two TLC plates, allowing each spot to dry before applying the next. Apply the sample at a point about 2 cm from the bottom of the plate and about 2 cm in from the right-hand side.
4. To the bulk of the lecithin solution in the sample bottle, add pancreatin solution (1.0 mL). Close the bottle with its stopper and shake the bottle well to form a creamy emulsion. Enclose the bottle in your fist and continue shaking.
5. After 15 min remove a sample from the incubation mixture and apply it as a spot at a point 2 cm in from the left-hand side of each TLC plate. Try to apply the same size sample as that of the lecithin solution.
6. Repeat step 4 after 30 minutes, placing the sample about 3 cm away from the 15 min sample.
7. During the incubation period, apply samples of egg yolk solution, lysolecithin solution and oleic acid solution to each TLC plate. The spots should be approximately 3 cm apart. Try to apply the same size sample of each and keep the sample spots as small as possible.
8. Separate the non-polar lipids by chromatography of one of the plates in Solvent B and the polar lipids in Solvent A. This should take 30 min – 1 h.
9. When solvent B has risen to within a few centimeters to the top of the TLC plate, remove the plate and mark the solvent front.
10. Allow the solvent to evaporate from the plate.
11. Stain the lipids by placing the plate for 20 min in a tank with iodine vapour.
12. Mark the position of each spot as quickly as possible after removing the plate from the iodine vapour, the stain gradually fades.
    - Calculate the Rf value for each component and try and identify them following the Rf values:
    - Cholesterol ether: 0.8 – 0.9
    - Triacylglyceride: 0.4 – 0.6
    - Fatty acid: 0.1. – 0.3
    - Cholesterol: 0.05
    - Phospholipid: 0
13. Repeat steps 9 and 10 for the polar lipids on plate A

Discussion

Comment on your results, indicating whether they agree with the mode of action of phospholipase A proposed in the introduction. Include the TLC results for your samples of egg yolk and lecithin in the discussion of the previous experiment

Questions

1. Why is lysolecithin only weakly stained with iodine vapour, whereas oleic acid of similar concentration is heavily stained?
2. Why is it important to have the incubation mixture well buffered
3. Lysolecithin is a powerful detergent and it is a product of the reaction. Would you expect it to stimulate or inhibit the enzymatic reaction, and why?
4. What is the natural detergent that emulsifies the lipids in the human intestines and where is it produced?

PRACTICAL FOUR

THE EFFECT OF DIET ON RAT LIVER ENZYMES

Levels of a number of cytoplasmic enzymes are highly sensitive to alterations of dietary or endocrine state. You will assay the activity of four differently fed rats. You will assay the blood glucose levels.

The four rats have received the following treatments;

1. Maintained on normal pellet diet
2. Fasted for three days before being killed
3. Fed for ten days on meat (high protein diet)
4. Fasted for three days, then re-fed on high sucrose diet for four days

In addition to vital salts and vitamin, the normal pellet diet consists roughly of:

- Carbohydrates 70% (mainly starch)
- Protein 20%
- Lipid l0%

The high sucrose diet contained

- Carbohydrate 70% (35% sucrose and 35% starch)
- Protein 20%
- Lipid 10%

The meat diet contained, as a percentage of dry wt

- Protein 80%
- Lipid 15%
- Carbohydrates < 2% (less than 2%)

ENZYMES ASSAYS

Fresh liver is homogenised with 9 volumes of 0.154 M KCL (isotonic media). The bulk of the homogenate is centrifuged for 10 min. at 15,000g. The supernatant, which contains all the cytoplasmic enzymes, is used in the first three assays described below; these are all spectrophotometric procedures involving NAD and NADP-linked systems. For glucose-6-phosphate assay the whole homogenate is used.

(i) Malic enzyme,

This enzyme catalyses the reduction of cytoplasmic $NADP^+$, as follows;

$$\text{Malate} + \text{NADP}^+ \xrightarrow[\text{Mg}^{2+}]{\text{MDH}} \text{Pyruvate} + CO_2 + \text{NADPH} + H^+$$

The enzyme is associated with lipogenesis.

Procedure

In a spectronic tube add

Reagent	**Volume in mls**
0.2 M Tris HCl, pH 7.4	1.0
0.1 M $MgCl_2$	0.1
$NADP^+$ (5mg/ml)	0.5
Enzyme (extract)	0.15
Water	
Total volume	

Make up the volume to 2.9 ml with water, mix and leave in the spectrophotometer to warm up for 2 mins.

Measure any increase in OD at 340 nm occurring before addition of substrate

Add 0.1 ml of 0.1 M DL-malate, mix and read the OD at 340 nm at half minute intervals for 5 minutes.

Question

From the rates of increase of OD corrected for any blank, calculate the activity of the enzyme in the livers as µmoles/min/g weight

ii) Citrate cleavage enzyme (citrate lyase)

This is another enzyme which is involved in lipogenesis. It splits citrate, in the presence of ATP and CoA, to acetyl-CoA and oxaloacetate. In the assay system used, the oxaloacetate formed oxidises $NADH + H^+$ in the presence of the added excess MDH.

$$\text{Citrate} + \text{ATP} + \text{CoASH} \xrightarrow[\text{Mg}^{2+}]{\text{Citrate lyase}} \text{Acetyl-CoA} + \text{Oxaloacetate} \xrightarrow[\text{MDH}]{\text{NAD}^+} \text{Malate}$$

Into a spectronic tube add the following reagents

Reagent	Volume
0.2 M Tris HCl, pH 7.4	1.0 ml
0.1 M Magnesium chloride	0.1 ml
Citrate, 0.1 M	0.5 ml
GSH, 0.01 M	100 µl
CoASH (5 mg/ml)	25 µl
NADH (2.5 mg/ml)	100 µl
MDH	5 µl
Enzyme (extract)	0.15 ml
Water	
Total volume	

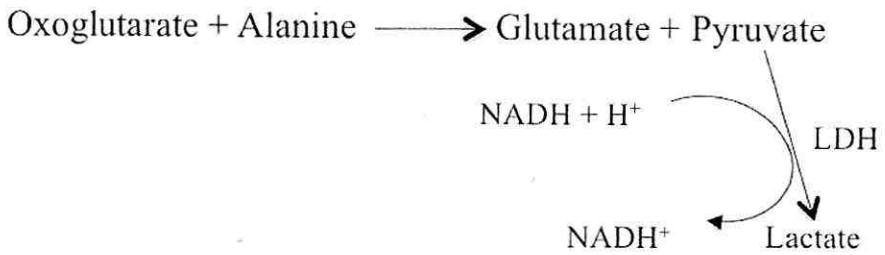
Make up the volume to 2.9 ml with water, mix and allow the tube to warm up in the spectrophotometer for 2 min. Measure any slow "blank" rate OD increase at 340 nm.
Add 0.1 ml 0.05 M ATP and record the OD at one-minute intervals for 5 min (every 30 seconds, in extracts from the liver of the sucrose re-fed rat).

Question

Correct for the blank rate and calculate enzyme activity as µmoles/min/g wet weight of liver.

(iii) Glutamate pyruvate transaminase (GPT)

This enzyme is assayed in the direction of pyruvate formation, the pyruvate formed being trapped by reduction, in the presence of excess LDH, with corresponding oxidation of NADH.



Mix in a spectronic tube

- GPT buffer (0.1 M phosphate, pH 7.5, containing 0.2 alanine)

- NADH (5 mg/ml) 0.1ml
- LDH 5 µl
- Enzyme (high speed supernatant from liver diluted x l0 water) 0.05ml

After allowing at least 2 min. to warm up in the spectronic read OD at 340nm and note any 'blank' rate of O.D. decrease.

Add 0.1 ml 2 -oxoglutarate (0.2 M), mix, read OD at half-minute intervals and determine the new rate of O.D. decrease.

Question

From the OD/min, corrected for blank if necessary, calculate the GPT activity of the liver as µMoles/min./g wet weight.

PRACTICAL FIVE

TO DETERMINE THE EFFECT OF β-HYDROXY BUTYRATE ON GLUCONEOGENESIS IN RAT KIDNEY CORTEX SLICES

The kidney cortex, like the liver, has high gluconeogenic activity. For studies with incubated slices, this tissue has certain advantages over liver. It does not accumulate glycogen; therefore, the carbohydrate produced from some precursor such as lactate all appears in the medium as glucose, and the complication of having to check glycogen levels in the slices before and after incubation is avoided. Moreover, it is easier to cut thin, firm slices from kidney cortex than from the liver.

In this experiment kidney cortex slices will be incubated in phosphate buffered saline medium gassed with oxygen. The rate of production of glucose from 2.5 mM lactate will be determined with and without the addition of 0.2M. β- Hydroxytutyrate.

Procedure: Individual incubation experiment will be carried out by groups of six students

Kidney cortex slices are prepared from rats that have been fed a high protein diet for three days. The slices are washed and placed in ice-cold krebs-Ringer phosphate buffered saline medium. For each incubation experiment about 50 mg slices are placed in 3.9 ml krebs-Ringer medium in 25ml flasks.

Prepare four flasks for addition of slices as follows. (vol. in mls)

	1	2	3	4
Krebs phosphate buffered medium	3.9	3.9	-	-
Phosphate krebs/medium containing 2.5 mM DL-Lactate	-	-	3.9	3.9
DL- β-hydroxybutyrate (0.2M)	-	0.1	-	0.1
Water	0.1	-	0.1	-
Total volume				

1. Place the flasks in an ice-bath.
2. Quickly blot and weigh the slices then add them to the flasks.
3. Gas each flask with oxygen for 1 min, seal it and then incubate with vigorous shaking at 40° C for one hour.
4. At the end of the incubations, cool the flasks in an ice-bath, then collect the medium from the slices.

5. To 3.0 ml of each medium, add 0.3ml - PCA (23% w/v) add 3N-PCA (23% w/v) wait for ten minutes and then centrifuge.

Assay for glucose and lactate in each medium

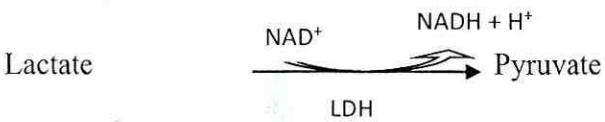
These assays will be carried out in duplicate

Assay of glucose

1. To I ml of each PCA supernatant add 3.0 ml glucose reagent.
2. Also, add 3.0ml reagent to 1ml water and to 0.2 ml standard glucose (0.1 mg/ml) made up to l ml with water.
3. Incubate at room temperature for 40-60min. and read the optical density of the experimental samples against the water blank at 436nm (note the incubation time).

Assay of lactate

You will need to assay the original content of lactate containing incubation medium, as well as the residual lactate content of the medium in flasks 3 and 4 after incubation. Before carrying out the lactate assay on the original medium, first add 3ml to 0.3 ml 3N-PCA



Make up, in test tubes a test system for each medium to be assayed together with one blank, as follows:

	Blank	**Test**
0.5 ml Glycine, pH 9.0, containing 0.4M hydrazine	2.9 ml	2.9 ml
PCA supernatant	-	0.1 ml
PCA (2%)	0.1 ml	-
LDH	5µl	5µl
NAD^+ (20 mM)	0.2 ml	0.2 ml
Total volume		

Incubate the tubes at 25°C for one hour. Then read the OD at 340nm - run against the blank. The reading gives the NAD+ reduction corresponding to lactate.

Questions

1. Determine the dilution factors for each assay
2. Calculate, for each incubation, the rate of glucose production and the rate of lactate utilization as μmoles per g wet weight of tissue per hour.

Notes on the assay of glucose using glucose oxidase.

The glucose oxide reagent contains, per ml, 2.5 mg crude glucose oxides, 30 μg peroxidase and 50 μg dianisidine, in 0.5 M phosphate buffer, pH 7.0

D-glucose in the sample (0 - 40 μg) reacts quantitatively with oxgen in a reaction catalyzed by oxides

$$\text{D-Glucose} + O_2 \longrightarrow \text{D-gluconolactone} + H_2O_2$$

Hydrogen peroxide then reacts with dianisidine (DH2), oxidising it to a brown coloured compound (D) The enzyme is peroxidase

$$\text{D-Glucose} + DH_2 \longrightarrow D + H_2O$$

Notes on the assay of L-Lactate using LDH

L-lactate is made to react quantitatively with NAD+, and reduction to NADH is recorded at 340 nm . The equilibrium position of the reaction

$$\text{Pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{Lactate} + NAD^+$$

To encourage it to go more rapidly to the left, a high pH buffer (Low H^+) is used in the assay. Moreover, we included hydrazine in the system, this compound reacts with pyruvate to form a hydrazone and thus acts as a trap.

PRACTICAL SIX

TO DETERMINE THE EFFECT OF INSULIN ON GLUCOSE UPTAKE BY ISOLATED RAT MUSCLE

A convenient muscle to use in this type of study is the diaphragm, which is of a convenient size yet thin enough to present little diffusion problem when incubated in a buffered salt medium, gassed with oxygen and containing appropriate substrates. The two latero-ventral areas of the muscle are used. Thus, from the same animal, practically identical 'hemidiaphragms' can be obtained for use in experimental and control incubations.

Procedure

Diaphragms are removed from 20 hr starved rats immediately after death, and washed in ice-cold saline. Hemidiaphragms are then carefully dissected and placed in ice-cold Krebs-Ringer Bicarbonate buffer, previously gassed with O_2/5% CO_2 and containing glucose (1.5 mg/ml)

To a 25 ml conical flask (control) add 1.0ml ice cold glucose bicarbonate medium. To a second flask add 1.0 ml of the same medium, also containing insulin (0.1 U/ml). Quickly blot and weigh the hemidiaphragms and place one in each flask. Gas both again with O_2/5% CO_2 for 1 min seal then incubate, with shaking, 40°C for 45 min. After incubation transfer both flasks to ice-water, quickly remove 0.5 ml medium from each and dilute to 5.0 ml with 3% perchloric acid. Take also 0.5 ml of the original glucose-containing bicarbonate medium, and dilute to 5.0 ml with 3% perchloric acid.

Determine glucose concentration in each of the PCA-treated sample as follows

1. To 0.2 ml of each PCA-treated sample add 3.0 ml glucose reagent.
2. Also add glucose reagent to 0.2 ml standard glucose (0.1 mg/ml)
3. Prepare a blank containing 0.2 ml water and 3.0 ml glucose reagent.
4. After 40-60 Min read optical densities against the blank at 436nm.
5. Perform all glucose assays in duplicate.

Questions

1. Determine the dilution factor for each assay
2. Calculate the glucose concentration of the medium in each flask before and after incubation
3. Calculate the rate of glucose taken up by each muscle as mg/g wet weight/h.

PRACTICAL SEVEN

ASSAY OF METABOLITES IN PERFUSED RAT HEART

To assay tissue metabolites levels it is imperative to arrest metabolism almost instantaneously, and fix these levels. Stress such as oxygen lack for example could cause changes in the level of a metabolite such as ATP in 1-2 sec or less.

The tissue to be assayed is therefore frozen rapidly, by immersion in acetone cooled with solid CO_2, or better still by freezing between metal tongs cooled to the temperature of liquid nitrogen. The frozen tissue is powdered whilst still frozen, then extracted with ice-cold percloric acid, in which most metabolites are soluble. After centrifuging, the extract is neutralized with KOH which precipitates perchlorate as the insoluble potassium salt. The neutral extract can then be used in enzymatic assays

Procedure

Hearts are perfused at 35°C through the coronary circulation by drip-through with krebs' bicarbonate Buffered medium containing glucose (1 mg/ml), insulin (0.05 U/ml) with or without β-OH Butyrate (5 mM). After 10 min. the heart is rapidly frozen by immersion in acetone/solid CO_2:

The frozen heart is placed in a mortar precooled with liquid nitrogen covered with more nitrogen and ground rapidly to a fine powder. This can be kept temporarily on solid CO_2 if necessary. The powder is then weighed and transferred to a known volume of ice-cold 3% percloric acid (about 3 ml per heart) Stirred rapidly with a fine glass rod and centrifuged.

The supernatants are neutralised carefully to pH 6.5-pH 7.0 with N-KOH, after adding 0.1 ml 0.4 M Phosphate to give some buffering capacity and prevent 'overshooting'. The exact volume of KOH used is recorded.

You are provided with neutralised PCA extracts for the assay of

i) citrate
ii) acetyl-camitine
iii) glucose-6-phosphate
iv) ATP

Metabolite assays to be performed using the Zeiss or the unicam SP 1800 UV spectrophotometer.

Citrate to cuvette add	
Triethanolamine buffer	(0.1ml, pH 7.6, cont. 10mg)
$ZnS0_4$ per 100 ml)	1.0ml
NADH (5 mg/ml)	50 µl
Extract	0.3ml
MDH	2µl
Water to	2ml

Record the O.D 340 mix in µ citrate lyase, until the O.D is again constant (a few min.) read again and record the O.D change that is due to citrate in the · extract sample.

The assay involves the following reactions

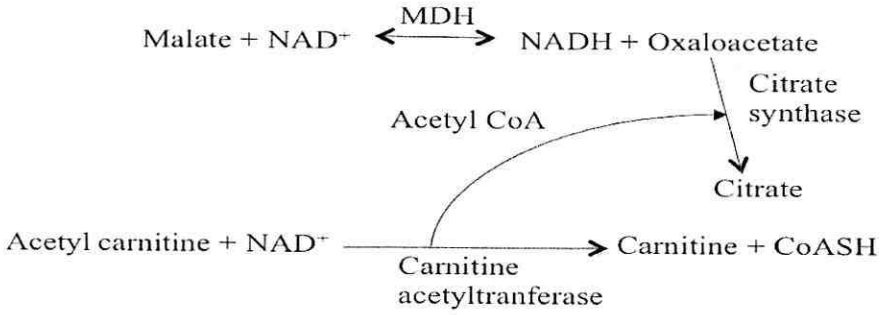
Acetyl carnitine

To a cuvette add:

Tris HCL (0.1 m, pH 7.8 containing 1mM EDTA)	0.2ml
Malate (1.0M)	25µl
NAD (10 mg/ml)	50µl
CoA.SH (5 mg/ml)	25µl
Extract	1.0ml

1. Record the O.D. (E1)
2. Mix in 5µl MDH and when the O.D is constant again take the new reading (E2) the reaction has now equilibrated.
3. Mix in 5µl of the citrate synthase/carnitine acetyl-transferase mixture. Wait until the O.D is again constant (about 5-l 0 min), and again take the reading (E3).
4. Record the O.D. change (E3- E2) which is due to the reaction of the acetyl carnitine. (Acetyl carnitine utilisation and NAD reduction are not quite stoichiometric to calculate acetyl carnitine content more precisely we would have to employ 1 correction factor involving (E2 - E 1)

The assay involves the following reactions:-



One mole of OAA will be removed from the MDH equilibrium for every mole of acetyl-carnitine present. This will be regenerated by the re-equilibrium of the MDH reaction with reduction of NAD.

Acetyl-CoA will also react in the system causing NAD reduction when synthase is added; however, heart muscle contains very little acetyl-CoA compared with acetyl carnitine so interference can be ignored.

Glucose -6- Phosphate

To a cuvette add:

Tris- HCL (pH 7.4, 0.5 M containing 25mM $MgCl_2$	
NADP (5 mg/ml)	0.4ml
Extract	50 µl
Water to 2 ml	

Read the O.D at 340 n then add 2 µl Glucose-6-phosphate dehydrogenase and record the increase in O.D.

PRACTICAL EIGHT

DETERMINATION OF SERUM TRANSAMINASES ACTIVITIES

Animal cells contain a variety of animal transferase. Aspartate aminotransferase (AST) is present in both the cytoplasm and mitochondria of cells. AST is also commonly referred to as glutamate aminotransferase (GOT). In conditions associated with mild degree of tissue injury, the predominant form serum is from the cytoplasm although some mitochondria enzyme activity is also present.

Both AST and alanine aminotransferase (ALT) are normally present in human plasma, bile, cerebrospinal fluid and saliva but one is found in urine unless a kidney lesion is present. The common name for ALT is glutamate pyruvate transaminase (OPT). In this practical session you will assay the activities of both AST and ALT in a serum specimen.

Glutamate pyruvate transaminase activity assay

L Alanine: 2 - Oxoglutarate (alpha-ketoglutarate) aminotransferase catalyses the reaction:

L-Alanine + 2-Oxoglutarate ⟶ L-Glutamate + Pyruvate

Reaction of the pyruvate with 2,4-dinitrophenylhydrazine produces an intensely coloured hydrazone on the addition of sodium hydroxide. Measurement of optical density at 505 nm provides a measure of enzyme activity when compared with a standard graph.

Reagents:

- GPT Buffered substrate pH 7.4, 2-oxoglutaric acid, 2 mM
- a-alanine 200 mm, Phosphate buffer 0.1 M
- 2, 4-Dinitrophenylhydrazine reagent (1 mM) in 1.0 N hydrochloric acid.
- Pyruvate standard (2.0 mM) in distilled water.
- Sodium hydroxide 0.4 M (carbonate free)
- Aniline citrate reagent: Dissolve 5 g citric acid AR in 5 ml Aniline AP.

Procedure

Blood specimens should be carefully without haemolysis. The serum may be stored for short periods at 4°C. Haemolysed serum should not be used.

Calibration graph

Prepare seven tubes as follows:

Tube No.	Water (mls)	Pyruvate (std) (mls)	Buffered substrate (mls)	International GPT units/L
1	0.2	0	1.0	0
2	0.2	0.05	0.95	6.5
3	0.2	0.10	0.90	12.5
4	0.2	0.15	0.85	21.0
5	0.2	0.20	0.80	30.5
6	0.2	0.25	0.75	39.5
7	0.2	0.30	0.70	56

Mix well and incubate at 37^0C for 30 minutes and to each tube add

1 ml 2,4-Dinitrophenylhydrazine reagent (l mM). Mix well and incubate for a further 20 minutes.

Add to each tube.

10 ml sodium hydroxide solution 0.4ml (free from carbonate)

Mix well and allow to stand for 10 min.

Read the optical density of each solution against test tube number one at 505 nm in 1 cm cuvette. *(International GPT unit is given in the above table. From the optical densities obtained, plot a calibration curve against GPT international units per litre. The calibration graph is not linear).*

	TEST	CONTROL
Buffered substrate	1.0 ml	1.0 ml
Serum	0.2 ml	0
Mix well, incubate at 37^0C for 30 min and add 2,4-Dinitrophenylhydrazine reagent	1.0 ml	1.0 ml
Serum	0 ml	0.2 ml

Mix well and incubate for a further 20 min.

Add 10 ml of 0.4 M NaOH mix well and allow to stand for 10 min. and read the optical density at

505 nm against water blank. Serum GPT level in normal subjects lie within the limits 2-15 i. μ/1

Use of test in Diagnosis

The increase in serum L-alanine: 2 -oxoglutarate aminotransferase (GPT) is considered a more sensitive indicator of hepatitis and liver cell damage than serum L-aspartate. 2-0xoglutarate aminotransferase (GOT) as the former enzyme is found in higher concentrations in liver tissue than in heart muscle. Furthermore unless GOT is considerably increased, GPT levels do not usually increase in myocardial infarction. Hence GOT and GPT values can assist in the differential diagnosis of cardiac and liver diseases.

Determination of serum L-aspartate aspartate aminotransferase (GOT)

The enzyme also called L-Aspartate: 2-oxoglutarate aminotransferase catalyses reaction

L-aspartate + 2-Oxoglutarate ⟶ L-glutamate + Oxaloacetate

The unstable oxaloacetate is quantitatively decarboxylated to pyruvate with aniline citrate reaction of pyruvate with 2,4-dinitrophenylhydrazine produces an intensely coloured hydrazone on the addition of sodium hydroxide solution: Measurement of optical density at 505 nm provide a measure of enzyme activity when compared with a standard graph

Reagents

- GOT buffered substrate pH 7.4
- 2mm α-oxoglutaric acid
- 200 mM DL_Aspartic acid
    - M Phosphate buffer
- 2, 4-Dinitrophenylhydrazine reagent (lmM) in 1.0 hydrochloric acid.
- Pyruvate standard (2.0 mM) in distilled water.
- 0.4 M Sodium hydroxide (Carbonate free)
- Alanine citrate reagent

Dissolve 5 g citric acid, analytical reagent grade in 5 ml distilled water and add 5 ml aniline, analytical reagent grade.

Calibration graph

Prepare seven tubes as follows

Tube No	Water (mls)	2 mM Std Pyruvate (mls)	Buffered substrate (mls)	International GOT units/L
1	0.2	0.1	1.0	0.0
2	0.2	0.05	0.95	6.0
3	0.2	0.10	0.90	13.0
4	0.2	0.15	0.85	22.0
5	0.2	0.20	0.80	30.5
6	0.2	0.25	0.75	39.5
7	0.2	0.30	0.70	51

1 Incubate at 37°C for 30 minutes

2 Into each tube add:

1.0 ml of 2,4-dinitrophenylhydrazine reagent (1mM) and incubate for a further 20 min, remove from water bath and to each tube add:

3 10.0 ml 0.4 M Sodium hydroxide. Mix well and allow to stand for 10 minutes.

4 Read the absorbance of each solution against test tube number one at 505 nm.

5 Subtract absorbance value obtained for tube 1 from the values obtained from the other tubes and from the absorbance obtained, plot a calibration curve.

Procedure

Serum or plasma should be separated from blood components. The serum may be stored for short periods at 4°C.

For each serum to be assayed prepare two tubes as follows:

	TEST	CONTROL
Buffered substrate. Place in water bath and leave for 3 min. to reach 37°C	1.0 mls	1.0 mls
Serum	0.2 mls	0.0 mls
Incubate at 37°C for 1 hr and add; Aniline Citrate reagent	1 drop	1 drop
Serum	0.0 mls	0.2 mls
Leave in water for 5 minutes then add 2.4 dinitrophenylhydrazine	1.0 mls	1.0 mls
Leave in the water bath for 15 minutes then remove from the bath and add 0.4M NaOH	10 mls	10 mls

Mix the contents of each tube and allow to stand for 10 minutes and read the absorbance at 505 nm against a water blank.

Serum GOT in normal subjects lie within the limits 4-20 IU per liter.

Use of test in diagnosis

The level of the enzyme L-aspartate: 2-oxoglutarate aminotransferase (GOT) increases significantly over the normal in myocardial infarction, hepatic necrosis and other diseases such as active cirrhosis. In a sample, the ratio of GOT to alkaline phosphatase is useful in the differential diagnosis of jaundice.

Questions

1 Write the reaction between pyruvate and 2, 4-dinitrophenylydrazine. Is the reaction specific?

2 What are the compounds in serum that could possibly interfere with the enzyme assay?

3 What are some of the experimental causes of

- False elevated activities of ALT and AST
- False decreased activities of ALT and AST

4 Explain the role of cofactors in the above two experiments

5 Why should haemolysed serum not be used for ALT and AST assay?

6 Explain briefly why the method you have used above is now no longer utilized in a diagnosis.

7 Give the principle of the more appropriate method.

8 Outline the clinical disease conditions that are associated with elevated levels of Serum transaminases

PRACTICAL NINE

DETERMINATION OF SERUM ALKALINE PHOSPHATASE AND ACID PHOSPHATASE

The phosphatases belong to the class of enzymes called hydrolases. They hydrolyse phosphoryl ester bonds, as well as compounds containing peptide, amide, hemiacetal and similar bonds. The phosphates have low specificity. Certain enzymes such as alkaline phosphatase will hydrolyse monoesters of phosphoric acid and metaphosphoric acid such as ATP. Clinically it has been possible to recognize three types of phosphatases. There are alkaline phosphatase (in serum, liver, bone, placenta and intestines with optimum pH of 10), acid phosphatase (in serum, prostrate and liver with optimum pH of 4 .9- 5 .0) and red blood cell phosphatase with optimum pH of 5.5 - 6.0. In this practical session you will assay the activities of both serum alkaline phosphatase and acid phosphatase.

Alkaline phosphatase activity assay

In the presence of magnesium ions alkaline phosphate catalyses the reaction

4-nitrophenyl phosphate $\longrightarrow$ 4-nitrophenol + phosphate

At pH 10.5, the activity is almost entirely due to alkaline phosphate. On addition of excess alkali the reaction is stopped and the liberated 4-nitrophenol simultaneously is converted to its yellow coloured sodium complex. The optical density is determined at 400nm: Comparison with a standard graph provides a measure of alkaline phosphatases activity.

Reagents

- Buffered substrate (pH10.5). Dissolve 373 mg glycine,10mg $MgCl2.6H_20$ and 165 mg Sodium p-nitrophenylphosphate dissolve in about 40 ml of 1M NaOH and dilute to 100 ml with double distilled water (**check pH**)
- 4-Nitrophenol standard solution. Dissolve 696 mg 4-nitrophenol in 0.02 M NaOH and make up to 1 litre with double distilled water. Dilute 10 ml of this solution to 1000 ml with 0 .02 M NaOH.
- Sodium Hydroxide 0.02 M

Calibration graph

Prepare six tubes as follows:

Tube No	4-nitrophenol solution	0.002M NaOH	Milli international units/L
1	0.5	10.6	16.6
2	2.0	10.1	33.3
3	1.5	9.6	50.3
4	2.5	8.6	83.3
5	3.5	7.6	117.0
6	4.5	6.6	150.0

Mix well and read the absorbance at 400 nm against water blank and plot against the corresponding enzyme activity measured in milli-international unit s per ml.

Procedure

1. Serum should be free from haemolysis. Plasma from anti-coagulated blood should not be used.

- The alkaline phosphate of human serum is stable for eight days at room temperature and for several weeks at 4°C.

For each serum to be assayed prepare two tubes as follows:

	TEST (in mls)	SERUM (in mls)
Buffered substrate	1.0	1.0
Incubate at 37°C for five minutes and add serum	0.1	0.0
Mix well and incubate at 37°C for 30 minutes		
Add 0.02 N NaOH	10.0	10.0
Serum	0.0	0.1
Total volume		

2 Mix the contents of each tube and read the optical densities at 400 nm against a water blank.

3 Subtract the serum blank absorbance from the test sample and hence find the alkaline phosphatase activity by reference to the calibration curve.

4 Serum that gives more than 150 milli-intemational units should be diluted with distilled water and re-assayed.

Use of test in diagnosis

The determination of serum alkaline phosphate (orthophosphoric monoester phosphydrolase) is of considerable importance in the diagnosis of disease of the bone and liver. Elevation of serum enzyme activity occurs in obstructive jaundice, Paget's disease, rickets and osteomalacia but reference should be made to relevant literature.

Determination of serum acid phosphatase

Reagent & materials

- Buffered substrate (pH 5.5). Dissolve 0.41 g citric acid; 1.125 g sodium citrate and 165 mg Sodium-p -nitrophenylphosphate in 100 ml double distilled water.
- 4-nitrophenol standard solution. Dissolve 696 mg 4-nitrophenol in 0.02M NaOH and make upto 1 litre. Dilute l0 ml of this solution to 1000 ml with 0.02 M NaOH.
- 0.005 M sodium hydroxide
- Serum
- Haemolysate

Procedure:

Prepare six tubes as follows:

Tube No	Std nitrophenol solution (ml)	0.05 M NaOH solution (ml)	Milli international units/mL
1	0.5	5.7	8.3
2	1.0	5.2	16.6
3	1.5	4.7	25.0
4	2.5	3.7	41.5
5	3.5	2.7	58.5
6	4.5	1.7	75

Mix well

Take the absorbance readings at 400 nm against water blank and draw a calibration graph.

Prepare four more tubes as follows:

Substrate	Test Serum	Serum Blank	Haemolysate Test	Haemolysate Blank	Substrate Blank
Buffered substrate (mls)	1.0	1.0	1.0	1.0	1.0
Incubate at 37°C then add					
Serum (mls)	0.2	0.0	0.0	0.0	0.0
Haemolysate (mls)	0.0	0.0	0.2	0.0	0.0
Mix, incubate at 37°C for 30 min. then add					
0.05 M NaOH (ml)	5.0	5.0	5.0	5.0	5.0
Serum	0.0	0.2	0.0	0.0	0.0
Haemolysate	0.0	0.0	0.0	0.2	0.0
Water	0.0	0.0	0.0	0.0	0.2

1. Mix the contents of each tube and read the optical densities at 400 nm against water blank.
2. Subtract the serum blank optical density and the substrate optical density from the test optical density hence find 'Total' acid phosphatase activity by reference to the calibration graph.
3. Similarly, subtract the haemolysate absorbance reading and the substrate optical density and also find 'TOTAL' acid phosphatase.

Questions

1. What are the isoenzymes of alkaline phosphatase?
2. Consider the definition of an international unit in this experiment with the S.I unit of enzyme activity and explain the differences.
3. What are the possible non-pathological causes of
   - An observed increased
   - Decreased serum alkaline phosphatase activity
4. What are the possible clinical indications for a doctor to request for serum alkaline phosphatase in:
   - A child of less than 7 years
   - An adult man of 30 years
   - A pregnant woman of 35 years
   - A man of 60 years

PRACTICAL TEN

AN INVESTIGATION OF THE FACTORS THAT AFFECT THE ACTIVITY OF ALKALINE PHOSPHATASE

Phosphatases (or phosphohydrolases) are enzymes which catalyse the hydrolysis of orthophosphate monoesters (the substrate) to inorganic phosphate and an alcohol (the products). They are found in all cells. You will use an alkaline phosphatase prepared from intestinal mucosa.

The velocity of the reaction is a measure of the rate of disappearance of the suhstrate or of the appearance of the products. The amount of substrate or product present at any time during the reaction is usually determined colorimetrically. This is of course a simple procedure if either the substrate or product are naturally coloured substances, or if a svnthetic substrate is used, which contains a coloured group. 4- nitrophenyl phosphate (4NPP) is a suitable synthetic substrate for phosphatases. The reaction it undergoes is:-

$$O_2N.C6H_4.OPO^-_3 + H_2O \longrightarrow O_2N.C_6H_4OH + HPO_4^{2-}$$

4-NPP ⟶ 4-nitrophenol (4-NP)

Phosphate acts as an inhibitor of the enzyme-catalysed reaction.

The product, 4NP, is colourless, in its undissociated form, but ionises in strongly alkaline solution to give the 4-nitrophenolate anion, which is yellow (absorption maximum 400 nm). Its concentration can therefore be measured directly, in a photometer. An added advantage is that addition of the alkali also stops the enzyme- catalysed reaction. The colour is stable, which means that you can continue with the experiments even if a spectrophotometer is not immediately available.

But make certain all your test-tubes are properly labelled. Cleanliness and efficiency are essential, if your experiments are to be successful.

Reagents and materials

- Alkaline phosphatase (0.1 mg protein/ml, in water).
- 4-nitrophenyl phosphate (4.0 mM in water) 4NPP
- 4-nitrophenol (0.1 mM in 0.1 M NaOH) (4 NP)
- Glycine-NaOH buffer (0.1 M, pH 9.9)

- Glycine-NaOH buffers (0.1 M, pH 8.4, 8.9, 9.4, 9.9, 10.4 & 10.9).
- NaOH (0.1 M, in water)
- Glycine-NaOH buffer (0.1 M, pH 9.9, containing 2 .0 mM NaH_2PO_4)

Procedure

Make sure you know what you are doing before you begin

A. Preparation of standard curve for 4-Nitrophenol

Prepare single labelled test-tubes containing 5.0 mls of: 0.1, 0.02, 0.03 and 0.04 mM 4NP in 0.1 M NaOH. (Note: 1.0 mls of 4NP stock solution + 4 mls of 0.1 M NaOH gives a final 4NP concentration of 0.02 mM).

Read the absorbance of each tube in the photometer at 400 nm, ***against a 0.1 M NaOH blank***

Construct a standard curve by plotting the measured absorbance against the amount of 4NP present, as µmoles in 5 mls

B. The effect of pH on the activity of alkaline phosphates

1. Take 6 clean test-tubes and label them with the pH values of the buffers you will use (8.4-10.9).
2. To each tube add: 0.5 mls stock 4NPP solution (4 mM in water) and 1.0 mls of the appropriate buffer.
3. Prepare a BLANK, in another tube by adding: 1.0 mls of buffer (pH 9.9), 0.5 mls of 4NPP and 0.5 mls of water.
4. Place the tubes (in a rack labelled with your initials) into a water bath at 37°C, and leave to warm for a couple of minutes. e.g 3 minutes.
5. Start the reaction by adding to each tube (except for the BLANK), 0.5 mls of enzyme solution. Note the time.
6. After 30 minutes, stop the reaction in each tube (including the BLANK) by the addition of: 3.0 ml 0.1 M NaOH.
7. Remove the tubes from the water-bath and measure the absorbance of each at 400 nm against the blank.

Questions

1. Convert the absorbance into µmoles 4NP by use of your standard curve.

2. Plot a graph of µlmoles against absorbance using your standard curve.
3. Plot a graph of µmoles 4NP produced, against pH.
4. What is the optimum pH of alkaline phosphatase?

C. The time course of the reaction

1. To a small conical flask, add 4.0 mls stock substrate solution and 8.0 mls buffer (pH 9.9). Place flask in a water bath at 37°C.
2. Number 6 test-tubes and add to each 3.0 mls of 0.1 M NaOH.
3. Start the reaction by the addition to the flask of: 4.0mls of enzyme solution.
4. At intervals (0, 10, 20, 30, 40 and 60 minutes), remove 2.0 ml samples from the flask and add each to the appropriate test-tube. Mix thoroughly.
5. Read the absorbance of the tubes at 400 nm using the *0' minutes'*. tubes as the BLANK. Plot a graph of µ moles 4NP produced against time, in minutes.

Questions:

1) What is the initial velocity of the reaction, in µmoles 4NP produced/minute?

2) Over what period of time is the reaction linear?

D. The effect of enzyme concentration on reaction rate

Set up the following tubes:

Tube Number	1	2	3	4
Substrate in mls	0.5	0.5	0.5	0.5
Buffer (mls) pH 9.9	1.0	1.0	1.0	1.0
Water (mls)	0	0.2	0.4	0.5

Incubate the tubes at 37°C for several minutes e.g. 3 minutes then begin the reaction by addition of the *correct volume* of enzyme to each tube.

Enzyme (mls) 0.5 0.3 0.1 0

Stop the reaction after exactly 30 min. by the addition of 3.0 mls sodium hydroxide solution to each tube

Read the absorbance of the tubes at 400 nm against tube 4. Convert the measured absorbances to umoles 4NP, using your standard curve. (This tells you the number of µmoles 4NP produced in 30 minutes).

Now calculate the initial velocity, V, of the reaction, in μ moles 4NP produced/minute. Work out by how many times the enzyme has been diluted in each tube, and from this calculate the enzyme concentration, (mg/protein/ml) in the assay solution, for each tube.
Using these results, plot a graph of V against (E).

Questions

1. Is the reaction dependent upon enzyme concentration, under conditions in which the substrate is 'saturating'? Illustrate your answer with the appropriate rate equation, and explanation of its meaning.
2. How many units/mls of alkaline phosphatase are present in the stock enzyme solution? (1 unit of enzyme activity is the amount of enzyme that will catalyse the transformation of 1μmole of substrate/minute. Therefore, if 1 ml of enzyme transforms 0.02 μmoles/minute the solution contains 0.02 units/ml).
3. If specific activity is defined as the number of units of activity per mg of protein, what is the specific activity of the stock phosphatase solution?

E. The effect of substrate concentration on reaction rate

To 5 labeled test-tubes, add: 1.0 mls of buffer (pH 9.9). Then add water and substrate as follows:

Tube Number	1	2	3	4	5
Stock Substrate (mls)	0.5	0.4	0.3	0.2	0.1
Water (mls)	-	0.1	0.2	0.3	0.4

1. Incubate the tubes at 37°C for 3 minutes then start the reaction by the addition of: *05mls of enzyme solution.*
2. Whilst the reaction proceeds, use the time to calculate the substrate concentration as moles/litre (M), in the incubation mixture, for each pair of tubes (tube 1 contains 10^{-3} moles/L substrate).
3. Stop the reaction after 30 min by addition of 3.0 mls of 0.1 M NaOH.
4. Read the absorbance of the solution at 400 nm using 0.1 N NaOH as the blank.
5. Plot graphs of V, the initial velocity μmoles 4NP produced/min) against (S), the (substrate concentration and of 1/V against 1/[S]. Take care to have correct units. This second graph is called a Linewever-Burk plot.

Questions

1. From the second plot, estimate the values of K_m, and V_{max} for the alkaline phosphatase under the conditions specified in your assay system.
2. What is the enzyme concentration (mg/ml) in the assay system? What would be the [value of V_{max} assuming an enzyme concentration of 1.0 mg/ml? Will the of K_m, also be different at this concentration?
3. How does the extrapolated value of V_{max}. from your graph compare with the value obtained in section C, in which a saturating concentration of substrate was present?
4. Explain the difference in values.

F. The effect of an inhibitor upon the reaction rate

This experiment is performed exactly as section E, but the buffer to be used contains 2.0 mM inorganic phosphate.

Calculate 1/V for each tube and plot your results on the same graph as in section E.

Questions

1. Is the inhibition by phosphate competitive or non-competitive? What does this mean in physical terms?
2. What is the inhibition constant for phosphate? (units are moles.1^{-1}. This can be calculated from the graph, since the point at which the line intersects with the x – axis =

$$K_m \frac{1}{1 + \frac{[I]}{K_i}}$$

3. Remember that the concentration of phosphate in the stock solution is 2.0 mM. It is diluted by addition of substrate and enzyme in the assay mixture.
4. What is its concentration in the assay mixture? Use this value in calculating Ki.

G. Other experiments

If you complete the experiments given above before the end of the session, there are several others that you could try, such as the effect of temperature on the reaction rate, and the effect of inorganic ions such as Mg (II). As the lecturer in charge for details.

PRACTICAL ELEVEN

PROTEIN (WESTERN) BLOTTING WITH IMMUNO DETECTION

Protein blotting simply involves transferring protein bands from an acrylamide gel onto a more stable and immobilising medium, such as nitrocellulose paper. Once transferred to the immobilising medium, a variety of analytical procedures may be carried out on the proteins which otherwise would have proved difficult or impossible in the gel. Such procedures may include hybridization with labelled DNA or RNA probes, detection with antibodies (as used in this experiment), detection by specific staining procedures, autoradiographic assay, etc. The approach is exactly analogous to the method used to transfer DNA from agarose gels (The Southern Blot)- and has been given the name 'Western' blotting.

There are a number of advantages of working with a protein blot rather than the original gel; these include:

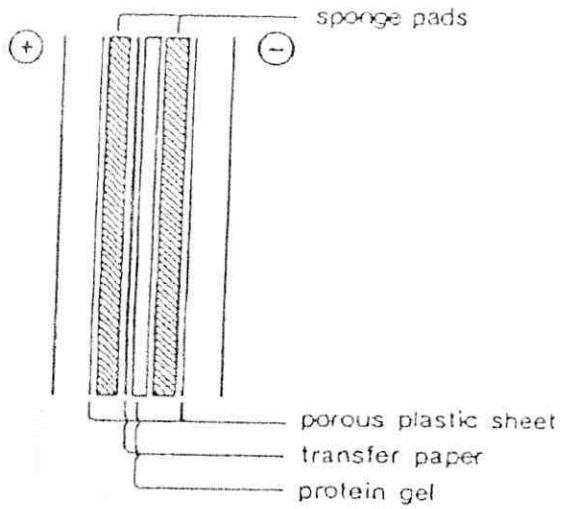
- Rapid staining/destaining: once transferred, staining and destaining can be achieved within 5 minutes.
- There is no ampholyte staining when analysing I.E.F. gels.
- Samples in preparative gels may be located rapidly.
- Low concentrations of samples can be detected since they are not spread throughout the thickness of a gel, but 'concentrated' at the surface of the paper. As a consequence, low specific activity probes can be used or lower concentrations of expensive or scarce probes are needed.
- The 'blot' is a convenient permanent record of the gel run.
- There are two ways of carrying out a protein blot, either by electroblotting or capillary blotting.

Electroblotting

In this method proteins are transferred from the gel to the immobilizing medium by electrophoresis (see Figure 1). This has the advantage of reducing transfer time to between 30 minutes and a few hours. The decreased transfer time also helps reduce any diffusion effects. The gel to be transferred is placed on top of a sponge pad saturated in buffer. The immobilizing medium is placed on top of the gel followed by a second sponge pad. This sandwich is then supported between two rigid porous plastic sheets (we get ours from Gallenkamp) and held together with two e/eastic bands. The sandwich is then placed between parallel electrodes in a buffer reservoir and an electrical current

passed. (The sandwich must be placed such that the immobilizing medium is between the gel and the anode.) Electrophoresis is carried out at 8-10 V/cm (with respect to electrode separation, 0.3-2.0 amp) for 1-4 hours. Where possible buffer should be circulated or cooled to minimise heating effects. Semi-dry blotting is an alternative version of electro blotting, where gels are electro blotted using pads of wet filter paper between the electrodes.

Figure 1



Capillary Blotting

For capillary blotting (see Figure 2), two or three sheets of absorbent filter paper are saturated with transfer buffer and connected to buffer reservoirs. The gel to be blotted is then placed on top of the filter paper and the transfer paper (nitrocellulose) placed on top of the gel. Two or three layers of dry blotting paper, several layers of paper towels (disposable nappies) and a weight are then stacked above the transfer paper and gel. Blotting is then allowed to take place for 24 to 48 hours, during which time the protein bands are carried from the gel to the nitrocellulose paper where they bind strongly.

Once protein blotting is completed, the nitrocellulose sheet may be analysed immediately, but it may also be stored indefinitely until a suitable time is available for analysis. The transferred proteins may be investigated by using any of the methods described above.

For the immunological detection of protein blots the nitrocellulose paper is firstly washed with Tween 80 to block remaining binding sites on the nitrocellulose. (The following discussion is based

on the assumption that one is identifying vine serum albumin.) The paper is then washed with a solution of rabbit anti- bovine serum albumin (first antibody). This antibody will obviously bind to any album in on the nitrocellulose sheet. When excess antibody has been removed by repeated saline washes, the paper is washed with a solution of goat anti-rabbit lgG conjugated to either horseradish peroxidase or alkaline phosphatase (second antibody). This second antibody will bind to any first antibody-albumin complex present on the paper. Following repeated saline washes to remove excess antibody, the paper is placed in an appropriate substrate solution for the conjugated enzyme. Substrates are chosen that have a coloured end-product. The appearance of a coloured band on the nitrocellulose sheet, therefore, determines the position of the bovine serum albumin on this sheet. Comparison of this nitrocellulose sheet stained for protein, or the original stained gel, allows the albumin band to be identified within the separated protein pattern. This procedure is shown in diagrammatic form in Figure

Figure 2: Atypical arrangement for capillary blotting

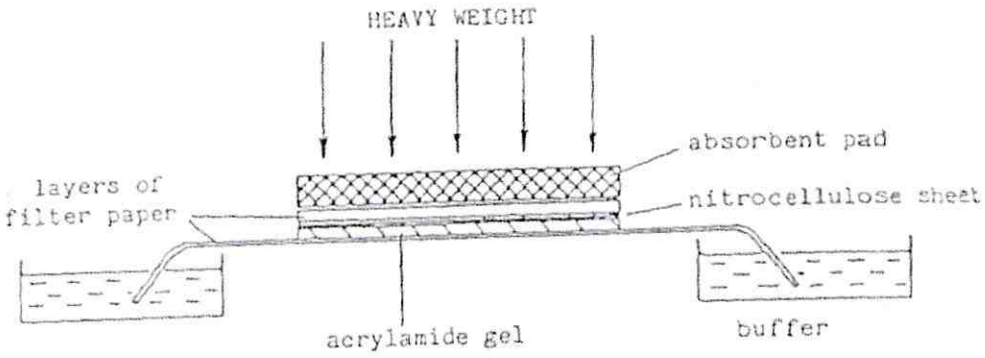
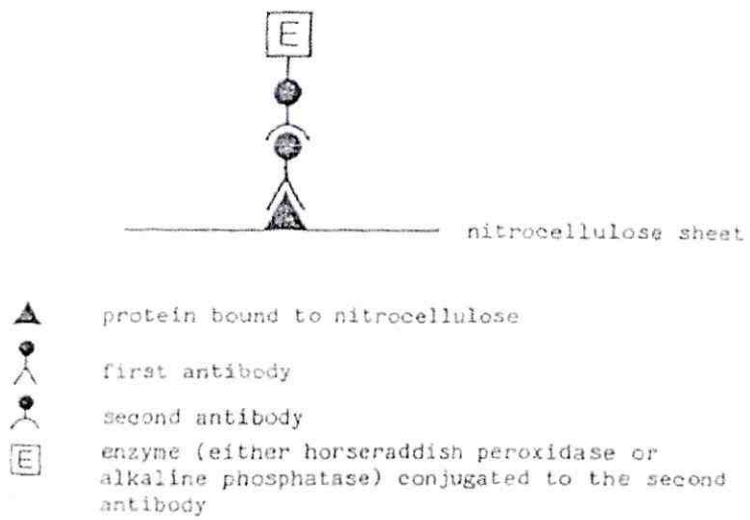


Figure 3: A schematic representation of the double-antibody technique to identify proteins on nitrocelellulose



3.

Reagents

1. Blotting Buffer (25 mM Tris, 192 mM glycine, 20%methanol, pH 8)

Tris	15.14g
Glycine	72.07 g

Methanol 1L

{To 5 dm^3 with distilled water (stable for weeks at 4^0C)}

2. Protein Stain: 0.2% Ponceau S in 10% acetic acid
3. Destain: Water
4. Phosphate buffered saline (PBS)+ Tween:

   NaCI 8.0 g

   KCI 0.2 g

   Na_2HP04 1.15g

   KH_2P04 0.2 g

   Tween 80 0.5 ml

   {to 1 dm 3 with distilled water (Stable for weeks at 4oC)}

5. 2% Tween 80 in PBS
6. A 1:400 dilution of rabbit anti-bovine serum albumin in PBS, containing 2% polyethylene glycol6000 and 0.05% Nonidet NP-40.
7. A 1:1,000 dilution of goat anti-rabbit lgG (conjugated to either horseradish peroxidase or alkaline phosphatase) in PBS, containing 2% polyethylene glycol 6000 and 0.05% Nonidet NP-40.
8. Enzyme substrates

   Horse radish peroxidase substrates: two possible substrates are described below:

   (a) Staining with 3-amino-9-ethylcarbazole

   Prepare a 1% stock solution of 3-amino-9-carbazole in acetone (store at 4°C) NB: 3-amino-9-carbazole is a possible carcinogen-use with care.

The substrate is prepared when required by mixing the following

0.8 ml stock solution

20.0 ml 0.05 M sodium acetate buffer, pH 5.0

10.0 µl 30% hydrogen peroxide

(b) Staining with 4-chloro-1-naphthol

Prepare a stock solution of 4-chloro-1-naphthol (3mg/ml) in methanol and store at 20 °C.

The substrate is prepared when required by mixing the following:

4.0 ml stock solution

20.0 ml 0.05 M Tris/HCI, pH 7.4 or Tris-buffered saline

10.0 µl 30% hydrogen peroxide

Substrate (a) gives a reddish-brown insoluble dye deposit in the presence of horseradish peroxidase.

Substrate (b) gives a blue deposit.

Alkaline Phosphatase Substrates:

5-Bromo-4-chloro-3-indolyl phosphate (BCIP)

In this method the enzyme converts the BCIP to the corresponding indoxyl compound, which precipitates, tautomerizes to a ketone, oxidizes and dimerizes to form a blue indigo, which is deposited in the nitrocellulose.

Dissolve the BCIP in a small volume of DMSO, then dilute to 2 mg/ml in 1 M ethanolamine, pH 9.8 containing 10 mM $MgCl_2$. (Alternatively 100mM $NaHCO_3$, pH 9.5 containing 10 mM $MgCl_2$ may be used).

BCIP with Nitro blue tetrazolium (NBT)

In this method the hydrogen ion released from dimerization (see above) reduces the NBT salt to the corresponding diformazan, which is intensely purple. This compound is therefore deposited along with the indigo giving an enhanced signal. To produce the substrate solution, mix the following:-

9.0 ml 0.15 M Veronal acetate buffer, pH 9.6

0.5 ml stock solution (5 mg/ml) of BCIP in dimethylformamide (keep stock solution at 20^0C)

1.0 ml (1 mg/ml) solution of NBT in veronal acetate buffer (prepare fresh as required)

20.0 µl 2 M $MgCl_2$

(3) **4-Methylumbelliferyl phosphate**

This substrate is converted to 4-methylumbelliferone, which is detected as a bluish- white fluorescent band using UV (360 nm)light.

The sub-strate is dissolved at 0.1 mg/ml in diethanolamine, 1mM MgCI2, pH 9.8.

(4) **Naphthol ASMX-phosphate plus Fast red TR salt**

Mix equal volumes of naphthol ASMX phosphate (0.4 mg/ml in distilled water) and Fast red

TR salt (6 mg/ml in 0.2 M Tris-HCI, pH 8.2). Both compounds are poorly soluble, and each should be stirred for 20 minutes before mixing. When mixed, filter the solution, then use directly. The reaction is terminated by washing the nitrocellulose sheet in distilled water.

Practical Procedure for Capillary Blotting

1. Assemble five layers of Whatman no. 1 filter paper as shown in Figure 13 and thoroughly wet them with blotting buffer. Carefully lay the gel to be blotted (without its stacking gel) on this wetted filter paper. The filter paper must, of course, be at least as wide as the gel being blotted.
2. Cut a piece of nitrocellulose paper exactly the size of the gel to be blotted and wet it by floating on blotting buffer. Carefully lay this wetted nitrocellulose paper over the gel, taking care not to trap any air bubbles. If the surface of the gel has dried out at all, it is easier to wet the top of the gel with blotting buffer before layering the nitrocellulose paper.
3. On top of the nitrocellulose paper, lay five layers of Whatman 3MM filter paper, cut to the same size as the gel, followed by a double pad of absorbent material (e.g. disposable nappies) also cut to the same size as the gel. To the top of the sandwich add a heavy weight and leave for 24 hours. NB. Care must be taken that the absorbent materials above the gel do not make contact with the lower layer of wetted filter paper, otherwise the flow of buffer will by-pass the gel rather than passing through the gel.
4. After blotting for 24 hours, dismantle the sandwich and recover the nitrocellulose paper. This may then be processed directly as described below.

Practical Procedure for Electroblotting

1. Following electrophoresis, rinse the gels in transfer buffer prior to blotting to facilitate the removal of electrophoresis buffer salts and detergents. The length of time required for equilibration is dependent on the gel thickness. It will be about 15 minutes for a 0.75 mm SDS-PAGE gel.
2. Cut the membrane to the dimensions of the gel. Wet the membrane by slowly sliding it at a 45° angle into transfer buffer and allowing it to soak for 15-30 minutes. Complete wetting of the membrane is important to insure proper binding.

Note: To avoid membrane contamination, always use forceps or wear gloves when handling membranes. If wetting is not complete after soaking in buffer, heat just to boiling, then remove from heat. Alternatively, sonicate the membrane in the solution. Also, the membrane may be immersed in methanol briefly, then soaked in buffer for 5-10 minutes prior to use.

3. Cut filter paper to the dimensions of the gel sandwich. Two pieces of filter paper per gel are needed for each gel/membrane sandwich. Completely saturate the filter paper by soaking in transfer buffer.
4. Saturate the fiber pads in transfer buffer.
5. Fill the buffer tank with transfer buffer.
6. Open the gel holder by sliding and lifting the latch. Note that one panel of the holder is clear and the other panel is tinted smoky gray. The clear panel is the anode (+) side, and the gray panel is the cathode (-) side. The cathode itself is identified by a black disk on the electrode panel on the inside wall of the Trans-Blot tank, and the anode is identified by a red disk. Place the opened gel holder in a shallow vessel. The clear panel should then rest at an angel against the wall of the vessel.
7. Place a pre-wetted fiber pad on the gray panel of the holder.
8. Place a piece of saturated filter paper on top of the pad.
9. Place the pre-equilibrated gel on top of the filter paper.
10. Place a pre-wetted transfer membrane on top of the gel. This is best done by holding the transfer sheet at opposite ends so that the center portion is sagging, and allowing the center portion to contact the gel first. Gradually lower the ends. This process will tend to expel any bubbles. Carefully rub the transfer sheet from side to side to push out all air bubbles and displace excess liquid between gel and paper. Bubbles can be removed by first rubbing with fingers, then by "roller- pin" exclusion using a pipet or test tube while holding the membrane and gel in position with your free gloved hand.
11. Complete the sandwich by placing a piece of saturated filter over the membrane(s). Again, remove air bubbles by rolling a glass pipet over the filter paper.
12. Place a saturated fiber pad on top of the filter paper.
13. Close the gel holder and hold it firmly (to keep the gel/membrane sandwich from moving laterally and disrupting good contact). Secure the latch. Place the gel holder in the Trans-Blot tank so that the gray panel of the holder is on the cathode side of the tank. The cathode is indicated by a black disc on the electrode panel (on the inside wall of the tank) and the anode side is indicated by the red disc. If only one gel is to be transferred, insert the sandwich in the center slot.
14. Ensure the buffer tank is filled with transfer buffer to the bottom of the red anode disc, and add a stir bar to the cell. Put the lid in place. Plug the unit into the power supply. Normal transfer polarity is from the cathode to the anode, ie, red wire to red outlet and black wire to black outlet on the power supply. Run at 500 mA, 1-4 hours.

Procedure for Semi-dry blotting

The 'sandwich' is prepared using three sheets of coated filter paper on both the anode and cathode side. Filter paper must be cut to the size of the gel (ie. do not use a full sheet covering the whole electrode as used in electroblotting). Run at 10V (250-300 mA) for 2 hr.

Detection of Protein on blots

Following blotting, but before probing the blot, the success of the transfer can be determined by transiently staining the blot with Ponceau S where proteins are seen as red bands on a white background. Stain the blot in Ponceaus for 1-2 mins then rigorously wash with repeated changes of water. Background stain should wash out leaving red-stained proteins on a white background. Further washing should remove all the protein stains. However, if any stains remains it will soon be removed when the blot is put into blocking solution.

Immunodetection of blots

1. Place the nitrocellulose paper in a small plastic dish and wash it, with gentle shaking, in blocking solution (20 ml) for 30 minutes or longer (overnight is suitable). This will block remaining binding sites on the nictrocellulose sheet. In all washing steps, take care that the nitrocellulose sheet is mobile in the washing solution, and not stuck to the bottom of the plastic dish.
2. Pour off the Tween solution and wash the nitrocellulose sheet in a solution of first antibody (10 ml) for 60 minutes.
3. Remove the first antibody solution, and wash the nitrocellulose sheet (4 x 5 minutes) in PBS-Tween (4 x 20 ml). This should remove all traces of any free first antibody.
4. Wash the nitrocellulose sheet in second antibody (10 ml) for 30 minutes, then remove this solution and wash the sheet (4 x 5 minutes) in PBS-Tween (4 x 20 ml).
5. Wash the sheet in freshly-prepared substrate solution. Colour bands (blue or red, depending on the enzyme and substrate used) should appear on the nitrocellulose sheet within a few minutes. When sufficient colour has developed, wash the sheet in distilled water and blot dry between two sheets of filter paper. Alternatively, ECL detection can be used as described on the next page.

NB: Sodium azide is not included in the PBS-Tween as this inhibits horseradish peroxide.

Alternative Detection Systems

Although enzymes are commonly used as markers for second antibodies, other markers can also be used. These include:

^{125}labelled second antibody, binding to the blot is detected by autoradiography.

Fluoresceine isothiocyanate labelled second antibody. This fluorescent label is detected by exposing the blot to ultra violet light.

^{125}labelled Protein A Protein A is purified from Staphylococcus aureus and specifically binds to the Fe region of lgG molecules. 125llabelled Protein A is therefore used instead of a second antibody, and binding to the blot is detected by autoradiography.

Gold-labelled second antibodies. Second antibodies (anti-species lgG) coated with minute gold particles are commercially available. These are directly visible as a red colour when they bind to the primary antibody on the blot.

Biotinylated second antibodies. Biotin is a small molecular weight vitamin that binds strongly to the egg protein avidin (Ko = 1o-15 M). The blot is incubated with biotinylated second antibody, then further incubated with enzyme conjugated avidin. Since multiple biotin molecules can be linked to a single antibody molecule, many enzyme linked avidin molecules can bind to a single biotinylated antibody molecule, thus providing an enhancement of the signal. The enzyme used is usually alkaline phosphatase or horseradish peroxidase.

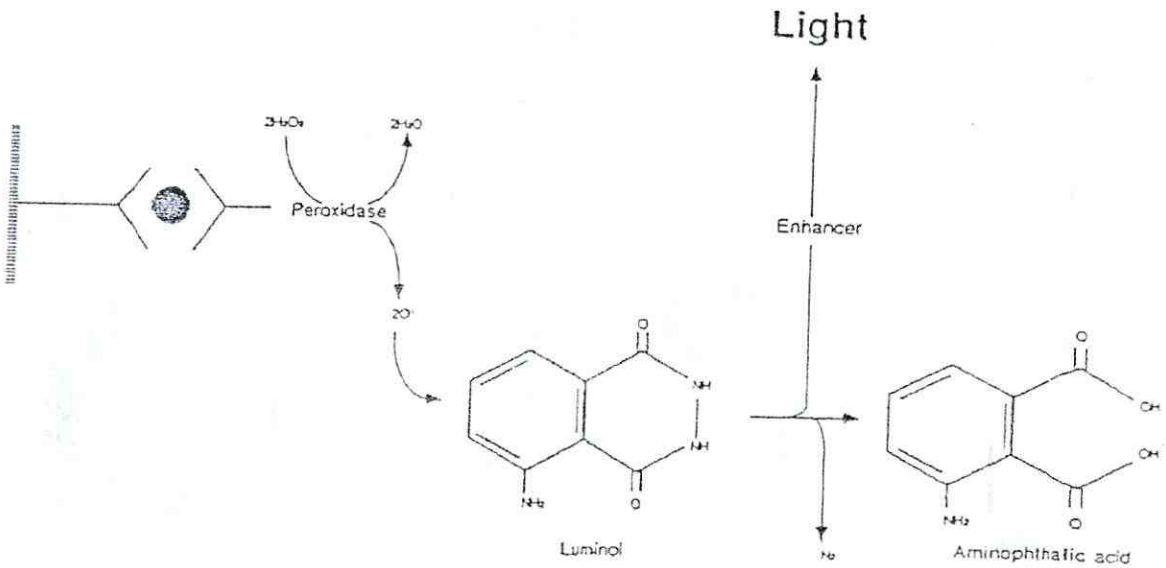
In addition to the use of labelled antibodies or proteins, other probes are sometimes used. For example, radioactively-labelled DNA can be used to detect DNA-binding proteins on a blot. The blot is firstly incubated in a solution of radiolabelled DNA, then washed, and an autoradiogram of the blot made. T he presence of radioactive bands, detected on the autoradiogram, identifies the positions of the DNA-binding proteins on the blot.

ENHANCED CHEMILUMINESCENCE (ECL)

An alternative approach to the detection of horseradish peroxidase is to use the method of enhanced chemiluminescence. In the presence of hydrogen peroxide and the chemiluminescent substrate luminal (Fig 4) horseradish peroxidase oxidises the luminal with concomitant production of light, the

intensity of which is increased 1000-fold by the presence of a chemical enhancer. The light emission can be detected by exposing the blot to a photographic film. Comparable substrates are now available for use with alkaline phosphatase -linked antibodies. ECL is 10-100 times more sensitive then using a chromogenic subsrate for the enzyme.

Figure 4



PRACTICAL TWELVE

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA}

Enzyme-linked immunosorbent assay (ELISA) was first introduced in the early 1970s and is a highly sensitive method for assaying either antigens or antibodies. In principle the assay is analogous to radio-immunoassay (RIA), the only major difference being the use of an enzyme rather than an isotope.

The general principle is as follows:
An antigen or antibody is linked to a solid phase surface, usually the inside of the wells of a Microplate (96 well round-bottomed plate).

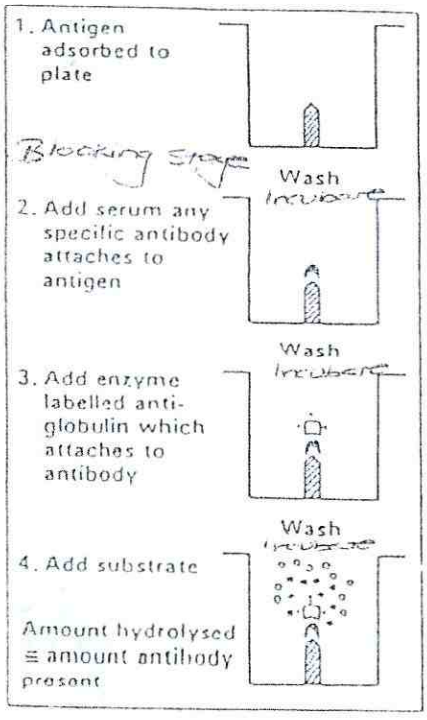
This 'sensitised' carrier surface binds the corresponding antibody/antigen from the test solution.

An enzyme-labelled specific antibody is attached to this antigen-antibody complex.

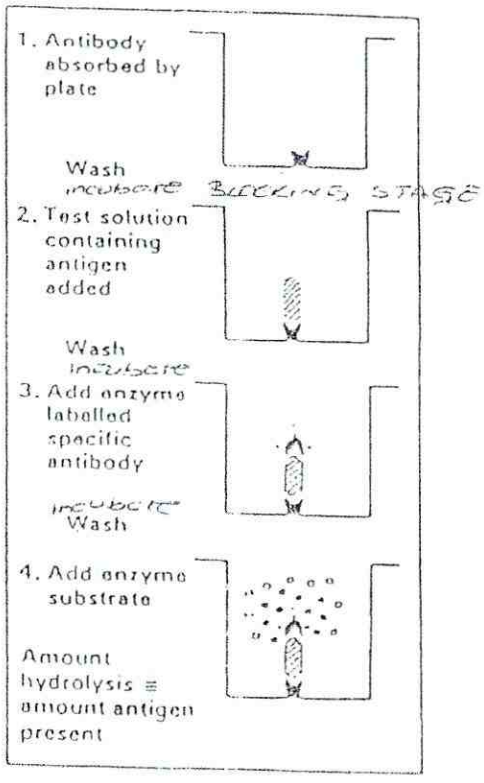
This labelled antibody, is then detected by the colour change resulting from the hydrolysis of an added enzyme substrate. The optical density of the final colour is directly proportional to the amount of unknown antibody or antigen in the test solution.

There are a number of possible permutations of this basic procedure. They can be broadly divided into competitive and non-competitive assays and some of the variations are shown in Figures 20 and 21. The sensitivity of the ELISA often approaches that achieved by radio- immunoassay and, consequently, the method has found considerable use in the clinical field, particularly for assaying antigen or antibodies in serum samples.

Figure 1

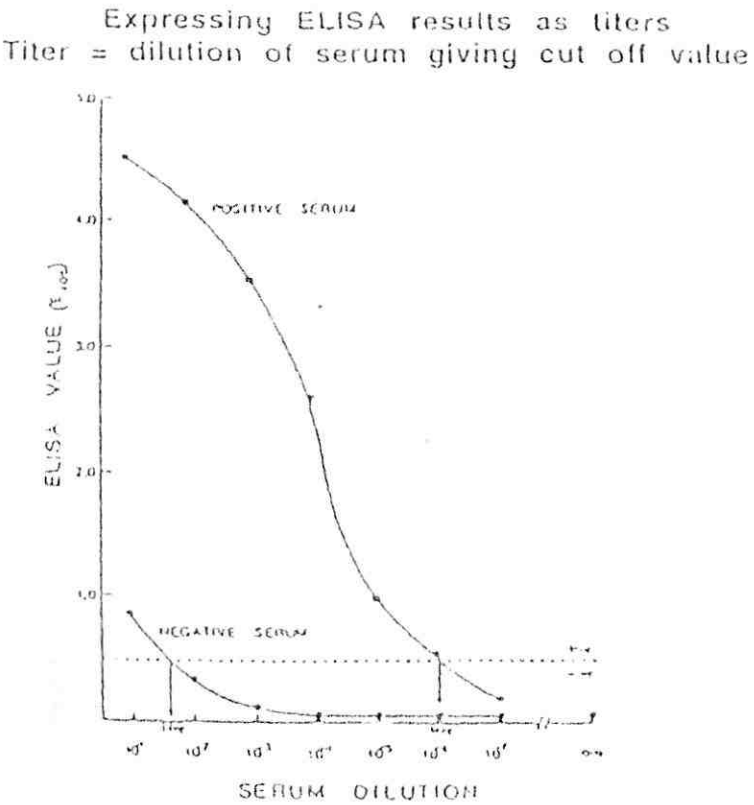


a) Double antibody sandwich method for detecting antibodies in a serum sample



b) Double antibody sandwich method for detecting antigen in a serum sample

Figure 2



MATERIALS

Coating Buffer:

1.5 g	Na_2CO_3
2.93 g	$NaHCO_3$ to 1 dm3, pH 9.9 (no adjustment)
0.2 g	NaN_3

Store at 4^0C for no longer that two weeks

PBS-TWEEN, pH 7.4:

8.0 g	NaCl
0.2 g	KH_2PO_4
1.15 g	Na_2HPO_4
0.2 g	KCl
0.5 ml	Tween 80

(0.2 g NaN_3, Optional)

10% diethanolamine buffer

97.0 ml	diethanolamine	
800 ml	Water	adjust pH to 9.8 with 1 M HCl
0.2 g	NaN_3	then make up to 1 dm^3
100 g	$MgCl_2.6H_2O$	

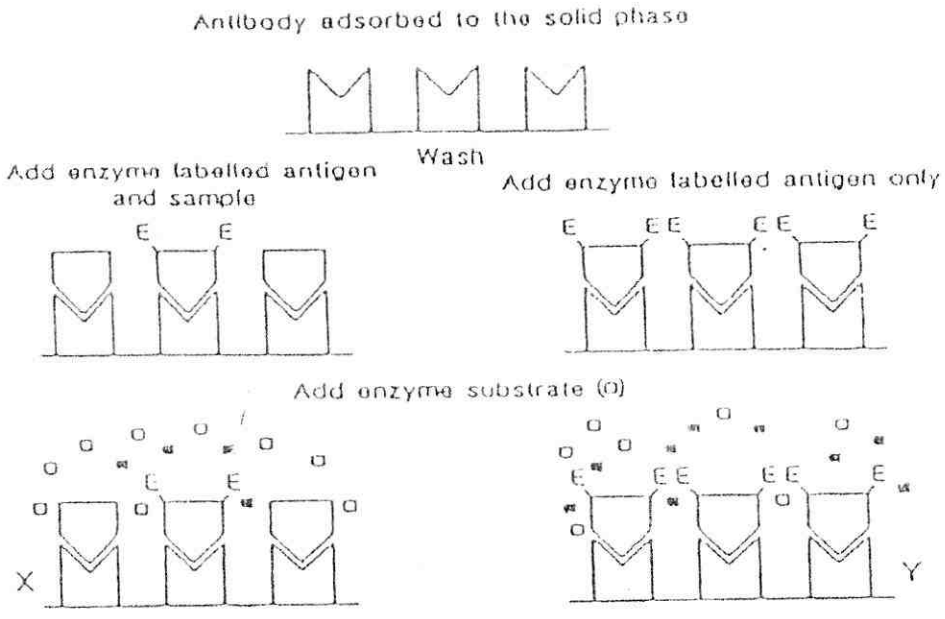
(Alternatively, 100 mM $NaHCO_3$ containing 10 mM $MgCl_2$ may be used)

Alkaline phosphate substrate.

Dissolve one phosphatase substrate (p-nitrophenylphosphate disodium; Sigma104) tablet (5 mg) in 5 ml of diethanolamine buffer.

Competitive ELISA

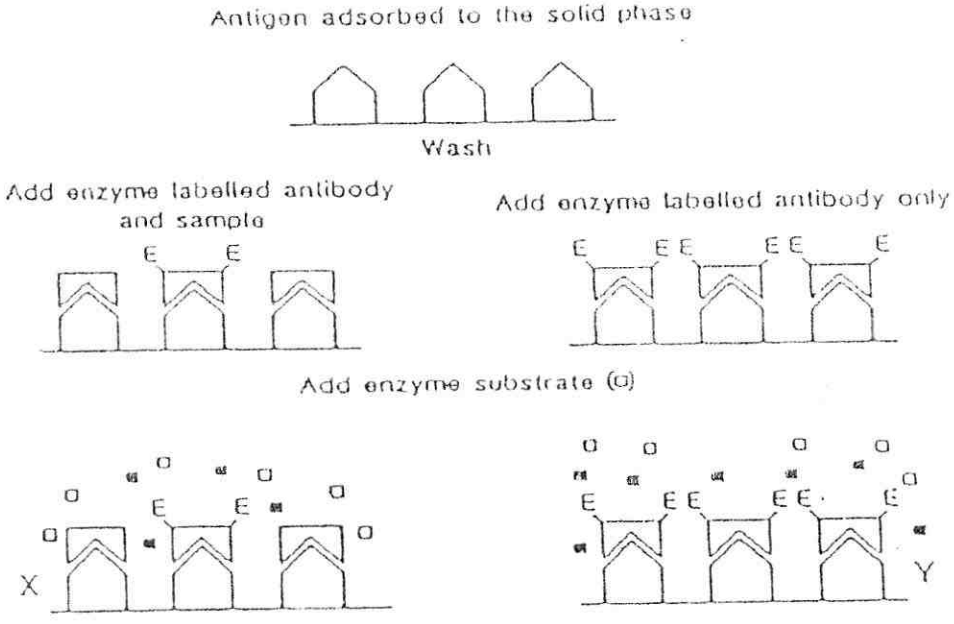
Detection of Antigen



(Product) (∝) = (Enzyme labelled antigen)

The difference between X and Y = (Antigen) present in the sample

Detection of Antibody



(Product) (∝) = (Enzyme labelled antibody)

The difference between X and Y = (Antibody) present in the sample

Notes

The major application of ELISA at present is in immunodiagnosis of infectious diseases. Such applications include:

- Measurement of viral antigens. e.g. Ebola virus, rift valley fever virus, dengue virus etc
- Measurement of bacterial and mycotic antigens, e.g. brucella antigens, salmonella antigens, aflatoxin, etc.
- Measurement of parasite antigens, e.g. *Trypanosoma cruzi, Schistosmna mansoni*
- The measurement of antibodies to viruses, e.g. measles, herpes, rabies, etc.
- The measurement of antibodies to bacteria, e.g. Brucella, Salmonella, Legionella, diphtheria toxoid, tetanus toxoid, etc.
- The measurement of antibodies to parasites, e.g. malaria, liver flukes, etc.

Blood and serum constituents present in non-infectious diseases can also be measured by ELISA. These include hormones (e.g/ HCG, insulin, oestrogen), tumour markers (e.g. alpha foetal protein}, haemolytical factors (e.g. Factor VIII, fibrinogen) and indicators such as rheumatoid factor.

In the following procedure rabbit serum is investigated for the presence of anti-bovine serum albumin antibodies using a double antibody sandwich technique. The procedure is represented diagrammatically in Figure 3.

Procedure

In this experiment we will analyse (**in duplicate**) two rabbit serum samples for the presence of antibodies to BSA. One rabbit was immunized will BSA (test serum) while the other one was not immunized with BSA (normal rabbit serum or negative control). In addition, serum known to have anti-BSA antibodies will be assayed (positive control). Serial dilutions will be used to show how an antibody titre can be obtained.

1. You are provided with a stock solution of bovine serum albumin (BSA) (20μg/ml) in coating buffer containing 0.1% gelatin. Add 100 μl of this solution to wells 2- 12, inclusive, in rows B to H and to row H. Cover the plate and leave at 4°C overnight. During this time the BSA will be adsorbed onto the surface of the well.
2. Empty wells in one swift swing action, bang 2 to 5 times on paper towels. Wash the wells by

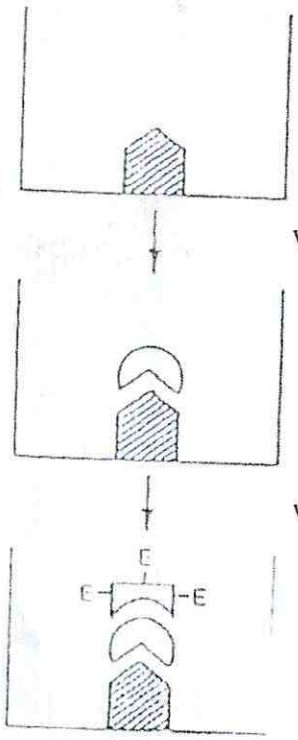
filling them with PBS-Tween, empty and bang a few times on paper towel. Repeat this step three to five times

3. Add to all wells 100 µl of 5% fat-free skim milk in PBS-Tween and block for 1 hr at room temperature. **Wash as in step 2 above**
4. To all wells, numbered 2- 12, add 100 µl of PBS-Tween.
5. To well B2 add 100 µl of a 1:100 dilution of the test serum (diluted in PBS-Tween). Mix well by drawing into the pipette tip a number of times (approximately 6 times), then transfer 100 µl to well B3. Continue in this way until you reach B12, when you will finally withdraw 100 µl of liquid and discard it. **A series of two-fold dilutions of the serum sample is thus achieved across the plate.**
6. Repeat the same procedure in row C (i.e. a duplicate of row B).
7. Carry out the same procedure on rows D and E but using the control rabbit serum.
8. Carry out the same procedure on rows G and H but using the positive control serum.
9. When steps 5 - 8 are completed, cover the plate and incubate at room temperature for two hours, with gentle agitation every 15 minutes.
10. Wash the plates three times with PBS-Tween as described in 2 above.
11. Prepare a fresh 1:1000 dilution of alkaline phosphatase conjugated anti-rabbit-lgG in PBS-Tween. Add 100 µl to each well (except no. 1). Cover and incubate at room temperature for one hour, with gentle agitation of the plate every 15 minutes.
12. Wash all wells three times with PBS-Tween.
13. Ensure that the enzyme substrate is at room temperature. Add 100 µl of enzyme substrate to each well (including now, column 1)
14. **Keep in the dark for 30 minutes or more at room temperature till color develops**
15. If colour development is slow, plates may be incubated at 37^0C to increase the enzyme reaction. Alternatively, some workers prefer to leave plates to develop colour slowly at 4 ^{0}C overnight.
16. Stop further color development by adding 50 µl 3M NaOH to all wells
17. Read and record absorbance at 450 nm

Questions

1. Calculate the average OD for each sample for each dilution
2. Determine the P/N ratio for the test samples
3. Did the test serum contain antibodies to BSA? Why?

Figure 3. Diagrammatic representation of the procedure used in this experiment



1. Wells coated with BSA

WASH X 3

2. Wells incubated with dilutions of rabbit serum
Any anti-BSA antibodies in the serum will
bind to the BSA in the wells

WASH X 3

3. Wells incubated with goat anti-rabbit IgG
labeled with alkaline phosphatase. This will
bind to any antigen-antibody complex present

WASH X 3

3. Add substrate. The amount of colour liberated in
a fixed time is a measure of the amount of enzyme
present, and therefore a measure of the amount of
anti-BSA antibodies present.